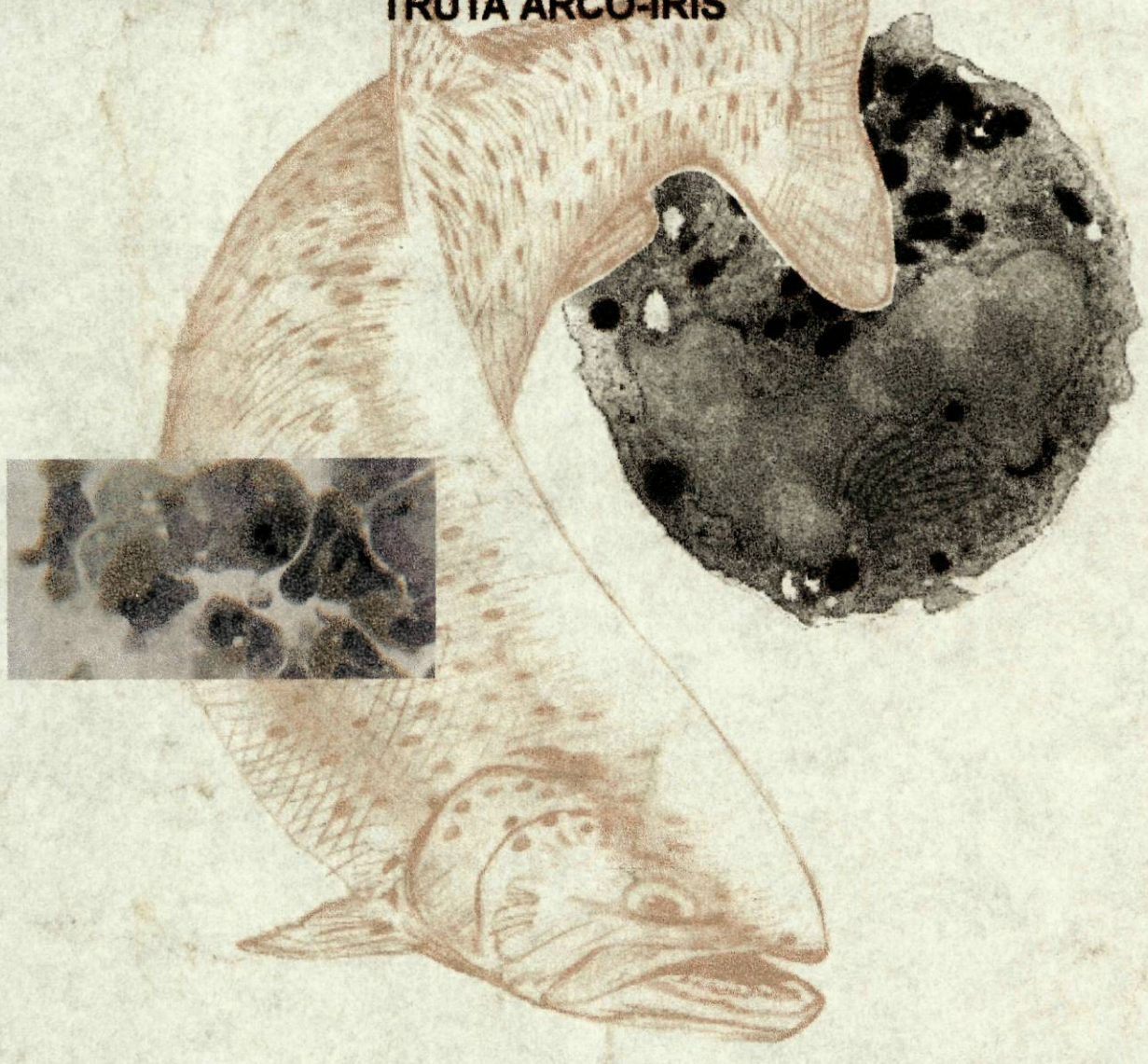
António Manuel dos Santos Afonso

CARACTERÍSTICAS CITOQUÍMICAS, PADRÕES DE MOBILIZAÇÃO E INTERACÇÃO DE NEUTRÓFILOS E MACRÓFAGOS NA INFECÇÃO EXPERIMENTAL DA TRUTA ARCO-ÍRIS



Porto 1998

António Manuel dos Santos Afonso

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Dissertação de candidatura ao grau de Doutor em Ciências Biomédicas, apresentada ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto, sob a orientação do Doutor Manuel Teixeira da Silva e co-orientação do Prof. Doutor Nuno Rodrigues Grande.

Departamento de Anatomia Normal

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Porto, 1998

Aos meus pais,

ao meu filho Rui, à Manela e às suas meninas

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De acordo com o número 2 do Artigo 8º do Decreto-Lei nº 388/70, esta dissertação inclui resultados contidos em artigos científicos, alguns dos quais originalmente publicados em revistas internacionais. No cumprimento do disposto no referido Decreto-Lei, o autor desta dissertação declara ser da sua responsabilidade a execução das experiências realizadas nestes trabalhos e ainda que participou no planeamento destes estudos, assim como na interpretação, na discussão dos resultados obtidos e na redacção dos manuscritos.

Trabalhos publicados ou em publicação:

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Afonso, A. & Silva, M. T. (1998). Macrophage/neutrophil interactions in the sea bass (*Dicentrarchus labax*).

Afonso, A. & Silva, M. T. (1998). Neutrophil apoptosis in rainbow trout (*Oncorhynchus mykiss*). An *in vitro* study.

PREFÁCIO

Os trabalhos que compõem esta dissertação versam o tema geral Imunologia da Infecção em peixes.

Várias opções tiveram de ser tomadas em relação à investigação que é incluída na presente dissertação.

Escolheu-se um salmonídeo como modelo animal, pois esta família de peixes é a mais utilizada em pisciculturas em Portugal, como no resto da Europa e América, tanto em água doce como em água salgada. Dentro dos salmonídeos optou-se pela truta arco-íris pois trata-se de uma espécie largamente cultivada em água doce no norte de Portugal. Apresentava-se, por isso, como um animal de laboratório facilmente acessível e a um custo razoável para os trabalhos que nos propúnhamos fazer, tendo em conta o grande número de exemplares necessários para se poderem estabelecer cinéticas celulares com valores estatisticamente válidos em condições normais ou de infecção experimental. Por outro lado, as condições de estabulação e experimentação em peixes que foi necessário criar à partida no Laboratório de Imunologia Comparada do IBMC, recomendavam a opção por um peixe de água doce dada a distância ao mar e o que isso implicava em custos e viabilidade.

A opção pelo emprego da truta arco-íris cultivada em água doce reflecte-se também nas espécies bacterianas que foram utilizadas na presente investigação.

Dentro do tema Imunologia da Infecção em peixes, escolhemos estudar a biologia celular da resposta inflamatória que acompanha a infecção, e dos fagócitos nela envolvidos, com particular interesse sobre o papel do neutrófilo. Esta escolha permitiu aproveitar a grande experiência que o laboratório onde trabalhámos tem nesta área e obter resultados que permitem uma comparação directa de vários aspectos das respostas à infecção, entre mamíferos e peixes.

A preparação da presente dissertação contou com a larga experiência que o Laboratório de Imunologia da Infecção do Instituto de Biologia Molecular e Celular tem relativamente a este tema em mamíferos, e também com a estreita colaboração que tem sido mantida com o Departamento de Zoologia da Universidade de Aberdeen, com o Departamento de Biologia Celular e Anatomia da Universidade de León, com o Departamento de Microbiologia da Universidade de Santiago de Compostela e com o "Marine Laboratory" de Aberdeen.

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INTRODUÇÃO GERAL

A revisão da literatura que se faz nesta secção, logicamente orientada de acordo com o tema central da presente dissertação, refere-se às publicações relevantes sobre o papel dos fagócitos nas infecções, com ênfase nas infecções de salmonídeos.

I. Pescas, aquacultura e imunologia da infecção nos peixes

As estatísticas a nível mundial das capturas efectuadas pela pesca mostram claramente que, após um crescimento contínuo ao longo das últimas décadas, e apesar do contínuo aumento do esforço de pesca, essas capturas estabilizaram, com valores de cerca de 91 milhões de toneladas em 1994 e 1995, sendo 7 milhões de toneladas provenientes de águas interiores e 84 milhões de toneladas provenientes do mar (FAO, 1996).

A aquacultura, embora seja uma actividade produtiva com milhares de anos, com particular importância nos países orientais, na Europa Ocidental e na América, apenas neste século se tornou uma actividade industrial com relevo no fornecimento de proteína animal para consumo humano.

O desenvolvimento da aquacultura só foi possível devido ao avanço científico e tecnológico, e ao investimento feito nesta área da produção. Assim, a aquacultura continua em lento mas progressivo aumento de produção, representando já 19% da produção aquática mundial com cerca de 21.3 milhões de toneladas em 1995, sendo 14.6 milhões de toneladas de águas interiores e 6.7 milhões de toneladas de aquacultura marinha (FAO, 1996).

A aquacultura pressupõe a existência de populações animais confinadas a um ambiente restrito, geralmente com elevada densidade populacional e simultaneamente com aporte de alimento fornecido pelo aquacultor aos animais estabulados.

As condições de vida das populações existentes em regime de aquacultura, em comparação com as das populações existentes em ambiente natural, se, por um lado, são favoráveis em termos de crescimento e de economia de espaço, por se encontrarem concentradas e confinadas, por outro, implicam uma maior promiscuidade entre os indivíduos que a constituem. Isto favorece a dispersão dos microrganismos que lhes estão associados, podendo estes ser, ou tornar-se em determinadas circunstâncias, patogénicos para as populações em causa. Acresce ainda que a estabulação implica em muitos casos o manuseamento dos indivíduos. Ora, tanto o confinamento como a manipulação causam estados de "stress" que contribuem para o aumento da sua

susceptibilidade aos microrganismos (Ellis, 1981; Pickering, 1981; Angelidis, 1987; Anderson, 1990).

Assim, surtos de infecção mais ou menos graves são uma característica intrínseca da aquacultura. Estes surtos podem ir de fenómenos esporádicos sem grande mortalidade, a infecções agudas ou crónicas graves, que causam a perda de elevadas percentagens da população em cultura. A estas patologias está ainda ligado o fenómeno não menos grave dos estados de patologia sub-clínica, com a consequente existência de portadores assintomáticos.

A aquacultura, ao tornar-se uma indústria ao nível mundial, implicando a selecção de espécies e o apuramento de raças e variedades mais propícias para determinados tipos de produção, desencadeou uma permuta de indivíduos ou ovos, que tem contribuído para a disseminação para vastas áreas do globo de muitas das patologias inicialmente endémicas de certas regiões. Acresce ainda a permanente possibilidade de contaminação do meio ambiente a partir de aquaculturas infectadas, onde as espécies selvagens aí existentes entram em contacto com agentes patogénicos com os quais jamais contactaram, podendo esse contacto ser catastrófico (Fryer & Bartholomew, 1996).

Nesta, como noutras áreas da produção, cedo se vieram a introduzir desinfectantes, antibióticos e outros biocidas para o tratamento ou prevenção de surtos infecciosos, sendo muitos destes tratamentos eficazes por adição do fármaco na ração ou por imersão dos animais num banho com elevada concentração do agente profiláctico ou terapêutico.

Mas, também na aquacultura, a utilização de antibióticos veio induzir o aparecimento de microrganismos progressivamente mais resistentes aos quimioterápicos, com o inevitável aumento das doses e a passagem a fármacos mais fortes ou mesmo a misturas de vários destes agentes químicos.

A dramática continuidade entre os sistemas industriais e o meio ambiente, tem originado um protesto cada dia mais intenso e generalizado dos grupos ecologistas e dos cidadãos em geral, para que se faça um esforço no sentido de se reduzirem ao mínimo os impactos que os sistemas industriais têm sobre a fauna e a flora selvagem e, finalmente, nas condições de vida da população humana.

Surge assim, também para a aquacultura, a necessidade de se criarem sistemas de segurança sanitária e de diagnóstico mais eficazes, de se aperfeiçoarem as intervenções profilácticas e terapêuticas, de se desenvolverem novas vacinas contra as mais perigosas patologias infecciosas, de se demarcarem as zonas afectadas e as zonas isentas de cada uma dessas patologias e de se impor o tratamento dos efluentes para que, por um lado se reduzam os riscos de produção e por outro, para que a poluição e a contaminação do meio ambiente sejam evitadas.

Muitas das dificuldades existentes em solucionar os problemas que se acabam de expor estão relacionadas com o pouco desenvolvimento em áreas das ciências biológicas viradas para os animais aquáticos.

Podemos incluir entre essas áreas de investigação fundamental a ecologia, a microbiologia, a fisiologia e a imunologia, as quais se dedicam ao estudo dos mecanismos biológicos relacionados com a poluição e contaminação do meio ambiente, com os estados patológicos ou de risco, ou ainda, com a resistência ou susceptibilidade de certas espécies aquáticas a determinados agentes patogénicos. O avanço no conhecimento nessas áreas permitirá desenvolver a investigação aplicada e as tecnologias mais adequadas para a resolução dos problemas discutidos atrás.

Um ponto central neste contexto tem a ver com o conhecimento dos processos imunológicos que são postos em jogo nos mecanismos de defesa contra a infecção em peixes. O progresso nesse conhecimento é fundamental para que a intervenção profiláctica ou curativa venha a ter maior sucesso.

A presente dissertação centra-se especialmente no estudo de alguns aspectos referentes ao papel dos fagócitos naqueles mecanismos de defesa na truta arco-íris.

II. Os fagócitos como elementos essenciais na defesa dos metazoários contra a infecção

1. Metchnikoff

É conhecido desde há muito que as características físicas e de comportamento evidenciadas por cada grupo, espécie ou indivíduo, como a tolerância a factores ambientais, capacidade reprodutora, agressividade, territorialismo, competição e predação são fundamentais para a sua sobrevivência. No que respeita aos estados de doença, nomeadamente os que estão associados a infecções, muito está ainda por ser compreendido.

Durante milénios, desconheceu-se a existência de seres microscópicos, a sua relação com os restantes seres vivos e particularmente a sua relação com muitas das patologias que sempre afligiram a humanidade. Esse desconhecimento, aliado ao desconhecimento da organização celular dos organismos, bem como de muitos outros aspectos da Biologia, foi a base da incapacidade de resolução de estados patológicos, muitos deles associados à ocorrência de epidemias que dizimaram grande parte da população humana, com a atribuição de tais males a castigos divinos, a conflitos metafísicos entre a vida e a morte, ou simplesmente à sina de cada um.

Só nos finais do Séculos XIX, com Pasteur e Koch, se chega ao conhecimento da origem bacteriana de muitas das doenças mais disseminadas pelo mundo. Os trabalhos pioneiros destes e de outros cientistas dessa época estão intimamente ligados ao nascimento da Imunologia como disciplina autónoma.

Conforme descrito por Kruif (1953), Metchnikoff, há cerca de um Século, ao estudar esponjas e estrelas-do-mar, observou células que, embora fazendo parte dos tecidos, possuiam uma certa autonomia, projectando parte do seu protoplasma deslocando-se de forma semelhante a amibas.

Metchnikoff, após injectar partículas de carmim nas larvas transparentes das estrelas-do-mar a fim de observar o que se passava no seu interior, descobre que essas células migradoras se deslocam em direcção às partículas de carmim e as ingerem. Foi baseado na observação da actividade destas células, as quais baptizou de fagócitos, que aquele cientista desenvolveu a teoria da fagocitose (Kruif, 1953).

A investigação desencadeada por Metchnikoff veio introduzir novos conceitos fundamentais para os alicerces da Imunologia, mostrando a intervenção celular directa nas defesas imunológicas e nomeadamente que os fagócitos integram a primeira linha de defesa contra os microrganismos (Silverstein, 1993).

Por ser um adepto das teorias evolucionistas, para Metchnikoff a doença era entendida como a consequência da relação entre um parasita e um hospedeiro, e portanto, uma forma de competição, implicando isto uma pressão selectiva sobre ambos os intervenientes (Kruif, 1953).

Hoje é patente que a sobrevivência dos seres pluricelulares (os metazoários) é permanentemente posta em causa pela multidão de seres microscópicos patogénicos, para alguns dos quais os primeiros não possuem defesas eficazes. As reflexões de Lederberg (1997) sobre a luta pela sobrevivência dos seres humanos contra os microrganismos e a sua coevolução, podem ser, na verdade, ampliadas aos metazoários em geral.

2. Uma visão filogenética e comparativa da fagocitose e dos fagócitos

Em comparação com o enorme esforço posto na investigação da Imunologia virada para a causa humana, na tentativa de se encontrarem formas de luta contra a doença, o esforço dedicado ao estudo mais amplo dessa disciplina, englobando todos os grupos animais, tem sido reduzido.

Ao passar-se de uma abordagem antropocêntrica daquela ciência para a escala zoológica, depara-se com a existência de um número enorme de grupos animais, muitos

deles englobando um elevado número de espécies, vivendo nos mais variados ecossistemas, e por isso sujeitos, desde a sua origem, a pressões selectivas muito diferentes. É, portanto, de esperar que nos níveis inferiores da escala zoológica, os processos de defesa a cargo do sistema imunológico tenham também particularidades que tornam o esforço de compreensão desses processos uma tarefa à partida mais vasta e, por isso, com avanços mais limitados.

No entanto, tem havido algum esforço no sentido de se conhecerem os aspectos mais relevantes da Imunologia aos diferentes níveis filogenéticos, esforço esse da responsabilidade dos estudiosos da Imunologia Comparativa.

Não se trata aqui apenas de uma actividade ditada pela mera curiosidade científica, pois a cultura de muitas espécies, quer de vertebrados quer de invertebrados, é hoje fonte insubstituível de alimentação humana.

O conhecimento da filogenia do sistema imunológico depara com a enorme dificuldade da inexistência da maioria dos grupos animais que, ao longo da vida na Terra, a foram ocupando e que se encontram hoje extintos. Apenas nos é possível analisar os descendentes que sobreviveram até hoje e que são o resultado da evolução, não sendo estes necessariamente iguais aos seus antecessores, por muito pouco que tenham evoluído (Du Pasquier, 1993).

Dada a universalidade e a permanência de pressões selectivas sobre os metazoários exercidas pelos agentes infecciosos, podemos esperar que todos os metazoários possuam um sistema de defesa contra infecções (Du Pasquier, 1993). É interessante salientar, no contexto da presente dissertação, que os fagócitos participam nos mecanismos imunológicos que operam contra as infecções por agentes infecciosos celulares em todos os metazoários (Du Pasquier, 1989; Horton & Ratcliffe, 1996).

2.1. A fagocitose e os fagócitos nos invertebrados

Ao contrário dos mamíferos, sobre os quais existe um grande conhecimento sobre as células do sistema imunológico, nos invertebrados os conhecimentos sobre a origem, desenvolvimento, estrutura e função desse sistema são escassos.

As células envolvidas no sistema imunológico dos invertebrados dividem-se em dois grupos: as células circulantes (celomócitos) e uma variedade de células fixas, podendo estas encontrar-se distribuídas pelos vários territórios do organismo e, em alguns casos, também localizadas em órgãos hematopoiéticos/fagocíticos (Ratcliffe *et al.*, 1985).

A enorme variedade de invertebrados tem dificultado a classificação dos celomócitos. Por exemplo, até há pouco tempo, só para os insectos existiam cerca de 70 nomes para os 6 a 9 tipos de células sanguíneas. Do ponto de vista funcional, as células

sanguíneas dos invertebrados foram agrupadas por Ratcliffe *et al.* (1985) nos seguintes tipos: células progenitoras, células fagocíticas, células hemostáticas, células nutritivas e células pigmentares.

Após a descoberta por Metchnikoff da existência de células fagocíticas nos tecidos dos invertebrados, vários nomes lhes foram dados, como: arqueócitos, macrófagos, amebócitos, granulócitos, monócitos, plasmatócitos, celomócitos e células granulares. Alguns destes fagócitos têm um aspecto hialino, outros possuem grânulos citoplasmáticos. Os fagócitos dos invertebrados, além de estarem claramente envolvidos na defesa contra microrganismos patogénicos, através da ingestão e morte destes, estão também ao serviço da coagulação e da cicatrização.

A quimiotaxia e a adesão, por constituírem os passos essenciais que antecedem a ocorrência das reacções imunológicas, têm sido estudadas nos fagócitos dos diferentes grupos de invertebrados. O processo de adesão, em muitos destes grupos, parece ser facilitado pela existência de aglutininas presentes no soro que podem envolver as partículas estranhas e funcionar como opsoninas.

Após a ingestão dos microrganismos invasores, com a formação do fagossoma, dá-se a fusão deste com os lisossomas, nos quais existem várias enzimas hidrolíticas como a fosfatase ácida e esterases não específicas, e peroxidases. Sobre o processo de morte desses microrganismos e a sua digestão intracelular pouco se sabe, sendo os dados conhecidos relativos aos moluscos e aos artrópodes. Várias espécies de bactérias foram testadas *in vitro* nestes grupos confirmando-se a sua morte intracelular. Estes processos são, no essencial, comuns aos conhecidos nos vertebrados. No entanto, o sistema peroxidase-H_2O_2-haleto não foi encontrado no pequeno número de artrópodes em que foi procurado (Ratcliffe *et al.*, 1985).

Por outro lado, a descoberta da presença de lisozima, aglutininas e de factores do sistema da profenoloxidase no soro, os quais são, pelo menos parcialmente, produzidos pelos leucócitos, veio realçar a interrelação entre os factores humorais e celulares a este nível (Ratcliffe *et al.*, 1985). Outra forma de morte de parasitas invasivos parece estar relacionada com o sistema da profenoloxidase. Alguns autores atribuem a este sistema, onde a melanina ou os seus precursores intervêm, uma acção microbicida (Ratcliffe *et al.*, 1985).

Além dos fagócitos circulantes, muitos invertebrados possuem os chamados órgãos fagocíticos com células fagocíticas fixas, nas quais podemos incluir os cloragogénios dos anelídeos, e as células reticulares e os podócitos dos moluscos gastrópodes. Estes órgãos foram também identificados nos insectos, nos equinodermes e nos urocordados (Ratcliffe *et al.*, 1985).

2.2. A fagocitose e os fagócitos nos vertebrados

2.2.1. Caracterização dos fagócitos dos vertebrados

Logicamente, a maioria do conhecimento nesta área refere-se aos mamíferos. Por isso, a descrição que se segue utiliza sobretudo dados referentes a esse grupo taxonómico. Sempre que adequado, referem-se dados que têm a ver com peixes, nomeadamente com a truta arco-íris.

Como se disse, a fagocitose é o mecanismo celular de ingestão de matéria particulada ao serviço da defesa contra infecções por agentes como bactérias e parasitas, mais amplamente distribuído ao longo de todos os phyla do reino animal (Van Oss, 1986; Adams & Hamilton, 1992; Greenberg & Silverstein, 1993). Também para os vertebrados, incluindo os peixes, a fagocitose integra a primeira linha de defesa contra agentes infecciosos que tenham ultrapassado as barreiras naturais (Greenberg & Silverstein, 1993).

Por outro lado, qualquer que seja a complexidade dos mecanismos imunológicos postos em jogo no processo de defesa contra agentes infecciosos, a fagocitose é também o último evento após qualquer processo infeccioso, pois cabe aos fagócitos retirar e digerir todos os restos celulares, próprios ou não, de modo a restabelecer a normalidade.

Pelo facto de existirem células que, embora não sendo especializadas nessa função, possuem alguma capacidade fagocítica, como os fibroblastos e células endoteliais nos mamíferos (Douglas & Hassan, 1991) e as células musculares nos peixes (Ferguson, 1975), Rabinowitz chamou fagócitos profissionais aos macrófagos e neutrófilos dos vertebrados.

Os fagócitos profissionais são células cuja função era tida como a de interiorizar partículas, nomeadamente microrganismos invasores, de os matar e de os digerir, repondo a homeostase do sistema. No entanto, esta visão clássica tem vindo a ser modificada, dado que, como veremos, os fagócitos, incluindo os neutrófilos, desempenham importantes funções independentes da fagocitose.

Existem diferenças nas características dos fagócitos dos diferentes grupos de animais, incluindo dentro dos vertebrados (Ratcliffe *et al.*, 1985; Rowley & Ratcliffe, 1988). O grande avanço no conhecimento dos fagócitos em mamíferos, relativamente aos restantes grupos animais, levou à adopção, ou pelo menos à tentativa de adopção, da nomenclatura dos fagócitos de mamíferos para os restantes grupos de vertebrados.

Os vários fagócitos dos diversos vertebrados possuem diferentes características em vários aspectos, incluindo a sua capacidade fagocítica e intervenção na resposta inflamatória e, mesmo nos casos em que é possível estabelecer um certo grau de homologia, estes estão presentes em proporções diferentes.

Os fagócitos profissionais nos mamíferos são os macrófagos e os granulócitos neutrófilos, aos quais Metchnikoff havia chamado macrófagos e micrófagos, respectivamente. Nos peixes, como provavelmente em todo o Reino Animal, os macrófagos, em conjunto com os neutrófilos (ou o granulócito fagocítico equivalente), constituem o principal sistema antimicrobiano.

Se nos peixes a identificação dos macrófagos não tem levantado grande controvérsia, já para os granulócitos tal não acontece (Yui & Yamazaki, 1986; Hine, 1992), o que não é de estranhar dado o elevado número de espécies de peixes (cerca de 21 700), pertencentes a três Classes, ocupando os mais variados ecossistemas (Fänge, 1994).

Os critérios básicos de identificação dos diferentes tipos leucocitários podem ser ontogenéticos, morfológicos e funcionais (Ellis, 1977). O conhecimento sobre as diferentes populações leucocitárias dos peixes tem sido revisto (Ellis, 1977) e com especial atenção ao caso dos granulócitos (Ainsworth, 1992; Hine, 1992).

As inconsistências na nomenclatura e as diferenças nos resultados obtidos no estudo dos granulócitos nos diferentes laboratórios, levou a que as comparações dentro de espécies sejam difíceis e as comparações entre espécies praticamente impossíveis (Ainsworsth, 1992). Este autor refere ainda que muitas das diferenças encontradas se devem à atenção que cada laboratório prestou às condições ambientais e fisiológicas impostas aos animais de experiência, as quais podem alterar, quer a morfologia quer as funções celulares. A estas causas de variação dos resultados, pode acrescentar-se a utilização de técnicas diferentes e a adaptação dos protocolos para cada espécie, as quais muitas vezes não são explicitadas nas publicações respectivas.

Nos peixes, as diferentes classes (Ciclóstomos, Condríctios e Osteíctios) possuem granulócitos com características próprias, que são muitas vezes exclusivas de um só grupo taxonómico, podendo mesmo encontrar-se diferentes granulócitos dentro de um grupo. Apesar dessas diferenças, tem sido constatado que nos peixes, pelo menos um dos tipos de granulócitos presentes é fagocítico (Hine, 1992).

Para além dos neutrófilos, tema que será abordado mais adiante, a identificação nos peixes dos restantes granulócitos, basófilos e eosinófilos têm sido fonte de grande controvérsia, conforme discutido por Ellis (1977). Células com grânulos eosinofílicos têm sido descritas no sangue e em vários tecidos de peixes. Hine (1992), sistematiza os diferentes granulócitos dos peixes cartilagíneos, admitindo a existência de neutrófilos, heterófilos, basófilos e de dois tipos de eosinófilos, os de grânulos homogéneos ("homogenous granule eosinophil" (HGE)) e os que possuem grânulos com um

bastonete axial ("axial rod eosinophil" (ARE)). O mesmo autor refere para os teleósteos que a maioria das espécies possui neutrófilos com características semelhantes aos dos mamíferos e refere que os eosinófilos são do tipo dos HGE anteriormente referidos. Estes leucócitos, quando encontrados extravascularmente são chamados células granulares eosinofílicas ("eosinophilic granule cells" (EGC)) (Hine, 1992). Estas células são encontradas em muitas espécies de peixes em diferentes números e com diferente distribuição pelos tecidos, estando a sua relação com a resposta inflamatória, assim como a sua capacidade fagocítica, ainda em estudo.

Vários tipos de granulócitos foram descritos em diferentes espécies de peixes. Na mucosa intestinal da carpa foram identificados quatro tipos diferentes de granulócitos; no entanto, excluindo os neutrófilos, parece tratar-se de diferentes estados de desenvolvimento da mesma linha celular semelhante aos mastócitos dos mamíferos (Rombout *et al.*, 1989). Os resultados sobre o estudo das EGC do lúcio (*Esox lucius*) apontam no mesmo sentido (Reite, 1996)

Em exsudados peritoneais de tilápia e carpa, Suzuki (1986) descreve células com características semelhantes às das EGC.

A dualidade célula eosinofílica/neutrófilo e o seu significado evolutivo tem sido especulado (Hine & Wain, 1988; Hine, 1992). Na opinião destes autores, células eosinofílicas e neutrófilos seriam importantes como células com capacidade antimicrobiana mas, no caso dos peixes, os granulócitos eosinofílicos seriam pouco eficazes como fagócitos ou mesmo incapazes de fagocitar, o que terá levado à especialização, nas espécies mais evoluídas dos teleósteos, do neutrófilo como granulócito fagocítico.

O já longo esforço dedicado ao estudo do **macrófago**, que, como se disse, começou no fim do século passado com Metchnikoff, tem levado ao estabelecimento progressivo de vários conceitos. Um desses conceitos é o do Sistema Fagocítico Mononuclear.

O conceito subjacente a este Sistema foi proposto por van Furth (van Furth *et al.*, 1972; van Furth, 1992) para substituir outro conceito que foi sendo abandonado, o do Sistema Reticuloendotelial, proposto por Aschoff em 1924 (Douglas & Hassan, 1991). Os macrófagos estão presentes em todos os territórios do organismo dos vertebrados. Como veremos, esses fagócitos residentes derivam de precursores (presentes nos orgãos hematopoiéticos e no sangue - os monócitos), que se diferenciam nesses territórios passando a células maduras, os macrófagos. O conjunto dos macrófagos residentes dos

tecidos e cavidades naturais e seus precursores constituem, no conceito de van Furth, o Sistema Fagocítico Mononuclear.

Os macrófagos são células de vida longa. Nos mamíferos eles podem permanecer nos tecidos e cavidades naturais durante meses (Greenberg & Silverstein, 1993). As funções mais marcantes dos macrófagos na defesa contra agentes infecciosos têm a ver com a fagocitose e com a regulação da resposta imunológica.

Por via da sua distribuição por todo o organismo, o macrófago é o primeiro tipo celular a entrar em contacto com qualquer microrganismo que tenha vencido as barreiras físicas do hospedeiro. Esta função de "sentinela" levou à proposta do termo custócito para os macrófagos residentes dos tecidos (Goerdt *et al.*, 1996). Após fagocitar, o macrófago desencadeia uma série de actividades que podem resultar na morte e digestão do agente infeccioso, como a fusão fagossoma/lisossoma, a explosão respiratória com a produção de radicais de oxigénio e, ainda, a produção de radicais de azoto. Estas actividades antimicrobianas dependem da explosão respiratória e de moléculas armazenadas nos grânulos citoplasmáticos, que são muito menos numerosos que os presentes nos neutrófilos. É de salientar que os mecanismos antimicrobianos dos macrófagos são menos potentes que os dos neutrófilos. Assim, nos mamíferos, moléculas com actividade antimicrobiana como a lactoferrina, mieloperoxidase e defensinas são constituintes dos neutrófilos mas faltam nos macrófagos (Falloon & Gallin, 1988; Ganz *et al.*, 1990; Elsbach & Weiss, 1992; Ogata *et al.*, 1992; Borregaard *et al.*, 1993; Edwards, 1994; Densen *et al.*, 1995).

A fagocitose de agentes microbianos pelo macrófago pode representar, pelo contrário, um passo decisivo no estabelecimento da infecção, quando se trata de um parasita intracelular com capacidade de resistir aos mecanismos antimicrobianos do macrófago e de se multiplicar no ambiente deste fagócito, hostil para outros microrganismos (Hall & Joiner, 1991). São exemplos desta situação, as infecções por micobactérias nos mamíferos e pela renibactéria (*Renibacterium salmoninarum)* nos salmonídeos. O facto de, em geral, os agentes infecciosos intracelulares infectarem os macrófagos, levou ao conceito clássico de que os macrófagos são os únicos fagócitos envolvidos nos mecanismos de defesa contra esses agentes (Adams & Hamilton, 1984). Veremos que este conceito não é já considerado correcto.

Para além da fagocitose, e muitas vezes em paralelo com ela, os macrófagos secretam mediadores químicos que vão desencadear a resposta inflamatória, como os factores quimiotácticos responsáveis pela chamada de novos fagócitos e a activação de sistemas humorais com actividade microbicida, como o sistema complemento, revisto para os mamíferos, entre outros autores, por Allison (1978), Nathan (1987) e Adams &

Hamilton (1992) e para os peixes por Secombes & Fletcher (1992). Como veremos, os macrófagos possuem ainda funções acessórias, ao funcionarem como células apresentadoras de antigénios, que vão participar na defesa imunológica específica.

No estudo dos monócitos/macrófagos no peixe adoptou-se desde sempre a nomenclatura estabelecida para os mamíferos.

Os monócitos dos peixe possuem as seguintes características citoquímicas: fosfatase ácida e PAS positivos, fosfatase alcalina variável com a espécie e quanto à peroxidase foram obtidos resultados variáveis entre espécies e mesmo entre diferentes populações da mesma espécie (Rowley *et al.*,1988). Os macrófagos apresentam uma relação núcleo/citoplasma diminuída e bordos mais irregulares em relação aos monócitos. Rowley *et al.* (1988) chamaram-lhes células fixas, por oposição às circulantes. Na solha (*Pleuronectes platessa*) os macrófagos encontram-se distribuidos pelo rim, baço, timo, coração, mesentério e fluido peritoneal (Ellis,1976). Esta distribuição, veio a verificar-se ser geral para os teleósteos.

Um tipo especial de macrófago que existe em répteis, anfíbios e, sobretudo, em peixes é o **melano-macrófago**. São células com a morfologia geral dos macrófagos, contendo pigmento castanho composto principalmente por melanina, hemosiderina e lipofuscina, em proporções variáveis com a espécie. Na truta arco-íris a melanina é o principal pigmento (Zapata & Cooper, 1990). Estes macrófagos associam-se em agregados nos orgãos hematopoiéticos de muitos peixes, os Centros Melano-Macrofágicos (Herraez & Zapata, 1986 e 1991). Nos salmonídeos, os melano-macrófagos não formam esses centros e encontram-se dispersos pelo rim, baço e fígado (Zapata & Cooper, 1990).

A função exacta deste tipo de macrófago não é ainda conhecida (Secombes & Fletcher, 1992). A presença de restos de eritrócitos sugere que eles participam no processo de degradação dos glóbulos vermelhos envelhecidos (Micale & Perdichizzi, 1990). Tem sido levantada a possibilidade dos melano-macrófagos estarem também envolvidos nos mecanismos de defesa contra infecções (Roberts, 1975; Ellis, 1976) porque a melanina pode intervir na produção de H_2O_2, a qual participa na formação de radicais livres de oxigénio, pela acção da mieloperoxidase (Zapata & Cooper, 1990).

Nos estudos que fazem parte desta dissertação a presença de melano-macrófagos foi raramente detectada, não havendo, por isso, referência nos nossos resultados a este tipo celular.

O **neutrófilo** é um leucócito com abundantes grânulos que, a pH neutro, coram mal pelos corantes ácidos e básicos mostrando assim um citoplasma translúcido, ligeiramente acinzentado. Nos mamíferos, estes granulócitos são células com o núcleo

polimórfico e daí a designação também utilizada de neutrófilos polimorfonucleares (PMNs). Nos peixes este termo é inadequado para os neutrófilos de um elevado número de espécies, dado que muitas possuem granulócitos com capacidade fagocítica que, à semelhança do chamados mononucleares, também possuem um núcleo não polimórfico, mas que nem por isso podem ser englobados nos leucócitos mononucleares. São exemplos desta situação os neutrófilos da solha, *Pleuronectes platessa* (Ellis, 1976; Ferguson, 1976), da tilápia, *Oriochromis niloticus* (Suzuki, 1986) e da carpa, *Cyprinus carpio* (Suzuki, 1986).

Granulócitos de aves e répteis e também os neutrófilos de coelho possuem grânulos com ponto isoeléctrico alto, que coram ligeiramente de vermelho em meio alcalino. Por isso, estes têm sido chamados heterófilos (Ellis, 1977; Hine, 1992), nomenclatura que muitos autores adoptaram para os neutrófilos dos peixes.

Os tipos de grânulos dos neutrófilos ou granulócitos homólogos, e o conteúdo desses grânulos, não são necessariamente iguais nos diferentes grupos de vertebrados. Estão descritos nos neutrófilos dos mamíferos três tipos de grânulos (primários ou azurófilos, secundários ou específicos e grânulos de gelatinase), além das vesículas secretoras ricas em fosfatase alcalina (Borregaard *et al.*, 1993).

Os grânulos primários ou azurófilos são também chamados grânulos peroxidase positivos, pois com técnicas histoquímicas estes grânulos apresentam forte actividade peroxidásica. Nestes grânulos existe uma enorme variedade de proteínas com actividade antimicrobiana, e muitas delas, ao contrário da mieloperoxidase, actuam de forma independente do oxigénio (Elsbach & Weiss, 1992; Edwards, 1994).

A mieloperoxidase é uma enzima com reconhecida importância nos mamíferos, pois participa numa cascata de reacções de oxidação/redução, na qual são produzidos vários intermediários reactivos do oxigénio (Edwards, 1994; Densen *et al.*, 1995).

A explosão respiratória que acompanha a fagocitose, consiste numa modificação do tipo e magnitude da respiração, da qual resulta a redução do oxigénio pelo sistema transmembranar da NADPH oxidase, com a formação de vários produtos tóxicos. Esta explosão respiratória pode ser desencadeada pelas partículas fagocitadas (bactérias, restos celulares, etc.) e por factores solúveis (peptídeos, lectinas, factores do complemento, ionóforos de cálcio, leucotrienos, endotoxinas, detergentes e outros). Este tema foi revisto para os mamíferos por Klebanoff (1988 e 1992), por Densen *et al.* (1995) e por Clark, (1990) e para os teleósteos por Chung & Secombes (1987b).

O primeiro intermediário activo formado na explosão respiratória é o anião superóxido (O_2^-), o qual sofre uma dismutação, quer de forma espontânea, quer pela acção da enzima superóxido dismutase (SOD), formando oxigénio e peróxido de hidrogénio (H_2O_2). O oxigénio singleto ($^1O_2$) é também formado. A partir do H_2O_2, com

a intervenção da mieloperoxidase e de um haleto (geralmente o Cl^-) forma-se um hipoaleto (neste caso o HOCl). Esta reacção ocorre também com o I^- e com o Br^-. Estes hipoaletos são moléculas com uma elevada actividade antimicrobiana (Klebanoff, 1988).

A peroxidase está também presente em vários tipos de granulócitos de peixes, dependendo da espécie considerada (Ainsworth, 1992). Os neutrófilos de todas as espécies pertencentes à família dos salmonídeos são peroxidase-positivos, enquanto os granulócitos eosinofílicos são peroxidase-negativos (Hine & Wain, 1988).

Têm sido descritos grânulos com características peculiares nos neutrófilos de algumas espécies de peixes (Fujii, 1981; Kurata *et al.*, 1995), mas apenas em alguns teleósteos modernos, os acantopterígios, foram descritos neutrófilos contendo mais que um tipo de grânulo (Suzuki, 1986; Meseguer *et al.*, 1993).

Ao contrário dos macrófagos, os neutrófilos são células de vida curta. No Homem duram cerca de 24 a 48 horas (Bainton, 1991). Uma característica fundamental dos neutrófilos é possuírem grande número de grânulos citoplasmáticos, cujo conteúdo tem uma potente actividade microbicida e citotóxica. Como foi referido, as moléculas do neutrófilo envolvidas em mecanismos antimicrobianos são mais diversificadas que as dos macrófagos. Assim, como vimos, nos mamíferos, moléculas como a lactoferrina, mieloperoxidase e defensinas são constituintes dos neutrófilos mas faltam nos macrófagos (Falloon & Gallin, 1988; Ganz *et al.*, 1990; Elsbach & Weiss, 1992; Ogata *et al.*, 1992; Borregaard *et al.*, 1993; Edwards, 1994; Densen *et al.*, 1995). Como será discutido mais adiante, é importante salientar que o fagócito que primeiro encontra microrganismos invasores -o macrófago, seja comparativamente pobre em mecanismos moleculares antimicrobianos, enquanto o neutrófilo, mais rico nessas moléculas, não se encontra distribuido pelos tecidos em condições normais.

Os neutrófilos são capazes de fagocitar microrganismos segundo processos comuns aos do macrófago. Após fagocitose e inclusão do agente fagocitado num fagossoma, o neutrófilo é capaz de desgranular, de forma a pôr em contacto com o agente fagocitado o seu conteúdo granular. Classicamente, e tendo em conta resultados obtidos em mamíferos, os neutrófilos são considerados como os fagócitos com a tarefa de fagocitar e eliminar os parasitas extracelulares (Campbell, 1990; Klebanoff, 1992; Edwards, 1994). Como será abordado no ponto 2.2.10, esta visão tem vindo a ser modificada.

É reconhecida uma elevada citotoxicidade para os neutrófilos de mamíferos (Cochrane, 1968; Edelson & Cohn, 1973; Henson & Johnston, 1987; Klebanoff, 1991; Klebanoff, 1992). Essa citotoxicidade deriva do facto de os mecanismos antimicrobianos envolverem moléculas com actividade antibiológica geral, que afectam, portanto, não só as entidades biológicas que são agentes infecciosos, mas também, as células do próprio hospedeiro. Em relação aos granulócitos dos peixes, e dado que eles utilizam

mecanismos idênticos aos dos mamíferos, também há citotoxicidade associada à sua actividade antimicrobiana (Fänge, 1994).

2.2.2. Caracterização dos fagócitos dos salmonídeos

A informação sobre os leucócitos dos salmonídeos encontra-se incluida na bibliografia dedicada ao estudo dos leucócitos dos peixes em geral (Ellis, 1977; MacArthur & Fletcher, 1985; Rowley *et al.*, 1988; Ainsworth, 1992; Hine, 1992).

Nos salmonídeos, como noutros grupos taxonómicos, são identificados dois tipos de leucócitos fagocíticos, os macrófagos e os granulócitos neutrófilos.

Como nos mamíferos, os **macrófagos dos salmonídeos** são células que derivam dos monócitos e são residentes dos tecidos e cavidades naturais.

Foi comprovada a existência de monócitos no sangue das trutas, (Yûki, 1957; Klontz, 1972; Ellis, 1977; Cannon *et al.*, 1980), embora em alguns estudos estes não tenham sido encontrados (McCarthy *et al.*, 1973)

Os macrófagos dos salmonídeos são células mononucleares, geralmente com o núcleo em forma de rim, com o citoplasma azul e agranular, quando corado pelos corantes de tipo Romanowsky. Estruturalmente semelhantes aos macrófagos de outras espécies, com retículo endoplasmático rugoso, um complexo de Golgi activo, centríolos geralmente situados na concavidade do núcleo e vesículas granulares derivadas do complexo de Golgi. São encontrados com frequência heterofagossomas, os quais atestam a característica fagocítica destes leucócitos e também vesículas pinocíticas. Possuem extensões protoplásmicas, particularmente quando se encontram activados (Ellis, 1977; MacArthur & Fletcher, 1985). À semelhança do que se conhece para os mamíferos, também nos peixes existem dificuldades na diferenciação entre os macrófagos pequenos e os linfócitos grandes (Ellis, 1977).

Em relação aos testes enzimáticos os macrófagos dos salmonídeos são positivos para a esterase, a fosfatase ácida e a 5'-nucleotidase, e negativos para a fosfatase alcalina e para a peroxidase (Zelikoff & Enane, 1992; Enane *et al.*, 1993; Press *et al.*, 1994; Brattgjerd *et al.*, 1995).

Os **neutrófilos** são os granulócitos fagocíticos dos salmonídeos. São os fagócitos cujo número mais rapidamente aumenta nos locais lesados ou infectados (Finn & Nielson, 1971). Possuem numerosos grânulos peroxidase-positivos (Yûki, 1957; Hine & Wain, 1988) e são também fosfatase ácida e fosfatase alcalina-positivos (Hine *et al.*, 1987)

Trabalhos *in vitro* mostraram que, também nos salmonídeos, os neutrófilos são capazes de migrar, fagocitar e produzir radicais livres de oxigénio, sob a acção de *Aeromonas salmonicida* e que estes mecanismos são incrementados após opsonização (Lamas & Ellis, 1994a). Também se verificou que os neutrófilos têm a capacidade de promover a fusão fagossoma/lisossoma, e assim matar e degradar bactérias patogénicas; no entanto, a sua fraca capacidade fagocítica *in vitro*, o aparecimento em microscopia electrónica de neutrofilos sem conteúdo granular e a morte de alguns destes neutrófilos após fagocitarem bactérias, deixou ainda dúvidas sobre qual o papel dos neutrófilos na resposta à infecção (Lamas & Ellis, 1994b).

No sangue da truta arco-íris têm sido encontradas percentagens de neutrófilos, relativamente ao total de leucócitos que variam entre 4.9 % e os 9.8% (Finn & Nielson,1971; Eiras *et al.*, 1983; Alvarez *et al.*, 1988).

Embora seja conhecido que os neutrófilos estão presentes no baço e no rim dos salmonídeos, não havia até à obtenção dos nossos resultados qualquer referência a valores para neutrófilos fora da circulação. Nos vários trabalhos que descrevem a população leucocitária da cavidade peritoneal em repouso da truta arco-íris, tanto Zelikoff & Enane, (1992) como Enane *et al.* (1993) referem a ausência de neutrófilos.

Vários granulócitos para além dos neutrófilos, têm sido descritos nos salmonídeos como é o caso dos eosinófilos; no entanto, a existência de basófilos encontra-se ainda em discussão (Ellis, 1977; Ainsworth, 1992; Hine, 1992).

No que respeita aos granulócitos eosinofílicos ("Eosinophilic Granule Cells", EGC) dos salmonídeos, sabe-se que são frequentes em vários tecidos, incluindo brânquias e parede intestinal (Ellis, 1977; Kent *et al.*, 1993; Powell *et al.*, 1993). Estudos *in virto* verificaram, a partir dos vinte dias de incubação de culturas primárias de brânquia de truta arco-íris infectadas com *R. salmoninarum*, o aparecimento de vários tipos de células leucocitárias, incluindo EGC, as quais se desenvolem em relação com o tecido conjuntivo presente (Flaño, 1995 e Flaño *et al.*, 1996b). Este grupo fez uma detalhada caracterização da maturação e desgranulação deste tipo celular, mostrando a sua semelhança com os mastócitos dos mamíferos.

Também é conhecido que na brânquia da truta arco-íris as EGC estão presentes no tecido do arco branquial, associadas ao endotélio dos capilares ou ainda livres no lumen capilar. Não foi, no entanto, encontrada evidência da relação deste tipo celular com a infecção por bactérias nem com o "stress" osmótico, não existindo normalmente eosinófilos ou EGC na circulação sanguínea naquela espécie (Powell *et al.*, 1990).

2.2.3. Origem e produção dos fagócitos

Os macrófagos e granulócitos são leucócitos que resultam de uma complexa maturação que, conforme o tipo de organização biológica a que pertencem, se desenrola em diferentes compartimentos, mas que genericamente se pode assumir como ocorrendo nos órgãos hematopoiéticos, passando depois à circulação sanguínea e finalmente aos tecidos. Para o caso dos Salmonídeos, a hematopoiese ocorre na porção anterior do rim (pronefros) (Ainsworth, 1992; Zapata & Cooper, 1990) e, em menor escala, no baço (Ainsworth, 1992).

Todos os leucócitos provêm de uma linha celular pluripotencial presente nos órgãos hematopoiéticos. A partir destas células, por divisões sucessivas e diferenciação vão surgir várias linhagens leucocitárias. Nos mamíferos, onde este ponto tem sido mais estudado, os macrófagos e os neutrófilos derivam de uma célula progenitora comum (a célula "stem" mielo-monocítica), que não dá origem aos outros dois tipos de granulócitos, os eosinófilos e basófilos (Metcalf, 1989). Isto aponta para a proximidade que existe entre os neutrófilos e macrófagos, ponto que será debatido mais adiante. Não se sabe se nos peixes a origem dos dois fagócitos profissionais também é comum. Como vimos, no caso dos macrófagos há uma célula intermediária entre o macrófago maduro dos tecidos e a célula progenitora dos orgãos hematopoiéticos: o monócito.

2.2.4. Mobilização dos fagócitos

Após atingirem o estado de monócito e neutrófilo maduro, os dois fagócitos acumulam-se nos orgãos hematopoiéticos e no sangue, onde permanecem durante algum tempo como "pools" de reserva. Desses "pools" podem ser mobilizados para os tecidos ou cavidades corporais, em resposta à presença de factores quimiotácticos do hospedeiro ou de microrganismos. Além disso, monócitos das reservas são periodicamente recrutados para substituição dos macrófagos que morrem nos vários territórios do organismo (van Furth, 1992).

Nos locais inflamatórios verifica-se uma vasodilatação que facilita a passagem dos neutrófilos e monócitos para fora da circulação. Este processo, mediado pela expressão de receptores específicos, inicia-se pela adesão do neutrófilo e do monócito ao endotélio dos capilares após a sua marginação, à qual se segue a chamada diapedese, ou seja a passagem dos fagócitos através do endotélio dos vasos sanguíneos (Huang & Silverstein, 1992).

Nos tecidos, os monócitos diferenciam-se até à fase de macrófago maduro, constituindo o macrófago residente que, como vimos, está presente em números significativos em todos os territórios do organismo dos vertebrados (van Furth, 1992; Goerdt *et al.*, 1996). Nos mamíferos, muito poucos neutrófilos são encontrados, em

condições normais, nos tecidos e cavidades naturais (Cohn, 1962a,b; de Bakker, 1983; Lehrer *et al.*, 1988; Haslett *et al.*, 1989; Silva *et al.*, 1989; van Furth, 1992; Edwards, 1994; Pestana, 1994; Densen *et al.*, 1995; Kubicka *et al.*, 1996). No que se refere à truta arco-íris, não há informação quantitativa sobre a distribuição dos macrófagos e neutrófilos pelos tecidos e cavidades naturais.

2.2.5. Opsonização

O processo de fagocitose é mediado por receptores, entre os quais os receptores para factores do sistema do complemento e para a porção Fc dos anticorpos (Adams & Hamilton, 1992; Wright, 1992; Greenberg & Silverstein, 1993). A presença destes receptores nos fagócitos dos salmonídeos também foi evidenciada (Sakai, 1984). Esta mediação da ingestão de microrganismos pelos fagócitos durante a resposta inflamatória através da acção daqueles factores do soro, a que se chama opsoninas, é bem conhecida em mamíferos (Wright, 1992). Nos peixes o efeito da opsonização, quer pela activação do sistema complemento, quer por anticorpos, também é conhecido (Michel *et al.*, 1990; Engstad & Robertsen, 1993; Lamas & Ellis, 1994a), havendo evidências funcionais para a sua existência, bem como para a existência de receptores para componentes do complemento e para o fragmento Fc dos anticorpos na superfície dos fagócitos (Sakai, 1992; Secombes & Fletcher, 1992; Hollebecq *et al.*, 1995).

2.2.6. Os processos intra-fagocíticos de combate a agentes infecciosos

A fagocitose de agentes infecciosos leva à sua inclusão em vesículas revestidas por membrana derivada da membrana plasmática -os fagossomas. Este processo permite ao fagócito pôr no seu interior a partícula fagocítica, retirando-a do meio extracelular e simultaneamente mantendo-a separada dos restantes componentes citoplasmáticos de modo a poder fazer actuar sobre ela as suas moléculas microbicidas num ambiente restrito e isolado. Isto reduz os efeitos nocivos para o hospedeiro da utilização de moléculas altamente citotóxicas.

Após a internalização dos agentes infecciosos pelos fagócitos desenvolve-se uma série de acontecimentos que, caso as defesas do hospedeiro superem as do microrganismo invasor, levam à morte e degradação deste. Um desses acontecimentos é a fusão fagossoma/lisossoma.

Os lisossomas são componentes citoplasmáticos granulares dos fagócitos (os grânulos) que contêm uma enorme variedade de moléculas microbicidas e degradativas. Imediatamente após a formação do fagossoma, ou mesmo durante a sua formação, inicia-se a fusão dos lisossomas com os fagossomas, formando-se os fagolisossomas, no

interior dos quais as enzimas hidrolíticas são capazes de destruir e digerir a maioria das bactérias.

O primeiro fenómeno intra-fagolisossomal é a rápida variação de pH. Primeiro o fagolisossoma torna-se alcalino por um curto espaço de tempo, ao qual se segue o abaixamento persistente do pH que deriva do facto de muitas das moléculas então aí formadas serem acídicas (Kaufmann, 1993). Os mecanismos de formação de radicais activos de oxigénio ocorrem nos fagolisossomas, tanto em mamíferos como em peixes (Chung & Secombes, 1987b; Klebanoff, 1992; Sharp & Secombes, 1992; Smith, 1994) e de azoto (Ding *et al.*, 1988; Klebanoff, 1992; Denis, 1994; Wang *et al.*, 1995).

Por seu lado, alguns microrganismos possuem entre os vários factores de virulência, a resistência ou escape aos mecanismos acima referidos. Consoante se trata de parasitas intra ou extracelulares, estes promovem ou inibem a sua fagocitose. (Hall & Joiner, 1991; Olivier *et al.*, 1992; Gotschlich, 1993). Depois ou durante a fagocitose alguns microrganismos promovem a sua saída do fagossoma ou do fagolisossoma para o citoplasma, ficando assim livres da acção do conteúdo citotóxico granular. Os que permanecem intra-vacuolares podem desencadear mecanismos de resistência às moléculas antimicrobianas do hospedeiro ou produzir enzimas que inactivam essas mesmas moléculas. Está descrito que a *Renibacterium salmoninarum* consegue escapar do fagossoma para o citoplasma (Gutenberger *et al.*, 1997). Várias espécies de *Aeromonas* possuem a camada A na sua parede externa e produzem enzimas extracelulares e toxinas, o que dificulta a sua fagocitose. Após serem fagocitadas, estas bactérias produzem várias enzimas extremamente activas como proteases, hemolisinas, e uma potente catalase que interfere com a explosão respiratória (Olivier *et al.*, 1992; Sharp & Secombes, 1993).

2.2.7. Regulação da actividade dos fagócitos

Ao considerarem-se os mecanismos de defesa dos peixes contra agentes infecciosos, é necessário ter em atenção alguns aspectos deste grupo de vertebrados. Trata-se de animais poiquilotérmicos, nos quais as funções celulares são susceptíveis de ser alteradas pelas variações da temperatura ambiente (Bly & Clem, 1992; Suzuki & Iida, 1992; Vallejo *et al.*, 1992; Zapata *et al.*, 1992; Hardie *et al.*, 1994; Pxytycz & Józkowicz, 1994).

Em relação à actividade dos fagócitos é importante lembrar que ela depende do grau de estimulação em que se encontram. Muito adequadamente, os macrófagos residentes dos tecidos e cavidades naturais dos vertebrados estão num estado quiescente, dado que, em geral, não estão envolvidos em actividades de defesa ou reparação, mas

antes aparecem como sentinelas em alerta permanente. Já os fagócitos envolvidos na resposta inflamatória que acompanha a infecção são sempre fagócitos estimulados.

Nos anos 60, o grupo de Mackaness (1969), trabalhando em mamíferos, definiu como "activado" o macrófago estimulado capaz de matar células tumorais e microrganismos patogénicos. Este conceito foi posteriormente alargado a outras funções celulares, típicas deste leucócito quando estimulado.

O estado de estimulação dos macrófagos pode ser progressivamente ampliado, desde a quiescência do macrófago residente até à estimulação máxima. Vários graus de estimulação são reconhecidos para os fagócitos como: residentes, responsivos, sensibilizados e activados (Adams & Hamilton, 1984).

Vários factores são capazes de estimular os macrófagos (intermediários inflamatórios e citoquinas produzidas pelo hospedeiro, ou substâncias produzidas por microrganismos). Os macrófagos dos peixes, como os dos mamíferos, são susceptíveis de serem activados, quer *in vivo*, quer *in vitro* (Chung & Secombes, 1987a; Secombes, 1987; Chung *et al.*, 1988), distendendo-se, aumentando a sua aderência a superfícies plásticas ou de vidro, fagocitando mais e mais rapidamente, desencadeando a explosão respiratória com produção de anião superóxido e de outros radicais de oxigénio, e aumentando a sua capacidade bactericida e larvicida (Secombes & Fletcher, 1992).

O estudo da estimulação do neutrófilo é muito mais recente (Steinbeck & Roth, 1989; Becker, 1990). A resposta dos neutrófilos a factores estimuladores ou activadores que sobre eles actuam é idêntica à dos macrófagos e muitas vezes complementar e recíproca, com a inter-estimulação destes dois fagócitos. Na verdade, sabe-se que, nos mamíferos, em situações inflamatórias, cada um dos fagócitos é capaz de secretar citoquinas com actividade estimuladora sobre o outro (ver adiante Secção "Interacção entre macrófagos e neutrófilos").

A activação dos fagócitos dos salmonídeos tem sido estudada particularmente em macrófagos, quer *in vivo*, quer *in vitro*.

Os fagócitos dos peixes, incluindo os salmonídeos, podem ser activados tanto por moléculas biológicas como o lipopolissacarídeo da parede das bactérias Gram-negativas (LPS) (Secombes, 1994; Solem *et al.*, 1995), muramil-dipeptídeo (MDP) (Kodama *et al.*, 1993), acetato de forbol miristato (PMA), IFNγ, TNFα, Zymozan, ou por moléculas sintéticas (f-MLP) ou ainda adjuvantes, microrganismos vivos ou mortos (Secombes, 1994), e mesmo células autólogas (Secombes, 1986).

A activação dos fagócitos tem sido observada em vários salmonídeos por vários autores, avaliando diferentes aspectos da activação. Assim, do ponto de vista bioquímico, aumentam a proteína celular e a fosfatase ácida (Enane *et al.*, 1993), e diminui a 5'-nucleotidase (Enane *et al.*, 1993; Hepkema & Secombes, 1994). Do ponto

de vista funcional, aumentam o volume celular, o espalhamento e a aderência ao vidro e ao plástico (Zelikoff & Enane, 1992), a quimiotaxia (Kodama *et al.*, 1993; Weeks-Perkins & Ellis, 1995), a capacidade fagocítica (Kodama *et al.*, 1993; Secombes, 1994), a explosão respiratória, avaliada quer pela quimioluminescência (Yamada *et al.*, 1988; Bayne & Levy, 1991; Sakai *et al.*, 1995a), quer pela produção de radicais livres de oxigénio (O_2^-, H_2O_2, $HO^\cdot$) (Chung & Secombes, 1987b; Bayne & Levy, 1991; Sakai *et al.*, 1995b; Solem *et al.*, 1995).

Como foi referido acima, a actividade dos fagócitos dos peixes é altamente dependente da temperatura da água em que vivem. Temperaturas fora da zona de valores a que o peixe está aclimatado (quer abaixo, quer acima) em geral inibem as funções dos fagócitos; uma descida rápida da temperatura reduz a magnitude da explosão respiratória e da fagocitose e actividade antimicrobiana dos fagócitos da truta arco-íris(Secombes & Fletcher, 1992; Hardie *et al.*, 1994). Descidas lentas da temperatura podem permitir a aclimatação dos fagócitos a essas temperaturas com reduzida diminuição das suas funções (Secombes & Fletcher, 1992).

Como acontece a outros níveis de organização biológica, incluindo no Homem, situações de "stress", de carência alimentar e de doença, podem diminuir a actividade do sistema imunológico dos peixes, nomeadamente dos seus fagócitos (Ellis, 1981; Secombes & Fletcher, 1992). A poluição das águas é, também, um factor com efeitos supressores sobre a actividade dos fagócitos dos peixes (Secombes & Fletcher, 1992).

2.2.8. Actividades acessórias dos fagócitos

Os macrófagos dos peixes possuem funções acessórias importantes, como apresentação de antigénios e iniciação de respostas inflamatórias (Secombes & Fletcher, 1992; Vallejo *et al.*, 1990). No entanto, conforme revisto recentemente por Secombes *et al.* (1996), ainda se sabe pouco sobre os aspectos moleculares dessas actividades macrofágicas.

No caso dos neutrófilos, e conforme será discutido na secção seguinte, só recentemente se começou a valorizar as suas funções acessórias, isto é, não fagocíticas.

2.2.9. A inflamação

A inflamação no Homem é um fenómeno que ao longo dos anos foi interpretado de diferentes maneiras. Nos finais do Século XIX, a maioria dos enfermos sujeitos a qualquer intervenção cirúrgica apresentava uma inexorável sequência de fenómenos (rubor, inchaço, acumulação de pus, dor e febre) constituindo estes sinais a inflamação que acompanhava a infecção.

Simultaneamente, o pus era considerado como a tradução fisiológica da capacidade defensiva do organismo, necessário ao processo curativo, mas, nem para a inflamação nem para a infecção, havia uma explicação concludente.

Após ter conhecimento dos trabalhos de Pasteur, Lister percebe a origem microbiana de tais fenómenos e aplica ácido fénico sobre os ferimentos ou em nebulizações durante as suas intervenções cirúrgicas, com o fim de reduzir os riscos de infecção, criando assim o conceito de assépsia.

Metchnikoff nos seus estudos sobre a fagocitose, demonstra que a inflamação era de facto um mecanismo evolutivo destinado a proteger o hospedeiro, passando então a falar-se de resposta inflamatória como componente dos mecanismos de defesa contra a infecção, na qual os fagócitos intervêm (Silverstein, 1993).

A inflamação é hoje reconhecida como uma resposta protectora do hospedeiro, não específica relativamente ao agente inflamatório, onde intervêm vários leucócitos e factores humorais, nomeadamente factores do complemento e outras proteínas séricas, bem como várias citoquinas como o TNF α, o IFN γ, a IL-1, IL-6, etc. (Bonta *et al.*, 1991; Suzuki & Iida, 1992; Secombes & Fletcher, 1992; Smith, 1994; Male, 1996).

A resposta inflamatória à infecção é um mecanismo que o hospedeiro desencadeia com o objectivo de ajudar a combater essa mesma infecção. No entanto, quando esta resposta é excessiva pode tornar-se ela própria factor de doença como consequência da patologia associada à actividade citotóxica de vários componentes da resposta inflamatória. Um exemplo deste fenómeno é a tuberculose pulmonar humana em que a permanente chamada e consequente acumulação de neutrófilos nos tecidos infectados leva à necrose do tecido pulmonar.

2.2.10. Interacção entre macrófagos e neutrófilos

Resultados recentes obtidos em mamíferos, nomeadamente neste laboratório (Silva *et al.*, 1989), apontam para uma visão mais ampla e integrada das funções dos dois fagócitos profissionais nos mecanismos de defesa contra infecções. Essa visão ultrapassa o conceito inicial de que os macrófagos seriam os fagócitos envolvidos na eliminação dos parasitas intracelulares enquanto os neutrófilos só participariam no combate aos parasitas extracelulares, e de que essas participações seriam só por intermédio da fagocitose, morte e eliminação dos agentes infectantes. Os dados recentes que vieram originar a necessidade de rever aquele conceito clássico têm a ver, inclusivamente, com aspectos celulares e moleculares da interacção e cooperação entre os dois fagócitos nas infecções em geral, que seguidamente serão revistos.

O primeiro dado com relevância para o ponto em discussão foi a observação da ocorrência de uma neutrofilia persistente e significativa na cavidade peritoneal de

ratinhos infectados intra-peritonealmente com micobactérias, agentes infecciosos que se multiplicam dentro de macrófagos (Silva *et al.*, 1989). Esta resposta neutrofílica apresentava aspectos interessantes: i- A presença de neutrófilos num foco infeccioso em que as bactérias eram intra-macrofágicas e, portanto, sem a possibilidade de os neutrófilos fagocitarem o agente infeccioso; esta observação apontava para a participação dos neutrófilos no processo infeccioso por uma via que não a clássica de fagocitose e eliminação directa do agente infeccioso. ii- A fagocitose de neutrófilos pelos macrófagos. Com base nestas e outras observações posteriores (Appelberg, 1992a,b,c; Appelberg *et al.*, 1994; Pestana, 1994; Sousa, 1994), foi proposto que os neutrófilos participariam nos mecanismos de defesa contra infecções intra-macrofágicas através de um mecanismo alternativo do classicamente atribuído a este fagócito: a interacção nos focos infecciosos entre os dois fagócitos com a transferência para o macrófago infectado de componentes do neutrófilo; essa transferência amplificaria a reduzida capacidade antimicrobiana do macrófago pelo fornecimento de componentes neutrofílicos que faltam ao macrófago (Silva *et al.*, 1989). Como dissemos, o neutrófilo é mais rico em moléculas com actividade antimicrobiana que o macrófago, que não produz, por exemplo, lactoferrina, mieloperoxidase e defensinas (Falloon & Gallin, 1988; Ganz *et al.*, 1990; Elsbach & Weiss, 1992; Ogata *et al.*, 1992; Borregaard *et al.*, 1993; Edwards, 1994; Densen *et al.*, 1995). É sabido que o macrófago é capaz de captar e utilizar em actividades antimicrobianas pelo menos algumas destas moléculas (Heifets *et al.*, 1980; Nogueira *et al.*, 1982; Shellito *et al.*, 1987; Leung & Goren, 1989; Maródi *et al.*, 1998) e foi verificado neste laboratório que, *in vitro*, o fornecimento de componentes neutrofílicos a macrófagos infectados com micobactérias (Silva *et al.*, 1989), ou toxoplasmas (Sousa, 1994) ampliava a actividade antimicrobiana dos macrófagos.

Há evidência para uma outra modalidade de cooperação entre neutrófilos e macrófagos. Como vimos acima, o macrófago é capaz de captar, e utilizar nos mecanismos antimicrobianos, a mieloperoxidase. Lincoln *et al.* (1995) mostraram que essa molécula pode, adicionalmente, activar o macrófago através da sua ligação a receptores da superfície do macrófago. Curiosamente, a activação do macrófago por este mecanismo parece ocorrer mesmo quando está eliminada a actividade enzimática da peroxidase (Lefkowitz *et al.*, 1997), sugerindo que a activação ocorre por simples ocupação dos receptores. Estes receptores macrofágicos para a peroxidase neutrofílica são os receptores para a manose (Shepherd & Hoidal, 1990; Lefkowitz *et al.*, 1997).

Por outro lado, começou a perceber-se recentemente que o neutrófilo não é uma célula terminal de reduzida actividade biossintética, unicamente envolvida na fagocitose e eliminação de agentes infecciosos, conforme a visão clássica da sua fisiologia. Pelo contrário, resultados obtidos em mamíferos mostram que este fagócito é capaz de produzir e secretar várias citoquinas, e muitas dessas moléculas actuam sobre o outro fagócito, o macrófago. Teremos, assim, mais uma modalidade de cooperação

macrófago/neutrófilo. A produção de citoquinas pelos neutrófilos de mamíferos, revista extensivamente por Lloyd & Oppenheim (1992), Cassatella (1996) e Romani *et al.* (1996), inclui a produção de IL-1, TGFβ, IL-6, TNFα, MIP-1a, MIP-1b, GM-CSF, M-CSF, IL-8, IL-10 e IL-12 (Lloyd & Oppenheim, 1992; Cassatella, 1995; Cassatella *et al.*, 1995). Estas citoquinas,, como Lloyd & Oppenheim (1992) referem, são factores não envolvidos na função efectora (ou eferente) do neutrófilo, mas antes, moléculas que intervêm na chamada fase aferente da resposta imunológica. Muitas dessas citoquinas participam na modulação de uma resposta imunológica protectora por interacções com outras células do sistema imunológico, incluindo macrófagos. Por exemplo, a IL-1 é uma citoquina estimuladora que induz a síntese e a libertação de outras citoquinas (IL-6, IL-8 e GM-CSF), de factores da fase aguda e estimuladora de linfócitos B e T. Tem uma actividade "feedback" positiva, para a produção de mais IL-1 e, simultaneamente, chama mais neutrófilos, macrófagos e linfócitos (Cassatella, 1995).

Mostrou-se que nos mamíferos os neutrófilos circulantes produzem TNFα, quando estimulados com LPS, GM-CSF ou *Candida albicans* (Romani *et al.*, 1996). O TNFα, à semelhança da IL-1 e da IL-6, é uma citoquina pró-inflamatória e tem uma actividade concertada com a IL-1 na modulação da resposta à infecção microbiana, estando implicado nas lesões tecidulares que acompanham a inflamação (Chaplin & Hogquist, 1992).

Muitas das citoquinas referidas produzidas por neutrófilos (IL-1, IL-6, G-CSF e GM-CSF), fazem aumentar o "pool" de progenitores do neutrófilo e simultaneamente estimulam as funções dos neutrófilos maduros, nomeadamente quanto à fagocitose e geração de radicais livres de oxigénio (Lloyd & Oppenheim, 1992). Também se mostrou que neutrófilos estimulados com GM-CSF sintetizam e libertam M-CSF. Os neutrófilos chegados inicialmente ao local inflamatório vão, assim, ter uma acção estimuladora para a produção medular de monócitos e de activação sobre os monócitos que posteriormente forem chegando ao local inflamatório. É interessante, no presente contexto, referir que monócitos e macrófagos produzem factores quimiotácticos e activadores para neutrófilos (Lehrer *et al.*, 1988; Appelberg, 1992a,b,c; Musso *et al.*, 1998) enquanto, por outro lado, neutrófilos secretam quimoquinas para monócitos/macrófagos (Taub *et al.*, 1996). Isto significa que cada um dos dois fagócitos profissionais têm a capacidade de atrair aos locais inflamatórios/infecciosos o outro fagócito.

A acção imunomoduladora do neutrófilo mostrou-se ainda mais complexa quando se verificou que citoquinas com características supressoras, como a IL-10, também foram detectadas nos neutrófilos (Nill *et al.*, 1995; Romani *et al.*, 1996).

Uma vez que o neutrófilo é o leucócito com maior taxa de produção nos mamíferos (1.0×10^{11} neutrófilos produzidos por dia num Homem adulto normal), e provavelmente também em muitos outros grupos animais, incluindo os peixes, e

constituindo a maioria das células infiltradas nos locais inflamatórios, então a produção de uma citoquina por neutrófilos, mesmo em pequenas quantidades por célula, terá um impacto biológico considerável (Lloyd & Oppenheim, 1992). Isto, em conjunto com a possibilidade de o tempo de semi-vida dos neutrófilos poder ser aumentado em situações inflamatórias (Lee *et al.*, 1993), aumenta a possibilidade dos neutrófilos terem uma acção moduladora significativa.

Nos peixes, vários trabalhos têm demonstrado actividades imunológicas de factores tipo citoquinas equivalentes às que são conhecidas nos mamíferos, como MIF, MAF, IL-1, IL-2, Interferãos, TNF, TGF ou CSF. Além disso, têm sido observadas reactividades cruzadas entre sobrenadantes de leucócitos de peixes e células de mamíferos, bem como entre citoquinas de mamíferos e células de peixes (Secombes, 1991; Secombes *et al.*, 1996).

No entanto, como Secombes e colaboradores (1996) referem como conclusão no seu artigo de revisão, embora seja evidente que factores com bioactividade de citoquinas tenham um papel importante na resposta leucocitária em peixes, a falta de reagentes purificados de peixe torna difícil obter conclusões sobre as características moleculares destes factores e sobre a sua origem. Apenas um autor (Kemenade *et al.*, 1995) refere a produção de citoquinas pelos neutrófilos de carpa, concretamente a IL-1, mas, como utilizou nesse estudo populações não purificadas de neutrófilos (devido à contaminação com macrófagos), não pôde atribuir inequivocamente a origem daquela citoquina a esses fagócitos.

Em conclusão, a ocorrência nos mamíferos de dois tipos de fagócitos com participação nos mecanismos de defesa contra a infecção é acompanhada pela ocorrência de várias modalidades de interacção e cooperação entre esses dois fagócitos profissionais. Este aspecto muito interessante da participação dos dois fagócitos nos mecanismos de defesa contra infecções estava por abordar no caso dos peixes, onde, como vimos, os dois fagócitos estão presentes. O estudo deste ponto é um dos temas centrais da presente dissertação.

No contexto da interacção/cooperação entre os macrófagos e os neutrófilos, será importante relembrar aqui que os dois fagócitos provêm de uma célula precursora comum.

Em **resumo**, comparando as características já conhecidas e referidas atrás da fagocitose e dos fagócitos nos mamíferos e nos teleósteos, pode dizer-se que elas são, no essencial, muito semelhantes. Nomeadamente, naqueles dois níveis de organização:

- há dois tipos de fagócitos (macrófagos e neutrófilos);
- os dois fagócitos são produzidos em orgãos hematopoiéticos e acumulam-se em "pools" de reserva nesses orgãos e na corrente sanguínea;

- desses "pools" os fagócitos migram para locais inflamatórios atraídos por factores quimiotácticos semelhantes (como produtos bacterianos, factores do complemento, eicosanoides, quimoquinas, etc.);
- a fagocitose de agentes infecciosos é promovida por opsonização por factores do sistema do complemento e por anticorpos devido à presença na superfície dos fagócitos de receptores para os factores do complemento e para anticorpos;
- agentes infecciosos internalizados nos fagócitos por via da fagocitose localizam-se em fagossomas, e nos casos de sucesso por parte do fagócito, são mortos por mecanismos microbicidas essencialmente idênticos, incluindo processos dependentes e independentes do oxigénio. Esses mecanismos dependem de moléculas semelhantes nos mamíferos e nos peixes, presentes em grânulos citoplasmáticos;
- os dois fagócitos podem ser activados, com aumento das capacidades funcionais, nomeadamente actividades antimicrobianas, por acção de citoquinas, como o IFNγ. No caso dos teleósteos, por serem animais poiquilotérmicos, a actividade dos fagócitos pode ser aumentada ou diminuida por variações da temperatura ambiente;
- muitas das actividades biológicas atribuídas a citoquinas, descritas em mamíferos têm sido encontradas em teleósteos, como é o caso das IL-1, IL-2, TGF, IFNγ, TNFα, CSFs, etc.;
- os macrófagos são células com capacidade de apresentação de antigénios e de desencadeamento de várias respostas, incluindo a inflamatória, muito importantes nos mecanismos de defesa contra infecções e de reparação de lesões.

É ainda de salientar que muitos destes aspectos comuns aos fagócitos e à fagocitose nos mamíferos e teleósteos, e que se aplicam a outros vertebrados, também têm sido descritos em invertebrados. Como já foi salientado, os fagócitos são os únicos leucócitos comuns a todos os metazoários (Tam *et al.*, 1976; Ratcliffe *et al.*, 1985; Greenberg & Silverstein, 1993).

III. Bactérias causadoras de infecções importantes nos salmonídeos em água doce.

As espécies aquáticas têm que fazer frente a uma enorme variedade de agentes patogénicos, incluindo vírus, bactérias e parasitas. É sabido que a maioria das bactérias patogénicas para os animais aquáticos são Gram-negativas (Ratcliffe *et al.*, 1985), sendo exemplos destas para o caso dos salmonídeos a *Aeromonas salmonicida*, agente etiológico da furunculose e a *Yersinia ruckeri*, agente da doença da boca vermelha ("Entheric Redmouth" (ERM)).

A ***Y. ruckeri*** é um bacilo Gram-negativo, geralmente móvel, com flagelação perítrica, oxidase negativo, fermentador da glucose. Recentemente foi feita uma actualização dos métodos de classificação das estirpes de *Y. ruckeri*, com base nos perfis de LPS e das proteínas de membrana (Romalde *et al.*, 1993), os quais vieram reorganizar a classificação dos anteriores 5 serotipos em 4, reunindo os serotipos I e III na nova designação O1, mantendo o serotipo II como O2, e passando a denominar como O3 e O4 os V e VI, respectivamente. No seu trabalho em truticulturas portuguesas, Sousa (1996) verificou a ocorrência de infecções por *Y. ruckeri* pertencentes aos serotipos O1 e O3 de Romalde *et al.* (1993).

A obtenção de uma vacina para a prevenção de infecções por *Y. ruckeri* não levantou tantas dificuldades como para outros agentes infecciosos dos salmonídeos (por exemplo *A. salmonicida*) tendo-se verificado que a imersão num banho com a bactéria morta era eficaz, ou mesmo que a simples imersão dos peixes num banho contendo o LPS da sua parede era suficiente para originar uma resistência efectiva e prolongada. Tal facilidade veio originar um certo desinteresse pelo estudo desta bactéria, o que significa um maior desconhecimento sobre o seu papel na interacção com os potenciais hospedeiros. Apesar do que foi dito, esta espécie de bactéria continua a ser a causa de elevadas mortalidades em truticultura.

Algumas espécies de bactérias Gram-positivas são também importantes como agentes infecciosos de salmonídeos. A ***Renibacterium salmoninarum*** (Sanders & Fryer, 1980), responsável pela doença bacteriana do rim ("bacterial kidney disease" (BKD)), tem particular importância dado que esta patologia, que se apresenta muitas vezes sob a forma crónica ou sub-clínica, é bastante resistente aos antibióticos e não é, ainda, evitável pelo uso de uma vacina eficaz (Munro & Bruno, 1988).

Trata-se de um bacilo curto e de pequena dimensão, fortemente Gram-positivo, não esporulado, imóvel, não capsulado; ocorre muitas vezes em pares ou em pequenas cadeias (Fryer & Sanders, 1981). É uma bactéria de crescimento lento em meios de cultura bacteriológicos e requer meios ricos suplementados com L-cisteína (Evelyn, 1977; Austin & Austin, 1987). Meios com carvão activado vieram possibilitar ao seu crescimento sem a utilização de soro fetal bovino (Daly & Stevenson, 1985).

A BKD foi descrita pela primeira vez em 1930 em salmões do rio Dee, na Escócia, sendo apelidada de "Dee disease" (Fryer & Sanders, 1981; Austin & Austin, 1987). A sua ocorrência parecia inicialmente restringir-se a águas doces, mas verificou-se que causa elevadas perdas também em água salgada (Fryer & Sanders, 1981), de forma particularmente preocupante para populações selvagens (Sakai *et al.*, 1992; Elliott *et al.*, 1997) ou de "sea ranching".

Muitos ensaios de quimioterapia contra esta infecção têm sido realizados, mas os resultados obtidos mostram que a utilização de antibióticos, além de dispendiosa, não leva à eliminação completa do parasita (Evelyn, 1993). Assim, o estudos para a produção de uma vacina têm continuado (Munro & Bruno, 1988; Evelyn, 1993).

Embora a vacinação resulte na produção de anticorpos contra a bactéria (Sakai *et al.*, 1993), estes não são efectivos na defesa do hospedeiro. Recentemente Pascho *et al.* (1997), em oposição aos resultados de Sakai *et al.* (1989, 1993), verificaram que peixes vacinados com a bactéria morta pelo formol sem adjuvante apresentavam maior sobrevivência após imersão em altas concentrações de bactéria que o controlo ou quando a vacinação era feita com mistura em Adjuvante Incompleto de Freund.

A BKD, assinalada em muitos países da Europa, América no Norte, Chile e Japão, continua a ser uma das mais importantes doenças que afecta a família dos salmonídeos, em água doce e no mar, tanto em aquacultura como em meio ambiente natural (Rockey *et al.*, 1991a; Sakai *et al.*, 1992; Evenden *et al.*, 1993). Esta infecção é considerada o problema principal na cultura nas primeiras fases de vida dos salmonídeos, particularmente para as culturas de salmão, pois, após as perdas em episódios da doença, muitos dos sobreviventes mostram-se mais susceptíveis à passagem para água salgada (Moles, 1997). Não há notícia da ocorrência desta doença em Portugal (Sousa, 1996).

A sua ocorrência em aquacultura de águas doces surge de forma sazonal, sendo os meses entre Fevereiro e Junho os mais críticos na Escócia (Bruno, 1986b).

É uma doença com transmissão horizontal e vertical (Fryer & Sanders, 1981), onde a possibilidade dos reprodutores serem portadores assintomáticos assume uma importância especial, pois estes são responsáveis pela transmissão vertical, através da contaminação dos ovos. Existe ainda a hipótese de contaminação através de invertebrados aquáticos que, captando bactérias em suspensão nas proximidades das aquaculturas, podem posteriormente vir a contaminar outros indivíduos (Sakai & Kobayashi, 1992; Paclibare *et al.*, 1994).

Tem sido demonstrada a possibilidade de transmissão horizontal por várias vias (Evenden *et al.*, 1993), embora não seja certo qual a via de transmissão natural. Por outro lado, sabe-se que na transmissão vertical um número muito baixo de bactérias, ou mesmo apenas uma, parece ser suficiente para que a transmissão ocorra (Brown *et al.*, 1990). Ao contrário, os ensaios feitos em relação à transmissão horizontal mostraram

que é necessário um número elevado de bactérias para que a contaminação ocorra (Evenden *et al.*, 1993).

Esta bactéria foi reconhecida como um parasita intracelular que causa uma infecção sistémica na fase final da infecção. A sua presença foi inicialmente verificada nos fagócitos (Young & Chapman, 1978; Bruno, 1986a), mas veio a confirmar-se que outras células, como células sinusoidais, parenquimatosas e reticulares dos órgãos hematopoiéticos, melano-macrófagos e células de barreira também podem ser infectadas (Flaño, 1992; Flaño, 1995; Flaño *et al.*, 1996a).

A presença da bactéria nos fagócitos é tida como um factor de dispersão da bactéria pelo hospedeiro (Evenden *et al.*, 1993), mas foi também observada a presença de bactérias livres na circulação sanguínea nas primeiras fases após a infecção intra-peritoneal (Flaño *et al.*, 1996a). Resultados recentes (Gutenberger *et al.*, 1997) parecem confirmar o crescimento intra-macrofágico desta bactéria, mas parece que também pode proliferar nas serosas dos tecidos moles (Bruno, 1986a).

A renibactéria tem afinidade especial para o tecido renal, onde surgem as primeiras lesões características da doença, sob a forma de granulomas. Posteriormente, lesões fibróticas são encontradas em todos os órgãos dos peixes infectados. Nas fases terminais da doença ocorre uma destruição maciça dos órgãos internos (Wood & Yasutake, 1956; Bruno, 1986a). É assinalável a ausência de sintomas marcados de doença até esta fase da infecção.

O facto de o rim ser o principal órgão hematopoiético dos peixes, levanta a hipótese de que a interacção da renibactéria com este tecido, nas primeiras fases da infecção, pode levar à destruição do tecido hematopoiético (Wood & Yasutake, 1956). A destruição renal progride, atingindo a sua porção posterior, obliterando todo o órgão e impedindo o seu normal funcionamento (Wood & Yasutake, 1956; Bruno, 1986a; Villena *et al.*, 1989).

No intestino, verifica-se uma inflamação com a participação de células eosinofílicas granulares (EGC) nos peixes moribundos. Outros tecidos são também afectados, nomeadamente no fígado, baço, brânquias, cérebro, vasos sanguíneos e timo (Wood & Yasutake, 1956).

As estirpes patogénicas de *R. salmoninarum* secretam grandes quantidades de uma proteína com um peso molecular de 57 kilodaltons. Esta proteína parece ser responsável pela hidrofobicidade da bactéria, e é secretada quer *in vitro* (Getchell *et al.*, 1985), quer *in vivo* (Turaga *et al.*, 1987). Vários estudos têm demonstrado que a produção desta proteína é um importante factor de virulência desta intrigante espécie de bactéria patogénica para os salmonídeos (Getchell *et al.*, 1985; Daly & Stevenson, 1987; Turaga *et al.*, 1987; Bruno, 1988; Daly & Stevenson, 1990; Rockey *et al.*, 1991b; Fredriksen *et al.*, 1997). Têm sido as características peculiares desta bactéria, aliadas à importância de

que se reveste na aquacultura e nos "stocks" selvagens de salmonídeos que nos levaram a dar-lhe uma especial atenção, pelo que é hoje um tema central dos nossos estudos.

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OBJECTIVOS

OBJECTIVOS

O objectivo central do conjunto de trabalhos que integram esta tese foi o de aprofundar o conhecimento sobre a interacção parasita/hospedeiro na infecção na truta arco-íris (*Oncorhynchus mykiss*). Foi dada particular atenção à biologia celular da resposta inflamatória que acompanha a infecção no que respeita ao papel dos dois fagócitos profissionais, o neutrófilo e o macrófago.

Como modelo utilizámos a infecção experimental da cavidade peritoneal. Este é um modelo largamente utilizado em trabalhos sobre a resposta inflamatória em ratinhos no laboratório onde esta dissertação foi preparada, por ter reconhecidas vantagens relativamente a outros modelos; além disso, como será abordado na Discussão Geral, as respostas leucocitárias que ocorrem na cavidade peritoneal são representativas das observadas noutros territórios do hospedeiro. A injecção de agentes flogísticos e a colheita de leucócitos foi levada a cabo na cavidade peritoneal da truta, após adaptação das técnicas anteriormente desenvolvidas para o ratinho.

Dado que, conforme referido na Discussão Geral, a informação disponível sobre os leucócitos da cavidade peritoneal da truta arco-íris em condições de repouso era escassa e controversa, a primeira fase do trabalho foi dedicada à análise desse aspecto. A informação sobre aquela população leucocitária é essencial para o conhecimento da localização dos fagócitos em condições normais e para a correcta interpretação das respostas celulares em condições inflamatórias. Para atingir esse objectivo, analizámos quantitativa e qualitativamente (morfologia e citoquímica por microscopia óptica e electrónica) a população leucocitária da cavidade peritoneal de trutas normais.

De seguida fomos estudar as características citoquímicas, os padrões de mobilização e a interacção de macrófagos e neutrófilos em situações de inflamação peritoneal, transitória ou persistente. Utilizámos como agentes inflamatórios não bacterianos a caseína e o Adjuvante Incompleto de Freund; como agentes bacterianos seleccionamos a *Yersinia ruckeri* e a *Renibacterium salmoninarum*, dado serem, como referido na Introdução, agentes infecciosos muito importantes para os salmonídeos em água doce. A renibactéria foi alvo de particular interesse por: i- ser um parasita intracelular patogénico específico para os salmonídeos; ii- tendo em conta alguns dados da literatura, originar respostas imunológicas peculiares e iii- porque dados preliminares obtidos no início da nossa investigação haviam mostrado uma resposta neutrofílica aberrante após a sua injecção intraperitoneal em trutas.

Na análise dos exsudados peritoneais inflamatórios foram utilizados métodos citológicos e citoquímicos, tanto em microscopia óptica como em microscopia

electrónica. Um dos objectivos dos estudos citoquímicos foi a caracterização precisa dos dois tipos de fagócitos durante a inflamação peritoneal.

Tendo em conta resultados obtidos em mamíferos, nomeadamente no laboratório onde esta dissertação foi preparada, fomos estudar as interacções entre macrófagos e neutrófilos, durante a resposta inflamatória na truta arco-íris, no sentido de tentar compreender a utilização pelo hospedeiro de dois fagócitos nos mecanismos de defesa contra a infecção.

Uma vez que os dados sobre a capacidade fagocítica dos neutrófilos de salmonídeos eram contraditórios, foi ainda comparada a capacidade fagocítica de macrófagos e neutrófilos durante a resposta inflamatória às bactérias em estudo e avaliado este fenómeno em diferentes condições de estimulação prévia da cavidade peritoneal.

Dado que nos mamíferos um aspecto central da interacção macrófago/neutrófilo tem a ver com a eliminação dos neutrófilos senescentes via apoptose, e consequente fagocitose pelos macrófagos dos neutrófilos apoptóticos, este fenómeno foi analisado nos exsudados peritoneais inflamatórios da truta.

A observação inicial de que na truta o neutrófilo é o único leucócito produtor de peroxidase, levou à quantificação da actividade peroxidásica presente em exsudados peritoneais, ao longo da infecção, procurando ultrapassar algumas limitações dos métodos citológicos para a quantificação de leucócitos. Procurou-se, também, avaliar o grau de desgranulação dos neutrófilos durante a inflamação, quantificando separadamente a actividade peroxidásica intra e extracelular.

TRABALHO EXPERIMENTAL

Capítulo 1

A população leucocitária da cavidade peritoneal não estimulada da truta arco-íris

1.1. Afonso, A., Ellis, A.E. & Silva, M. T. (1997). The leucocyte population of the unstimulated peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*). *Fish and Shellfish Immunology.* **7**: 335-348.

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The leucocyte population of the unstimulated peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*)

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The leucocyte population of the resting (unstimulated) peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*) weighing from 100 to 200 g and kept at 10, 15 or 20° C was analysed. The values for total leucocytes were not significantly different for the three temperatures studied, the average being $4{\cdot}35 \pm 1{\cdot}76 \times 10^6$ per cavity. In performing differential counts of Wright-stained preparations, 9 to 13% of the cells could not be characterised with confidence (because large lymphocytes and small macrophages are impossible to distinguish), 33 to 39% were macrophages, 46 to 53% were small lymphocytes and 1·3 to 2·2% were neutrophil granulocytes. Electron microscopy observations suggest that most of the cells which were difficult to characterise by light microscopy would be small macrophages. Thrombocytes (identified by electron microscopy) and eosinophilic granular cells (EGC) were very rarely seen. Light microscopy cytochemistry showed that: (1) macrophages and some lymphocytes were non-specific esterase positive; (2) macrophages and a few lymphocytes were acid phosphatase positive; (3) macrophages and lymphocytes were alkaline phosphatase negative and peroxidase negative; and (4) neutrophils were alkaline phosphatase and peroxidase positive. This latter characteristic allows for the clear distinction between macrophages and neutrophils, the strong, granular peroxidase staining being particularly evident. Transmission electron microscopy of ultrathin sections of samples processed for peroxidase activity showed that the neutrophil cytoplasmic granules, irrespective of size and shape, were positive, while all the other leucocytes were negative. The characteristic peroxidase staining of the nuclear and rough endoplasmic cisternae found in mammalian resident peritoneal macrophages was not seen in the resident macrophages of the resting rainbow trout peritoneal cavity. Ultrastructural cytochemistry for acid phosphatase showed positively stained cytoplasmic granules in the macrophages, in neutrophils and in a few small lymphocytes.

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Key words: rainbow trout leucocytes, peritoneal leucocytes, trout leucocyte cytochemistry.

I. Introduction

The intraperitoneal (i.p.) inoculation of infectious agents in mice has been extensively used in this laboratory as a model to analyse the leucocyte

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response to infection (Silva *et al.*, 1989; Appelberg *et al.*, 1991). One advantage of such a model derives from the fact that, in contrast with what occurs in solid organs, the peritoneal cell response that follows the i.p. inoculation of micro-organisms can be easily studied both qualitatively and quantitatively. This is so because the leucocytes in the inflammatory peritoneal exudate remain largely as a dispersed population which is, therefore, amenable to precise methods of cytological analysis (Silva *et al.*, 1989).

Assuming that such an advantage would also exist in the study of infection in fish, in the present study preliminary studies on infection in salmonids were undertaken using the peritoneal model and the results confirmed that the above assumption is correct. Very restricted data have been reported for the resting peritoneal leucocyte population of salmonids (Zelikoff & Enane, 1992; Enane *et al.*, 1993; Jørgensen *et al.*, 1993). While the paper by Jørgensen *et al.* (1993) on peritoneal inflammatory responses to phlogistic agents in Atlantic salmon does not include values for the resting cavity, the work by Zelikoff & Enane (1992) and by Enane *et al.* (1993) reports the controversial finding that the resident peritoneal population of rainbow trout would be entirely made up of macrophages. Here, the leucocyte population was analysed in detail in the unstimulated peritoneal cavity of rainbow trout. The data now obtained will serve as a baseline for the detailed analysis of the intraperitoneal cell response to infection in rainbow trout.

II. Materials and Methods

FISH

Sexually immature rainbow trout (*Oncorhynchus mykiss*) weighing between 30 and 200 g purchased from a commercial farm were used in this study. The animals were maintained in recirculating aerated water, at the temperature indicated below, in 700 l fibreglass tanks. Water quality was maintained with mechanical and biological filtration and fish were fed *ad libitum* on commercial pellets. Experimental groups of fish maintained at 10, 15 or 20° C ($\pm$ 1° C) were studied. The fish were maintained for at least 15 days in the same tank and at the required temperature before the study. The results to be reported in the present paper regard only healthy fish, as concluded from their activity, exterior appearance and, *a posteriori*, by necropsy observation.

COLLECTION OF PERITONEAL LEUCOCYTES

The peritoneal cells were collected by a procedure adapted from the one in use in our laboratory for mice (Silva *et al.*, 1989). In this method the peritoneal cavity is not opened at any time. The fish were transferred to a container with about 10 l of water at the same temperature as that in the aquarium and containing 0·6 ml l^{-1} of ethylene glycol monophenyl ether (Merck), and the anaesthesia was prolonged until the death of the animal. The mucus on the abdominal side of the fish was cleaned with ethanol. To avoid contaminating the peritoneal cells with blood the fish was exsanguinated either by inserting a 19 gauge needle into the caudal vessels or by cutting the dorsal aorta. The

anal opening was clamped with surgery forceps to prevent the loss of injected fluid through the abdominal pore (see Discussion). Using a 25 gauge needle, phosphate-buffered saline (PBS) with osmotic strength adjusted to 310–340 mOsm l^{-1} and supplemented with 20 U heparin (Choay) ml^{-1} was injected i.p. in the ventral midline, midway between the pelvic and pectoral fins; the volume of PBS to be injected was adjusted to the size of the fish (see Discussion) and was usually 2 to 5 ml. This was followed by the injection of air (about the same volume as PBS) through the same needle. The abdominal area was then massaged for about 30 s to disperse the peritoneal cells in the injected PBS. As much as possible of the intraperitoneal PBS containing suspended cells was collected with a syringe fitted with a 19 gauge needle and placed in ice until processed. Haemorrhagic peritoneal washings were discarded.

STUDY OF PERITONEAL LEUCOCYTES

The peritoneal cell suspension was analysed within minutes after collection for several morphological and cytochemical parameters as follows.

Total and differential cell counts

Thirty-three trout weighing from 30 to 240 g were used to study the total number of peritoneal leucocytes. In 24 of the above fish weighing from 100 to 200 g, total and differential leucocyte counts were performed. Total cell counts were performed with a haemocytometer. Cytospin preparations were made with a Shandon Cytospin 2 using a volume of peritoneal lavage PBS depending on the cell concentration, in order to obtain adequate numbers of cells per slide. The cytospins were stained with Wright's stain (Haemacolor, Merck) after fixation for 1 min with formol-ethanol (10 ml of 37% formaldehyde and 90 ml of absolute ethanol) (Kaplow, 1965). Differential counts were carried out in these preparations by counting between 300 and 500 cells and were based on previous descriptions of fish leucocyte morphology (Ellis, 1977; Ainsworth, 1992; Hine, 1992; Jørgensen *et al.*, 1993). The values were analysed by Student's *t*-test. The correlation between trout size and number of total peritoneal leucocytes was analysed by the Statworks ANOVA program.

Electron microscopy

The peritoneal leucocyte suspension in PBS was fixed at 4° C by the addition of 9 volumes of paraformaldehyde-glutaraldehyde fixative (Silva & Macedo, 1983; Silva *et al.*, 1987) to 1 volume of suspension. After at least 4 h the cells were washed twice with 50 mM cacodylate buffer (pH 6·4) and then fixed at room temperature for 2 h with 1% OsO_4 in veronal-acetate buffer supplemented with calcium chloride (Silva & Macedo, 1983) and post-fixed at room temperature with 1% aqueous uranyl acetate for 1 h (Silva *et al.*, 1971). After dehydration in ethanol the samples were embedded in Epon and ultrathin sections were cut with an LKB Ultratome III microtome. Routinely, the sections were doubly contrasted with uranyl acetate-lead citrate (Silva *et al.*, 1987). Observations and micrographs were done with a Zeiss EM 9 electron

microscope. The different types of leucocytes were characterised following previously reported ultrastructural descriptions of fish leucocytes (Ellis, 1977; Cannon *et al.*, 1980; Thuvander *et al.*, 1987; Ainsworth, 1992; Hine, 1992; Slierendrecht *et al.*, 1995).

Light microscopic cytochemistry

Cytospin preparations obtained as described above were stained for the following enzyme activities: peroxidase by the method of Kaplow (Kaplow, 1965); acid phosphatase by the method of Barka & Anderson (1962); alkaline phosphatase by the method of Burstone (1958) using naphthol AS-Bi phosphate as substrate; and alpha-naphthyl acetate esterase (ANAE; non-specific esterase) by the method of Pearse (1972). Controls were performed as follows: peroxidase, omitting hydrogen peroxide; acid phosphatase, omitting the substrate; and alkaline phosphatase, omitting the substrate or including 1 mM levamisole in the incubation mixture.

Ultrastructural cytochemistry

Cells were stained for peroxidase activity by the diamino-benzidine method of Robbins *et al.* (1971), and for acid phosphatase activity by the method of Petty *et al.* (1985), using trimetaphosphate as substrate. Controls were performed by omitting hydrogen peroxide (peroxidase) and trimetaphosphate (acid phosphatase).

III. Results

TOTAL AND DIFFERENTIAL LEUCOCYTE COUNTS

The results, obtained by combining the light and electron microscopic studies, show that the leucocytes of the unstimulated peritoneal cavity of rainbow trout are macrophages (Figs 1, 2 and 6), lymphocytes (Figs 1, 3, 5 and 7), granulocytes and thrombocytes. Thrombocytes were identified by electron microscopy, and were found to be quite rare. Granulocytes were mostly neutrophils (Figs 1, 4), eosinophilic granular cells (EGC) being very rarely seen. In all fish a variable proportion of small mononuclear leucocytes (up to 13% of total leucocytes) could not be characterised by light microscopy with confidence and were recorded as non-classified (NC) cells. The electron microscope observations suggest that most of these small mononuclear cells would be small macrophages.

In Wright-stained preparations the distinction between neutrophils and macrophages can be difficult. This is so because the nuclear shape of some neutrophils is not as multilobular as in mammalian neutrophils and, in addition, some macrophages have a rather polymorph nucleus. However, the cytochemical characteristics of these leucocytes, reported below, allow for the clear distinction between them.

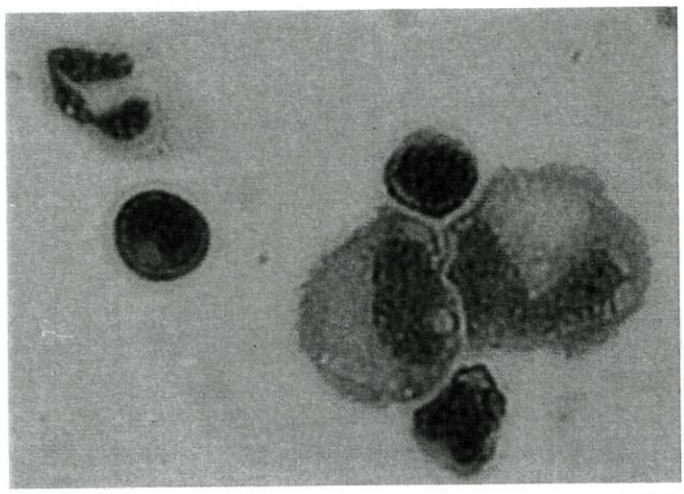


Fig. 1. Light microscopy of a Wright-stained cytospin preparation of peritoneal leucocytes, showing one neutrophil with a multilobulated nucleus, two macrophages (large mononuclear cells) and 3 lymphocytes (small mononuclear cells). x 1000.

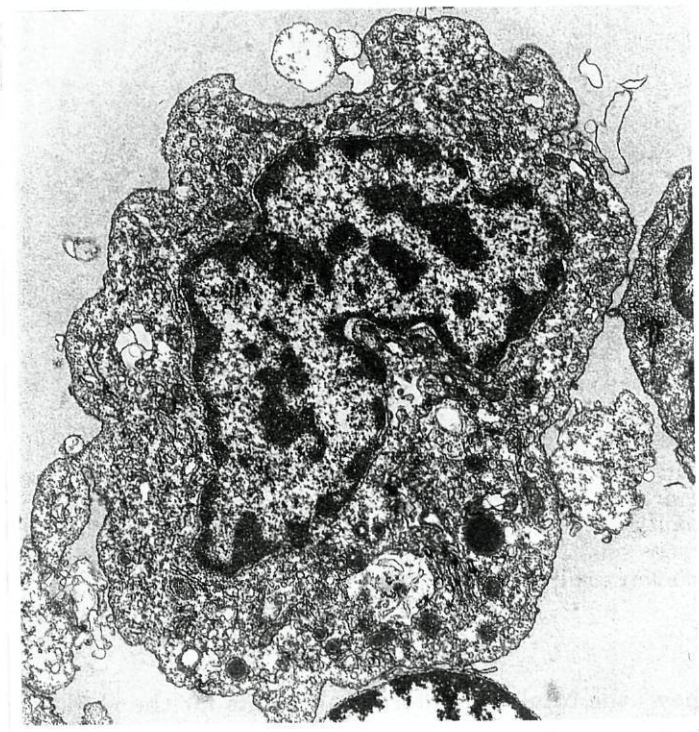


Fig. 2. A macrophage in a sample processed for conventional electron microscopy. The cytoplasm contains granules, phagocytic vesicles, a few mitochondria and cisternae of endoplasmic reticulum. Section double contrasted with uranyl-lead. x 16 000.

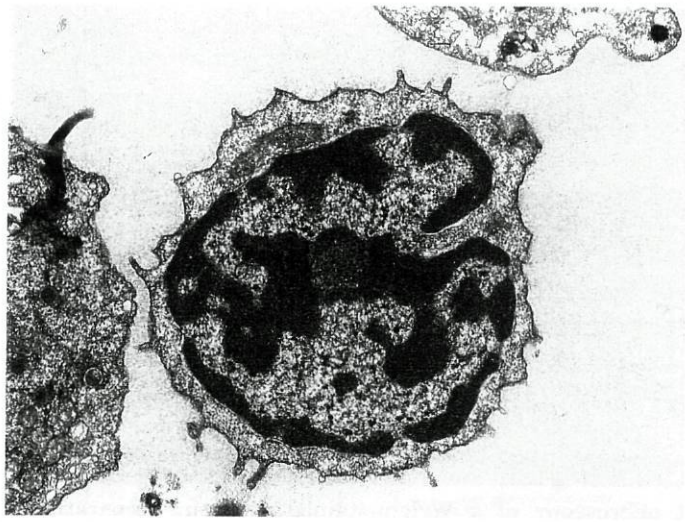


Fig. 3. Same sample as in Fig. 2, showing a small mononuclear leucocyte with the characteristics of a lymphocyte. Notice the high nucleus/cytoplasm ratio. The cytoplasm contains ribosomes and one mitochondrion. Section contrasted with uranyl and lead. x 16 000.

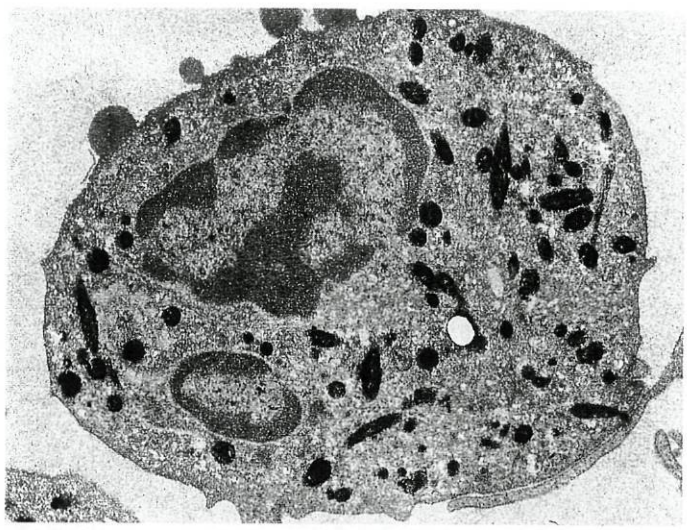


Fig. 4. A neutrophil in a sample processed for ultrastructural cytochemistry for peroxidase activity. Note the presence of abundant peroxidase-positive (electrondense) cytoplasmic granules. Some of the granules have an elongated shape and some are very small. The cytoplasmic matrix has a holey structure due to the presence of many small light patches (see text). Section contrasted with lead citrate. x 16 000

Table 1 shows the total and differential counts for the predominant leucocytes of the unstimulated peritoneal cavity of rainbow trout. No significant differences were found for total and differential leucocyte numbers as related to the three temperatures studied.



Fig. 5. A lymphocyte from the same sample as in Fig. 4. No peroxidase-positive granules are present. Notice the high nucleus/cytoplasm ratio. A few mitochondria are present in the narrow cytoplasm. Section contrasted with lead citrate. x 17 800.

The correlation between cell number and weight of the fish is shown in Fig. 8. Although cell number was usually higher in heavier fish, the correlation between the two parameters is rather low. This last point is likely to be due not only to the high individual variation in the number of peritoneal cells but also to the narrow spectrum of weights studied.

CYTOCHEMISTRY

The results for the enzymatic cytochemical characterisation by light microscopy showed that most macrophages were ANAE-positive, including some very strongly-positive; the proportion of the macrophages showing strongly positive staining for ANAE activity increases in inflammatory peritoneal exudates (unpublished observations). Most lymphocytes were ANAE-negative but a few which were slightly positive were always found; the same applies to acid phosphatase activity. The only absolute enzymatic markers were peroxidase (Fig. 2) and alkaline phosphatase activities; 100% of neutrophils were positive for both enzymes while all the other cell types found in the non-stimulated peritoneal fluid were negative. Since the peroxidase staining of neutrophil granules is very strong, this technique can be regarded as a specific and efficient stain for these phagocytes.

By transmission electron microscopy, peroxidase activity was found in neutrophilic granules of all sizes and configurations (Fig. 4). From the many electron micrographs obtained during the present study it seems that all cytoplasmic granules of neutrophils are peroxidase-positive. All the other leucocytes were negative (a lymphocyte is shown in Fig. 5). Ultrastructural cytochemistry for acid phosphatase was strongly positive in most macrophages

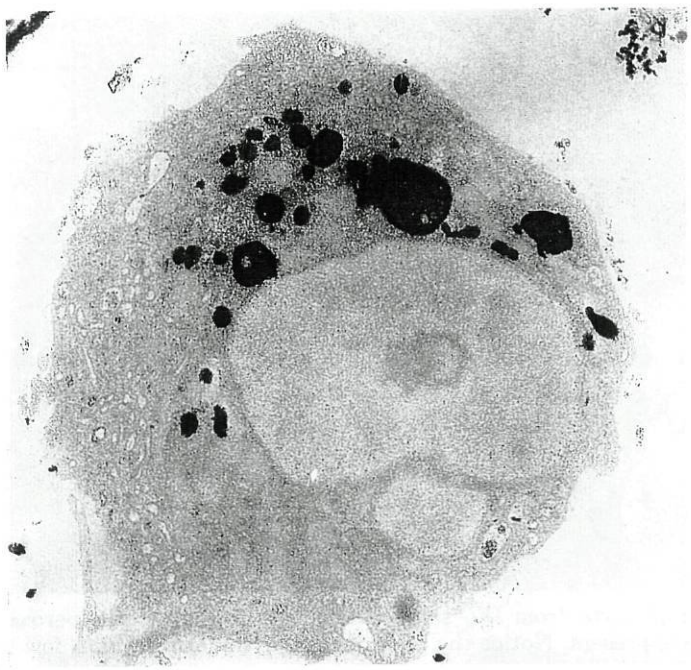


Fig. 6. A macrophage in a sample processed for ultrastructural cytochemistry for acid phosphatase. The macrophage has many acid phosphatase-positive (electron-dense) cytoplasmic granules, some of them of large diameter. Section contrasted with lead citrate. x 17 600.



Fig. 7. A lymphocyte in the same sample as in Fig. 6. The lymphocyte has some acid phosphatase-positive (electron-dense) cytoplasmic granules in a cell pole. Section contrasted with lead citrate. x 16 000.

where the enzymatic activity was seen in lysosomes (Fig. 6). This intense labelling of the lysosomal compartment of macrophages can be used to quantitatively study the phagosome-lysosome fusion as in the case of infected

Table 1. Peritoneal leucocyte population of rainbow trout maintained at different temperatures

Temperature	Weight (g)	Total cells	Macrophages	Lymphocytes	Neutrophils	Non-classified
10° C	155·83 ± 33·18	460·64 ± 214·60	152·90 ± 79·90 (33·19%)	243·55 ± 174·29 (52·87%)	6·09 ± 4·29 (1·32%)	58·10 ± 69·63 (12·61%)
15° C	159·16 ± 38·95	425·07 ± 158·63	164·51 ± 59·79 (38·70%)	195·71 ± 116·42 (46·04%)	9·46 ± 5·15 (2·23%)	55·39 ± 36·61 (13·03%)
20° C	122·80 ± 5·02	383·17 ± 181·85	141·36 ± 53·48 (36·89%)	202·56 ± 121·78 (52·86%)	12·55 ± 6·19 (1·62%)	26·70 ± 13·79 (8·63%)

Values represent the mean+S.D. of the number ($\times 10^{-4}$) of leucocytes per peritoneal cavity. In brackets are the % of the different leucocyte types. Groups of eight fish were studied at each of the 3 temperatures indicated. No significant differences were found ($P>0·065$) for the comparison between the values for fish weight and for total leucocytes, macrophages, lymphocytes, neutrophils and non-classified cells at different temperatures.

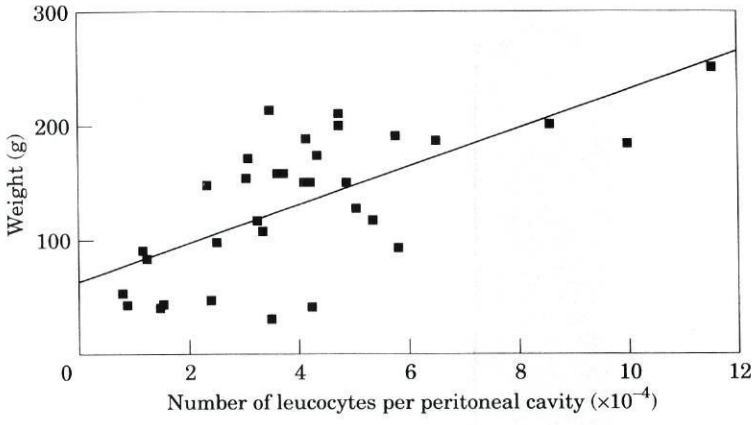


Fig. 8. Correlation between peritoneal total cell number and size of rainbow trout. $N=33$. Correlation coefficient=0·6688.

mouse macrophages (Silva *et al.*, 1987). Neutrophils had acid phosphatase-positive granules and a few lymphocytes showed positively-stained cytoplasmic granules, usually with a polar location (Fig. 7).

In lead-contrasted sections of samples processed for the peroxidase activity, the cytoplasm of the neutrophils showed a holey aspect due to the presence of many small light patches (Fig. 4). Such holes correspond to unstained polysaccharide granules (Silva & Afonso, unpublished).

IV. Discussion

The present study shows that the peritoneal cavity of rainbow trout has a resident peritoneal leucocyte population that can easily be collected and that is amenable to precise quantitative analysis. There are, however, problems that have to be taken into consideration when using the peritoneal cavity of trout for studies on leucocytes. One relates to the peculiar anatomical characteristics of the peritoneal cavity of salmonids (George *et al.*, 1982) which opens to the exterior through abdominal pores close to the urinary and rectal openings. Due to this peculiar anatomical feature, if too much volume of fluid is injected into the peritoneal cavity of trout, the resulting intraperitoneal high pressure opens the abdominal pores and part of the fluid is lost. To collect the peritoneal cells, therefore, volumes of PBS and air were adjusted to the size of the fish in order to avoid this problem.

Another problem relates to the lack of an oviduct in female salmonids where eggs are released into the peritoneal cavity prior to their exit at spawning via the genital pore. Non-spawned eggs degrade in the peritoneal cavity, and this induces inflammation (Afonso & Silva, unpublished). Consequently, for studies on infection involving the peritoneal cavity of salmonids, either sexually immature fish or fish fully recovered from the sexual-related peritoneal inflammation should be used.

The present results show that the predominant cells in the unstimulated peritoneal cavity of rainbow trout are lymphocytes, macrophages and, to a

lesser extent, neutrophils. The fact that thrombocytes were only rarely found shows that the resting peritoneal population is at variance with the blood leucocyte population, which besides the above three types of leucocytes contains a large proportion of thrombocytes.

Neutrophils and macrophages can be difficult to distinguish in conventional light microscopic preparations because the characteristic difference between the nuclear shape found in mammalian macrophages and neutrophils may not be so marked in trout phagocytes. This is particularly so in inflammatory peritoneal exudates (unpublished observations). However, the two phagocytes can be easily and specifically labelled for light microscopy by alkaline phosphatase and peroxidase staining. The latter is especially interesting because it stains intensely the neutrophil cytoplasmic granules and, therefore, makes it easy to detect these phagocytes even in low power microscopy.

One conclusion from the present study is that the distinction between the two types of leucocytes that make up more than 97% of the cells in the resting peritoneal cavity of rainbow trout—the lymphocytes and the macrophages—cannot always be done with confidence by light microscopy. This is so because: (1) large lymphocytes are easily confused with small macrophages in preparations stained by the Romanowsky-type stains; (2) no satisfactory antigenic markers are available to specifically label these mononuclear cells; (3) as shown in the present work for four cytochemical stains, no satisfactory enzymatic markers are available for their distinction, in fact, some macrophages are only very slightly positive for ANAE, an activity present in some lymphocytes. The same applies to acid phosphatase which is present in macrophages but also in cytoplasmic granules of some lymphocytes. In the present work cells that could not be safely distinguished as macrophages or lymphocytes by light microscopy were denoted as non-classified (NC) leucocytes.

On the other hand, transmission electron microscopy of ultrathin sections was found to be reliable in the characterisation of neutrophils, macrophages, lymphocytes (except very large ones) and thrombocytes, and the use of this technique reduced the proportion of leucocytes which were difficult to characterise as compared to light microscopy. As stressed in the report on catfish blood leucocytes by Cannon *et al.* (1980), large lymphocytes still may be difficult to distinguish from macrophages on the basis of fine structure. The present ultrastructural observations suggest that the actual proportion of macrophages would be higher than that obtained by light microscopy, with most of the leucocytes included in the NC group being small macrophages. Additionally, they show that thrombocytes are very rare in the unstimulated peritoneal leucocyte population of rainbow trout. Quantitative electron microscopy, however, is time consuming because it requires that many cells be counted in preparations made from different blocks to compensate for the heterogeneous distribution of the leucocytes in the pelleted samples (Silva, unpublished results). Differential counts based on electron microscopic observations were not, therefore, attempted in the present study.

One interesting observation is that resident macrophages of the peritoneal cavity of rainbow trout are peroxidase-negative both by light and electron

microscopy. In the mouse, rat and guinea pig, most of the resident peritoneal macrophages, although peroxidase-negative in light microscopy stainings, show by ultrastructural cytochemistry a peroxidase-positive staining of the perinuclear and rough endoplasmic reticulum cisternae (Daems *et al.*, 1973; Beelen *et al.*, 1979; De Bakker *et al.*, 1985), a pattern that we did not observe in the rainbow trout resident peritoneal macrophages. In this context, it is worth noting that while human, guinea pig, rat and mouse blood monocytes contain cytoplasmic granules positive for peroxidase (van Furth *et al.*, 1970; Nichols *et al.*, 1971; Beelen *et al.*, 1979), we found rainbow trout monocytes to be negative (unpublished observations). In this respect, trout monocytes are like rabbit monocytes (Nichols *et al.*, 1971).

Some mononuclear leucocytes with ultrastructural characteristics of lymphocytes were observed to contain acid phosphatase-positive cytoplasmic granules. It is possible that these cells correspond to non-specific cytotoxic cells (NCC) or Natural Killer (NK) cells because the above ultrastructural and cytochemical characteristics are similar to those described for mammalian NK cells (Kay & Douglas, 1991). It has been reported, however, that rainbow trout NCC are not granular (Greenlee *et al.*, 1991). Further studies are required to clarify this point.

The present results show that the resident (resting) peritoneal leucocyte population of rainbow trout is not much different from that present in mice (De Bakker *et al.*, 1985; Silva *et al.*, 1989; Appelberg *et al.*, 1991) and humans (Kubicka *et al.*, 1996). In fact, and unlike the results from another laboratory (Zelikoff & Enane, 1992; Enane *et al.*, 1993), the predominant leucocytes in the resting peritoneal cavity of rainbow trout are neutrophils, lymphocytes and macrophages, the two mononuclear cells representing more than 97% of total leucocytes.

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Capítulo 2

A resposta neutrofílica e macrofágica peritoneal da truta arco-íris à injecção intraperitoneal de agentes flogísticos

2.1. Afonso, A., Lousada, S., Silva, J., Ellis, A. E. & Silva, M. T. (1998). Neutrophil and macrophage responses to inflammation in the peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*). A light and electron microscopic cytochemical study. *Diseases of Aquatic Organisms.* (Em publicação).

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Neutrophil and macrophage responses to inflammation in the peritoneal cavity of rainbow trout *Oncorhynchus mykiss*. A light and electron microscopic cytochemical study

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ABSTRACT: The neutrophil and macrophage responses that accompany inflammation were studied in the peritoneal cavity of rainbow trout *Oncorhynchus mykiss* using light and electron microscopic cytochemistry. Neutrophils of inflammatory peritoneal exudates were alpha-naphthyl butyrate esterase-negative, peroxidase-positive and rich in cytoplasmic glycogen granules. Macrophages were poor in glycogen, esterase-positive and usually peroxidase-negative. Some peroxidase-positive macrophages were due to the transfer to macrophages of neutrophilic peroxidase. The ultrastructural double labelling for glycogen/peroxidase or esterase/peroxidase was most useful for precisely characterising neutrophils and macrophages in the inflamed peritoneal cavities and for correctly labelling peroxidase-positive macrophages. Intraperitoneal injection of casein, Incomplete Freund's Adjuvant (IFA) and live or formol-killed *Yersinia ruckeri* resulted in a rapid influx of neutrophils, peaking at 24 to 48 h post-injection and reaching values, in the case of live bacteria, 500× those in the resting, unstimulated peritoneal cavity. Peritoneal macrophages also increased, but the response was slower (peak at 5 d) and with more modest increases in number (7.5×). Neutrophil and mononuclear cells returned to normal values after 15 d in the case of casein and bacteria, but continued above base values 30 d after the injection of IFA. Conversely, after the injection of phosphate buffered saline, India ink or with sham-injections, very moderate neutrophil and macrophage responses subsided in a few hours. Phagocytosis of bacteria was studied by light microscopy of preparations stained for peroxidase by a new method which allows for the simultaneous observation of intracellular bacteria and peroxidase staining. When *Y. ruckeri* was injected into resting peritoneal cavities, bacteria were ingested by the resident macrophages. When the bacteria were injected into cavities with high numbers of neutrophils (due to the previous injection of casein), more neutrophils than macrophages contained bacteria. Results show that the macrophages are the resident phagocytes of the peritoneal cavity of trout, while neutrophils are present in that body cavity in significant numbers only in situations of inflammation and only as long as the inflammation persists.

KEY WORDS: Rainbow trout leukocytes · Rainbow trout peritoneal leukocytes · Rainbow trout neutrophils · Neutrophils in inflammation · Cytochemistry of trout phagocytes

INTRODUCTION

Phagocytosis, killing and degradation of invading microrganisms by specialised phagocytes, is a crucial process in the defense of vertebrates against some infections (Mackaness 1969, Ellis 1977, Adams & Hamilton 1984, Edwards & Kirkpatrick 1986, Lehrer et al. 1988, Hine 1992, Brown 1995). It is interesting that mammals, teleosts and other higher eukaryotes utilize 2 types of phagocytic cells—neutrophils and macrophages—in the defense against infection (Rowley et

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al. 1988, Secombes & Fletcher 1992, Brown 1995). From studies conducted with mammals, data on the behaviour of each of the 2 phagocytes during inflammation, including that caused by infection, can be summarised as follows (Lehrer et al. 1988, Haslett et al. 1989, Silva et al. 1989, van Furth 1992, Densen et al. 1995, Kubicka et al. 1996). Neutrophils are present in large numbers in blood and hematopoietic organ pools as a reserve, and, under normal conditions, are rare in the tissues and body cavities. In contrast, macrophages are present in all body compartments, and, therefore, are the first phagocytes to encounter the invading microrganisms when superficial barriers are breached. Neutrophils from the reserve pools are quickly attracted to the infectious foci by microbial products and by chemotactic agents produced by immune cells including macrophages (Lehrer et al. 1988, Appelberg 1992). Monocytes, the blood circulating precursors of tissue macrophages, are also recruited to infectious sites, where they increase the initial macrophage population.

Researchers at this laboratory have been interested in investigating whether a pattern of distribution and mobilization of the 2 phagocytes similar to that described above for mammals occurs in fish. In the present paper we expand upon previous observations of the leucocyte population of the resting peritoneal cavity of rainbow trout *Oncorhynchus mykiss* (Afonso et al. 1997) by analyzing the response of neutrophils and macrophages during inflammation. The peritoneal model (Silva et al. 1989, Appelberg et al. 1991, Pestana 1994, Sousa 1994) was used because, in contrast to what occurs in solid organs, the peritoneal cell response that accompanies intraperitoneal inflammation can be easily studied both qualitatively and quantitatively. This is so because the leucocytes in peritoneal exudates largely remain a dispersed population, which is, therefore, amenable to precise methods of cytological analysis (Silva et al. 1989, Afonso et al. 1997). Moreover, the phagocyte response to inflammation in the peritoneal cavity is representative of that seen in tissues (van Furth 1992).

Sometimes, with conventional cytological procedures, it is difficult to distinguish neutrophils and macrophages in the inflammatory peritoneal exudates. Therefore, light and electron microscopic cytochemical methods were used to analyze the peritoneal phagocyte response to the presence of infectious agents.

The present study extends our knowledge gained from previous observations (Afonso et al. 1997) and shows that, in rainbow trout, neutrophils and macrophages display different patterns of localization in the normal host and of mobilization into inflamed areas. These patterns are similar to those seen in mammals.

MATERIALS AND METHODS

Experimental fish. Sexually immature rainbow trout weighing between 100 and 200 g, purchased from a commercial farm, were maintained in recirculating aerated fresh water, at 15°C, and were fed ad libitum on commercial pellets. The fish to be injected with phlogistic agents were transferred to 600 l fibreglass tanks under the same conditions as above, where they were maintained for at least 15 d before the study. Only healthy fish, as indicated by their activity and exterior appearance, were used.

Growth, harvesting and treatment of bacterial agents. The strain FP-3 of *Yersinia ruckeri* type 1 was kindly supplied by Prof. J. Barja (University of Santiago de Compostela, Spain). Details of the preparation of bacterial suspensions are given elsewhere (Afonso et al. 1998). Preliminary infectivity tests showed that, for the conditions used in this work, this bacterium does not induce a progressive infection. Formol-killed bacteria were prepared by treating suspensions in phosphate buffered saline (PBS) adjusted to 310 mOsm l^{-1} with 8% formaldehyde for 3 d, followed by 5 washings with PBS. Plating of the formol-treated bacteria showed 100% killing. For fish inoculation, concentrated, frozen suspensions of live or killed bacteria were thawed at room temperature and diluted to the desired concentrations (see below) with PBS.

Intraperitoneal injection of phlogistic agents. The following agents were injected intraperitoneally (i.p.) in volumes of 100 µl: 0.4% India ink in PBS; 12% casein in water; undiluted Incomplete Freund's Adjuvant (IFA); suspensions of live or formol-killed *Yersinia ruckeri* (5×10^7 bacteria per fish).

Live *Yersinia ruckeri* was also injected i.p. in trout with peritoneal cavities previously inflamed by the i.p. injection of 100 µl of 12% casein in water 48 h before the bacterial challenge. The inoculum (3×10^8 per fish) was higher than that administered to fish with resting peritoneal cavities because the number of phagocytes (macrophages plus neutrophils) was higher in the peritoneal cavities of fish previously injected with casein (144.48×10^6) as compared to untreated fish (1.74×10^6).

Intraperitoneal injections were carried out as described (Afonso et al. 1997). Control fish were injected i.p. with PBS or subjected to a sham injection by inserting the needle into the peritoneal cavity without injection.

Collection of peritoneal leucocytes. The peritoneal cells were collected at intervals (see Tables 1 & 2, and Fig. 7) after the i.p. injections using a procedure previously described in detail (Afonso et al. 1997). The peritoneal cell suspension was analysed within minutes after collection for several morphological and cytochemical parameters as follows.

Light microscopy of peritoneal leucocytes. Total cell counts were performed with a haemocytometer. Cytospin preparations were made with a Shandon Cytospin 2. The cytospins were stained with Wright's stain (Haemacolor, Merck) after fixation for 1 min with formol-ethanol (10 ml of 37% formaldehyde and 90 ml of absolute ethanol) (Kaplow 1965). The leucocytes in the peritoneal exudates were differentially counted as neutrophils and mononuclear cells, except in some situations when mononuclear leucocytes were further distinguished as macrophages and lymphocytes (see 'Results'). As described previously for the resting peritoneal leucocyte population of rainbow trout (Afonso et al. 1997), some peritoneal mononuclear leucocytes could not be classified with certainty. These cells, referred to as 'non-classified leucocytes' (Afonso et al. 1997), were present in the inflammatory peritoneal exudates studied here in percentages well below (less than 5% of total leucocytes) those reported for the resting peritoneal cavities. These cells were included in the class 'mononuclear leucocytes'.

Electron microscopy of peritoneal leucocytes. The peritoneal leucocyte suspension in PBS was processed for electron microscopy as described (Silva et al. 1971, Silva & Macedo 1983, Silva et al. 1987, Afonso et al. 1997).

To enhance the electron density of glycogen, some samples were processed using 2% OsO_4 in 0.1 M cacodylate buffer pH 7.3, supplemented with 3% potassium ferrocyanide (Karnowsky 1971, Robinson et al. 1982) as fixative.

Observations and micrographs were done with a Zeiss EM 10C electron microscope.

Cytochemistry. For peroxidase detection by light microscopy 2 methods were used. One is the classic technique of Kaplow (1965). This method is quite efficient but has 2 major disadvantages, namely: (1) the peroxidase staining is quite light-sensitive and fades in a few days, and (2) it stains the peroxidase-positive sites a very deep blue, making it difficult to visualize bacteria inside neutrophils.

Because of these drawbacks, a new method was devised which is referred to in the present paper as Antonow's method. It is carried out as follows:

(1) Fixation of the cytospin preparations is done with formol-ethanol as in Kaplow's method (1965).

(2) After rinsing with running tap water for a few seconds, the preparations were treated with a 1/30 dilution (in water) of Kaplow's reagent (without safranin) for 5 to 10 min at room temperature. The diluted reagent was prepared fresh or kept frozen.

(3) To counterstain the preparations the Wright or the Gram stain was used.

For ultrastructural detection of peroxidase activity the method of Robbins et al. (1971) was followed. Controls were carried out for all techniques by omitting hydrogen peroxide from the incubation mixtures.

To test the resistance of trout neutrophilic peroxidase to cyanide the method described by Horton et al. (1996) was used; the incubation reagent contained 8 mM NaCN.

Ultrastructural detection of alpha-naphthyl butyrate (ANB) esterase was carried out as described by Bozdech & Bainton (1981), with postfixation with 1% osmium tetroxide in distilled water with pH adjusted to 7.6 to heighten the contrast of the reaction product (Bozdech & Bainton 1981). In some samples, the ANB esterase staining was followed by the peroxidase technique carried out as indicated above (Robbins et al. 1971).

The ultrastructural staining of cytoplasmic polysaccharides, most likely glycogen, was carried out using the original method of Thiéry (1967), or a modification (Silva & Macedo 1987). Thiéry staining was applied to ultrathin sections of samples processed as described above for conventional electron microscopy or for peroxidase ultrastructural cytochemistry.

Study of blood monocytes. Monocytes in the blood of normal rainbow trout were studied in concentrated blood leucocytes obtained by the method described by Thuvander et al. (1987) and applied to heparinised venous blood collected from the caudal vein. These leucocyte preparations were analysed for peroxidase, esterase and glycogen using the methods described above for peritoneal leucocytes.

RESULTS

Cytochemistry for peroxidase of inflammatory peritoneal neutrophils and macrophages

Kaplow's technique strongly stains peroxidase in cytoplasmic granules of inflammatory neutrophils (Figs. 1a, b). The dark-blue staining is very intense and is most useful for detecting very reduced amounts of the enzyme activity, as was the case for some macrophages in the inflamed cavities which showed a few small peroxidase-positive cytoplasmic granules (Fig. 1b).

Utilization of Antonow's technique to detect peroxidase activity results in a green staining of the neutrophil cytoplasmic granules (Fig. 1c–f). Individual granules are sometimes visible (Fig. 1c), but, more frequently, a more or less homogeneously stained cytoplasm is present (Fig. 1d–f). The specificity of the staining is the same as with the conventional Kaplow method, being abolish by omitting hydrogen peroxide from the incubation mixture.

The green peroxidase staining is light insensitive and remains stable for more than 1 yr (the time tested so far). In contrast to what happens with the conventional Kaplow method, staining using Antonow's technique

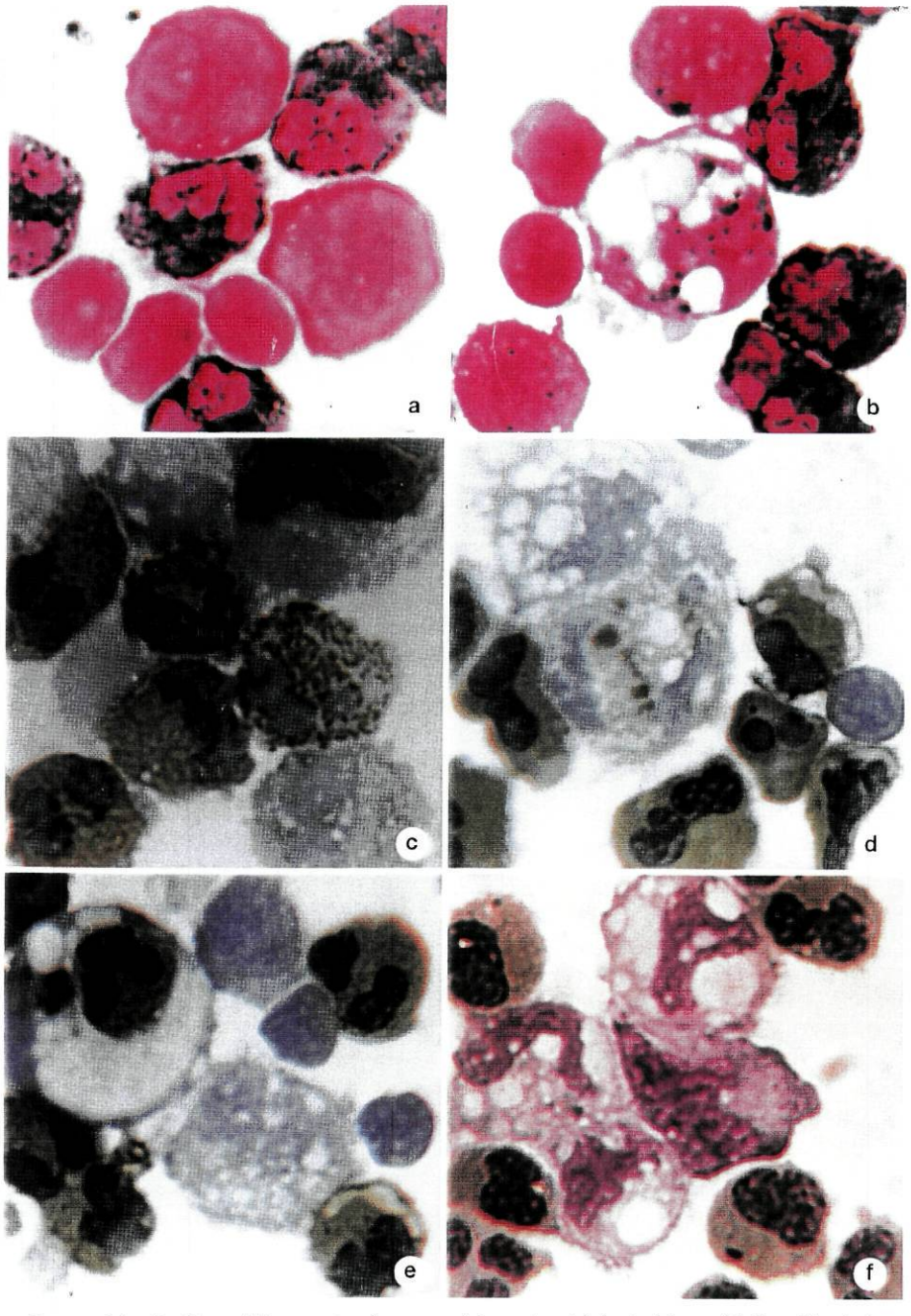


Fig. 1. (a) *Oncorhynchus mykiss*. Peritoneal leucocytes from a rainbow trout injected i.p. with live *Yersinia ruckeri*. Sample collected 24 h post-injection and stained for peroxidase using Kaplow's method. The neutrophils have abundant peroxidase-positive (blue) granules. Mononuclear cells, including 2 macrophages, are peroxidase-negative. ×850. (b) Same sample as in (a), also stained for peroxidase using Kaplow's method. The neutrophils have abundant peroxidase-positive granules. The 3 macrophages have a few peroxidase-positive granules. Two lymphocytes are peroxidase-negative. ×850. (c) Peritoneal cells from a fish injected i.p. with live *Y. ruckeri*. Sample collected 2 d after injection and stained for peroxidase using Antonow's method (Wright counterstaining). One neutrophil shows numerous, well-defined, peroxidase-positive (green) cytoplasmic granules. The other neutrophils have a more compact cytoplasmic peroxidase staining. The macrophages present in the field are peroxidase-negative. ×850. (d) Peritoneal cells from a fish injected i.p. with live *Y. ruckeri*. Sample collected 2 d after injection and stained for peroxidase using Antonow's method (Wright counterstaining). Several neutrophils (one with nuclear alterations suggestive of apoptosis) show an intense peroxidase-positive (green) staining. One macrophage is peroxidase-negative, whereas the other has a few peroxidase-positive inclusions. ×850. (e) Same sample as in (d), also stained for peroxidase using Antonow's method (Wright counterstaining). Notice the macrophage which had phagocytosed a neutrophil. The other macrophage is peroxidase-negative. ×850. (f) Peritoneal cells from a fish injected i.p. with live *Y. ruckeri*. Sample collected 24 h after injection and stained for peroxidase using Antonow's method (Wright countersaining). Notice a bacterium inside a neutrophil. The macrophages present in this field are peroxidase-negative. ×850

allows for a clear visualization of intraneutrophilic bacteria, whether the counterstain is carried out with the Wright or with the Gram stain. In the former case bacteria appear blue against a green background (Fig. 1f). In the case of Gram-positive bacteria, where the Gram stain is used, bacteria appear blue-black against a green background (not shown). Because the counterstaining can be done with the Wright stain, identification of leucocytes is easier with the Antonow's than with Kaplow's method, which uses safranin as a counterstain (cf. Fig. 1a, b).

Some inflammatory mononuclear leucocytes with morphological characteristics typical of macrophages contain peroxidase activity (Fig. 1b, d, e, and see Figs. 4 & 6). The patterns of peroxidase staining were typically different for neutrophils and peroxidase-positive macrophages. In neutrophils, the staining was quite homogeneous, activity being present in abundant granules which filled most of the cytoplasm of the phagocyte (Fig. 1a–f). In peroxidase-positive macrophages, the staining was rather heterogeneous, showing a range of characteristics from the presence of discrete small peroxidase-positive granules (Fig. 1b) to the presence of small (Fig. 1d) or large vacuoles filled with peroxidase-positive material or even intact neutrophils with peroxidase-positive granules (Fig. 1e).

The peroxidase staining of trout neutrophilic granules was resistant to 8 mM NaCN when assayed by the diaminobenzidine technique described by Horton et al. (1996).

Inflammatory neutrophils were seen by electron microscopy to contain numerous peroxidase-positive granules of varying sizes and shapes (Figs. 2 & 3). When bacteria were present inside neutrophilic phagocytic vacuoles, peroxidase activity was also present in the vacuoles (Fig. 2), indicating that fusion of cytoplasmic granules with the phagosome had occurred.

Polysaccharide granules of neutrophils and macrophages

Abundant glycogen granules were consistently seen in the cytoplasm of neutrophils when adequate techniques were used (Fig. 3). In macrophages these inclusions

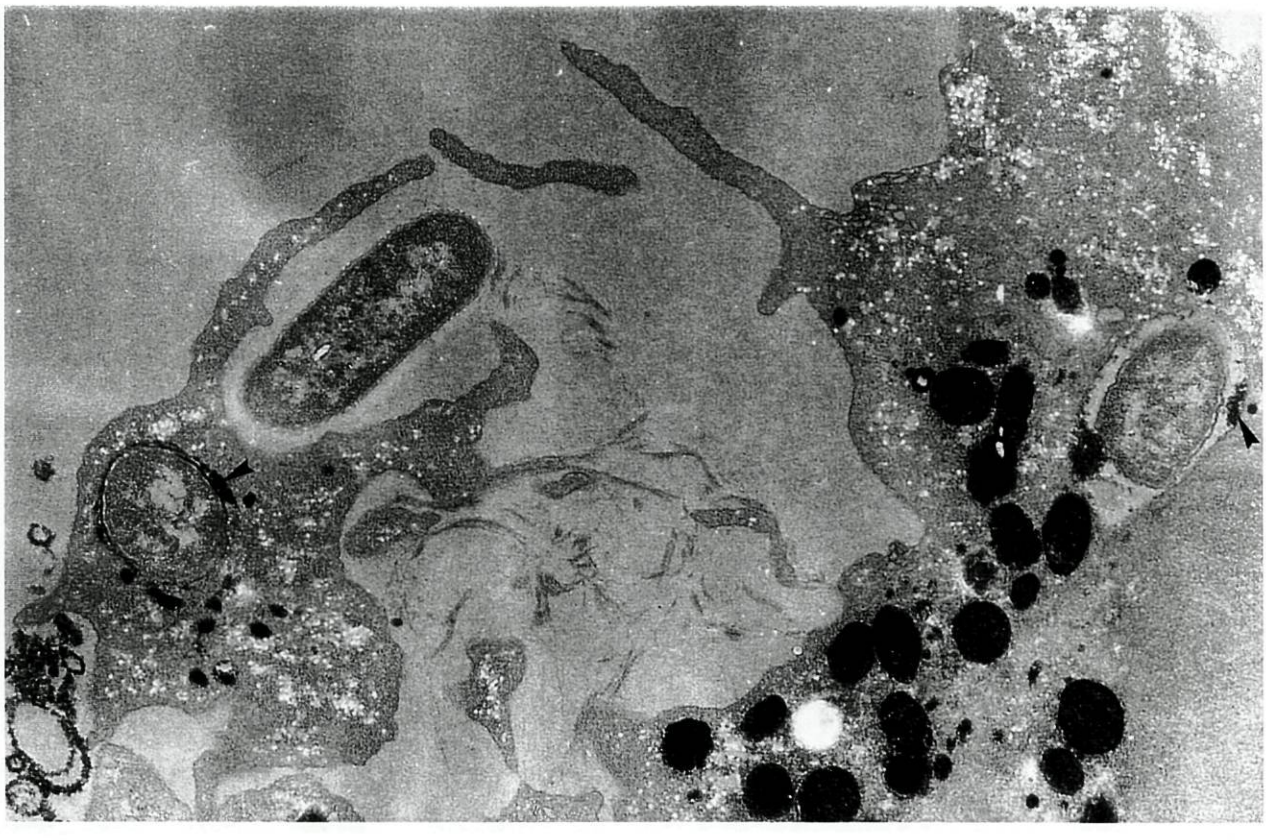


Fig. 2. Two neutrophil fragments in a peritoneal exudate of a trout inoculated i.p. with live *Yersinia ruckeri* 16 h before the harvesting of the peritoneal leucocytes. Sample processed for peroxidase and post-fixed with osmium tetroxide in acetate-veronal buffer. Notice the peroxidase-positive neutrophil granules and peroxidatic reaction in 2 phagocytic vacuoles containing bacteria (arrowheads). The neutrophilic cytoplasm has numerous small electron-transparent areas, which represent unstained glycogen granules. One bacterium was in the process of being phagocytosed. Section contrasted with lead citrate. ×13 640■

were always scarce (Fig. 4), except when, occasionally, clusters of polysaccharide granules were found in phagocytic vacuoles or in intramacrophagic neutrophils.

Ultrastructural detection of ANB-esterase

Electron microscopy of rainbow trout peritoneal phagocytes stained for the detection of ANB esterase showed, as reported for mammals (Bozdech & Bainton 1981), a characteristic dense staining of the surface of macrophages whereas neutrophils were negative. The simultaneous staining for esterase and peroxidase showed resident macrophages of the resting peritoneal cavity and some macrophages in the inflamed cavities with an esterase-positive surface and no peroxidase-positive sites (Fig. 5), and neutrophils with peroxidase-positive cytoplasmic granules and an esterase-negative cell surface (Fig. 6). Some macrophages in the inflammatory peritoneal exudate labelled by the typical esterase-positive surface were found to have peroxidase-positive cytoplasmic vacuoles and/or granules (Fig. 6, arrow), thus confirming the observations reported above of inflammatory macrophages with peroxidase-positive sites.

Kinetics of peritoneal neutrophil response to phlogistic agents

The injection of casein, IFA and bacteria (Fig. 7A–D) resulted in strong inflammatory responses compared to PBS (Fig. 7E), carbon and sham injections. In the latter 3 situations, small perturbations occurred in neutrophil numbers soon after treatment but values had returned to normal after several hours. Data from PBS injected fish (Fig. 7E) are representative of fish injected with India ink and sham-injected fish (not shown).

Neutrophilic responses to strong inflammation (casein, IFA and bacteria) are shown in Fig. 7A–D. In the case of live bacteria, neutrophil influx into the peritoneal cavity peaked at 24 to 48 h and resulted in a 500-fold increase in number. Macrophage responses (Fig. 7A–D) peaked at 2 to 4 d and the increase was comparatively more modest (7.5×). In the case of IFA and bacteria, at 48 h the neutrophil numbers were greater than the macrophage number; casein neutrophil numbers were below but close to those of macrophages. The extensive influx of neutrophils into the peritoneal cavities injected with bacteria had almost completely subsided 8 d after the i.p. inoculation (Fig. 7C, D). In the 3 situations of extensive inflammation the number of peritoneal leucocytes had returned to normal after 15 d, except in the case of IFA, when after 30 d the values were still slightly above normal (Fig. 7A–D).

Rainbow trout inoculated with 5×10^7 live *Yersinia ruckeri* recovered from the infection, as judged from the disappearance of the bacteria from the peritoneal exudates 4 d post-inoculation and from the absence of clinical signs of infection.

Phagocytosis of bacteria by neutrophils and macrophages

The distribution of phagocytosed bacteria in the 2 phagocytes of the peritoneal cavity of rainbow trout

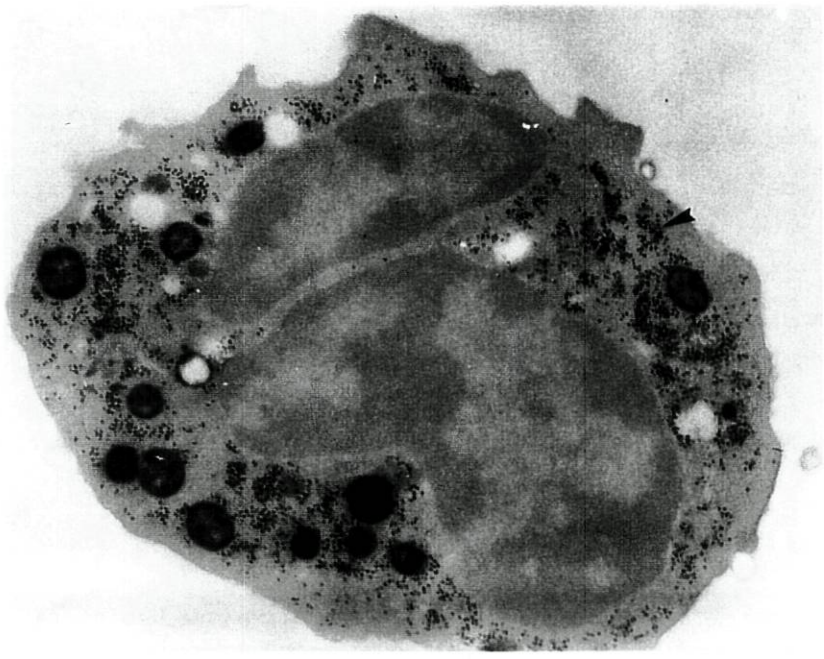


Fig. 3. Neutrophil in the peritoneal exudate of a trout 44 h after the injection of Incomplete Freund's Adjuvant (IFA). Sample processed for peroxidase and section stained for glycogen using Thiéry's method. Notice the peroxidase-positive cytoplasmic granules and the Periodic Acid-Schiff (PAS)-positive glycogen inclusions (arrowhead). ×15 120

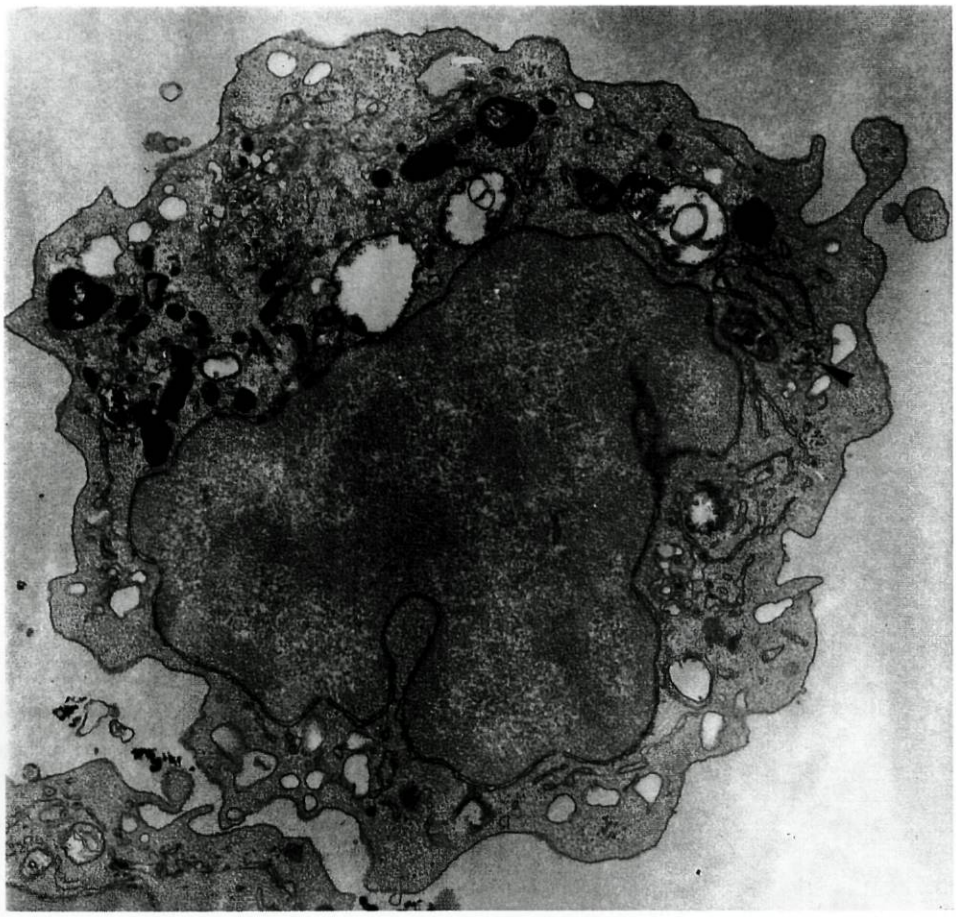


Fig. 4. Electron micrograph of a macrophage in the peritoneal exudate of a rainbow trout 20 h after i.p. inoculation with live *Yersinia ruckeri*. Sample processed for peroxidase and post-fixed with osmium tetroxide supplemented with potassium ferrocyanide. Notice the presence of peroxidase-positive cytoplasmic granules and vacuoles. Only a few scattered glycogen granules are visible in the cytoplasm (arrowhead). Section contrasted with lead citrate. ×13 600

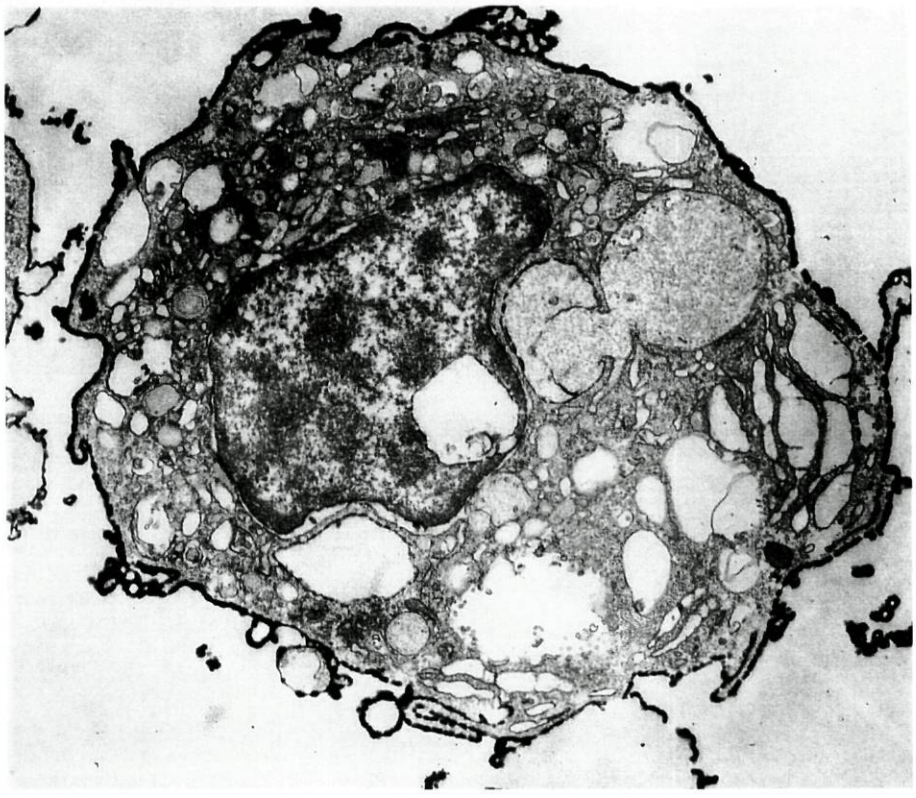


Fig. 5. Macrophage from an unstimulated peritoneal cavity of a rainbow trout. Sample processed for a alpha-naphthyl butyrate (ANB) esterase followed by peroxidase. Note the strongly esterase-positive cell surface and absence of peroxidase staining of the cytoplasmic granules. Section contrasted with lead citrate. ×9000

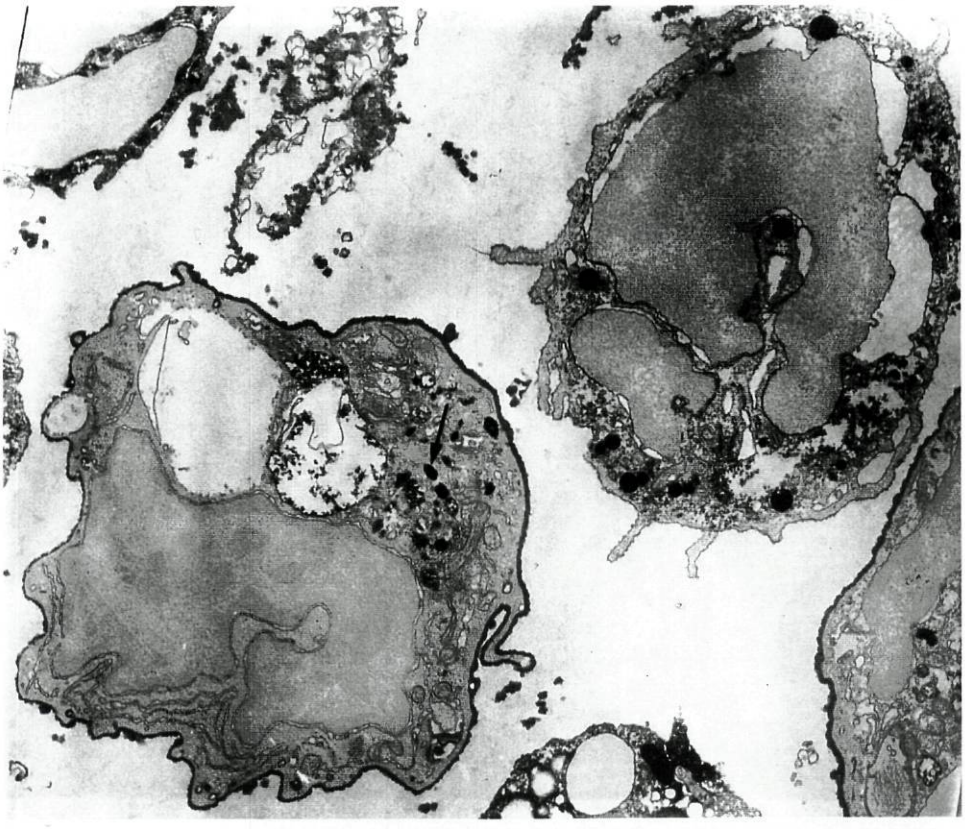


Fig. 6. Same sample as in Fig. 4 but processed for ANB esterase followed by peroxidase staining. Notice the neutrophil (right-hand side) with peroxidase-positive granules and no esterase staining of the cell surface. A macrophage, labelled by a strong esterase staining on its surface, contains several peroxidase-positive cytoplasmic granules (arrow) similar to those present in the neutrophil, and a large vacuole with peroxidase-positive material. Section stained with lead citrate. ×7600

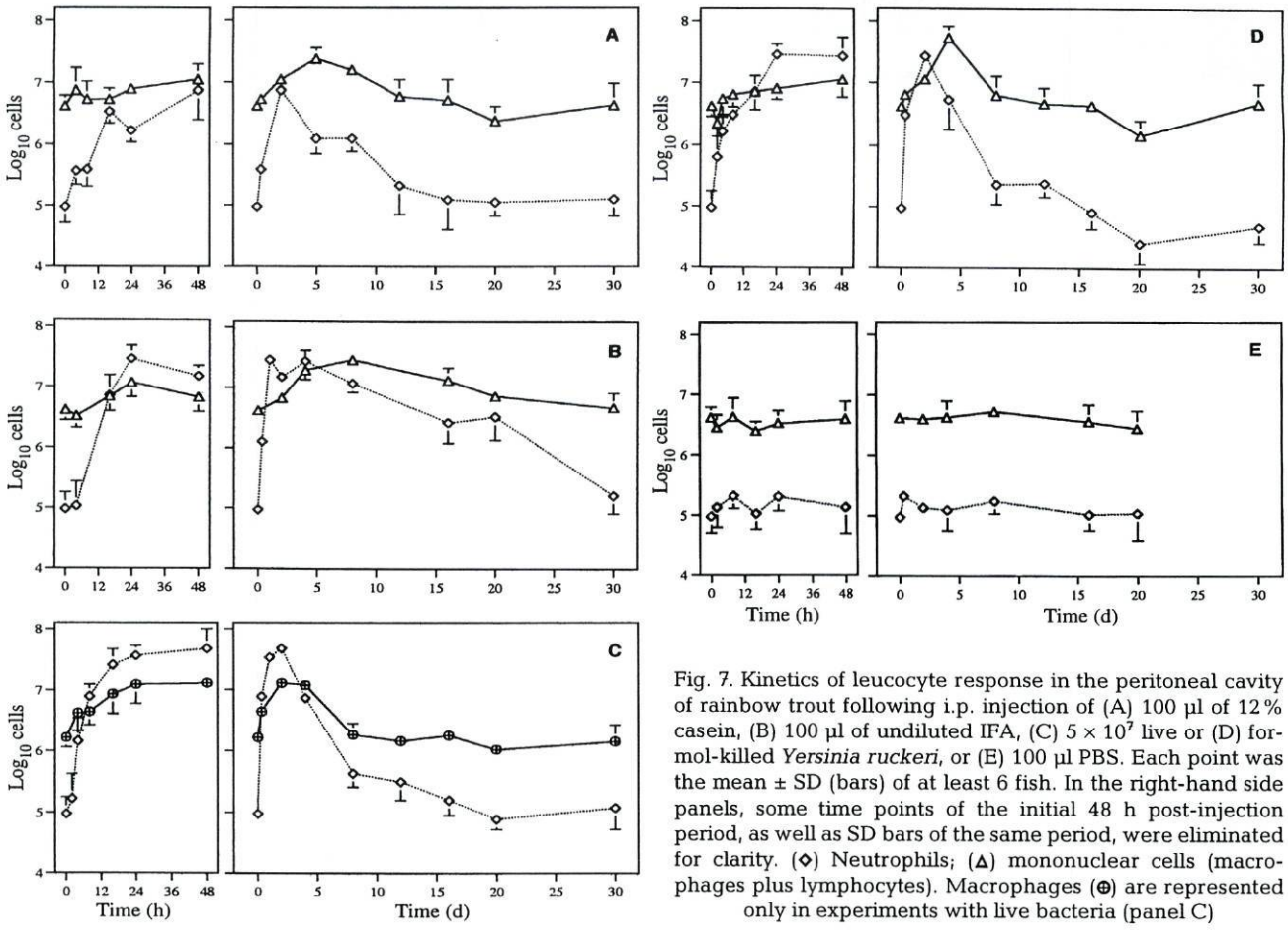


Fig. 7. Kinetics of leucocyte response in the peritoneal cavity of rainbow trout following i.p. injection of (A) 100 µl of 12% casein, (B) 100 µl of undiluted IFA, (C) 5×10^7 live or (D) formol-killed *Yersinia ruckeri*, or (E) 100 µl PBS. Each point was the mean ± SD (bars) of at least 6 fish. In the right-hand side panels, some time points of the initial 48 h post-injection period, as well as SD bars of the same period, were eliminated for clarity. (◇) Neutrophils; (△) mononuclear cells (macrophages plus lymphocytes). Macrophages (⊕) are represented only in experiments with live bacteria (panel C)

was analysed in cytospin preparations stained using Antonow's technique. Table 1 shows that following the injection of *Yersinia ruckeri* into the resting peritoneal cavity significantly more macrophages than neutrophils had ingested bacteria. However, this distribution of bacteria in the 2 phagocytes was changed when bacteria were injected into peritoneal cavities rendered inflamed by the previous injection of casein. In this case, a higher number of neutrophils than macrophages with ingested microrganisms was found, with percentages of neutrophils with bacteria approaching those of macrophages with bacteria (Table 2).

Cytochemistry of blood monocytes

Blood monocytes from normal trout were found by light and electron microscopy to be peroxidase-negative, esterase-positive and poor in glycogen granules (not shown).

DISCUSSION

One interesting aspect regarding the participation of phagocytosis in defence mechanisms against infection in trout, as in other vertebrates, is the utilization of 2 leukocytes with phagocytic capacities, the neutrophil and the macrophage. To obtain reliable, quantitative data on the participation of each of these phagocytes in antimicrobial mechanisms, an unequivocal distinction between them is necessary. Since neutrophils and macrophages in inflammatory sites in fish can be impossible to distinguish using simple morphological criteria, the present study used cytochemical techniques, including ultrastructural double-labellings (peroxidase followed by periodic acid-Schiff reaction, PAS, and peroxidase following ANB esterase).

Table 1. Number of peritoneal macrophages and neutrophils with bacteria after i.p. inoculation of 5×10^7 live *Yersinia ruckeri* into the unstimulated peritoneal cavity of rainbow trout (same experiment as in Fig. 7C). N = 6. Number of macrophages or neutrophils with bacteria per 100 total macrophages or neutrophils given in parentheses. p-values (Student's *t*-test) for the comparison between number of macrophages with bacteria and number of neutrophils with bacteria were $p < 0.005$ for the 3 time points studied

Time after bacterial challenge (h)	Macrophages $\times 10^4 \pm$ SD	(%)	Neutrophils $\times 10^4 \pm$ SD	(%)
4	79.312 ± 48.43	(19.2)	8.683 ± 6.20	(6.0)
8	66.372 ± 32.23	(15.17)	14.839 ± 6.44	(1.9)
24	88.596 ± 52.80	(7.04)	41.840 ± 16.48	(1.15)

Table 2. Number of peritoneal macrophages and neutrophils with bacteria in the peritoneal cavity of rainbow trout injected i.p. with live 3×10^8 *Yersinia ruckeri* 48 h after i.p. injection of 100 µl of 12% casein. N = 5. Number of macrophages or neutrophils with bacteria per 100 total macrophages or neutrophils given in parentheses. p-values (Student's *t*-test) for the comparison between number of macrophages with bacteria and number of neutrophils with bacteria were $p = 0.360$ (6 h), $p = 0.144$ (8 h) and $p < 0.05$ (24 h). p-values for the comparison of number of neutrophils with bacteria in Table 1 with number of neutrophils with bacteria in Table 2 were $p < 0.005$ for 8 and 24 h

Time after bacterial challenge (h)	Macrophages $\times 10^4 \pm$ SD	(%)	Neutrophils $\times 10^4 \pm$ SD	(%)
6	105.96 ± 25.05	(18.0)	126.66 ± 40.35	(15.4)
8	177.89 ± 72.75	(23.0)	257.24 ± 81.83	(19.9)
24	418.71 ± 60.10	(22.7)	570.34 ± 102.58	(8.2)

Our results show that peroxidase staining is an essential label for neutrophils. The classical method of Kaplow is quite efficient for the purpose of detecting peroxidase activity in leukocytes, but the intense, dark-blue staining of neutrophil granules makes it difficult or impossible to visualize intraneutrophilic bacteria. Antonow's method as described herein, which results in a green staining of peroxidase-positive sites, allows for a clear visualization of both peroxidase activity and bacteria. Other advantages of Antonow's method over Kaplow's are the possibility of using Wright counterstaining, which makes it easier to classify leukocytes, and this method's insensitivity to light and long-term stability, which allows for the stocking of preparations over long periods of time.

In contrast to that which is reported for mammalian neutrophilic myeloperoxidase (Horton et al. 1996), we found that trout neutrophilic peroxidase is cyanide-resistant. In this respect, peroxidase of trout neutrophils behaves like mammalian eosinophil peroxidase (Horton et al. 1996).

The present results show that peritoneal inflammatory macrophages, in contrast to resting resident macrophages (Afonso et al. 1997) and blood monocytes (present results), can have peroxidase activity. This might complicate the differential labelling of neutrophils and macrophages in inflamed areas. Our study shows, however, that a an unambiguous distinction between the 2 phagocytes, even when macrophages are peroxidase-positive, can be achieved. Firstly, as described above, the patterns of peroxidase positivity in light microscopic preparations are different for neutrophils and macrophages. Secondly, the ultrastructural double-labellings (peroxidase + PAS, ANB esterase + peroxidase) allow for a clear characterization of peroxidase-positive phagocytes: neutrophils are strongly

peroxidase-positive, esterase-negative and rich in glycogen granules, while peroxidase-positive macrophages are, like the peroxidase-negative macrophages and blood monocytes, esterase-positive and poor in glycogen granules. The double-labellings moreover, permit, the characterization of small portions of phagocytes, when most of the cellular details are not present in the picture as in the case with tangential sections (cf. Fig. 2). Details about the occurrence of peroxidase-positive macrophages in inflammatory peritoneal exudates of rainbow trout are given elsewhere (Afonso et al. 1998).

Taking advantage of the capacity of Antonow's technique for the simultaneous visualization of peroxidase activity and bacteria, we found that both the neutrophil and the macrophage of rainbow trout, given the opportunity, phagocytose bacteria. The much higher percentage of macrophages with bacteria compared to neutrophils when *Yersinia ruckeri* was injected into resting peritoneal cavities can be explained by the high predominance of the mononuclear phagocytes in the population receiving the injected bacteria. The opportunity for phagocytosis by the neutrophils that are attracted to the cavity following the injection of the bacteria will largely depend on the number of bacteria still extracellular at the time of neutrophil arrival. Such an opportunity will, obviously, be higher when the total number of bacteria injected is above the phagocytic capacity of the resident peritoneal macrophages. It is interesting that, when the injection of bacteria is made into cavities with a high number of neutrophils, these phagocytes extensively ingested bacteria, confirming that the actual phagocytic index for the 2 phagocytes in a given situation significantly reflects the opportunity they have to encounter the infectious agent.

The results from previous (Afonso et al. 1997) and present studies show that in rainbow trout, as in the mouse, the 2 professional phagocytes display different patterns of localization in the normal host and mobilization into inflamed areas. The scant neutrophil population of the resting, non-inflamed peritoneal cavity of rainbow trout (Afonso et al. 1997) is quickly increased in intense inflammatory situations by an extensive influx of neutrophils attracted from the reserve pools in hematopoietic organs and blood. The important permanent population of resident peritoneal macrophages (Afonso et al. 1997) increases, as well, although less extensively and more slowly than the neutrophil population. This increase in macrophage number is presumably due to an influx of monocytes from the reserve pools, as is well documented for the mouse (DeBakker et al. 1985, Appelberg et al. 1991, Pestana 1994). It is worth stressing that at some early points in time after the injection of bacteria and IFA the number of neutrophils was greater than the number of macrophages. The observation that 8 d after the inoculation of *Yersinia ruckeri*, when the fish had already recovered from the infection, the number of peritoneal neutrophils was very close to normal shows that neutrophil numbers can return rather quickly to basal values. This is explained by the short lifespan of neutrophils and represents an advantage for the host in the sense that the potentially harmful presence of these cytotoxic leukocytes in the tissues is shortened. The finding that 30 d after the i.p. injection of IFA neutrophil numbers were still above normal may well be interpreted as resulting from the very slow elimination of the adjuvant's mineral oil maintaining the inflammatory situation. This shows that an analysis of the peritoneal neutrophilic response may be useful for the study of new adjuvant substances.

Since the mobilization of phagocytes into the peritoneal cavity is similar to that found for tissues (van Furth 1992), the present results indicate, therefore, that in fish, like in mammals (Lehrer et al. 1988, Hasslet et al. 1989, Silva et al. 1989, van Furth 1992, Densen et al. 1995, Kubicka et al. 1996), macrophages are the tissue resident phagocytes while the neutrophils are present in tissues in significant numbers only in situations of inflammation and only as long as the inflammation persists.

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Peculiar leukocyte response to the intraperitoneal injection of virulent *Renibacterium salmoninarum* in rainbow trout (*Oncorhynchus mykiss*)

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(Unpublished manuscript)

ABSTRACT

The intraperitoneal (i.p.) injection of *Renibacterium salmoninarum* strain FT-10, virulent for salmonid fish, in rainbow trout was not followed by the usual early neutrophil influx seen with other bacteria, including the non-virulent renibacterial strain MT-239. Moreover, the i.p. injection of the virulent renibacteria induced, after the initial 8 days, an extensive aggregation of peritoneal leukocytes. Such an aggregation was not found when the non-virulent strain was used. The formation of these cell clumps precluded counting of the leukocyte by conventional cytological methods in exudates collected after the first 8 days of infection. Therfore, to indirectly quantify the neutrophil response during the 60 days of the infection we used a technique for peroxidase assays in inflammatory peritoneal exudates we previously adapted to fish.

The present results showed a very good correlation ($r^2 = 0.926$) between the number of neutrophils and peroxidase activity in peritoneal exudates collected during the first 8 days of infection, when no leukocyte clumps were present. The use of the peroxidase technique showed that a persistent and important neutrophilia was maintained from day 8 after infection with the virulent *R. salmoninarum* strain until the end of the experiment (60 days), when the fish started to die.

INTRODUCTION

Renibacterium salmoninarum is the agent of bacterial kidney disease (Sanders & Fryer, 1980), a systemic, granulomatous infection of salmonid fish. This infection, with a worldwide distribution, has important economic consequences because it is one of the most important infection of hatchery-reared salmonids (Fryer & Sanders, 1981). Additionally, the disease shows interesting microbiological and immunological aspects. In fact, renibacteria are obligate pathogens specific for salmonids (Sanders & Fryer, 1980), with an intracellular growth (Young & Chapman, 1978; Bruno, 1986; Flaño *et al.*, 1996a, b; Gutenberger *et al.*, 1997). The microrganism is difficult to cultivate in bacteriological media and is a very slow grower (Evelyn, 1993). It usually produces a

slowly progressing infection (Fryer & Sanders, 1981; Austin & Austin, 1987) which is resistant to chemotherapy (Elliot *et al.*, 1989), and no effective vaccine has been produced so far (Munro & Bruno, 1988). The disease is spread among susceptible fish by horizontal as well as vertical transmission (Evelyn, 1993). Finally, the virulent strains, but not the avirulent ones, produce huge amounts of an auto-aggregating surface protein (p57) with agglutinating properties for leukocytes of salmonid fish and erythrocytes of several animals (Wiens & Kaattari, 1991; Bandin *et al*, 1989), and with a suppressive action on antibody production in salmonids (Turaga, Wiens & Kaattari, 1987a, b; Fredriksen *et al.*, 1997).

During our previous studies on the neutrophil response to intraperitoneal inflammation induced by bacteria (Afonso *et al.*, 1998a,b) we found that the peritoneal leukocyte reponse following the intraperitoneal injection (i.p.) of a virulent strain of *R. salmoninarum* could not be quantified beyond the initial 8 days by conventional cytological methods due to the formation of abundant and large cell clumps. That situation prompted us to use the peroxidase assay previously developed for the indirect quantification of neutrophil responses in inflammatory foci (Afonso *et al.*, 1998c).

In the present paper the kinetics of neutrophil influxes into the peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*) following the i. p. inoculation of *R. salmoninarum* is described. We found that the neutrophil response induced by this Gram-positive bacterium can be evaluated by the peroxidase technique and also that the response induced by a virulent strain of the bacterium was at variance from that seen with other bacterial agents, including a non-virulent strain of renibacteria.

MATERIALS AND METHODS

Experimental fish

Sexually immature rainbow trout weighing between 100 and 200g, were purchased from a commercial farm. The animals were maintained in recirculating aerated fresh water, at 10^{o}C, in 7000 l fibreglass tanks. Water quality was maintained with mechanical and biological filtration and fish were fed *ad libitum* on commercial pellets.

The fish to be injected were transferred to 600 l tanks with water at the same temperature, where they were maintained for at least 15 days before the study. Only healthy fish, as concluded from their activity and external appearance were used for the i.p. injections.

Growth, harvesting and treatment of bacterial agents

A virulent strain of *R. salmoninarum* (strain FT-10) was kindly supplied by Prof. J. Barja (University of Santiago de Compostela, Spain). The low virulence strain MT-239 was kindly supplied by Dr. D. W. Bruno (Marine Laboratory, Aberdeen). To prepare the suspensions, bacteria grown on KDM 2 medium (Evelyn, 1977) for 15 days at 15°C were suspended in L-15 Leibovitz medium (Gibco BRL) with 5 % foetal bovine serum, divided in aliquots and frozen at -70°C. The inocula to be injected were prepared by washing and resuspending the above aliquots in phosphate buffered saline (PBS) with osmolality adjusted to 310 mOsm $^{-1}$ /l, at 1 x 10^9 bacteria ml^{-1}. Bacteria were counted in a Helber Bacteria Counting Chamber (Fisons Scientific Equipment, Loughborough, Leics., UK).

Intraperitoneal injection of bacteria

Suspensions of bacteria were injected into the peritoneal cavity of groups of fish under anaesthesia (0.3ml ethylene glycol monophenyl ether per l, Merck), in volumes of 100µl using a 25G needle. Immediately after the i.p. injection the fish were returned to their tank.

Control fish were injected i. p. with 100µl of PBS.

Collection of peritoneal exudates

The peritoneal exudates were collected from non-injected fish and, at intervals, after the i.p. injections by a procedure previously described in detail (Afonso *et al.*, 1997, 1998b). Briefly, after killing the fish by anaesthetic overdose and exsanguination, 5ml of 50mM sodium acetate buffer, pH 5.4, supplemented with 0.3M sucrose

(Bozeman *et al.*, 1990) and 10 units ml^{-1} heparin (Choay), or 5ml of PBS with heparin (Afonso *et al.*, 1997, 1998b), was injected i.p. in the ventral midline followed by injection through the same needle of about 1 ml of air. The abdominal area was massaged for about 30 seconds to disperse the peritoneal cells in the injected buffer and the cells in suspension were then collected with a syringe fitted with a 19 G needle.

Peritoneal cell suspensions were placed on ice until processed for cytological, bacteriological and peroxidase assays. Haemorrhagic peritoneal exudates were discarded.

Morphological study of peritoneal leukocytes

The peritoneal cell suspensions were analysed within minutes after collection for several morphological and cytochemical parameters as follows.

Cytology

Total cell counts were performed with a hemocytometer. Cytospin preparations were made with a Shandon Cytospin 2 using a volume of peritoneal lavage depending on the cell concentration, in order to obtain adequate numbers of cells per slide. The cytospins were stained with Wright´s stain (Haemacolor, Merck) after fixation for 1 min with formol-ethanol (10ml of 37 % formaldehyde and 90ml of absolute ethanol) (Kaplow, 1965).

Bacteria were stained by the Gram´s method, using commercially available reagents.

For transmission electron microscopy of ultrathin sections, previously described methods were used (Silva & Macedo, 1983, Silva *et al.*, 1987).

Cytochemistry

The method of Kaplow (Kaplow, 1965) or the Antonow´s technique (Afonso *et al.*, 1998a) were used for the detection of peroxidase activity of peritoneal leukocytes in cytospins.

Spectrophotometric peroxidase assay

The tetramethylbenzidine (TMB) method of Bozeman *et al.* (1990) with minor alterations (Afonso *et al.*, 1998 c) was used for the quantitative assay of peroxidase activity in peritoneal exudates. Since in rainbow trout neutrophils are the only leukocytes with endogenous peroxidase activity (Afonso *et al.*, 1997, 1998a), neutrophilic peroxidase can be measured in suspensions containing neutrophils together with the other leukocytes.

The method was applied to total peritoneal exudates or, separately, to their leukocyte population and extracellular fluid as described before (Afonso *et al.*, 1998 c).

Absorbance readings for peroxidase activity of peritoneal leukocyte suspensions are expressed as ΔOD_{655} min^{-1}.

Statistics

Values were analysed by the Student´s *t*-Test. Correlation coefficients (r^2) were calculated with the Staworks ANOVA Program.

RESULTS

Cytology of peritoneal exudates following i. p. injection of *R. salmoninarum.*

Following the i.p. injection of *R. salmoninarum* (both strains), the bacteria were quickly phagocytosed by the resident peritoneal macrophages. This was seen both in cytospin preparations of peritoneal exudates stained for peroxidase activity by the Antonow´s technique (Fig 1 and 2), and by electron microscopy (Fig. 9-11). In samples collected at 4h after the i.p. injection of the virulent strain and processed for ultrastructural analysis, renibacteria were already seen inside macrophagic phagosomes (Fig. 10 and 12). As previously described for *Yersinia ruckeri* (Afonso *et al.*, 1998a), only very rare neutrophils were seen with phagocytosed renibacteria during the initial phases of the infection. In very advanced stages of infection by the virulent strain numerous free bacteria were frequently seen (Fig. 3 and 4; see also Fig. 28).

The cytological study of peritoneal exudates collected at intervals from trout injected i.p. with the virulent strain of *R. salmoninarum* (FT-10) showed that, from day 8 post-injection until the end of the experiment (60 days), numerous cell clumps were present (Fig. 6-8). That these clumps were not artifacts produced after the collection of the exudates, but rather formed inside the peritoneal cavity, was concluded from the observation of their presence in the peritoneal cell suspensions just after their collection and before any processing.

The clumps were seen by electron microscopy of thin sections to consist of all the leukocyte types found in the peritoneal cavity of rainbow trout, that is, macrophages, neutrophils and lymphocytes. In many of the clumps the leukocytes were tightly packed with no intercellular spaces (Fig. 13-18). Intramacrophagic bacteria were frequently seen in these cell clumps (Fig. 13-16). The presence of neutrophils in the cell aggregates, barely discernible by light microscopy of preparations stained by the peroxidase methods, was well visualized by ultrastructural cytochemistry (Fig. 17 and 18).

With the non-virulent *R. salmoninarum* (strain MT-239), no cell clumps were seen during the 30 days of the experiment (Fig.5).

Correlation between neutrophil numbers and peroxidase activity

Fig. 19 shows the kinetics of cellular peroxidase activity and the number of neutrophils (as counted by cytological methods) in the peritoneal cavities of trout during the initial 8 days following the i. p. injection of 1×10^8 *R. salmoninarum* (strain FT-10). As previously found for other situations (Afonso *et al.*, 1998c), peritoneal cell-associated peroxidase activity closely followed the number of neutrophils; the r^2 for the correlation between those two parameters was 0.926.

The peroxidase assays in the exudates from fish infected with virulent renibacteria showed that the amount of peroxidase per neutrophil (PAN) (Afonso *et al.*, 1998c) varied very little in the period of time during which neutrophils could be counted by cytological methods (the initial 8 days) (Fig. 20). This was at variance with the results found with *Y. ruckeri* (Afonso *et al.*, 1998c) (Fig. 20).

Kinetics of peritoneal neutrophil responses following the i. p. injection of virulent and non-virulent *R. salmoninarum* in trout

Because the cell aggregates were very abundant and contained high numbers of peritoneal cells, cell counts by the usual cytological techniques could not, obviously, be accurately carried out after day 8. Therefore, after this time point, the number of neutrophils in the peritoneal cavities of fish injected with FT-10 strain of *R. salmoninarum* was calculated from the values of cell-associated peroxidase activity.

Fig. 21 shows the kinetics of the neutrophil responses to the i.p. injection of 1 x 10^8 *R. salmoninarum* (strains FT-10 and MT-239) in rainbow trout.

Evolution of the intraperitoneal infection following the i.p. injection of virulent and non-virulent *R. salmoninarum*

Because the growth of *R. salmoninarum* in bacteriological media is not reliable enough to allow the use of CFU counts (Gutenberger *et al.*, 1991), the progress of the i.p. infection was analysed by fish survival rates and by microscopic observation of the peritoneal exudates collected at various time intervals.

With strain MT-239 no bacteria were seen after the initial 48 hours following the i.p. injection.

In the case of the virulent strain FT-10, bacteria were consistently seen inside macrophages in samples collected at intervals during the 60 days of infection studied (Fig. 1, 2 and 22-28). At the later time points, numerous free bacteria were also visible (Fig. 3, 4 and 28). Because these extracellular bacteria were seen among cellular debris (Fig. 28) it is likely that they were released by phagocyte lysis. Interestingly, these extracellular bacteria were seen to be surrounded by an outer membrane (Fig. 29) which is not seen in bacteria grown in bacteriological media. This membrane most likely represents the phagosomal membrane which is kept apposed to the bacteria upon phagocyte lysis. When renibacteria freshly isolated from the peritoneal exudates with

large numbers of extracellular bacteria were cultivated *in vitro* in Mueller-Hinton medium, portions of that outer membrane were still attached to the bacterial surface after 8 days of culture (Fig. 30).

As reported above, with infection by strain FT-10, the numerous cell clumps seen after 8 days of infection frequently contained intramacrophagic bacteria (Fig. 13-16).

Fish started to die after 60 days of infection with the virulent strain FT-10. No mortality or morbidity was found in fish injected with strain MT-239.

DISCUSSION

One novel observation reported in the present study is the presence of abundant, large aggregates of leukocytes in the peritoneal cavities of trout injected i.p. with the virulent strain of *R. salmoninarum*. These cell clumps appeared after the initial 8 days of infection and persisted until the end of the experiment at 60 days. No such clumps were seen with the non-virulent strain. This leukoagglutination most likely is the effect of the activity of p 57, a protein produced and excreted in large amounts by the virulent renibacteria (Bruno, 1988), but not by the MT-239, non-virulent strain (Bruno, 1988). In fact, as shown by Wiens *et al.* (1991), that protein agglutinates salmonid leukocytes *in vitro*.

The present study shows that the peroxidase assay is a reliable technique for the indirect quantification of the neutrophil response that follows the i.p. injection of a virulent strain of *R. salmoninarum* in trout. Therefore, that technique allowed the quantification of the influx of neutrophils to the peritoneal cavity during the entire course of the infection, despite the occurrence of abundant, large aggregates of peritoneal leukocytes at the later times of the infection.

An interesting finding in the experiments with the virulent strain of renibacteria was the absence of the strong neutrophilia that has been found at 24-48 hours in all other instances of peritoneal infection both in trout and mice. Very importantly, the non-

virulent strain of renibacteria produced the usual neutrophil early response, although of lower magnitude and delayed as compared, for example, with that seen with *Y. ruckeri*.

Work in progress is aimed at disclosing the mechanism for the absence of the initial neutrophilic influx seen with the virulent renibacteria. Although the possibility exists for an effect of the low temperature at which the experiments were carried out (10^{o}C) -because this is the optimum temperature for the renibacterial infection- the fact that the non-virulent strain produced an early neutrophilic response at that temperature has to be considered. It is possible that virulence of *R. salmoninarum* for trout is associated with some capacity to delay the initial neutrophilic response which is typical of all the other similar situations studied. The determination of the peroxidase activity per neutrophil (PAN) in the peritoneal samples collected before the starting of leukocyte aggregation (up to 8 days) gave values only slightly above those of blood neutrophils of normal trout. The PAN values remained unchanged during the first 8 days. This is at variance with what had previously been found with the transient, acute, inflammation induced by the i.p. injection of *Y. ruckeri* in trout. (Afonso *et al.*, 1998c). In this later situation, the PAN values increased significantly at 24 and 48h after the bacterial injection, when the neutrophilia was at its highest, and decreased thereafter to values bellow the basal ones at day 6, when the neutrophil response was subsiding. This difference in the evolution of the PAN values between the two types of infection correlates well with the characteristics of each of the two neutrophil responses: acute and transient in the case of *Y. ruckeri*, and slow and persistent in the case of *R. salmoninarum*.

In the context of the discussion of this point, it has to be stressed that, as discussed above, the virulent strain of renibacteria, but not the non-virulent one, has the ability to aggregate leukocytes of salmonids due to the activity of p 57, as shown in *in vitro* tests (Wiens *et al.*, 1991) and *in vivo* (present results). We found, however, no evidence for the formation of leukocyte aggregates during the initial 8 days of infection, at least, no such cell clumps were found in the collected peritoneal exudates; and preliminary

observations showed it to be very unlikely that cell aggregates would be adhering to the peritoneal wall.

The virulent renibacteria induced, instead, a rather late neutrophil influx, which was very important and persisted to the end of the experiment, at 60 days, when the fish started to die from the progressive infection. It is interesting that the initial neutrophil response induced by the non-virulent renibacteria started to subside at 4 days after the i.p. inoculation, the neutrophils reaching almost normal after 20 days.

In conclusion, the peculiar neutrophilic response induced by the virulent *R. salmoninarum* strain provided an excellent opportunity to further test the applicability of the peroxidase technique as an indirect method for the quantitative assay of neutrophil numbers in inflammatory exudates.

Work is in progress in this Laboratory to take advantage from the very interesting model that the peritoneal infection by renibacteria in salmonids represents for the study of the role of neutrophils in the infection by intracellular bacteria. Namely, we are interested to know i- why the initial peak of neutrophilia is missing in the host response to the infection by the virulent strain; ii- if this absence of neutrophil early influx is responsible for the installation of the progressive infection; iii- why the persistent and important neutrophilic response that goes on after the initial 8 days of infection has no protective effect against the infection.

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LEGENDS TO THE FIGURES

Fig. 1 and 2

Peritoneal cells 12h after the i. p. injection of 1x 10^8 *R. salmoninarum*, strain FT-10. Cytospin stained for peroxidase activity by the Antonow technique. Several bacteria are present inside peroxidase-negative macrophages. The neutrophils are peroxidase-positive (green). x 680.

Fig. 3 and 4

Peritoneal cells 60 days after the i. p. injection of 1x 10^8 *R. salmoninarum*, strain FT-10. Cytospin stained by the Antonow technique. Notice the numerous extracellular bacteria, some of them in dense clumps. x 680.

Fig. 5 and 6

Low power photograph of cytospin preparations of trout peritoneal exudates collected 15 days after the i. p. injection of 1 x 10^8 *R. salmoninarum* strain MT-239 (Fig. 5) or FT-10 (Fig.6).

Fig. 7 and 8

Leukocyte aggregates in peritoneal exudates collected 15 days after the i.p. injection of 1x 10^8 *R. salmoninarum*, strain FT-10. Cytospin stained by the Antonow technique. x 45.

Fig. 9-12

Intramacrophagic renibacteria in a peritoneal exudate collected 4 h after the i.p. injection of 1x 10^8 *R. salmoninarum*, strain FT-10. The bacteria are inside phagosomes. Some of the phagosomes are fused with lysosomes (arrows in Fig. 9). At high magnification, the phagosomal membranes are clearly seen (arrows in Fig. 10 and 12). Sections stained with uranyl-lead . Fig. 9: x 24 500. Fig. 10: x 78 200. Fig. 11: x 19 600. Fig. 12: x 72 000.

Fig. 13-18

Electron microscopic images of sectioned leukocyte aggregates in peritoneal exudates collected at different times from trout injected i.p. with 1 x 10^8 *R. salmoninarum* , strain FT-10. Fig. 13 and 14: 11 days of infection. Fig. 15,17 and 18: 20 days of infection. Fig. 16: 60 days of infection. The samples in Fig. 17 and 18 were processed for the ultrastructural detection of peroxidase activity, and neutrophils are recognized by their peroxidase-positive cytoplasmic granules. Intramacrophagic bacteria are present in Fig.13, 14, 15 and 16. Sections contrasted with uranyl-lead, except Fig. 17 and 18 which were stained with lead alone. Fig. 13: x 11 600. Fig. 14: x 7 400. Fig. 15: x 7 400. Fig. 16: x 8 800. Fig. 17: x 5 700. Fig. 18: x 7 200.

Fig. 19

Number of neutrophils and peroxidase activity (both per peritoneal cavity) during the first 8 days following the i. p. injection of 1 x 10^8 *R. salmoninarum* (strain FT-10).

Fig. 20

Peroxidase activity per neutrophil (PAN) expressed as ΔOD_{655} min^{-1} x 10^{-6}, in peritoneal exudates collected during the first 8 days following the i. p. injection of 1 x 10^8 *R. salmoninarum* (strain FT-10). For comparison, the PAN values in peritoneal exudates collected during the initial 6 days after the i. p. injection of 5 x 10^7 *Y. ruckeri* are shown (values taken from Afonso *et al.*, 1998c). The baseline represents the value for blood neutrophils of normal rainbow trout (values taken from Afonso *et al.*, 1998c). * Denotes statistically significant differences ($P<0.05$).

Fig. 21

Kinetics of neutrophil response in the peritoneal cavities of rainbow trout injected i. p. with 1 x 10^8 *R. salmoninarum*, strains FT- 10 and MT-239. + denotes the

value for neutrophils at 48h after the i. p. injection of 5 x 10^7 *Y. ruckeri* in rainbow trout kept at 15°C.

Fig. 22-27

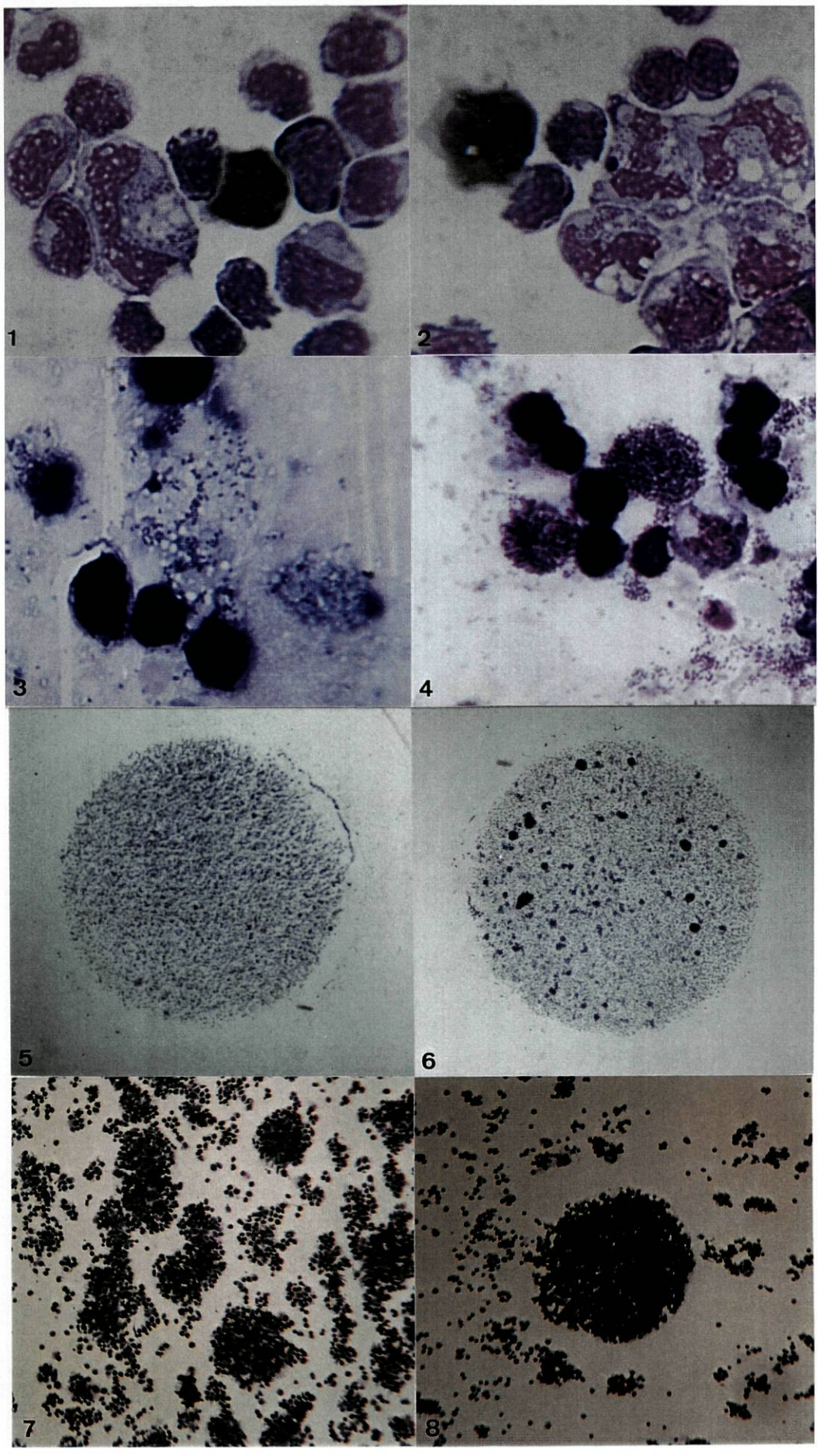
Ultrastructural images of renibacteria inside macrophages in peritoneal exudates of rainbow trout injected i. p. with 1 x 10^8 *R. salmoninarum*, strain FT- 10. Fig. 22: 1 day post-injection. Fig. 22 and 23 (x 11 700): 3 days. Fig. 24 (x 24 500): 13 days. Fig. 25 (x 11 600) and 26 (x 24 300): 21 days. Fig. 27 (x 43 500): 60 days.

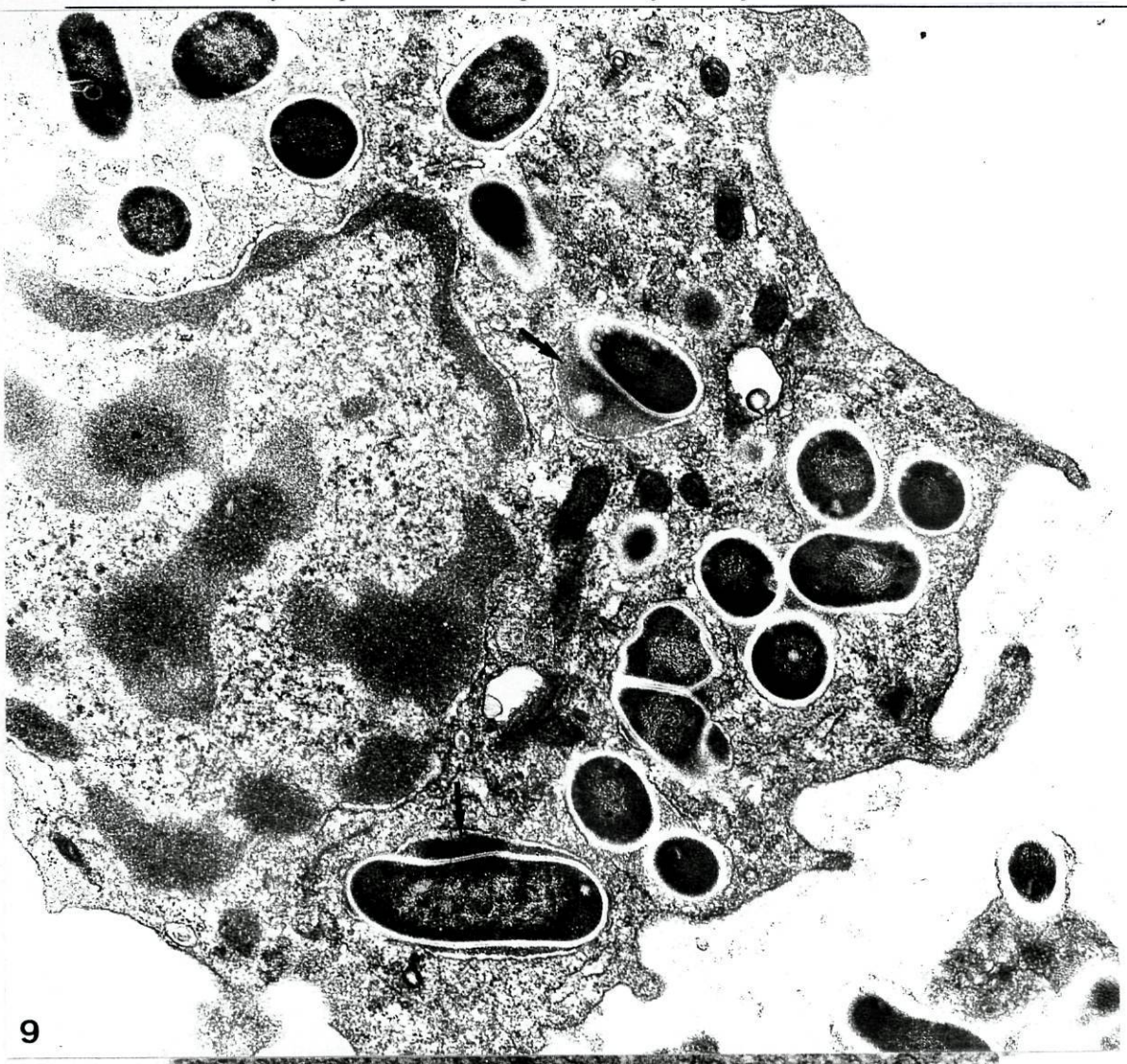
Fig. 28 and 29

Ultrastructural images of extracellular bacteria in a peritoneal exudate of a rainbow trout injected i. p. with 1 x 10^8 *R. salmoninarum*, strain FT- 10, 30 days before. The bacteria are among cell debris and show normal ultrastructural characteristics and some of them are under division. At the higher magnification (Fig. 29) the presence of an outer membrane surrounding the bacteria is clearly visible (arrow). Fig. 28: x 10 800. Fig. 29: x 81 600.

Fig. 30

R. salmoninarum, strain FT-10, from the sample in Fig. 30 and 31, 8 days after culture in Mueller-Hinton medium. Portions of the outer membrane present in the bacteria of the inoculum (and shown in Fig. 29) are still present (arrow). x 73 600.

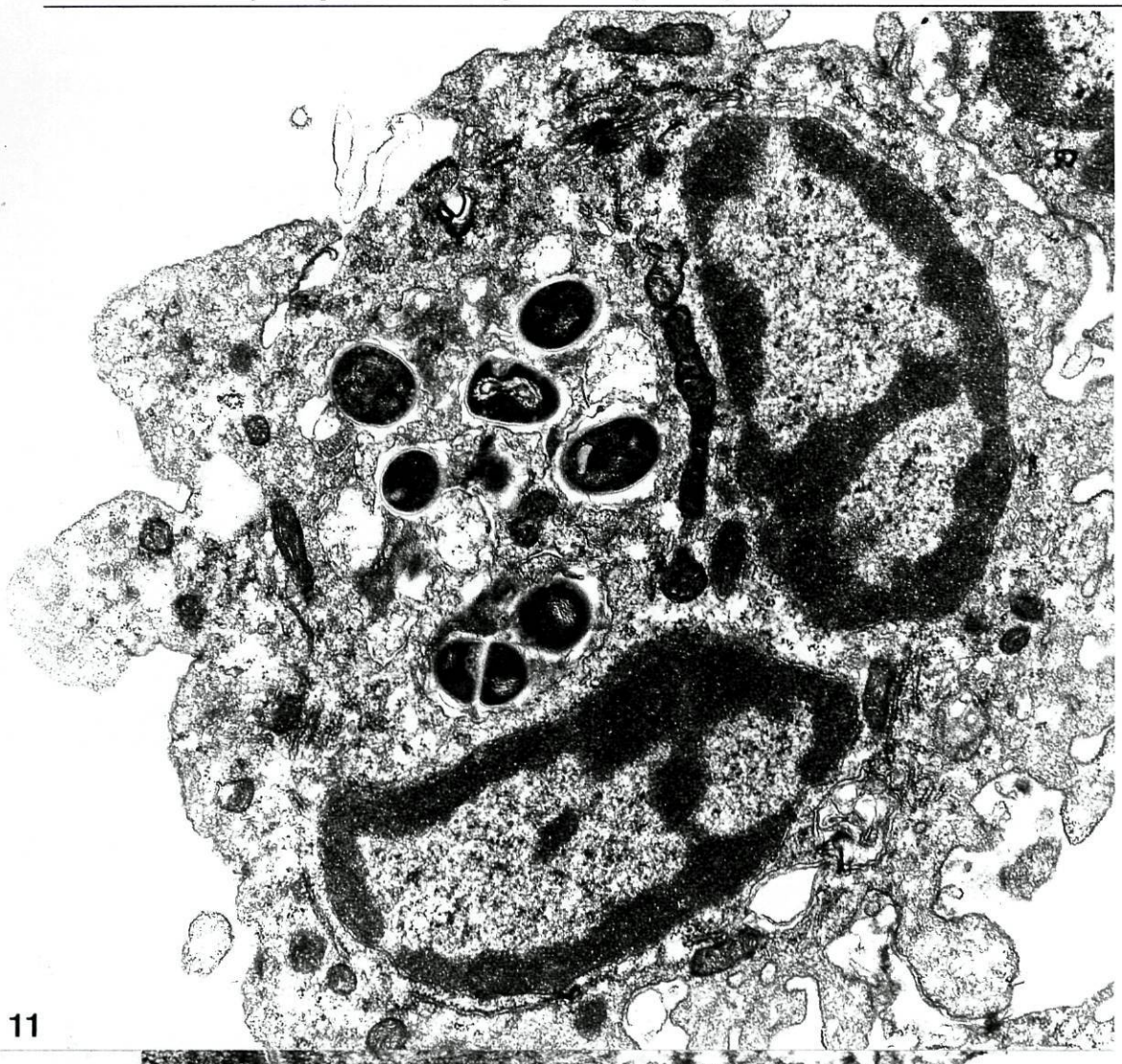

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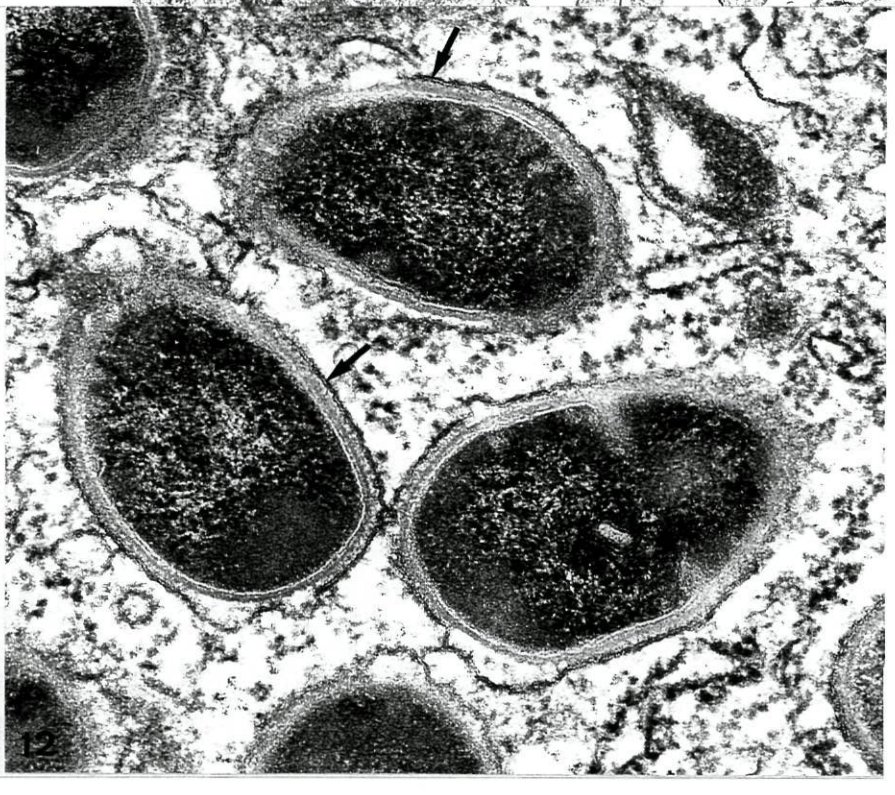
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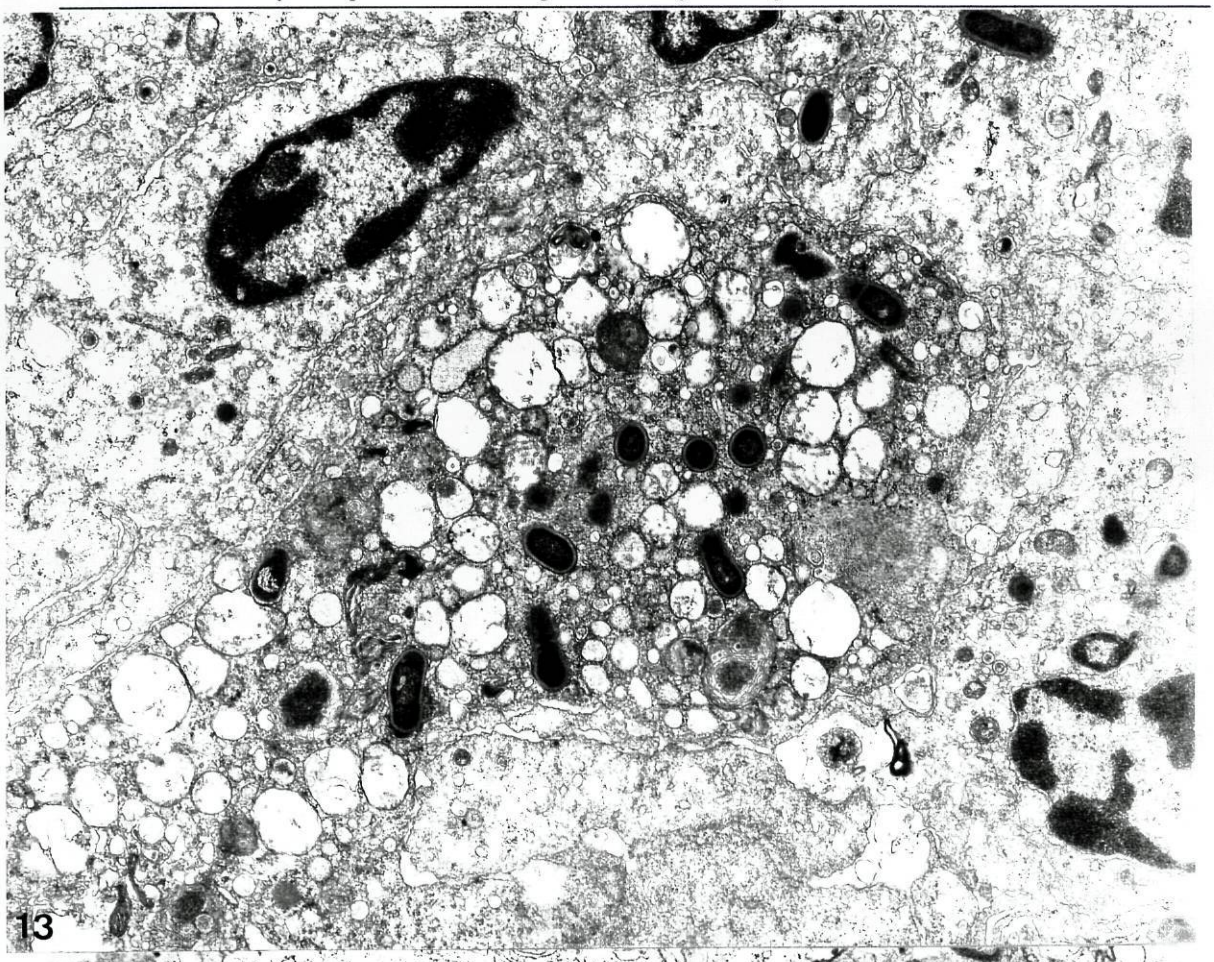
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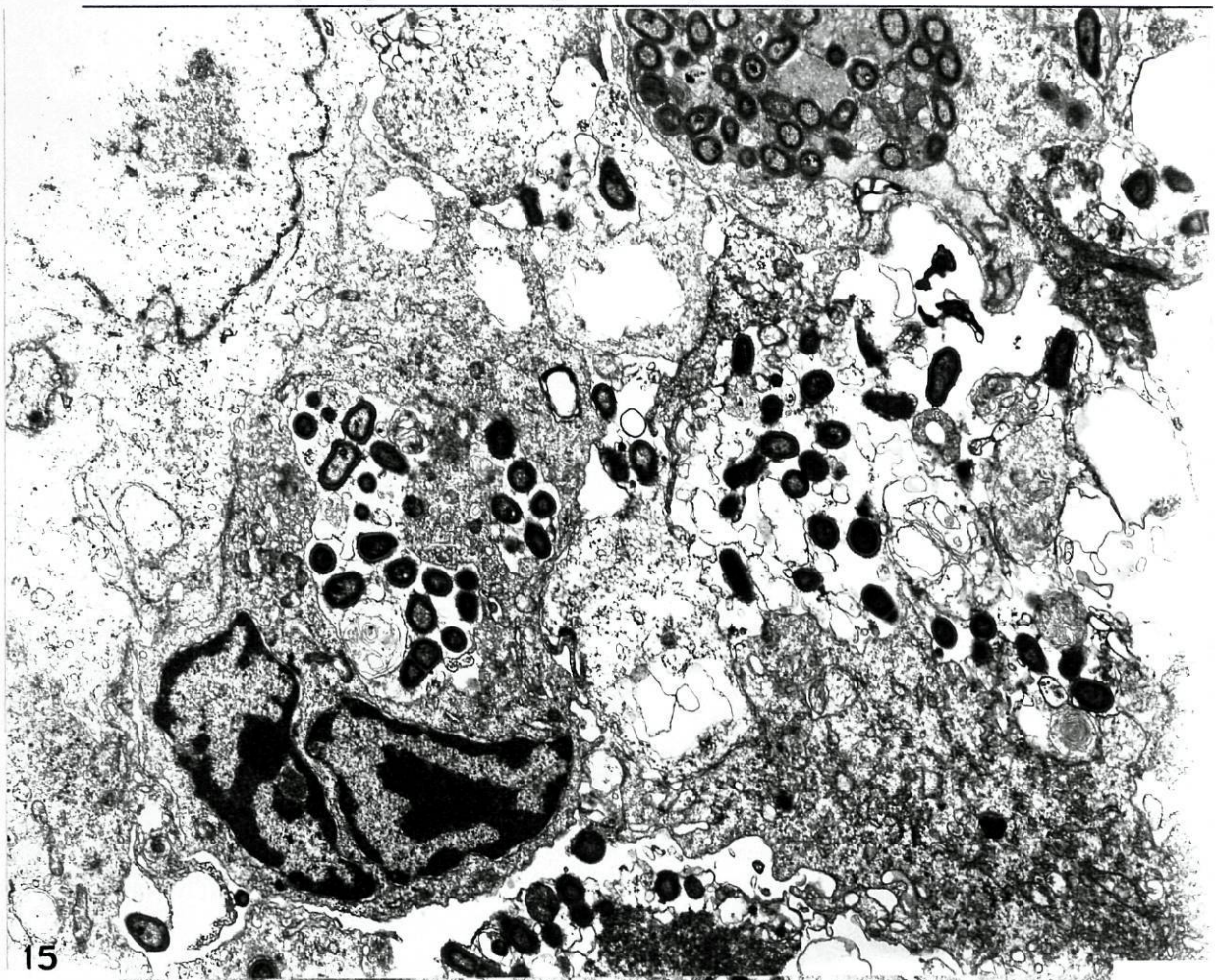
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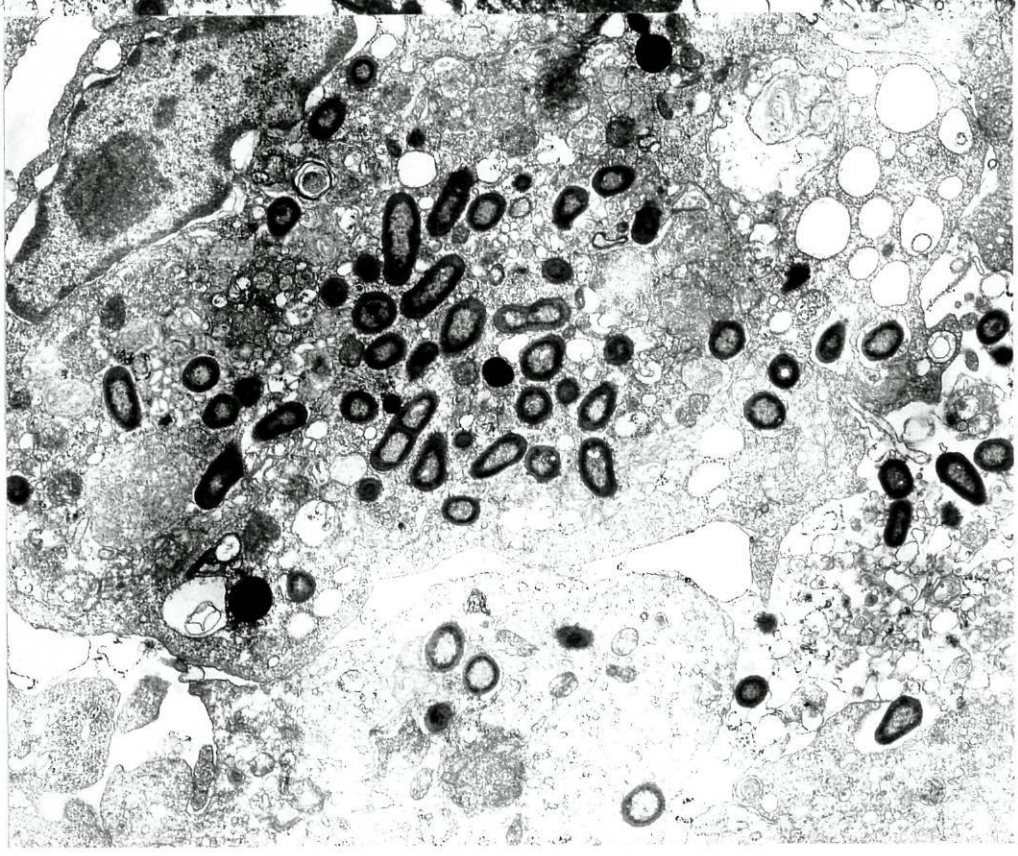
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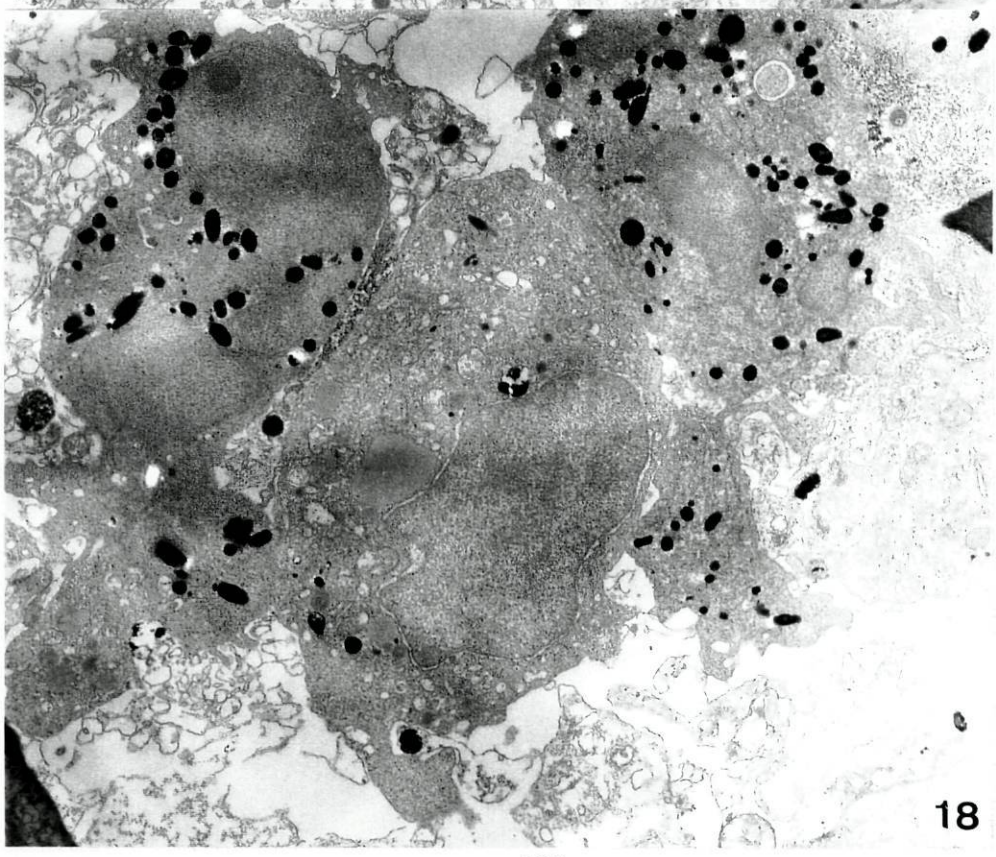
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17



18

Fig. 19

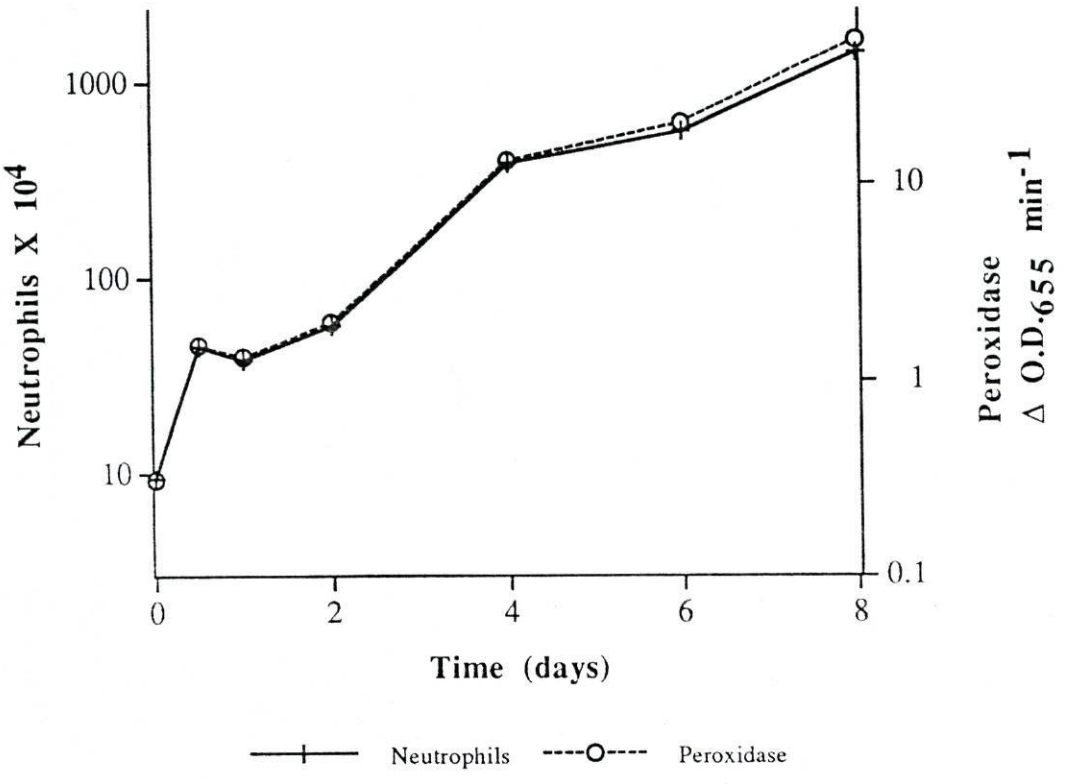

Neutrophils X 10^4
1000
100
10
Peroxidase
Δ O.D.$_{655}$ min^{-1}
10
1
0.1
0
2
4
6
8
Time (days)
Neutrophils
Peroxidase

Fig. 20

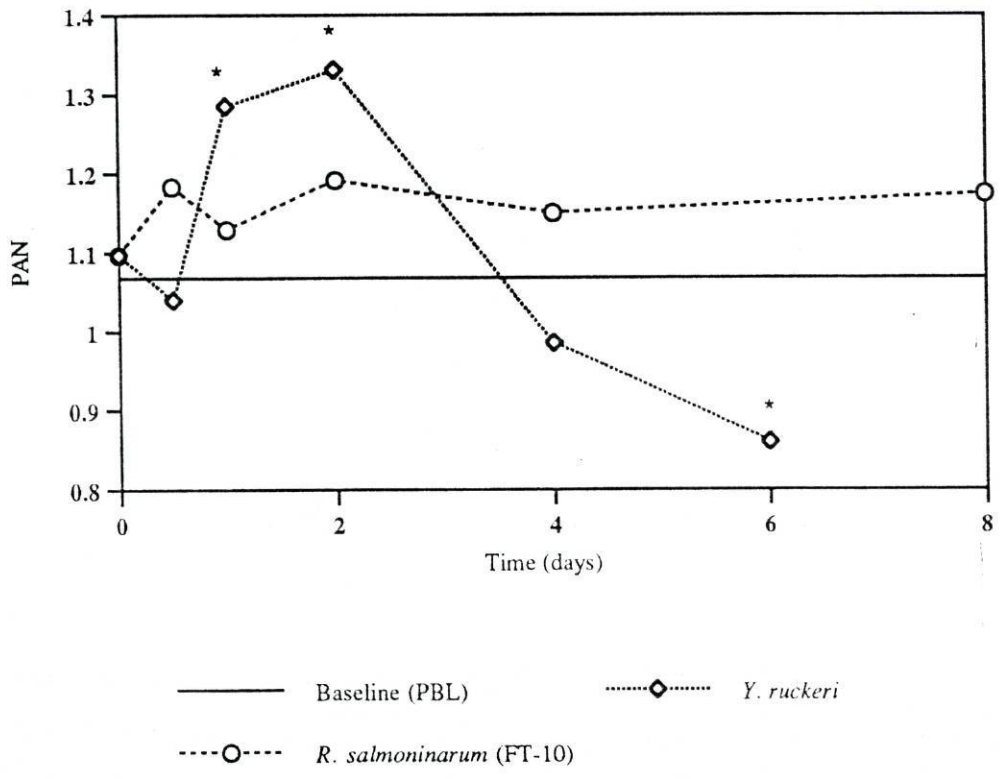
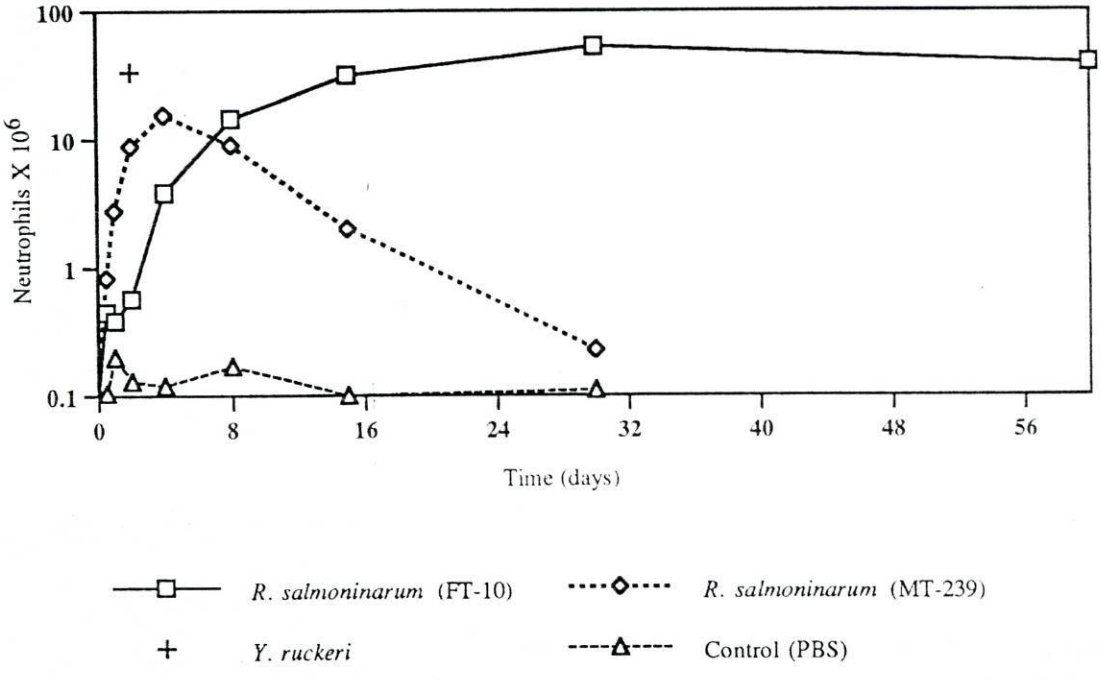
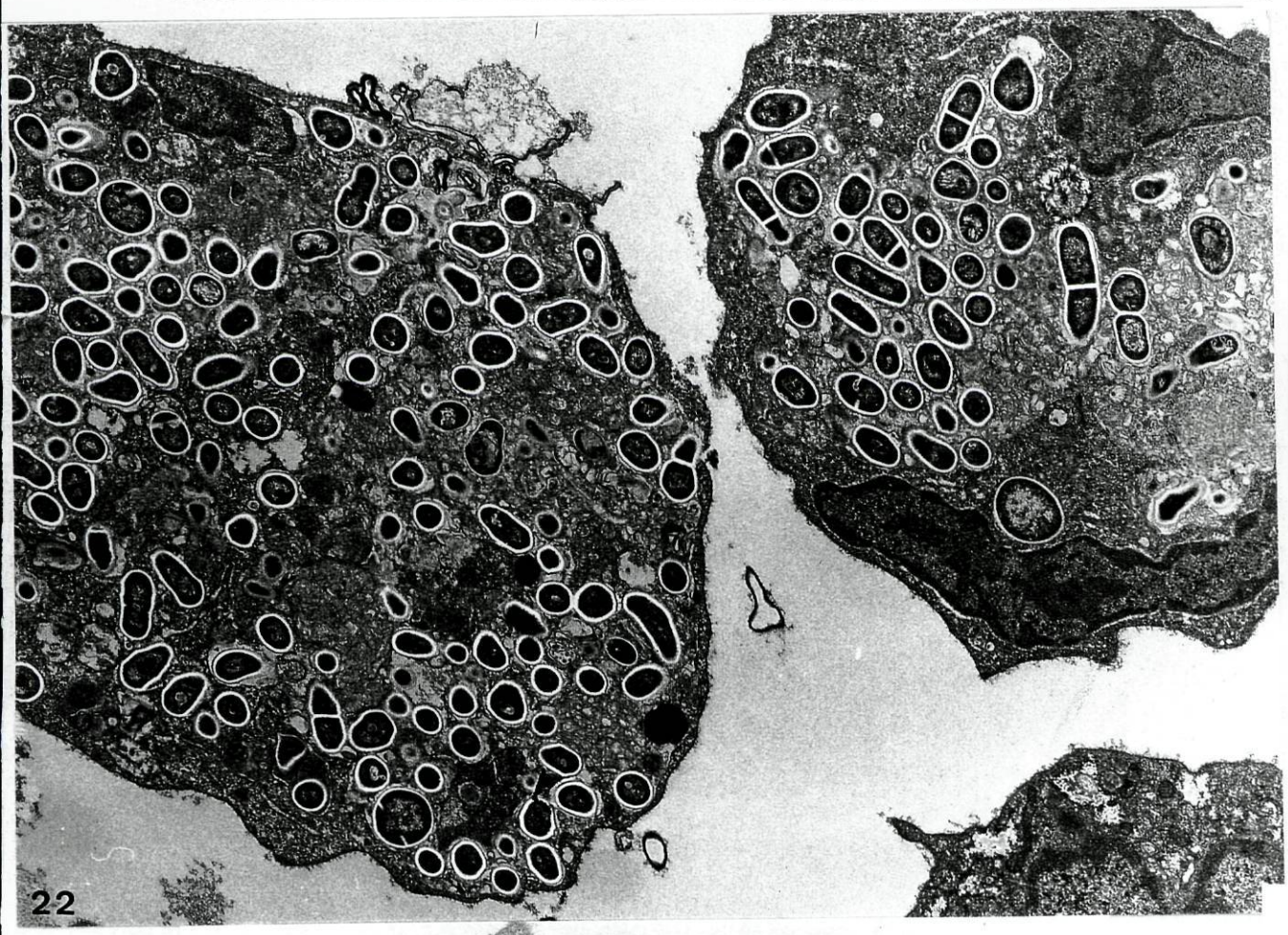
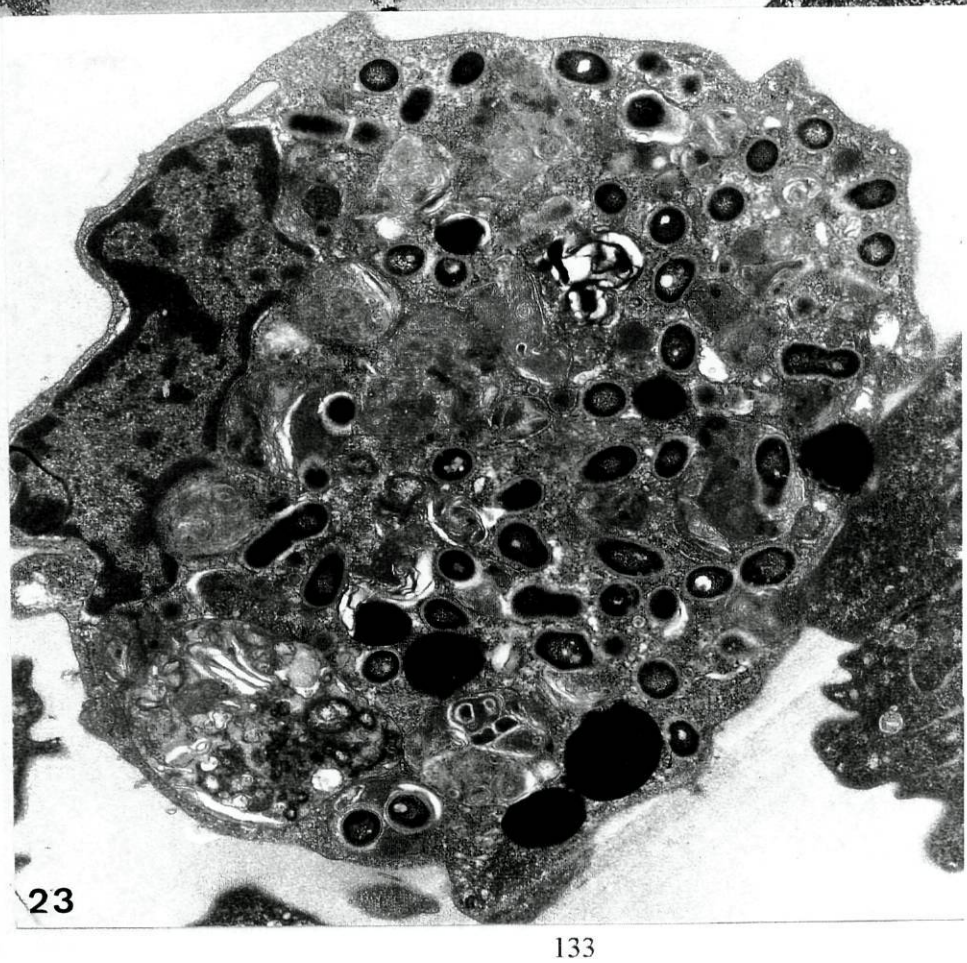

1.4
1.3
1.2
1.1
1
0.9
0.8
PAN
0
2
4
6
8
Time (days)
Baseline (PBL)
Y. ruckeri
R. salmoninarum (FT-10)

Fig.21

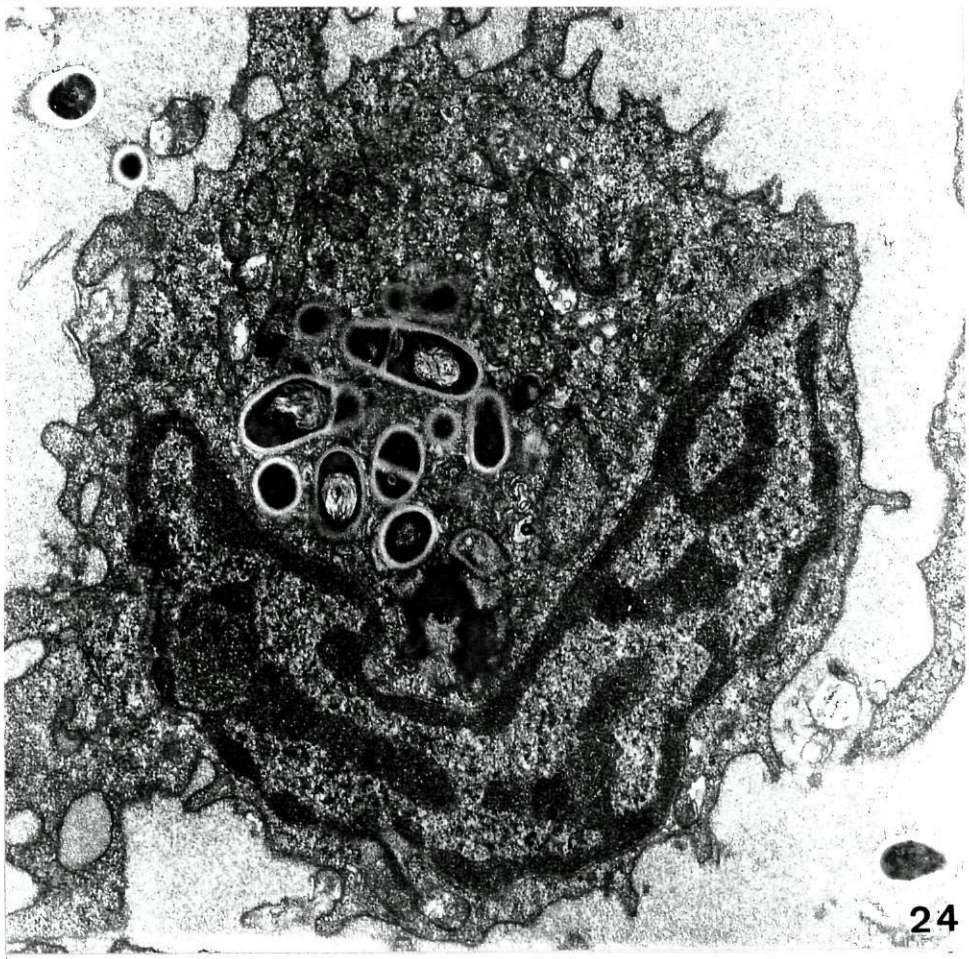

100
10
1
0.1
Neutrophils X 10^6
0
8
16
24
32
40
48
56
Time (days)
R. salmoninarum (FT-10)
R. salmoninarum (MT-239)
Y. ruckeri
Control (PBS)



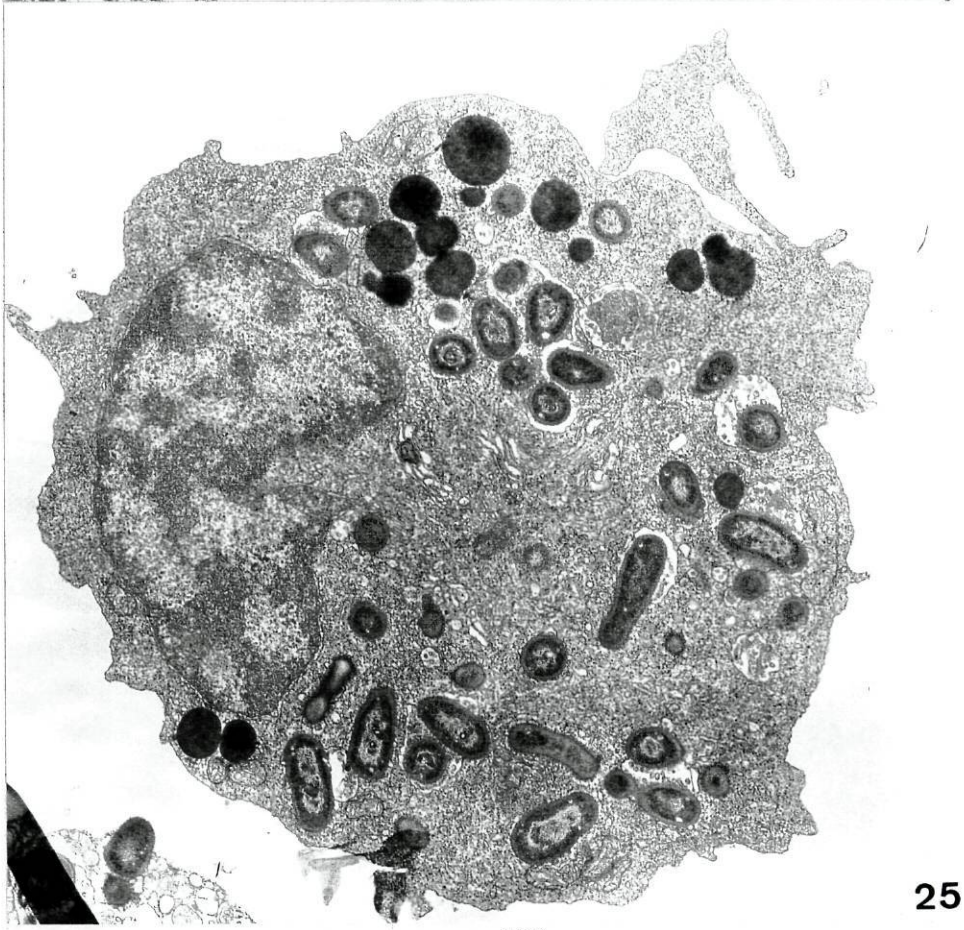
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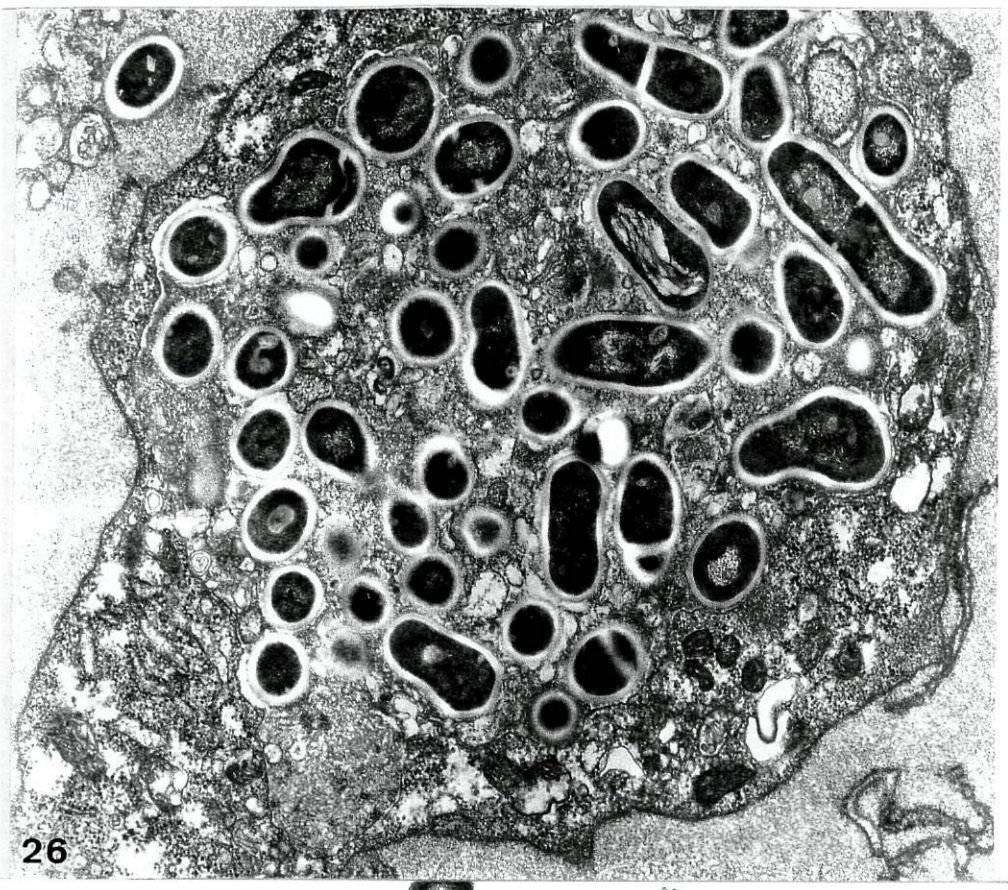
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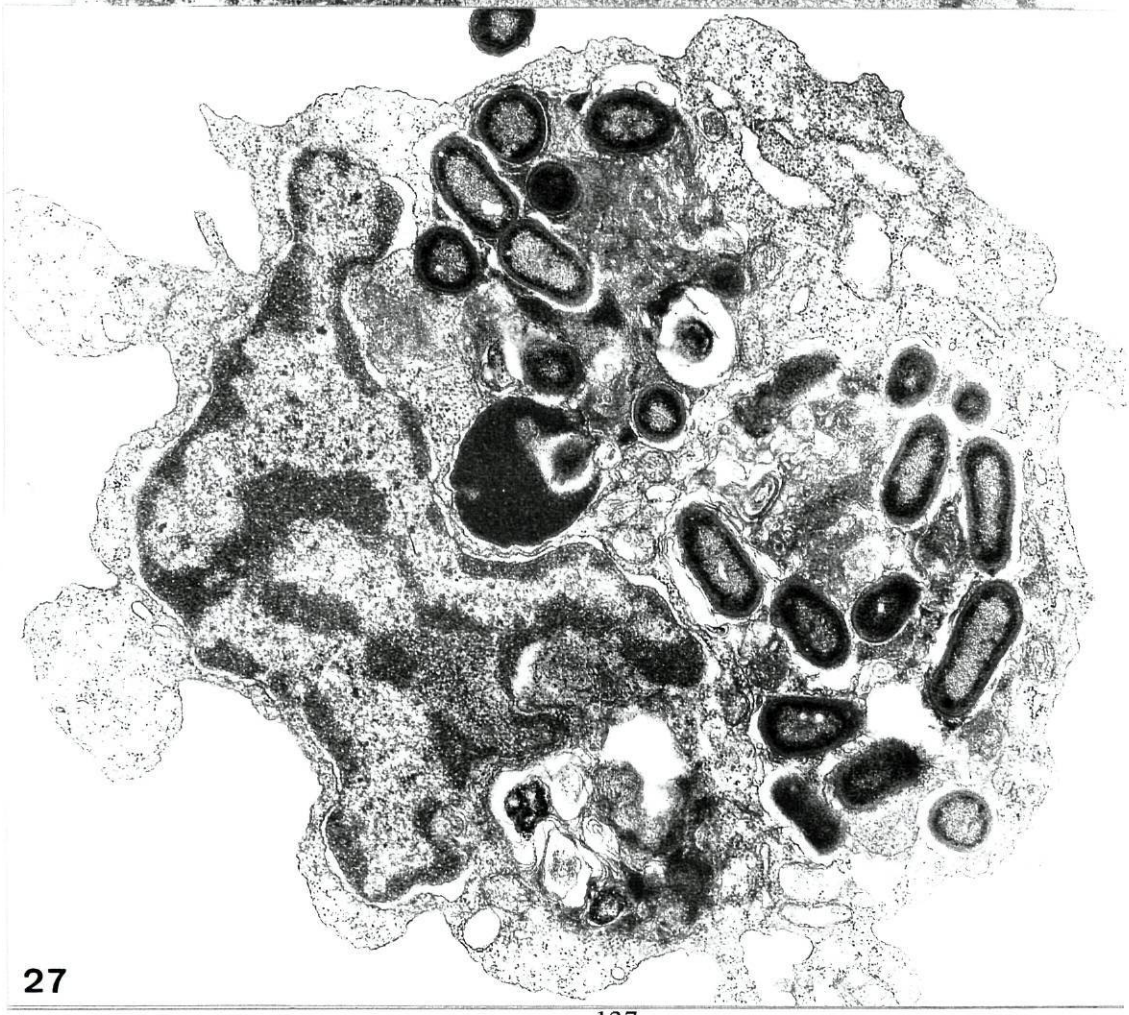
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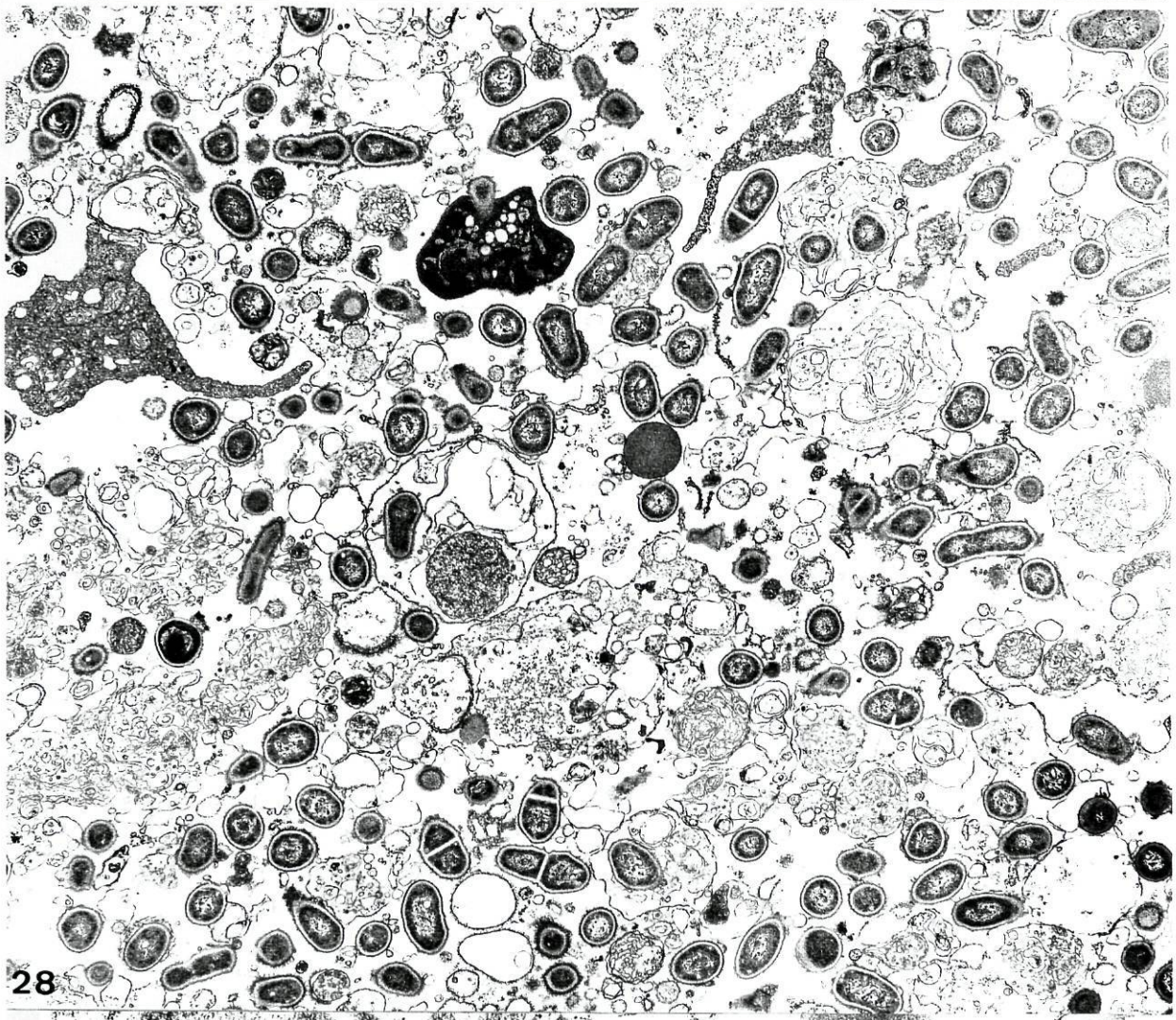
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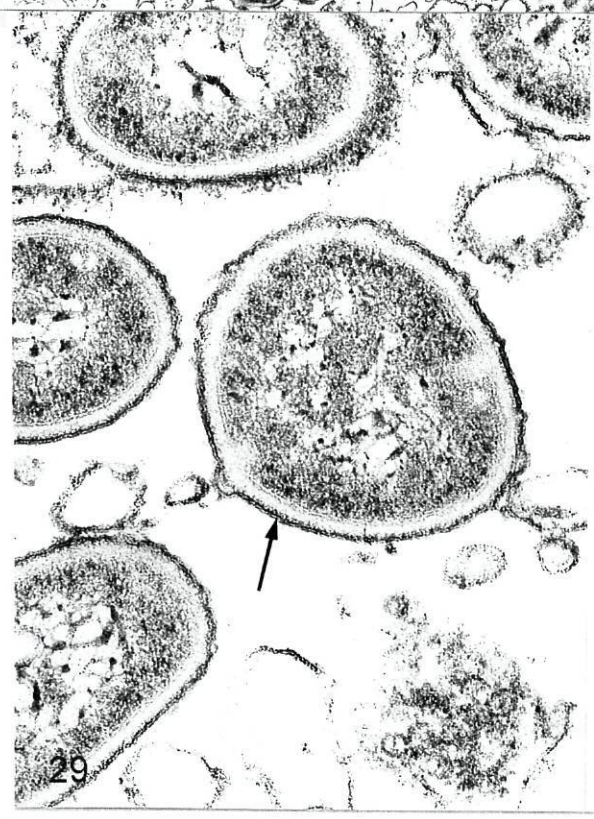
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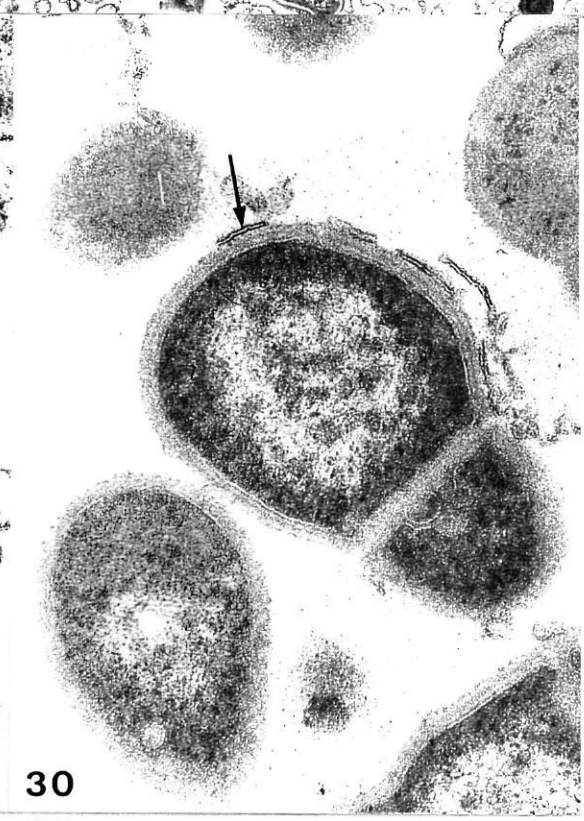
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2.3. Afonso, A., Oliveira, C., Ellis, A. E. & Silva, M. T. (1998). Peroxidase activity as a measure of neutrophil populations in inflammatory peritoneal exudates of rainbow trout (*Oncorhynchus mykiss* Walbaum). *Journal of Fish Diseases.* (Em publicação).

Peroxidase activity as a measure of neutrophil populations in inflammatory peritoneal exudates of rainbow trout (*Oncorhynchus mykiss* Walbaum)

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ABSTRACT

In rainbow trout, the only leukocytes with endogenous peroxidase are the neutrophils, where the enzyme occurs in great amounts. The usefulness of a quantitative peroxidase assay as an indirect evaluation of neutrophil influxes into inflamed territories was, therefore, considered. The assay, using tetramethylbenzidine, was applied to exudates from peritoneal cavities of rainbow trout inflamed by intraperitoneal injection of *Yersinia ruckeri*. Most of the peroxidase activity measured in the inflamed peritoneal cavities was cell-associated, with values for extracellular peroxidase below 5 % of total peroxidase. No free peroxidase activity was detected in resting cavities. The kinetics of neutrophil numbers and peroxidase activity in peritoneal exudates during the 6 days of inflammation studied followed identical patterns, with a correlation coefficient of 0.996. This shows that the peroxidase assay can be used as an indirect, quantitative evaluation of neutrophil responses during peritoneal inflammation.

INTRODUCTION

In vertebrates, one crucial mechamism in the host defence against infection is phagocytosis, killing and degradation of the invading microrganism (Ellis 1977; Adams & Hamilton 1984; Hine 1992; Secombes & Fletcher 1992; Brown 1995). Killing of infectious agents within phagocytic leukocytes involves, among others, antimicrobial oxidant systems (Klebanoff 1988). One of the most important of these systems is the peroxidase + halide + hydrogen peroxide (Klebanoff 1991) system.

It was recently shown that neutrophils are the only cells in the leukocyte population of resting or inflamed peritoneal cavities of rainbow trout with endogenous peroxidase activity (Afonso *et al.* 1997; 1998b). In fact, while mammalian monocytes are peroxidase-positive (Daems; Poelmann & Brederoo 1973; Klebanoff 1991), monocytes are peroxidase-negative in trout (Afonso *et al.* 1998b), and the peroxidase-positive sites seen in some macrophages in inflammatory peritoneal exudates represent neutrophil peroxidase internalised by macrophages (Afonso *et al.* 1998a,b). Moreover, being

extremely abundant in trout neutrophils (Hine & Wain 1988b; Afonso *et al.* 1997, 1998b), as is the case with other Vertebrates (Matheson, Wong & Travis 1981; Klebanoff 1988), peroxidase is a very sensitive marker for the neutrophil.

It was considered, therefore, that quantitation of peroxidase activity in inflamed exudates would be a useful, indirect, albeit specific measure of neutrophil inflammatory responses. In the present paper the results of a study adapting the Bozeman, Learn & Thomas (1990) technique for the quantitative assay of mammalian granulocyte peroxidase to the model of bacteria-induced peritoneal inflammation in rainbow trout (Afonso *et al.* 1998a,b) are described.

The present study show that neutrophilic influxes which accompany peritoneal inflammation due to intraperitoneal injection of live or killed *Yersinia ruckeri* can be reliably evaluated by measuring peroxidase activity in the peritoneal exudates.

MATERIALS AND METHODS

Experimental fish

Sexually immature rainbow trout weighing between 100 and 250g, were purchased from a commercial farm (Castro & Cabero, Lda, Paredes de Coura, Portugal). The animals were maintained in recirculating aerated fresh water, at 15°C, in 7000 l fibreglass tanks. Water quality was maintained with mechanical and biological filtration and fish were fed *ad libitum* on commercial pellets. The fish to be injected were transferred to 600 l tanks where they were maintained for at least 8 days in the same conditions as above before the study. Only healthy fish, as concluded from their activity and exterior appearance were used for the present study.

Growth, harvesting and treatment of bacteria

The strain FP-3 of *Yersinia ruckeri* type 1 was kindly supplied by Prof. J. Barja (University of Santiago de Compostela, Spain). To prepare the suspensions, the bacteria were grown in Tryptic Soy Broth (TSB) (Difco, Detroit, Michigan, USA) at 18°C for 2

days. The bacteria were collected by centrifugation and suspended in TSB with 15 % glycerol, divided in aliquots and frozen at -70°C.

Formol-killed bacteria were prepared by treating suspensions in phosphate buffered saline (PBS) with 8 % formaldehyde for 3 days, followed by 5 washings with PBS. Plating of the formol-treated bacteria showed 100 % killing. Final suspensions of killed bacteria were prepared in TSB and frozen as described for live bacteria.

For intraperitoneal (i.p.) injection, concentrated, frozen suspensions of live or killed bacteria were thawed at room temperature and diluted with PBS with osmolality adjusted to 310 mOsm l^{-1} to 5 x 10^8 bacteria ml^{-1}. Bacteria were counted in a Helber Bacteria Counting Chamber (Fisons Scientific Equipment, Loughborough, Leics., UK).

Intraperitoneal injection of bacteria

Suspensions of bacteria in PBS (100µl) were injected into the peritoneal cavity of fish under anaesthesia (0.3ml ethylene glycol monophenyl ether l^{-1}, Merck, Hohenbrunn, D-85662, Germany), using a 25G needle. Fish injected i. p. with 100µl of PBS served as controls for the injection of bacteria. Immediately after the i.p. injection the fish were returned to their tank.

Preparation of leukocyte suspensions containing neutrophils

Two kinds of neutrophils were used in the present study: i- resting (non-activated) neutrophils were obtained from the blood and peritoneal cavities of non-injected trout; ii- inflammatory (activated) neutrophils were collected from peritoneal cavities of trout injected i.p with bacteria. Neutrophil activation in inflammation has been discussed by Becker (1990).

Collection of peripheral blood leukocytes

Heparinised venous blood was collected from the caudal vein of normal rainbow trout and the leukocytes were isolated by the method described by Thuvander, Norrgren & Fossum (1987). Neutrophils were not separated from the other leukocytes in order to

minimize the loss of peroxidase that occurs during the separation procedures (Sengelov, Kjeldsen & Borregaard 1993). Since blood monocytes do not contain endogenous peroxidase (Afonso *et al.* 1998b), all the enzyme activity found in suspensions of total blood leukocytes represented neutrophilic peroxidase. Final suspensions of the blood leukocytes were made in the peroxidase assay buffer (50mM sodium acetate buffer, pH 5.4, supplemented with 0.3M sucrose) as recommended by Bozeman, Learn & Thomas (1990), and processed as described below.

Collection of peritoneal exudates

The peritoneal exudates were collected from non-injected fish and, at intervals, from fish injected i.p. with PBS or bacteria. The procedure previously described in detail (Afonso *et al.* 1997) was used for the collection, with a modification of the collecting buffer. This modification was introduced in order to have from the beginning the peritoneal leukocytes suspended in the buffer used for the peroxidase assay (Bozeman *et al.* 1990). Pilot experiments showed that the use of this buffer instead of the PBS used in previous cytological and cytochemical studies (Afonso *et al.* 1997, 1998b), although slightly detrimental for morphological quality, did not alter the results regarding the leukocyte total and differential counts. Therefore, cell suspensions in the peroxidase assay buffer were also used for the cytological studies. After sacrificing the fish by an anaesthetic overdose and by exsanguination, 5ml of 50mM sodium acetate buffer, pH 5.4, supplemented with 0.3M sucrose (Bozeman *et al.* 1990) and 10 units heparin ml^{-1} (Choay) was injected i.p. in the ventral midline followed by injection through the same needle of about 1 ml of air. The abdominal area was massaged for about 30 seconds to disperse the peritoneal cells in the injected buffer and the cells in suspension were then collected with a syringe fitted with a 19G needle and placed on ice until processed for cytology and peroxidase assay. Haemorrhagic peritoneal exudates were discarded.

Morphological study of peritoneal and blood leukocytes

The peritoneal and blood cell suspensions were analysed within minutes after collection for several morphological and cytochemical parameters as follows.

Total cell counts were performed with a haemocytometer. For differential leukocyte counts cytospin preparations were made with a Shandon Cytospin 2 (Astmoor, Runcorn, Cheshire WA7 1PR, England) using a volume of peritoneal lavage depending on the cell concentration, in order to obtain adequate numbers of cells per slide. The cytospins were stained with Wright's stain (Haemacolor, Merck, Hohenbrunn, D-85662, Germany) after fixation for 1min with formol-ethanol (10ml of 37 % formaldehyde and 90ml of absolute ethanol) (Kaplow 1965).

The method of Kaplow (1965) or the Antonow's technique (Afonso *et al.* 1998b) were used for the cytochemical detection of peroxidase activity of peritoneal leukocytes in cytospins.

Spectrophotometric peroxidase assay

The method of Bozeman *et al.* (1990), using 3, 3′, 5, 5′- tetramethylbenzidine (TMB) and based on the observations by Andrews & Krinsky (1982) and Suzuki, Ota, Sasagawa, Sakatani & Fujikura (1983), was used to quantitate the peroxidase activity in peritoneal exudates and suspensions of blood leukocytes.

In the case of peritoneal exudates, the method was applied to total peritoneal exudates or, separately, to their leukocyte population and extracellular fluid. For this, the peritoneal exudates were centrifuged in an Eppendorf microcentrifuge at 7000 *g* for 1 min at 4°C and the supernatants separated from the cell pellet. These three types of peroxidase activity are referred to as total peroxidase, leukocyte peroxidase and free peroxidase, respectively.

For leukocyte and total peroxidase assays the leukocytes were lysed by treatment of leukocyte suspensions with Triton X-100, as proposed by Bozeman *et al.* (1990). The effect of increasing concentrations of detergent in the incubation mixture containing 10^5 neutrophils on enzyme readings was the same as that described by Bozeman *et al.* (1990)

for human neutrophils, with a plateau reached at Triton X-100 concentration of 100µg ml^{-1}. This concentration was used thereafter with all samples.

Since neutrophil peroxidase activity is stable in suspensions stored frozen at -70°C (King, Jefferson & Thomas 1997), the peritoneal samples from several fish and time points were stored at that temperature. The samples were then processed together as follows.

After thawing at room temperature, the samples were resuspended in ice-cold 50mM acetate buffer, pH 5.4 supplemented with Triton X-100 and sonicated on ice in an Ultrasonic processor W373 (Heat Systems, Ultrasonics, Inc., Farmingdal, New York, USA), for 5s, using a microtip and energy setting at position 4.

The incubation mixture consisted of the ready-to-use TMB reagent from Sigma (cat. T 8665), plus either the leukocyte suspension or supernatant. For each assay, serial dilutions of the samples were made in 50mM acetate buffer, pH 5.4 supplemented with Triton X-100.

Volumes in the final incubation mixture were: 500µl of 50mM acetate buffer, pH 5.4, 100µl of sample and 250µl of TMB reagent. As reported by Suzuki *et al.* (1983), the reaction was linear over more than 3min. Incubations were carried out at 37°C. for 3min when the reaction was stopped by placing the samples on ice with concomitant addition of 1 vol. of ice-cold 0.2M acetic acid, as described by Bozeman *et al.* 1990. The samples were clarified by centrifugation in the Eppendorf microcentrifuge mentioned before at 7000 g for 1 min at 4°C and readings were done at an absorbance of 655nm in a Shimadzu spectrophotometer, model UV-1201 (Shimadzu Corporation, Tokyo, Japan). Absorbance readings between 0.150 and 0.800 were used for the calculations. The readable leukocyte suspensions contained between 4×10^4 and 30×10^4 neutrophils ml^{-1}.

Since benzidine and hydrogen peroxide react with haemoglobin (Rüegg & Jungi 1988), and inflammatory peritoneal exudates sometimes contain erythrocytes (Afonso *et al.* 1998b), we analysed the influence of contaminating red blood cells on peroxidase activity readings. This was done either by using peritoneal exudates with known numbers of erythrocytes, as counted in a haemocytometer, or adding to blood-free exudates

known numbers of erythocytes from heparinised blood collected from the caudal vein as described above.

As a standard for the peroxidase assay, horseradish peroxidase (HRP) (Sigma P 8250) was assayed using the same conditions as those for the leukocyte suspensions.

Peroxidase activity is expressed as ΔOD_{655} min^{-1}. Peroxidase activities of peripheral blood or peritoneal leukocyte suspensions, together with the corresponding neutrophil counts, were used to calculate (i)-the correlation coefficient (r^2) between number of neutrophils in the samples added to the peroxidase assay mixture and the OD, and (ii)-peroxidase activity per neutrophil (PAN).

Statistics

Values were analysed by the Student's *t*-Test. Correlation coefficients (r^2) were calculated with the Microsoft Exel 5 Program.

RESULTS

Horseradish Peroxidase (HRP)

Fig. 1 shows the absorbance readings of 6 independent tests for 5 concentrations of horseradish peroxidase. The correlation coefficient was 0.982.

The presence of increasing concentrations of Triton X-100 in the incubation mixture increased the readings for HRP activity, as found for human granulocyte peroxidases (Bozeman *et al.* 1990). The highest increase was 12.5 % with detergent concentration of 100µg ml^{-1}.

Effect of contaminating erythrocytes or bacteria present in the leukocyte suspensions

Using peritoneal leukocyte suspensions with added red blood cells, the presence of erythocytes in concentrations up to 40 % of the number of neutrophils did not influence the peroxidase readings at OD_{655}. The presence of erythrocytes in numbers representing

66 % or 100 % of the number of neutrophils, increased the peroxidase readings by less than 10 % (data not shown). Since haemorrhagic peritoneal exudates were discarded, it can be concluded that the peroxidase readings obtained were not affected by the presence of red blood cells.

No peroxidase activity was found in *Y. ruckeri* suspensions (not shown) and, therefore, the presence of bacteria in peritoneal exudates did not influence peroxidase readings.

Peroxidase activity of resting and inflammatory neutrophils

Non-inflammatory neutrophils were studied in suspensions of total blood leukocytes from non-injected trout and of total leukocytes from resting peritoneal cavities. Several dilutions of each sample were tested, containing neutrophils over the range 4-30 x 10^4 ml^{-1}.

Fig. 2 shows the correlation between the number of blood neutrophils and peroxidase activity. From these values a correlation coefficient of 0.918 and a PAN of $1.068 \pm 0.188 \times 10^{-6}$ were obtained.

A correlation coefficient of 0.897 was obtained between the number of resting neutrophils and peroxidase readings. From these values a PAN of $1.097 \pm 0.373 \times 10^{-6}$ was calculated. This value is not statistically different from that for PAN of blood neutrophils ($P>0.25$). No free peroxidase was detected in the resting peritoneal cavities.

Inflammatory peritoneal neutrophils

Infectivity tests showed that the living bacterial inoculum used in the present work was not pathogenic for the trout, as judged from the observation that bacteria were not present in the peritoneal exudates 4 days after the inoculation (data not shown) and from the absence of clinical signs. Two results are in accordance with this evolution of the infection: (i)-the influx of neutrophils to the peritoneal cavity in response to the i. p. injection of bacteria was transient, with neutrophil numbers well below the maximum value at 6 d after inoculation (Table 1; Fig. 3A) and very close to normal at 15 days

(Afonso *et al.* 1998b); (ii)-the peritoneal neutrophil influx to the inoculation of live bacteria was not much different from that seen with killed bacteria (Table 1; Fig. 3A).

Suspensions with known numbers of neutrophils collected at intervals from peritoneal cavities of trout injected i. p. with 5 x 10^7 live or formol-killed *Y. ruckeri*, or with PBS (control) were assayed for peroxidase activity. The PAN values for the several peritoneal samples collected at intervals after the injections (Table 1 and Fig. 4) show that, at the onset of the inflammation (12h post-injection of live bacteria, and 12 and 24h for killed bacteria), the PAN was similar ($P>0.25$) to that of blood or resting peritoneal neutrophils. Then, the values for PAN increased (maximum values at 2 days were 121.3 % or 112.9 % of resting peritoneal neutrophils for fish injected with live or killed bacteria, respectively). At 6 days the values for PAN were significantly below the basal value (78.4 % or 86.7 % of the initial after injection of live or killed bacteria, respectively). Variations in PAN were smaller and occurred later for fish injected with killed bacteria as compared to live bacteria.

It was previously shown that the mild inflammation that follows the i. p. injection of PBS in rainbow trout is accompanied by a small peak of neutrophil influx at 8h post-injection (Afonso *et al.* 1998b). Values for total peritoneal peroxidase and PAN at that time point were 0.133 ± 0.055 and $1.103 \pm 0.240 \times 10^{-6}$, respectively. The value for PAN is not statistically different from that of resting peritoneal or blood neutrophils ($P> 0.25$). Free peroxidase was undetectable.

Kinetics of peroxidase activity in inflamed peritoneal cavities

Table 1 and Fig. 3B show the values obtained for total peroxidase in the peritoneal cavities of trout injected i. p. with bacteria.

A correlation coefficient of 0.996 was found when comparing the values for neutrophil numbers and for peroxidase activities over the 6 days of the experiment.

In accordance with this correlation, a good match between neutrophil numbers and peroxidase activity was obtained when neutrophil numbers and peroxidase activities (Table 1 and Fig. 3) were represented as percentages (Fig. 5). The slight deviations

between the two curves and the relative positions of such deviations reflect the variations in PAN shown in Fig.4.

No free peroxidase activity was detectable in peritoneal cavities of non-injected fish nor in fish injected with PBS. The maximum value (0.983 ΔOD min^{-1} per peritoneal cavity) was obtained in samples collected 24h after the injection of live bacteria, a value that represents a small proportion of total peroxidase (less than 5 %). At 6 days after the injection of live bacteria free peroxidase decreased to 0.003 ΔOD min^{-1} per peritoneal cavity, which represents 0.1 % of total peroxidase.

DISCUSSION

The present results show that the TMB technique developed by Bozeman *et al.* (1990) for the quantitative assay of peroxidase activity in human granulocytes is also applicable to peritoneal exudates of inflamed cavities of rainbow trout. Good points include: (i)-the technique is simple, using a reaction that is linear during several minutes, which allows the use of only one reading; (ii)-it is very sensitive; (iii)-contrary to other techniques, it uses a non-carcinogenic reagent (Suzuki *et al.* 1983; Bozeman *et al.* 1990); (iv)-it can be applied to samples stored frozen; (v)-there was a good correlation coefficient when comparing absorbance readings for peroxidase activity with number of blood neutrophils or resting peritoneal neutrophils in the range of 4 to 30 x 10^4 neutrophils ml^{-1} in the final sample. Moreover, the presence of contaminating erythrocytes only affected peroxidase readings when the red blood cells were present in very high numbers.

Using the values for peroxidase activity and for the number of neutrophils in the corresponding samples a value was obtained for the peroxidase activity per neutrophil (PAN). Such a value was not significantly different for blood neutrophils and resting peritoneal neutrophils, which is not surprising because at both locations the neutrophils are not activated.

When applying the TMB technique to the transiently inflamed peritoneal exudates of trout injected i. p. with bacteria, a very close match between number of neutrophils and

peroxidase activity was found, showing that the peroxidase assay is a reliable measure of neutrophil responses in the present model. The small deviations observed when correlating the graphs of neutrophil numbers versus peroxidase activities correspond to the slight variations in the PAN of neutrophils in inflamed cavities which occur after the initial 12h or 24h post-injection of live or killed bacteria, respectively. Such variations represent an increase in PAN that, although small, is significant at 24 and 48h after the injection of live bacteria and at 48h for killed bacteria. This would indicate an actual increase in the amount or activity of peroxidase in the inflammatory as compared to the resting neutrophils. Cytological analysis of the samples, however, did not give evidence for that but, rather, yielded evidence for an alternative interpretation. Namely, the increase in PAN under discussion could be due to the phagocytosis of neutrophils and neutrophil components by macrophages, which is known to occur in that situation (Afonso *et al.* 1998a). Since the peroxidase of neutrophils acquired by macrophages will also participate in the assays of the leukocyte peroxidase, a higher value for peroxidase per neutrophil would, therefore, be obtained. At 4 days after the injection of bacteria, when inflammation was being resolved, the values for PAN decreased to close to normal and a further reduction occurred at 6 days. This reduction could be interpreted as due to the degradation of peroxidase taken by macrophages and/or to a decrease in the actual concentration of the enzyme in the remaining neutrophils. It is known that peroxidase activity in inflammatory neutrophils of the eel decreases in comparison with non-inflammatory neutrophils (Hine & Wain 1988a).

In contrast with the above discussed variations in the PAN values, at 12h after the i. p. injection of live or killed bacteria, as well as at 24h after the injection of killed bacteria, the PAN values are not statistically different from those of blood or resting peritoneal neutrophils. This indicates that little change occurred in the granular compartment of attracted neutrophils in the initial phases of inflammation induced by bacteria in the present model. This would be in agreement with the finding that very few neutrophils phagocytosed bacteria after the i. p. injection of 5 x 10^7 *Y. ruckeri*, the majority of them being taken by the resident peritoneal macrophages before the arrival of the attracted neutrophils (Afonso *et al.* 1998b). It is known that one important stimulus

for peroxidase secretion by neutrophils is phagocytosis (Bradley, Christensen & Rothstein 1982, Densen, Clark & Nauseef 1995; King *et al.* 1997).

The present results show that most of the peroxidase activity measured in inflamed peritoneal cavities is cell-associated. In fact, the maximum value found for free peroxidase represented only 4.16 % of total peroxidase, and this occurred in very inflamed peritoneal cavities (24h after the i.p. injection of live *Y. ruckeri*). In the resting peritoneal cavities no free peroxidase activity could be detected, and at 6 days post-injection of live bacteria, when the peritoneal inflammation was subsiding, free peroxidase was only 0.1 % of the total. The finding that low levels of free peroxidase were measured during the bacteria-induced peritoneal inflammation is in accordance with the observation above discussed that incoming neutrophils attracted to the peritoneal cavity after the injection of 5 x 10^7 *Y. ruckeri* were not actively phagocytosing the bacteria (Afonso *et al.* 1998b). Moreover, it is well documented that peroxidase, contrary to other components of neutrophilic granules, is released by inflammatory human neutrophils in small amounts (Sengelov *et al.* 1993; Sengelov, Follin, Kjeldsen, Lollike, Dahlgren & Borregaard 1995). Additionally, it has been shown that the released, extracellular peroxidase does not persist, at least with enzymatic activity, for long periods of time. This is because: (i)-Free peroxidase is rapidly inactivated by the oxygen radicals generated by reactions catalysed by the peroxidase itself (Bradley *et al.* 1982; Edwards, Nurcombe & Hart 1987; King *et al.* 1997). ii- The peroxidase molecule is taken up by leukocytes, especially macrophages, present in the inflamed exudates (Shellito, Sniezek & Warnock 1987; Leung & Goren 1989; Lincoln, Lefkowitz, Cain, Castro, Mills, Lefkowitz, Moguilevsky & Bollen 1995); it has been shown that the macrophage mannose receptor is involved in the uptake (Shepherd & Hoidal 1990; Lefkowitz, Lincoln, Lefkowitz, Bollen & Moguilevsky 1997). It follows, therefore, that free peroxidase measurements are probably an underestimation, the amounts of peroxidase actually secreted or lost by neutrophils being above the values which were measured. To better understand this point, it would be interesting to assay peroxidase by methods not dependent on its enzymatic activity, for example by immunochemistry. This is not, unfortunately, possible in the present situation because no antibodies against trout

neutrophil peroxidase are available. It is noteworthy, however, that in a study with inflammatory human neutrophils, when free peroxidase was assayed by non-enzymatic methods, only small amounts of peroxidase were detected (Leffel & Spitznagel 1975). Since active peroxidase has important cytotoxicity (Edelson & Cohn 1973; Klebanoff & Clark 1975; Klebanoff 1991), the occurrence of extracellular peroxidase at low concentrations would represent an advantage for the host.

In conclusion, the present results show that the quantitative assay for peroxidase in peritoneal exudates of rainbow trout with transient peritoneal inflammation can be used as a simple, reliable indication about the neutrophil responses that accompany inflammation. Work in progress is evaluating the use of the assay in inflammatory situations where neutrophil counts are problematic and in situations of sustained inflammation due to progressive infection.

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LEGENDS TO THE FIGURES

Fig. 1

Correlation between concentration of horseradish peroxidase and absorbance readings for peroxidase activity. For each enzyme concentration 6 independent readings were done. Correlation coefficient = 0.982.

Fig. 2

Correlation between number of blood neutrophils and absorbance readings for peroxidase activity. Correlation coefficient = 0.918.

Fig. 3

Kinetics of neutrophil numbers per peritoneal cavity (A) and of total peroxidase activity (expressed as $\Delta OD\ min^{-1}$) per peritoneal cavity (B) of normal rainbow trout (day 0) and trout injected i. p. with 5×10^7 live (open symbols) or dead (closed symbols) *Y. ruckeri* after 0.5, 1, 2, 4 and 6 days.

Fig. 4

Variation in the PAN values (peroxidase activity per neutrophil; expressed as $\Delta OD\ min^{-1} \times 10^{-6}$), during the 6 days of peritoneal inflammation induced by 5×10^7 live (closed circles) or formol-killed (open circles) *Y. ruckeri*. The baseline represents the PAN value for blood neutrophils.

* Denotes differences statistically significant ($P < 0.05$).

Fig. 5

Kinetics of neutrophil numbers per peritoneal cavity (squares) and of total peroxidase activity (expressed as $\Delta OD\ min^{-1}$) per peritoneal cavity (circles) of normal rainbow trout (day 0) and trout injected i. p. with 5×10^7 live (A) or dead (B) *Y. ruckeri* (days 0.5, 1, 2, 4 and 6). Values represented as percentage of the normal (day 0).

Table 1

Values for neutrophil numbers and total peroxidase activity (expressed as ΔOD_{655} min^{-1}) per peritoneal cavity used to construct Fig. 3, 4 and 5.

PAN = Peroxidase activity per neutrophil.

N = number of fish per sample.

ND - not done.

Fig. 1

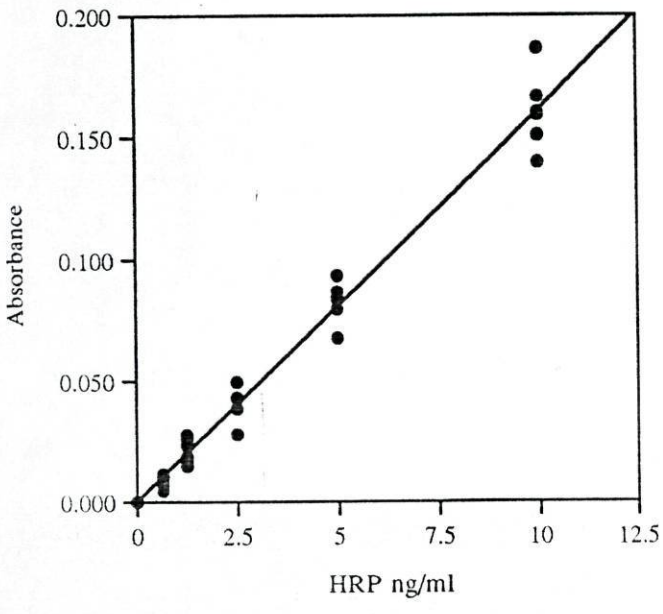

0.200
0.150
0.100
0.050
0.000
Absorbance
0
2.5
5
7.5
10
12.5
HRP ng/ml

Fig. 2

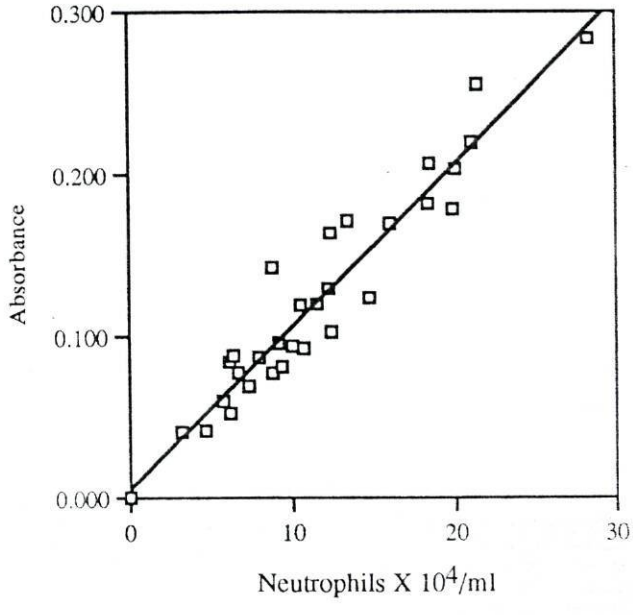

0.300
0.200
0.100
0.000
Absorbance
0
10
20
30
Neutrophils X 10^4/ml

Fig 3A

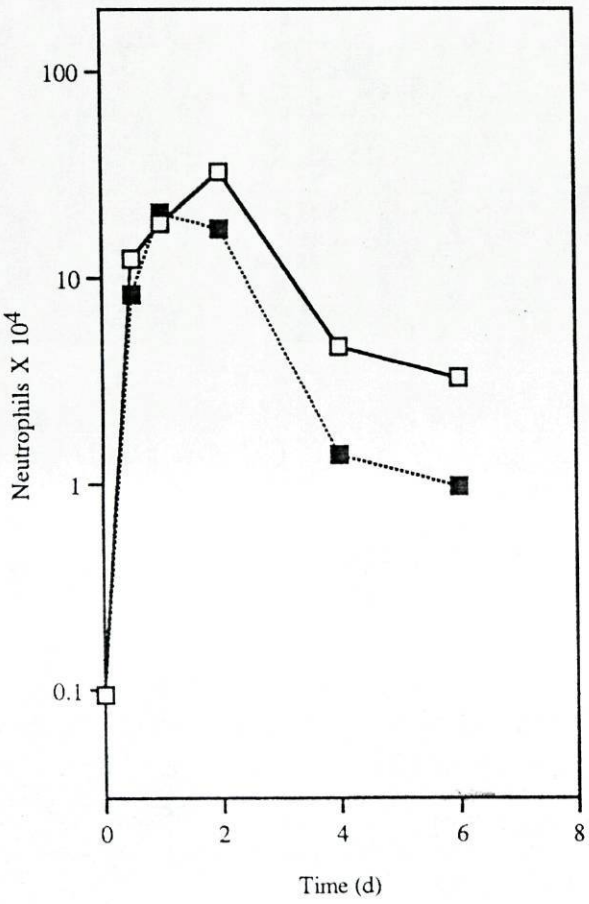

100
10
1
0.1
Neutrophils X 10^4
0
2
4
6
8
Time (d)

Fig 3B

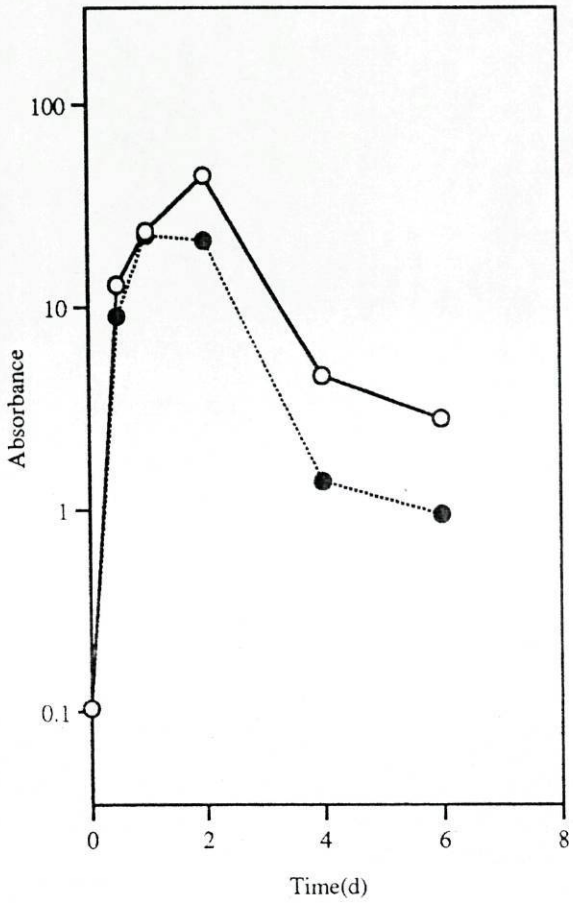

100
10
1
0.1
Absorbance
0
2
4
6
8
Time(d)

Fig. 4

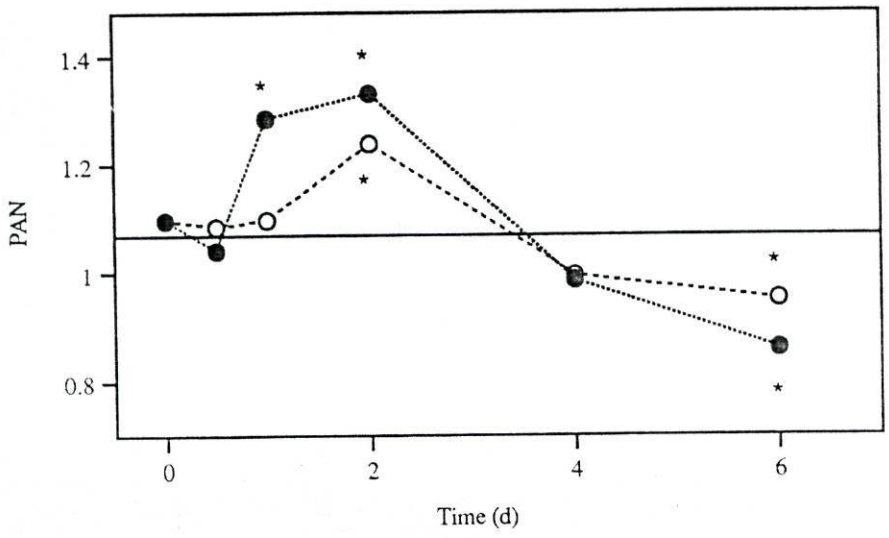


Fig. 5A

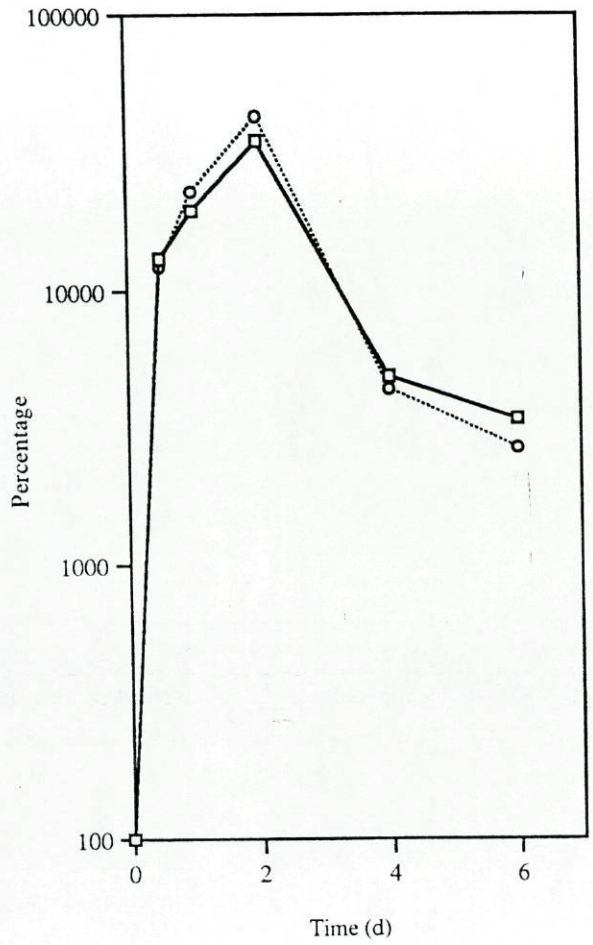

100000
10000
1000
100
Percentage
0
2
4
6
Time (d)

Fig. 5B

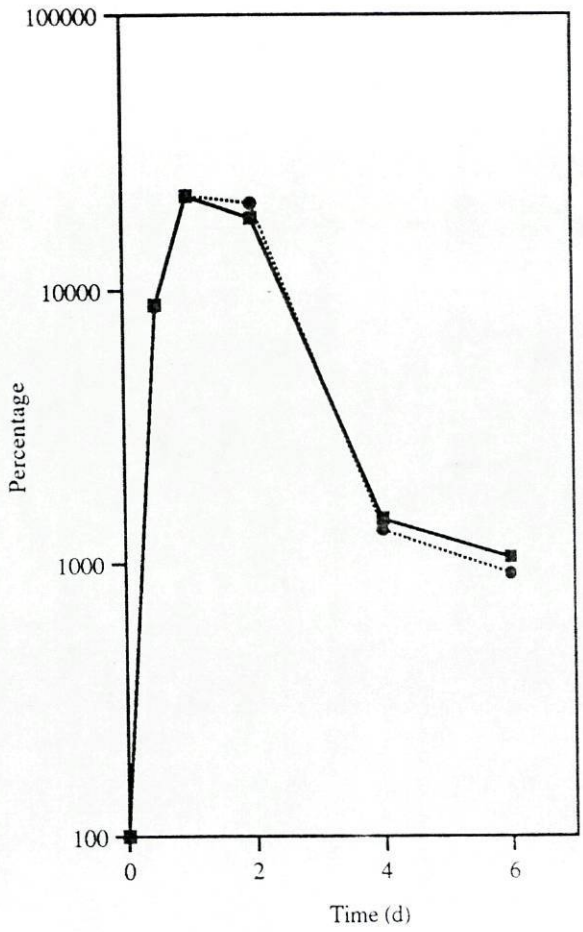

100000
10000
1000
100
Percentage
0
2
4
6
Time (d)

Table 1

Sample		Neutrophils ($X10^6$)	Total PO	PAN ($X\ 10^{-6}$)	N
Resting peritoneal leukocytes		0.0946 ± 0.0515	0.104 ± 0.054	1.097 ± 0.373	14
PBS 8 h		0.1206 ± 0.0431	0.133 ± 0.055	1.103 ± 0.240	8
Live *Y. ruckeri*	12 h	12.412 ± 8.382	12.926 ± 9.525	1.041 ± 0.230	15
" " "	24 h	18.386 ± 11.257	23.627 ± 16.497	1.285 ± 0.251	27
" " "	48 h	32.781 ± 15.017	44.556 ± 24.582	1.331 ± 0.399	18
" " "	4 d	4.650 ± 2.946	4.585 ± 3.128	0.986 ± 0.395	8
" " "	6 d	3.262 ± 1.680	2.806 ± 1.916	0.860 ± 0.210	9
Killed *Y. ruckeri*	12 h	8.362 ± 5.618	9.074 ± 6.791	1.085 ± 0.243	11
" " "	24 h	20.641 ± 20.330	22.642 ± 21.424	1.097 ± 0.262	22
" " "	48 h	17.196 ± 21.422	21.298 ± 28.266	1.238 ± 0.280	9
" " "	4 d	1.384 ± 0.654	1.377 ± 0.570	0.995 ± 0.111	11
" " "	6d	0.998 ± 0.262	0.949 ± 0.200	0.951 ± 0.069	6

2.4. Afonso, A., Macedo, P. M. & Silva, M. T. (1998). Glycogen granules in resting and inflammatory rainbow trout phagocytes. An ultrastructural study. (Manuscrito inédito).

Glycogen granules in resting and inflammatory rainbow trout phagocytes. An ultrastructural study

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(Unpublished manuscript)

ABSTRACT

Glycogen granules in the cytoplasm of blood and peritoneal neutrophils and macrophages of rainbow trout were studied by electron microscopy after staining with osmium tetroxide-potassium ferrocyanide or by a periodic acid Schiff ultrastructural technique (Thiéry's method). The glycogen deposits appeared as dense granules with a diameter, in the case of neutrophils, of 35.72±3.20nm.

The semi-quantitative morphometric study of samples processed for the detection of peroxidase or esterase activities (to specifically label neutrophils and macrophages), and simultaneously stained for the specific detection of glycogen by the Thiéry's method, showed that inflammatory neutrophils were richer in glycogen granules as compared to resting neutrophils. Glycogen granules could not be found in resting (resident) peritoneal macrophages but were found in inflammatory macrophages. In these macrophages the granules were in smaller amounts than in neutrophils present in the same samples. Two populations of glycogen granules with different diameters were seen in inflammatory macrophages. One population consisted of granules significantly smaller than those of neutrophils. The other was identical to those of neutrophils and may represent neutrophil glycogen transferred to the macrophages.

INTRODUCTION

Phagocytosis, killing and degradation of pathogenic microrganisms is a fundamental mechanism of host defense against infection in animals. Two distinct professional phagocytes are present in many hosts, the macrophage and the neutrophil. Glycogen is a storing molecule to be used as a source for glucose in several cells, and, in the case of neutrophils and macrophages, glycogen-derived glucose is utilized for the respiratory burst that accompanies phagocytosis (Scott, 1968; Wilkinson, 1977; Weisdorf *et al.*, 1982).

It has been reported that Inflammatory neutrophils of mammals (Scott & Cooper, 1974, Robinson *et al.*, 1982) and the eel (Nagamura & Wakabayasi, 1985; Hine & Wain, 1988) contain more glycogen than resting neutrophils. This increase in the storage molecule would be useful for the metabolic activities of the neutrophils phagocytosing under the anaerobic conditions which frequently are faced by the phagocytes (Suzuki & Iida, 1992).

A few reports deal with the presence of glycogen in fish phagocytes, but, as reviewed by Ellis (1977), MacArthur & Fletcher (1985) and by Rowley *et al.* (1988), most of the work regards light microscopic studies. Extending our previous results regarding the cytochemical characterization of resting and inflammatory rainbow trout phagocytes, here we analyse by ultrastructural methods the glycogen pools of neutrophils and macrophages in the blood and peritoneal cavities.

MATERIALS AND METHODS

Fish

Sexually immature rainbow trout weighing between 100 and 200g, were purchased from a commercial farm. The animals were maintained in recirculating aerated fresh water, at 15°C, in 7000 l fibreglass tanks. Water quality was maintained with mechanical and biological filtration and fish were fed *ad libitum* on commercial pellets. The fish to be injected with phlogistic agents were transferred to 600 l fibreglass tanks under the same conditions as above, where they were maintained for at least 15 days before the study. Only healthy fish, as indicated by their activity and exterior appearance, were used.

Phagocytes

The present results regard phagocytes under resting or inflammatory situations. Resting neutrophils and monocytes/macrophages were studied in the leukocyte populations of blood and of resting peritoneal cavities of normal, non-injected, rainbow trout. Inflammatory neutrophils and macrophages were collected from peritoneal cavities rendered inflamed by the intraperitoneal (i.p.) injection of bacterial or non-bacterial phlogistic agents. The peritoneal phagocyte responses following the i.p. injection of the agents used in this study were previously described in detail (Afonso *et al.*, 1998b).

Intraperitoneal injection of phlogistic agents. The following agents were injected i.p. in volumes of 100µl: undiluted Incomplete Freund's Adjuvant (IFA); suspensions of live or formol-killed *Yersinia ruckeri* (5×10^7 bacteria per fish). The strain FP-3 of *Y. ruckeri* type 1 was kindly supplied by Prof. J. Barja (University of Santiago de Compostela, Spain). Suspensions of live or formol-killed bacteria to be injected into the peritoneal cavities of trout were prepared as described (Afonso *et al.*, 1998a,b). In some experiments, 24h after the i.p. injection of killed 5×10^7 *Y. ruckeri*, fish were injected i.p. with 4×10^8 live *Y. ruckeri* to promote the phagocytosis of bacteria by the neutrophils (Afonso *et al.*, 1998b).

All agents were injected as described (Afonso *et al.*,1997), into the peritoneal cavity of fish under anaesthesia with 0.3ml ethylene glycol monophenyl ether (Merck) l^{-1}. Immediately after the i.p. injection the fish were returned to their tank.

Collection of peritoneal leukocytes. The peritoneal cells were collected from untreated trout and at intervals (see Results) after the i.p. injections by a procedure previously described in detail (Afonso *et al.*, 1997; 1998b). The peritoneal cell suspensions were analysed within minutes after collection for several morphological and cytochemical parameters as follows.

Study of blood leukocytes. Leukocytes in the blood of normal rainbow trout or of trout i.p. injected with 2 bacterial inocula, were studied in concentrated blood leukocyte suspensions obtained by the method of Thuvander *et al.* (1987) applied to heparinised blood collected from the caudal vein. These leukocyte preparations were analysed for peroxidase and glycogen by the methods described below.

Electron microscopy of leukocytes. Blood or peritoneal leukocyte suspensions in PBS were centrifuged at 1000 *g* for 10min and the pellet was fixed by the following methods. (i)- Routinely, samples were fixed by our standard procedure as described by Silva & Macedo (1983, 1987). This includes: a prefixation with 4 % paraformaldehyde-1.25 % glutaraldehyde-10mM calcium in 50mM cacodylate buffer (pH 6.4) at 4°C; after at least 4h, the cells were washed twice with 50mM cacodylate buffer supplemented with 10mM calcium chloride (pH 6.4), then fixed at room temperature for 2h with 1 % OsO_4 in veronal-acetate buffer (pH 6.4) supplemented with 10mM calcium chloride; a postfixation, at room temperature, with 1 % aqueous uranyl acetate for 1h (Silva *et al.*, 1971) followed. (ii)- Fixation with 2.5 % glutaraldehyde in 50mM cacodylate buffer (pH 7.3) for 2-4h, followed by 2 % OsO_4 in 0.1M cacodylate buffer pH 7.3, supplemented with 3 % potassium ferrocyanide (Karnowsky, 1971; Robinson *et al.*, 1982).

After dehydration in ethanol, the samples were embedded in Epon and ultrathin sections were cut with an LKB Ultratome III microtome. The sections were contrasted with uranyl acetate followed by lead citrate (Silva *et al.*, 1987) or with lead citrate alone, as indicated in the legends to the figures. Observations and micrographs were done with a Zeiss EM 10C electron microscope.

Ultrastructural cytochemistry. Detection of peroxidase activity by electron microscopy was carried out by the method of Robbins *et al.* (1971), with an initial fixation with 0.25 % glutaraldehyde.

Ultrastructural detection of alpha-naphthyl butyrate (ANB) esterase was carried out as described by Bozdech and Bainton (1981), with fixation with 1 % osmium tetroxide in distilled water with pH adjusted to 7.6 to heighten the contrast of the reaction product (Bozdech & Bainton, 1981). In some samples, the ANB esterase staining was followed by the peroxidase technique carried out as indicated above (Robbins *et al.*, 1971).

The ultrastructural staining of cytoplasmic polysaccharides was carried out using the original method of Thiéry (1967), or a modification (Silva & Macedo, 1987). These techniques, which specifically label, at the electron microscope level, periodic acid Schiff (PAS)-positive molecules, were applied to sections of samples fixed by the methods described above for conventional electron microscopy or processed for peroxidase or ANB esterase ultrastructural cytochemistry. For the Thiéry staining, the sections were mounted on gold grids and were sequentially treated with: 1 % aqueous

periodic acid for 30min, 0.2 % thiocarbohydrazide in 20 % acetic acid for 2h, and 1 % aqueous silver proteinate (Thiéry, 1967) or 1 % silver vitelinate (Silva & Macedo, 1987) for 30min in the dark.

Morphometry

To semi-quantitatively measure the amount of glycogen in neutrophils and macrophages, electron micrograph prints of Thiéry-stained sections of samples processed for the detection of peroxidase activity were made at x 20,000. The number of granules in cytoplasmic portions of median sections of the phagocytes was scored and converted to number of granules/μm^2.

Glucose assay in plasma and peritoneal exudates

Glucose concentrations were determined by the hexokinase method using a commercial kit, in exudates of inflamed peritoneal cavities and in plasma from heparinised venous blood of the same fish (n = 5), collected as described above. The determinations were carried out in trout that were injected i.p. with the 2 consecutive inocula of *Y. ruckeri*, as described above.

Statistics

Values were analysed by the Student´s *t*-Test.

RESULTS

Glycogen granules now observed in the cytoplasmic matrix of neutrophils and macrophages were of the b type (Drochmans, 1962) (Fig. 1-6). The diameter of neutrophil glycogen granules in ultrathin sections of samples stained by the Thiéry´s technique was 35.72 ± 3.20 nm (n = 36). Inflammatory macrophages in these samples contained granules with the same size as those of neutrophils (Fig. 3) as well as smaller granules (25.53 ± 3.81 nm; n = 14; $P<0.001$; Fig. 4). Occasionally, clusters of polysaccharide granules were found inside macrophages in inflamed peritoneal cavities (Fig. 5), sometimes in phagocytic vacuoles or in intramacrophagic neutrophils, as reported elsewhere (Afonso *et al.*, 1998a). These granules were of the larger size (Fig. 5).

Differences in glycogen amount and distribution in resting and inflammatory phagocytes

As previously reported (Afonso *et al.*, 1998,b), the distinction between neutrophils and macrophages, in peritoneal cavities of rainbow trout, particularly in inflamed cavities, is sometimes difficult. We have previously shown that trout

neutrophil glycogen can be stained by the Thiéry's technique applied to sections of samples processed for the ultrastructural detection of peroxidase or esterase activities, with the simultaneous staining of glycogen and peroxidase (cf. Fig. 1, 3 and 4), or glycogen and esterase (Afonso *et al.*, 1998b). This double labelling was now used for the comparative study of polysaccharide inclusions in the two phagocytes.

Abundant glycogen granules were consistently seen in the cytoplasm of resting or inflammatory neutrophils (Figs. 1-3 and 6). Inflammatory peritoneal neutrophils contained more glycogen granules than resting peritoneal neutrophils or neutrophils in the peripheral blood of non-injected or injected trout (Table 1); moreover, while in peritoneal resting neutrophils, and in blood neutrophils of injected or non-injected fish, the glycogen granules were evenly distributed in the cytoplasmic matrix (Fig. 1), in neutrophils from inflamed peritoneal cavities clusters of glycogen granules were usually present (Fig. 2 and 6). In the samples of inflamed peritoneal cavities, neutrophil glycogen particles were more abundant when the peritoneal cavities were injected with IFA or with a second bacterial inoculum (Table 1).

Neutrophils in the blood had amounts of glycogen granules which were not statistically different for non-injected and i.p. injected trout (Table 1).

In macrophages, glycogen granules were not seen in resting peritoneal populations and were scarce in inflammatory macrophages (Table 1). This significant difference in the glycogen content of the two phagocytes explains the rather strong red staining (PAS-positivity) seen in neutrophils by light microscopy of cytospins stained with the Schiff reagent; macrophages appear PAS-negative (not shown).

Glucose concentration in plasma and inflamed peritoneal exudates

Glucose concentration in plasma of 5 trout injected with the 2 consecutive inocula of *Y. ruckeri* was 187.6 ± 73.3mg/100ml. In this situation of intense peritoneal inflammation, small amounts of ascitic fluid were present. The glucose concentrations were determined in the peritoneal exudates of the same fish, collected after the i.p. injection of 2.0ml of PBS and then corrected for the volume of the ascites. The glucose concentration was 148.0 ± 42.7 mg/100ml of the ascite. This value is not statistically different from that of plasm ($P = 0.434$).

DISCUSSION

The present study of glycogen in trout leukocytes used a semi-quantitative morphometric assay. This technique has the advantage, over the chemical assay, of allowing the analysis of individual leukocytes, whereas the chemical method only gives an averaged value for the total leukocyte population. Moreover, with the use of double-

labelling techniques (peroxidase/glycogen and esterase/glycogen) the precise differential characterization of glycogen of neutrophils and macrophages was achieved.

Glycogen granules could not be found in resting peritoneal macrophages, and in inflammatory peritoneal macrophages smaller amounts were found as compared to neutrophils. In mammals, macrophages are known to have small reserves of glycogen compared to neutrophils (Carr & Daems, 1980; Parmley, 1988); the same was reported for the phagocytes of the plaice (Ellis, 1976) and the channel catfish (Cannon *et al.*, 1980). This difference in glycogen content of the two phagocytes is in accordance with neutrophils being primarily glycolytic cells and macrophages more capable of producing ATP via oxidative phosphorilation (Kilpatrick *et al.*, 1990).

No significant differences in glycogen content were now found between blood and resting peritoneal neutrophils. This result, in accordance with the observation that peroxidase content in these two neutrophil populations was not, as well, different (Afonso *et al.*, submitted), may be taken as an indication that the small population of neutrophils found in resting peritoneal cavities of rainbow trout is not activated.

As compared to resting peritoneal neutrophils, the glycogen amount was found to be slightly higher in neutrophils in the inflamed peritoneal cavity 24h after the i.p. injection of killed *Y. ruckeri* and much higher when a second injection of live bacteria was done into the already inflamed cavities. A high glycogen content was also found in neutrophils 44h after the i.p. injection of IFA. These results have to be related to the following observations. As recently reported (Afonso *et al.*, 1998b), following the i.p. injection of 5 x 10^7 *Y. ruckeri* in rainbow trout, the neutrophils attracted to the peritoneal cavity by the injection of the bacteria are not phagocytozing the microrganisms. This is so because the resting peritoneal cavity of trout contains a very important population of resident macrophages but very low numbers of neutrophils (Afonso *et al.*, 1997), these numbers increasing significantly only several hours after the i.p. inoculation of bacteria (Afonso *et al.*, 1998b). Consequently, the injected bacteria are taken by the resident macrophages before the arrival of the attracted neutrophils (Afonso *et al.*, 1998b). However, when the bacteria are injected into peritoneal cavities already containing high numbers of neutrophils (by the previous injection of inflammatory agents) neutrophils phagocytoze the micorganisms at a high rate (Afonso *et al.*, 1998b). On the other hand, we found that the mineral oil of the IFA is taken up by the macrophages and by the incoming neutrophils because, for the amount of IFA injected, not all the oil could be taken by the resident macrophages (unpublished results). It seems, therefore, that the neutrophils attracted to the peritoneal cavities when the bacteria are taken by the macrophages would be engaged in a sort of non-phagocytic activity which asks for lower amounts of reserve polysaccharide as compared with situations where the leukocyte has the opportunity to phagocytoze. Glycogen deposits in neutrophils are used up in the respiratory burst that accompanies phagocytosis of microrganisms (Scott, 1968; Wilkinson, 1977; Weisdorf *et al.*, 1982) and the higher

glycogen content of neutrophils engaged in phagocytosis would be useful. It may appear, therefore, strange the present observation that the amount of glycogen is not decreased in neutrophils with ingested bacteria. This observation is, however, in accordance with the results showing that neutrophil glycogen content only decreases when phagocytosis is carried out in the absence of glucose (Lane & Lamkin, 1985), but not when glucose is available (Stossel & Cooper, 1970), and that levels of glucose comparable to those in blood were now found in peritoneal exudates in the situation where neutrophils were phagocytosing bacteria.

In mammals and fish, circulating blood neutrophils and mature neutrophils of the haematopoietic organ pools, are resting cells available to migrate to foci of inflammation (Densen *et al.*, 1995; Suzuki & Iida, 1992). In guinea-pigs with the peritoneal cavity rendered inflamed by the i. p. injection of casein or LPS, Scott and Cooper (1974) found that at 24h post-injection, the amount of glycogen of peritoneal neutrophils was increased 7 fold, while, at the same time, blood neutrophils showed only a 1.6 fold increase in the content of the polysaccharide. This observation is in accordance with those of Wulff (1962) and of Robinson *et al.* (1982), and with our finding that neutrophils in the blood of trout with peritoneal cavities intensely inflamed by 2 consecutive i.p. injections of bacteria, have blood neutrophils with glycogen granules in amounts similar to those of the non-injected fish. These results indicate that the increase in glycogen content of the inflammatory neutrophils observed in the inflamed peritoneal cavities of mammals (Scott & Cooper, 1974; Robinson *et al.*, 1982) and fish (present results) occurs during the migration from the blood or in the inflammatory foci, rather than in the hematopoietic tissues or peripheral blood. However, Nagamura & Wakabayashi (1985) and Hine & Wain (1988) described the increase in PAS staining of blood neutrophils of the eel after the injection of bacteria or bacterial products. These conflicting results probably are explained by the use of a very archaic teleost.

The polysaccharide granules we found in inflammatory macrophages were of two types. One has a diameter smaller than that of neutrophil granules. The other seems to represent neutrophil glycogen transferred to macrophages. This would occur through the same mechanisms described for the transfer of other neutrophil components, like peroxidase (Silva *et al.*, 1989; Sousa, 1994; Afonso *et al.*, 1998a). It is tempting to consider that the glycogen particles transferred from neutrophils to macrophages would be used by the macrophages for their own metabolic activities, as was sugested long ago by Rebuck *et al.* (1968).

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TABLE 1

Semi-quantitative morphometric assay of amount of glycogen cytoplasmic granules in rainbow trout phagocytes

PHAGOCYTE	N° OF GRANULES PER μm^2
Non-inflammatory phagocytes	
Peripheral blood neutrophils in non-injected trout	50.80±13.52
Peripheral blood neutrophils in injected trout	44.45±12.28
Resting peritoneal neutrophils	61.61±18.27
Resting peritoneal macrophages	not detectable
Inflammatory phagocytes	
Peritoneal neutrophils	
Killed *Y. ruckeri* 24h	74.77±22.52
Killed *Y. ruckeri* 24h+ live *Y. ruckeri* 4h	121.80±42.98
IFA 44h	94.57±29.98
Peritoneal macrophages	
IFA 44h	14.05±4.86

Statistical analysis (Student´s *t* test)
Comparison:
Blood neutrophils (non-injected fish)/resting peritoneal neutrophils P=0.264
Blood neutrophils (non-injected fish/blood neutrophils (injected fish) P=0.324
Resting peritoneal neutrophils/neutrophils *Y. ruckeri* 24h P=0.339
Blood neutrophils/neutrophils *Y. ruckeri* 24h P=0.043
Resting peritoneal neutrophils/Neutrophils *Y. ruckeri* 24h+*Y.ruckeri* 4h P=0.02
Blood neutrophils/neutrophils IFA 44h P=0.002
Resting peritoneal neutrophils/neutrophils IFA 44h P=0.049
Macrophages IFA 24h/neutrophils IFA 44h P=0.001

LEGENDS TO THE FIGURES

In all figures Bar = 1μm

Fig. 1

Neutrophil in a resting peritoneal cavity, fixed with 0.25 % glutaraldehyde, processed for peroxidase and fixed with OsO_4. Section stained by the Thiéry's technique. Non-clustered PAS-positive granules are uniformly distributed in the cytoplasmic matrix of the neutrophil. Several peroxidase-positive cytoplasmic granules are present.

Fig. 2

A-Neutrophil in the peritoneal exudate of a rainbow trout injected 24h before with live 5 x 10^7 *Yersinia ruckeri*. Sample fixed with 0.25 % glutaraldehyde, processed for the detection of peroxidase activity and then fixed with OsO_4 supplemented with potassium ferrocyanide. No postfixation with uranyl acetate. Notice the peroxidase-positive granules and the clusters of densely contrasted glycogen granules (arrows). Section contrasted with lead citrate.

B-Enlarged part of Fig. 2 showing clusters of densely contrasted glycogen granules.

Fig. 3

High magnification showing portions of a neutrophil (left) and of a macrophage in the same sample as in Fig. 2. Sample fixed with 0.25 % glutaraldehyde, processed for the detection of peroxidase activity and fixed with OsO_4. No postfixation with uranyl acetate. Section stained by the Thiéry's technique. Notice the numerous PAS-positive granules in the cytoplasm of the neutrophil. The macrophage shows granules similar to those of the neutrophil, but in a smaller amount.

Fig. 4

Peritoneal exudate of a rainbow trout injected 44h before with Incomplete Freund's Adjuvant (IFA). Sample fixed with 0.25 % glutaraldehyde, processed for the detection of peroxidase activity and postfixed with OsO_4. The pictures shows portions of a neutrophil (top; with peroxidase-positive granules) and of a macrophage. Section stained by the Thiéry's method. Notice the clusters of abundant PAS-positive granules in the neutrophil. A few, PAS-positive granules are present in the macrophage; these granules are smaller than those in the neutrophil.

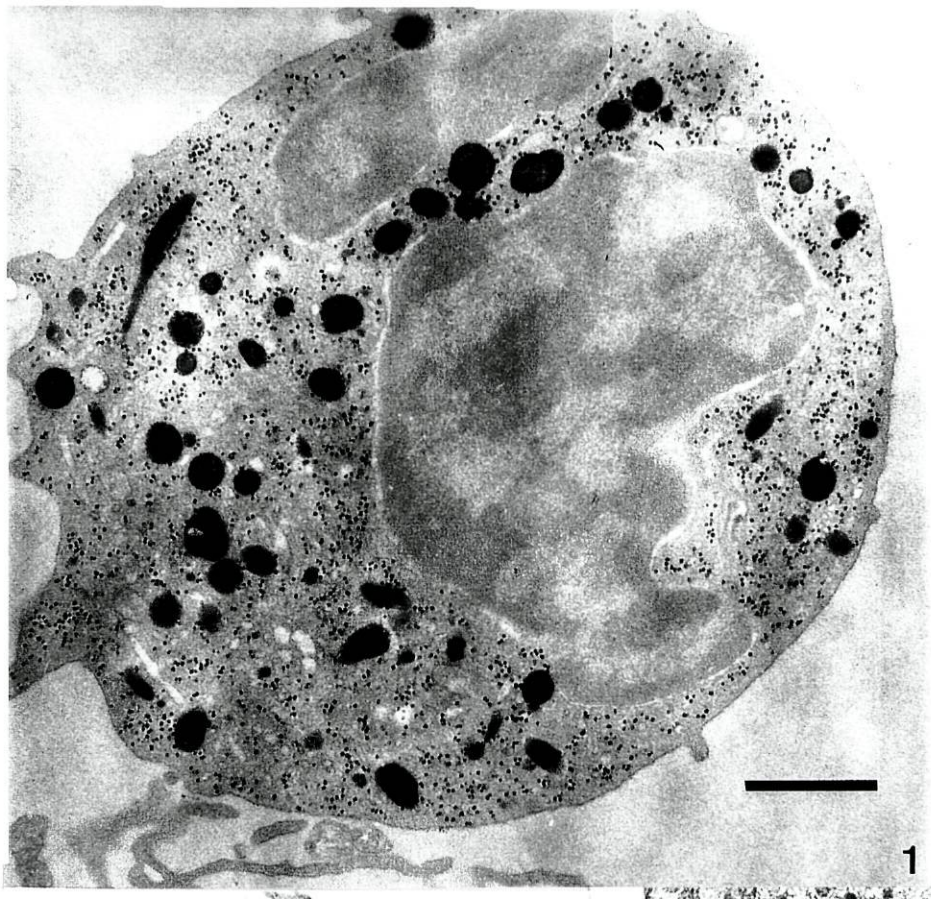
Fig. 5

Same sample as in Fig. 2. Fixation with 0.25 % glutaraldehyde, and sample processed for the detection of peroxidase activity and postfixed with OsO_4 supplemented with potassium ferrocyanide. The picture shows a macrophage with a large cluster of glycogen granules (arrow). Sites of peroxidase activity are present (arrowheads), including a granule in the glycogen deposit. Section contrasted with lead citrate.

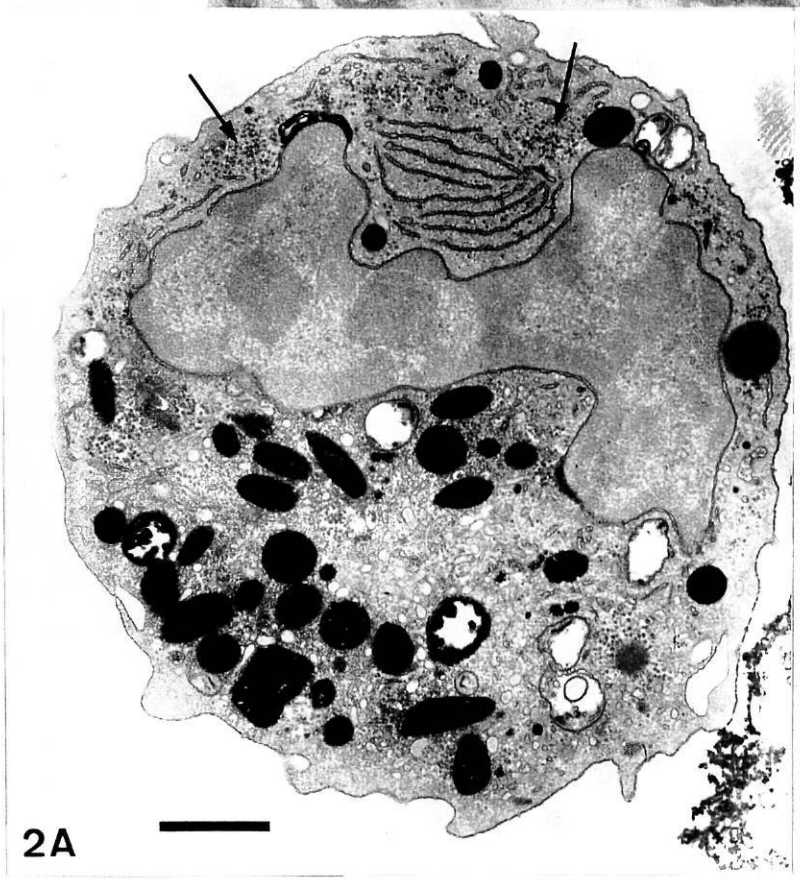
Fig. 6

Part of a neutrophil in the same sample as in Fig. 4. Sample fixed by protocol (i), and section stained by the Thiéry´s technique. Clusters of abundant PAS-positive granules are present in the cytoplasmic matrix of the neutrophil.

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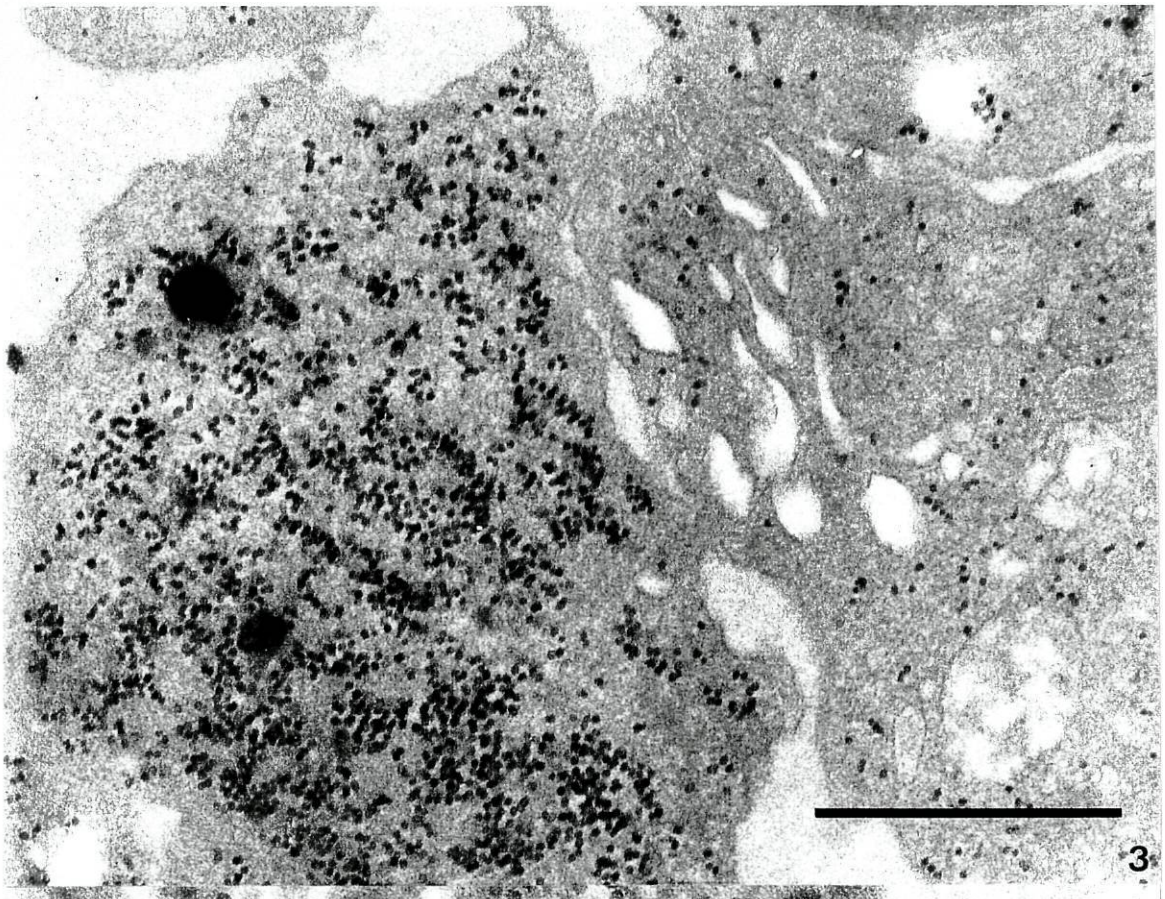
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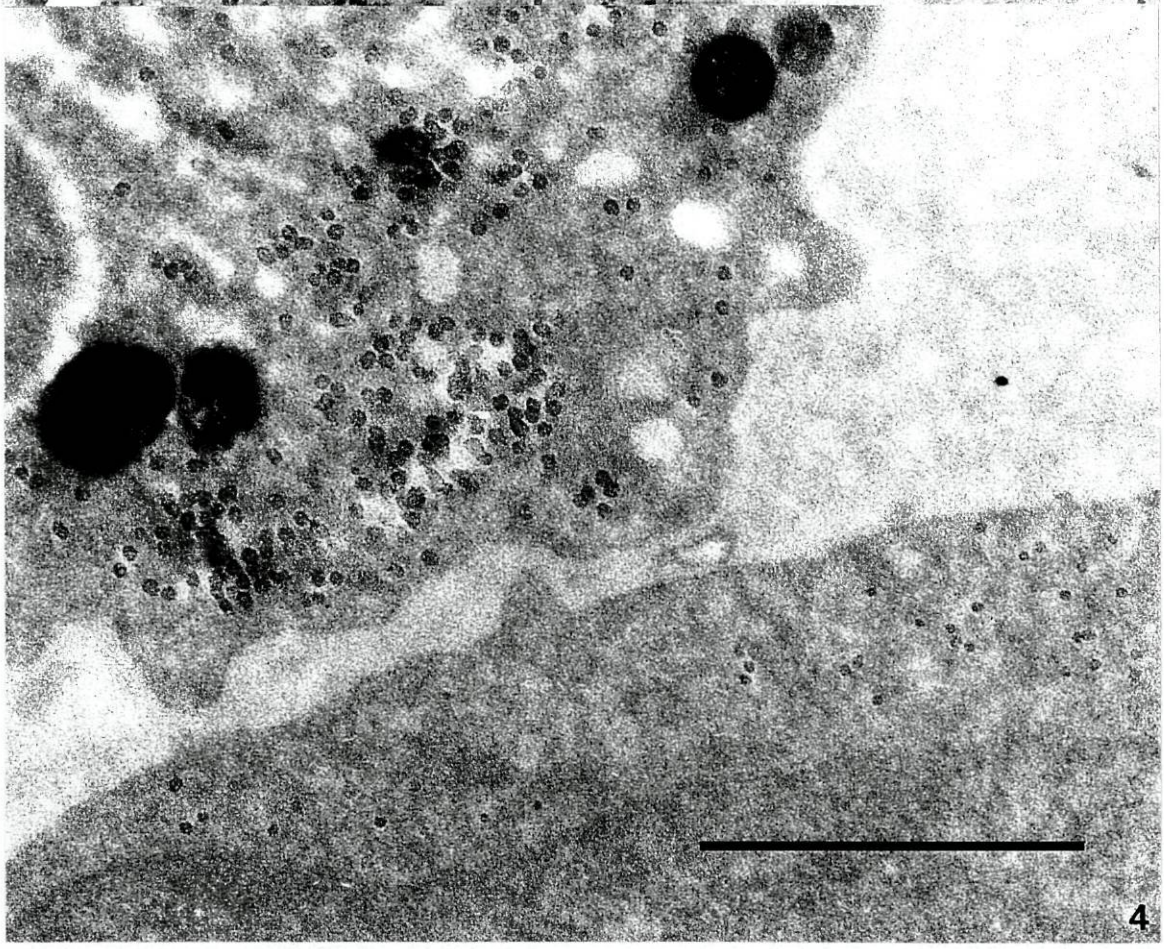


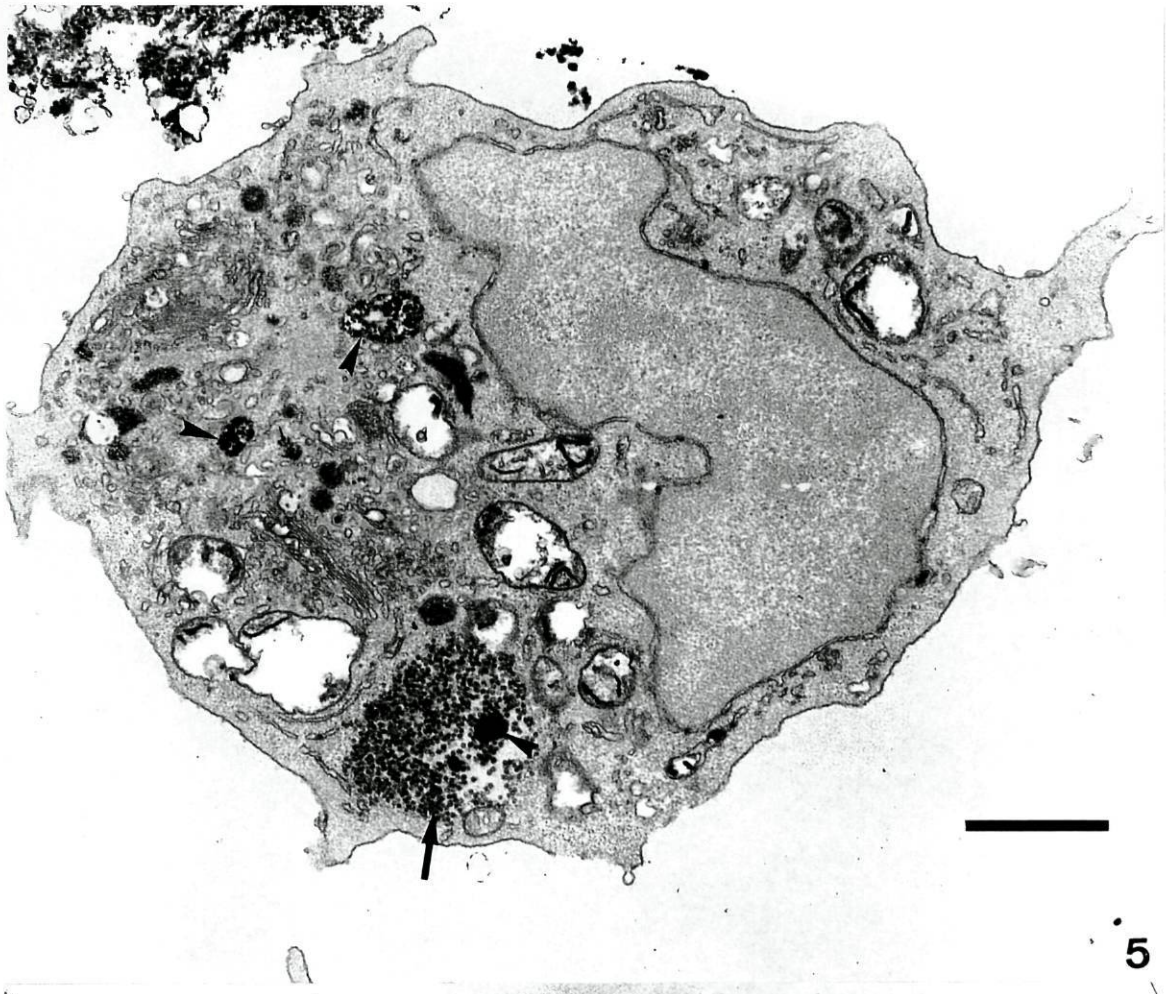
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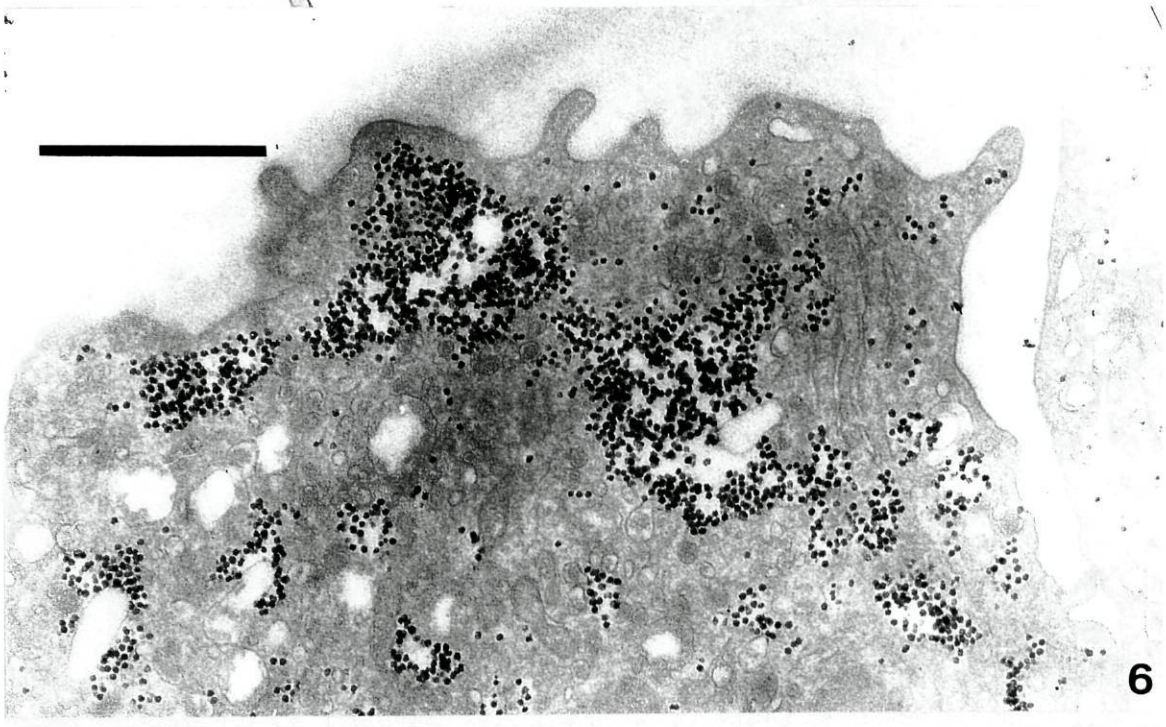
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Capítulo 3

A interacção entre macrófagos e neutrófilos na cavidade peritoneal inflamada da truta arco-íris e do robalo

3.1. Afonso, A., Silva, J., Lousada, S., Ellis, A. E. & Silva, M. T. (1998). Uptake of neutrophils and neutrophilic componentes by macrophages in the inflamed peritoneal cavity of rainbow trout *(Oncorhynchus mykiss*). *Fish and Shellfish Immunology.* **8**: 319-338.

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Uptake of neutrophils and neutrophilic components by macrophages in the inflamed peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*)

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The interaction between neutrophils and macrophages in peritoneal inflammatory exudates in rainbow trout injected intraperitoneally with several bacterial and non-bacterial phlogistic agents was studied by light and electron microscopic histochemistry. The transfer of neutrophilic components to macrophages was demonstrated by the presence in macrophages of myeloperoxidase, an enzyme of the neutrophil not produced by rainbow trout monocytes and macrophages. Two mechanisms observed for the transfer were phagocytosis of intact neutrophils and phagocytosis of apoptotic bodies released from inflammatory neutrophils. Neutrophils with phagocytosed bacteria were also found to be ingested by macrophages, with the consequent transfer of neutrophilic components and bacteria to the macrophages. Two other mechanisms of transfer are considered. One involves the intake by the macrophage of neutrophilic granules or granular molecules released by lysing or degranulating neutrophils, respectively. The other may be through gap junctions which were observed between adhering inflammatory neutrophils and macrophages.
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Key words: rainbow trout, peritoneal leucocytes, neutrophil/macrophage interaction, phagocytes in fish infection.

I. Introduction

A crucial mechanism of defence in vertebrates is phagocytosis, killing and degradation of invading microorganisms by neutrophils and macrophages (Suzuki, 1986; Lehrer, 1988; Ainsworth, 1992; Secombes & Fletcher, 1992; Densen *et al.*, 1995). Studies carried out on mammals indicate that phagocytes display different patterns of localisation in the normal host and mobilisation into inflamed territories (Lehrer, 1988; Haslett *et al.*, 1989; Silva *et al.*, 1989; van Furth, 1992; Densen *et al.*, 1995). Such patterns can be summarised as follows. Neutrophils are present in large numbers in blood and haematopoietic organs as reserve pools, and, under normal conditions, are rare in the

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tissues and body cavities. In contrast, macrophages are present in all body compartments and constitute the first phagocytes which invading microorganisms encounter. Neutrophils from the reserve pools are quickly and extensively attracted to the infectious foci by microbial products and by cytokines produced by immune cells including macrophages (Lehrer, 1988; Appelberg, 1992*a*,*b*,*c*). Monocytes, the blood circulating precursors of tissue macrophages, are also recruited to infectious sites, although at a slower rate than neutrophils, where they increase the initial macrophage population; cytokines produced by neutrophils are known to attract monocytes (Taub *et al.*, 1996). It follows, therefore, that each of the two phagocytes has the capacity to attract the other. Shortly after the onset of an infection, the phagocyte population at the infectious foci is enlarged and composed of both macrophages and neutrophils. Previous studies have dealt with the interactions between the two phagocytes in those sites in mammals, using the peritoneal cavity of mice (Silva *et al.*, 1989; Sousa, 1994).

The peritoneal model of infection, both in mammals (Silva *et al.*, 1989; Appelberg *et al.*, 1991; Sousa, 1994) and fish (Afonso *et al.*, 1997, 1998), provides a clear example of the distinct patterns of distribution and mobilisation of the two phagocytes referred to above. In the case of fish, it has been shown that the sterile peritoneal cavity of rainbow trout contains a significant population of resident macrophages but very few neutrophils (Afonso *et al.*, 1997), though the latter are quickly and extensively attracted to the cavity when microorganisms are injected intraperitoneally (Afonso *et al.*, 1998).

Classically, macrophages have been reported as the only phagocytes implicated in the killing of so-called intracellular parasites, because these phagocytes are the host cell for such pathogens. On the other hand, neutrophils are considered as effector phagocytes for extracellular parasites only, their role being viewed as irrelevant against the parasites growing intracellularly in macrophages because in such a residence these parasites are not accessible to phagocytosis and killing by neutrophils (Mackaness, 1969; Adams & Hamilton, 1984; Edwards & Kirkpatrick, 1986).

Since the initial finding of a persistent presence of significant numbers of neutrophils in the peritoneal cavity of mice intraperitoneally infected with mycobacteria (Silva *et al.*, 1989; Appelberg *et al.*, 1991), an intracellular parasite growing inside macrophages has been of interest to researchers investigating the possible roles of neutrophils in defence mechanisms against intramacrophagic pathogens. In particular the possibility was proposed of a neutrophil participation in the defence against intramacrophagic infection by an indirect mechanism alternative to the classical direct activity via phagocytosis and killing of infectious agents. Such a mechanism would involve the interaction between neutrophils and macrophages in the infectious foci, with the phagocytosis of the neutrophil, or of neutrophilic components, by the infected macrophage (Silva *et al.*, 1989; Sousa, 1994).

In fish, both types of phagocytes are known to be present and to operate in infectious situations through processes essentially identical to those reported for mammals (Ellis, 1977; Ainsworth, 1992; Hine, 1992; Secombes & Fletcher, 1992; Afonso *et al.*, 1998). The present paper describes and discusses some facets of the interaction between neutrophils and macrophages in rainbow

trout (*Oncorhynchus mykiss*) using the peritoneal model of inflammation (Silva *et al.*, 1989; Sousa, 1994; Afonso *et al.*, 1997) which produces exudates where the two professional phagocytes co-exist (Afonso *et al.*, 1998).

II. Materials and Methods

EXPERIMENTAL FISH

Sexually immature rainbow trout weighing between 100 and 200 g were purchased from a commercial farm. The animals were maintained in recirculating aerated fresh water, at 15° C, in 7000 litre fibreglass tanks. Water quality was maintained with mechanical and biological filtration and fish were fed ad libitum on commercial pellets. The fish to be injected were transferred to 600 litre tanks where they were maintained for at least 15 days in the same conditions as above before the study. Only healthy fish, as concluded from their activity and exterior appearance, were used for the i.p. injections.

GROWTH, HARVESTING AND TREATMENT OF BACTERIAL AGENTS

The strain FP-3 of *Yersinia ruckeri* type 1 was kindly supplied by Prof. J. Barja (University of Santiago de Compostela, Spain). To prepare the suspensions, the bacteria were grown in Tryptic Soy Broth (TSB) (Difco) at 18° C until the middle of the exponential phase, using as inoculum bacteria repeatedly passaged on Tryptic Soy Agar. The bacteria were collected from the broth by centrifugation and suspended in TSB with 15% glycerol to the indicated concentrations, divided in aliquots and frozen at − 70° C. Final suspensions of bacteria in phosphate buffered saline (PBS) with osmolality adjusted to 310 mOsm l^{-1} were adjusted to 5×10^8 bacterial ml^{-1} by counting in a Helber Bacteria Counting Chamber (Fisons Scientific Equipment, Loughborough, U.K.). Preliminary infectivity tests showed that, for the conditions used in this work, this inoculum is not pathogenic for trout.

Renibacterium salmoninarum (strain FT-10) was kindly supplied by Prof. J. Barja (University of Santiago de Compostela, Spain). To prepare the suspensions, bacteria grown on KDM 2 medium (Evelyn, 1977) for 15 days at 15° C were suspended in L-15 medium (Gibco BRL) with 5% foetal bovine serum, divided in aliquots and frozen at − 70° C. The inocula to be injected were prepared by washing and resuspending the above aliquots in PBS, at 1×10^9 bacteria ml^{-1}. Bacteria were counted in a chamber as described above for *Y. ruckeri*. Under the conditions described, this inoculum of *R. salmoninarum* was found to induce a progressive, fatal, infection.

INTRAPERITONEAL INJECTION OF PHLOGISTIC AGENTS

The following agents were used: 12% casein in water, undiluted Incomplete Freund's Adjuvant (IFA), and suspensions of bacteria in PBS. All agents were

injected into the peritoneal cavity of fish under anaesthesia (0·3 ml ethylene glycol monophenyl ether l^{-1}, Merck), in volumes of 100 μl using a 25 G needle. Immediately after the i.p. injection the fish were returned to their tank. Control fish were injected i.p. with PBS.

COLLECTION OF PERITONEAL LEUCOCYTES

The peritoneal cells were collected at intervals (see Results) after the i.p. injections by a procedure previously described in Afonso *et al.* (1997). Briefly, after killing the fish by anaesthetic overdose and exsanguination, 5 ml of PBS containing 20 units ml^{-1} heparin (Choay) was injected i.p. in the ventral midline followed by injection through the same needle of about 1 ml of air. The abdominal area was massaged for about 30 s to disperse the peritoneal cells in the injected PBS and the cells in suspension were then collected with a syringe fitted with a 19 G needle and placed on ice until processed.

STUDY OF PERITONEAL LEUCOCYTES

The peritoneal cell suspension was analysed within minutes after collection for several morphological and cytochemical parameters as follows.

Light microscopy

Total cell counts were performed with a haemocytometer. Cytospin preparations were made with a Shandon Cytospin 2 using a volume of peritoneal lavage depending on the cell concentration, in order to obtain adequate numbers of cells per slide. The cytospins were stained with Wright's stain (Haemacolor, Merck) after fixation for 1 min with formol–ethanol (10 ml of 37% formaldehyde and 90 ml of absolute ethanol) (Kaplow, 1965), and leucocyte differential counts were performed as described (Afonso *et al.*, 1997).

Electron microscopy

The peritoneal leucocyte suspension in PBS was centrifuged at 1000 *g* and the pellet was fixed with 4% paraformaldehyde–1·25% glutaraldehyde–10 mM calcium fixative (Silva & Macedo, 1983; Silva *et al.*, 1987) at 4° C. After a minimum of 4 h the cells were washed twice with 50 mM cacodylate buffer supplemented with 10 mM calcium chloride (pH 6·4) and then fixed at room temperature for 2 h with 1% OsO_4 in veronal–acetate buffer supplemented with 10 mM calcium chloride (Silva & Macedo, 1983) and postfixed at room temperature with 1% aqueous uranyl acetate for 1 h (Silva *et al.*, 1971). After dehydration in ethanol the samples were embedded in Epon and ultrathin sections were cut with an LKB Ultratome III microtome. Routinely, the sections were contrasted with uranyl acetate followed by lead citrate (Silva *et al.*, 1987). Observations and micrographs were done with a Zeiss EM 10 C electron microscope.

Cytochemistry

Peroxidase. For light microscopy the method of Kaplow (1965) or a modification of it (Antonow's technique) were used. Antonow's method was carried out as follows (Afonso *et al.*, 1998):

(i) Fixation of the cytospin preparations was carried out with formol–ethanol using the Kaplow method (Kaplow, 1965).

(ii) After rinsing with running tap water for a few seconds, the preparations were treated with a 1/30 dilution (in water) of the Kaplow's reagent (without safranin) for 5–10 min at room temperature. The diluted reagent was prepared fresh or kept frozen.

(iii) To counterstain the preparations the Wright or the Gram stain was used.

In the case of fish injected i.p. with *Y. ruckeri*, the number of peritoneal macrophages positive for peroxidase was counted. For this purpose, peritoneal cells from groups of six fish were collected at intervals (see Fig. 11) until 16 days after the bacterial inoculation. The cells were processed, as described above, for leucocyte counts and staining of peroxidase. The numbers of total macrophages, peroxidase-positive macrophages and neutrophils were counted in at least 200 leucocytes per fish.

For ultrastructural studies the method of Robbins *et al.* (1971) was used. Controls involved omitting hydrogen peroxide from the incubation mixtures.

Esterase. Ultrastructural detection of alpha-naphthyl butyrate (ANB) esterase was carried out as described by Bozdech and Bainton (1981). In some samples this was followed by the peroxidase technique carried out as indicated above (Robbins *et al.*, 1971).

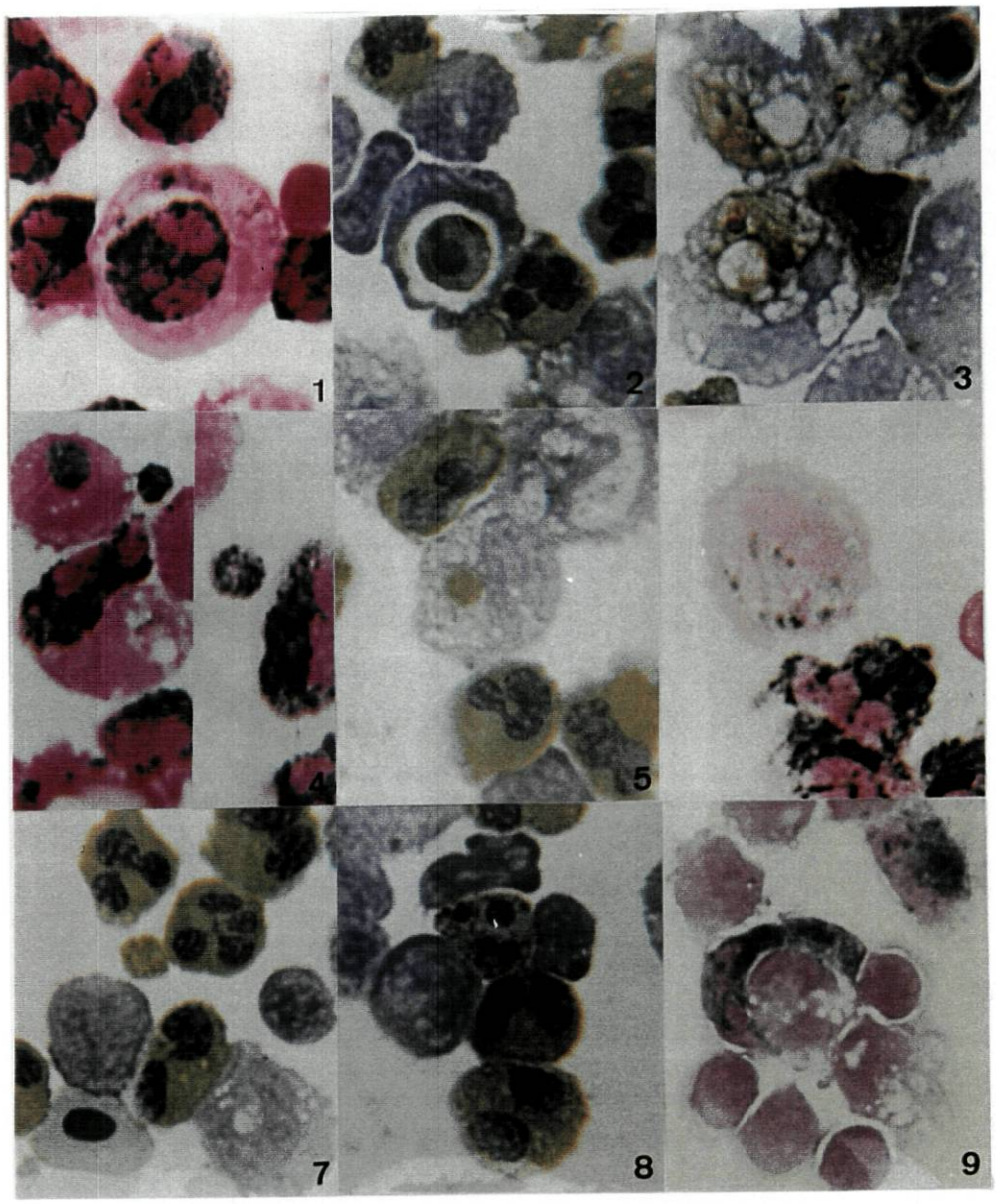
Polysaccharide staining. The ultrastructural staining of cytoplasmic polysaccharides was carried out using Thiéry's method (Silva & Macedo, 1987; Thiéry, 1967) applied to ultrathin sections of samples processed as described above for conventional electron microscopy or for ultrastructural cytochemistry for peroxidase.

III. Results

INFLAMMATORY PERITONEAL EXUDATE

The i.p. injection of the phlogistic agents used in the present study induces neutrophilic and monocytic/macrophagic responses of variable magnitude which were previously described in detail (Afonso *et al.*, 1998). Therefore, the peritoneal exudates analysed contained predominantly phagocytes which were of three types: resident macrophages, incoming exudate macrophages (monocytes) and neutrophils (mostly incoming).

As previously reported in detail (Afonso *et al.*, 1997, 1998) the rainbow trout neutrophils were peroxidase-positive (Figs 1–9), ANB esterase-negative (Fig. 10) and contained large amounts of intracytoplasmic glycogen. On the other hand, blood monocytes and peritoneal resident macrophages were ANB esterase-positive, peroxidase-negative and contained few glycogen granules (Afonso *et al.*, 1997, 1998).



Figs 1-9. Cytospin preparation of leucocytes in the peritoneal exudate of a rainbow trout 48 h after the intraperitoneal inoculation of 5 x 10^7 Y. *ruckeri.* Preparation stained for peroxidase using Kaplow's (Figs 1, 4, 6, 9) and Antonow's (Figs 2, 3, 5, 7, 8) technique. Fig. 1. Note the abundant peroxidase-positive (blue) neutrophil granules. A macrophage contains an intact neutrophil and a few small peroxidasepositive cytoplasmic granules. Fig. 2. Note the peroxidase-positive (green) cytoplasm of the neutrophils. A macrophage contains an apoptotic neutrophil. Fig. 3. Macrophages with large areas containing peroxidase activity. Fig. 4. Three neutrophil apoptotic bodies including one inside a macrophage. Fig. 5. Macrophage with a neutrophil apoptotic body. Fig. 6. Macrophage containing several small peroxidase-positive granules. Fig. 7. Neutrophil apoptotic body in contact with a neutrophil. Fig. 8. One neutrophil has the nuclear characteristics of apoptosis. Fig. 9. Note the neutrophil embracing a macrophage. All magnifications x 1000.

Contrary to the resident macrophages of the unstimulated peritoneal cavity of rainbow trout (Afonso *et al.*, 1997), some large mononuclear leucocytes of the cavity inflamed by the injection of all the phlogistic agents studied with morphology of macrophages contained peroxidase-positive material (Figs 1–6, 10). That these peroxidase-positive leucocytes were macrophages was confirmed by using the simultaneous staining for esterase/peroxidase (Fig. 10) or peroxidase/glycogen, as described in detail elsewhere (Afonso *et al.*, 1998).

The patterns of peroxidase positivity inside macrophages found in light microscopy preparations can be grouped as follows: type 1—positivity due to the granules of intact neutrophils (Fig. 1), including apoptotic neutrophils (Fig. 2), phagocytosed by macrophages (see below); type 2—clumps of peroxidase-positive material in large vacuoles (Fig. 3); type 3—clumps of positive granules inside small vacuoles (Figs 4 and 5); and type 4—dispersed and usually scarce small granules (Fig. 6). Ultrastructural images corresponding to the above patterns were also observed; some examples are shown in Figs 12, 13 and 16.

Quantification of the peroxidase-positive peritoneal macrophages was carried out in light microscopy preparations stained by the Antonow's method in the case of i.p. inoculation of *Y. ruckeri* (Fig. 11). This quantification shows that the kinetics of peroxidase-positive macrophages followed that of incoming neutrophils, and that at 24 h post-inoculation most of the macrophages contained peroxidase activity. Throughout the period of time studied (Fig. 11), most of the peroxidase-positive macrophages were of the type 4 described above. In the control fish injected i.p. with PBS the values for peroxidase-positive macrophages were very low (not shown).

PHAGOCYTOSIS OF NEUTROPHILS BY MACROPHAGES

In the exudates of fish injected with the phlogistic agents used, where the two types of professional phagocytes were co-existing, the regular presence of macrophages which had internalised neutrophils was recorded, both by light microscopy (Figs 1 and 2) and at the ultrastructural level (Figs 12 and 13). That the phagocytosed cells inside the macrophages were in fact neutrophils could be determined by their typical morphology in Wright-stained cytospins or because of the presence of the characteristic cytoplasmic granules and abundant glycogen particles by electron microscopy, and by the peroxidase-positivity of the cytoplasmic granules of the internalised cells.

In the peritoneal cavity of trout inoculated with *R. salmoninarum*, macrophages were found which had phagocytosed neutrophils containing bacteria (Fig. 13).

NEUTROPHIL APOPTOSIS

Neutrophils with nuclear alterations typical of apoptosis were observed in the inflamed peritoneal exudates (Fig. 8). Preliminary observations with the Apo-Direct kit for apoptosis detection (Phoenix Flow Systems, San Diego, CA, U.S.A.) showed that these nuclear abnormalities corresponded to DNA

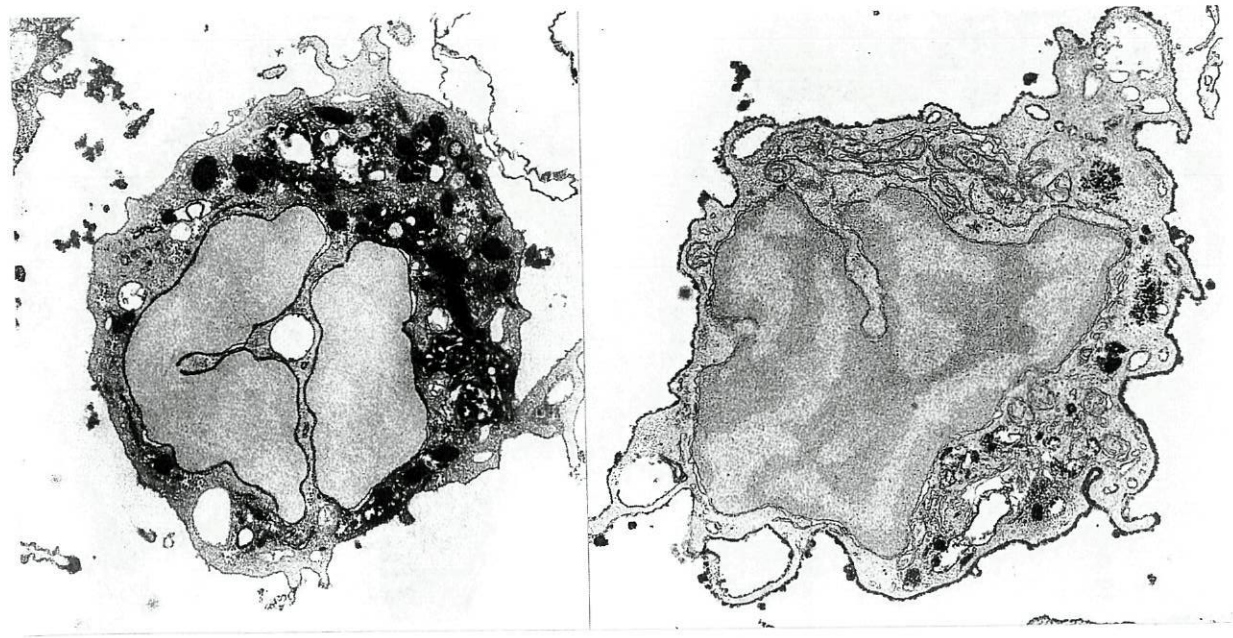


Fig. 10. A neutrophil and a macrophage in a peritoneal exudate of a rainbow trout collected 48h after inoculation of $5x10^7$ *Y. ruckeri*. Sample processes for ABN - esterase followed by peroxidase stainings. The neutrophil has several peroxidase-positive cytoplasmatic granules and no esterase staining. The macrophage shows the typical esterase staining and no esterase staining. The macrophage shows the typical esterase stainig of its cell surface and a few peroxidase-positive cytoplasmatic granules and vacuoles. Section contrasted with lead citrate. x 9000.

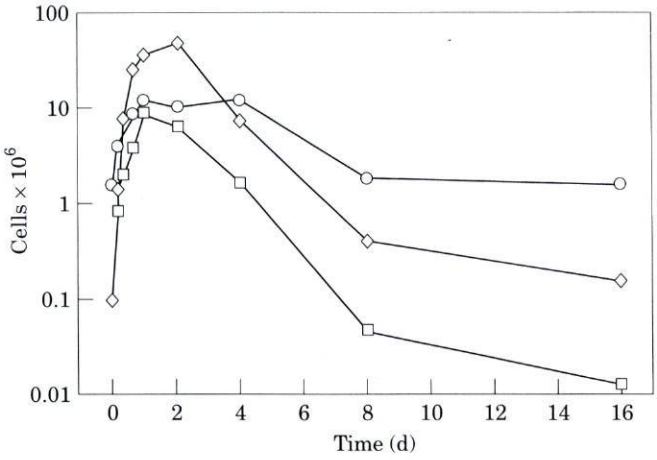


Fig. 11. Kinetics of total macrophages, peroxidase-positive macrophages, and neutrophils in the peritoneal cavity of rainbow trout injected intraperitoneally with 5×10^7 *Y. ruckeri*. (○) Total macrophages. (□) Peroxidase-positive macrophages. (◇) Neutrophils. Six fish per time point were studied.

fragmentation, a hallmark of apoptosis (Cohen *et al.*, 1992). The apoptotic neutrophils were occasionally seen shedding apoptotic bodies (Fig. 14). These structures were identified by light and electron microscopy by their subcellular size, spherical shape and by the presence of peroxidase-positive granules (Figs 4, 7, 15, 16). Free neutrophil apoptotic bodies were also observed in the inflammatory peritoneal exudates (Figs 4, 7, 15) and macrophages containing neutrophil apoptotic bodies were regularly observed in all the situations of intraperitoneal inflammation studied (Figs 4, 5, 16).

In the case of peritoneal inflammation induced by bacterial agents, apoptotic bodies containing bacteria were also observed both free and in macrophages (Fig. 16).

ADHERENCE OF NEUTROPHILS TO MACROPHAGES

The phagocytosis of neutrophils by macrophages, as reported above, appeared to be initiated by the two phagocytes adhering together, followed by the progressive surrounding of the neutrophil by the macrophage prior to its internalisation. However, another type of close contact between the two phagocytes was observed, namely an interaction where the macrophage was surrounded by the neutrophil, as if the neutrophil were phagocytosing the macrophage (Fig. 9), though this was never found to occur. It appears that some specificity exists in the above association because: (i) neutrophils were never seen surrounding other leucocytes besides the macrophages; and (ii) only in inflammatory situations was such an interaction found; never in normal, unstimulated peritoneal exudates where, as reported previously, a few neutrophils are nevertheless present (Afonso *et al.*, 1997). Sometimes the macrophage embraced by a neutrophil contained peroxidase-positive material. By electron microscopy cell adhesions with the characteristics of gap junctions (Fig. 17) were occasionally found in neutrophil/macrophage pairs.

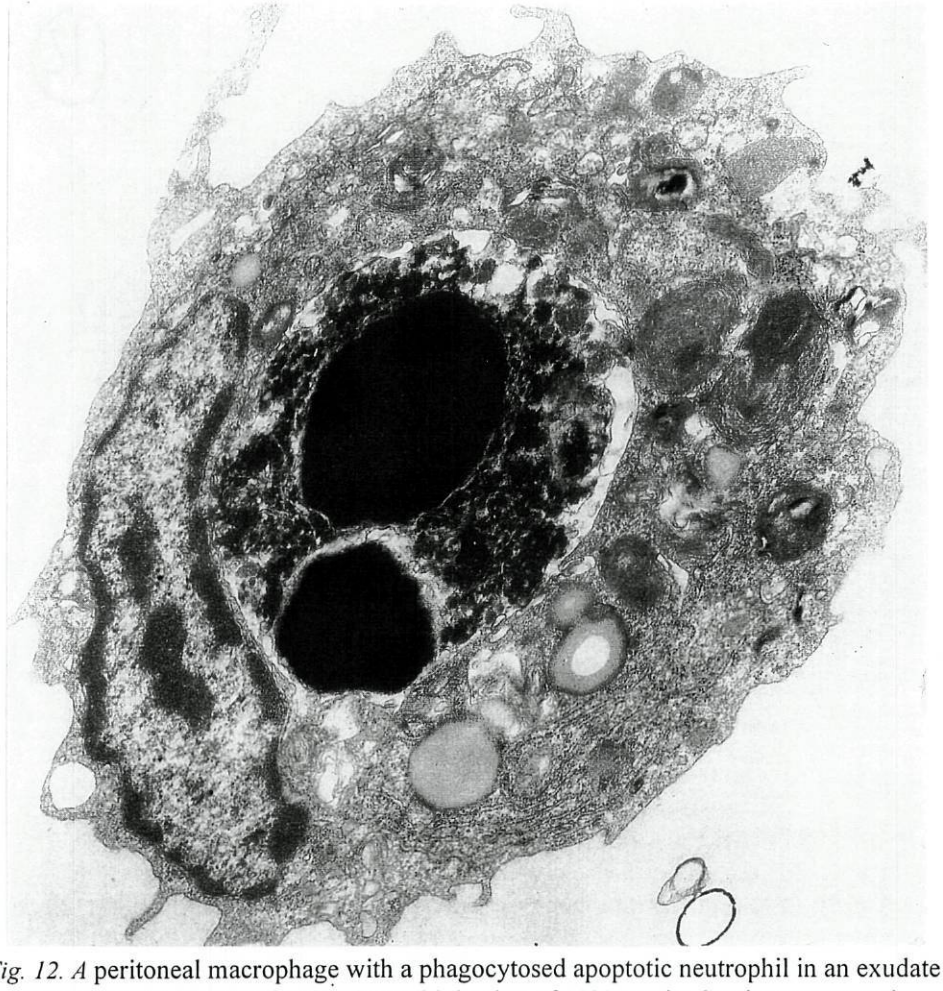


Fig. 12. A peritoneal macrophage with a phagocytosed apoptotic neutrophil in an exudate collected 40 h after the intraperitoneal injection of 12% casein. Section contrasted with uranyl-lead. x 13 600.

IV. Discussion

The present results show that in fish, as in mammals (Brewer, 1964; Parmely *et* al., 1981; Newman *et* al., 1982; Sanui *et* al., 1982; Savill *et* al., 1989a,b; Silva *et* al., 1989; Sousa, 1994), the macrophage and the neutrophil present together in an inflammatory site display interactions which are likely to have important physiological consequences.

As previously described in detail (Afonso *et al.,* 1997, 1998), the use of cytochemical methods has made it possible to clearly and specifically label neutrophils in the peritoneal cavity of rainbow trout because these phagocytes have abundant peroxidase-positive granules and abundant intracytoplasmic glycogen inclusions, and are negative for ANB esterase staining. The resident peritoneal macrophages of rainbow trout are peroxidase-negative when assayed either by light or electron microscopy (Afonso *et al.,* 1997, 1998). Blood monocytes were also found to be peroxidase-negative and, therefore, the

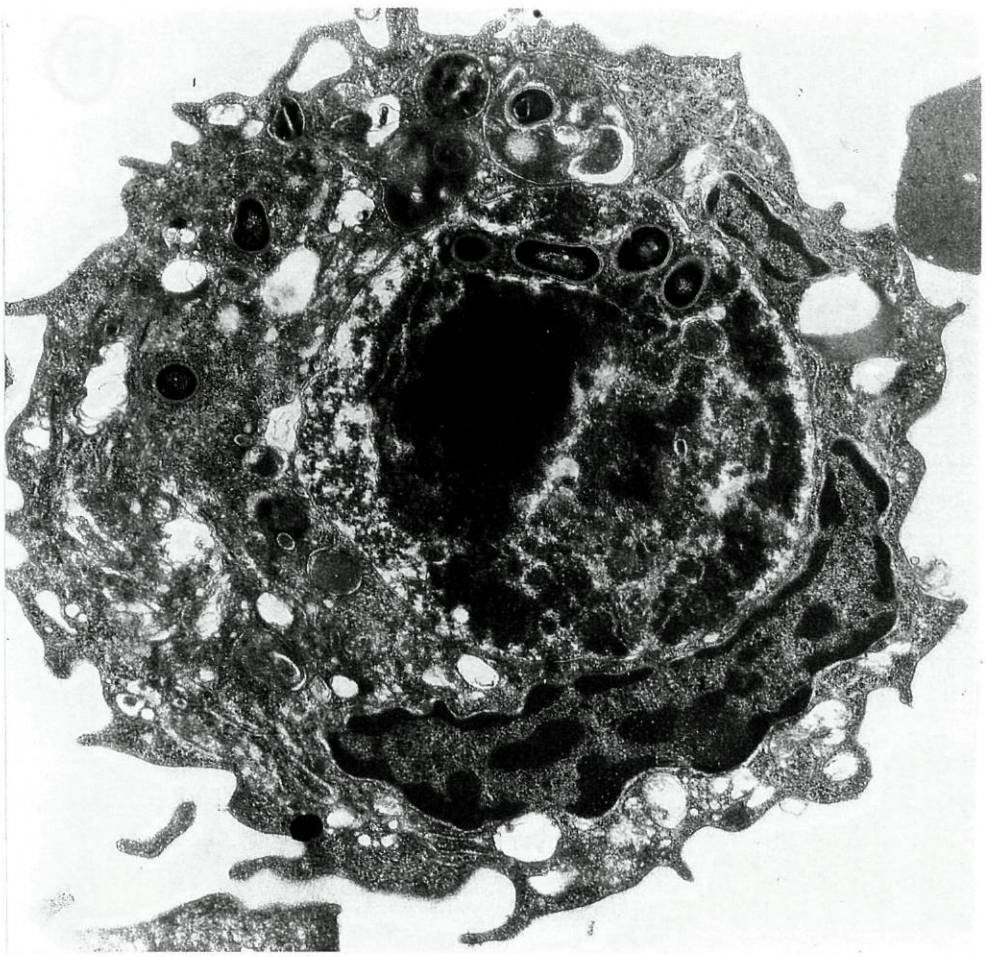


Fig. 13. A peritoneal macrophage with a phagocytosed neutrophil in an exudate of a trout inoculated 6 d before with 1 x 10^8 *R. salmoninarum.* Notice the presence of bacteria in the cytoplasms of both the macrophage and the neutrophil. Section contrasted with uranyl-lead x 12 800.

incoming (exudate) monocytes in the inflamed peritoneal cavity must also be devoid of peroxidase activity (Afonso *et al.,* 1998). Since the neutrophils are the only peroxidase-positive leucocyte in the peritoneal exudate, the peroxidase activity can be used as a specific marker for neutrophilic material. In the present study of inflammatory exudate cells we found some peroxidasepositive large mononuclear leucocytes with the morphology of macrophages. That these leucocytes are indeed macrophages is confirmed by two other cytochemical characteristics: their ANB esterase positivity and scarcity of intracytoplasmic glycogen granules. Consequently, the presence of peroxidase activity in macrophages of the inflamed peritoneal cavity of rainbow trout is an indication of the occurrence of transfer of neutrophilic material to the macrophages. The present observations show two obvious mechanisms for such a transfer: the phagocytosis by the macrophage of intact neutrophils and of neutrophil apoptotic bodies.

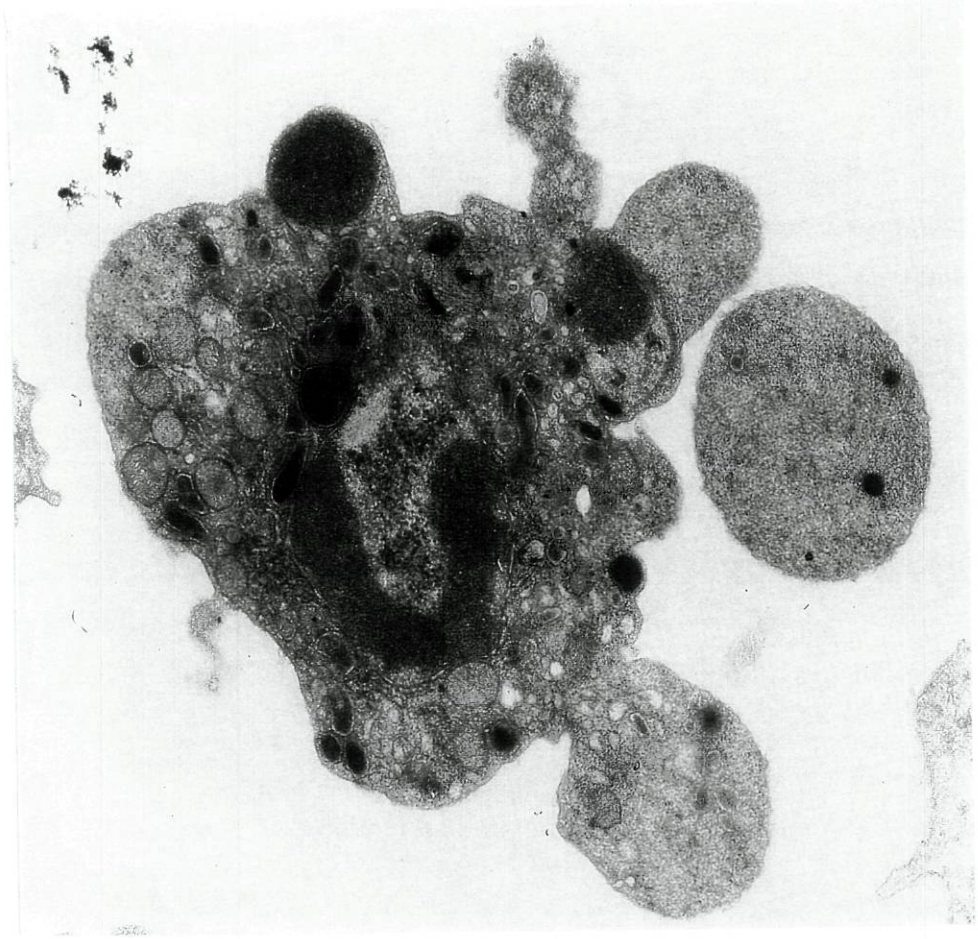


Fig. 14. An apoptotic neutrophil shedding apoptotic bodies in an exudate collected 40h after the intraperitoneal injection of 12% casein. Section contrasted with uranyl-lead. x 23 600.

Four patterns of peroxidase positivity were observed in macrophages present in the inflammatory peritoneal exudates. Type 1 refers to the presence of neutrophils sufficiently intact to be detected by Wright counter-staining when using Antonow's method. Most probably, this pattern shows neutrophils which had been phagocytosed by the macrophage very shortly before sampling. In fact, in mammals, it is known that the phagocytosed neutrophils are quickly degraded by the hydrolytic enzymes of the macrophage lysosomes (Newman *et al.*, 1982; Haslett *et al.*, 1989), with the resulting loss of the normal morphology of the neutrophil. However, the peroxidase activity remains for some time after the loss of normal neutrophil morphology (Heifets *et al.*, 1980) and thus, the presence of peroxidase-positive material in large vacuoles inside the macrophages (pattern type 2) is an indication that those vacuoles are phagosomes with remnants of a phagocytosed neutrophil in advanced degradation.

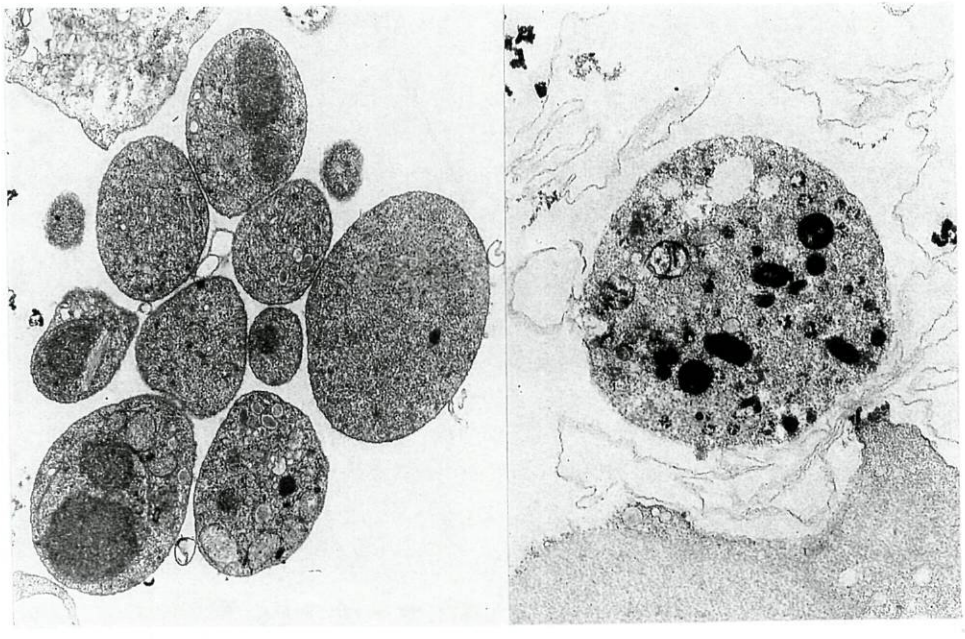


Fig. 15. Free neutrophil apoptotic bodies in the peritoneal cavity of a rainbow trout injected intraperitoneally with 5 x 10' Y. *ruckeri.* On the left conventional electron microscopy; section contrasted with uranyl-lead. x 11600. On the right, sample processed for peroxidase; section contrasted with lead. x 18 900. Note the presence of neutrophil granules with peroxide activity.

It is known that senescent mammalian neutrophils in inflammatory foci undergo apoptosis (Haslett *et al.,* 1989; Savill *et al.,* 1989a,b). One feature of this process is the release of apoptotic bodies, sub-cellular particles rich in neutrophil granules that are taken up by macrophages (Hanker & Giammara, 1983; Cohen *et al.,* 1992). The presence of neutrophil apoptotic bodies inside rainbow trout macrophages observed here represents the type 3 pattern of peroxidase positivity in macrophages.

Whether other types of transfer of neutrophilic components to the macrophage, besides those discussed above, are occurring in inflamed sites of rainbow trout is not known with certainty, but some possibilities have to be considered. One regards the neutrophil/macrophage interaction here described whereby the two phagocytes appear closely adherent with the possible participation of intercellular junctions. The interpretation that this interaction could lead to material transfer is supported by two observations. Firstly, the adherence of neutrophils was preferentially to macrophages; and, secondly, the peroxidase activity was sometimes found in the macrophages associated with neutrophils. It is interesting that the adherence of neutrophils to macrophages was seen only in inflammatory exudates but never in the normal, unstimulated peritoneal cavity. The finding of the junctions of transmission electron microscopy has been, so far, occasional, but it is possible that it might occur more frequently than it appears, because the chances of the plane of section passing through the actual location of the junction are quite small The occurrence of adhesions between neutrophils



Fig. 16. A peritoneal macrophage with phagocytosed neutrophil apoptotic bodies (arrows) in an exudate of a trout inoculated 12 d before with 1 x 10^8 *R. salmoninarum.* One of the apoptotic bodies (*) contains bacteria. Section contrasted with lead. x 14 500.

and macrophages reported here for inflammatory exudates in rainbow trout has not been described, to our knowledge, in mammals.

Another possibility for the transfer of neutrophilic material to the macrophages is the uptake by the macrophage of neutrophil granules or molecules. Phagocytosis of neutrophilic granules and of neutrophilic granular molecules by macrophages has been reported for rodents (Heifets *et al.,* 1980; Shellito *et al.,* 1987; Leung & Goren, 1989; Sousa, 1994). The neutrophil granules can be released by neutrophils lysing at the inflammatory sites due to leucotoxins produced by infectious agents (Cochrane, 1968; Henson & Johnston, 1987). It is



Fig. 17. (a) Adherent neutrophil and macrophage in the peritoneal exudate of a rainbow trout injected intraperitoneally with 5×10^7 *Y. ruckeri* 24 h before. Sample processed for detection of peroxidase activity. Section contrasted with lead. Note the numerous peroxidase-positive granules in the cytoplasm of the neutrophil. In the cytoplasm of the macrophage a few granules show peroxidase activity. × 9000. (b) High magnification of the region of contact indicated in (a) showing intercellular junctions with the characteristics of gap junctions. × 60 800.

also known that, at inflammatory sites, activated neutrophils secrete molecules stored in their granules through the process known as degranulation, and that at those sites neutrophilic molecules, like myeloperoxidase, lactoferrin and defensins, can be present extracellularly (Estensen *et al.*, 1974; Goldstein *et al.*, 1975; Pryzwansky & Bréton-Gorius, 1983; Ganz, 1987; Shellito *et al.*, 1987; Falloon & Gallin, 1988; Leung & Goren, 1989; Esaguy *et al.*, 1991; Densen *et al.*, 1995). The occurrence, also reported here, of macrophages with discrete peroxidase-positive granules (pattern type 4) may well correspond to the transfer of neutrophilic granules or molecules.

The phagocytosis by macrophages of intact neutrophils in inflammatory foci was described a century ago by Metchnikoff in tadpoles, and thereafter it was found to occur in mammals, including man (Brewer, 1964; Parmley *et al.*, 1981; Newman *et al.*, 1982; Sanui *et al.*, 1982; Shellito *et al.*, 1987; Silva *et al.*, 1989; Sousa, 1994). However, the physiological importance of such an event is not yet completely understood. On the one hand, it has been described as a mechanism of disposing of senescent neutrophils by macrophages and thus

limiting tissue damage in acute inflammation and in the resolution of inflammation (Haslett *et al.*, 1989; Savill *et al.*, 1989*a*,*b*; Cohen *et al.*, 1992). On the other hand, the phagocytosis of neutrophils by macrophages at infectious foci may have another consequence. Namely, the uptake of the neutrophils by infected macrophages would have the additional advantage of enhancing the capacity of the macrophage to cope with infection (Silva *et al.*, 1989; Sousa, 1994). Such an increased antimicrobial capacity can result from the acquisition by the macrophage of antimicrobial molecules present in the neutrophil and lacking in the macrophage (Heifets *et al.*, 1980; Locksley *et al.*, 1982; Nogueira *et al.*, 1982; Lima & Kierszenbaum, 1985; Shellito *et al.*, 1987; Leung & Goren, 1989; Silva *et al.*, 1989; Sousa, 1994). That some antimicrobial molecules operating in neutrophils, like myeloperoxidase, lactoferrin and defensins, are not present in mammalian macrophages is well documented (Falloon & Gallin, 1988; Ganz *et al.*, 1990; Ogata *et al.*, 1992; Borregaard *et al.*, 1993; Densen *et al.*, 1995), as is the utilisation in antimicrobial activities of some of those molecules when transferred to macrophages (Heifets *et al.*, 1980; Nogueira *et al.*, 1982).

One interesting aspect regarding the phagocytosis of neutrophils by macrophages in infected peritoneal exudates described in this paper regards the ingestion of infected neutrophils, and of neutrophil apoptotic bodies, containing bacteria, by the macrophages, as already reported in experimental mycobacteriosis in mice (Silva *et al.*, 1989). This phagocytosis results in the transfer to the macrophages of bacteria initially phagocytosed by the neutrophils. When the host is able to control the growth of the invading microorganism, it is likely that the macrophage's antimicrobial and degradative activities will be exerted on these transferred parasites. Since macrophages are much more long-lived as compared to neutrophils, such a transfer can be advantageous to the host. Moreover, it is likely that the antimicrobial molecules of the neutrophil transferred to the macrophages with the bacteria will participate in the attack on the microorganism. In fact, it has been shown that neutrophilic molecules ingested by macrophages are mobilised to parasite-containing macrophage phagosomes (Leung & Goren, 1989; Sousa, 1994) and are used in the macrophage's antimicrobial processes (Heifets *et al.*, 1980; Nogueira *et al.*, 1982; Silva *et al.*, 1989; Sousa, 1994). On the other hand, in the instances where the parasite resists the host's defence mechanisms, the transfer of the infectious agent to the macrophage through the phagocytosis by this phagocyte of infected neutrophils or neutrophilic apoptotic bodies may be an efficient way for the agent to spread in the organism.

A new picture is emerging for the physiology of the mammalian neutrophil. Recent results show that the neutrophil is not just a terminal effector cell solely engaged in the killing of extracellular pathogens. Rather, the neutrophil appears as a leucocyte that also participates in the defence mechanisms against intracellular pathogens (Silva *et al.*, 1989; Conlan & North, 1991, 1992, 1994; Rogers & Unanue, 1993; Appelberg *et al.*, 1994, 1995; Czuprynski *et al.*, 1994*a*,*b*; Sjostedt *et al.*, 1994; Sousa, 1994; Barteneva *et al.*, 1996; Newman *et al.*, 1997). Moreover, neutrophils have been shown to be biosynthetically active leucocytes producing and excreting several cytokines that participate in immune mechanisms, including the modulation of macrophage activity

(Lloyd & Oppenheim, 1992; Cassatella, 1995; Cassatella *et al.*, 1995). The occurrence of transfer of neutrophilic components to macrophages, described here, is evidence for the view that in fish, like in mammals, cooperation between neutrophils and macrophages would occur as part of the defence mechanism against parasites growing inside macrophages. Whether such a transfer occurs in fish as well as in mammals (Silva *et al.*, 1989; Sousa, 1994), actually enhancing the capacity of trout macrophages against fish pathogens remains to be directly demonstrated and is currently under investigation in this laboratory.

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Macrophage/neutrophil interactions in sea bass (*Dicentrarchus labrax*)

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(Unpublished manuscript)

ABSTRACT

Inflammatory peritoneal exudates of sea bass (*Dicentrarchus labrax*) previously injected by the intraperitoneal route with *Yersinia ruckeri* were collected at 12, 24, 48 and 96h and processed for the detection of myeloperoxidase activity and ultrastructural analysis of leukocytes. Numerous neutrophils and macrophages were present at all time points studied. Apoptotic neutrophils were seen, mainly in samples 48 and 96h after inoculation of the bacteria. Extensive transfer of peroxidase from neutrophils to macrophages was observed; this transfer was more important in the samples collected at 48 and 96h post-inoculation. The transfer was due to the phagocytosis by the macrophages of intact neutrophils and neutrophil apoptotic bodies. Transfer to macrophages of neutrophils containing phagocytosed bacteria was also observed.

INTRODUCTION

Previous studies from this laboratory on the interaction between macrophages and neutrophils in inflamed peritoneal exudates of rainbow trout have shown the occurrence of the transfer of neutrophils and neutrophilic components to macrophages (Afonso *et al.*, 1998b). In order to see whether such a transfer, which was shown to occur also in mice (Silva *et al.*, 1989; Sousa, 1994), also occurs in other fish, we studied the interaction between the two professional phagocytes in the inflamed peritoneal cavity of sea bass (*Dicentrarchus labrax*). The leukocyte population of the resting peritoneal cavity of sea bass, and the peritoneal neutrophil response to the presence of bacteria were also analysed.

MATERIALS AND METHODS

Fish

Sexually immature sea bass weighing between 54 and 120g, were purchased from a commercial farm. The animals were maintained in recirculating aerated sea water, at 17^{o}C. Water quality was maintained with mechanical and biological filtration and fish were fed *ad libitum* on commercial pellets. The fish to be injected with phlogistic agents

were transferred to 300 l fibreglass tanks under the same conditions as above, where they were maintained for at least 15 days before the study. Only healthy fish, as indicated by their activity and exterior appearance, were used.

Intraperitoneal injection of bacteria

The strain FP-3 of *Yersinia ruckeri* type 1 was kindly supplied by Prof. J. Barja (University of Santiago de Compostela, Spain). Suspensions in phosphate buffered saline (PBS) of live bacteria to be injected into the peritoneal cavities of sea bass were prepared as described (Afonso *et al.*, 1988a,b).

The bacteria (5 x 10^7 in 100µl of PBS per fish) were injected as described (Afonso *et al.*,1998a), into the peritoneal cavity of fish under anaesthesia with 0.3ml ethylene glycol monophenyl ether l^{-1} (Merck). Immediately after the i.p. injection the fish were returned to their tank. Control fish were injected i.p. with 100µl of PBS.

Collection and study of peritoneal leucocytes

The peritoneal cells were collected from undisturbed peritoneal cavities and 12, 24, 48 and 96h after the i.p. injections, by a procedure previously described in detail (Afonso *et al.*, 1997; 1998a). The peritoneal cell suspensions were analysed within minutes after collection for several morphological and cytochemical parameters as follows.

Light microscopy

Total cell counts were performed with a haemocytometer. Cytospin preparations were made with a Shandon Cytospin 2 using a volume of peritoneal lavage depending on the cell concentration, in order to obtain adequate numbers of cells per slide. The cytospins were stained with Wright's stain (Haemacolor, Merck) after fixation for 1min with formol-ethanol (10ml of 37 % formaldehyde and 90ml of absolute ethanol) (Kaplow, 1965), and leukocyte differential counts were performed as described (Afonso *et al.*, 1997; 1998a).

Cytochemistry for peroxidase was carried out by the method of Kaplow (Kaplow, 1965) or a modification of it (Antonow's technique; Afonso *et al.*, 1998a).

Electron microscopy of leucocytes

Peritoneal leukocyte suspensions in PBS were centrifuged at 1000*g* for 10min and the pellet was fixed by our standard procedure as described elsewhere (Silva & Macedo, 1983; Silva *et al.*, 1987), using paraformaldehyde- glutaraldehyde- calcium prefixation, followed by OsO_4 supplemented with 10mM calcium chloride, and a postfixation with uranyl acetate (Silva *et al.*, 1971).

After dehydration in ethanol, the samples were embedded in Epon and ultrathin sections were cut with an LKB Ultratome III microtome. Routinely, the sections were contrasted with uranyl acetate followed by lead citrate (Silva *et al.*, 1987) or with lead citrate alone, as indicated in the legends to the figures. Observations and micrographs were done with a Zeiss EM 10C electron microscope.

RESULTS

i- The resting peritoneal cavity of sea bass was found to contain a leukocyte population consisting predominantly of mononuclear cells, a few peroxidase-positive neutrophilic granulocytes and peroxidase-negative eosinophilic granular cells (EGC) (Fig. 1 and 2).

Macrophages were typically peroxidase-negative (Fig. 1 and 2).

ii- In the peritoneal exudates inflamed by the i.p. injection of *Y. ruckeri* a very strong neutrophilic response was seen (Fig. 17).

Bacteria were seen inside macrophages (Fig. 3) and neutrophils (Fig. 4). The light microscopic cytological study of these inflammatory exudates showed the presence of numerous macrophages with peroxidase activity (Fig. 7-12). This intramacrophagic activity showed the same patterns as described for rainbow trout (Afonso *et al.*, 1998b): type 1- positivity due to the presence of intact neutrophils recognized by the abundant peroxidase-positive granules (Fig. 7, 11, 12), phagocytosed by macrophages; type 2- clumps of peroxidase-positive material in large vacuoles (Fig. 9 and 10); type 3- clumps of positive granules inside small vacuoles (Fig 8); and type 4- dispersed small granules (not shown). The very large vacuoles with peroxidase activity (pattern type 2) were

rather frequent at 48-96h; peroxidase activity in these vacuoles ranged from quite strong (macrophage in the right side of Fig. 10) to rather faint (macrophages in the left side of Fig. 9 and 10) and in between (macrophage in the right side of Fig. 9). As in the case of rainbow trout (Afonso *et al.*, 1998b), macrophages that had phagocytosed neutrophils which, in turn, had phagocytosed bacteria, were frequently seen (Fig. 11 and 12).

iii-Apoptotic neutrophils (Fig. 5 and 6) and neutrophil apoptotic bodies were frequently seen in the inflamed peritoneal exudates, mainly in the samples collected at 48 or 96h post-inoculation of bacteria. The neutrophil apoptotic bodies were seen either free or inside macrophages (Fig. 8), originating the pattern type 3 referred to above.

iv-Preliminary ultrastructural observations showed images (Fig. 13-16) corresponding to those obtained by light microscopy and described above. Examples of macrophages containing neutrophilic material are reported in Fig. 15 and 16. Fig. 13 shows an intramacrophagic neutrophilic apoptotic body together with remnants of a neutrophil. A macrophage containing a neutrophil with bacteria is shown in Fig. 15. This image is the ultrastructural counterpart of Fig. 11, while the macrophage in Fig. 14 is the equivalent of those in Fig. 9 and 10.

DISCUSSION

Our preliminary observations regarding the resident peritoneal leukocyte population in sea bass are in acordance to those described by Meseguer *et al.* (1993).

The results now reported regarding the interaction between the 2 phagocytes in inflammatory foci, are similar to those we described for inflammatory peritoneal exudates of rainbow trout (Afonso *et al.*, 1998b). As in trout, the resting peritoneal cavity of sea bass contains many macrophages and few neutrophils. Secondly, resident macrophages are peroxidase-negative.

The present preliminary results show that in sea bass, as described before for the mouse (Silva *et al.*, 1989; Sousa, 1994) and rainbow trout (Afonso *et al.*, 1998b), peroxidase is transferred from neutrophils to macrophages when the two phagocytes coexist in inflammatory exudates. Moreover, the transfer to macrophages of bacteria

inside neutrophils was, as well, observed in the sea bass model, as described before for the mouse (Silva *et al.*, 1989) and rainbow trout (Afonso *et al.*, 1998b).

The present results indicate that mechanisms of cooperation similar to those extensively described and discussed before for trout (Afonso *et al.*, 1998b), operate between the two professional phagocytes present in infectious foci in sea bass.

ACKNOWLEDGEMENTS

The authors are grateful to Prof. A. Ellis (Marine Laboratory, Aberdeen, U.K.) for continuous help and support, to Prof. Alberto Villena and Dr. Jorge Pedrosa for helpful discussions and to Prof. António C. Castro for supplying the fish used in the present work.

The excellent technical assistance provided by Mrs. Paula M. Macedo, Miss Irene Barros and Mr. Adão Silva is gratefully acknowledged.

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LEGENDS TO THE FIGURES

Fig. 1-12: Peroxidase staining (Antonow's technique) of peritoneal leukocytes of sea bass. Counterstaining by the Wright stain. Peroxidase activity is stained green-brown. x 900.

Fig. 1 and 2: Leukocyte population of resting peritoneal cavities, showing a few neutrophils (peroxidase-positive). Macrophages (M) are peroxidase-negative. Peroxidase-negative granulocytes are also present (G). The small mononuclear leukocytes are lymphocytes and thrombocytes.

Fig. 3 and 4: Bacteria inside phagocytes. In Fig. 3 a peroxidase-negative macrophage is shown with bacteria in a vacuole. In Fig. 4 a neutrophil shows peroxidase-positive cytoplasmic granules and bacteria. Samples collected at 12h after intraperitoneal injection of *Y. ruckeri*.

Fig. 5 and 6: Apoptotic neutrophils with fragmented, dense nuclei and peroxidase-positive granules in peritoneal exudates 48h after intraperitoneal injection of *Y. ruckeri*.

Fig. 7: A macrophage with a phagocytosed neutrophil. Peritoneal exudate collected 24h after intraperitoneal injection of *Y. ruckeri*.

Fig. 8: A macrophage with a neutrophil apoptotic body and a few internal bacteria in a sample collected 48h after intraperitoneal injection of *Y. ruckeri*.

Fig. 9 and 10: Macrophages with very large vacuoles containing bacteria and peroxidase. Sample collected 48h after intraperitoneal injection of *Y. ruckeri*.

Fig. 11 and 12: Macrophages with neutrophils which had phagocytosed bacteria. Sample collected 24h after intraperitoneal injection of *Y. ruckeri*.

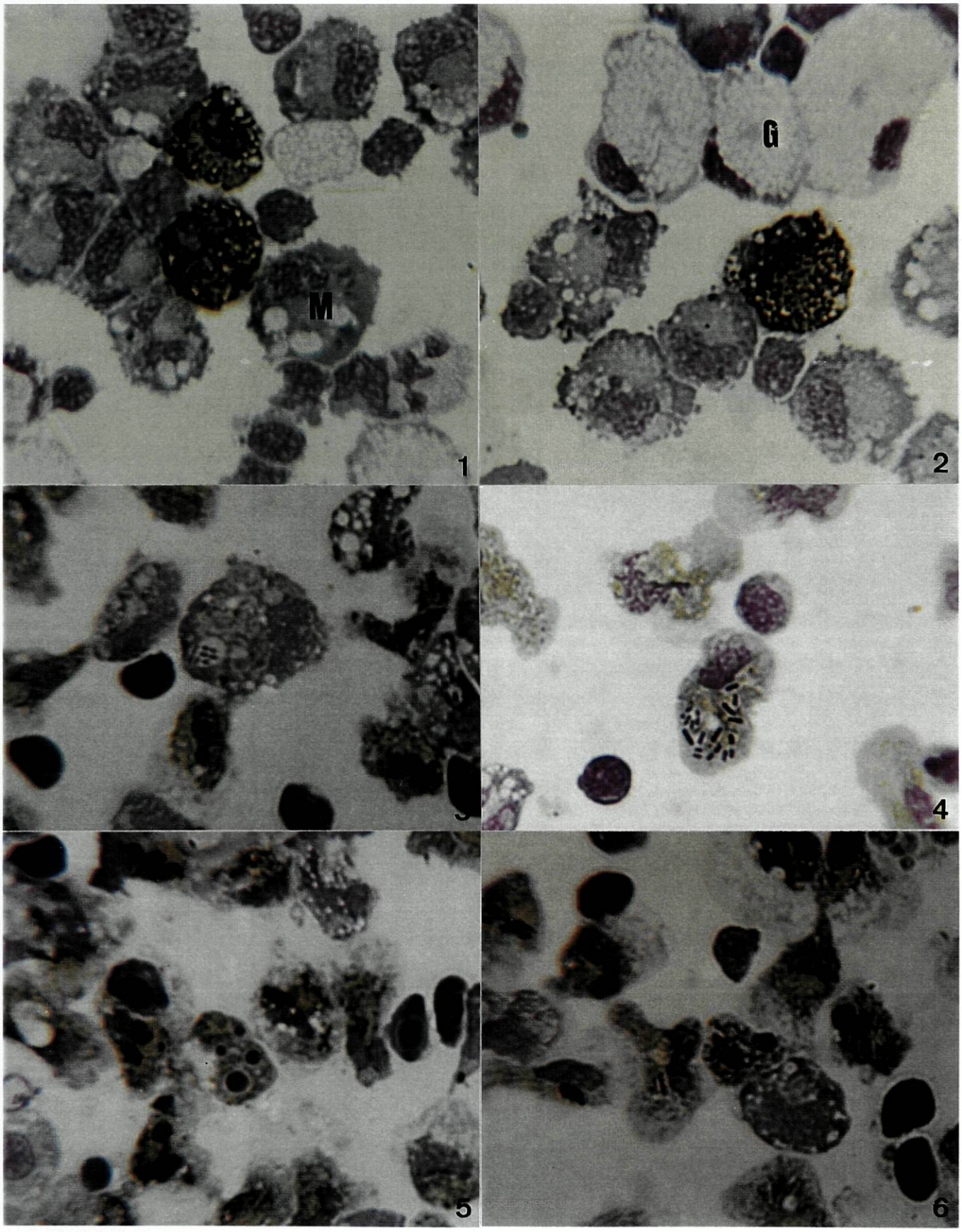
Fig. 13-16: Transmission electron microscopy of ultrathin sections of sea bass peritoneal macrophages. Sections contrasted with uranyl acetate followed by lead citrate. Bar: 1μm.

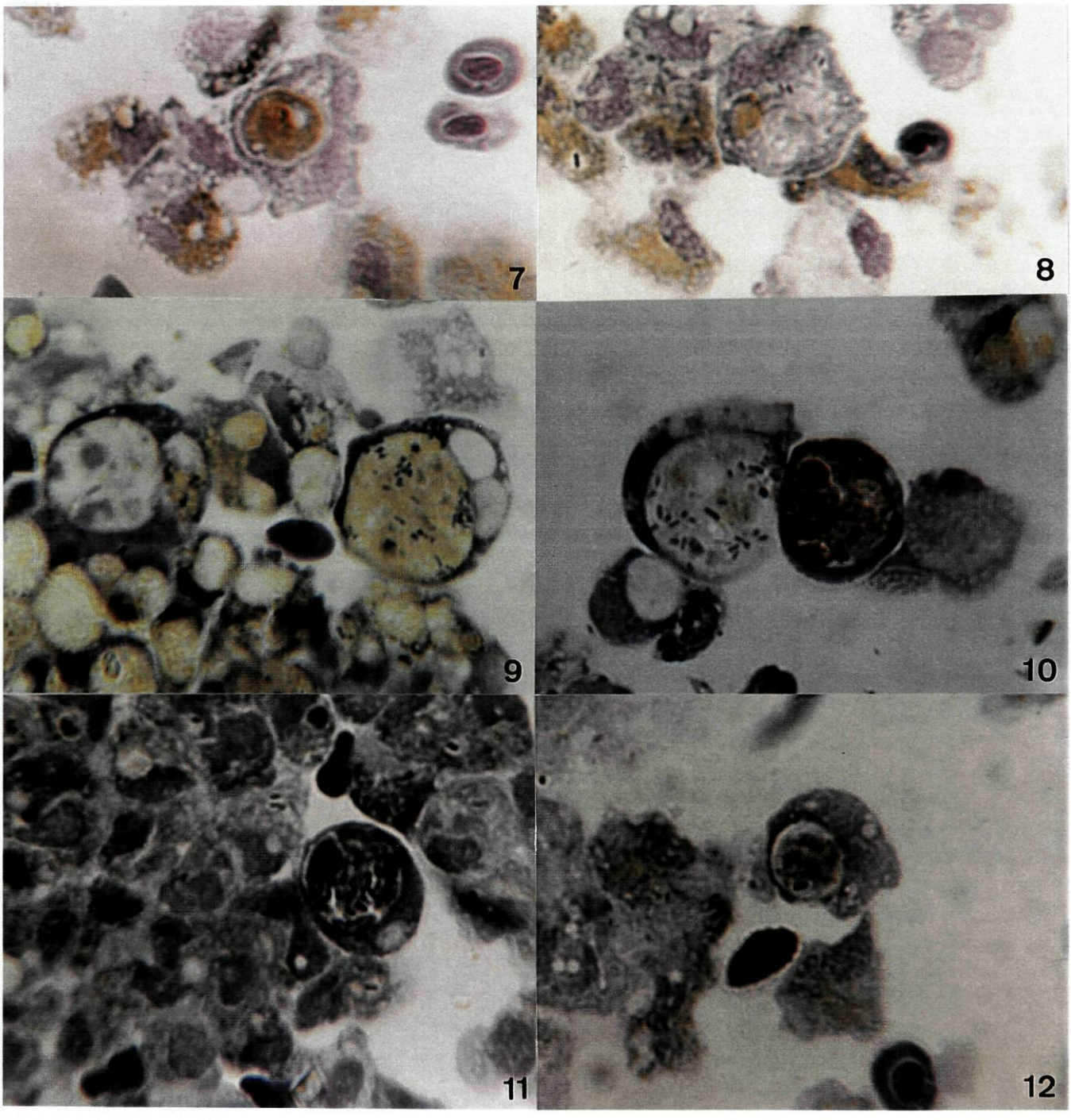
Fig. 13: A macrophage with the remnants of a neutrophil and a neutrophil apoptotic body (top).

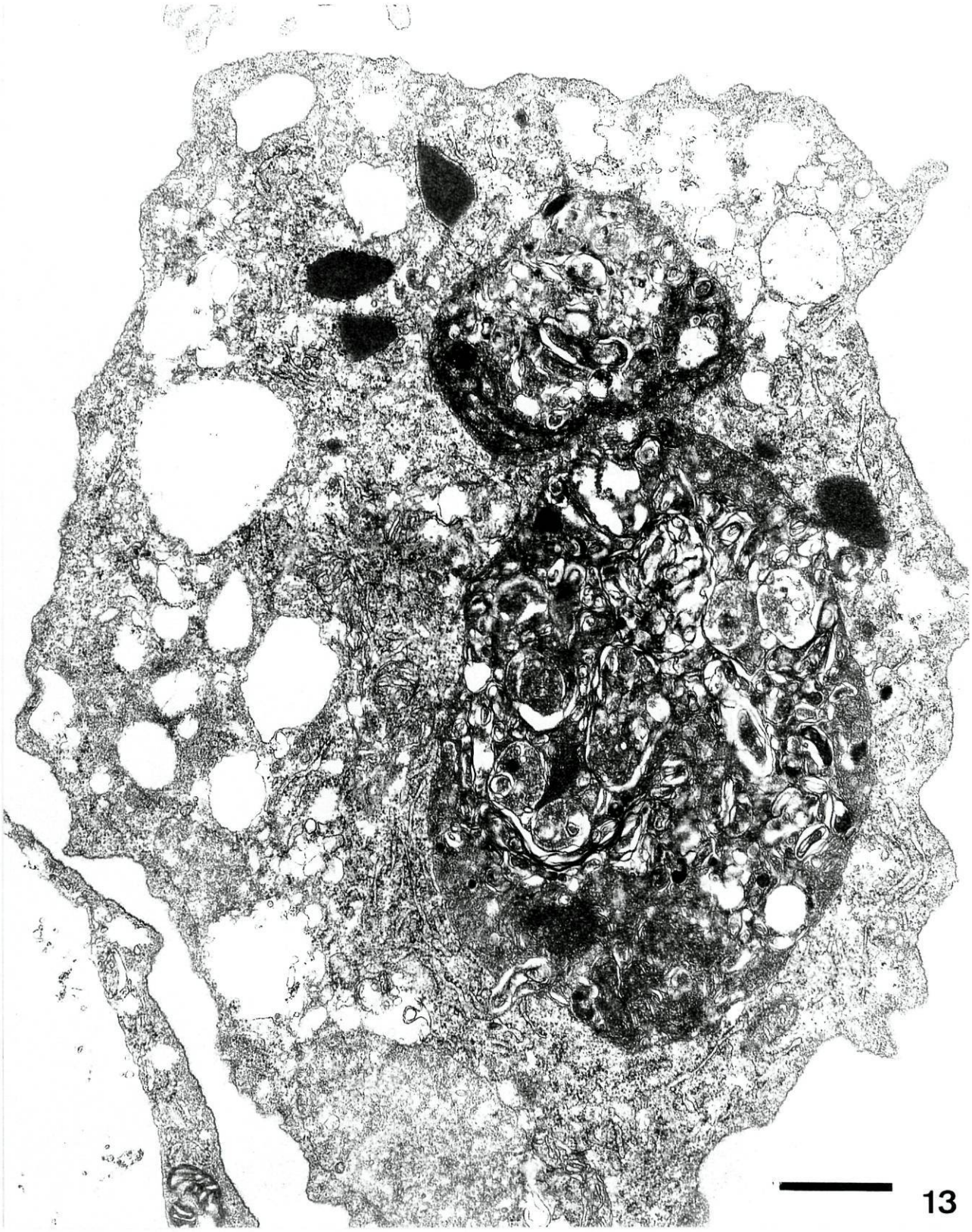
Fig. 14: A macrophage with a very large vacuole where cell debris and bacteria are present. This image is similar to that of the macrophage in the left side of Fig. 10.

Fig. 15 and 16: Macrophages with remnants of neutrophils. The intramacrophagic neutrophil in Fig. 11 had previously phagocytosed several bacteria.

Fig. 17: Kinetics of neutrophil response to the i.p. injection of *Y. ruckeri* or PBS (control) in sea bass.


M
1
G
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3
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5
6


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13


14


15

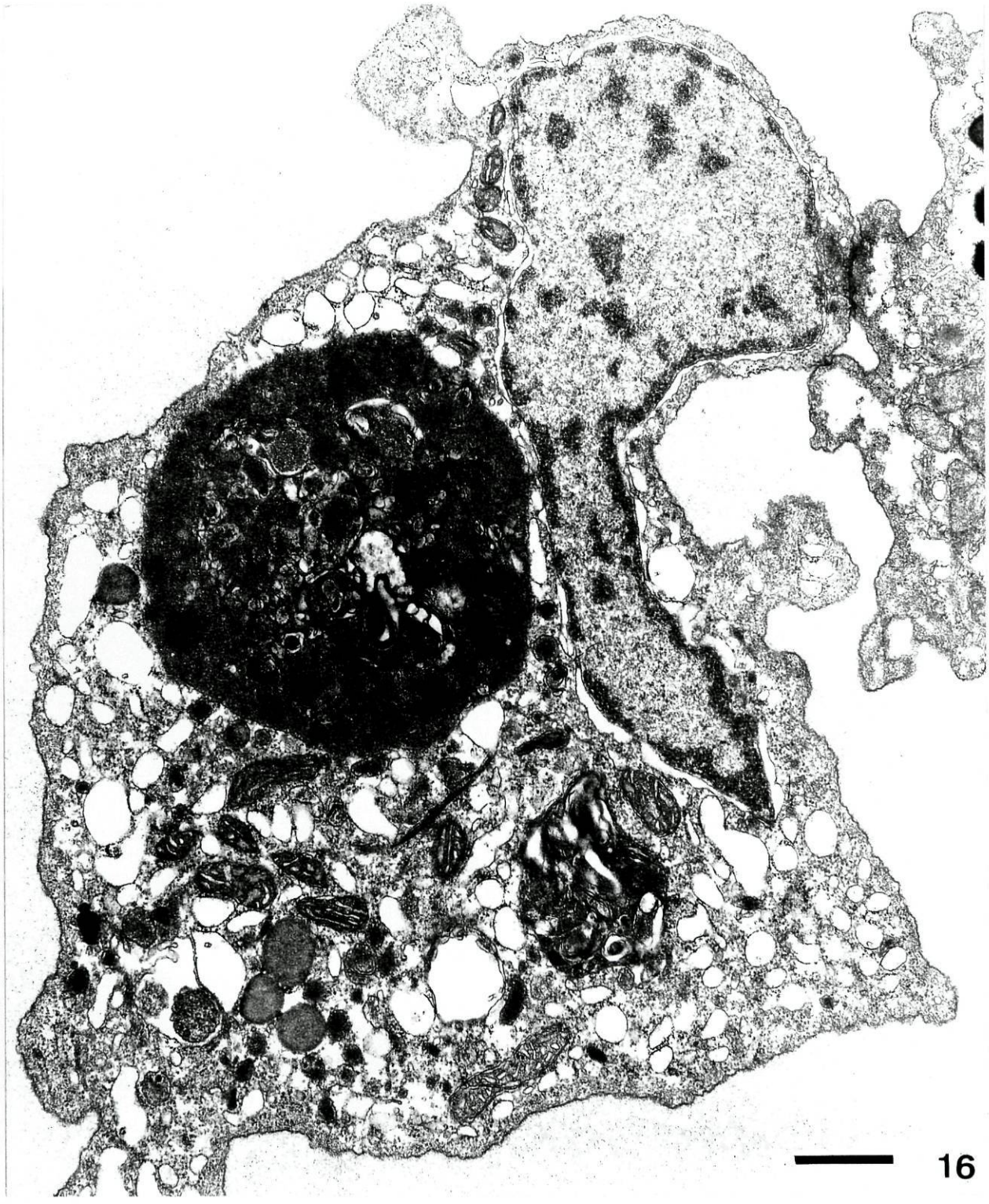
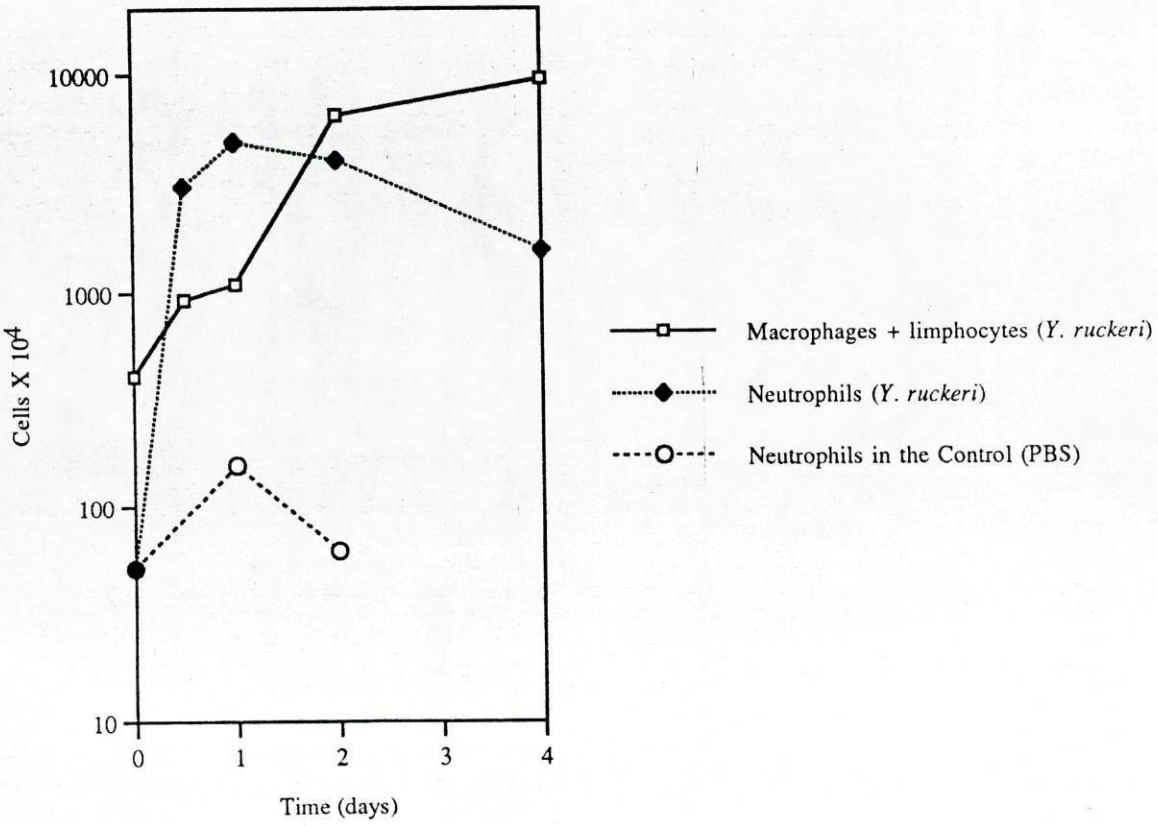

16

Fig. 17


10000
1000
100
10
Cells X 10^4
0
1
2
3
4
Time (days)
Macrophages + limphocytes (*Y. ruckeri*)
Neutrophils (*Y. ruckeri*)
Neutrophils in the Control (PBS)

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Neutrophil apoptosis in rainbow trout (*Oncorhynchus mykiss*). An *in vitro* study

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(Unpublished manuscript)

ABSTRACT

Extensive neutrophil apoptosis was observed upon *in vitro* incubation of inflammatory peritoneal exudates of rainbow trout containing high numbers of neutrophils and macrophages together with lymphocytes. The inflammatory exudates were produced by the intraperitoneal injection of killed *Yersinia ruckeri*. Apoptotic neutrophils were characterized by typical nuclear morphological alterations and by fluorescence staining for nicked DNA. Extensive production of neutrophil apoptotic bodies was observed. Both apoptotic neutrophils and neutrophil apoptotic bodies were extensively phagocytosed by macrophages. This represents a mechanism of transfer of neutrophil components to macrophages.

Essentially, neutrophil apoptosis in rainbow trout is similar to that described for mammalian neutrophils. This represents the first description of the phenomenon in fish.

INTRODUCTION

The phagocytosis by macrophages of intact neutrophils in inflammatory foci was described a century ago by Metchnikoff in tadpoles, and thereafter it was found to occur in mammals, including man (Brewer, 1964; Newman *et al.*, 1982; Parmley *et al.*, 1981; Sanui *et al.*, 1982; Shellito *et al.*, 1987; Silva *et al.*, 1989; Sousa, 1994). We have shown that in rainbow trout (Afonso *et al.*, 1998b) and in sea bass (Afonso & Silva, 1998) this event also occurs in inflammatory peritoneal exudates where the two phagocytes coexist. As reviewed elsewhere (Afonso *et al.*, 1998b), the physiologic importance of such an event is not yet completely understood. It has been taken as representing a mechanism of neutrophil disposal. This role of macrophages in disposing of neutrophils has been extensively studied in mammals (Haslett *et al.*, 1989; Savill *et al.*, 1989a) but not in fish. In mice and man it has been shown that aging neutrophils present in inflammatory exudates undergo a process of programmed cell death (apoptosis) which results in surface changes of the neutrophil that lead to their recognition, phagocytosis and degradation by macrophages (Haslett *et al.*, 1989; Savill *et al.*, 1989a). One significant advantage of this phagocytosis by the macrophage of senescent but still intact (apoptotic) neutrophils results from the prevention of the lysis

of dying neutrophils which would, otherwise, result in the release of the highly cytotoxic components (Cochrane, 1968; Henson & Johnston, 1987) of this phagocyte. Neutrophil death occurs naturally at the end of its short life or it can be induced by the leukocidal activity of some pathogenic microrganisms (Cochrane, 1968; Henson & Johnston, 1987). The phagocytosis of senescent neutrophils by macrophages is, therefore, a mechanism important in the limitation of the tissue damage in acute inflammation and in the resolution of inflammation (Cohen *et al.*, 1992; Haslett *et al.*, 1989; Savill *et al.*, 1989a).

As briefly reported and documented elsewhere (Afonso *et al.*, 1998b; Afonso & Silva, 1998), we found evidence for the occurrence of neutrophil apoptosis in inflamed peritoneal exudates of both rainbow trout and sea bass. This prompted us to study in more detail this phenomenon in rainbow trout. For this purpose, inflammatory peritoneal exudates containing high numbers of neutrophils (Afonso *et al.*, 1998a) together with macrophages and lymphocytes, were incubated *in vitro* for several days to allow for the induction of neutrophil apoptosis (Savill *et al.*, 1989b).

MATERIAL AND METHODS

Fish

Sexually immature rainbow trout weighing between 100 and 200g, were purchased from a commercial farm. The animals were maintained in recirculating aerated fresh water, at 15^{o}C, in 7000 l fibreglass tanks. Water quality was maintained with mechanical and biological filtration and fish were fed *ad libitum* on commercial pellets. The fish to be injected with bacteria were transferred to 600 l fibreglass tanks under the same conditions as above, where they were maintained for at least 15 days before the study. Only healthy fish, as indicated by their activity and external appearance, were used.

Inflammatory peritoneal exudates

Peritoneal exudates of trout injected intraperitoneally (i.p.) with phlogistic agents were produced and collected as previously described (Afonso *et al.*, 1997, 1998a), except that, to collect the inflammatory leukocyte population, cell culture L15 Leibovitz

medium (Gibco, BRL) supplemented with 10 % bovine foetal serum, was used. The results now presented were obtained with exudates produced by the i.p. injection of suspensions of formol-killed *Yersinia ruckeri*, prepared as described elsewhere (Afonso *et al.*, 1998a). Exudates were collected 20 hours after the i.p. injection.

Incubation *in vitro*

The suspensions of peritoneal leukocytes were adjusted to 10^6 cells/ml with cell culture L15 Leibovitz medium supplemented with 10 % bovine foetal serum and incubated at 18°C with shaking. At intervals (see Results) samples were collected and processed as follows.

Cytological studies

These studies were carried out using morphological and cytochemical techniques for light and electron micoscopy. These techniques included routine leukocyte total and differential counts as previously described (Afonso *et al.*, 1997, 1998a). Detection of peroxidase activity by light and electron microscopy was by the Antonow's (Afonso *et al.*, 1998a) and Robbins *et al.* (1971) techniques, respectively. Fluorescence labelling of cells under apoptosis was carried out with the commercial kit Apodirect (Phoenix Flow Systems). Electron microscopy methods were as previously described (Silva *et al.*, 1971, 1987; Silva & Macedo, 1983).

RESULTS AND DISCUSSION

The light microscopic study carried out on leukocyte suspensions incubated *in vitro* for different periods of time showed the occurrence of neutrophil apoptosis. This was already seen in 12h samples, and increased with incubation time.

Neutrophil apoptosis was detected both by the characteristic nuclear alterations (Cohen *et al.*, 1992; Savill *et al.*, 1989b) as well as by the specific method involving fluorescence microscopy for detection of nicked DNA. The general features of neutrophil apoptosis now observed with inflammatory trout neutrophils are similar to those previously described for mammalian neutrophils (Cohen *et al.*, 1992; Savill *et al.*,

1989b). They include: i- Condensation and fragmentation of nuclei (Fig. 3-6); these nuclei stain with the technique for the detection of nicked DNA. ii-Formation of neutrophil apoptotic bodies (Hanker & Giammara, 1983; Cohen *et al.*, 1992) (Fig. 8-13). These sub-cellular bodies were identified by their size, spherical shape and by the presence of peroxidase-positive granules. Some contained nuclear fragments (Fig. 10 and 11), but most were devoided of nuclei. These bodies were seen either shedding from neutrophils (Fig. 9), free (Fig. 8-12) or within macrophages (Fig. 6-9). Furthermore, macrophages with internalized apoptotic neutrophils were seen (Fig. 6 and 7). The phagocytosis of neutrophil apoptotic bodies had been described in mammals (Hanker & Giammara, 1983).

One technical aspect related to the detection of neutrophil apoptotic bodies in exudates deserves special consideration. The current practice using cytospin preparations for light microscopy cytological studies of exudates has a critical drawback: most apoptotic bodies are not present in the final preparations. This is due to the small size of these sub-cellular structures, which are lost during the centrifugation through absortion by the filter paper used to take out the fluid where the cells are suspended. The result of this is that only a partial view of the process is obtained when using the cytospin method. We found that centrifugation of peritoneal inflammatory exudates at 1000*g* for 30min allowed for the adequate sampling of apoptotic bodies. This technique was used for the ultrastructural studies now reported.

In conclusion, the present study showed that in rainbow trout inflammatory/senescent neutrophils undergo programed cell death (apoptosis) with general characteristics similar to those described for mammalian neutrophils.

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The authors are grateful to Prof. A. Ellis (Marine Laboratory, Aberdeen, U.K.) for continuous help and support, to Prof. Alberto Villena and Dr. Jorge Pedrosa for helpful discussions and to Prof. António C. Castro for supplying the fish used in the present work.

The excellent technical assistance provided by Mrs. Paula M. Macedo, Miss Irene Barros and Mr. Adão Silva is gratefully acknowledged.

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LEGENDS TO THE FIGURES

Fig. 1-9: Peroxidase staining (Antonow's technique) of inflammatory peritoneal leukocytes of rainbow trout suspended in cell culture L15 Leibovitz medium (Gibco, BRL) supplemented with 10 % of bovine foetal serum and incubated *in vitro* for several periods of time. Counterstaining by the Wright stain. x 870.

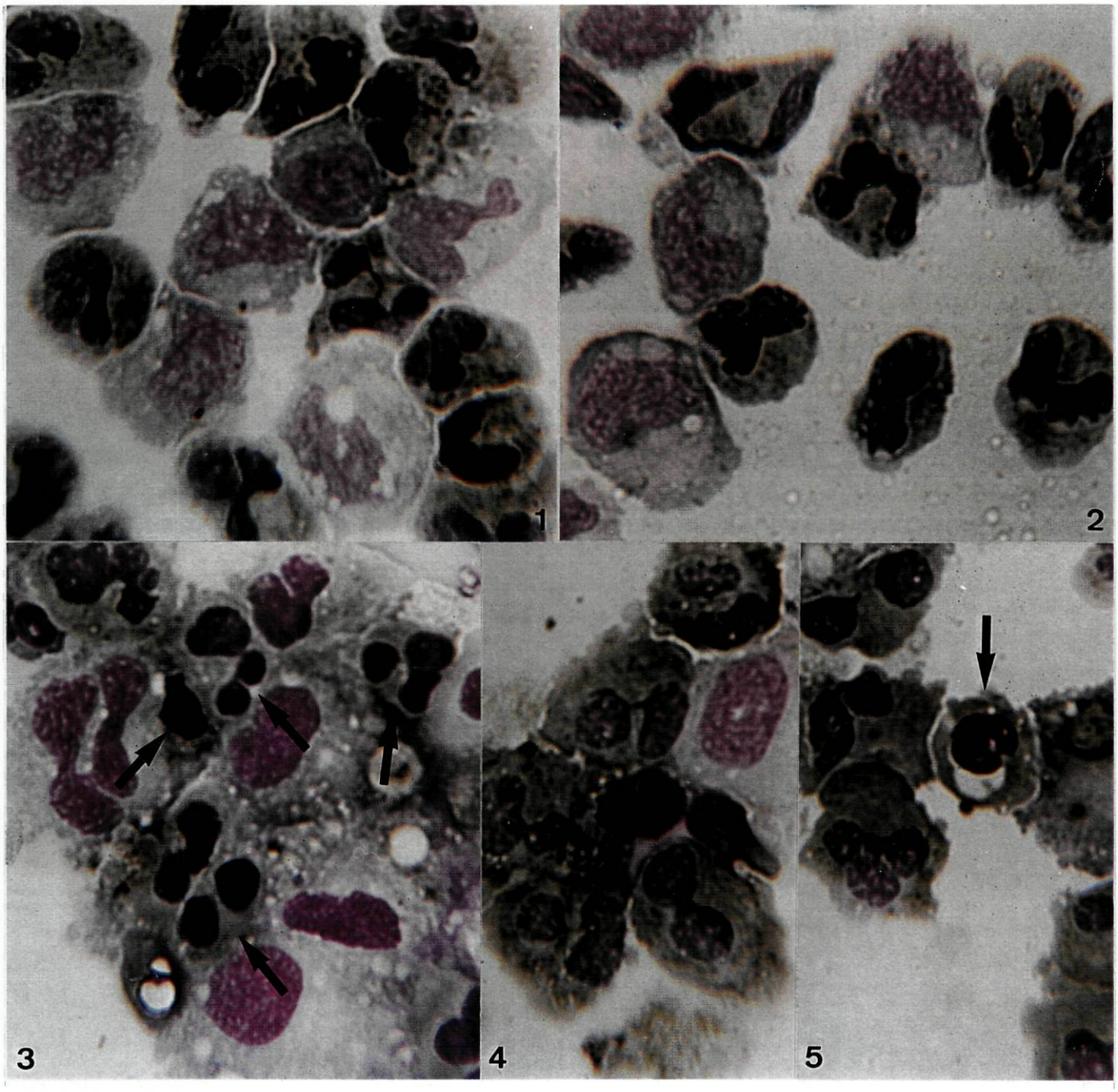
Fig. 1 and 2: Leukocytes at 0 time point. Several peroxidase-positive (green-brown reaction) neutrophils and macrophages (peroxidase-negative) are present.

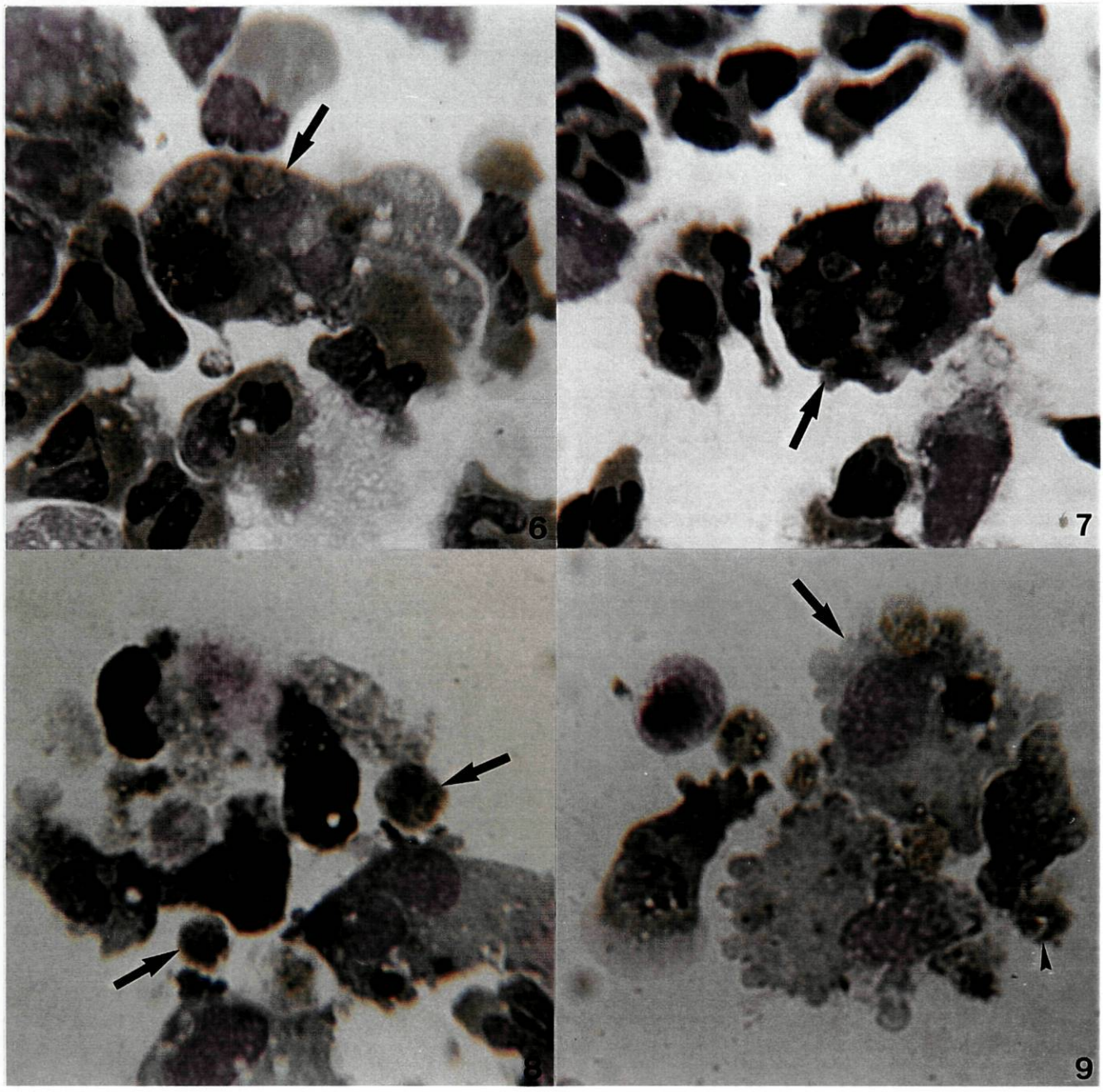
Fig. 3, 4 and 5: 12h of incubation. Several neutrophils with nuclear alterations typical of apoptosis are present (arrows).

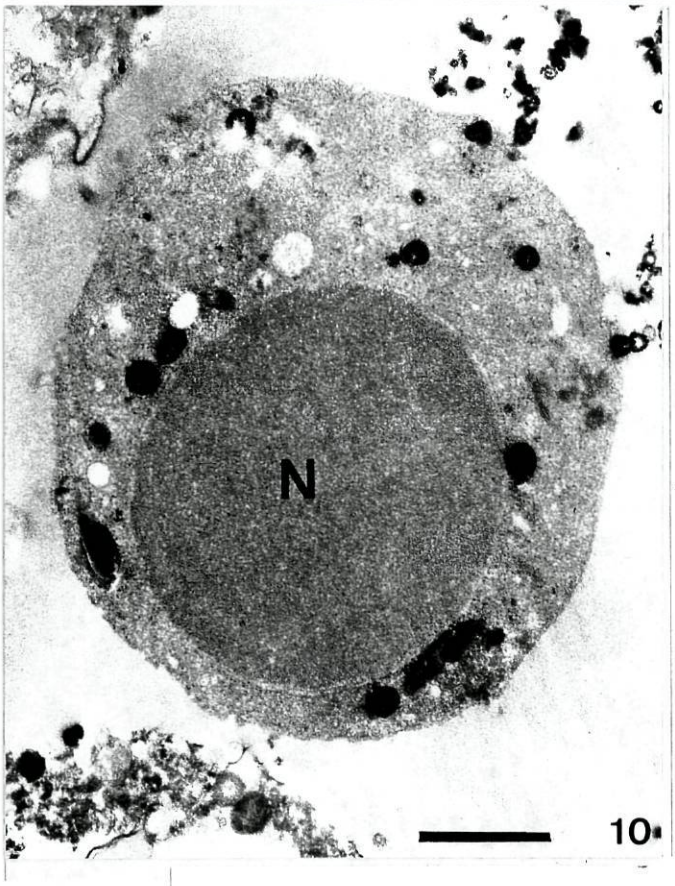
Fig. 6 and 7: 48h of incubation. Two macrophages with internalized apoptotic neutrophils are shown (arrows). These macrophages also phagocytosed neutrophil, peroxidase-positive, apoptotic bodies.

Fig. 8 and 9: 96h of incubation. Several neutrophilic apoptotic bodies are present. Those labelled with arrows in Fig. 8 are free. In Fig. 9, one macrophage contains one neutrophil apoptotic body, and several other bodies are adhering to the phagocyte.

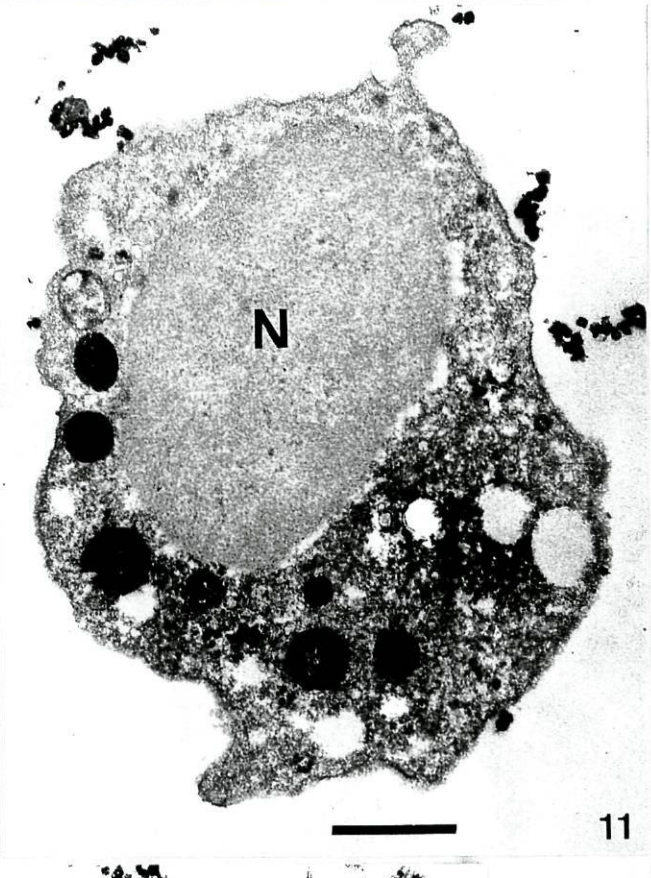
Fig. 10-12: Ultrastructural detection of peroxidase activity in neutrophil apoptotic bodies collected at 48h from the *in vitro*-incubated peritoneal cells. In Fig. 10 and 11 the apoptotic bodies contain nuclear fragments (N). Peroxidase-positive cytoplasmic granules are present in the three figures. Bar: 1μm.


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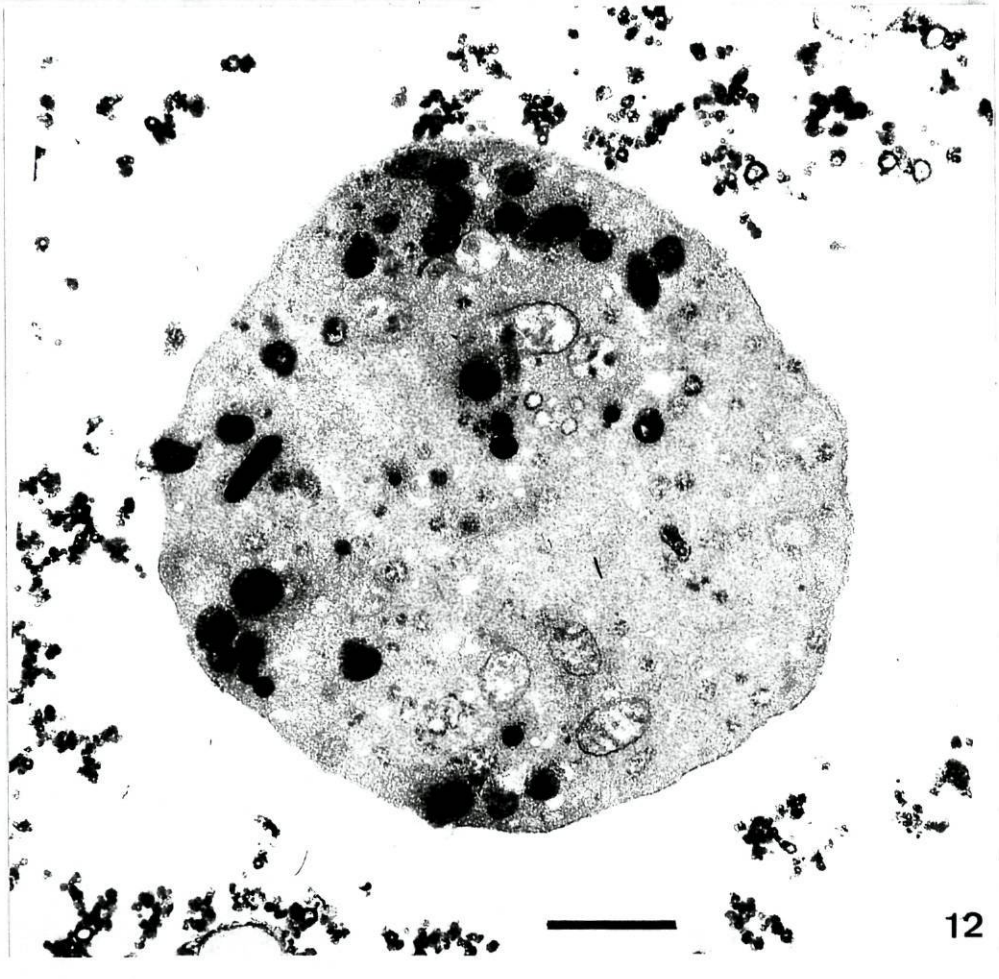

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DISCUSSÃO GERAL

Um mecanismo essencial na defesa contra a infecção nos animais é a fagocitose, morte e degradação dos microrganismos invasores pelos neutrófilos e macrófagos (Suzuki, 1986; Lehrer *et al.*, 1988; Ainsworth, 1992; Secombes & Fletcher, 1992; Densen *et al.*, 1995).

Estudos desenvolvidos em mamíferos mostraram que aqueles dois fagócitos têm padrões diferentes de localização nos territórios do hospedeiro normal assim como de mobilização para focos inflamados, nomeadamente, como consequência de infecção (Lehrer *et al.*, 1988; Haslett *et al.*, 1989; Silva *et al.*, 1989; Van Furth, 1992; Densen *et al.*, 1995). Esses padrões podem ser resumidos da seguinte maneira: os neutrófilos estão presentes em grande número no sangue e orgãos hematopoiéticos como "pools" de reserva e, em condições normais, são raros nos tecidos e cavidades naturais. Pelo contrário, os macrófagos estão presentes em todos os compartimentos e, por isso, são o primeiro fagócito que os microrganismos invasores encontram. Os neutrófilos são rápida e extensivamente recrutados das reservas para os locais infectados, pela actividade quimiotáctica de produtos microbianos, de factores extracelulares e citoquinas produzidas por células do hospedeiro, incluindo macrófagos (Lehrer *et al.*, 1988; Appelberg, 1992a,b,c). Os monócitos circulantes, precursores dos macrófagos dos tecidos, também são recrutados para os locais inflamados indo aumentar a população de macrófagos inicialmente presentes, embora o seu incremento ocorra em menor grau, e a sua chegada aconteça de forma mais lenta que o dos neutrófilos; citoquinas produzidas por neutrófilos participam nessa chamada (Taub *et al.*, 1996). Por conseguinte, verifica-se que cada um dos fagócitos profissionis tem a capacidade de atrair o outro aos locais inflamados/infectados. O resultado é que, pouco tempo depois do início do processo inflamatório, os locais infectados contêm uma população leucocitária em que coexistem números elevados de macrófagos e neutrófilos. Estudos anteriores deste laboratório abordaram a análise das interacções entre os dois fagócitos nos focos inflamatórios usando o modelo da cavidade peritoneal do ratinho (Pestana, 1994; Silva *et al.*, 1989; Sousa, 1994).

1. O modelo peritoneal

A escolha do modelo que utiliza a cavidade peritoneal para o presente estudo da inflamação, nomeadamente da que acompanha a infecção, teve a ver com a experiência anterior do laboratório em que os trabalhos incluídos nesta dissertação foram realizados. Essa experiência, obtida com estudos em ratinhos, tinha mostrado inequivocamente as vantagens daquele modelo para estudos de respostas leucocitárias na inflamação e infecção, permitindo inclusivamente a demonstração, pela primeira vez, da ocorrência de

uma resposta neutrofílica sustentada durante a infecção por micobactérias (Silva *et al.*, 1989). Uma das vantagens do modelo peritoneal em relação aos modelos convencionais, que estudavam por métodos histológicos as respostas leucocitárias em orgão sólidos, como o baço, fígado, gânglios, etc., deriva do facto de os exsudados peritoneais serem habitualmente compostos por leucócitos dispersos, o que torna fácil e seguro a quantificação em termos de leucócitos totais e diferenciais. Na única situação em que encontrámos exsudados peritoneais com abundantes agregados leucocitários que inviabilizaram a citologia quantitativa (em tempos avançados de infecção intraperitoneal da truta arco-íris com *Renibacterium salmoninarum* (trabalho 2.2, Capítulo 2)), mas mais uma vez o modelo peritoneal apresentou vantagens. Na verdade, foi fácil e seguro avaliar da resposta neutrofílica através do método indirecto, mas específico, do doseamento da actividade peroxidásica celular (trabalho 2.3, Capítulo 2). Os exsudados peritoneais são particularmente acessíveis a esta metodologia, em comparação com o que é de esperar em estudos com orgãos sólidos.

Além daquela vantagem, a utilização da cavidade peritoneal como modelo em estudos da resposta inflamatória apresenta outro aspecto vantajoso importante no caso dos peixes: a cavidade peritoneal é uma via importante para a inoculação de vacinas (Finn & Nielson, 1971). Acresce que a resposta leucocitária que ocorre em infecções experimentais na cavidade peritoneal é representativa da que acontece noutros territórios (van Furth, 1992).

A adaptação do modelo peritoneal ao caso particular das trutas levantou à partida algumas dificuldades, particularmente porque, ao contrário dos ratinhos, naquela espécie não é possível separar a parede abdominal do folheto peritoneal, não sendo assim possível visualizar as vísceras no momento da colheita dos exsudados. Esta dificuldade foi ultrapassada pela palpação e afastamento dos orgãos abdominais do local da picada. Ao mesmo tempo, a parede abdominal das trutas é bastante espessa e vascularizada, o que leva facilmente à contaminação dos exsudados com sangue. Assim, optou-se por se exsanguinar os animais antes da colheita por secção da aorta dorsal, e por colher os exsudados peritoneais com agulha e seringa em vez de se abrir a cavidade abdominal para esse fim.

Outro problema enfrentado na aplicação do modelo peritoneal às trutas tem a ver com uma particularidade anatómica destes peixes. Na verdade, a cavidade peritoneal dos salmonídeos abre para o exterior através de um poro abdominal (George *et al.*, 1982). Embora este poro se encontre normalmente fechado, a injecção intraperitoneal de volumes demasiadamente altos de líquido pode ocasionar a abertura do poro com a consequente perda para o exterior de parte do líquido injectado. Por outro lado, as fêmeas dos salmonídeos têm uma peculiar organização da gónada. Primeiro, não desenvolvem oviductos, segundo, na fase final da maturação a gónada rompe-se sendo os óvulos

libertados para a cavidade peritoneal antes da sua saída para o exterior pelo poro genital (Lagler *et al.*, 1977; George *et al.*, 1982). Verificou-se que esta passagem dos óvulos pela cavidade peritoneal origina uma situação de inflamação intraperitoneal (observações não publicadas). Por esta razão, e porque no presente trabalho se utilisaram machos e fêmeas de trutas arco-íris, seleccionaram-se sempre peixes adultos sexualmente imaturos.

Conforme descrito nos trabalhos 2.1 e 2.2 (Capítulo 2), a utilização do modelo peritoneal permitiu pela primeira vez analisar detalhadamente de maneira quantitativa a resposta fagocítica inflamatória, transitória ou persistente, incluindo a que acompanha a infecção em peixes.

2. Novos métodos aplicados ao estudo dos leucócitos na inflamação

Alguns aspectos técnicos originais, referentes a métodos utilizados pela primeira vez nos estudos incluídos nesta dissertação, merecem discussão detalhada. Essas técnicas permitiram a obtenção de dados quantitativos precisos sobre as cinéticas celulares que acompanham a inflamação e a análise quantitativa da capacidade fagocítica de neutrófilos e macrófagos.

O problema técnico central para a obtenção de dados quantitativos seguros sobre as respostas neutrofílicas e macrofágicas durante a inflamação peritoneal na truta arco-íris, foi o de distinguir neutrófilos e macrófagos. Esta distinção, que já levantava alguns problemas nas cavidades peritoneais em repouso, uma vez que alguns macrófagos podem apresentar um núcleo polimorfo, levantou novos problemas nas cavidades inflamadas. Um desses problemas resulta da ocorrência de actividade peroxidásica em alguns macrófagos inflamatórios, deixando, portanto, de ser segura a identificação dos neutrófilos por essa reacção citoquímica. Para resolver este problema, recorreu-se à marcação dupla dos leucócitos peritoneais por citoquímica ultrastrutural. Essa marcação mostrou que os neutrófilos eram peroxidase-positivos e esterase (alfa naftil butirato)-negativos, enquanto os macrófagos, incluindo os peroxidase-positivos, eram esterase-positivos. Além disso, foi aplicada a marcação ultrastrutural dupla da peroxidase/glicogénio e da esterase/glicogénio. Estas técnicas mostraram que os neutrófilos (peroxidase-positivos) são esterase-negativos e muito ricos em grânulos de glicogénio, enquanto os macrófagos, incluindo os peroxidase-positivos, são esterase-positivos e pobres em glicogénio. Uma das vantagens destas marcações citoquímicas múltiplas é a possibilidade de identificar macrófagos e neutrófilos por microscopia electrónica, mesmo quando só uma pequena parte da célula é visível (por exemplo, Fig. 2, trabalho 2.1 (Capítulo 2).

A detecção citoquímica da actividade peroxidásica mostrou-se fundamental para a caracterização dos neutrófilos e para a avaliação da transferência de componentes neutrofílicos para os macrófagos. Para essa técnica citoquímica em microscopia óptica

foi desenvolvido um método novo (Antonow), baseado no método clássico de Kaplow (1965), com as seguintes vantagens sobre os métodos existentes: i- possibilidade de visualizar bactérias intracelulares em neutrófilos com as granulações citoplasmáticas coradas para a detecção da peroxidase; ii- possibilidade de usar como coloração de contraste os corantes tipo Romanowsky, o que facilita a identificação dos vários tipos leucocitários; iii- possibilidade de rever as preparações durante mais de 1 ano depois da sua coloração, dada a estabilidade do produto da reacção citoquímica. A nova técnica para a peroxidase permitiu um estudo quantitativo da fagocitose de bactérias por neutrófilos e macrófagos na cavidade peritoneal inflamada de truta arco-íris.

3. População leucocitária da cavidade peritoneal em repouso da truta arco-íris

Um passo indispensável na aplicação do modelo peritoneal à truta foi a definição da população peritoneal basal, isto é, da cavidade peritoneal não estimulada pela injecção intraperitoneal de agentes flogísticos, poi tal definição estava por fazer de maneira satisfatória. Na verdade, um grupo, ao estudar cinéticas leucocitárias peritoneais na truta arco-íris, não refere os valores basais (Jorgensen *et al.*, 1993) e outro refere a presença de 100 % de macrófagos (Zelikoff & Enane, 1992; Enane *et al.*, 1993). Conforme descrito no trabalho 1.1 (Capítulo 1) estes resultados não foram confirmados pelas nossas observações preliminares.

Os nossos estudos mostraram que, ao contrário do descrito por aqueles autores, a cavidade peritoneal da truta arco-íris não estimulada possui uma população celular residente composta por linfócitos, macrófagos e neutrófilos. Esta população é, portanto, semelhante à existente nas cavidades peritoneais em repouso dos mamíferos (Cohn, 1962a,b; de Bakker, 1983; Silva *et al.*, 1989; Pestana, 1994) incluindo o homem (Kubicka *et al.*, 1996). Trombócitos e Células Eosinofílicas Granulares (EGC) só muito raramente foram observadas. O número de macrófagos era de cerca de 1.5×10^6 isto é, cerca de 40 % dos leucócitos totais. O número de neutrófilos era de cerca de 9.5×10^4, representando apenas 1.3 a 2.2 % do total de leucócitos.

No ratinho a percentagem de neutrófilos na cavidade peritoneal em repouso é mais baixa (de Bakker, 1983; Pestana, 1994) do que a que encontrámos na truta, podendo mesmo esses fagócitos estar ausentes, se os ratinhos forem mantidos em condições de especial cuidado em termos de contacto com agentes infecciosos (de Bakker, 1983). Dado que as trutas utilizadas nos nossos estudos não estavam em condições de esterilidade, e tendo em conta a particularidade anatómica acima discutida (a existência do poro abdominal), é possível que o número comparativamente elevado de neutrófilos nas cavidades peritoneais em repouso reflicta uma maior frequência de "encontros" com microrganismos, em comparação com o que se passa no ratinho.

No sentido de confirmar se os valores obtidos para a população peritoneal em repouso em animais mantidos no laboratório se verificavam para os salmonídeos, em condições de cultura próximas das encontradas em ambiente natural, foram analisados os exsudados de trutas fário (*Salmo trutta*) mantidas em tanques com água corrente natural no Posto Aquícola na Serra do Marão, cujos "stocks" são provenientes de reprodutores selvagens.

O estudo da população peritoneal destas trutas fário de peso próximo do das arco-íris que estudámos, mostrou valores de neutrófilos de 13.2 x 10^4 por cavidade peritoneal, correspondendo a 1.8 % do total de leucócitos. Estes valores são muito próximos dos encontrados nas trutas arco-íris mantidas no nosso laboratório.

Trabalho futuro deverá mostrar se os muito baixos números de neutrófilos agora descritos para a cavidade peritoneal não inflamada das trutas arco-íris e fário representam o padrão geral para os teleósteos. O único dado que encontrámos na Literatura refere à volta de 5 % de neutrófilos no robalo (*Dicentrarchus labrax*) e 22 % na dourada (*Sparus aurata*) (Meseguer *et al.*, 1993). O valor para o robalo não se afasta muito do encontrado nas trutas. O valor alto descrito para a dourada carece de confirmação, uma vez que as contagens foram baseadas em métodos morfológicos que não incluiam citoquímica, e os autores daquele trabalho admitem a presença na cavidade peritoneal da dourada de uma população de neutrófilos do tipo dos encontrados no sangue periférico muito inferior aos referidos 22 %.

4. A resposta inflamatória

Conforme descrito nos trabalhos 2.1 e 2.2 (Capítulo 2), a resposta dos leucócitos fagocíticos à inflamação aguda produzida na cavidade peritoneal da truta arco-íris pela injecção intraperitoneal de agentes flogísticos, é caracterizada pela subida acentuada e precoce no número de neutrófilos, seguida mais tarde por uma subida menor no número de macrófagos. Foi excepção a este padrão a resposta neutrofílica à injecção intraperitoneal da estirpe virulenta de *R. salmoninarum* (FT-10).

Foi verificado que durante a resposta inflamatória peritoneal o número de neutrófilos pode aumentar cerca de 500 vezes relativamente ao valor basal em 24-48 horas, passando muitas vezes este tipo celular a ser o leucócito mais abundante. Esta subida é substancial porque, conforme discutido atrás e consta do trabalho 1.1 do Cap. 1, antes da indução da inflamação os neutrófilos estão presentes na cavidade peritoneal da truta em números muito reduzidos.

A resposta mais lenta dos macrófagos origina valores máximos atingidos geralmente 2 ou 3 dias mais tarde que o dos neutrófilos. Por outro lado, o aumento relativo do número de macrófagos é também menor, atingindo nos casos extremos cerca de 7,5 vezes o valor observado em repouso.

Estas subidas no número de neutrófilos e macrófagos são, no essencial, semelhantes às descritas na cavidade peritoneal do ratinho após a injecção de agentes flogísticos, bacterianos ou não (de Bakker, 1983; Appelberg & Silva, 1989; Silva *et al.*, 1989). Uma diferença interessante tem a ver com a menor rapidez do influxo peritoneal de neutrófilos no caso da truta, o que poderá resultar da temperatura inferior a que operam os leucócitos no peixe.

Nos casos em que a inflamação foi resolvida a curto prazo (a inflamação transitória induzida pela caseína ou por *Y. ruckeri* morta ou viva de virulência reduzida), a normalização dos valores do número de neutrófilos ocorreu 15 dias depois das injecções intraperitoneais e aos 8 dias esses valores já eram próximos dos normais. Quando a presença do agente inflamatório foi persistente, a neutrofilia prolongou-se por muito mais tempo. Assim, com a injecção do IFA -óleo mineral de difícil degradação- a neutrofilia era ainda muito elevada aos 20 dias. Com a injecção da estirpe virulenta de *R. salmoninarum* (FT-10), que produz infecção persistente até ao fim do período experimental estudado (60 dias), encontrámos uma resposta neutrofílica sustentada até ao final desse período. Pelo contrário, após a injecção de renibactéria não virulenta (estirpe MT-239), os valores de neutrófilos peritoneais estavam em acentuada descida aos 15 dias após a injecção e perto dos valores normais aos 30 dias (trabalho 2.2, Capítulo 2).

Estes resultados mostram que a quantificação da resposta neutrofílica é uma medida adequada para a avaliação da intensidade, progresso e resolução da inflamação.

Estudos semi-quantitativos pouco precisos haviam sugerido anteriormente este tipo de padrão de resposta inflamatória dos dois fagócitos profissionais nos peixes (Finn & Nielson, 1971; Thuvander *et al.*, 1987; Suzuki & Iida, 1992).

Tendo em conta que as respostas leucocitárias observadas na cavidade peritoneal são representativas das que ocorrem noutros territórios (van Furth, 1992), estes resultados da evolução das respostas inflamatórias dos dois fagócitos profissionais, em conjunto com os referentes à cavidade peritoneal em repouso, mostram que na truta arco-íris, como nos mamíferos (de Bakker, 1983; Silva *et al.*, 1989; Pestana, 1994; Kubicka *et al.*, 1996), os macrófagos são os fagócitos residentes dos tecidos e que os neutrófilos são raros nos territórios não inflamados. Em situações de inflamação os neutrófilos são rapidamente chamados aos locais inflamados, seguindo-se um pouco mais tarde um aumento do número de macrófagos. Os neutrófilos permanecem no local inflamado só enquanto a inflamação persiste.

5. A fagocitose na inflamação de origem bacteriana

O desenvolvimento da técnica Antonow para a detecção da actividade peroxidásica em neutrófilos permitiu, conforme discutido atrás, a visualização simultânea da peroxidase e de bactérias intraneutrofílicas. A aplicação daquela técnica ao estudo da

fagocitose durante as infecções intraperitoneais permitiu verificar que, após a injecção de inóculos contendo menos de 10^8 bactérias (no caso da *Y. ruckeri*), a grande maioria dos microrganismos injectados é fagocitada pelos macrófagos residentes. Os neutrófilos entretanto chegados à cavidade peritoneal não encontram bactérias livres em número significativo, pelo que a sua oportunidade para os fagocitar é mínima. A verificação de que esta reduzida participação dos neutrófilos na fagocitose de bactérias inoculadas na cavidade peritoneal não é devida a uma baixa capacidade fagocítica intrínseca, mas antes à falta de bactérias livres, foi conseguida pela observação de que, quando as bactérias foram inoculadas em cavidades com elevados números de neutrófilos (pela injecção prévia de agentes inflamatórios), a sua fagocitose pelos neutrófilos se fez a um regime idêntico ao observado para os macrófagos (trabalho 2.1, Capítulo 2).

Se fosse tido em conta o conceito clássico da fisiologia do neutrófilo como leucócito puramente fagocítico, estes resultados apontariam para uma aparente inutilidade da chamada daqueles leucócitos. Mas, conforme discutido na Introdução, e tendo em conta resultados obtidos em mamíferos, o neutrófilo não é mais visto como uma célula unicamente envolvida na fagocitose de agentes infecciosos. Resultados desta dissertação, nomeadamente a observação da presença de números elevados de neutrófilos em situações em que eles não participam como fagócitos, levam a considerar que, também nos peixes, os neutrófilos poderão desempenhar funções a que chamaremos as funções não fagocíticas. Era já conhecido há bastante tempo para os mamíferos que os macrófagos têm actividades não fagocíticas (Adams & Hamilton, 1992), mas o reconhecimento, que referiremos adiante, de que também os neutrófilos desempenham funções não fagocíticas importantes é bem mais recente.

6. Uma visão moderna da participação de neutrófilos e macrófagos nos mecanismos de defesa contra infecções

Um dos aspectos da visão clássica das funções do neutrófilo, conforme foi descrito na Introdução, era a de que esta é uma célula que, após ter a sua maturação nos órgãos hematopoiéticos, vai aparecer envolvida na resposta inflamatória como um "saco carregado de enzimas", sem qualquer outra função que não seja o transporte do seu conteúdo, o qual põe em contacto com os microrganismos que deram origem à sua migração após fagocitose e desgranulação para o fagossoma.

Esta concepção acerca do neutrófilo tem vindo a mudar como resultado da investigação que nos últimos tempos tem sido desenvolvida em mamíferos, e muitas actividades não fagocíticas importantes têm vindo a ser atribuidas ao neutrófilo. A sua actividade não é já encarada só como puramente fagocítica e dependente da modulação desempenhada por macrófagos ou outras células efectoras na inflamação, mas é-lhe reconhecido também um papel regulador dessa mesma inflamação. Sabe-se hoje que o neutrófilo possui uma capacidade moduladora da resposta imunológica, nomeadamente

pela síntese de variados factores, incluindo de citoquinas (Lloyd & Oppenheim, 1992; Cassatella, 1995, 1996; Romani *et al*, 1996). A cooperação com o macrófago na resposta inflamatória, agora verificada para o caso dos peixes, é parte do leque de actividades do neutrófilo.

A participação dos neutrófilos nos mecanismos de defesa contra a infecção está a ser vista, portanto, como muito mais complexa e multifacetada do que o conceito clássico considerava. Neste contexto, um dos aspectos recentemente desenvolvidos tem a ver com a participação importante dos neutrófilos nos mecanismos de defesa contra parasitas intracelulares, incluindo intramacrofágicos. A disponibilização recente de um anticorpo monoclonal capaz de depletar eficazmente o ratinho de neutrófilos, permitiu demonstrar claramente que aqueles fagócitos são essenciais para os mecanismos de resistência contra a infecção por vários parasitas intracelulares, incluindo *Listeria monocytogenes* (Conlan & North, 1991; Conlan & North, 1992; Rogers & Unanue, 1993; Appelberg *et al.*, 1994; Conlan & North, 1994; Czuprynski *et al.*, 1994a,b; Rakhmilevich, 1995; Leal *et al.*, 1996; Conlan, 1997), *Mycobacterium avium* (Appelberg *et al.*, 1995; Pedrosa *et al.*, 1996), *Mycobacterium tuberculosis* (Pedrosa *et al.*, 1996), *Toxoplasma gondii* (Sayles & Johnson, 1996), *Salmonella* (Conlan & North, 1992; Conlan, 1997; Vassiloyanakopoulos *et al.*, 1998), *Francisella tularensis* (Conlan & North, 1992; Conlan, 1997), *Chlamydia trachomatis* (Barteneva *et al.*, 1996), *Yersinia enterocolitica* (Conlan, 1997) e *Candida albicans* (Jensen *et al.*, 1994; Romani *et al.*, 1997a,b). Uma limitação importante destes trabalhos, em que foram utilizados ratinhos neutropénicos, é a de que, embora os resultados obtidos mostrem claramente a importância do neutrófilo no combate contra aquelas infecções, não esclarecem o(s) mecanismo(s) da participação daquele leucócito. É de admitir que essa participação opere através de mecanismos vários e talvez simultâneos, como a amplificação da capacidade anti-microbiana dos macrófagos (por transferência de componentes neutrofílicos com forte actividade anti-microbiana que faltem no macrófago), recrutamento de monócitos para os locais infectados (Lloyd & Oppenheim, 1992), activação dos macrófagos por moléculas produzidas por neutrófilo, como a mieloperoxidase (Lincoln *et al*, 1995) e citoquinas, incluindo a IL-12 (Cassatella *et al*, 1995).

Os resultados dos estudos da inflamação peritoneal realizados pormenorizadamente na truta arco-íris e, mais preliminarmente no robalo, e incluídos na presente dissertação, mostram que também nestes teleósteos há interacções entre os dois fagócitos profissionais e que também neste grupo taxonómico os neutrófilos estão envolvidos em funções não fagocíticas.

O tipo de interacção entre os dois fagócitos que observámos nos exsudados peritoneais inflamados de truta e de robalo, exsudados em que os dois fagócitos coexistem em número elevado, foi a transferência de neutrófilos e de material neutrofílico para os macrófagos. Essa transferência origina a ocorrência, nos exsudados inflamatórios, de macrófagos positivos para a peroxidase. A aquisição de actividade

peroxidásica em macrófagos no decurso da resposta inflamatória, na truta arco-íris e no robalo, foi descrita por nós em primeira mão (trabalhos 2.1, Capítulo 2 e 3.1 e 3.2, Capítulo 3).

A transferência de componentes neutrofílicos para os macrófagos foi detectada pela presença dentro de macrófagos de neutrófilos suficientemente intactos para serem identificados por métodos citológicos usuais, e pela presença em macrófagos de actividade peroxidásica e de grânulos de glicogénio. A transferência para os macrófagos de componentes neutrofílicos também foi observada como resultado da fagocitose pelo macrófago de corpos apoptóticos neutrofílicos. A ocorrência de ligações tipo "gap junctions" observada agora pela primeira vez, entre neutrófilos e macrófagos nos exsudados peritoneais inflamatórios de truta arco-íris, parece representar mais outra modalidade de transferência de material neutrofílico para os macrófagos, modalidade não descrita em fagócitos de mamíferos. No sentido de confirmar se a presença de junções entre macrófagos e neutrófilos aderentes, acima referida, pode promover o trânsito de moléculas entre os dois fagócitos, será interessante ensaiar as técnicas usadas por Alves *et al.* (1995). Estes autores usaram, para o estudo do trânsito de moléculas através de ligações entre células epiteliais do timo de mamíferos, a injecção *in vitro* de fluorocromos numa das células do par e ensaios de "patch-clamp".

É de salientar que as observações descritas nos trabalhos incluídos na presente dissertação referentes à transferência de material neutrofílico para os macrófagos foram facilitadas pelo facto de na truta arco-íris, ao contrário do que acontece nos mamíferos, o neutrófilo ser o único leucócito com actividade peroxidásica endógena.

A fagocitose de neutrófilos e corpos apoptóticos de neutrófilos contendo bactérias, agora descrita em trutas e robalos, representa um mecanismo de transferência para o macrófago de bactérias inicialmente fagocitadas pelos neutrófilos. Esta transferência poderá representar uma vantagem adicional para o hospedeiro, inclusivamente porque o macrófago é uma célula de vida muito mais longa que a do neutrófilo.

A avaliação directa da participação do neutrófilo nas infecções dos salmonídeos, nomeadamente pelo parasita intracelular *R. salmoninarum,* será possível apenas quando for desenvolvida uma tecnologia para a eficaz indução de neutropenia selectiva.

Estas observações mostram que interacções neutrófilo/macrófago descritas inicialmente em mamíferos também ocorrem extensivamente nos peixes agora estudados. Embora, como propõe Klein (1989), talvez não se deva encarar a Imunologia dos invertebrados a pensar no que se conhece nos vertebrados, é tentador considerar como muito provável que, nos invertebrados que possuam macrófagos e neutrófilos como fagócitos profissionais, a interacção/cooperação entre esses fagócitos também ocorrerá.

As diferenças nas características funcionais, na distribuição territorial e nos padrões de mobilização dos dois fagócitos profissionais da truta, o macrófago e o neutrófilo,

discutidas atrás, sugerem que a utilização por este hospedeiro de dois fagócitos poderá obedecer a uma estratégia, que resumiremos a seguir. A presente interpretação para essa estratégia na utilização de dois fagócitos pela truta é, obviamente, semelhante à já proposta por este laboratório para o caso dos mamíferos (Silva *et al.*, 1989; Pestana, 1994; Sousa, 1994; Silva, 1997), uma vez que, no essencial, as duas situações são equivalentes.

A estratégia em discussão terá características de **segurança, rapidez, economia e eficácia.** A segurança deste sistema parece estar relacionada com o perigo pontencial que os neutrófilos representam para o hospedeiro, por serem células com um conteúdo muito rico em moléculas altamente citotóxicas, conforme referido na Introdução. Por isso, é um factor de segurança ter este fagócito, em condições normais, nos "pools" de reserva do sangue e orgãos hematopoiéticos, onde ele permanece, até ser necessário, num estado de quiescência. Pelo contrário, o fagócito residente de todos os territórios, o macrófago, é uma célula, conforme debatido na Introdução, muito mais pobre em moléculas citotóxicas e, por isso, menos agressiva para as células desses territórios. A rapidez e eficácia da resposta é conseguida pelas características dos neutrófilos que acorrem de forma pronta e em grande número ao local da infecção em resposta a factores quimiotácticos, migrando rapidamente para os locais onde estes foram produzidos. Aí os neutrófilos desenvolvem funções que vão desde a fagocitose e morte de microrganismos (quando há microrganismos extracelulares) à cooperação com o macrófago, fornecendo-lhe moléculas com elevada actividade antimicrobiana que o macrófago residente não possui, e ainda produzindo e segregando factores moduladores da resposta imunológica capazes de atrair e activar o outro fagócito. Conforme referido na Introdução, é interessante que cada um dos dois fagócitos seja capaz de atrair e activar o outro, numa estratégia de cooperação mútua.

O facto de os macrófagos residentes serem células com baixa actividade e de longa duração, podendo, assim como os neutrófilos, ser estimulados apenas aquando da necessidade de intervenção, representa uma forma de reduzir os custos energéticos e o risco de citotoxicidade para o hospedeiro na defesa contra as infecções, isto é, de melhorar a economia e aumentar a segurança do sistema de defesa. A eficácia de tal estratégia está na possibilidade de responder rapidamente a qualquer estímulo sem ter de manter situações de risco para o próprio e evitar consumo de energia, de localizar a sua acção aos locais inflamados e finalmente, porque a célula citotóxica é de vida curta, voltar rapidamente a um estado de repouso após debelar o foco infeccioso ou irritativo.

Um ponto interessante a salientar nesta discussão sobre a utilização de dois fagócitos pela truta e muitos outros hospedeiros é a origem comum desses dois fagócitos. Pelo que se sabe nos mamíferos (Metcalf, 1989), o neutrófilo e o macrófago provêm de uma célula precursora comum, enquanto curiosamente os outros dois granulócitos (o eosinófilo e o basófilo) não provêm dessa célula. Esta origem comum parece reflectir-se no que há de comum, no essencial, nos dois fagócitos. As diferenças que os especializam

aquando da suas funções à periferia foram adquiridas pelo processo de diferenciação que decorreu durante a sua maturação.

O passo final na reposição da normalidade, quando a infecção e a inflamação são resolvidas a favor do hospedeiro, é uma função essencialmente do macrófago, ao qual cabe fagocitar e digerir os restos dos microrganismos e das células mortas, alteradas ou em apoptose que resultaram dos processos desencadeados na resposta à infecção.

Será interessante investigar até que ponto, na escala filogenética animal, nomeadamente nos invertebrados, existem condições para a ocorrência de uma estratégia baseada na utilização de dois fagócitos profissionais como a que temos vindo a discutir para mamíferos e teleósteos. Tendo em conta o que foi dito atrás, tal estratégia requere não só a existência de dois fagócitos com as características funcionais específicas dos neutrófilos e macrófagos dos vertebrados, mas ainda um tipo de organização anatómica compatível com a separação de territórios incluindo "pools" de reserva, circulação sanguínea, cavidades naturais e orgãos.

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RESUMO E CONCLUSÕES

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A aquacultura representa em Portugal, e em muitos outros países, uma actividade industrial de relevo no fornecimento de proteína animal para consumo humano. As condições inerentes à aquacultura industrial são altamente propícias à ocorrência de infecções, as quais representam um sério problema com elevados custos económicos. Surge assim a necessidade de se aperfeiçoarem as intervenções profilácticas e terapêuticas e de se desenvolverem novas vacinas contra as mais perigosas patologias.

A maioria das dificuldades existentes em solucionar estes problemas estão relacionadas com o pequeno grau de desenvolvimento em áreas das ciências biológicas viradas para os animais aquáticos. Um ponto central neste contexto tem a ver com o conhecimento dos processos imunológicos que são postos em jogo nos mecanismos de defesa contra a infecção em peixes. O progresso nesse conhecimento é fundamental para que a intervenção profiláctica ou curativa venha a ter maior sucesso.

A presente dissertação centra-se no estudo de alguns aspectos referentes ao papel dos fagócitos naqueles mecanismos de defesa na espécie mais utilizada em Portugal para piscicultura: a truta arco-íris.

Nos trabalhos incluídos na presente dissertação utilizou-se a cavidade peritoneal da truta para estudar as respostas neutrofílica e macrofágica durante a inflamação induzida pela injecção intraperitoneal (i.p.) de vários agentes flogísticos bacterianos e não bacterianos. Para esse estudo adaptou-se à truta a técnica desenvolvida neste laboratório para a produção de inflamação na cavidade peritoneal do ratinho e para a análise citológica e citoquímica das respostas leucocitárias que acompanham a inflamação.

Utilizaram-se trutas arco-íris de 30 a 200g, sexualmente imaturas, fornecidas por uma truticultura comercial e mantidas em aquários neste laboratório. O estudo da população leucocitária peritoneal de trutas não injectadas ou injectadas com vários agentes flogísticos incluiu agentes bacterianos (*Yersinia ruckeri* viva e morta pelo formol, e *Renibacterium salmoninarum*) e não bacterianos (caseína e Adjuvante Incompleto de Freund (IFA)). Estirpes de virulência diferente foram utilizadas para a *R. salmoninarum*.

O estudo dos exsudados peritoneais foi feito por várias técnicas de microscopia óptica e electrónica, incluindo algumas desenvolvidas pela primeira vez durante este estudo.

As técnicas de microscopia óptica incluiram: contagem total e diferencial de leucócitos; citoquímica para a peroxidase, fosfatase ácida, fosfatase alcalina, alfa-naftil-acetato esterase e glicogénio. Para a peroxidase foi desenvolvido um método novo (Antonow) com as seguintes vantagens sobre os métodos existentes: i- possibilidade de

visualizar bactérias intracelulares em neutrófilos com as granulações citoplasmáticas coradas para a detecção da peroxidase; ii- possibilidade de usar como coloração de contraste os métodos tipo Romanowsky, o que facilita a identificação dos vários tipos leucocitários; iii- possibilidade de rever as preparações durante mais de 1 ano depois da sua coloração, dada a estabilidade do produto da reacção citoquímica. A nova técnica para a peroxidase permitiu um estudo quantitativo da fagocitose de bactérias por neutrófilos e macrófagos na cavidade peritoneal inflamada da truta arco-íris.

As técnicas de microscopia electrónica de transmissão (cortes ultra-finos) utilizaram métodos de preparação convencionais para estudos morfológicos ultrastruturais e métodos de citoquímica para detecção da actividade da peroxidase, alfa-naftil-butirato, esterase, fosfatase ácida e alcalina, e para detecção de moléculas PAS-positivas (glicogénio). Foram usadas marcações duplas, aplicando sucessivamente à mesma amostra a técnica da esterase seguida da peroxidase e, pela primeira vez, as técnicas da peroxidase ou esterase seguidas da detecção do glicogénio. Estas marcações múltiplas permitiram a caracterização segura de neutrófilos e macrófagos nos exsudados inflamatórios.

Métodos morfométricos foram aplicados a amostras preparadas sequencialmente para a peroxidase e para a detecção específica de glicogénio para quantificar os depósitos de glicogénio em neutrófilos e macrófagos.

A avaliação da evolução das infecções peritoneais foi feita por observação microscópica, incluindo ultrastrutural.

A actividade peroxidásica foi avaliada quantitativamente em cavidades peritoneais em repouso e em exsudados peritoneais de cavidades inflamadas pelos agentes descritos acima por um método espectrofotométrico que utiliza a tetrametilbenzidina.

Resultados entretanto obtidos levaram a estudos preliminares da resposta inflamatória peritoneal do robalo (*Dicentrarchus labrax*) à injecção i.p. das bactérias acima indicadas, e da apoptose do neutrófilo *in vitro* em exsudados peritoneais inflamatórios de truta arco-íris.

O estudo citoquímico de neutrófilos e monócitos do sangue da truta arco-íris foi feito em suspensões concentradas de leucócitos isolados do sangue venoso por punção da veia caudal.

Os resultados obtidos levaram às seguintes **conclusões gerais.**

1 - A cavidade peritoneal da truta arco-íris não estimulada possui uma **população celular residente** composta por linfócitos, macrófagos e neutrófilos. Trombócitos e Células Eosinofílicas Granulares (EGC) só muito raramente foram

observadas. Esta população de leucócitos é relativamente homogénea para indivíduos do mesmo tamanho. Não se verificou a ocorrência de variações significativas para animais mantidos a 10ºC, 15ºC ou 20ºC.

A identificação precisa por microscopia óptica dos diferentes leucócitos presentes na cavidade peritoneal em repouso apresenta algumas dificuldades. A diferenciação entre macrófagos pequenos e linfócitos grandes, à semelhança do que acontece com os mamíferos, é frequentemente impossível pelos métodos citológicos de coloração correntes. Este problema é ainda complicado pelo facto de haver actividades enzimáticas comuns a macrófagos e alguns linfócitos, como é o caso da esterase e fosfatase ácida. A análise ultrastrutural permitiu concluir que aqueles leucócitos difíceis de caracterizar são, na maioria, macrófagos pequenos.

Os neutrófilos constituiam 1.3 a 2.2 % do total de leucócitos da cavidade peritoneal não estimulada. A identificação de neutrófilos por microscopia óptica convencional pode ser difícil uma vez que alguns macrófagos podem apresentar um núcleo polimorfo. A caracterização segura dos neutrófilos foi conseguida pela detecção da actividade peroxidásica, uma vez que este leucócito foi o único leucócito com essa característica citoquímica encontrado na cavidade peritoneal não estimulada. A análise por citoquímica ultrastrutural mostrou que todos os grânulos citoplasmáticos dos neutrófilos de truta arco-íris possuem peroxidase.*

Além disso, estes fagócitos são muito ricos em grânulos de glicogénio. A quantificação por morfometria aplicada a microfotografias de amostras processadas para a detecção ultrastrutural dupla dos grânulos de glicogénio e de peroxidase, mostrou que o conteúdo nesses grânulos não era significativamente diferente para neutrófilos da cavidade peritoneal em repouso e do sangue periférico de trutas não injectadas.

Os macrófagos peritoneais residentes não continham grânulos de glicogénio nem actividade peroxidásica nas cisternas nuclear e do retículo endoplasmático. Estas duas características estão em contraste com o que se passa com os macrófagos residentes da cavidade peritoneal dos mamíferos.

As características citoquímicas que encontrámos para os leucócitos peritoneais da cavidade peritoneal não estimulada da truta arco-íris estão resumidas no Quadro 1.

Ao contrário do que se passa com os mamíferos, a actividade peroxidásica dos grânulos dos neutrófilos da truta arco-íris mostrou-se resistente a baixas concentrações de cianeto. Neste aspecto a peroxidase da truta é semelhante à peroxidase dos eosinófilos de mamíferos.

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* Resultados não documentados nesta dissertação, mostraram que um outro compartimento vacuolar está presente nos neutrófilos de truta, formado por vesículas secretoras fosfatase alcalina-positivas. Este compartimento é altamente mobilizável por fusão destas vesículas com a membrana ciplasmática, levando à deposição da enzima na superfície do neutrófilo.

Em exsudados inflamatórios as células granulares eosinofílicas (EGC) apenas apareceram esporadicamente e nunca encontrámos evidência de que estas células fossem fagocíticas. Por outro lado, quando estavam presentes em número razoável, era frequente a observação de grânulos livres e fagocitados pelos macrófagos.

Quadro 1

Características citoquímicas dos leucócitos da cavidade peritoneal da truta arco-íris

CARACTERÍSTICA	MACRÓFAGOS		NEUTRÓFILOS		LINFÓCITOS
	repouso	inflam.	repouso	inflam.	
peroxidase	-	- ou +	+++	+++	-
fosfatase alcalina	-	-	++	++	-
fosfatase ácida	+	+++	+	++	- ou +
alfa-naftil esterase	++	+++	-	-	- ou +
grânulos de glicogénio	-	+	+++	++++	ND

ND = não determinado

As EGC da truta arco-íris eram células esféricas, com núcleo geralmente excêntrico e com o citoplasma quase completamente ocupado pelos grânulos eosinofílicos, muitas vezes com um tom alaranjado nas preparações coradas pelo Wright. Em termos citoquímicos estas células eram peroxidase, esterase e fosfatase ácida negativas.

2 - Ao contrário do que está descrito para os monócitos do sangue circulante de vários mamíferos, os **monócitos circulantes da truta arco-íris** mostraram-se peroxidase-negativos.

3 - A **resposta inflamatória** peritoneal foi estudada após a injecção intraperitoneal de vários agentes flogísticos bacterianos e não bacterianos atrás referidos.

A caracterização dos dois fagócitos nas cavidades peritoneais inflamadas levantou novos problemas em comparação com o estudo das cavidades em repouso. Um desses problemas resulta da ocorrência de actividade peroxidásica em alguns macrófagos inflamatórios, complicando, portanto, a identificação dos neutrófilos por essa reacção citoquímica. Para resolver este problema, recorreu-se à marcação dupla dos leucócitos

peritoneais por citoquímica ultrastrutural. Essa marcação mostrou que os neutrófilos eram peroxidase-positivos e esterase-negativos, enquanto os macrófagos peroxidase-positivos eram esterase-positivos. Além disso, aplicámos pela primeira vez, a marcação ultrastrutural dupla da peroxidase seguida pela do glicogénio, e da esterase seguida pela do glicogénio. Estas técnicas mostraram que os neutrófilos são peroxidase-positivos, esterase-negativos e muito ricos em grânulos de glicogénio, enquanto os macrófagos peroxidase-positivos são esterase-positivos e pobres em glicogénio.

No Quadro 1 estão indicadas as características citoquímicas que encontrámos para os macrófagos e neutrófilos peritoneais inflamatórios da truta arco-íris.

4 - O estudo ultrastrutural do **glicogénio** dos fagócitos da truta arco-íris contrastados por métodos específicos, mostrou que esta molécula de reserva tem uma estrutura granular tipo β com um diâmetro de 35.72 ± 3.20nm, semelhante à dos grânulos de glicogénio do músculo e dos neutrófilos de mamífero. Verificou-se que o número de grânulos de glicogénio dos neutrófilos peritoneais presentes em situação normal aumentam significativamente nos estados inflamatórios que acompanham a injecção intraperitoneal de agentes flogísticos. Esse aumento foi maior nos casos em que os neutrófilos inflamatórios peritoneais foram chamados a fagocitar bactérias ou IFA. Nestas situações de hiper-inflamação peritoneal, o conteúdo em grânulos de glicogénio dos neutrófilos do sangue não era superior ao dos neutrófilos de trutas não injectadas, indicando que o aumento do conteúdo naquela molécula de reserva ocorre depois da saída dos neutrófilos da corrente sanguínea para o local inflamado.

Não se encontrou evidência de que o glicogénio dos neutrófilos que fagocitam IFA ou bactérias seja utilizado na explosão respiratória. Esta observação pode ser explicada pela presença de concentrações de glucose nas cavidades peritoneais inflamadas semelhantes às encontradas no sangue periférico.

Foi encontrada evidência da transferência de glicogénio dos neutrófilos para os macrófagos nas cavidades peritoneais inflamadas.

5 - Em termos de resposta dos dois fagócitos à inflamação, ela foi, com excepção da que acompanha a injecção de *R. salmoninarum* virulenta, caracterizada por uma subida precoce e acentuada no número de neutrófilos, seguida de uma subida mais tardia do número de macrófagos. A magnitude das respostas inflamatórias dos dois fagócitos foi dependente do tipo de estímulo.

Durante a resposta inflamatória peritoneal os neutrófilos podem aumentar cerca de 500 vezes o valor basal em 24-48 horas, passando muitas vezes a ser o leucócito mais abundante.

A resposta mais lenta dos macrófagos origina valores máximos atingidos geralmente 2 ou 3 dias mais tarde que o dos neutrófilos. Por outro lado, o aumento relativo dos macrófagos é também menor, atingindo nos casos extremos cerca de 7,5 vezes o valor em repouso.

Nos casos em que a inflamação foi transitoriamente induzida por agentes flogísticos não bacterianos (caseína) ou por *Y. ruckeri* morta ou viva de virulência reduzida, a normalização dos valores dos neutrófilos ocorre 15 dias depois das injecções intraperitoneais e aos 8 dias esses valores já são próximos dos normais. Quando a inflamação foi persistente a neutrofilia prolongou-se por muito mais tempo. Assim, com a injecção do IFA -óleo mineral de difícil degradação- a neutrofilia era ainda muito alta aos 20-30 dias. Com a injecção da estirpe virulenta de *R. salmoninarum*, que produz infecção persistente até ao fim do tempo do estudo (60 dias), encontrámos uma resposta neutrofílica sustentada até esse tempo. Pelo contrário, após a injecção de renibactéria não virulenta, os valores de neutrófilos peritoneais estavam em acentuada descida aos 15 dias e perto dos valores normais aos 30 dias.

6 - Durante a resposta inflamatória à injecção intraperitoneal de *Y. ruckeri* na truta arco-íris o grau de **fagocitose** para os 2 fagócitos mostrou-se dependente do acesso dos fagócitos aos microrganismos. Verificámos que pós a injecção de bactérias na cavidade peritoneal em repouso, a maioria do inóculo (quando este contém menos de 10^8 bactérias no caso da *Y. ruckeri*) é fagocitado pelos macrófagos residentes, e que os neutrófilos entretanto chegados à cavidade peritoneal não encontram, por isso, bactérias livres em quantidades significativas. Este resultado mostra que no início da infecção o macrófago é o fagócito principal. Pelo contrário, quando as bactérias foram injectadas em cavidades peritoneais com números elevados de neutrófilos (pela injecção prévia de caseína ou bactérias) o nível de fagocitose pelos neutrófilos foi elevado.

7 - A resposta inflamatória à injecção intraperitoneal da truta arco-íris com uma estirpe virulenta de *R. salmoninarum* tem características peculiares que não se verificam para nenhuma outra espécie de bactéria já ensaiada, quer em mamíferos, quer em peixes. Verificámos que a resposta à injecção com uma estirpe virulenta de renibactéria não é acompanhada do habitual acentuado pico precoce de neutrófilos, mas a partir dos 4 dias

desenvolveu-se uma resposta inflamatória crónica com aumento de neutrófilos até um patamar elevado, que se manteve até a morte dos peixes 2 a 3 meses mais tarde.

Pelo contrário, a estirpe de renibactéria de virulência reduzida originou um pico de neutrofilia peritoneal precoce após inoculação i.p. em trutas arco-íris.

8 - A contagem diferencial dos leucócitos presentes na cavidade peritoneal durante a inflamação aí induzida, mostra que o número de neutrófilos presente num território é uma medida adequada da intensidade, progressão e resolução da inflamação.

9 - Verificou-se a ocorrência de **apoptose nos neutrófilos** nos exsudados peritoneais inflamatórios. Esta apoptose é acompanhada pela produção e libertação de corpos apoptóticos. Essas estruturas sub-celulares neutrofílicas, ricas em grânulos peroxidase-positivos, foram frequentemente vistas dentro de macrófagos.

Ensaios preliminares mostraram que ocorre extensa apoptose dos neutrófilos quando exsudados peritoneais inflamatórios da truta arco-íris foram incubados *in vitro* em meio de cultura de células. Essa apoptose era acompanhada de extensa produção de corpos apoptóticos. Tanto os neutrófilos como os corpos apoptóticos do neutrófilo foram fagocitados pelos macrófagos presentes nas suspensões leucocitárias.

Estas são as primeiras descrições referentes à apoptose nos neutrófilos de peixes.

10 - Em exsudados peritoneais colhidos após a primeira semana de infecção com *R. salmoninarum*, verificámos haver dificuldade na quantificação e diferenciação dos leucócitos, incluindo a dos neutrófilos, pela presença de grande número de aglomerados celulares. Isto veio impôr a necessidade de utilização de outro método de quantificar a presença de neutrófilos. Dado que, na truta, o neutrófilo é o único leucócito com peroxidase endógena, a avaliação quantitativa da resposta neutrofílica foi conseguida pela **quantificação da actividade peroxidásica**. Esse método espectrofotométrico, que utiliza a tetrametilbenzidina, mostrou uma excelente correlação entre as contagens de neutrófilos e a actividade peroxidásica quando aplicado a suspensões leucocitárias dispersas. A aplicação deste método ao estudo da resposta neutrofílica à injecção intraperitoneal de renibactéria virulenta permitiu verificar a ocorrência de uma resposta neutrofílica sustentada (após os primeiros 8 dias) se prolonga até aos 2 meses.

11 - Adicionalmente, a utilização do método de doseamento da actividade peroxidásica em exsudados peritoneais, permitindo a quantificação da peroxidase intra e

extracelular, veio mostrar que aquela actividade enzimática é predominantemente intracelular. A actividade extracelular foi indetectável nas cavidades peritoneais de trutas não estimuladas e atingiu um máximo de só 5 % da peroxidase total às 24 horas de infecção com *Y. ruckeri* viva, regredindo novamente para níveis extremamente baixos nos tempos seguintes de infecção.

12 - O método de doseamento da actividade peroxidásica permitiu ainda quantificar a peroxidase por neutrófilo (PAN) em várias situações (repouso e inflamação peritoneal). Os valores de PAN foram semelhantes para neutrófilos do sangue periférico e da cavidade peritoneal de trutas não injectadas, indicando que os neutrófilos presentes nas cavidades de trutas não injectadas não estão estimulados. A injecção de uma dose baixa de *Y. ruckeri* viva de baixa virulência ou morta induz uma inflamação transitória na qual a actividade peroxidásica por neutrófilo (PAN) apresenta pequenas variações. Um aumento às 24-48h relativamente aos valores obtidos para os neutrófilos do sangue periférico ou da cavidade peritoneal em repouso é seguido de uma redução progressiva após três ou quatro dias. O aumento verificado durante os primeiros dias parece ser devido à presença de peroxidase nos macrófagos inflamatórios. Nos tempos mais longos, o decréscimo dos valores de actividade peroxidásica parece devido à perda de actividade da enzima contida nos neutrófilos. No caso da atenuada resposta neutrofílica característica dos primeiros dias após a injecção i.p. da estirpe virulenta de *R. salmoninarum*, os valores de PAN não apresentaram as variações acima referidas para a inflamação induzida por *Y. ruckeri*.

13 - Nos exsudados peritoneais inflamatórios, em que coexistem elevados números de neutrófilos e de macrófagos, verificámos a ocorrência de transferência de neutrófilos e de material neutrofílico para os macrófagos. Essa transferência foi detectada pela presença dentro de macrófagos de neutrófilos suficientemente intactos para serem identificados por métodos citológicos usuais, e pela presença em macrófagos de actividade peroxidásica e de grânulos de glicogénio. Verificámos que a transferência para os macrófagos de componentes neutrofílicos também ocorre pela fagocitose pelo macrófago de corpos apoptóticos neutrofílicos. A ocorrência de ligações tipo "gap junctions" observada agora pela primeira vez, entre neutrófilos e macrófagos nos exsudados peritoneais inflamatórios, parece representar outra modalidade de transferência de material neutrofílico para os macrófagos.

A fagocitose de neutrófilos e corpos apoptóticos de neutrófilos contendo bactérias, agora descrita, representa um mecanismo de transferência para o macrófago de bactérias inicialmente fagocitadas pelos neutrófilos.

Aspectos semelhantes de interacção macrófago/neutrófilo foram observados em exsudados peritoneais de robalos injectados intraperitonealmente com bactérias.

Em resumo, a presente dissertação contribui para o avanço no conhecimento dos mecanismos de defesa contra infecções em peixes, nomeadamente no que diz respeito ao papel dos dois fagócitos profissionais - neutrófilos e macrófagos.

Tendo em conta que o que se passa na cavidade peritoneal (no que se refere à localização territorial daqueles fagócitos e à sua mobilização para locais inflamados, nomeadamente por infecção) é representativo do que acontece noutros territórios, podemos concluir que, na truta arco-íris: i- nos tecidos e cavidades naturais normais, os macrófagos são os fagócitos residentes e os neutrófilos são muito pouco abundantes. ii- em situações de infecção, numerosos neutrófilos são rapidamente chamados dos "pools" de reserva, havendo um pico muito elevado de neutrófilos às 24-48h após o início da infecção. Sendo o fagócito residente dos tecidos, o macrófago é o primeiro fagócito a encontrar microrganismos invasores quando estes ultrapassam as barreiras naturais, e é, portanto, o fagócito que internaliza esses invasores. Curiosamente, os neutrófilos são chamados precocemente e em grandes números aos focos infecciosos, mesmo quando não há já microrganismos para fagocitar. Esta observação aponta para a existência de funções neutrofílicas não fagocíticas.

Na truta arco-íris, os neutrófilos são os únicos leucócitos com actividade peroxidásica endógena. Por isso, a observada presença de peroxidase em macrófagos nas cavidades peritoneais de truta inflamadas por agentes flogísticos bacterianos e não bacterianos, representa uma aquisição pelos macrófagos de componentes neutrofílicos. Verificámos que esta transferência de materiais neutrofílicos para macrófagos se faz por mecanismos semelhantes aos descritos em mamíferos (fagocitose de neutrófilos e de corpos apoptóticos neutrofílicos). A fagocitose de grânulos de neutrófilos e endocitose de moléculas neutrofílicas livres poderão constituir mecanismos adicionais de transferência. A passagem de moléculas do neutrófilo para o macrófago através de junções intercelulares tipo "gap junctions" formadas entre neutrófilos e macrófagos aderentes entre si poderá representar um mecanismo novo de transferência de moléculas entre fagócitos. Verificámos que, como nos mamíferos, a transferência de neutrófilos e

corpos apoptóticos neutrofílicos para macrófagos origina também a transferência de bactérias, quando os neutrófilos ou corpos apoptóticos neutrofílicos contêm bactérias.

No seu conjunto, os resultados acima resumidos mostram que, na truta arco-íris, os dois fagócitos profissionais, os neutrófilos e os macrófagos, têm padrões de localização, mobilização e interacção semelhantes aos descritos para os mamíferos. Estudos adicionais são necessários para esclarecer se o mesmo acontece noutros níveis de organização biológica animal.

RÉSUMÉ ET CONCLUSIONS

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L'aquaculture représente au Portugal et dans plusieurs pays une activité industrielle d'une grande importance dans la fourniture de proteines animales destinées à la consommation humaine. Les conditions inhérentes à l'aquaculture industrielle sont très favorables au développement d'infections qui représentent un sérieux problème économiquement trés coûteux. Il y a alors une nécessité à perfectionner les interventions prophilactiques et thérapeutiques et de développer de nouveaux vaccins contre les pathologies les plus dangereuses affectant les poissons.

La plupart des difficultés existantes pour résoudre ces problèmes sont dûes au petit niveau de developpement des sciences biologiques dédiées aux animaux aquatiques. Un point central dans ce contexte est la connaissance des procéssus immunologiques qui sont en jeu dans les mécanismes de défense contre les infections des poissons. Le progrès sur ce sujet est fondamental pour que l'intervention prophilactique ou curative ait un grand succès.

Cette dissertation se centre sur l' étude de quelques aspects concernant le rôle des phagocytes dans ces mécanismes de défense dans l'espèce de poissons la plus utilisée au Portugal dans l'aquaculture: la truite arc-en-ciel.

Dans les travaux décrits dans cette dissertation, on a utilisé la cavité péritonéale de la truite pour étudier les réponses des neutrophiles et des macrophages pendant l'inflammation induite par l'injection intrapéritonéale (i.p.) de plusieurs agents phlogistiques bactériens et non bactériens. Pour cette étude on a adopté à la truite les protocoles developpés dans notre laboratoire pour induire l'inflammation dans la cavité péritonéale du souriceau et pour l'analyse cytologique et cytochimique des réponses leucocytaires qui accompagnent cette inflammation.

On a utilisé des truites arc-en-ciel de 30 à 200g, sexuellement imatures, provenant d'une culture commerciale de truites et tenues dans un aquarium au laboratoire. Dans l'étude de la population leucocytaire péritonéale de truites non injectées ou injectées avec plusieurs agents phlogistiques, on a utilisé des agentes bactériens (*Yersinia ruckeri* vivante et morte par le formol et *Renibacterium salmoninarum*) et non bactériens (caséine et adjuvant incomplet de Freund (IFA)). Des souches de virulence différente ont été utilisées pour la *R. salmoninarum*.

L'étude des exsudés péritonéaux a été faite utilisant plusieurs téchniques de microscopie optique et éléctronique, dont certaines ont été dévelopées pour la première fois dans cette étude.

Les téchniques de microscopie optique étaient: quantification totale et différentielle des leucocytes, cytochimie à la peroxydase, phosphatase acide,

phosphatase alcaline, alpha-naphthyl-acetate esterase et glycogène. Pour la peroxydase on a développé une nouvelle méthode (Antonow) avec les avantages suivants sur les méthodes existantes: i - la possibilité de visualiser des bactéries à l'interieur des neutrophiles malgrès la présence des granules cytoplasmiques colorées en utilisant la détection de la peroxydase; ii - la possibilité d'utiliser comme coloration de contraste les méthodes du type Romanowski, ce qui facilite l'identification des différents types leucocytaires; iii - la possibilité de revoir les préparations pendant plus d'un an, dûe à la stabilité du produit de réaction cytochimique. La nouvelle téchnique pour la peroxydase a permis de quantifier la phagocytose des bactéries par les neutrophiles et les macrophages dans la cavité péritonéale inflammée de la truite arc-en-ciel.

Pour la microscopie éléctronique à transmission (coupes ultrafines) on a utilisé des méthodes de préparation conventionnelles pour les études morphologiques ultrastructurelles et des méthodes de cytochimie pour la détection de l'activité peroxydase, alph-naphthyl-butirate, esterase, phosphatase acide et alcaline et pour la détection de molécules PAS-positives (glycogène). On a utilisé le double marquage appliquant de suite au même échantillon la téchnique de l'esterase suivie de la peroxydase, et pour la première fois, les téchniques de peroxydase ou d'esterase suivies de la détection du glycogène. Ces marquages multiples ont permis une meilleure caractérisation des neutrophiles et des macrophages dans les exsudés inflammatoires.

Des méthodes morphométriques ont été appliquées à des échantillons préparés pour la déctection spécifique de la peroxydase et le glycogène afin de quantifier les dépots de glycogène dans les neutrophiles et les macrophages.

L'analyse de l'évolution des infections péritoneales a été faite par l'observation microscopique et ultrastructurelle.

L'activité de la peroxydase a été évaluée quantitativement dans les cavités péritonéales au repos et dans les exsudés péritoneaux des cavités inflammées pas les agents décrits plus haut par une méthode spectrophotométrique qui utilise la tetramethylbenzidine.

Les résultats obtenus nous ont conduit à effectuer des études préliminaires de la réponse inflammatoire péritonéale du bar (*Dicentrarchus labrax*) à l'injection i. p. des bactéries et de l'apoptose *in vitro* des neutrophiles dans les exsudés péritoneaux inflammatoires de la truite arc-en-ciel.

L'étude cytochimique des neutrophiles et monocytes du sang de la truite arc-en-ciel a été faite dans des suspensions concentrées de leucocytes isolés du sang veineux par ponction de la veine caudale.

Les résultats obtenus nous ont permis de faire les **conclusions générales** suivantes:

1 - La cavité péritonéale non stimulée de la truite arc-en-ciel a une **population cellulaire résidente** composée par les lymphocytes, les macrophages et les neutrophiles. Les thrombocytes et les cellules éosinophiliques granulées (EGC) ont été très rarement observées. Cette population de leucocytes est relativement homogène chez les individus de même taille. On n'a pas observé de variations significatives chez des animaux tenus à 10°C, 15°C ou 20°C.

L'identification précise par microscopie optique des différents leucocytes présents dans la cavité péritonéale au repos présente quelques difficultés. La différenciation entre macrophages petits et lymphocytes grands, comme pour les mammifères, est souvent impossible par des méthodes cytologiques de coloration habituelles. Ce problème est aussi compliqué à cause de l'existence d'activités enzymatiques communes aux macrophages et à quelques lymphocytes, comme l'esterase et la phosphatase acide. L'analyse ultrastructurale a permis de conclure que les leucocytes mononucleaires de caracterisation difficile sont en majorité des macrophages petits.

Les neutrophiles constituaient 1.3 à 2.2 % du total de leucocytes de la cavité peritoneale non stimulée de truite.L'identification des neutrophiles par microscopie optique conventionelle peut être difficile parce que certains macrophages peuvent présenter un noyau polymorphe. La caractérisation sûre des neutrophiles a été possible grâce à la détection de l'activité de la peroxydase, puisque ce leucocyte a été le seul trouvé dans la cavité péritonéale non stimulée ayant cette caractéristique cytochimique. L'analyse par cytochimie ultrastructurale a montré que tous les granules du cytoplasme des neutrophiles de la truite arc-en-ciel ont de la peroxydase.[1]

En plus, les neutrophiles sont très riches en granules de glycogène. La quantification par morphométrie appliquée à des micrographies après la détection ultrastructurale à la fois des granules de glycogène et de peroxydase, a démontré que le contenu en granules de glycogène n'était pas significativement différent dans les neutrophiles de la cavité péritonéale au repos et du sang périphérique des truites non injéctées.

Les macrophages péritoneaux résidents ne contenaient pas de granules de glycogène et n'avaient pas une activité peroxydase dans les cisternes du noyau et du réticulum endoplasmique. Ces deux caractéristiques sont en contraste avec ce qui se passe avec les macrophages résidents de la cavité péritonéale des mammifères.

[1] Des résultats préliminaires, non documentés dans cette dissertation, ont démontré la présence dans les neutrophils de truite arc-en-ciel d'un compartment formé par des vacuoles trés riches en phosphatase alcaline, independant du compartiment granulaire peroxydase-positif. Ce compartiment est très mobilisable par fusion des vacuoles du cytoplasme avec la membrane plasmique du neutrophile.

Les caractéristiques que nous avons observées pour les leucocytes péritonéaux de la cavité péritonéale non stimulée de la truite arc-en-ciel sont résumées dans le Tableau 1.

Tableau 1

Caractéristiques cytochimiques des leucocytes péritoneaux de la cavité péritonéal de la truite arc-en-ciel.

CARACTÉRISTIQUES	MACROPHAGES		NEUTROPHILES		LYMPHOCYTES
	repos	inflam.	repos	inflam.	
Peroxydase	-	- ou +	+++	+++	-
Phosfatase alcaline	-	-	++	++	-
Phosfatase acide	+	+++	+	++	- ou +
Alpha-naphtil esterase	++	+++	-	-	- ou +
Granules de glycogène	-	+	+++	++++	ND

ND = non déterminé

Contrairement à ce qui se passe chez les mammifères, l'activité de la peroxydase des granules des neutrophiles de la truite arc-en-ciel est résistente à baisses concentrations de cyanure. Sur ce point, la peroxydase de la truite est semblable à la peroxydase des eosinophiles des mammifères.

Dans les exsudés inflammatoires, les cellules granulaires eosinophiliques (EGC) surviennent seulement sporadiquement et on n'a jamais trouvé l'evidence que ces cellules étaient phagocytiques. Par contre, quand elles étaient présentes en nombre raisonnable, on voyait souvent ces granules libres et phagocytés par les macrophages. Les EGC étaient des cellules sphériques avec un noyau généralement excentrique et avec le cytoplasme près que completement occupé par les granules eosinophiliques de couleur orange dans les préparations colorées par le Wright. Cytochimiquement, ces cellules étaient peroxydase, esterase et phosphatase acide-négatives.

2 - Contrairement à ce qui est décrit pour les monocytes du sang circulant de plusieurs mammifères, les **monocytes circulants de la truite arc-en-ciel** sont peroxydase-négatifs.

3 - **La réponse inflammatoire** péritonéale a été étudiée après injection intrapéritonéale de plusieurs agents phlogistiques bactériens et non bactériens. La caractérisation des deux phagocytes dans les cavités péritoneales inflammées a originé de nouveaux problèmes en comparaison avec l'étude des cavités au repos. Un de ces problèmes résulte de la présence d'activité de la peroxydase dans quelques macrophages inflammatoires, ce qui complique l'identification des neutrophiles par cette réaction chimique. Pour résoudre ce problème, on a utilisé le double marquage des leucocytes péritoneaux par cytochimie ultrastructurale. Ce marquage a démontré que les neutrophiles étaient peroxydase-positifs et esterase (alpha-naphthyl-butirate)-négatifs alors que les macrophages peroxydase-positifs étaient esterase-positifs. Nous avons aussi appliqué, pour la première fois, le double marquage ultrastructurale de la peroxydase et glycogène et de l'esterase et glycogène. Ces téchniques ont démontré que les neutrophiles etaient peroxydase-positifs, esterase-negatifs et très riches en granules de glycogène alors que les macrophages peroxydase-positifs sont esterase-positifs et pauvres en glycogène.

Les caractéristiques cytochimiques que nous avons trouvées pour les macrophages et neutrophiles péritoneaux inflammatoires de la truite arc-en-ciel sont résumées dans le Tableau 1.

4 - L'étude ultrastructurale du **glycogène** des phagocytes de la truite arc-en-ciel contrastés par des méthodes spécifiques, a démontré que cette molécule de réserve a une structure granulaire type β avec un diamètre de 35.72 ± 3.20nm, semblable à des granules de glycogène du muscle et des neutrophiles de mammifère.

On a observé que le nombre de granules de glycogène des neutrophiles péritoneaux présents en situation normale, augmente significativement dans les états inflammatoires qui acompagnent l'injection intrapéritonéale d'agents phlogistiques. Cette augmentation a été plus grande lorsque les neutrophiles inflammatoires péritoneaux ont été appelés pour phagocyter les bactéries ou l'IFA. Dans ces situations d'hyper-inflammation péritonéale, le contenu en granules de glycogène des neutrophiles du sang n'était pas supérieur à celui des neutrophiles de truites non injéctées, indiquant que l'augmentation du contenu en cette molécule de réserve a lieu après la sortie des neutrophiles du sang vers le site inflammé.

On n'a trouvé aucune évidence de l'utilisation dans l'explosion respiratoire du glycogène des neutrophiles qui phagocytent IFA ou bactéries. Cette observation peut être

expliquée par la présence de concentrations en glucose, dans les cavités péritoneales inflammées, comparables à celles trouvées dans le sang périphérique.

On a pu observer le transfert du glycogène des neutrophiles vers les macrophages dans les cavités péritoneales inflammées.

5 - La réponse des deux phagocytes à l'inflammation a été, à l'exception de celle qui accompagne l'injection de *R. salmoninarum*, caractérisée par une augmentation précoce et accentuée dans le nombre de neutrophiles, suivie d'une augmentation un peu plus tard du nombre de macrophages. La grandeur des réponses inflammatoires des deux phagocytes dépendait du type de stimulation.

Pendant la réponse inflammatoire péritonéale les neutrophiles ont pu augmenter au moins 500 fois la valeur basale en 24-48h, représentant souvent les leucocytes les plus abondants.

La réponse plus lente des macrophages a donné origine à des valeurs maximales généralement atteintes deux ou trois jours plus tard que les neutrophiles. Par contre, l'augmentation relative des macrophages est aussi faible, ne dépassant pas 7.5 fois la valeur au repos.

Quand l'inflammation a été induite transitoirement par des agents phlogistiques non bactériens (Caseine) ou par *Y. ruckeri* morte ou de virulence réduite, la normalisation des valeurs des neutrophiles survenait 15 jours après injection intrapéritoneale, et dès le 8^{e} jour ces valeurs étaient déjà très comparables aux valeurs normales. Quand l'inflammation est persistente, la neutrophilie se maintient plus de temps. Alors, avec l'injection du IFA -huile minérale de dégradation difficile- la neutrophilie était encore très haute aprés 20-30 jours. Avec l'injection d'une souche virulente de *R salmoninarum* , qui produit une infection persistente jusqu'à la fin du temps d'étude (60 jours), nous avons trouvé une réponse neutrophilique soutenue pendant tout ce temps. Par contre, après l'injection de *R. salmoninarum* non virulente les valeurs des neutrophiles peritoneaux étaient en grande décroissance depuis 15 jours et près des valeurs normales au bouf de 30 jours aprés.

6 - Pendant la réponse inflammatoire à l'injection intrapéritonéale de *Y. ruckeri* dans la truite arc-en-ciel le degré de phagocytose pour les deux phagocytes dépend de l'accès des phagocytes aux microorganismes. Aprés l'injection des bactéries dans la cavité péritonéale au repos, la majorité de l'inoculat (quand il contient moins de 10^{8} bactéries dans le cas de *Y. ruckeri*) est phagocyté par les macrophages residents; cependant les neutrophiles qui arrivent à la cavité péritonéale ne trouvent pas de bactéries libres en quantités significatives.

Ce résultat nous démontre qu'au début de l'infection le macrophage est le principal phagocyte. Par contre, quand les bactéries sont injectées dans les cavités péritoneales

ayant un grand nombre de neutrophiles (par l'injection préalable de caseíne ou de bactéries) le niveau de phagocytose par les neutrophiles était plus élevé.

7 - La réponse inflammatoire à l'injection intrapéritonéale de la truite arc-en-ciel avec *R. salmoninarum* a des caractéristiques particulières qui ne se vérifient avec aucune autre espèce de bactéries déjà étudiée ches les mammifères ou les poissons. La réponse à l'injection d'une souche virulente de renibacterium n'est pas accompagnée de l'important et habituel pic précoce de neutrophiles, mais après quatre jours une réponse inflammatoire chronique se développe avec une augmentation de neutrophiles jusqu'à un plateau, qui se maintient jusqu'à la mort des poissons deux ou trois mois plutard. Par contre, la souche de renibacterium de virulence réduite a originé des pics de neutrophilie précoce après l'inoculation i.p. des truite arc-en-ciel.

8 - La quantification différentielle des leucocytes présents dans la cavité péritonéale pendant l'inflammation induite nous démontre que le nombre de neutrophiles présents dans un territoire est un indicateur convenable de l'intensité, la progression et la résolution de l'inflammation.

9 - Dans les exsudés péritoneaux inflammatoires on a verifié l'entrée en **apoptose des neutrophiles**. Cette apoptose s'accompagne de la prodution et libération de corps apoptotiques. Ces structures sub-cellulaires du neutrophile, riches en granules peroxydase-positifs, ont été vues dans les macrophages.

Des essais préliminaires ont démontré une entrée massive en apoptose des neutrophiles quand des exsudés péritoneaux inflammatoires de la truite arc-en-ciel ont été incubés *in vitro* dans un milieu de culture de cellules. Cette apoptose s'accompagne d'une grande production de corps apoptotiques. Les neutrophiles, comme les corps apoptotiques du neutrophile, ont été phagocytés par les macrophages présents dans les suspensions leucocytaires.

C'est la première description de l'apoptose des neutrophiles chez les poissons.

10 - Dans les exsudés péritoneaux recueillis après la première semaine de l'infection avec *R. salmoninarum*, on a verifié l'existence de difficultés dans la quantification et différenciation des leucocytes, et aussi des neutrophiles, à cause de la présence d'un grand nombre d'agglomérats cellulaires. Alors il y avait la nécéssité d'utiliser uneautre méthode pour quantifier la présence de neutrophiles. Comme dans la truite le neutrophile est l'unique leucocyte avec activité peroxydasique endogène, l'évaluation quantitative de la réponse neutrophilique a été possible grâce à la **quantification de l'activité peroxydase**. Cette méthode spectrophotométrique qui utilise la tétra-methyl benzidine, a demontré une excellente corrélation entre le nombre de neutrophiles et l'activité peroxydase quand elle est appliquée à des suspensions

leucocytaires dispersés. L'application de cette méthode à l'étude de la réponse neutrophilique à l'injection intrapéritonéale de *R. salmoninarum* a possibilité la verification d'une réponse neutrophilique soutenue (aprés les premiers 8 jours) qui se prolonge jusquà 2-3 mois.

11- En outre, l'utilisation de la méthode du dosage de l'activité de la peroxydase dans les exsudés péritoneaux, a permis la quantification de la peroxydase intra et extracellulare. Ceci a permis de montrer que l'activité enzymatique est surtout intracellulaire. L'activité extracellulaire n'a pas été détectée dans les cavités péritoneales des truites non stimulées atteignant un maximum de seulement 5 % de la peroxydase totale après 24h d'infection avec *Y. ruckeri* vivante, etrevenant de nouveau à des niveaux très bas dans le temps qui suit l'infection.

12 - La méthode de dosage de l'activité peroxydasique a permis aussi de quantifier la peroxydase par neutrophile (PAN) dans plusieurs conditions (repos et inflammation péritonéale). Les valeurs de PAN ont été semblables pour les neutrophiles du sang périphérique et de la cavité péritonéale de truites non injectées, indiquant que les neutrophiles présents dans les cavités de truites non injectées ne sont pas stimulés. L'injection d'une petite dose de *Y. ruckeri* vivante de virulence réduite ou morte a provoqué une inflammation transitoire dans laquelle l'activité peroxydasique par neutrophile (PAN) présente de petites variations. Une augmentation dans les 24-48h relativement aux valeurs obtenues pour les neutrophiles du sang périphérique ou de la cavité péritonéale au repos, est suivie d'une réduction progressive après 3 ou 4 jours. L'augmentation verifiée pendant les premiers jours, serait dûe à la présence de peroxydase dans les macrophages inflammatoires. Aprés un temps plus longs, la réduction des valeurs de l'activité peroxydasique se doit, on pense, à la perte d'activité de l'enzyme contenue dans les neutrophiles. Dans la situation de réponse neutrophilique atténuée, caractéristique des premiers jours après l'injection i.p. de la souche virulente de *R. salmoninarum,* les valeurs de PAN n'ont pas présenté les variations mentionées ci-dessus pour l'inflammation due a *Y. ruckeri.*

13 - Dans les exsudés péritoneaux inflammatoires, où coexistent en nombre élévé des neutrophiles et des macrophages, nous avons vérifié l'existence de transfert de neutrophiles et de matériel neutrophilique vers les macrophages. Ce transfert a été detecté par la présence dans des macrophages de neutrophiles suffisamment intacts pour être identifiés par les méthodes cytologiques usuelles, et par la présence dans les macrophages d'activité peroxydasique et de granules de glycogène. Le transfert dans les macrophages de composants du neutrophile se fait aussi par la phagocytose par le macrophage de corps apoptotiques du neutrophile. L'existence de liaisons du type "gap junctions" observées maintenant pour la première fois, entre neutrophiles et

macrophages dans les exsudés péritoneaux inflammatoires, semble représenter une nouvelle modalité de transfert du matériel neutrophilique vers les macrophages.

La phagocytose des neutrophiles et des corps apoptotiques de neutrophiles ayant des bactéries, ici décrites, représente un mécanisme de transfert vers le macrophage de bactéries initialement phagocytées par les neutrophiles.

Des aspects similaires d'interaction macrophage/neutrophile ont été observés dans des exsudés péritoneaux du bar injéctés intrapéritonéalement avec des bactéries.

En **résumé**, cette dissertation a permis de mieux connaitre les mécanismes de défense contre les infections de poissons, en particulier de ceux liés aux deux phagocytes professionnels - les neutrophiles et les macrophages.

Comme ce qui se passe dans la cavité péritonéale, en ce qui concerne la localisation territoriale de ces phagocytes et leur mobilisation vers les locaux inflammés, notamment par infection, c'est réprésentatif de ce qui se passe dans d'autres territoires, nous pouvons conclure que, dans la truite arc-en-ciel: i- Dans les tissues normaux, les macrophages sont les phagocytes résidents et les neutrophiles sont rares. ii- En cas d'infection, beaucoup de neutrophiles sont appelés rapidement des "pools" de réserve, ayant un pic très élevé de neutrophiles 24-48h après le début de l'infection. Comme phagocyte résident des tissus, le macrophage est le premier phagocyte qui trouve et phagocyte les microorganismes envahisseurs qui dépassent les barrières naturelles. Curieusement, les neutrophiles sont appelés précocement et en grande quantité aux foyers d'infection, même quand il n'y a plus de microorganismes à phagocyter. Cette observation signale l'existence de fonctions non phagocytiques du neutrophile.

Chez la truite arc-en-ciel, les neutrophiles sont les seuls leucocytes avec activité peroxydasique endogène. C'est pour cela que la présence de peroxydase dans les macrophages des cavités péritonéales de truites inflammées par des agents phlogistiques bactériens et non bactériens, représente une acquisition par les macrophages de composants du neutrophile. Ce transfert de matériaux du neutrophile vers les macrophages se fait par des mécanismes semblables à ceux décrits pour les mammifères (phagocytose de neutrophiles et de corps apoptotiques du neutrophile et probablement phagocytose de granules de neutrophiles et endocytose de molécules du neutrophile libres) ou encore par un mécanisme nouveau, à travers les jonctions inter-cellulaires du type "gap junctions" entre macrophages et neutrophiles adhérents. On a verifié que, comme pour les mammifères, le transfert de neutrophiles et corps apoptotiques du neutrophile vers les macrophages origine aussi le transfert de bactéries, quand les neutrophiles ou les porps apoptotiques du neutrophile ont des bactéries.

L'ensemble des résultats ici résumés, montre que chez la truite arc-en-ciel les deux phagocytes professionels, les neutrophiles et les macrophages, ont des modèles de localization, mobilization et interaction semblables a ceux décrits pour les mammifères. D'autres études sont nécessaires pour comprendre si le même se verifie à d'autres niveaux d´organisation animale.

ABSTRACT AND CONCLUSIONS

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Aquaculture is a relevant industry in Portugal as well as worldwide as a food supply. The inherent conditions in industrial aquaculture are highly propitious for the occurrence of infections which represents a problem with serious biological and economic costs. Therefore prophylactic and therapeutic interventions including vaccination should be improved to offset the most dangerous infectious diseases of fish.

Many of the existing difficulties to resolve are related to the poor development in the areas of the biological sciences dealing with aquatic animals. A central point in this context has to do with the knowledge of the immunological processes which are put into play in the defence against infection in fish. Progress in that area is fundamental in order that any prophylactic or curative practice would achieve the greatest success.

The central focus of this thesis regards some aspects of the role of phagocytes in the defence mechanisms in rainbow trout, which is the most important fish species in aquaculture in Portugal.

The peritoneal cavity was chosen as the experimental model to study the inflammation induced by bacterial and non-bacterial phlogistic agents. The peritoneal technique previously developed in this laboratory in the mouse model, was adapted to produce inflammation in rainbow trout and to analyse the leukocyte response, using cytological and cytochemical methods.

Sexually immature rainbow trout weighing 30 to 200g, were supplied by a commercial aquaculture and maintained in aquaria facilities in this laboratory. The study of peritoneal leukocyte populations was done both in non injected fish and in fish injected with different phlogistic agents, including bacteria (live or formol-killed *Yersinia ruckeri* and *Renibacterium salmoninarum*) and non-bacterial agents (casein, Incomplete Freund's Adjuvant (IFA)). Strains of *R. salmoninarum* with different virulence were used.

Techniques using the light and electron microscopy were applied to the study of the peritoneal exudates. Some of these techniques were original, being developed and applied for the first time during this study.

The light microscopy techniques included: Total and differential leukocyte counts; cytochemical stains for peroxidase, acid phosphatase, alkaline phosphatase, alpha-naphthyl-acetate esterase and glycogen. A new method was developed for the staining of peroxidase (Antonow) which has important advantages such as: i- visualisation of intra-neutrophilic bacteria, simultaneous with the stain for the peroxidase-positive granules; ii- the use of Romanoswsky methods as counterstain, making the identification of different leukocyte types easier; iii- preparations can be seen during more than a year due to the

stability of the staining. The new peroxidase technique allowed the quantitative study of bacterial phagocytosis by neutrophils and macrophages, in the inflamed peritoneal cavity of rainbow trout.

Current techniques of transmission electron microscopy were used to the study of morphology, and cytochemical methods were applied to detect peroxidase, alpha-naphthyl-butirate, esterase, acid and alkaline phosphatases and PAS-positive polysaccharide molecules (glycogen). Double staining techniques were applied to the same sample for the detection of esterase and peroxidase and, for the first time, peroxidase or esterase followed by glycogen. These double staining techniques allowed for precise characterisation of neutrophils and macrophages in inflammatory peritoneal exudates.

Morphometric methods were applied to samples prepared for the detection of peroxidase and for specific glycogen staining in order to quantify the amount of glycogen in neutrophils and macrophages.

Microscopic techniques, including ultrastructural techniques, were used to assess the evolution of infection.

A spectrophotometric method that uses tetramethylbenzidine was applied to quantify peroxidase activity in resting peritoneal exudates, or in peritoneal exudates inflamed by the injection of the above described agents.

Preliminary studies were carried out on the inflammatory response in the peritoneal cavity of sea bass (*Dicentrarchus labrax*) to the i.p. injection of bacteria, and on neutrophil apoptosis in inflammatory peritoneal exudates of trout.

The cytochemical study of rainbow trout blood neutrophils and monocytes was done in leukocyte concentrated suspensions of blood collected from the caudal vein.

The results obtained led to the following **general conclusions**:

1- The non-stimulated peritoneal cavity of rainbow trout contains a **resident leukocyte population** consisting of lymphocytes, macrophages and neutrophils. Thrombocytes and eosinophilic granule cells (EGC) were only rarely observed. This leukocyte population is homogeneous for fish of the same size. Fish maintained at different temperatures (10°C, 15°C and 20°C) did not show any significant differences in their peritoneal cell population.

Some difficulties were found to reliably identify by light microscopy leukocytes present in the non-stimulated peritoneal cavity. As in mammals, the differentiation between small macrophages and large lymphocytes is often impossible by the simple cytological methods. The problem is further compounded by the silmutaneous

occurrence of cytochemical reactions in macrophages and some lymphocytes, as is the case with acid phosphatase and esterase. Ultrastructural observations suggest however that most of the small mononuclear cells which were difficult to identify could be small macrophages.

In the non-stimulated peritoneal cavity 1.3 to 2.2 % of leukocytes were found to be neutrophils. The distinction between neutrophlis and macrophages by light microscopy can be difficult since the nuclear shape of some macrophages is rather polymorphic. However, the clear distinction of these leukocytes was achieved by the peroxidase stain because neutrophils are the only cell type that have this cytochemical characteristic in the non-stimulated peritoneal cavity. The ultrastructural cytochemical analysis showed that all cytoplasmic granules of rainbow trout neutrophils contain peroxidase. [+]

Morphometric quantification of glycogen granules, applied to micrographs stained for peroxidase and glycogen, showed that glycogen granule numbers in resting peritoneal neutrophils were not different from those in blood neutrophils collected from non-injected fish.

Resident peritoneal macrophages have neither glycogen granules nor peroxidase activity in their nuclear cisterns or endoplasmic reticulum. These characteristics contrast with that of peritoneal resident macrophages in mammals.

Table 1

Cytochemical characteristics of rainbow trout peritoneal leukocytes

CHARACTERISTIC	MACROPHAGES		NEUTROPHILS		LYMPHOCYTES
	resting	inflamed	resting	inflamed	
peroxidase	-	- or +	+++	+++	-
alkaline phosphatase	-	-	++	++	-
acid phosphatase	+	+++	+	++	- or +
alpha-naphthyl esterase	++	+++	-	-	- or +
glycogen granules	-	+	+++	++++	ND

ND = not determined

[+] We found, by preliminary ultrastructural observations, not documented in the present thesis, that alkaline phosphatase activity is present in a vacuolar compartment distinct from the peroxidase-positive granular compartment. The alkaline phosphatase-positive vacuoles were found to be highly mobilizable by fusion with the cytoplasmic membrane with the consequent localization of the enzyme at the neutrophil surface.

The cytochemical characteristics we found for leukocytes in the peritoneal cavity of non-stimulated fish are shown in Table 1.

Contrary to what occurs in mammals, the rainbow trout neutrophilic peroxidase activity is resistant to low concentrations of cyanide. In this way, trout neutrophilic peroxidase resembles mammal eosinophilic peroxidase.

In inflammatory exudates the EGC only appeared occasionally and we did not find any evidence for phagocytic activity in this cell type. When EGC were present in fair numbers, their granules were seen dispersed extracellularly and inside macrophages.

EGC were round cells with central or eccentric round nuclei, with cytoplasm full of granules. When these cells were stained with the Wright's stain, their granules appeared red/orange. Cytochemically, EGC were peroxidase, esterase and acid phosphatase-negative.

2 - Contrary to what is described for mammals, we found that **trout peripheral blood monocytes** are peroxidase negative.

3 - The inflammatory peritoneal response was studied after intraperitoneal injection of various phlogistic agents -both bacterial and non bacterial- already referred to above.

The characterisation of the two phagocytes in the inflamed peritoneal cavities raised new problems in comparison to the study of the cavities at rest. One of those problems is due to the occurrence of peroxidase activity in some inflammatory macrophages making impossible, therefore, the absolute identification of the neutrophils by this cytochemical reaction. In order to solve this problem, double labelling of peritoneal leukocytes by ultrastructural cytochemical techniques was used. That labelling showed that neutrophils were peroxidase-positive and esterase (alpha-naphthyl butyrate)-negative, whereas the peroxidase-positive macrophages were esterase-positive. Furthermore, we applied, for the first time, the double ultrastructural labelling of peroxidase and glycogen and of esterase and glycogen. These techniques showed that the neutrophils were peroxidase-positive, esterase-negative and very rich in glycogen granules while the peroxidase positive macrophages were esterase-positive and with fewer glycogen granules.

The cytochemical characteristics we found for the inflammatory peritoneal macrophages and neutrophils of the rainbow trout are summarised in Table 1.

4 - The study of **glycogen** of phagocytes of rainbow trout labelled by specific methods showed that this reserve molecule has a type β granular structure, with a diameter of 35.72 $\pm$ 3.20nm, being similar to the glycogen granules of mammalian neutrophils and muscle. We found that the number of glycogen granules in peritoneal

neutrophils increased significantly in the inflammatory situations. That increase was greater in cases in which inflammatory peritoneal neutrophils were recruited to ingest bacteria or IFA. In the case of peritoneal hyperinflammation, the glycogen granule content of blood neutrophils was not increased as compared to neutrophils of non-injected trout. This indicates that the increase in the content of this reserve molecule occurs after neutrophils leave the blood stream to accumulate in the inflamed area.

There was no evidence that the glycogen of the neutrophils which ingested IFA or bacteria was utilised in the respiratory burst. This observation can be explained by the occurrence of concentrations of glucose in the inflamed peritoneal cavities similar to those of peripheral blood.

Evidence was found of the transfer of glycogen from the neutrophils to the macrophages in the inflamed peritoneal cavities.

5 - With the exception of the response to the injection of virulent *R. salmoninarum*, the **kinetics** of the influx of the two phagocytes during inflammation was characterised by a very early marked rise in the number of neutrophils followed by a slower rise in the number of macrophages.

The magnitude of the inflammatory response of these two phagocytes was dependent on the type of stimulus.

During the peritoneal inflammatory response the neutrophil numbers can increase around 500 times above the baseline in 24-48 hours, becoming the most abundant leukocyte.

The slower recruitment of macrophages attained maximum values 2 or 3 days later as compared to neutrophils. The increase in macrophage numbers was slower than with neutrophils, attaining in extreme cases about 7.5 times the value found at rest.

In transitory inflammation (induced by casein or by *Y. ruckeri* of low virulence or by formol-killed *Y. ruckeri*), the normalisation of the values of neutrophil numbers occurred 15 days after the intraperitoneal injection and at 8 days those values were already close to normal. When the inflammation was persistent, the neutrophilia was prolonged for much more time, as was the case with the injection of IFA -a mineral oil difficult to degrade- when the neutrophil recruitment was maintained for 20-30 days.

With the injection of a virulent strain of *R. salmoninarum* which produces persistent infection to the end of the study (60 days), we found a sustained neutrophil response up to that time. On the contrary, with the non-virulent renibacteria the number of peritoneal neutrophils were in marked decline 15 days after the injection and close to normal at 30 days.

6 - During the inflammatory response to the intraperitoneal injection of *Y. ruckeri*

in rainbow trout, the degree of **phagocytosis** by the two phagocytes is dependent on the access of the phagocytes to the microrganisms. After the injection of bacteria in the peritoneal cavity at rest, the largest amount of the inoculum (when this contains less than 10^8 bacteria in the case of *Y. ruckeri*) was ingested by the resident macrophages; the neutrophils which meanwhile have arrived in the peritoneal cavity do not find free bacteria in significant quantities.

This result shows that at the beginning of the infection the macrophage is the principal phagocyte. On the contrary, when the bacteria were injected into the peritoneal cavity with large numbers of neutrophils (induced by previous injection of casein or bacteria) the level of phagocytosis by neutrophils was elevated.

7 - The inflammatory response to intraperitoneal injection of rainbow trout with virulent *R. salmoninarum* showed peculiar characteristics which have not been previously seen with any other bacteria already tested, whether in mammals or fish: the response to the injection with a virulent strain was not accompanied by the usual early sharp peak of neutrophils, but, instead, after 4 days, a chronic inflammatory response developed with an increase of neutrophils to a high plateau which remained up to 2 or 3 months later, when the fish started to die. On the other hand, the i.p. injection of renibacteria with reduced virulence produced a transitory neutrophil response with an early neutrophil peak.

8 - The differential count of leukocytes present in the peritoneal cavity during inflammation, showed that the number of neutrophils present in the area is an adequate measure of the intensity, progression and resolution of the inflammation.

9 - The occurrence of **apoptosis in the neutrophils** was observed in the inflammatory peritoneal exudates of rainbow trout and sea bass. That apoptosis was seen to be accompanied by the production and release of apoptotic bodies. Those sub-cellular neutrophilic structures rich in peroxidase-positive granules were also seen inside the macrophages.

Preliminary experiments showed that extensive apoptosis of neutrophils occurred when inflammatory peritoneal exudates of rainbow trout were incubated *in vitro* in cell culture medium. Apoptosis was accompanied by an extensive production of apoptotic bodies. Both neutrophils and neutrophil apoptotic bodies were ingested by the macrophages present in the leukocyte suspensions.

These are the first references to apoptosis in the neutrophils of fish.

10 - Difficulties were met in differentiating and quantifying leukocytes, namely neutrophils, in peritoneal exudates harvested after the first week of infection with a virulent strain of *R. salmoninarum,* because of the occurrence of extensive leukocyte

clumping. This prompted us to look for another method to quantify neutrophils. Given that in trout the neutrophil is the only leukocyte with endogenous peroxidase activity, the quantitative evaluation of the neutrophil influx was accomplished by the **quantification of peroxidase activity.** This spectrophotometric method, which uses tetramethylbenzidine, when applied to a dispersed leukocyte suspension, showed a very good correlation between neutrophil counts and peroxidase activity. The application of this method to the study of the neutrophil response to intraperitoneal injection of virulent renibacteria showed the occurrence of a sustained neutrophil influx from the first 8 days up to 2 months.

11 - In addition, the quantification of peroxidase activity in peritoneal exudates, allowing the discrimination between intra- and extracellular peroxidases, showed that the enzymatic activity is predominantly intracellular. Extracellular activity was undetectable in the peritoneal cavities of non-stimulated trout and attained a maximum of only 5 % of total peroxidase in peritoneal exudates collected 24 hours after infection with live *Y. ruckeri* and regressing once again to very low levels in the latter period of infection.

12 - The quantitative method for peroxidase activity also permitted the quantification of the peroxidase per neutrophil (PAN) in various situations (at rest and during peritoneal inflammation). The values of PAN were similar for neutrophils from peripheral blood and from the peritoneal cavity of non-injected trout, indicating that the neutrophils present in the cavities of naive trout were not stimulated. The injection of a small dose of live *Y. ruckeri* of low virulence or of formol-killed *Y. ruckeri*, induced a transitory inflammation during which the peroxidase activity per neutrophils (PAN) showed only small variations. An increase at 24-48 hours relative to the values obtained for the neutrophils from peripheral blood or from the peritoneal cavity at rest, was followed by a progressive reduction after three or four days. The increase observed during the first days appears to be due to the presence of peroxidase in the inflammatory macrophages. The decrease in the values of peroxidase activity that occurred later on appears to be due to a loss of enzyme activity in the neutrophils. In the case of an attenuated neutrophil response, characteristic of the first days after i.p. injection of the virulent strain of *R. salmoninarum*, the values of PAN did not show increases similar to those found in the inflammation induced by *Y. ruckeri*.

13 - In the inflammatory peritoneal exudates in which elevated numbers of neutrophils and macrophages coexist, transfer of neutrophils and neutrophil material to the macrophage was found. That transfer was detected by the presence inside macrophages of neutrophils sufficiently intact to be identified by the usual cytological methods and also by the presence in macrophages of peroxidase activity and glycogen granules. The transfer of neutrophil components to the macrophages was found to be due, also, to phagocytosis by the macrophage of apoptotic neutrophil bodies. The

occurrence of intercellular junctions of the gap-junction type, observed now for the first time, between neutrophils and macrophages in inflammatory peritoneal exudates seems to represent a novel mechanism of transfer of neutrophilic material to the macrophage.

The observed phagocytosis of neutrophils and neutrophil apoptotic bodies containing bacteria represents a mode of transfer to the macrophage of bacteria initially ingested by neutrophils.

Aspects of macrophage/neutrophil interaction, similar to those described above for rainbow trout, were observed in peritoneal exudates of sea bass injected with bacteria by the intraperitoneal route.

In summary, the present dissertation contributes to the advancement in the knowledge of the mechanisms of defence against infections in fish, namely with respect to the role of the two professional phagocytes, the neutrophils and macrophages.

Since the phagocyte response to inflammation in the peritoneal cavity is representative of that seen in tissues, we can conclude that, in rainbow trout: i- in normal tissues and body cavities, macrophages are the resident phagocytes and the neutrophils are rare; ii- in areas of infection, numerous neutrophils are rapidly recruited from the reserve pools peaking at 24-48 hours after the beginning of the infection. As a constant resident phagocyte of the tissues, the macrophage is the first phagocyte to encounter and phagocytose invading organisms, when these breach the natural barriers. Interestingly, the neutrophils are attracted very early and in large numbers to infectious foci even when there are no microrganisms to ingest. This observation points to the existence of non-phagocytic functions in neutrophils.

In the rainbow trout, neutrophils are the only leukocytes with endogenous peroxidase activity. Because of this, the observed presence of peroxidase in macrophages from peritoneal cavities of trout inflamed by phlogistic agents, both bacterial and non-bacterial, represents an acquisition by macrophages of neutrophilic components. That transfer of neutrophilic materials to the macrophage was seen to involve mechanisms similar to those described in mammals, namely phagocytosis of neutrophils and of neutrophilic apoptotic bodies. Phagocytosis of neutrophil granules and endocytosis of free neutrophil molecules may be another mechanism for the transfer. The mobilization of neutrophil molecules to the macrophage through the intercellular junctions, now described in adhering neutrophils and macrophages, may represent a novel mechanism of transfer. The now observed transfer to macrophages of neutrophils, or neutrophil apoptotic bodies, containing bacteria represents a mechanism of transfer to the macrophage of bacteria initially phagocytosed by neutrophils.

Taken together, the above summarised results show that, in rainbow trout, the two professional phagocytes, neutrophils and macrophages, display patterns of localization, mobilization and interaction similar to those previously described for mammals.

Additional studies are necessary to disclose whether the same occurs in other levels of biological organization.