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The production of a macrophage-activating factor from rainbow trout *Salmo gairdneri* leucocytes

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SUMMARY

Rainbow trout head kidney and blood leucocytes are shown to be capable of secreting a soluble macrophage-activating factor (MAF) after stimulation with concanavalin A (Con A). The presence of phorbol myristate acetate (PMA) as a co-stimulant increased the production of MAF. Both respiratory burst activity (nitroblue tetrazolium, NBT, reduction and H_2O_2 production) and bactericidal activity were enhanced after incubation of resident or elicited macrophages with the MAF-containing supernatants for 48–72 hr. The target culture period before the addition of MAF did not affect their resposiveness, but a continuous presence of MAF was necessary for maximal stimulation.

INTRODUCTION

Soluble mediators released from T lymphocytes, collectively called lymphokines, have important amplifying/regulatory effects upon the immune response. Yet the phylogeny of lymphokine production has received little attention in comparison with the phylogeny of immunoglobulins or allograft rejection in the vertebrates. That fish produce antibodies and reject grafts has long been interpreted as representing the earliest manifestations of a co-ordinated humoral and cell-mediated immune system. So it might be anticipated that fish will also produce and secrete a range of regulatory lymphokines during their immune responses.

Most studies investigating lymhokine production in fish have looked at the production of a leucocyte migration-inhibition factor from antigen-sensitized leucocytes (Timur, 1975; Jayaraman, Mohan & Muthukkaruppan, 1979; Smith, McCarthy & Paterson, 1980; McKinney, McLeod & Sigel, 1981; Secombes, 1981), using the capillary tube technique. These studies have shown a positive inhibitory effect upon leucocyte migration but failed to show that soluble mediators are involved and to identify positively the target cells. Several recent studies, however, have demonstrated convincingly that fish leucocytes can release soluble factors that have effects upon particular types of leucocytes. Supernatants from mitogen or alloantigen mixed leucocyte reaction (MLR)-stimulated leucocyte cultures have a growth-promoting activity on purified phytohaemagglutinin (PHA)-activated lymphoblasts but not virgin leucocytes (Caspi & Avtalion, 1984; Grondel & Harmsen, 1984), analogous to the effect of IL-2, suggesting that interleukins exist in fish. Similarly supernatants from mitogen and specific antigen-stimulated leucocytes have been shown to be abe to attract leucocytes (Howell, 1987) and to increase the spreading, vacuolation and adherence of purified macrophages in culture (Smith & Braun-Nesje, 1982; Secombes, 1987). These latter findings were particularly interesting as fish macrophages can be activated *in vivo* to give an enhanced bactericidal activity (Olivier, Eaton & Campbell, 1986; Chung & Secombes, 1987), but there have been no studies of the production of a macrophage-activating factor (MAF) in fish. This prompted the present study to investigate if such supernatants possess the ability to activate fish macrophages *in vitro*, as determined by their ability to increase the release of reactive oxygen species and to increase the bactericidal activity of rainbow trout macrophages in culture.

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MATERIALS AND METHODS

The production of lymphokine (MAF)-containing supernatants

Lymphokine (LK)-containing supernatants were prepared initially as described by Secombes (1987). Rainbow trout *Salmo gairdneri* Richardson blood or head kidney (HK) cell suspensions were diluted in L-15 medium (Gibco, Paisley, Renfrewshire) containing 10 μ/ml heparin, 1% penicillin/streptomycin (P/S) and 10% fetal calf serum (FCS) and layered over 51% Percoll density gradients. After centrifugation at 400 *g* for 40 min at 4°, the leucocytes were removed from the Percoll medium interface and a viable cell count made using 0·1% trypan blue. Tissue culture flasks (25 cm^2) were then seeded with $1{\cdot}25 \times 10^7$ or $2{\cdot}5 \times 10^7$ leucocytes in a total of 5 ml L-15 medium (i.e. $2{\cdot}5 \times 10^6$ or 5×10^6 cells/ml) containing 1% P/S, 5×10^{-5} M 2-mercaptoethanol (2-ME), and the cells pulsed for 3 hr at 18° with concanavalin A (Con A) ranging from 5 to 20 μg/ml. During this period the lymphocytes became slightly adherent (Pick & Kotkes, 1977), enabling the removal of the supernatants containing Con A without disturbing the cells, and the mono-

layer was then gently washed five times with sterile phosphate-buffered saline (PBS) to remove any residual Con A. After the final wash, the monolayers were supplemented with L-15 medium containing 1% P/S, 10% FCS and 5×10^{-5} M 2-ME and cultured for 24 hr or 48 hr before harvesting the supernatants. The supernatants were stored at −20° until use. Such supernatants possessed undetectable amounts of Con A as determined by haemagglutination (Secombes, 1987). Supernatants collected from leucocytes that had not been pulsed with Con A but were cultured for the appropriate time were used as controls.

Phorbol esters are known to enhance LK (IL-2) production in fish (Caspi & Avtalion, 1984) and interferon-gamma (IFN-γ) (MAF) production in mammals (Yip *et al.*, 1981, 1982) and so phorbol myristate acetate (PMA; Sigma) was used as a co-stimulant to attempt to increase the production of a macrophage-activating factor in this study. Various concentrations of PMA were used, ranging from 1 to 1000 ng/ml, and were added at the same time as Con A at 10 μg/ml. After the 3-hr pulse in serum-free medium, the monolayers of leucocytes were washed five times with PBS to remove residual Con A and PMA. These supernatants were collected and stored as for the Con A-stimulated cultures.

Detection of MAF-containing supernatants

Elicited or HK macrophages from rainbow trout were prepared as described previously (Chung & Secombes, 1987, 1988). Elicited cells were collected from the peritoneal cavity of fish that had received an i.p. injection of Freund's incomplete adjuvant (Difco, Detroit, MI) 10 days earlier. HK leucocyte suspensions were enriched for macrophages using a 34–51% Percoll density gradient. In both cases the macrophages were then purified by adherence to microtitre plates for 2 hr, followed by removal of the non-adherent cells. The macrophages were then cultured overnight before addition of the supernatants unless otherwise stated. LK-containing and control supernatants were normally diluted 1:4 or 1:8 in L-15 medium, 1% P/S, 10% FCS and 5×10^{-5}M 2-ME before addition to the target macrophages, although due to batch variation it was found to be necessary to determine the optimal dilution of each LK-containing supernatant using serial dilutions from 1:2 to 1:512 in the NBT assay (see below). The target macrophages were incubated with the supernatants for 24–72 hr, or for 72 hr with a change of supernatant after 48 hr. In one experiment pulse exposure of the macrophages to the supernatants was carried out for comparison with continuous exposure. Here the macrophages were incubated with the supernatants for 4 hr, 24 hr or 48 hr. After the 4-hr and 24-hr pulse the supernatants were removed and the macrophages washed and cultured for 44 hr and 24 hr, respectively (i.e. 48 hr in total) before being assayed.

Following incubation with the supernatants assays for respiratory burst or bactericidal activity were performed. The reduction of nitroblue tetrazolium (NBT; Sigma) and the horseradish peroxidase-dependent oxidation of phenol red, induced by stimulation of the macrophages with PMA, were used to measure the production of superoxide anion and hydrogen peroxide, respectively (Chung & Secombes, 1988). The macrophages were covered with 100 μl of 1 mg/ml NBT or 0·2 mg/ml phenol red solution and incubated for 30 min in the presence of 1 μg/ml PMA. For the NBT assay the reaction was stoppped by fixing the cells with methanol, and after washing in 70% methanol the reduced formazan was solubilized in KOH/DMSO (Sigma) and the optical density determined in a multiscan spectrophotometer at 620 nm. The phenol red assay was stopped by the addition of 10 μl 1 N NaOH and the optical density determined as for the NBT assay. The bactericidal assay was carried out as described by Graham, Jeffries & Secombes (1988) using two strains of the fish pathogen *Aeromonas salmonicida*; the relatively non-virulent strain 004 (Ellis, Burrows & Stapleton 1988) and the virulent strain 184 (Adams *et al.*, 1987). Briefly, the bacteria were diluted to a starting concentration of 10^8 cells/ml and serially diluted in tryptic soy broth (TSB; Difco) ready for use. After washing the macrophage monolayers to remove all traces of P/S, 20 μl of each dilution of bacteria were added to each well and the microtitre plate was then centrifuged at 150 *g* for 5 min. The macrophages were killed immediately by addition of 50 μl 0·2% Tween 20 in distilled water or were incubated for 5 hr at 18° to allow killing of the bacteria to occur before macrophage lysis. One-hundred microlitres of TSB were then added to each well to support an overnight (16 hr) growth of the surviving bacteria before addition of 10 μl 5 mg/ml MTT (Sigma, Poole, Dorset) and determination of the optical densities at 600 nm. The data were adjusted to give a killing index (KI) by dividing the readings from macrophages incubated for 5 hr with a particular concentration of bacteria (T_5h) by those from macrophages killed immediately. The lower the index below 1·0 the more bacteria (T_0h) had been killed.

RESULTS

Optimization of LK production

Both HK and blood leucocytes could be stimulated with Con A to release a lymphokine that significantly increased the reduction of NBT by HK macrophage targets. With HK supernatants (Fig. 1) it can be seen that stimulation of LK production was generally optimal with 10 μg/ml Con A and with the higher leucocyte number (5×10^6 cells/ml). In addition, supernatants from leucocytes incubated for 48 hr had consistently higher levels of activity than those from 24-hr cultures. The incubation time with the targets was also found to be important. Macrophages incubated continuously with the LK supernatants for 24 hr or 72 hr did not show a significant increase in NBT reduction compared with control supernatant-treated macrophages, whereas those incubated for 48 hr did. At 72 hr any increases may have been obscured by the relatively high control values seen at this time, typical of HK macrophages after several days of culture, although the levels of LK-treated cells were clearly lower than at 48 hr. However, in later studies (Fig. 2) it was found that the level of activity induced by 48 hr could be maintained or even increased at 72 hr by replacing the LK-containing supernatant after 48 hr. The length of time that the targets were in culture before addition of the LK was not so critical. In experiments where the supernatants were added to the macrophages immediately after removal of the non-adherent cells, no difference in the level of stimulation was seen compared with macrophages left in culture overnight before addition of the supernatants. Indeed, even macrophages in culture for 4 days before their use could be stimulated with the LK supernatants to give significantly increased NBT reduction. Essentially the same results as these were found when using the blood leucocyte supernatants.

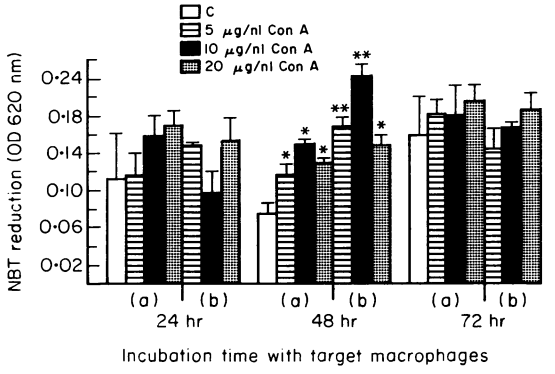

Figure 1. Reduction of NBT by HK macrophages in the presence of 1 μg/ml PMA, after incubation of the targets for varying times with 48-hr supernatants from HK leucocytes alone (C) or stimulated with different concentrations of Con A. The leucocytes were cultured at $2{\cdot}5 \times 10^6$ cells/ml (a) or 5×10^6 cells/ml (b). Results are means plus standard error of triplicate readings after 30 min. * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$ compared with the control.

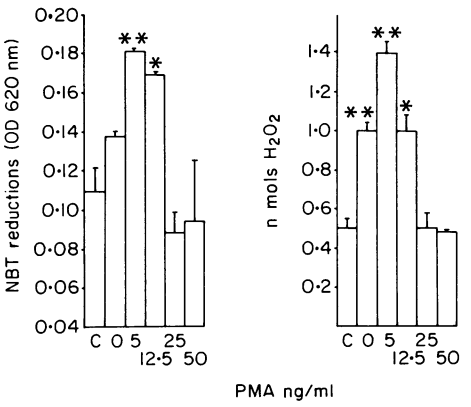

Figure 3. Reduction of NBT and production of H_2O_2 by HK macrophages in the presence of 1 μg/ml PMA, after a 48-hr incubation with 48-hr supernatants from HK leucocytes alone (C) or stimulated with 10 μg/ml Con A and varying concentrations of PMA. Results are means plus SE of triplicate readings after 30 min. * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$ compared with the control.

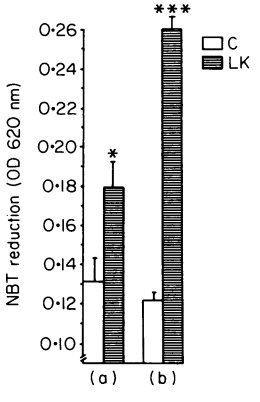

Figure 2. Reduction of NBT by HK macrophages in the presence of 1 μg/ml PMA, after incubation for 48 hr (a) or 72 hr (b) with control (C) or lymphokine (LK)-containing supernatants from 48-hr HK leucocyte cultures stimulated with 10 μg/ml Con A and 5 ng/ml PMA. The supernatants were changed at 48 hr in (b). Results are means plus SE of triplicate readings after 30 min. * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$ compared with the control.

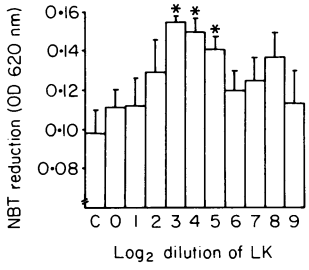

Figure 4. Reduction of NBT by HK macrophages in the presence of 1 μg/ml PMA, after a 48-hr incubation with control (C) or lymphokine (LK)-containing supernatants obtained as in Fig. 2. The LK supernatant was two-fold diluted from neat (0) to 1:512 (9). Results are means plus SE of triplicate readings after 30 min. * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$ compared with the control.

Stimulation of LK release was significantly higher using PMA with Con A compared with Con A alone (Fig. 3). The optimal concentration of PMA was 5 ng/ml for both the NBT and hydrogen peroxide assays. Acid phosphatase levels could also be significantly increased with LK supernatants from PMA/Con A-stimulated leucocytes but here the highest levels were noted at 25 ng/ml PMA (S. Graham, personal observation). Elicited macrophage targets assayed for NBT reduction in response to the LK supernatants responded in much the same way as the HK macrophages. One exception was that 24-hr supernatants collected from PMA/Con A-treated leucocytes were not stimulatory to elicited macrophages after 48 hr in culture, whereas they did cause a significant increase in NBT reduction with HK macrophages. Forty-eight-hour supernatants were stimulatory to both target types.

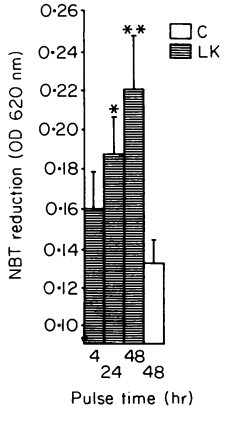

Figure 5. Reduction of NBT by HK macrophages in the presence of 1 μg/ml PMA, after 48 hr continuous exposure to control (C) or lymphokine (LK)-containing supernatants, or after a pulse exposure to LK for 4 hr and 24 hr followed by incubation in medium for 44 hr and 24 hr, respectively. C and LK supernatants were obtained as in Fig. 2. Results are means plus SE of triplicate readings after 30 min. * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$ compared with the control.

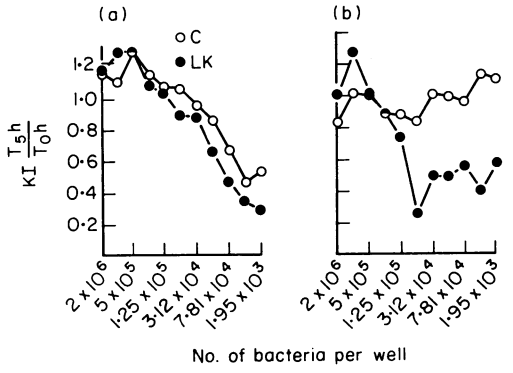

Figure 6. Bactericidal activity of HK macrophages after 48-hr exposure to control (C) or lymphokine (LK)-containing supernatants obtained as in Fig. 2. Macrophages were incubated with two-fold dilutions of *A. salmonicida* strain 004 (a) or strain 184 (b) for 0 hr and 5 hr and the results expressed as a killing index (T_5h MTT reduction/T_0h MTT reduction). The lower the killing index below 1·0 the more bacteria had been killed.

In order to ensure that this enhancement of macrophage stimulation was due to increased LK and not to contaminating PMA, a sample of LK-containing supernatant was dialysed overnight at 4° against PBS and filter sterilized. A non-dialysed but filter-sterilized sample of the same preparation was used as a positive control and a 5 ng/ml PMA solution was dialysed and filtered as a negative control. Activity was retained in the LK-containing supernatants after dialysis, whereas the dialysed PMA solution gave no significant increases after 48 hr incubation with HK macrophages.

A typical dilution curve for a LK-containing supernatant is shown in Fig. 4. In this sample the optimum dilution giving maximum stimulation for NBT reduction was found to be 1:8 and the activity being lost above 1:32. This test was performed for each batch of LK made before use in further assays.

Pulse exposure

In order to investigate if LK had to be present continuously for macrophage activation to occur, an experiment was carried out using pulsed exposures to the supernatants (Fig. 5). A 4-hr pulse followed by 44 hr in culture did not give a significant increase in NBT reduction. A 24-hr exposure to the LK supernatants followed by 24 hr in culture gave a small (just significant at the 95% probability level) degree of stimulation, but the largest increase was seen using continuous exposure to the LK for the duration of the experiment.

The effect of LK on the bactericidal activity of macrophages

The optimal conditions for the production and detection of LK, determined using the NBT assay, were used to investigate the effects of the supernatants upon the bactericidal activity of macrophage targets. Forty-eight-hour supernatants from PMA/Con A-stimulated leucocytes were incubated with HK macrophages for 48 hr prior to the assay. As can be seen in Fig. 6a, both LK-treated and control macrophages were able to effect killing of the relatively non-virulent strain of *A. salmonicida* (004), with increasing efficiency at lower bacterial concentrations. An increase in the killing ability of the LK-treated cells, as denoted by smaller KI values, was seen at most bacterial concentrations but this increase was not particularly large. However, when using the virulent strain 184 a marked difference between the killing ability of LK-treated and control macrophages was seen (Fig. 6b). Control supernatant-treated macrophages were generally unable to kill this strain (KIs of approximately 1·0), whereas LK-treated macrophages could effect substantial killing at bacterial concentrations below 5×10^5 bacteria per well. Normally about 10^5 macrophages are present per well so at this concentration there is about a 5:1 target to effector ratio.

DISCUSSION

Activated macrophages are defined functionally as cells having an increased abilty to kill micro-organisms and tumour cells, with the former being considered the most important functional characteristic (Nathan, 1986). The results from this study clearly show that rainbow trout leucocytes stimulated with Con A release a soluble factor that has this effect, enhancing the ability of macrophages to kill the fish bacterial pathogen *A. salmonicida*. Both an increased bactericidal activity for a relatively non-virulent strain and the ability to kill a virulent strain were acquired in the LK-treated macrophages. Therefore this LK is a macrophage-activating factor (MAF). That fish leucocytes do secrete a macrophage-activating lymphokine is not surprising in view of the previously observed *in vivo* activation of salmonid macrophages (Olivier *et al.*, 1987; Chung & Secombes, 1987).

MAF production was optimal using PMA as a co-stimulant with Con A, similar to that seen for mammalian MAF (IFN-γ) production. Macrophages incubated with MAF for 48 hr showed significantly increased respiratory burst activity (O_2^- and H_2O_2) as well as enhanced microbicidal activity. Such reactive oxygen species (ROS) have potent microbicidal activity (Babior, 1984; Lowrie, Jackett & Andrew, 1985) and the close correlation between the production of ROS by trout macrophages, especially of H_2O_2 (Chung & Secombes, 1987), and their ability to kill micro-organisms suggests that they also have an important role in the microbicidal mechanisms of fish phagocytes. Whether this increase in respiratory burst activity can be accounted for by an increase in the kinetic parameters of NADPH oxidase and elevated cellular levels of NADPH as seen in mammals (Adams & Hamilton, 1984) will be interesting to determine.

Longer culture (>48 hr) with MAF supernatants led to a decrease in macrophage activity unless the supernatants were changed. Similarly, in a morphological study we have shown that these supernatants significantly increase macrophage spreading and that a noticeable improvement in spreading is observed after 72 hr of culture if the medium is changed daily rather than being left for the entire period (Secombes, 1987). Possibly the MAF is exhausted from these supernatants by 48 hr or its activity wanes within this time.

The time in culture before addition of MAF did not have an effect upon the responsiveness of the macrophages to the LK, unlike some mammalian studies (Nacy *et al.*, 1984). However, a continuous presence of MAF was necessary to achieve maximal stimulation of the macrophages. This may reflect the activation of a greater number of macrophages or an increased activity of individual activated cells, possibly associated with a need for

multiple cycles of receptor–ligand complex internalization as noted for the induction of tumour cell killing (Evered, Nugent & O'Connor, 1986). Little difference in responsiveness to MAF was observed between resident (HK) and elicited macrophage targets using 48-hr leucocyte supernatants, but there was a suggestion that elicited macrophages were less sensitive to MAF than HK macrophages using 24-hr supernatants. These supernatants probably contained less MAF activity than 48-hr supernatants as they were less stimulatory to HK macrophages, although still able to cause a significant increase in NBT reduction. However, they were not stimulatory for elicited macrophages, possibly due to differential target sensitivity. This is in agreement with murine studies where microbicidal activity of resident macrophages is equal to or greater than that of elicited macrophages after LK treatment, dependent upon the pathogen used in the killing assay (Nacy *et al.*, 1984).

That lymphokines with the ability to affect both specific and non-specific effector functions exist in fish demonstrates the importance of such regulatory factors in immune responses throughout the vertebrates. Whether these factors have a common origin or are the result of convergent evolution in the different vertebrate classes awaits a more comprehensive analysis of the molecules from the lower vertebrates. To this end further studies are in progress to give a preliminary characterization of teleost MAF.

ACKNOWLEDGMENTS

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