Immunology 1984 **52** 477

Phylogeny of interleukins: growth factors produced by leucocytes of the cyprinid fish, *Cyprinus carpio* L.

J. L. GRONDEL & E. G. M. HARMSEN *Department of Experimental Animal Morphology & Cell Biology and Department of Animal Husbandry, Agricultural University, Wageningen, The Netherlands*

Accepted for publication 23 January 1984

Summary. Supernatants of phytohaemagglutinin (PHA)-activated pronephric leucocytes from carp (*Cyprinus carpio* L.) contain a lymphocyte growth factor which can induce a proliferative response of purified lymphoblasts but not freshly isolated leucocytes. The growth-promoting activity can be reduced by absorbing the supernatant with mitogen-activated blasts. In addition, increased incorporation of tritiated thymidine into PHA-activated blast cells is also induced by supernatants from two-way mixed leucocyte cultures. The data show that even at the evolutionary level of teleost fish, amplifying/regulatory leucocyte products exist. It is suggested that these factors play as important a role in the regulation of the immune response in fish as they do in mammals.

INTRODUCTION

Soluble factors formed by distinct populations of leucocytes play a pivotal role in the regulation of the immune response. In mammals, these regulatory products can be detected in supernatants of cultures of allogeneic leucocytes and mitogen-stimulated leucocytes (Gillis *et al.*, 1978; Andersson *et al.*, 1979; Miller *et al.*, 1980; Susskind & Faanes, 1981; Kern *et al.*, 1981). Depending on the assay system used, two factors, interleukin-l (IL-l) and interleukin-2 (IL-2) can be distinguished. IL-1 is a macrophage-derived product (Smith *et al.*, 1980) that, together with mitogen or antigen, triggers the T-helper cell to produce IL-2 (Smith *et al.*, 1980; Farrar *et al.*, 1980). IL-2 exerts its activity only on activated cells (e.g. blasts) and maintains their proliferative capacity (Smith *et al.*, 1979). IL-2 influences antibody responses to T-dependent antigens positively (Leibson, Marrack & Kappler, 1981) and supports the maturation of cytotoxic precursor cells into effectors (Shaw *et al.*, 1978; Wagner *et al.*, 1980).

Correspondence: Dr J. L. Grondel, Agricultural University, Dept. of Experimental Animal Morphology & Cell Biology, Zodiac, P.O. Box 338, 6700 AH Wageningen, The Netherlands.

Although there is a relatively great phylogenetic distance between mammals and fish, this latter group of ectothermic vertebrates is characterized by well-developed humoral and cellular (Corbel, 1975) immune systems. Thymus, spleen, pronephros and mesonephros are the major lymphoid organs; bone marrow and lymph nodes are lacking. In carp, the pronephros and mesonephros are the major sites of antibody formation. The spleen plays a minor role (Rijkers, Frederix-Wolters & van Muiswinkel, 1980). Despite our knowledge of many facets of the immune system of fish, the existence and the role of modulating interleukin-like factors of fish has not been examined.

The purpose of the present study was to trace the phylogenetic ancestry of such amplifying/regulatory factors by determining whether they are produced by fish leucocytes.

MATERIALS AND METHODS

Animals

Outbred carp, 6–8 months of age, were raised in our laboratory. They were kept in aquaria with aerated running tapwater at a temperature of $22 \pm 1°$. The animals were fed daily with pelleted dry food (K30, Trouw and Co, Putten, The Netherlands) by means of a 'Scharflinger' automatic feeder.

Cell cultures

Standard culture medium (RPMI-1640, Flow, Irvine, Scotland), diluted to carp tonicity (270 mosmol), was buffered with 2·1 g/litre $NaHCO_3$ (pH 7·4) and was supplemented with 2 mM L-glutamine (Merck, Darmstadt, F.R.G.), 100 μg/ml Streptomycin (Serva, Heidelberg, F.R.G.) and 100 IU/ml penicillin-G (Serva). This standard medium was further supplemented with 10% heat-inactivated pooled carp serum (PCS). Pronephric leucocytes were prepared as described earlier (Grondel & Boesten, 1982). In brief, MS-222 (Sandoz, Basel, Switzerland)-anaesthetized animals were bled before dissection to reduce contamination of the leucocyte suspensions with erythrocytes. Single cell suspensions were obtained by teasing the tissue (in serum-free culture medium) through a nylon sieve. The cells were washed twice in ice-cold culture medium without serum and cell viability was assessed using the trypan blue exclusion method. Cells were cultured in round-bottom microtitre plates (M24 ART, Greiner, Nürtingen, F.R.G.) in a volume of 0·2 ml per well. Triplicate cultures were incubated at 29° in a humidified atmosphere of 5% CO_2 plus 95% air. DNA-synthesis was assayed by [^{3}H]-thymidine ([^{3}H]-TdR) incorporation after a 16 hr pulse with 0·4 μCi [^{3}H]-TdR per culture (specific activity 5 Ci/mmol, Radiochemical Centre, Amersham, U.K.). Cells were harvested onto glass-fibre strips (Bioproducts, Walkersville, U.S.A.) using a Multiple Automated Sample Harvester (Mash II, Dynatech, Nürtingen, F.R.G.). The filters were dried and radioactivity was determined by scintillation spectrometry. All results are expressed as the arithmetic mean c.p.m. $\pm$ SD observed in triplicate cultures.

Production of active supernatants

Active supernatants (ASN) were obtained by incubating a 5 ml pronephric leucocyte suspension (5×10^6 cells per ml culture medium) in 25 cm^2 tissue culture flasks (no. 3050, Costar, Cambridge, U.S.A.) for 3 hr in the presence of 50 μg/ml phytohaemagglutinin (PHA-P, Difco, Detroit, U.S.A.). After this incubation at 29°, the mitogen-containing medium was discarded and the adherent monolayer of cells was rinsed three times with fresh medium at room temperature. Subsequently, the mitogen-activated cells were cultured for another 20 hr with 5 ml culture medium. The supernatants were collected, centrifuged at 1000 *g* for 20 min at 4°, sterilized by filtering through 0·2 μm membrane filters (Gelman, Michigan, U.S.A.) and stored at $-20°$. Control supernatants (CSN) of non-mitogen-treated leucocyte cultures served as controls. An IL-2-containing supernatant of a mouse cell line (EL-4) was kindly provided by Dr W. A. Buurman, Department of Surgery, Biomedical Centre, State University of Limburg, The Netherlands.

Preparation of blasts and assay for growth activity

In previous studies, blasts were characterized by their change in density and size during the mitogen activation relative to non-mitogen-treated control cells, using continuous Percoll gradients and staining techniques (A. van den Ouweland, personal communication). In addition, pronephric leucocytes ($2{\cdot}5 \times 10^7$ cells) were cultured for 4 days in 25 cm^2 culture flasks (Costar) in a total volume of 5 ml culture medium supplemented with 50 μg/ml PHA. After the incubation period, the PHA-activated cells were mechanically agitated by pipette, harvested and washed twice. In addition, the cell suspension was layered over a Percoll cushion (Pharmacia, Uppsala, Sweden) with a density of 1·055 g/ml and centrifuged for 20 min at 800 *g* at 4°. Blasts, collected from the interface, were washed twice, resuspended in culture medium and diluted in the same medium to the appropriate cell concentration. These cells were then subcultured in the presence of ASN, CSN, mitogen or medium only. Growth activity was determined by [^{3}H]-thymidine uptake (see above section on 'Cell cultures').

RESULTS

The assay for growth factor activity of a given supernatant was based on the ability of a supernatant to promote growth of blasts in the absence of mitogen. Due to their active state, freshly isolated PHA blasts incorporated significant levels of tritiated thymidine. Therefore, the number of blast cells per well was titrated so as to provide an assay population that by itself, only incorporated low levels of thymidine. Cultures that contained 1×10^5 or $0{\cdot}5 \times 10^5$ blasts

incorporated significant levels of tritiated thymidine on days 1 and 2 of culture and significantly reduced levels on days 3–6. Cultures that contained $2{\cdot}5 \times 10^4$ blasts, however, incorporated only low levels of thymidine on all days tested. Therefore, this cell number was used in the assay throughout this study.

Supernatant fluids were added at the start of the blast cell culture. Maximal activity of the supernatants, at 50% final concentration (0·1 ml cell suspension and 0·1 ml supernatant), progressively declined upon serial dilution (data not shown). For this reason growth activity found at 50% supernatant concentration is presented for all experiments.

Supernatants of cultures of pronephric leucocytes that had been incubated with PHA for only 3 hr maintained proliferative activity of PHA blasts for at least 5 days. Figure 1 presents results from one of two experiments that involved two different supernatants. In these experiments, both supernatants of non-mitogen-treated leucocyte cultures as well as fresh medium served as controls. Figure 1 shows that both types of control cultures incorporated only low levels of tritiated thymidine during the experiment. PHA by itself was mitogenic for blasts, but significantly less so than the active supernatant. Based on these kinetic data and to increase the sensitivity to different supernatant fluids, blasts were harvested in the logarithmic-phase (on day 3 and/or 4 of culture) in the subsequent experiments rather than on day 5.

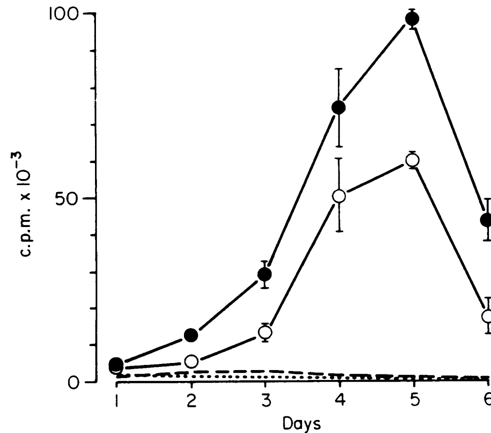

Figure 1. Kinetics of thymidine uptake into blast cells. Active supernatant (ASN) of PHA-activated pronephric leucocytes was tested on $2{\cdot}5 \times 10^4$ blasts per well at 50% final concentration (0·1 ml cell suspension and 0·1 ml ASN). PHA responsiveness was detected by culturing blast cells in the presence of a control supernatant (CSN) of non-mitogen-treated leucocyte culture supplemented with PHA (final concentration 50 μg/ml). Blasts cultured in CSN or medium only served as controls. ASN (●); CSN+PHA (○); CSN (– – –), fresh medium (· · · · ·).

Results suggest that leucocyte-formed growth factors, rather than any contaminating PHA, were responsible for the continued proliferation of PHA blasts. Titration experiments revealed that PHA agg-

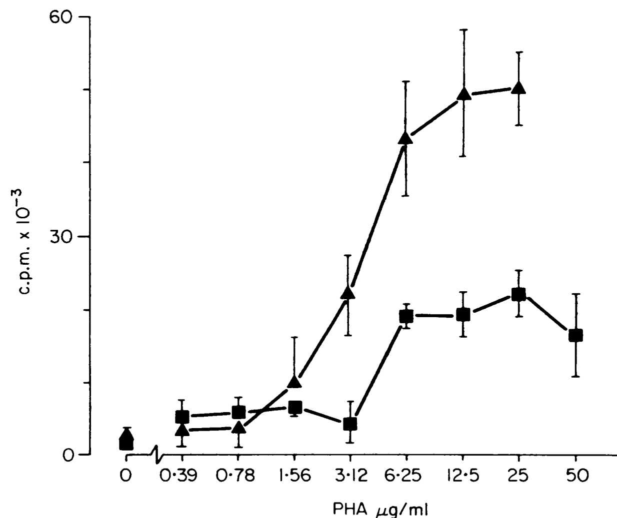

Figure 2. Proliferative response of blasts to various concentrations of PHA. Blast cells ($2{\cdot}5 \times 10^4$ per well) were cultured for 3 (■) and 4 (▲) days. Controls were cultured in medium without PHA.

lutinates sheep erythrocytes and is mitogenic for blast cells at concentrations higher than or equal to 0·39 μg/ml (mitogen titration in Fig. 2; agglutination data not shown). Since the four supernatants were neither haemagglutinating nor mitogenic for virgin pronephric leucocytes, any PHA contained in the supernatant was below the concentration detectable by these assays. As seen in Fig. 3, substances generated and released into the supernatant fluid during 24 and 48 hrs two-way mixed leucocyte cultures (MLC) also contained growth factors as assayed with PHA blasts. No mitogens were added to such cultures. It should be noted that MLC-generated supernatant fluid substances reported in Fig. 3 effected a 2·7-fold (24 hr MLC-supernatant) or 4·3-fold (48 hr MLC-supernatant) enhancement of tritiated thymidine incorporation compared with medium controls. Although significant, this increase was less than that seen for PHA-generated growth factors. The mixed leucocyte cultures were harvested on day 6 and stimulation indices around 40 and more were recorded. However, the highest levels of PHA stimulation are already achieved on the fourth day of culture. Therefore, it is conceivable that the difference between the growth activities can be explained by the polyclonal abilities of PHA. In contrast, an IL-2-containing supernatant of a mouse cell line (EL-4) was not able to activate carp blasts, indicating that these factors are probably specific for certain vertebrate groups.

Several observations suggest that blasts are the preferential targets of the growth factors. (i) The PHA-generated supernatants were mitogenic when added to PHA blasts. However, they were not stimulatory when added to cultures of freshly harvested (virgin) pronephric leucocytes (Fig. 4b). (ii) When an active supernatant supplemented with PHA (final concentration 50 μg/ml) was added to either virgin leucocytes or to PHA blasts, there was hardly any stimulation beyond that noted for the addition of PHA or supernatant separately (Fig. 4a). Adsorption

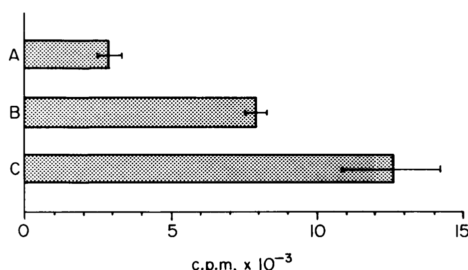

Figure 3. Proliferation of mitogen-activated blast cells in response to factors present in supernatant of a two-way mixed leucocyte culture. Pronephric leucocytes ($1{\cdot}25 \times 10^7$ cells) from each animal were cultured in 25 cm^2 flasks in a total volume of 5 ml culture medium. After 24 hr (B) and 48 hr (C) supernatants were harvested, centrifuged, filter-sterilized and tested at a final concentration of 50%. Control blasts were incubated with medium only (A). Cultures were harvested on day 3.

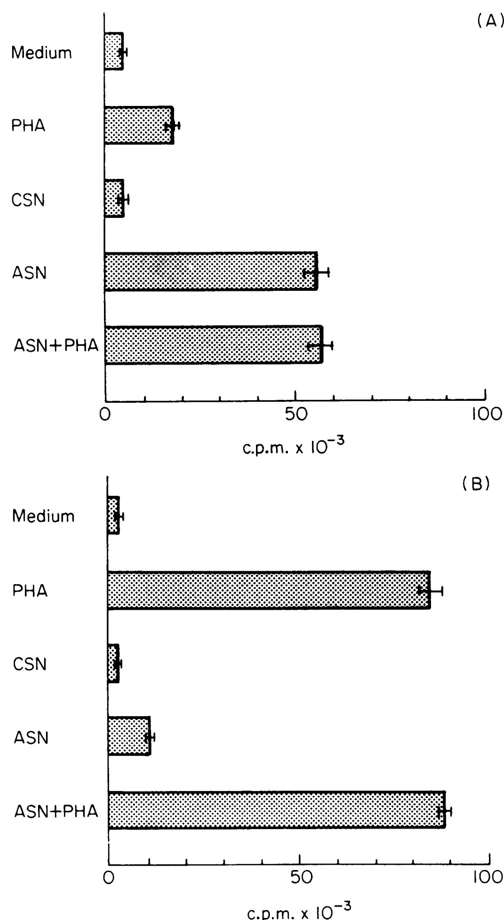

Figure 4. Effect of (active) supernatant (ASN) of PHA-stimulated pronephric leucocytes and control supernatants (CSN) or non-mitogen-treated leucocytes. Blast cells ($2{\cdot}5 \times 10^4$ per well, A) or virgin pronephric leucocytes ($2{\cdot}5 \times 10^5$ per well, B) were incubated for 3 (A) or 4 (B) days, respectively with medium only or medium supplemented with PHA (50 μg/ml), 50% CSN, 50% ASN or 50% ASN+PHA.

of a supernatant by PHA blasts reduced thymidine uptake by blasts by 36–48% in two experiments involving different supernatants. In contrast, incubation of one of these supernatants with virgin leucocytes did not significantly reduce the activity (Fig. 5).

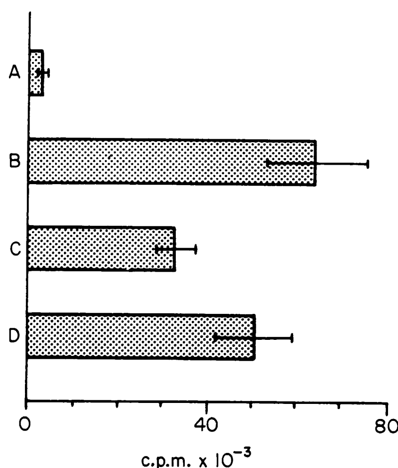

Figure 5. Effect of adsorption of growth promoting supernatants by PHA-stimulated blasts or virgin pronephric leucocytes. A cell-free supernatant (0·5 ml) was adsorbed by $2{\cdot}5 \times 10^7$ blasts (C) or by $2{\cdot}5 \times 10^7$ freshly prepared pronephric leucocytes (D) and was tested at a final concentration of 50% on $2{\cdot}5 \times 10^4$ blasts per well, cultured for 3 days. Adsorption was carried out by incubating the cells for 4 hr at 4°. The adsorbed supernatants were filter-sterilized after removing the cells by centrifugation. Blasts incubated with medium only (A) or with unadsorbed supernatant (B) served as controls. *P* value: for comparison between B and C, $<0{\cdot}01$; for B and D, not significant.

DISCUSSION

The study presented here was designed to investigate whether fish leucocytes form growth factors in response to mitogenic or allogeneic stimulation analogous to mammalian interleukins. For the production of the growth factor (or factors), a modification of the method described by Miller *et al.* (1980) for mice was used. Our procedure yields an active supernatant fluid free of mitogenic amounts of PHA without further processing. Freedom from mitogenic activity was determined by freshly isolated pronephric leucocytes being unable to respond to the active supernatant. This observation indicates both the absence of appropiate PHA concentrations as well as the inability of resting cells to respond to factors which sustain blast cell growth. The main problem in the procedures for the production of IL-2 in mammals is the presence of the lectin in the preparation and its possible interference with the growth-promoting activity. It was demonstrated that high levels of IL-2 were obtained by short-term treatment of murine and human lymphocytes with PHA or concanavalin A (Con A) (Spiess & Rosenberg, 1981; Wu *et al.*, 1982). Washing the IL-2-producing cells reduced the lectin concentration to levels which were insufficient to stimulate freshly isolated lymphocytes (Spiess & Rosenberg, 1981). Only activated lymphocytes were susceptible to IL-2.

In our experiments, supernatant fluids without mitogens were obtained by culturing allogeneic-stimulated carp leucocytes. The MLC-generated supernatant also contained a growth-promoting activity, although its activity was less compared to supernatants derived from PHA-stimulated carp leucocytes. It is conceivable that this difference relates to the polyclonal activation abilities of PHA.

For the detection of growth-promoting factors in supernatants lectin-activated blasts were used as indicator cells. Proliferation of those activated cells could be maintained for several days by addition of the growth factor. However, the indicator cells were still sensitive to PHA. Freshly isolated mouse T cell blasts also show a lectin responsiveness, but after repeated subculture those blasts become totally unresponsive to the mitogen used (Andersson *et al.*, 1979). At that time, their continuous proliferation will be dependent on the presence of IL-2.

The results of the adsorption experiments show that PHA-activated carp blasts can remove the growth factors from supernatants to a certain extent, suggesting the existence of receptors on these cells. This observation is consistent with the current view that only activated cells actually can adsorb IL-2 (Coutinho *et al.*, 1979; Kern *et al.*, 1981), whereas freshly isolated leucocytes remove only small amounts from the supernatants (Smith *et al.*, 1979).

Recently, factors with IL-2 activity have been reported for chickens (Schauenstein, Globerson & Wick, 1982). From a phylogenetic point of view, it was interesting to note the lack of any mutual cross-reactivity between murine and avian growth factors. Within mammals the absence of species specificity is reported (Gillis *et al.*, 1978; Coutinho *et al.*, 1979; Gillis, Smith & Watson, 1980). Mouse, rat and human IL-2 prep-

arations show identical biological activity when tested with a mouse assay system (Gillis *et al.*, 1980). On the other hand, the proliferation of human cells showed a preference for a homologous growth factor (Lafferty, Andrus & Prowse, 1980).

The results presented in this paper demonstrate that carp pronephric leucocytes produce soluble factors upon mitogenic or allogeneic stimulation. The abilities of these factors strongly resemble those of mammalian IL-2. However, the homology between these substances has yet to be determined biochemically. Whether the presence of macrophages and/or a soluble factor produced by these cells are required for the stimulation of carp leucocytes is under investigation.

ACKNOWLEDGMENTS

Prof. N. Cohen (Department of Microbiology, Division of Immunology, University of Rochester School of Medicin and Dentistry, Rochester, U.S.A.), Dr W. B. van Muiswinkel and Dr E. Egberts are gratefully thanked for their stimulating interest and advice during the performance of this study and preparation of the manuscript. We thank Mr W. J. A. Valen for drawing the illustrations.

REFERENCES

ANDERSSON J., GRÖNVIK K.O., LARSSON E.L. & COUTINHO A. (1979) Studies on T lymphocyte activation. I. Requirements for the mitogen-dependent production of T cell growth factors. *Eur. J. Immunol.* **9,** 581.

CORBEL M.J. (1975) The immune response in fish: a review. *J. Fish Biol.* **7,** 539.

COUTINHO A., LARSSON E.L., GRÖNVIK K.O. & ANDERSSON A. (1979) Studies on T lymphocyte activation. II. The target cells for concanavalin A-induced growth factors. *Eur. J. Immunol.* **9,** 587.

FARRAR J.J., MIZEL S.B., FULLER-FARRAR J., FARRAR W.L. & HILFIKER M.L. (1980) Macrophage-independent activation of helper T cells. I. Production of interleukin 2. *J. Immunol.* **125,** 793.

GILLIS S., FERM M.M., OU W. & SMITH K.A. (1978) T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* **120,** 2027.

GILLIS S., SMITH K.A. & WATSON J. (1980) Biochemical characterization of lymphocyte regulatory molecules. II. Purification of a class of rat and human lymphokines. *J. Immunol.* **124,** 1954.

GRONDEL J.L. & BOESTEN H.J.A.M. (1982) The influence of antibiotics on the immune system. I. Inhibition of the mitogenic leukocyte response *in vitro* by oxytetracycline. *Dev. Comp. Immunol.* Suppl. 2, 211.

KERN D.E., GILLIS S., OKADA M. & HENNEY C.S. (1981) The role of interleukin-2 (IL-2) in the differentiation of cytotoxic T cells: the effect of monoclonal anti-IL-2 antibody and absorption with IL-2 dependent T cell lines. *J. Immunol.* **127,** 1323.

LAFFERTY K.J., ANDRUS L. & PROWSE S.J. (1980) Role of lymphokine and antigen in the control of specific T cell responses. *Immunol. Rev.* **51,** 279.

LEIBSON H.J., MARRACK P. & KAPPLER J.W. (1981) B cell helper factors. I. Requirement for both interleukin 2 and another 40.000 mol wt factor. *J. exp. Med.* **154,** 1681.

MILLER R.G., LALANDE M.E., DERRY H. & PAETKAU V. (1980) Second signal requirements of cytotoxic T lymphocyte precursors. *Behring Inst. Mitt.* **67,** 41.

RIJKERS G.T., FREDERIX-WOLTERS E.M.H. & VAN MUISWINKEL W.B. (1980) The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*). *Immunology,* **41,** 91.

SCHAUENSTEIN K., GLOBERSON A. & WICK G. (1982) Avian lymphokines. I. Thymic cell growth factor in supernatants of mitogen stimulated chicken spleen cells. *Dev. Comp. Immunol.* **6,** 533.

SHAW J., MONTICONE V., MILLS G. & PAETKAU V. (1978) Effects of costimulator on immune responses *in vitro*. *J. Immunol.* **120,** 1974.

SMITH K.A., GILLIS S., BAKER P.E. & MCKENZIE D. (1979) T cell growth factor-mediated T cell proliferation. *Ann. N.Y. Acad. Sci.* **332,** 423.

SMITH K.A., LACHMAN L.B., OPPENHEIM J.J. & FAVATA M.F. (1980) The functional relationship of the interleukins. *J. exp. Med.* **151,** 1551.

SPIESS P.J. & ROSENBERG S.A. (1981) A simplified method for the production of murine T cell growth factor free of lectin. *J. immunol. Meth.* **42,** 213.

SUSSKIND B.M. & FAANES R.B. (1981) Effects of γ-irradiation on lymphocyte subpopulations participating in the development of the cytotoxic T lymphocyte response. *J. Immunol.* **127,** 1485.

WAGNER H., RÖLLINGHOFF M., PFIZENMAIER K., HARDT C. & JOHNSCHER G. (1980) T-T cell interactions during *in vitro* cytotoxic T lymphocyte (CTL) responses. II. Helper factor from activated Lyt 1^+ T cells in rate limiting (i) in T cell responses to nonimmunogenic alloantigen, (ii) in thymocyte responses to allogeneic stimulator cells, and (iii) recruits allo- or H-2-restricted CTL precursors from the Lyt 123^+ T subset. *J. Immunol.* **124,** 1058.

WU Y., ERNGERG J., MASUCCI M.G., JOHNSON D., KLEIN E. & KLEIN G. (1982) Human T cell growth factor (TCGF) produced by repeated stimulation of non-adherent human lymphocytes. *J. immunol. Meth.* **51,** 35.

Note added in proof: After this manuscript was accepted for publication, Caspi & Avtalion (1984) obtained results with respect to factors promoting the proliferation of carp T-like cells. In contrast to our results, they report that the proliferation of fish lymphoblasts can be sustained using IL-2 enriched preparations from mammalian lymphocytes.

CASPI R.R. & AVTALION R.R. (1984) Evidence for the existence of an IL-2 like lymphocyte growth promoting factor in a bony fish, *Cyprinus carpio*. *Dev. comp. Immunol.* (in press).