F cells. The R cells are probably also involved; however, the low incorporation observed in fractions 4 and 5, where R cells were numerous, seems to indicate that their role in protein synthesis is limited. The same conclusion applies to E cells. It is also possible that some R cells of the type found in fractions 8 and 9, with very few lipid vacuoles and higher density, synthesize protein more actively than old R cells. Taken together, the results indicate that the F cells are the major cell type implicated in protein synthesis in the decapod hepatopancreas (fig. 4) and support for the first time in vitro all the deductions made from ultrastructural studies on F cells [1, 9, 19].

Our results concerning the protein synthesis capacities of the various cell populations do not appear to correlate with those of Ahearn et al.[15], showing that the time course of $^3$H alanine uptake into purified R cells exhibits a sixfold higher rate of influx than that shown in F cell suspensions. However, the measured rates could be influenced by other factors. For example, the concentration of cold leucine in R cells may be higher than that in F cells. The intracellular pools in different cell types have to be determined.

The F cells are also known to store digestive enzymes in vacuoles before secretion[17]. Measurement of α-amylase activity confirms its presence in both of the F cell types. The difference of density between the two categories may correspond to the evolution of these cells toward B cells. Provided that care is taken only to pool animals which are all in the same physiological condition, dissociation and cell suspension techniques could offer a powerful tool for studying hormonal control of protein synthesizing activities, even in complex organs like the crustacean hepatopancreas.

Acknowledgments. This work was supported by a grant from the Ifremer to J. Y. Toullec. We thank Dr D. Soyez and Dr I. Robbins for critical reviews of the typescript and help with the translation.

1 Davis, L. E., and Burnett, A. L., Devl Biol. *10* (1964) 122.
2 Jacobs, W., Z. Zellforsch. *8* (1928) 1.
3 Hirsch, G. C., and Jacobs, W., Z. vergl. Physiol. *A 12* (1930) 524.
4 Al-Mohanna, S. Y., Nott, J. A., and Lane, D. J. W., Crustaceana *48* (1985) 260.
5 Al-Mohanna, S. Y., and Nott, J. A., J. mar. Biol. Ass. U.K. *67* (1987) 803.
6 Gibson, R., and Barker, P. L., Oceanogr. mar. Biol. A. Rev. *17* (1979) 285.
7 Dall, W., and Moriarty, D. J. W., in: Biology of Crustacea, vol. 5, p. 215. Academic Press, New York 1983.
8 Stanier, J. E., Woodhouse, M. A., and Griffin, R. L., Crustaceana *14* (1968) 56.
9 Loizzi, R. F., Z. Zellforsch. *113* (1971) 420.
10 Barker, P. L., and Gibson, R., J. exp. mar. Biol. Ecol. *26* (1977) 297.
11 Barker, P. L., and Gibson, R., J. exp. mar. Biol. Ecol. *32* (1978) 177.
12 Al-Mohanna, S. Y., and Nott, J. A., Mar. Biol. *95* (1987) 129.
13 Papathanassiou, E., and King, P. E., Comp. Biochem. Physiol. *77 A* (1984) 243.
14 Ogura, K., Annot. Zool. Japon *32* (1959) 597.
15 Ahearn, G. A., Monckton, E. A., Henry, A. E., and Botfield, M. C., Am. J. Physiol. *244* (1983) R 150.
16 Bunt, A. T., Crustaceana *15* (1968) 282.
17 Malcoste, R., van Wormhoudt, A., and Bellon, C., C. Acad. Sci., Paris *296* (1983) 597.
18 Hopkins, S. P., and Nott, J. A., J. mar. Biol. Ass. U.K. *60* (1980) 891.
19 Al-Mohanna, S. Y., Nott, J. A., and Lane, D. J. W., J. mar. Biol. Ass. U.K. *65* (1985) 901.
20 Al-Mohanna, S. Y., and Nott, J. A., J. mar. Biol. Ass. U.K. *66* (1986) 403.
21 Devillez, E. J., and Fyler, D. J., Can. J. Zool. *64* (1985) 81.
22 Drach, P., and Tchernigotvzeff, C., Vie Milieu *18* (1967) 595.
23 Parry, G., J. exp. Biol. *31* (1964) 601.
24 Bernfeld, P., Meth. Enzymol. *1* (1955) 149.
25 Richard, P., Thèse 3ème cycle, Marseille 1982.
26 Loret, S., van de Goor, N., and Devos, D., Oceanis *15* (1989) 419.
27 Ellender, R. D., Middelbrooks, B. L., and MacGuire, S. L., Hawaï Congress 1988.

0014-4754/92/030272-06$1.50 + 0.20/0


## Enzymatic basis for protection of fish embryos by the fertilization envelope

S. Kudo

*Department of Anatomy, Gunma University School of Medicine, Maebashi 371 (Japan)*
*Received 19 November 1990; accepted 16 August 1991*

*Abstract.* The mechanism by which the fertilization envelope (FE) is able to protect the embryo of fish until hatching is almost unknown, except for its function as a physical barrier. FE extract from activated or fertilized eggs of the fish *Salmo gairdneri* was demonstrated to contain enzyme activities using an agar plate enzyme assay. The enzymes apparently active were carboxymethylcellulase (cellulase; EC 3.2.1.4), laminaranase (endo-1,3(4)-β-glucanase; EC 3.2.1.6), carboxymethylchitinase (chitinase; EC 3.2.1.14), xylanase (endo-1,4-β-xylanase; EC 3.2.1.8), mannanase (mannan 1,2-(1,3)-α-mannosidase; EC 3.2.1.77), dextranase (EC 3.2.1.11), a protease and lysozyme (EC 3.2.1.17). The FE extract exerted an antifungal or fungicidal action on the fungus *Saprolegnia parasitica,* whereas an extract from the vitelline envelopes (VE) has no apparent enzyme activity nor antifungal or fungicidal action. Enzymes acquired by the FE through the cortical reaction may have an important defensive role, protecting the embryo against invaders or pathogens.
*Key words.* Fertilization envelope; enzyme activities; antifungal action.

It is well known that whenever freshwater fish are handled or even slightly bruised, fungus infection is likely to follow[1], and that fungus disease in fish may sometimes spread after outbreaks in fish farms and streams[2]. Of all the saprolegniacious fungi known to attack fish and fish eggs, *Saprolegnia parasitica* is most frequently implicated[1-3]. However, infection with *Saprolegnia* may be caused by some predisposing factor(s), such as bacterial infection[3-5], or water temperature, a fall in temperature being considered to be particularly conductive to outbreaks of the disease[1]. Even in the latter case, however, it is likely that the fungus would attack weakened skin sites possibly resulting from the drop in water temperature, as a secondary invader showing a close relationship with some unknown primary parasite, although there is experimental evidence that *S. parasitica* can act as a lethal primary parasite of healthy fish[6]. In addition to the detrimental effects of *Saprolegnia* on fish propagation, it is known that a single fungus-infected egg in masses of eggs may form a focus of infection that spreads rapidly to the surrounding eggs, many of which are thus wasted[3]. Prompt removal of fungus-infected fish and eggs, together with sterilization of aquaria, are therefore crucial in commercial fish farming.

The fertilization envelope (FE) is formed by transformation of the pre-existing vitelline envelope (VE) in conjunction with activity of cortical granule or alveolus exudates during the cortical reaction, which involves formation and toughening of the FE and the establishment of polyspermy blocking by degradation of sperm receptors of the VE surface[7-9]. In the fertilized or activated fish eggs, the most striking and intriguing alteration in the process of VE-FE transformation is seen in the outermost layer of the FE, which differs in ultrastructure, cytochemistry and immunohistochemistry from the VE outermost layer[10-14]. In addition, FE extracts from artificially activated fish eggs were found to exert a bactericidal action on the bacteria *Aeromonas hydrophila* or *Vibrio anguillarum*, isolated from a fish farm and a diseased fish, respectively, whereas VE extracts had no such action[11,13]. The main source of the FE extracts was the outermost layer, as confirmed by electron microscopy and agglutination tests of fish sperm or human B-type erythrocytes[11,13]. It is known that fungi do not grow on the FE surface enclosing the developing embryo, but do so rapidly on the FE residues after the embryo has hatched and on the FE enclosing a dead embryo; this suggests that the FE may have antifungal activity. This study was therefore designed to investigate whether FE extract from artificially activated eggs of the fish *Salmo gairdneri* shows antifungal enzyme activity against *S. parasitica* isolated from the body surface of a carp with mycosis. As reported here, FE extract was found to contain enzymes, known in plants as defense enzymes, that possess antifungal or fungicidal activity.

*Materials and methods*

Mature eggs of the fish *Salmo gairdneri* were obtained from Gunma Prefectural Fisheries Experimental Station in Maebashi. The FEs were obtained from artificially activated eggs by immersing the mature eggs in fresh water, or from fertilized eggs 2 weeks after fertilization, and purified as described previously[11,13]. FE residues (FER) from fertilized eggs of the same species were collected about 24 or 48 h after the embryo had hatched. VEs were obtained in a similar manner to FEs. VE, FE, and FER extracts were lyophilized after extraction by methods described previously[11,13].

An agar plate assay for enzyme activities was employed. Ten kinds of substrate were used as follows: barley $\beta$-glucan (Sigma) which consists of $\beta$-1,3(4)-glucan; lichenan (Sigma) which consists of $\beta$-1,3(4)-glucan; laminaran (insoluble; purchased from Serva) which consists of over 95% $\beta$-1,3-glucan; carboxymethylcellulose (CM-cellulose; Sigma); carboxymethylchitin (CM-chitin; Funakoshi Co., Japan); xylan (from oat spelts; Sigma) which consists of poly $\beta$-1,4-xylopyranose; *Micrococcus lysodeikticus* dried cells (Sigma) which are very sensitive to lysozyme; casein (Nakarai Chemicals, Japan); dextran (Sigma), and mannan (from *Saccharomyces cerevisiae*; Sigma). Each substrate (10 mg dry weight) (apart from *M. lysodeikticus* dried cells, dextran and mannan) was prepared by heating in 2 ml of 50 mM phosphate buffer (pH 7.0) containing 0.85% NaCl (PBS). Laminaran and CM-chitin were then centrifuged to remove insoluble elements (3000 rpm for 15–20 min). 2 ml of dissolved substrate or supernatant was mixed with 8 ml of 2% agar dissolved in PBS, and then spread on a Petri dish. When the agar had solidified, wells 4 mm in diameter were punched into the agar layer and filled with 10 µl of a lyophilized FE, FER or VE extract, or boiled FE extract (0.5, 1 or 2 mg/well) dissolved in PBS. Diameters of lysis haloes were photographed 16 h after incubation at 30 °C. FE extract was treated with pronase (Kaken, Japan) or trypsin type 1 (2 × crystallized, Sigma), to investigate possible inhibition of enzymatic activity: 24 mg of FE extract were incubated with pronase (ca. 90 PUK units) or trypsin (ca. 20000 BAEE units) in 50 mM PBS (pH 7.6) for 1 h at 37 °C. The solution of FE extract (2 mg/well) was subsequently boiled for 10 min to inactivate pronase or trypsin activity and then poured into wells in agar containing one of the substrates.

*S. parasitica*, previously grown in culture on Sabouraud dextrose agar (Eiken Chemicals Co., Japan) was used to determine the antifungal or fungicidal action of the FE or VE extract. Pieces of the fungus mycelium were suspended in 50 mM PBS (pH 7.0) containing 10 mg of a lyophilized FE or VE extract per ml of PBS, and the suspension was incubated at 30 °C with gentle shaking. Controls were mycelial fragments suspended in 50 mM PBS at 30 °C without FE or VE extract. Aliquots (0.1 ml) of all the suspensions were placed on Sabouraud dextrose agar in Petri dishes 6, 12, and 24 h after incubation. The

plates were photographed 48 h later at room temperature (25 °C).

*Results*

The agar plate enzyme assay demonstrated enzyme activity of the FE extract, a halo around the wells in the agar plates indicating degradation of ten kinds of substrates (fig. 1). The sizes of haloes produced around the wells were found to be dose-dependent. The active enzymes included carboxymethylcellulase (cellulase; EC 3.2.1.4), laminaranase (endo-1,3(4)-β-glucanase; EC 3.2.1.6), carboxymethylchitinase (chitinase; EC 3.2.1.14), xylanase (endo-1,4-β-xylanase; EC 3.2.1.8), dextranase (EC 3.2.1.11), mannanase (mannan 1,2-(1,3)-α-mannosidase; EC 3.2.1.77), a protease and lysozyme (EC 3.2.1.17), although the latter was relatively inactive. These findings suggest that the FE extract may be able to protect the embryo by attacking fungal cell walls. Boiling of the FE extract for 10 min had little effect on enzyme activity, except for that of lysozyme. There was no diminution of enzyme activity in FE extract obtained from FEs of fertilized eggs two weeks after fertilization, and even 24 h FER extracts still retained enzyme activity, although this was greatly reduced in comparison to that of the FE extract, a smaller halo forming around the wells (fig. 2). However, an extract from FERs collected 48 h after hatching was accompanied by a further reduction in enzyme activity. FE extract treated with pronase formed very small or no haloes around the wells in agar containing one of the substrates, whereas trypsinized FE extract did form haloes, comparable to those of the controls (fig. 2). In the agar containing *M. lysodeikticus* dried cells as a substrate, an obvious halo formation suggested that lysozyme might have been further activated by trypsinization (fig. 2G).

To determine whether the action of the FE extract is antifungal or fungicidal, a suspension of *S. parasitica* mycelium in FE extract was incubated with gentle shaking at 30 °C. Prolongation of the incubation time increased inhibition of hyphal growth from the mycelium: incubation for 6 h considerably inhibited the growth of hyphae; incubation for 12 h resulted in poor growth of short hyphae from a few mycelial pieces, which was evident even 48 h later, and incubation for 24 h resulted in complete inhibition of hyphal growth from all mycelial pieces (fig. 3). VE extract at the same concentration as that of the FE extract exerted neither antifungal nor fungicidal action (fig. 3), and no haloes were formed (data not shown). The pronase-treated and boiled FE extract exerted little effect on inhibition of hyphal growth, but the trypsinized and boiled FE extract was as efficient in this respect as was non-treated FE extract (fig. 3).

*Discussion*

Fungal cell walls consist of various polysaccharides, such as chitin, α-glucans, β-glucans and mannans[15], that are responsible for the strength and integrity of cell walls, often existing in the form of chemical complexes with proteins and other sugars or polysaccharides[16,17]. Each cell wall component appears to show a certain degree of stratification, as shown in a structural model for the cell wall[18]. Furthermore, it has been shown that β-1,3-glucanases and chitinases are capable not only of attacking the cell walls of pathogens in vitro[19–22] but also of inhibiting the growth of several fungi in the presence of a combination of both enzymes[23,24]. These data, together with the findings described here, suggest that enzymes in the FE extract may have acted synergistically on the cell wall of the fungus *S. parasitica* and exerted an anti-

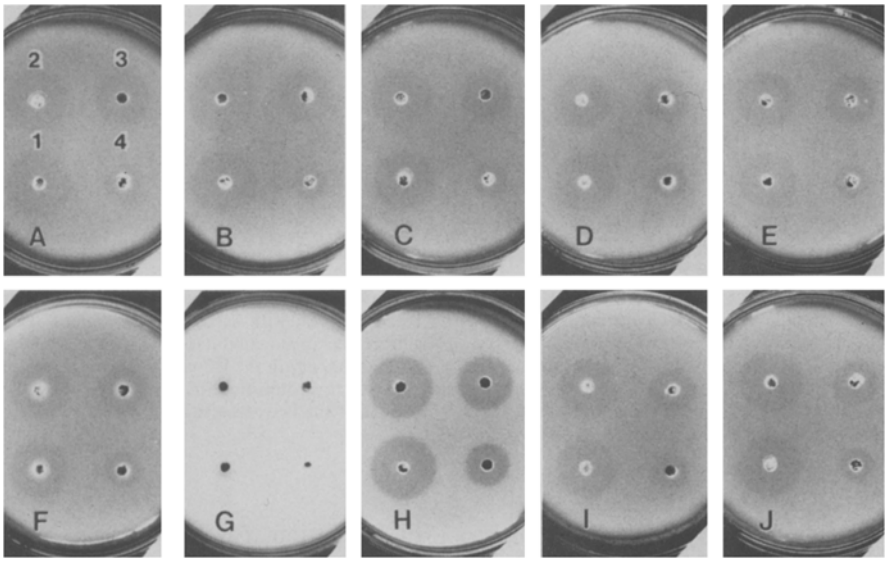


Figure 1. Agar plate assay for enzyme activity of FE extract. Each plate (A–J) contains a substrate, as follows: A, barley β-glucan; B, lichenan; C, laminaran; D, CM-cellulose; E, CM-chitin; F, xylan; G, *M. lysodeikticus* dried cells; H, casein; I, dextran; J, mannan. The numbered wells (1–4) in A contain 2-week FE extract (2 mg; from fertilized eggs 2 weeks after fertilization), FE extract (2 mg), FE extract (1 mg) and FE extract (0.5 mg), respectively. The other plate wells correspond to the numbered wells in A.

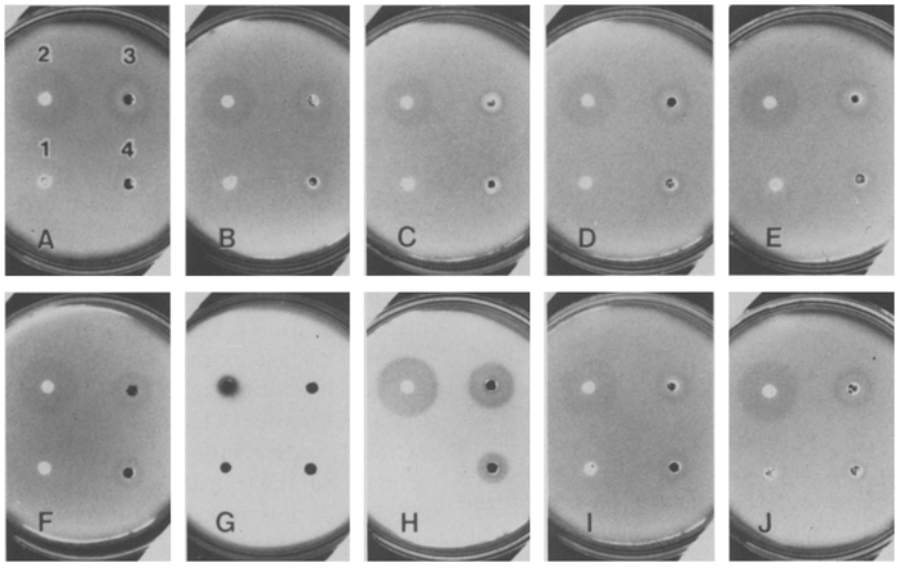


Figure 2. Agar plate assay for enzyme activity of pronase-treated or trypsinized and boiled FE extract, or FER extract. (A–J) as in fig. 1. The numbered wells (1–4) in A contain 2 mg of pronase-treated and boiled FE extract, trypsinized and boiled FE extract (not boiled in well 2 in G), 24 h-FER extract (24 h after hatching) and 48 h-FER extract (48 h after hatching), respectively. The other plate wells correspond to the numbered wells in A. In well 2 in G, the obvious halo formation suggests a further activation of lysozyme activity by trypsinization.

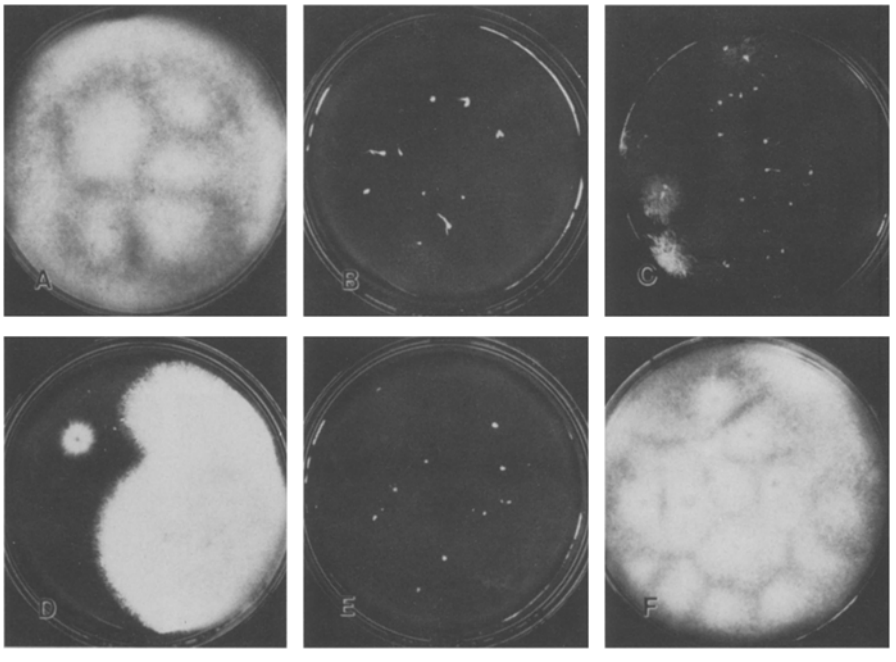


Figure 3. Antifungal or fungicidal effect of FE or VE extract on inhibition of hyphal growth from mycelial pieces. *A*, Mycelium incubated for 24 h with suspension containing VE extract: hyphal growth is very vigorous. *B*, Mycelium incubated for 24 h with suspension containing FE extract: no hyphal growth. *C*, Mycelium incubated for 12 h with suspension containing the FE extract: hyphal growth only in a few mycelia. *D*, Mycelium incubated for 24 h with suspension containing pronase-treated and boiled FE extract: very vigorous hyphal growth. *E*, Mycelium incubated for 24 h with suspension containing trypsinized and boiled FE extract: complete inhibition of hyphal growth, implying retention of enzyme activity in the FE extract. *F*, Control preparation incubated for 24 h with mycelium alone in 50 mM PBS (pH 7.0) without FE extract: the surface of the Petri dish is covered with hyphae.

fungal or fungicidal action by gradually digesting the main components responsible for structural integrity, starting at the outside and working inwards. Thus, prolongation of the incubation time of the FE extract and fungus would have enhanced the action.

In conclusion, FE extract from activated or fertilized eggs of *Salmo gairdneri* contains enzymes that defend the egg against the fungus that is the major cause of mycosis in fish and fish eggs [1, 25]. The mechanism by which the FE is able to protect the developing embryo from in-

vaders or pathogens until hatching is probably related to the enzymatic properties of the FE outermost layer, which are acquired through the cortical reaction, in addition to the function of the FE as a physical barrier. FE extracts have already been shown to exert a bactericidal activity [11, 13]. Further experiments are necessary to verify the presence of any antiviral defense mechanism. This information may lead to new insights into the function of the FE and the cortical alveoli.

1 Scott, W. W., and O'Bier, A. H., Prog. Fish-Cult. *24* (1962) 3.
2 Stuart, M. R., and Fuller, H. T., Nature *217* (1968) 90.
3 Kanouse, B. B., Mycologia *24* (1932) 431.
4 Egusa, S., Bull. Jap. Soc. Sci. Fish. *31* (1965) 517.
5 Egusa, S., and Nishikawa, T., Bull. Jap. Soc. Sci. Fish. *31* (1965) 804.
6 Hoshino, T., and Ookubo, N., J. Tokyo Univ. Fish. *42* (1956) 1.
7 Carroll, E. H. Jr, and Epel, D., Exp. Cell Res. *90* (1975) 429.
8 Bryan, J., J. Cell Biol. *44* (1970) 635.
9 Chandler, D. E., and Heuser, J., J. Cell Biol. *84* (1980) 618.
10 Kudo, S., Develop. Growth Differ. *24* (1982) 327.
11 Kudo, S., and Inoue, M., Zool. Sci. *3* (1986) 323.
12 Kudo, S., and Inoue, M., Regeneration and Development, p. 411. Okada Print. Publ. Co., Maebashi, Japan 1988.
13 Kudo, S., and Inoue, M., J. exp. Zool. *250* (1989) 219.
14 Kudo, S., Sato, M., and Inoue, M., J. exp. Zool. *245* (1988) 63.
15 Gorin, P. A. J., and Barreto-Bergter, E., The Polysaccharides, vol. 2, p. 365. Academic Press, New York 1983.
16 Chattaway, F. W., Holmes, M. R., and Barlow, A. J. E., J. gen. Microbiol. *51* (1968) 367.
17 Farkas, V., Microbiol. Rev. *43* (1979) 117.
18 Trinci, A. P. J., Sci. Prog. London *65* (1978) 75.
19 Young, D. H., and Pegg, G. F., Physiol. Plant Path. *19* (1981) 391.
20 Young, D. H., and Pegg, G. F., Physiol. Plant Path. *21* (1982) 411.
21 Boller, T., Gehri, A., Mauch, F., and Vögeli, U., Planta *157* (1983) 22.
22 Kombrink, E., Schröder, M., and Hahlbrock, K., Proc. natl Acad. Sci. USA *85* (1988) 782.
23 Skujins, J. J., Potgieter, H. J., and Alexander, M., Archs Biochem. Biophys. *111* (1965) 358.
24 Schlumbaum, A., Nature *324* (1986) 365.
25 Tiffney, W. N., J. Elisha Mitchell Sci. Soc. *55* (1939) 134.

0014-4754/92/030277-05$1.50 + 0.20/0


## Roles for interleukin-2 (IL-2) and IL-4 in the generation of allocytotoxic T cells in the primary and secondary responses in vitro

H. Suzuki, I. S. Tunru and A. Yokoyama

*First Department of Internal Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01 (Japan)*
*Received 23 April 1991; accepted 29 July 1991*

*Abstract.* Roles for interleukin-2 (IL-2) and IL-4 in the generation of murine allocytotoxine T lymphocytes (allo-CTL) in the primary and secondary responses were studied in vitro. The generation of allo-CTL in the primary response was inhibited by anti-IL-2 monoclonal antibody (mAb), but was not inhibited by anti-IL-4 mAb. On the other hand, the generation of allo-CTL in the secondary response was partially inhibited by either anti-IL-2 or anti-IL-4 mAb, and it was almost completely inhibited by the combination of two mAbs. $CD8^+$ cell-depleted splenocytes produced IL-2, but not IL-4, in response to alloantigens in the primary response, and these cells produced both IL-2 and IL-4 in the secondary response. Both exogenous IL-2 and IL-4 induced functionally active allo-CTL in the primary response from $CD4^+$ cell-depleted splenocytes when these cells were stimulated with T cell-depleted allogeneic cells. These results suggest that the allo-CTL induction in the primary response is IL:-2-dependent and secondary allo-CTL induction is both IL-2 and IL-4-dependent, because unprimed $CD4^+$ T cells produce IL-2, but not IL-4, whereas primed cells produce both IL-2 and IL-4 in response to alloantigens.
*Key words.* Interleukin-2; interleukin-4; cytotoxic T lymphocytes.

The growth and differentiation of cytotoxic cells are regulated by soluble factors, of which interleukin-2 (IL-2), a lymphokine produced by activated helper T cells, has been shown to play a major role in the generation of cytotoxic cells, such as allospecific cytotoxic T lymphocytes (CTL)[1] and lymphokine activated killer (LAK) cells[2]. On the other hand, IL-4, which is also produced by helper T cells, is demonstrated to have potency in the generation of cytotoxic cells, in addition to its effect on B cells. Murine IL-4 induces alloreactive CTL[3] and LAK cells[4] in in vitro cultures. In human systems, it was reported that IL-4 augmented the induction of allo-CTL in IL-2-supplemented cultures, whereas IL-2-mediated induction of LAK cells was inhibited by IL-4[5].

Although activated helper T cells produce both IL-2 and IL-4, the roles of these two lymphokines for allo-CTL induction in the primary and secondary responses are not well characterized. Recent studies have shown that murine helper T cells are composed of at least two subsets that can be distinguished on the basis of their patterns of lymphokine production[6]. One subset, termed Th1, pro-