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Identification of an interleukin 2-like substance as a factor cytotoxic to oligodendrocytes and associated with central nervous system regeneration

(cytotoxicity/lymphokines/nerve injury/nerve regeneration)

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ABSTRACT **Axons of the central nervous system in adult mammals do not regenerate spontaneously after injury, partly because of the presence of oligodendrocytes that inhibit axonal growth. This is not the case in lower vertebrates (e.g., in fish), where regeneration of the optic nerve does occur spontaneously and has been correlated with the presence of factors cytotoxic to oligodendrocytes. The present study provides evidence that the substance originating from the fish optic nerves, which is cytotoxic to oligodendrocytes, is an interleukin 2-like substance.**

Axons of the central nervous system (CNS) in lower vertebrates regenerate after injury (1–4). In contrast, in mammalian CNS the ability of axons to regenerate spontaneously is poor (5–8). The environment that surrounds the nerve has a decisive effect on the ability of the nerve to undergo regeneration of its injured axons. This conclusion is based on the observation that such axons are able to grow for some considerable distance into a peripheral nerve bridge grafted to injured nerves of the mammalian CNS *in vivo*. *In vitro*, injured mammalian CNS axons can grow into a mammalian peripheral nerve but not into a segment of a mammalian optic nerve (9–11). Among the cells that surround the injured nerve are astrocytes (potentially scar-forming cells), oligodendrocytes (potential inhibitors of axonal growth), microglia, and macrophages.

Considerable research interest has recently been focused on the oligodendrocytes. It was shown that mature oligodendrocytes create an environment that is nonpermissive for growth (9). Monoclonal antibodies directed against the inhibitory molecules were shown to neutralize the growth-inhibitory effect (10). In line with these observations is our finding that the high regenerative capacity of the fish optic nerve is correlated with the presence of a factor or factors cytotoxic to oligodendrocytes (12–14). This factor(s) was detected among the soluble substances derived from regenerating fish optic nerves, which were collectively termed conditioned medium (CM) and, when applied *in vivo*, helped to create conditions conducive to axonal growth in other species—e.g., in injured optic nerves of rabbit (15, 16). Recent studies in our laboratory suggest that the cytotoxic factor(s) in the fish might be associated with the postinjury inflammatory reaction—i.e., with the early invasion of injured nerves by blood-derived cells (14). In the present study, the cytotoxic factor was identified as an interleukin 2 (IL-2)-like molecule.

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MATERIALS AND METHODS

Preparation of Soluble Substances Derived from Regenerating Fish Optic Nerves. Carp (*Cyprinus carpio*, 800–1200 g; Tnuva, Israel) were anesthetized with 0.05% 3-aminobenzoic acid ethyl ester (Sigma), and their optic nerves were crushed with forceps (for 30 sec). Eight days later, the nerves were removed by dissection and incubated in serum-free medium for 1.5 hr (four nerve segments per 300 μl of medium) at 25°C (15). The resulting medium, defined as CM, was then collected, and its protein content was determined by the Bradford assay (17).

Preparation of Medium Conditioned by Fish Lymphocytes. Fish were anesthetized with 3-aminobenzoic acid ethyl ester (Sigma). Blood was withdrawn from the venous plexus of the eye orbit and was collected (usually 30 ml from two or three fish) from the socket into a tube containing heparin sulfate (100 units/ml; BDH Chemicals). The blood was diluted with an equal volume of phosphate-buffered saline (PBS) and allowed to stand for 5 min before being layered on top of the Percoll solution [30 ml of diluted blood was carefully layered on top of the solution in a 50-ml sterilized Corex (Corning) centrifuge tube by using a small pipet]. The tubes were capped and centrifuged in swinging buckets at 800 × *g* for 30 min at 20°C.

After centrifugation, leukocytes were found in the interphase, where they could easily be removed with a Pasteur pipet. The leukocytes were collected into a 50-ml tube and washed twice with PBS. The cells were suspended in a small volume of L-15 medium containing penicillin and streptomycin (100 units/ml) and then counted. The cells were diluted to 2×10^7 cells per ml, and 10-ml aliquots were placed into 75-cm^2 flasks (Falcon). The flasks were left for 1 hr at 20°C, and the nonadherent cells were removed and placed into a second flask. This was repeated, and after 1 hr the nonadherent cells were placed into a third flask. The first and second flasks were washed twice with 100 ml of PBS with vigorous shaking. The first flask contained a macrophage-enriched culture, and the third flask contained a lymphocyte-enriched culture. Ten milliliters of medium was added, and all flasks were incubated for 8 hr, after which all of the supernatants were collected and centrifuged at 2000 × *g* for 5–15 min. The supernatants were concentrated 25- to 100-fold in a Centricon or Amicon unit (Amicon). The samples were then stored at 4°C.

Preparation of Oligodendrocyte Cultures and Immunofluorescence Staining. Enriched oligodendrocyte cultures derived

Abbreviations: CM, conditioned medium; CNS, central nervous system; GalC, galactocerebroside; IL-1, -2, and -6, interleukins 1, 2, and 6; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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from neonatal rat brain were prepared as described (18, 19) and seeded on glass coverslips (13 mm; 3×10^5 cells per well) previously coated with poly(L-lysine) (20 mg/ml; Sigma). After 48 hr, the seeded cells were treated with the CM or with CM preincubated for 2 hr either with rabbit anti-human IL-2 antibodies (Genzyme) or with control antibodies (rabbit anti-neurofilaments). The number of mature oligodendrocytes—i.e., galactocerebroside (GalC)-positive cells—was determined by immunofluorescence as follows. Cells were thoroughly washed with Hanks' balanced salt solution containing 2% (vol/vol) fetal bovine serum, heat-inactivated, and incubated for 30 min at 37°C with 50 μl of the monoclonal anti-GalC antibodies [IgG3, hybridoma supernatant diluted 1:5 in Dulbecco's modified Eagle's medium (DMEM); Serotec]. This was followed by washing and further incubation with 50 μl of fluorescein-conjugated goat anti-mouse IgG3 (1:50 in DMEM) and then washing and fixation in methanol for 10 min at −20°C. At a final washing, the cells were coated with glycerol containing 22 mM 1,4-diazobicyclo[2.2.2]-octane. As controls we used coverslips that underwent the same staining procedure as that used for the primary antibodies, which were omitted. Coverslips were placed on the glass slides, sealed with nail polish, and stored at 4°C. Cells were counted over the entire coverslip.

Immunoblot (Western Blot) Analysis. Samples were electrophoresed on SDS/PAGE. The gel was blotted onto nitrocellulose for 2 hr at 200 mA. The nitrocellulose was incubated for 2 hr at 37°C with PBS containing 5% (vol/vol) milk and then washed in PBS.

The blot was incubated with IL-2 antibodies for 2 hr at 37°C and then washed three times, each time for 5 min, in PBS containing 0.05% Tween 20. Finally, the blot was incubated for 2 hr at 37°C with ^{125}I-labeled goat anti-rabbit antibodies (10^6 cpm/ml), washed three times, dried, and autoradiographed.

Affinity Purification of the Cytotoxic Factor(s). Mouse anti-human IL-2 antibodies (0.5 mg) were coupled to 0.1 ml of packed polyacrylhydrazide-agarose (Bio-Yeda) by the procedure of Wilchek and Miron (20). Purification was carried out as follows. CM (50 μl) was added to the anti-IL-2 antibody-coupled packed resin and incubated for 2 hr at 37°C. The supernatant was then collected, and the remaining resin was washed three times, each time with 1 ml of PBS. Elution was carried out at room temperature with 50 μl of 0.2 M glycine (pH 2.7) with shaking for 10 min. The eluted material was collected into 10 μl of 1 M Tris buffer (pH 8.0) and applied at the appropriate dilution to cultures of oligodendrocytes. Assessment of the cytotoxic effect of the original preparation and that of the eluted material was examined by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma), which determines the amount of surviving cells.

The assay was carried out as follows. Cells were treated with the CM alone or with the bound substances eluted from the anti-IL-2 antibody-conjugated column. Activities of the eluted substances and of the original preparation were assessed by the colorimetric method (21). The final dilutions of the eluted substances used in the assay corresponded to those of the crude CM. After incubation for 48 hr, 10 μl of MTT was added for 3 hr; the medium was then removed, and 100 μl of 0.04 M HCl in isopropanol was added. The cells were gently shaken until all crystals had dissolved, and their absorbance was recorded at 550 nm against absorbance at 600 nm as a reference.

RESULTS

Presence of an IL-2-Like Molecule Cytotoxic to Oligodendrocytes in Fish CM. In a previous study we suggested that the cytotoxic factor(s) present in medium conditioned by regenerating fish optic nerves may belong to the lymphokine family (13, 14). We investigated this possibility in the present study by examining whether treatment with antibodies against various cytokines would result in a loss of cytotoxic activity. Of the various antibodies tested—i.e., those directed against interleukins 6, 1, and 2 (IL-6, IL-1, and IL-2), platelet-derived growth factor (PDGF), and tumor necrosis factor, only the antibodies directed against IL-2 neutralized the cytotoxic effect of the CM. As for the presence of any of these factors in the fish CM, the binding assay revealed the presence of PDGF (22), which was shown independently to be devoid of any cytotoxic activity on oligodendrocytes (14). Negative results obtained for the other tested lymphokines do not necessarily imply their absence in the fish CM, as the results might derive from poor immunological cross-reactivity due to species differences. The results with IL-2 are shown in Fig. 1. After 48-hr of incubation, CM either alone or preincubated with rabbit polyclonal antibodies against recombinant human IL-2 was added to the oligodendrocyte cultures and incubated for a further 48 hr. Antibodies directed against neurofilaments were used as control. Oligodendrocytes were identified by immunofluorescence. Antibodies against recombinant mouse IL-2 neutralized the cytotoxic activity of the CM (Fig. 1). When the amount of antibodies was kept constant (0.1 μg of IgG fraction; 1 μg neutralizes 1 unit of IL-2) the neutralization achieved was a function of the concentration of CM applied, with complete neutralization at the lowest concentration tested—i.e., 0.2 μg of total protein per ml. A 10-fold higher amount of antibodies (1 μg of IgG fraction) resulted in neutralization of higher concentrations of CM (i.e., 0.5 and 5 μg/ml) (Fig. 1 *Inset*). According to these results, and based on the potency of the IL-2 antibodies used for neutralization in the present assay, 1 μg of the CM is estimated to contain 0.5–2 units of biologically active IL-2-like molecules.

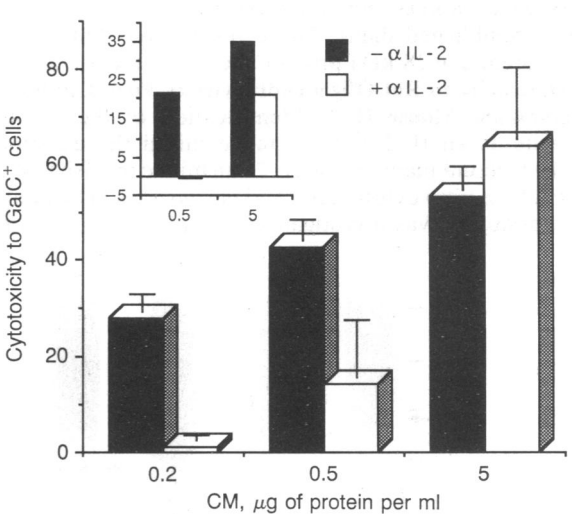

FIG. 1. Neutralization of the cytotoxic effect of fish soluble substances on oligodendrocytes by rabbit polyclonal antibodies directed against recombinant human IL-2 antibodies: assessment by immunofluorescence. The highest amount of the soluble substances derived from regenerating fish optic nerves (5 μg of protein) resulted in about 60% cytotoxicity and no neutralization with anti-IL-2 antibodies; 0.5 μg of CM caused 42% cytotoxicity, half of which could be neutralized by the antibodies; complete neutralization with the same amount of antibodies could be obtained when only 0.2 μg of the CM was applied. Results are expressed as percent cytotoxicity, in relation to cytotoxicity-free control cultures treated with the antibodies only (100% survival, no cytotoxicity). All experiments were repeated three times and were carried out with 0.1 μg of anti-IL-2 antibodies (IgG). The results of one experiment are given in this figure. The absolute total number of GalC$^+$ cells counted in each coverslip ranged from 300 to 500 in the various experiments. (*Inset*) Similar experiments carried out with 10-fold more anti-IL-2 antibodies—i.e., 1 μg of IgG.

Purification and Characterization of the IL-2-Like Molecule from Fish CM. To determine the size of the IL-2-like molecule, we subjected the CM to Western blot analysis by using monoclonal antibodies directed against human IL-2. These antibodies recognized a polypeptide of 20 kDa in a preparation of mouse recombinant IL-2. Fig. 2 shows the presence of a single IL-2 immunoreactive band at a molecular weight of 28 kDa. Since lymphocytes are known to produce IL-2, we compared the interaction of the same antibodies with fish lymphocytes under the same experimental conditions. A single IL-2 immunoreactive band of ≈14 kDa was observed (Fig. 2). No immunoreactivity bands were observed in any of the preparations when the antibodies were omitted. These results suggest that IL-2 or a molecule with which IL-2 antibodies cross-react might be responsible for the cytotoxic effect on oligodendrocytes.

This finding prompted us to purify the cytotoxic substances by the use of an IL-2 affinity column. CM was passed through an affinity column prepared from mouse monoclonal antibodies against IL-2, and the bound substances were eluted with 0.2 M glycine (pH 2.7). Fractions containing the IL-2-bound substances were tested for their cytotoxic effects on oligodendrocytes by the use of a colorimetric method for assessment of the number of oligodendrocytes and hence of the cytotoxicity (21). The IL-2-bound substances eluted from the column were cytotoxic to oligodendrocytes (Fig. 3). The small scale of the purification did not permit calculation of the specific activity of the eluate. However, in view of the known limits of the protein detection, we could estimate that the specific activity of the eluate was at least 10^3-fold higher than that of the CM. The eluate, which was found to retain the cytotoxic activity, was also subjected to Western blot analysis with anti-IL-2 antibodies, which revealed the presence of the original 28-kDa immunoreactive polypeptide (S.E. and M.S., unpublished data). These results thus link the IL-2 immunoreactive 28-kDa protein and the cytotoxic activity.

Cytotoxicity to Rat Oligodendrocytes *in Vitro* Exhibited by Recombinant Mouse IL-2. Identification of the cytotoxic molecule as an IL-2-like substance raised the question of whether recombinant mouse IL-2 can mimic the fish CM with respect to *in vitro* cytotoxicity and *in vivo* growth facilitation. This possibility was investigated by application of recombinant mouse IL-2 to cultures of rat oligodendrocytes. At high IL-2 concentrations, the number of mature oligodendrocytes, as reflected by the number of $GalC^+$ cells, was significantly reduced (88.6 ± 15.3% cytotoxicity at 150 units/ml; $P < 0.0005$ by analysis of variance) (Fig. 4). Representative micrographs showing the effects of recombinant mouse IL-2 on oligodendrocytes are shown in Fig. 5. The cells that were spared were processless (Fig. 5*b*) rather than process-bearing cells (Fig. 5*d*). It should be noted, however, that the amount of recombinant mouse IL-2 required for demonstrable cyto-

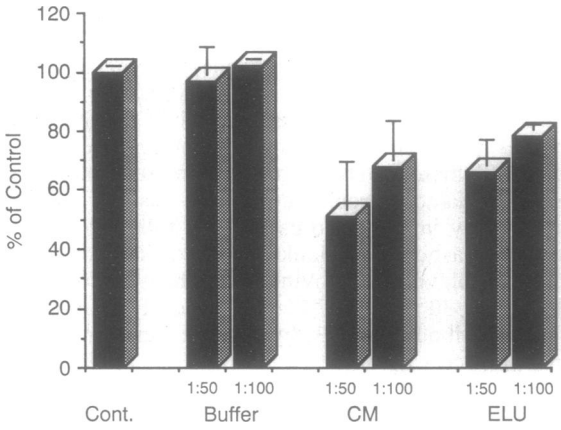

FIG. 3. Affinity purification of the IL-2-like factor from fish optic nerve CM. Cytotoxic activity on oligodendrocytes, assessed by the colorimetric MTT assay, was recovered from the fish optic nerves by elution of the substances bound to the anti-IL-2 antibodies, which had been coupled to the column. Controls were untreated cultures or cultures treated with medium containing the elution buffer (Buffer), thus ruling out the possibility that any cytotoxicity in the eluate might have resulted from the buffer used for elution of the bound material from the column. The experiment, which was repeated three times, was performed in triplicate, and the results are presented as means ± SD of the untreated culture values, representing 100% survival. Analyses by the repeated-measures method revealed that the effects of the eluted substances (ELU) differed significantly ($P < 0.05$) from those of the two corresponding control cultures.

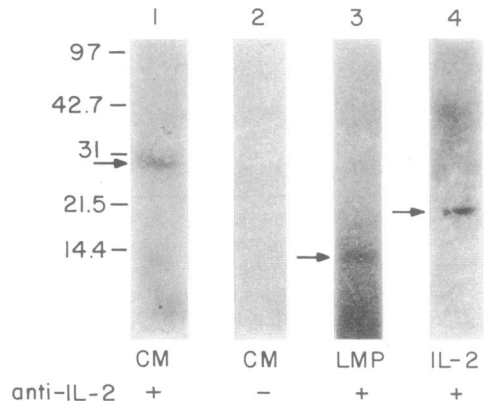

FIG. 2. Western blot analysis of the fish optic nerve soluble substances with mouse anti-human IL-2 monoclonal antibodies. Optic nerve CM (400 μg of protein) and media conditioned by fish blood lymphocytes (LMP, 16 μg) or mouse IL-2 (75 ng) were electrophoresed on SDS/PAGE. Lanes: 1, CM incubated with anti-IL-2 antibodies; 2, control slot containing CM treated with second antibodies only; 3, slot containing medium conditioned by fish LMP incubated with the anti-IL-2 antibodies; 4, slot containing recombinant mouse IL-2 incubated with the same antibodies. Arrows to the left of each lane (excluding lane 2) point to the IL-2 immunoreactive bands in each slot. Molecular weight markers were electrophoresed on the same gel and their positions are indicated.

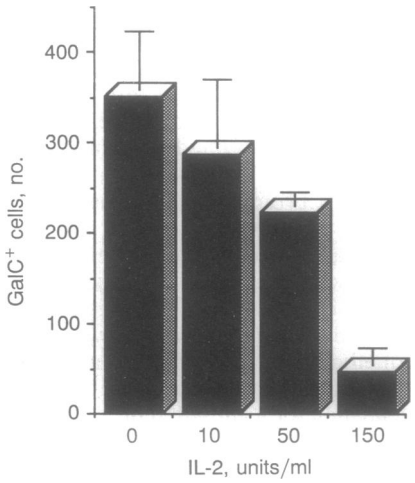

FIG. 4. Reduction in oligodendrocyte number by recombinant mouse IL-2 in rat brain oligodendrocyte cultures. Enriched cultures of rat brain oligodendrocytes were prepared on glass coverslips. Cells were treated, 48 hr after seeding, with recombinant mouse IL-2 (0–150 units/ml). The number of cells was assessed by the use of antibodies directed against GalC. The number of labeled cells was counted with a fluorescence microscope. Untreated cells were used as control (no cytotoxicity). Results of one experiment performed in triplicate are shown. The experiment was repeated three times, and the same dose-dependent effect was observed.

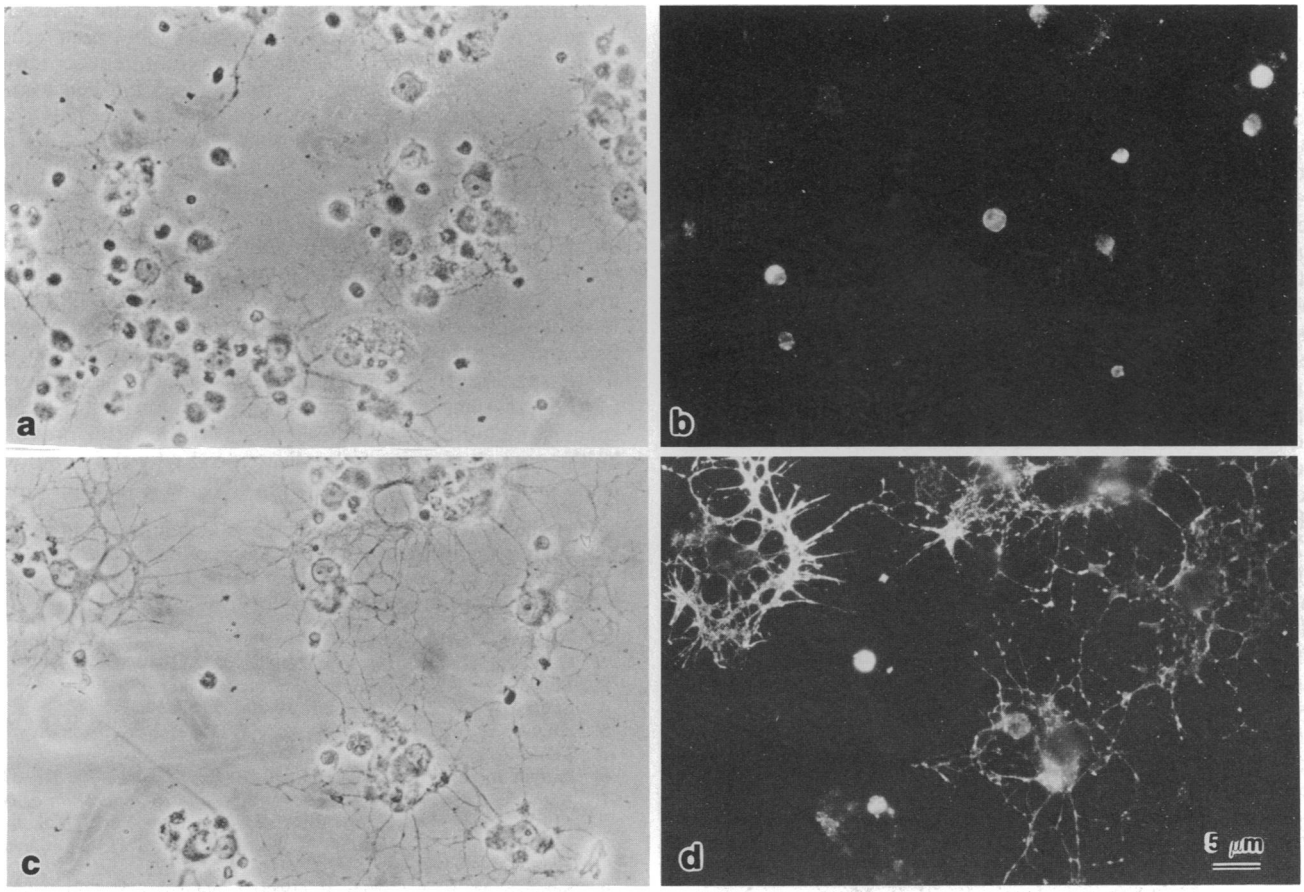

FIG. 5. Reduction in the number of mature oligodendrocytes (GalC-labeled) caused by recombinant mouse IL-2. Oligodendrocyte cultures were prepared as described in the legend to Fig. 1. Treatment with recombinant mouse IL-2 (100 units/ml) was carried out as described for Fig. 4; and staining, as described for Fig. 1. (*b* and *d*) Fluorescent micrographs of IL-2-treated (*b*) and untreated (*d*) cells, respectively, stained with anti-GalC antibodies. Respective phase micrographs of the fields shown in *b* and *d* are seen in *a* and *c*.

toxic activity on oligodendrocytes was far higher than the estimated amount in the CM. Therefore, it is possible that the IL-2 derived from fish has a higher affinity for rat oligodendrocytes than that of the recombinant mouse IL-2 for rat oligodendrocytes. To rule out the possibility that the observed effect of the IL-2 on oligodendrocytes is due to nonspecific cytotoxicity, we applied IL-2 also to cultures of astrocytes—i.e., rat brain monolayers of flat cells. As expected, no cytotoxicity was observed (Table 1).

DISCUSSION

The present study provides evidence that the fish-derived cytotoxic factor associated with regeneration is IL-2 or a closely related compound.

We have shown in the past that CM derived from regenerating fish optic nerves can facilitate axonal growth *in vivo* (15, 16). Independently, we showed that *in vitro* this preparation possesses a number of activities, including cytotoxicity to mature oligodendrocytes of both fish and rat (12–14). The observed correlation between the level of the cytotoxic factor and axonal regeneration in the fish strongly suggested an association between them (14). Moreover, the correlation between regeneration and spontaneous reduction in the number of oligodendrocytes in the fish (14) suggested that *in vivo* regeneration in the fish is related to and might benefit from the presence of a factor that is cytotoxic to oligodendrocytes. The latter association is based on the suggestion of Schwab and his colleagues (9, 10) that mature oligodendrocytes may interfere with axonal growth.

Table 1. IL-2 is not cytotoxic to rat brain astrocytes

Cells per well, no.	[^{3}H]Thymidine incorporation in the absence or presence [unit(s)/ml] of IL-2, cpm ± SD (% of untreated controls)					
	0	0.1	1	10	50	200
5,000	28,030 ± 518 (100 ± 2)	33,632 ± 2351 (120 ± 7)	31,084 ± 984 (110 ± 3)	33,384 ± 5181 (119 ± 16)	31,879 ± 2957 (113 ± 9)	30,248 ± 1052 (107 ± 3)
30,000	55,727 ± 5137 (100 ± 11)	60,345 ± 793 (108 ± 8)	58,231 ± 86 (105 ± 8)	62,328 ± 103 (112 ± 9)	56,601 ± 658 (102 ± 8)	57,380 ± 2771 (103 ± 9)

The monolayer of flat cells from dissociated brain cultures (described in Fig. 1), which remained after the oligodendrocytes had been shaken off overnight (Fig. 1), were further removed by trypsin (Sigma) at 300 units/ml in DMEM (Ca^{2+} and Mg^{2+} free) containing 0.5 mM ethylenediamine tetracetic acid (EDTA). After incubation for 5 min at 37°C, the cells were washed twice in DMEM and resuspended in DMEM containing 5% fetal bovine serum. In each well of microtiter plates previously coated with poly(L-lysine) at 20 μg/ml, 5000 or 30,000 cells were seeded in 100 μl of medium. Forty-eight hours after seeding, IL-2 (0–200 units/ml) or control medium was added to each well and incubation was continued for 48 hr. The effect was assessed by means of thymidine incorporation: 2 μCi (74 kBq) of [^{3}H]thymidine was added to each well and incubation was continued for 48 hr, after which cells in each well were harvested and assayed for radioactivity. Results are expressed by the average of triplicates ± SD. No inhibitory effect could be observed.

The goal of the present study was to identify the cytotoxic molecule(s) originating from the fish. That it is an IL-2-like molecule was confirmed by the following observations: (*i*) it was purified by affinity chromatography of anti-IL-2 antibodies, (*ii*) antibodies against IL-2 neutralized the cytotoxic activity of the CM derived from regenerating fish optic nerves, (*iii*) Western blot analysis revealed the presence of an IL-2 immunoreactive band of 28 kDa in the fish CM, and (*iv*) recombinant mouse IL-2 had a selective cytotoxic effect *in vitro* on oligodendrocytes but not on astrocytes. The apparently higher potency of the IL-2-like substances in the fish CM than that of the recombinant mouse IL-2 strongly suggests that the specificity of the activity is determined by the tissue and not by the species—i.e., the CNS-derived fish IL-2 is probably different from the recombinant IL-2, which is immune system-derived. These results also favor the likelihood that the active molecule is a modification of the immunoderived IL-2. This is further substantiated by our results, shown in Fig. 2, that the IL-2 immunoreactive substance in fish blood lymphocytes is a polypeptide of about 14 kDa, not a 28-kDa polypeptide as in the nerve. These two IL-2 immunoreactive polypeptides might represent products of a single gene and two transcripts that undergo alternative splicing. It is also possible that the two polypeptides represent a product of a single transcript that undergoes a post-translational modification. Our results to date strongly suggest that the molecule is an IL-2-like molecule, rather than a non-IL-2-like molecule that happens to cross-react immunologically with IL-2 (S.E. and M.S., unpublished data). The fact that fish lymphocyte CM resembles the immunoderived IL-2 of mammals would appear to support the notion that the antibodies used here recognize fish IL-2 and thus suggests that in the fish CM, the antibodies recognize a molecule that is similar to IL-2 but not identical with it. Moreover, immunological cross-reactivity was observed not only in the Western blot analysis but also in biological activity, ruling out the likelihood that the recognized molecules are unrelated.

The origin of the IL-2-like molecule identified in the CM might be resident CNS cells. Alternatively, it could be a product of invading blood cells (23, 24). IL-2 in the immune system is thought to be an important cytokine, responsible for either inhibition or progression of many immune responses (23, 25). In the nervous system, some of the observations related to the effects of IL-2 on oligodendrocytes appear to be contradictory (26–28). For example, Saneto and his colleagues (26, 27) showed that IL-2 inhibits proliferation of rat brain oligodendrocyte progenitors, whereas Benveniste and Merrill (28) showed that recombinant human IL-2 causes proliferation of rat brain oligodendrocytes.

An association between IL-2 and injury in the CNS in general and in the brain in particular has also been pointed out (29, 30). Nieto-Sampedro and Chardy (29) found IL-2 activity after brain injury. Similarly, Liang *et al.* (25) found IL-2 in brain lesions created by MPP^+ (1-methyl-4-phenylpyrimidine). In addition, up-regulation of IL-2 binding sites was observed in rat hippocampus as a result of injury (30). Nevertheless, no association between IL-2 and CNS regeneration has yet been suggested.

The observations reported in the present study suggest that an IL-2-like substance is a factor associated with regeneration. Other recent studies have also suggested that regeneration might be promoted by treatments that circumvent growth hindrance by oligodendrocytes—e.g., applications of a factor cytotoxic to oligodendrocytes such as tumor necrosis factor (30) or of antibodies directed against the oligodendrocyte-associated inhibitors (31, 32). The identification of an IL-2-like molecule as a regeneration factor could in itself be a major achievement because it would identify a crucial element needed in the development of a treatment for injured nerves. In addition, being a cytokine it could represent a factor that involves a sequence of inflammatory events—i.e., cross talk between the nervous and immune systems that might play a role in axonal regeneration. It remains to be discovered whether the IL-2-like molecules isolated from fish optic nerves can mimic completely the *in vivo* regenerative effect of the crude fish-derived preparation and, if so, whether its action *in vivo* is via elimination of oligodendrocytes. Moreover, it has still to be determined whether the IL-2-like compound found in the present study is unique to fish and is thus distinct from that in mammals, or is a tissue-specific IL-2-like compound—i.e., common to the CNS but distinct from the immune system-derived IL-2 of all species, including fish and mammals. If the latter proves to be the case, it remains to be discovered why the compound is not produced by the mammalian CNS, at least after injury.

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