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Conservation of Apoptosis as an Immune Regulatory Mechanism: Effects of Cortisol and Cortisone on Carp Lymphocytes

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This is the first study to show that apoptosis as an immune regulatory mechanism is conserved in fish, demonstrating its importance in maintaining immunological homeostasis. The data further show that this mechanism is subject to control by glucocorticosteroids. Carp plasma cortisol concentrations increase from 20 to 434 ng/ml and cortisone from 5 to 50 ng/ml within 9 min of the onset of handling stress. At basal steroid concentrations *in vitro,* cortisol, but not its conversion product cortisone, inhibits proliferation of peripheral blood lymphocytes (PBL), as measured by [^{3}H]thymidine incorporation. Induction of apoptosis in activated PBL is the apparent mechanism of cortisol action. In nonstimulated PBL cultures, apoptosis is induced by neglect (a lack of stimulating signals). Stimulation with LPS or PHA rescues lymphocytes from this type of apoptosis. Stimulated PBL populations, however, are sensitive to cortisol-induced apoptosis. Culture supernatants from activated PBL protect PBL from apoptosis by neglect, probably by supplying a growth signal. These supernatants, however, have no effect on cortisol-induced apoptosis. © 1997 Academic Press

INTRODUCTION

In mammals, bidirectional communication between the endocrine and immune systems through shared signal molecules and receptors is now well established (Blalock, 1994; Besedovsky & Del-Rey, 1996). Prominent examples are the activation of the hypothalamus–pituitary–adrenal (HPA) axis by interleukin-1 (IL-1) (Besedovsky, Del-Rey, Klusman, Furukawa, Monge-Arditi, & Kabierch, 1991) and the immunosuppressive effects of glucocorticosteroids (GS), the ultimate signal of the activated HPA axis (Cupps & Fauci, 1982). There are two hypotheses for the mechanism of immune suppression by GS. The first hypothesis is based on GS-induced suppression of cytokine production (Munck & Guyre, 1991); in this way, GS may interfere with initiation of the immune response. The second hypothesis is based on GS-induced apoptosis in immune cells (Wyllie, 1980; Cohen & Duke, 1984), resulting in impairment of the immune response. Apoptosis is a morphologically distinct process of programmed cell death, characterized by nuclear condensation and DNA fragmentation, common to the mammalian immune system (Schwartzman & Cidlowski, 1993; Penninger & Mak, 1994) and also detected in avian (Compton, Gibbes, & Swicegood, 1990) and amphibian (Ruben, Buchholz, Ahmadi, Johnson, Clothier, & Shiigi, 1994) thymocytes.

In bony fish, hematopoiesis and GS production are, in contrast to mammals, combined in one organ (the anterior or head kidney). Therefore, direct, paracrine interactions of the immune and endocrine systems are feasible. GS treatment is known to influence immune parameters in fish *in vivo,* e.g., reduced antibody titers (Wechsler, Mc Allister, Hetrick, & Anderson, 1986), reduced immunocompetence (Pickering &

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Pottinger, 1989; Houghton & Matthews; 1986), and redistribution of lymphocytes (Maule & Schreck, 1990) have been reported. Redistribution of lymphocytes was also measured following stress-induced increases in endogenous GS levels (Maule and Schreck, 1990). Cortisone present in fish plasma is most likely derived from cortisol, as teleost fish interrenal cells do not secrete cortisone (Patino, Redding, & Schreck, 1987) and cortisol is rapidly converted to cortisone in several fish tissues *in vivo* (Donaldson & Fagerlund, 1972; Patino, Schreck, & Redding, 1985). As this conversion can be a physiologically relevant regulation mechanism and cortisone may reach plasma and tissue concentrations higher than those of cortisol (Huang, Ke, Hwang, & Lo, 1983; Weisbart & Mc Gowan, 1984; Pottinger & Moran, 1993), we studied the direct, *in vitro* effects of both steroids on carp lymphocyte proliferation.

The mechanism of GS action on fish immune functions is still obscure; Tripp, Maule, Schreck, and Kaattari (1987) suggested a role for cytokines, whereas Iger, Balm, Jenner, and Wendelaar Bonga (1995) detected apoptotic leukocytes in the skin of cortisol-fed carp. Whether apoptosis as a regulator of immunological homeostasis is conserved within fish is unknown.

The aim of this study was to determine direct effects of physiological concentrations of both cortisol and its conversion product, cortisone, on carp peripheral blood leukocytes (PBL). To investigate the mechanism of action, the effect of cortisol on mitosis, necrosis, and apoptosis in PBL was analyzed using flow cytometry. To study a possible influence of cytokines on these processes, the effect of lymphocyte culture supernatants on GS-suppressed PBL was determined.

MATERIALS AND METHODS

Animals

Common carp, *Cyprinus carpio* L., were provided by "De Haar vissen," Agricultural University, Wageningen, The Netherlands. Fish were held at 23°C in recirculating, UV-treated water and were fed dry pellet food daily (Provimi, Rotterdam, The Netherlands). Individually marked adult fish, 8–18 months old and weighing around 200 g, were used in all experiments. Fish were anesthetised in 0.3 g/liter tricaine methane sulfonate (TMS; Cresent Research Chemicals, Phoenix, AZ) buffered with 0.6 g/liter sodium bicarbonate (Sigma, St. Louis, MO). Blood was collected by vena puncture of the caudal vessels.

Plasma Cortisol and Cortisone

Four groups of six fish were each kept in one aquarium and were sampled at approximately 11.00 pm. The catching procedure was designed to act as the stressor to raise plasma cortisol levels: fish were netted, anesthetised, and sampled one by one with 1.5-min intervals, inducing increasing "stress" with catch order. The last fish was resampled after 2 h to obtain an indication of the clearance time of plasma cortisol and cortisone. Cortisol was measured in full plasma by radioimmunoassay (RIA) using a polyclonal rabbit serum against cortisol-3-(*O*-carboxymethyl)oxime-BSA (Klinger, St Albans, UK), with less than 10% cross-reactivity to cortisone. Cortisol:cortisone ratios in plasma were determined using gas chromatography followed by mass spectroscopy (GC-MS) as described earlier (Vermeulen, Lambert, Lenczowski, & Goos, 1993). Briefly, steroids were extracted from 1-ml plasma samples using reversed-phase Sep Pak C18 cartridges (Waters, Milford, MA) following the manufacturer's

protocol. Steroids were eluted with diethylether and methoxime–trimethylsilyl derivatives were prepared. After hexane–acetonitril extraction, the steroids were dissolved in 20 μl hexane, of which 1 μl was injected for GC-MS analysis. Identification and quantitative ratio determination of cortisol and cortisone were based on characteristic mass fragments and retention times. Plasma cortisone levels were calculated from cortisol:cortisone ratios and absolute cortisol concentrations obtained by RIA.

Proliferation Measurements by [^{3}H]Thymidine Incorporation

PBL were isolated as described earlier (Verburg-van Kemenade, Weyts, Debets, & Flik, 1995). PBL were seeded in 96-well plates at 10^6 cells per well in 100 μl of 90% (v/v) RPMI 1640 medium in water to match carp osmolarity. Cells received no stimulus (controls), 1 μg/ml phytohemagglutinin (PHA; Difco, Detroit, MI), or 200 μg/ml lipopolysaccharide (LPS; *Escherichia coli:*B5 LPS, Difco) was added. Cells were incubated at 27°C and 5% CO_2 for 4 h, followed by addition of 100 μl culture medium (90% RPMI 1640 supplemented with 2 m*M* L-glutamine, 100,000 IU/liter penicillin-G (Sigma), 50 mg/liter streptomycin sulfate (Serva, Heidelberg, Germany), and 1% pooled carp serum (PCS)). The PCS used was derived from 10 adult carp and contained 40 ng/ml cortisol. Since the final PCS concentration in the cultures was 0.5%, the cortisol content in standard cultures was 0.2 ng/ml. Extra cortisol or cortisone was added in concentrations of 0.36–360 ng/ml. In time course experiments steroids were washed away after 2 or 16 h. Cultures were maintained for 72 h and subsequently labeled with 0.5 μCi/ml methyl-[^{3}H]thymidine (Amersham, UK) for 16 h and harvested with a Skatron semiautomatic cell harvester (Lier, Norway). Filters with retained cells were dried for 1 h at 50°C and counted in a Beckman LS 1701 scintillation counter using Beckman Ready Safe Scintillation Fluid.

Flow Cytometric Analysis of Mitosis, Necrosis, and Apoptosis

A monoclonal antibody (mAb) against the proliferating cell nuclear antigen (PCNA) was used (PC-10; DAKO, Denmark) to detect percentages of mitotic cells. PCNA protein is selectively present in the nucleus of cells in the S-phase of mitosis. It is the auxiliary protein of DNA polymerase δ and therefore necessary for DNA replication (Prelich, Tan, Kostura, Mathews, So, Downey, & Stillman, 1987; Bravo, Frank, Blundell, & MacDonald-Bravo, 1987). The anti-PCNA PC-10 mAb has been shown to be a S-phase probe (Landberg & Roos, 1991) and to react with carp (Alfei, Onali, Spano, Colombari, Altavista, & De- Vita, 1994). Apoptosis was measured by *in situ* labeling of DNA strand breaks, using TdT-mediated dUTP nick end labeling (TUNEL). Necrosis was measured by propidium iodide exclusion by healthy cells; 1 μg/ml propidium iodide was added to approximately 2×10^6 cells just before FACS analysis.

PBL were seeded in 24-well plates (5×10^6/500 μl/well) and received no stimulus (control) or were stimulated with either 1 μg/ml PHA or 200 μg/ml LPS. After 4 h, 500 μl of culture medium was added, with or without 36 ng/ml cortisol or cortisone, which was chosen as a physiological concentration. Cells were harvested after 4 h (controls only), 16 h, and 4 days and washed with PBS supplemented with 1% bovine serum albumin (BSA). For apoptosis measurements, 2×10^6 cells were fixed in 4% paraformaldehyde and labeled for DNA strand breaks with a TUNEL kit from Boehringer (Mannheim, Germany), strictly following the manufacturer's protocol. For mitosis measurements, 2×10^6 PBL were fixed in precooled (−20°C) methanol and left to stand for 15 min at room temperature, centrifuged at 700*g* for 5 min, and washed

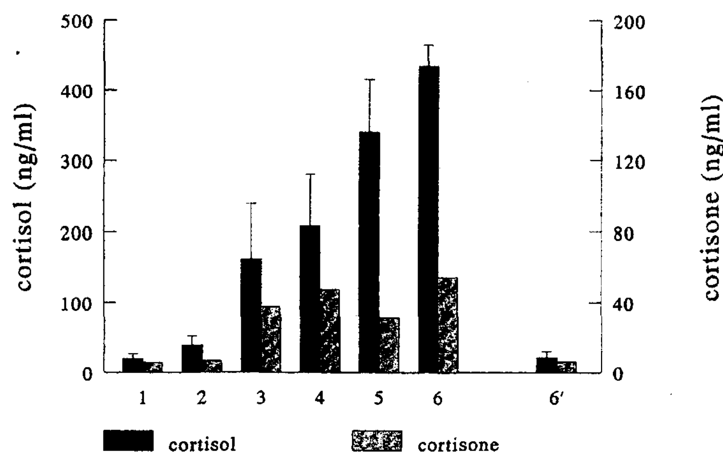

FIG. 1. Plasma cortisol and cortisone concentrations of carp caught and sampled one at a time, at 1.5-min intervals. On the *X*-axis is the catch order. Cortisol bars represent the means of four fish ± SE. Cortisone bars were calculated from cortisol:cortisone ratios measured for one group of fish. 6′ represents fish 6, resampled 2 h after the initial stressor.

twice with PBS + 1% BSA, followed by incubation with the FITC-conjugated PC-10 mAb against PCNA at 4°C for 30 min in the dark. Cells were washed twice with PBS + 1% BSA before measurement. Fluorescence intensities were measured on a FACStar flow cytometer (Beckton-Dickinson, Mountain View, CA). Only cells within the lymphocyte gate (Koumans-van Diepen, Taverne-Thiele, Rens, & Rombout, 1994) were used for further calculations.

PBL Culture Supernatants

PBL culture supernatants were isolated as described earlier (Verburg- van Kemenade, Daly, Groeneveld, & Wiegertjes, 1996). Shortly, following isolation, 10^7 PBL/ml were stimulated for 4 h in culture medium without PCS, supplemented with 10 μg/ml PHA. The PHA was washed away three times with medium, and PBL were cultured for 2 days in culture medium with 0.5% PCS. Supernatants were harvested, centrifuged in an Eppendorf centrifuge to remove cell debris, aliquoted, and kept at −20°C until use. Carp leukocyte culture supernatants have been shown to contain IL-2-like activity (Grondel & Harmsen, 1984). PBL were stimulated with LPS as described in the flow cytometry section and cultured for 16 h in the presence or absence of 20% (v/v) PBL culture supernatant and 36 ng/ml cortisol. Percentages of apoptotic PBL in these cultures were determined.

Statistics

Mean values of treatments were compared using the *t* test. Differences were considered significant when $p < .05$.

RESULTS

Plasma Cortisol and Cortisone in Control and Stressed Fish

Plasma cortisol and cortisone levels are depicted in Fig. 1. Basal carp plasma cortisol was 19 ng/ml and increased with catch order, reaching a maximum of 434

ng/ml in the 6th fish. A significant rise in plasma cortisol occurred within 6 min from catching the first fish from the tank. Basal carp plasma cortisone concentration was 5 ng/ml, rising to 50 ng/ml within 7.5 min of the onset of catching the first fish. Plasma cortisol and cortisone concentrations returned to basal levels within 2 h. Approximately 80% of cortisol in fish plasma is bound to GS binding globulins and 20% is present as unbound steroid (Flik & Perry, 1989). At half-maximum (200 ng/ml) cortisol levels, 40 ng/ml will be unbound. We therefore decided to use 10^{-7} M (36 ng/ml) cortisol in *in vitro* experiments.

Cortisol and Cortisone Effects on Proliferation in PBL Cultures

Addition of 36 ng/ml cortisol to the culture medium inhibited basal proliferation (controls) to 32%, LPS-stimulated proliferation to 39%, and PHA-stimulated proliferation to 50% after 4 days of culture (Fig. 2A). Although absolute counts measured after activation showed fish to fish differences, relative inhibition due to cortisol was always of the same order (see max. SE in Fig. 2). LPS-stimulated cells tended to be more sensitive to cortisol inhibition than PHA-stimulated cells at all concentrations. Significant inhibition of PBL proliferation was measured at cortisol levels as low as 3.6 ng/ml in all cultures. Cortisone had no effect on PBL proliferation (Fig. 2A). Inhibition of proliferation was time-dependent; incubation of PBL with 36 ng/ml cortisol for 2 h, 16 h, and for the whole culture time (4 days) caused basal proliferation to drop to 65%, 55, and 30%, respectively; LPS-stimulated proliferation to 86%, 46, and 33%; and PHA-stimulated proliferation to 79%, 65% and 45% (Fig. 2b), with no significant differences between control, LPS-, or PHA-stimulated populations.

Cortisol and Cortisone Effects on Percentages of Apoptotic, Necrotic, and Mitotic PBL

There are three possible mechanisms for cortisol-induced inhibition of PBL proliferation: a mitotic block, actual cell death, or both. To distinguish between these two possibilities, the effect of cortisol and cortisone on percentages of apoptotic, necrotic, and mitotic cells in PBL cultures was determined. Addition of cortisol significantly increased percentages of apoptotic PBL in LPS-stimulated cultures after 16 h (from 37 to 61%) and 4 days (from 24 to 58%, Fig. 3B). In PHA-stimulated cultures, cortisol had no effect on 16-h cultures. After 4 days of PHA-stimulated culture, the percentage of apoptotic PBL was significantly increased, from 22 to 36% (Fig. 3C). Cortisol had no effect on the percentage of apoptotic PBL in control cultures (Fig. 3A). In cultures without cortisol, stimulation significantly decreased percentages of apoptotic PBL in comparison to control cultures.

Percentages of necrotic PBL after 4 days of culture were approximately 10% in all cultures. Addition of cortisol had no effect on percentages of necrotic PBL (data not shown). Percentages of mitotic PBL were not significantly different in control (11%) and LPS-stimulated (7%) cultures after 4 days of culture, whereas in PHA-stimulated cultures, 52% of PBL were mitotic after 4 days. Addition of cortisol had no effect on percentages of mitotic PBL.

Culture in the presence of 36 ng/ml cortisone did not affect percentages of apoptotic, necrotic, or mitotic PBL after 4 days of culture (data not shown).

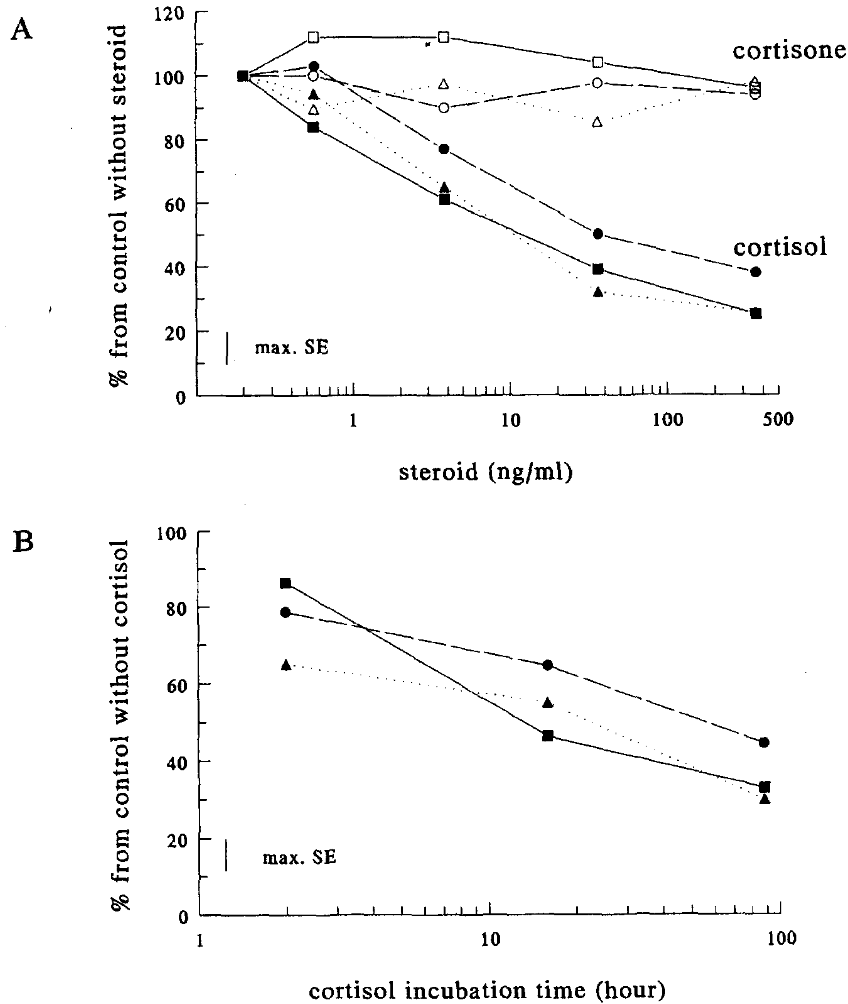

FIG. 2. Relative effect of cortisol (closed symbols) and cortisone (open symbols) on [^{3}H]thymidine incorporation by 4-day control (▲), LPS-stimulated (200 μg/ml, ■), or PHA-stimulated (1 μg/ml, ●) stimulated PBL. Control cultures without cortisol resulted in 1600 ± 722 cpm, LPS stimulation in 11,000 ± 5000 cpm, and PHA stimulation in 80,000 ± 19,000 cpm. Points represent means of six to eight fish and maximum SE is indicated (A). Effect of exposure time: cortisol (36 ng/ml) was washed away after 2 h (2h), after 16 h (16h), or was present for the whole culture time (88 h). Points represent means of four fish and maximum SE is indicated (B).

Effect of Culture Supernatants from PHA-Prestimulated PBL on PBL Apoptosis

Culture supernatants from prestimulated PBL reduced percentages of apoptotic PBL in 4-h control cultures from 11% to 3% (Fig. 4A). At 16 h of culture, PBL culture supernatants could no longer rescue control PBL from apoptosis. Therefore, to detect supernatant effects on cortisol-induced apoptosis, we looked at LPS-stimulated PBL, as cortisol affects these cultures within 16 h. Percentages of apoptotic cells in LPS-stimulated cultures after 16 h were slightly, but significantly decreased in the presence

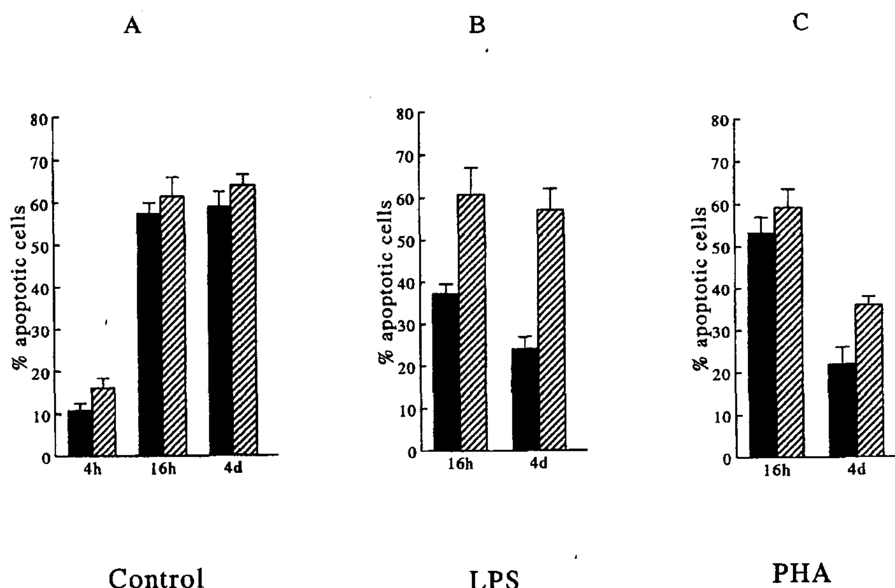

FIG. 3. Percentages of apoptotic PBL in 4-h (4h), 16-h (16h), and 4-day (4d) control (A), LPS-stimulated (200 μg/ml, B) or PHA-stimulated (1 μg/ml, C) cultures, cultured without (filled bars), or with 36 ng/ml cortisol (striped bars). Bars represent means of five fish ± SE.

of culture supernatant (Fig. 4B). The apoptosis-inducing effect of cortisol on these cells, however, was not significantly affected by PBL culture supernatants.

DISCUSSION

Cortisol and Cortisone in Vivo

Stress-induced increases in cortisone levels reported here are in line with those previously reported for carp (Huang et al., 1983; Barton & Iwama, 1991). Plasma cortisol levels, however, are higher and the rise in plasma cortisol is faster than reported for rainbow trout at continuous confinement (Pottinger and Moran, 1993) and coho salmon after short handling (Patino, Redding, & Schreck, 1987). Species-dependent differences in stress responses or cortisol/cortisone conversion rates, water temperatures, or the different stressors applied to the fish may explain these observed differences.

Cortisol and Cortisone in Vitro

Cortisol, at concentrations as low as 3.6 ng/ml (equivalent to approximately 20 ng/ml total plasma GS), directly inhibited proliferation of carp PBL *in vitro*. Inhibition of proliferation of LPS-stimulated PBL has also been shown in salmonids (Tripp et al., 1987; Espelid, Løkken, Steiro, & Bøgwald, 1996). The low concentration of cortisol that can inhibit carp PBL proliferation indicates that cortisol effects on the fish immune system are not necessarily linked to stress responses. Cortisol may be important in maintaining immunologic homeostasis. Our finding that cortisone has no effect on PBL proliferation indicates that the conversion of cortisol to cortisone may contribute to regulation of immunosuppression by cortisol. That is, the rate of prolifera-

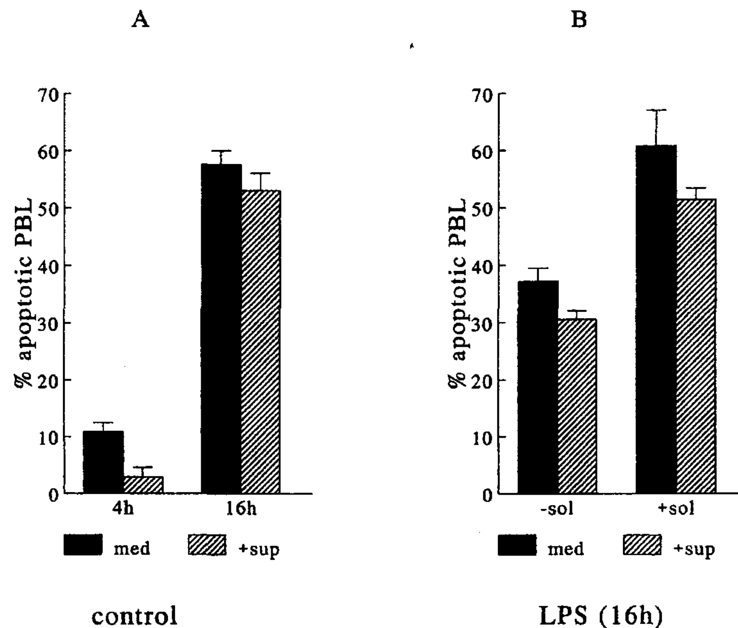

FIG. 4. Percentages of apoptotic PBL in 4-h (4h) and 16-h (16h) control cultures, cultured in normal medium (med) or in the presence of 20% (v/v) culture supernatant from PHA-prestimulated PBL (+sup) (A). Percentages of apoptotic LPS-stimulated PBL in the absence or presence of 36 ng/ml cortisol (sol) and 20% (v/v) culture supernatant. Bars represent means of three fish ± SE (B).

tion of carp PBL would depend not only on the immunological stimulus, but also on the release of cortisol and the conversion rate of cortisol to cortisone.

Mechanism of Action of Cortisol

Addition of cortisol did not alter percentages of mitotic PBL in culture, showing that inhibition of proliferation by cortisol is not due to a block of PBL mitosis. The low percentage of PCNA-expressing cells in LPS-stimulated cultures may be caused by their relative low proliferation as compared to PHA-stimulated cultures.

Cortisol-induced inhibition of PBL proliferation is therefore due to actual cell death, which can occur by either necrosis or apoptosis. Necrosis was not affected by cortisol. Cortisol, however, did increase percentages of apoptotic PBL in LPS-stimulated PBL and, to a lesser degree, in PHA-stimulated PBL. Therefore, we conclude that in fish cortisol effects on PBL are mediated by induction of apoptosis in these cells. In mammals, PHA and LPS are known to stimulate T- and B-lymphocytes, respectively. These mitogens have been applied to study lymphocyte heterogeneity of several teleost fish species (Clem, Miller, & Bly, 1991; Koumans-van Diepen, Harmsen, & Rombout, 1994). The observations that PHA stimulates Ig^- PBL and LPS stimulates Ig^+ PBL indicate similar T-B cell specificity for PHA and LPS in teleosts compared to mammals.

Cortisol-induced apoptosis in activated PBL can also explain the cortisol-induced inhibition of proliferation in nonstimulated (control) cultures; the few PBL that get activated just by culturing them may become sensitive to cortisol-induced apoptosis. The increase in apoptotic PBL in these cultures would be small, since there are only

a few activated cells, but inhibition of [^{3}H]thymidine incorporation can be significant as cells affected by cortisol are the ones responsible for the counts measured. Corroborating this hypothesis is the lack of a cortisol effect on catfish PBL apoptosis *in vitro* reported by Alford, Tomasso, Bodine, and Kendall (1994), which may be due to the fact that they measured apoptosis in nonstimulated cells. In mammals GS readily induce apoptosis in immature T and B lymphocytes, whereas mature lymphocytes are resistant to GS-induced apoptosis (Cohen & Duke, 1984). The fact that activation sensitizes mature mammalian lymphocytes to GS-induced apoptosis has only recently become clear (Brunetti, Martelli, Colasante, Piantelli, Musiani, & Aiello, 1995; Lanza, Scudeletti, Puppo, Bosco, Peirano, Filaci, Fecarotta, Vidali, & Indiveri, 1996).

Percentages of apoptotic cells increased with culture time, and high percentages of apoptotic PBL were detected in 16-h or 4-day control cultures. Apoptosis in these cultures was most likely caused by a lack of growth stimuli following the *in vivo-in vitro* transition. Withdrawal of positive signals induces apoptosis in mammalian lymphocytes (apoptosis by neglect) (Raff, 1992). LPS (and PHA) can protect PBL from this type of apoptosis. The protective effect of LPS is most likely due to actual rescue of cells from apoptosis as it is already detected after 16 h of culture, and at that time, LPS stimulation does not affect total cell numbers (pers. obs.). This indicates that the protective effect is not due to "dilution" of unstimulated (and dying) cells by newly formed, activated cells. Factors in culture supernatants from PHA-prestimulated carp PBL probably also supply growth stimuli to PBL, cultured either with or without cortisol, and thereby delay apoptosis by neglect. These supernatants, however, had no effect on cortisol-induced apoptosis. This indicates that a lack of PBL-derived factors is not the apoptosis signal, but that cortisol directly induces apoptosis in these PBL. Culture supernatants were used, as purified or recombinant fish cytokines are not available, carp cells do not cross-react with mammalian cytokines, and IL-2-like activity has been detected in carp leukocyte culture supernatants (Grondel & Harmsen, 1984). In man, reports on IL-2 protection from GS-induced apoptosis in activated PBL are contradictory; both a protective effect (Brunetti et al., 1995) and a lack of protection (Lanza et al., 1996) have been reported. In our experiments, apoptosis seems to be induced in different ways for nonstimulated and stimulated PBL: nonstimulated PBL may become apoptotic due to a lack of positive signals (death by neglect) and activated PBL are sensitive to cortisol-induced apoptosis.

Our data show that carp lymphocytes are subject to apoptotic processes. *In vitro,* physiological concentrations of cortisol increase the incidence of apoptosis, whereas the natural cortisol metabolite, cortisone, does not. These data show for the first time that apoptosis and its regulation by cortisol are similar in fish to that seen in other vertebrates, indicating that this is a conserved mechanism for immunological homeostasis.

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