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It has long been observed that stress can increase our susceptibility to infectious diseases and cancers. Recent experimental evidence suggests that this increased susceptibility is due to suppression of immunity. A variety of neuroendocrine products are released during periods of stress, and a large number of these are now known to influence leukocyte function *via* a receptor mediated process. Indeed, the capacity for neural and hormonal regulation of immunity in mammals is now well established. Direct neural control of immune reactivity is evidenced by the extensive autonomic innervation of lymphoid tissues, the effects of denervation on immunity, the presence of high affinity receptors for autonomic neurotransmitters on leukocyte membranes, and the multitude of leukocyte functions which can be altered by adrenergic and cholinergic agents *in vitro*.

This thesis demonstrates that the immune systems of certain teleosts are also susceptible to some degree of neural control. The

spleen of the coho salmon, *Oncorhynchus kisutch*, contains a rich adrenergic innervation. Depletion of peripheral catecholamine stores with 6-hydroxydopamine (6-OHDA) resulted in an increased number of antibody-secreting cells in the spleens of subsequently immunized fish. 6-OHDA treatment did not influence plasma cortisol levels, and did not affect the antibody response if administered one week after, rather than two days prior to, immunization.

Agonists of adrenergic and cholinergic receptors significantly influenced the *in vitro* activity of leukocytes from the rainbow trout, *Salmo gairdneri*. Agonists of beta-adrenergic receptors suppressed the *in vitro* antibody-secreting cell response and mitogen induced proliferation of leukocytes from the spleen, and the chemiluminescent response of pronephric leukocytes. The suppression induced by beta-receptor agonists could be blocked by beta-receptor antagonists. Agonists of alpha-adrenergic and cholinergic receptors enhanced both the *in vitro* antibody response and the chemiluminescent response. These effects were also blocked by the appropriate receptor antagonists. Alpha and cholinergic agonists had no effect on the mitogenic response. The alpha agonist-induced enhancement of the antibody response was found to be mediated by alpha-2 adrenergic receptors on rainbow trout splenic leukocytes.

Neural Regulation of Immunity in Teleosts

by

Craig M. Flory

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CHAPTER I

NEURAL MODULATION OF IMMUNITY IN TELEOSTS

INTRODUCTION

The internal defense system of mammals is remarkable in its complexity, and (at least at first sight) in its seemingly autonomous nature. This view of the immune system developed when it became evident that a variety of immune processes could be affected by immunocytes cultured *in vitro*, in isolation from other cells and tissues. This capacity (which has been essential for delineating the precise roles of leukocyte subsets and their secretory products) has led naturally to a focus on the regulatory influence of lymphoid cell products only, while ignoring other factors potentially capable of modulating immunocyte function *in vivo*. Numerous regulatory leukocytes and a growing list of cytokines are involved in the initiation, propagation, and subsequent modulation of immune reactivity. The complexity of these homeostatic mechanisms has strengthened this image of the immune system as completely self-regulating. This view, however, is no longer tenable. It is now clear that the cellular constituents of the immune system are (not surprisingly) subject to the same neural and hormonal regulatory mechanisms as other tissues. Despite a long history of anecdotal evidence for this, convincing experimental evidence has come about only recently.

STRESS AND IMMUNITY

Early notions that immunity could be influenced by the nervous system came from observations that depression, and various forms of stress, could influence human susceptibility to diseases and cancer (Solomon & Moos, 1964). Recent studies of such phenomena using various animal models have tended to focus on either how stress affects tumor development and growth (Sklar & Anisman, 1979; Riley, 1981), or how specific leukocyte functions are altered (Keller *et al.*, 1981; Stein, 1985); rarely have these two aspects been combined in the same study. Good correlations between stress, immune dysfunction, and enhanced tumor growth have been documented, however. For example, Shavit *et al.* (1985) reported that a foot shock stress in rats mediated both suppression of natural killer cell activity and enhancement of mammary tumor growth.

The precise affects of stress on immune competence depend on a variety of factors including the species involved, the form of stress, the age and history of the animal, and whether or not the stress is escapable (which gives an indication of how the ability to 'cope' influences the result). Despite this, some consistent patterns have emerged. Most striking are the findings that while acute stress usually results in immunosuppression (Monjan & Collector, 1977), chronic exposure leads to an adaptation to the stress, and even enhancement of the immune response (Solomon, 1969; Monjan & Collector, 1977; Jessop, Gale & Bayer, 1987). Furthermore, the suppressive effects of acute stress could be

ameliorated by prior exposure to chronic stress (Irwin & Livnat, 1987). Stress-induced alterations of immune capacity are not limited to the period of exposure to the stress. Significant immunosuppression one month after the termination of a six month chronic stress regime in rats was reported by Odio *et al.* (1986). This suppression was not the result of changes in T-lymphocyte numbers. While the mediators of these affects remain largely unknown, Keller *et al.* (1983), using adrenalectomized rats, found that factors other than glucocorticoids can play a role in the immunosuppressive effects of stress.

ASSOCIATIVE LEARNING AND THE IMMUNE SYSTEM

There is considerable interest at this time from both an academic and possible therapeutic standpoint in classical conditioning of the immune system. Work in this area, which actually began in the 1920's (see Ader, 1983 for review), probably constitutes the earliest line of investigation into nervous-immune system interactions. In the mid 1970's there was a revitalization of this field following the report by Ader and Cohen (1975) of a conditioned suppression of the antibody response in rats. This conditioning was achieved by pairing saccharin-spiked drinking water with intraperitoneal injections of cyclophosphamide (an immunosuppressant). Conditioned animals generated weaker immune responses than did controls (in which the exposure to saccharin and cyclophosphomide was not paired) when subsequently exposed to saccharin alone. This conditioning regime

was shown to prolong significantly the survival of mice with autoimmune diseases (Ader & Cohen, 1982).

This study was precipitated by the incidental observations of Ader (1974) of a high mortality rate in conditioned animals (when subsequently subjected to saccharrin) in an illness-induced taste aversion model of associative learning. The cyclophosphamide had been used originally as the unconditioned stimulus simply because it caused gastrointestinal upset; its immunosuppressive properties were not considered at the time. These results have been verified in several other labs (Gorczynski, Macrae & Kennedy, 1982; Neveu, Dantzer & Le Moal, 1986), and have also been repeated using anti-lymphocyte serum, which does not produce the potentially confounding side affects of cyclophosphamide (Kusnecov *et al.*, 1983). Cell mediated immune responses, including natural killer cell activity (Ghanta *et al.* 1987), are also amenable to conditioning (Gorczynski *et al.*, 1982; Bovbjerg, Ader & Cohen, 1984).

HORMONES AND IMMUNITY

The studies discussed so far beg the question of the cellular and biochemical mediators of these effects. By what mechanisms do stress, emotions, and associative learning influence immune capacity? There are two major pathways by which activity of the central nervous system can influence peripheral tissues: by the release of humoral products from the neuroendocrine system (or other endocrine glands influenced by these products) or by the direct

innervation of lymphoid tissues. The influence of endocrine products will be considered first. Although glucocorticoids are known to be important components of stress responses (Munck, Guyre & Holbrook, 1984), and have significant, largely suppressive effects on a variety of immune functions (Gillis, Crabtree & Smith, 1979; Snyder & Unanue, 1982), the focus of this discussion will be on the influence of other, less well known, immunomodulatory hormones.

Endogenous opiates (i.e., the endorphins and enkephalins) influence immunity (Wybran, 1985; Plotnikoff *et al.*, 1986), and mammalian leukocytes possess opiate receptors which are indistinguishable in their properties from those found on nerve cell membranes (Mehrishi & Mills, 1983; Blalock, Bost & Smith, 1985; Sharp *et al.*, 1987). The influences of endogenous opiates on immune processes include the augmentation of natural-killer cell activity (Mandler *et al.*, 1986), leukocyte interferon production (Brown & Van Epps, 1986), and lymphocyte proliferative responses (Gilman *et al.*, 1982). Adrenocorticotropic hormone (ACTH), which is derived from the same precursor peptide as the endogenous opiates, also affects lymphocyte function *via* specific cell-surface receptors (Johnson *et al.*, 1982; Alvarez-Mon, Kehrl & Fauci, 1985), and all of these compounds are released during periods of stress (Plotnikoff *et al.*, 1986). While the opiates are in general stimulatory (at least *in vitro*), they also appear to mediate the stress-induced suppression of immunity observed by Shavit *et al.* (1985).

Mammalian leukocytes also bear specific receptors for, and can be functionally altered by, a variety of other pituitary hormones. Growth hormone acts as a growth factor during lymphocyte activation (Snow, 1985), and enhances the mitogen-induced proliferation of splenic lymphocytes (Davila *et al.*, 1987). Argenine vasopressin and oxytocin are capable of replacing the interleukin-2 (IL-2) requirement in the production of gamma-interferon by mitogen-stimulated T lymphocytes (Johnson & Torres, 1985). Thyrotropin (TSH) augments the antibody response to sheep red blood cells (SRBC) (Kruger & Blalock, 1986).

Prolactin receptors have been identified on human T and B lymphocytes (Russell *et al.*, 1985). This molecule can enhance both antibody production and lymphocyte proliferation (Spangelo *et al.*, 1987). Bromocryptine (which causes an inhibition of prolactin secretion) prevents the induction of tumoricidal macrophages in mice, and prolactin replacement reverses this effect (Bernton, Meltzer & Holaday, 1988). This inhibition of macrophage activation, which is T-cell dependent, was determined to be the result of insufficient gamma-interferon production by T lymphocytes in prolactin-depleted animals. Melatonin, a product of the pineal gland, is able to reverse stress-induced suppression of antibody production and anti-viral activity (Pierpaoli & Maestroni, 1987). Melatonin levels vary widely over a twenty-four hour period, and the time of application was found to be critical in obtaining optimal results (evening treatments being the most effective).

Other hormones which have been implicated in the regulation of immunity include 1,25-dihydroxyvitamin D_3 (Tsoukas, Provvedini & Manolagas, 1984), thyroxin (Fabris, Muzzioli & Mocchegiani, 1982), the panreatic hormones insulin (Krug, Krug & Cuatrecasas, 1972) and glucagon (Bhathena *et al.*, 1981), and the gonadal steroids (see Grossman, 1984, for review). The influence of gonadal steroids on the immune system is evidenced by the sexual dimorphism of the immune response (with females generally having a more active immune system - and a greater predisposition to autoimmune diseases), the effects of gonadectomy and sex hormone replacement on immunity, and the immunosuppression induced by pregnancy (Grossman, 1984). This last point is of considerable importance since it appears to be the mechanism by which an organism carries a fetus, which, because of the paternal antigens it possesses, is really analogous to a foreign tissue graft (Suzuki & Tomasi, 1979).

The suppression of cell-mediated immunity during pregnancy is believed to be due largely to the heightened levels of estrogens. The affects of estrogens on lymphoid cells appear not to be direct, but rather through the induction of immunoregulatory thymic serum factors (Grossman & Roselle, 1983; Mattsson, 1983; Luster *et al.*, 1984).

LEUKOCYTE PRODUCTION OF 'NEUROENDOCRINE' HORMONES

Lymphoid cells are able to produce, as well as respond to, various 'neuroendocrine' hormones. Human alpha-interferon

preparations have been found to contain ACTH and endorphin-like activity, and human lymphocytes stained positive in an immunofluorescence assay for ACTH and gamma-endorphin (Smith & Blalock, 1981). In a subsequent paper, Smith, Meyer and Blalock (1982) reported an increase in corticosterone levels in hypophysectomized mice which were infected by the newcastle disease virus (NDV). Since the classical source of ACTH (the pituitary) had been removed, it was postulated that the adrenal stimulation was the result of lymphocyte-derived ACTH. NDV is a potent inducer of both interferon and ACTH production by lymphoid cells. Glucocorticoids normally depress ACTH production by the pituitary, and it was shown in this study that spleen cells from virally infected, but not control or dexamethasone treated, (and hypophysectomized) mice stained positive for ACTH. Dupont *et al.* (1984) described the case history of a patient with ectopic ACTH syndrome, and hypercortisolism, in which no ACTH-producing tumor could be found. ACTH levels were returned to normal, however, after the removal of some inflammatory tissue (which was laden with lymphocytes), supporting the physiological relevance of leukocyte-derived ACTH.

Endorphins, ACTH, and several other hormones are derived from a common precursor molecule, pro-opiomelanocortin (POMC). It has been documented that both monocytes (Lolait *et al.*, 1986) and lymphocytes (Westly *et al.*, 1986) can express the POMC gene, and that these cells contain mRNA for ACTH, endorphins, and enkephalins (Lolait *et al.*, 1986; Westly *et al.*, 1986; Zurawski *et al.*, 1986; Martin, Prystowsky & Angeletti, 1987). The structural

identity of these leukocyte products with their pituitary counterparts has since been confirmed (Harbour, Smith & Blalock, 1987). As alluded to previously leukocyte, as well as pituitary, production of ACTH and endorphins can be depressed by the synthetic glucocorticoid dexamethasone (Smith *et al.*, 1982). It turns out that the induction of ACTH synthesis is also controlled by similar mechanisms in both systems. Corticotropin releasing factor (CRF), the hypothalamic releasing hormone which induces the secretion of ACTH by cells of the pituitary, also induces leukocyte ACTH secretion (Smith *et al.*, 1986). Lipopolysaccharide (LPS) can also induce ACTH secretion by leukocytes. The ACTH induced by LPS, however, differs in size from the CRF-induced ACTH, suggesting that these two factors are activating different enzymes involved in the processing of POMC (Harbour *et al.*, 1987).

BRAIN LESIONS AND IMMUNITY

Another focus of research has been the influence on immunity of lesions to the hypothalamus (Keller *et al.*, *1980; Cross et al.*, 1984), or the neocortex (Biziere *et al.*, 1985), and of hypophysectomy (Berczi *et al.*, 1981; Cross *et al.*, 1982). While significant affects on *a* variety of immune functions have been reported, the mechanisms involved have not been elucidated, and are difficult to determine given the many possible side affects of such manipulations. Somewhat more controlled studies have employed the neurotoxin 6-hydroxydopamine (6-OHDA) to selectively destroy central

noradranergic pathways (Roszman & Brooks, 1985). In this particular study central (intra-cisternal) injections of 6-OHDA caused a suppression of the primary antibody response to SRBC. The potential mechanisms are still many, however, given that central noradrenergic systems are not only involved in regulating the secretion of hypothalamic releasing factors, but also send projections to the periphery.

IMMUNOREGULATION BY THE AUTONOMIC NERVOUS SYSTEM

We have seen that a large number of pituitary, adrenal, and gonadal hormones are capable of influencing immunity. As mentioned previously, the other potentially important route of communication between the central nervous and immune system is the direct autonomic innervation of lymphoid tissues. The details of this innervation, as well as the immunomodulatory affects of either surgical or chemical denervation, are discussed at length elsewhere (Flory, 1988a, chapter II of this thesis). While it is often assumed that the principal mechanism by which this innervation influences immunity is the direct effects of neurotransmitters on leukocyte function, other possibilities exist and must be considered.

One such alternative mechanism is the neural control of leukocyte trafficking through lymphoid organs. Ernstrom and Sandberg (1973) reported that intracardial injections of either norepinephrine or isoproterenol caused a significant mobilization of lymphocytes and granulocytes from the spleen of the guinea pig, and

that these effects could be blocked by specific adrenergic antagonists. Interestingly, chemical sympathectomy with 6-OHDA did not affect leukocyte release. Epinephrine administration also increased the dissemination of specific antibody-secreting cells from the spleens of immunized animals (Ernstrom & Soder, 1975). It has also been reported that adrenergic agents increase, while cholinergic agents decrease, the output of lymphocytes from lymph nodes in sheep (Moore, 1984; Moore *et al.*, 1987).

Adrenergic agents also affect the thymus. Phenylephrine enhanced and isoproterenol suppressed lymphopoiesis in fetal mouse thymic explants (Singh, 1979). Furthermore, isoproterenol and hydrocortisone acted synergistically to induce thymic atrophy (Durant, 1986). While humans are not amenable to the types of studies discussed above, several workers have investigated the effects of adrenergic administration on human peripheral blood leukocyte populations (Gader, 1974; Yu & Clements, 1976; Crary *et al.*, 1983). In the study by Crary *et al.* (1983) both an overall increase in circulating lymphocytes and altered ratios of T-lymphocyte subsets were observed. For the most part these studies have examined the effects of systemically administered agents, rather than how neural activity in these tissues affects cell trafficking. Nevertheless, they illustrate potential modes of action of neuronally-derived factors.

An important advance in establishing that autonomic neurotransmitters could have direct effects on leukocyte functions was the demonstration of the relevant receptors on leukocyte

membranes. High affinity beta-adrenergic receptors have been identified in mixed lymphocyte populations from humans (Williams, Snyderman & Lefkowitz, 1976) and mice (Johnson & Gordon, 1980). The receptors reported by Williams *et al* (1976), which were identified by [^{3}H] alprenolol binding, were present at densities of about 2000 sites/cell, and had an average affinity for [^{3}H] alprenolol of 10nM. Johnson and Gordon (1980) reported about 500 sites/cell and affinities of 1nM for dihydroalprenolol for the murine beta receptors. The beta-adrenergic receptor of human lymphocytes is of the beta-2 subclass (Conolly & Greenacre, 1977; Brodde *et al.*, 1981). Some authors have reported that T and B lymphocytes do not differ with regards to beta-receptor density (Bishopric, Cohen & Lefkowitz, 1980). Most studies indicate, however, that B-cells possess approximately twice the number of beta-receptors as T-cells (Krawietz *et al.*, 1982; Bidart *et al.*, 1983; Landmann *et al.*, 1984; Miles *et al.*, 1984). The roles of beta-receptors in thymocyte differentiation (Singh *et al.*, 1979; Rossi, 1987), and the changes in leukocyte beta-receptor numbers and affinity which occur with aging (Kohno, Cinader & Seeman, 1986), have also been investigated.

There is a dynamic modulation of leukocyte beta-receptors upon denervation or upon the *in vivo* administration of adrenergic agents. T and B lymphocytes from 6-OHDA treated mice exhibit increased receptor density (Miles *et al.*, 1984). The density of beta-receptors on human lymphocytes decreases after administration of beta-adrenergic agonists (Lee, 1978; Aarons *et al.*, 1983).

Conversely, treatment with adrenergic antagonists increases the beta-receptor numbers (Williams & Davies, 1984; Aarons & Molinoff, 1982). Mammalian lymphocytes also possess high affinity acetylcholine (Gordon, Cohen & Wilson, 1978; Maslinski, Grabczewska & Ryzewski, 1980), alpha adrenergic (McPherson & Summers, 1982), and dopamine receptors (Le Fur, Phan & Uzan, 1980; Ovadia & Abramsky, 1987).

It is also important to consider macrophages, mast cells, neutrophils, and other cell types (as well as lymphocytes) when trying to establish the cellular targets of autonomic neurotransmitters, or any other immunoregulatory agent. Beta-adrenergic (Abrass *et al.*, 1985), and muscarinic-cholinergic (Lopker *et al.*, 1980) receptors have been identified on mammalian macrophages. Stimulation of the monocyte beta-receptor induces the synthesis of complement proteins (Lappin & Whaley, 1982). Catecholamines and acetylcholine are also known to effect the release of histamine and other vasoactive amines from mast cells (Fantozzi *et al.*, 1978; Alm & Bloom, 1983). Alpha-adrenergic receptors have been found on human platelets (Cooper *et al.*, 1978), and human neutrophils possess high affinity beta-adrenergic receptors (Lad *et al.*, 1984).

Neutrophils and macrophages are vital components of an organism's first line of defense against invading pathogens, and macrophages serve also as accessory cells in lymphocyte-mediated responses. Mast cell products mediate allergic and other inflammatory responses, and can affect a variety of lymphocyte

functions. It is therefore imperative to consider these cell types as well when asking how neurotransmitters influence immune capacity; lymphocytes will not always be the primary target. The influence of catecholamines (or their analogs) on the *in vitro* generation of antibody-secreting cells in mice by SRBC (an antigen which requires the collaboration of multiple cell types in order to induce an immune response) is reviewed elsewhere (Flory, 1988b, chapter III of this thesis). In addition to the work dealing with catecholamines and immunity, there is also a good deal known about the immunoregulatory roles of the neuropeptides substance P and somatostatin (Payan & Goetzl, 1985), and vasoactive intestinal peptide (O'dorisio, Wood & O'dorisio, 1985).

IMMUNE-NEUROENDOCRINE FEEDBACK MECHANISMS

The discussion so far has focussed on how the nervous and endocrine systems influence immunity. However, communication between these two systems is truly bidirectional, and I would like now to discuss the neuroendocrine changes which take place during (and are the consequence of) an immune response. In 1975, Besedovsky and Sorkin reported that immunization of rats or mice with a variety of different T-dependent antigens resulted in increased plasma glucocorticoid levels at the peak of the immune response (as determined by numbers of plaque forming cells). In subsequent studies it was found that suppression of the response to unrelated antigens (sequential antigenic competition) could be overcome by

adrenalectomy, indicating the physiological relevance of the observed glucocorticoid increases (Besedovsky, del Rey & Sorkin, 1979). This adrenal response could be mimicked by supernatants from either Con A or allogeneically stimulated lymphocyte cultures (which contained less than 0.1% monocytes) (Besedovsky, del Rey & Sorkin, 1981). The substance which mediates this effect, glucocorticoid increasing factor (GIF), was isolated and found to bear no functional or structural similarities to known cytokines. It appears to act at the level of the hypothalamic-pituitary axis rather than directly on the adrenal gland (Besedovsky *et al.*, 1985a).

In another series of experiments immune responses were correlated with decreased norepinephrine levels in lymphoid organs (del Rey *et al.*, 1981), and the hypothalamus (Besedovsky *et al.*, 1983), and with increased neuronal activity in specific hypothalamic nuclei (Besedovsky *et al.*, 1977). The depletion of catecholamine in the periphery was restricted to lymphoid organs (and the adrenal medulla), and the degree of depletion was strongly correlated with the magnitude of the immune response (del Rey *et al.*, 1982).

In summing up these findings, Besedovsky, del Rey and Sorkin (1985b) proposed the following model for an immune-neuroendocrine feedback loop. The GIF secreted by stimulated lymphocytes functions to reduce the activity of inhibitory noradrenergic neurons in the hypothalamus (hence the reduced norepinephrine turnover rates observed here). This loss of inhibition allows increased activity, and output of releasing factors, by the hypothalamus. Carlson *et*

al. (1987) and others have shown that the decrease in hypothalamic norepinephrine occurs specifically in the paraventricular nucleus, from which corticotropin releasing factor (CRF) is derived. The CRF then stimulates the release of ACTH from the pituitary, which in turn induces glucocorticoid release from the adrenal cortex. It was suggested that this circuit may function to prevent excessive proliferation of lymphocytes with low affinity for antigen, or polyclonally stimulated lymphocytes, and thus help to prevent autoimmune and lymphoproliferative diseases (Besedovsky *et al.*, 1985b).

Similarly, the monokine IL-1 has been found to induce the secretion of ACTH (and other hormones) from the pituitary (Woloski *et al.*, 1985; Bernton *et al.*, 1987; Berkenbosch *et al.*, 1987; Sapolsky *et al.*, 1987). While the work of Sapolsky *et al.* (1987) and Berkenbosch *et al.* (1987) suggests that IL-1 is acting at the level of the hypothalamus (increasing CRF production), others have shown that IL-1 is able to stimulate pituitary cells directly (Woloski *et al.*, 1985; Bernton *et al.*, 1987). In support of the latter mechanism of action, IL-1 has been found to induce the expression of the POMC gene in pituitary cells (Brown, Smith & Blalock, 1987). Other hormones released by IL-1 include prolactin, thyrotropin, growth hormone, and leutenizing hormone (Bernton *et al.*, 1987).

Many new and intriguing concepts about the immune system have developed from research into neuroimmune interactions. One such concept, first proposed by Blalock (1984), is that the immune system

functions as a sensory organ for 'noncognitive' stimuli. We may not be consciously aware of infections, or tumors, but these may be 'sensed' by the immune system. As we have seen, leukocytes secrete products (opioids, GIF, IL-1, etc.) which can act centrally as well as peripherally. If different types of stimuli lead to the selective production of these factors, which then convey specific information to the CNS (concerning, for instance, the type of pathogen present), then feedback mechanisms from the CNS may in turn modify the subsequent immune response.

STRUCTURAL AND FUNCTIONAL SIMILARITIES BETWEEN THE NERVOUS AND IMMUNE SYSTEMS

A number of observations point to the close evolutionary and developmental ties (as well as functional interdependence) between the nervous and immune systems. Neurons and lymphocytes share a number of cell surface molecules, including Thy-1, L3T4, and OX-2 (Parnes & Hunkapillar, 1987). While the functional significance of these molecules is unknown, antibodies directed against the Thy-1 molecule induce both neural regeneration (Leifer *et al.*, 1984) and proliferation of T-lymphocytes (Gunter, Malek & Shevach, 1984). Thy-1, like other molecules which are restricted to the nervous system such as neuronal-cell adhesion molecule (N-CAM) and myelin associated glycoprotein, belong to the 'immunoglobulin superfamily' of structurally and evolutionarily related proteins (Parnes & Hunkapiller, 1987). The lymphokine IL-1, which is known to influence

the CNS in a number of ways (febrile response, ACTH secretion, Ia expression on glial cells), has now been localized in nerve fibers of the hypothalamus (Breder, Dinarello & Saper, 1988), where it may be functioning as an intrinsic neurotransmitter. The calcium and potassium channels of lymphocytes undergo changes in expression and function which are related to cellular development and behavior, and in this respect resemble the ion channels of the nervous system (Lewis & Cahalan, 1988). Nerve growth factor, a neurotrophic hormone of vital importance for the proper development of the nervous system, enhances the mitogen-induced proliferative response of splenic lymphocytes (Thorpe, Werrbach-Perez & Perez-Polo, 1987), and is chemotactic for neutrophils (Boyle *et al.*, 1985). Conversely, lectin-stimulated T cells secrete a molecule known as neuroleukin, which, along with enhancing immunoglobulin secretion, promotes the survival of cultured spinal and sensory neurons (Gurney *et al.*, 1986).

Directed exocytosis was once thought to be restricted to neurosecretory and other permanently polarized cells. It is now known, however, that both mast cells and cytotoxic T-cells degranulate preferentially in the region of the cell membrane where the stimulus occurred (Sitkovsky & Paul, 1988). T-helper cells also become polarized (reorient their microtubule organizing centers and Golgi apparatus towards the stimulus) and exhibit directed exocytosis of helper factors (Poo, Conrad & Janeway, 1988). Such 'mobile synapses' would greatly increase the degree of specificity with which various leukocyte subsets could interact, and, since this

communication is bidirectional (i.e. lymphocytes receive information in a similar manner), supports the concept of leukocytes functioning as 'mobile sensory cells' (Blalock, 1984).

A TELEOST MODEL OF NEUROIMMUNOREGULATION

This review illustrates the extent of efforts to characterize, in a small number of mammalian models, interactions between their neuroendocrine and immune systems. Work of this nature in the lower vertebrates is restricted, however, to a few studies demonstrating that adrenergic agents are able to influence antigen binding by splenic leukocytes of various amphibia (Hodgson *et al.*, 1978; Hodgson, Clothier & Balls, 1979), and that nerve terminals are present adjacent to lymphocytes in the jugular body of the frog (Zapata, Villena & Cooper, 1979). Teleosts, and especially salmonids, would make excellent models for research in neuroendocrine-immune system interactions. Salmonids have proven to be suitable models for research into various aspects of human health, including cancers (Hendricks *et al.*, 1980), and the similarities in teleost and mammalian immune systems provide a rational basis for extrapolation (Ellis, 1982). A teleost model of neuroimmunoregulation could also help to elucidate the ancestry and basic mechanisms involved in this phenomenon.

The salmonids are of significant economic importance in many parts of the world. As the pressures for larger and more efficient fish farming facilities increase, there will be an even greater need

to understand both the ways in which teleosts protect themselves from pathogenic organisms, as well as the mechanisms involved in stress-induced suppression of this resistance. Increased adrenergic activity is an important component of the stress response in fish as well as in mammals (Woodward & Smith, 1985). As we have seen, mammalian leukocytes bear specific receptors for, and can be functionally altered by, autonomic neurotransmitters, and the extensive innervation of mammalian lymphoid organs supports the physiological relevance of immunoregulation by neurotransmitters.

This thesis embodies the results of a series of investigations on the potential for autonomic regulation of immunity in two species of salmonid, the coho salmon (*Onchorynchus kisutch*) and the rainbow trout (*Salmo gairdneri*). Specific goals included elucidating the pattern of innervation in the salmonid spleen, determining the effect of denervation on the generation of an immune response, and establishing whether or not salmonid leukocytes bear cell-surface receptors for autonomic neurotransmitters. This last inquiry was approached by examining the effects of adrenergic and cholinergic agents in a variety of *in vitro* assays of leukocyte function.

CHAPTER II

Autonomic innervation of the spleen of the coho salmon, *Oncorhynchus kisutch*: A histochemical demonstration and preliminary assessment of its immunoregulatory role.

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ABSTRACT

Neuroendocrine regulation of the immune system, now a well established phenomenon in mammals, is effected in part through the autonomic innervation of lymphoid tissues. Sympathetic fibers specifically target lymphocyte-rich areas in mammalian lymphoid tissues, and their ablation or the administration of adrenergic agents can significantly alter immune responses. This study demonstrates that the spleen of the coho salmon is also richly innervated by adrenergic neurons. These neurons are seen to leave the vascular plexes - and any smooth muscle association - to enter the parenchyma of the spleen, where they often make close contact with perivascular leukocytes. Chemical denervation with

6-hydroxydopamine, which causes a severe depletion of tissue catecholamines, results in a significant enhancement of the anti-TNP-SRBC antibody-secreting cell response in the spleens of immunized fish. These results suggest that denervation is removing a constraint, in the form of inhibitory catecholamines, on the immune response. Increased sympathetic activity is an important component of the stress response in fish as well as in mammals, and may play a role in the subsequent down-regulation of immune capacity and increased susceptibility to disease often associated with fish aquaculture.

INTRODUCTION

The modulation of immunity *via* the neuroendocrine and autonomic nervous systems is now well documented in mammals (see Goetzl, 1985 for review). Despite a long history of anecdotal evidence for this functional association between the brain and the immune system, an understanding of the actual cellular and biochemical mechanisms of this interaction has been approached only recently. One focus of current research has been to elucidate the details of the extensive autonomic, largely sympathetic innervation of both primary and secondary lymphoid tissues. While much of this work has dealt with the spleen (Reilly *et al.*, 1979; Williams *et al.*, 1981; Ackerman *et al.*, 1986), other organs and tissues which have been examined in some detail include the thymus,

lymph nodes, bone marrow, appendix, and gut-associated lymphoid tissue (see Bulloch, 1985, and Felten *et al.*, 1985 for reviews). Sympathetic fibers within these tissues, apart from their expected smooth muscle associations, specifically target leukocyte-rich areas such as the periarteriolar lymphatic sheath (PALS) of the spleen (Felten *et al.*, 1985; Ackerman *et al.*, 1986).

These anatomical associations, and the well known correlations between leukocyte adrenergic (or cholinergic) receptor stimulation, cyclic nucleotide levels, and lymphocyte function *in vitro* (see Berczi, 1985, and Sanders & Munson, 1985a for reviews), strongly suggested an immunoregulatory role for this lymphoid tissue innervation. Evidence for such a role comes in part from studies which document the effects of surgical denervation of lymphoid organs, or general chemical destruction of sympathetic nerve endings (chemical sympathectomy), on immune responsiveness (see Livnat *et al.*, 1985; and Felten *et al.*, 1987a for reviews of this literature). Studies such as these point to a significant influence of autonomic neurotransmitters on immune capacity. This influence is further evidenced by the many studies demonstrating altered lymphocyte function following the *in vivo* administration of adrenergic agents (e.g. Crary *et al.*, 1983).

Except for a limited number of reports involving various amphibian models (Hodgson, Clothier & Balls, 1979; Zapata, Villena & Cooper, 1982), very little research of this nature has been undertaken using lower vertebrates. A good deal is now known, however, about the general anatomy and pharmacology of teleost

adrenergic systems (see Nilsson, 1984 for review). Work on the cod, *Gadus morhua*, has revealed that the teleost spleen receives both adrenergic and cholinergic nervous input *via* a branch of the anterior splanchnic nerve (Nilsson & Grove, 1974). While the effects of nerve stimulation, denervation, or the application of various adrenergic agents on processes such as splenic contraction in fish have been well documented (Nilsson & Grove, 1974), the possible anatomical and functional association between this splenic nervous tissue and cells of the immune system has not been studied. Teleosts, particularly salmonids, are widely used now as models for research into various aspects of human health. A teleost model of neuroimmunoregulation could also help elucidate the ancestry and basic mechanisms involved in this phenomenon, and should shed light on the potentially vital adrenergic component of stress-induced immunosuppression and mortality in fish - an important problem in fish aquaculture (Specker & Schreck, 1980).

MATERIALS AND METHODS

Experimental animals

Hatchery-bred coho salmon (*Oncorhynchus kisutch*) were housed in a flow-through, dechlorinated water system maintained at 12-14^{o}C. The coho, which ranged in size from 80-200 g, were fed to satiation daily with Oregon Moist Pellet, and exposed to a 12

h/12 h light dark cycle. All manipulations were carried out between 9 and 11 a.m. Prior to any manipulations fish were anesthetized in a benzocain bath consisting of 5 ml of a stock benzocaine solution (10% benzocaine in 95% EtOH) in 10 L of water.

Histochemistry

Adrenergic neurons and pronephric chromaffin cells were localized by the glyoxylic acid-induced histofluorescence technique of de la Torre (1980). Tissue sections were observed with a Zeiss epifluorescent microscope equipped with standard filter sets for fluorescein. Histological details and the identity of fluorescent structures were determined by studying alternate sections stained with hematoxylin and eosin (H&E).

Denervation

Chemical denervation was achieved by injections of the drug 6-Hydroxydopamine (6-OHDA-HBr, Sigma), which is known to cause a selective, reversible destruction of adrenergic nerve terminals in adult mammals. Since it is not certain that 6-OHDA affects only the adrenergic neurons of fish (see discussion) the term 'denervation' (usually reserved for surgical manipulations) will be used in place of 'sympathectomy.' The 6-OHDA, which was dissolved in 0.5 mM HCl (at 40 mg/ml) to prevent oxidation, was injected intramuscularly at doses of 10mg/kg body weight at 7 days, 15mg/kg at 6 days and

20mg/kg 2 days prior to immunization. This procedure has been shown to result in a significant reduction in splenic catecholamine levels in the cod, *Gadus morhua* (Abrahamsson & Nilsson, 1975). Controls were always anesthetized and sham denervated by intramuscular injections of 0.5 mM HCL only.

Immunizations

Forty eight hours after the last 6-OHDA injection the fish were immunized by injecting 300 ul of a 10% suspension of trinitrophenylated sheep erythrocytes (TNP-SRBC), prepared according to Rittenberg and Pratt (1969), into the dorsal sinus. The sinus was located by directing a 26g 1/2" needle anteriorly behind the last dorsal fin ray. Controls received saline injections.

Detection of the anti-TNP-SRBC response

Two weeks after immunization (time of peak response, data not shown) the numbers of specific antibody-producing (plaque-forming) cells were determined using the passive hemolytic plaque assay of Cunningham and Szenberg (1968) as modified for salmonids by Kaattari and Irwin (1985). Briefly, single cell suspensions from individual spleens were prepared by gently expressing the tissue, with frequent rinses, through a 100 mesh wire screen with a syringe plunger. The resulting suspension was then rinsed twice in L-15 medium (Gibco) containing 10% fetal calf serum. Cell viability, as

determined by trypan blue exclusion, always exceeded 95%. One hundred ul of the cell suspension, 25 ul of a 10% suspension of TNP-SRBC or SRBC in a modified barbital buffer (MBB) (Kaattari and Irwin, 1985) and 25 ul steelhead (*Salmo Gairdneri*) serum diluted 1:15 in MBB (complement source) was then mixed in individual wells of a 96 well microtiter plate. The contents of each well were then added to individual chambers, which were sealed with paraffin, and incubated for 2-3 hours at 16^{o}C before counting the plaques under a dissecting microscope.

Tissue catecholamines

The splenic levels of epinephrine and norepinephrine were quantified by high pressure liquid chromatography with electrochemical detection (HPLC-EC). Tissue sample preparation and HPLC-EC were according to the method of Trombly *et al.* (1986). Access to this equipment was kindly provided by Dr. Barbara Gordon at the University of Oregon.

Plasma cortisol

Plasma cortisol levels were quantified by radioimmunoassay according to Redding *et al.* (1984), by Samuel Bradford in the laboratory of Dr. Carl Schreck, Oregon State University.

RESULTS

Autonomic innervation of the coho spleen

The spleen of the coho salmon receives a rich adrenergic innervation (Fig. 1). While the adrenergic neurons are found mainly associated with the splenic vasculature, individual varicose fibers are also observed leaving the vascular plexes and entering the parenchyma of the spleen. These parenchymal fibers, which have no apparent smooth muscle association (as determined from H&E stained sections), often make close contact with aggregations of the yellow autofluorescent cells and melanomacrophages which are found clustered near the major blood vessels.

Chromaffin cells of the pronephros

As in the plaice (Grove *et al.*, 1972) and the cod (Abrahamsson & Nilsson, 1976), the chromaffin cells of the coho pronephros (Fig. 2) are located in the walls of the posterior cardinal vein which passes through this tissue.

Effects of chemical denervation with 6-OHDA on tissue catecholamine levels

Chemical denervation with 6-OHDA significantly reduced the splenic levels of both epinephrine and norepinephrine as determined

by HPLC-EC (Fig. 3). This reduction was also evidenced by a complete absence of specific fluorescence in the spleens of treated fish (not shown). Treatment with 6-OHDA did not however reduce fluorescence intensity in the pronephros, suggesting that the chromaffin cells of fish, as in mammals, are insensitive to this drug. In contrast to the cod, where epinephrine is the major splenic catecholamine (Abrahamsson & Nilsson, 1975), norepinephrine dominates in the spleen of the coho (Fig. 3).

Effects of denervation on the splenic plaque forming cell (PFC) response to T-SRBC.

The overall number and percentage of specific PFC were increased significantly in the spleens of fish denervated prior to immunization (Fig. 4). Whereas denervation prior to immunization resulted in an enhanced immune response, there was no effect if the denervation injections were begun one week after immunization (Fig. 5). The effect of 6-OHDA treatment seems therefore to be most critical during early stages of the immune response.

Plasma cortisol levels

Plasma cortisol was monitored to ascertain whether the immunological changes observed could be due to long term effects of denervation on circulating glucocorticoid levels. Cortisol levels did not however differ in the two groups when assayed one week after

denervation (Fig. 6). Whether earlier, more acute elevations in cortisol due to handling during the denervation procedure are affected by the presence of 6-OHDA is unknown.

DISCUSSION

This study has shown that the salmonid spleen contains an extensive adrenergic innervation, and that autonomic neurotransmitters may play an important role in the regulation of teleost, as well as mammalian, immune systems. The exact anatomical relationship between the adrenergic innervation of the coho spleen described here and the splenic lymphocytes remains to be established. Teleosts do not possess the well developed PALS characteristic of the mammalian spleen which, at least in rodents, is specifically targeted by sympathetic fibers (Felten *et al.*, 1985). Examinations of H&E stained sections of the coho spleen suggest that lymphoid aggregations are, however, closely associated with the blood vessels. Zapata (1982) also documented diffuse lymphoid tissue layers surrounding arteries and melanomacrophage centers in *Rutilus rutilus* and *Gobio gobio*, and Agius (1980) pointed to the anatomical and functional analogies between fish melanomacrophage centers and the germinal centers characteristic of mammalian secondary lymphoid tissues. These findings suggest that the melanomacrophage centers would be likely targets for neurons modulating lymphocyte and/or accessory cell function in fish, and

these regions do indeed appear to be well innervated. The yellow autofluorescent cells observed in the spleen of the coho salmon appear identical to those seen in mammalian lymphoid tissues. In mammals these cells, which have been tentatively identified as mast cells, are specifically targeted by sympathetic neurons (Williams *et al.*, 1981). Trabeculae and the smooth muscle often associated with them, which also tend to be well innervated in mammals (Felten *et al.*, 1985), are lacking or indistinct in salmonids.

By combined immunostaining for tyrosine hydroxylase (TH), a key enzyme in catecholamine biosynthesis (and therefore a good marker of sympathetic nerve fibers), and for a variety of leukocyte cell-surface markers, Felten *et al.* (1987b) have established that sympathetic (TH positive) fibers in the rat are closely associated with both T and B lymphocytes, and macrophages, in different compartments of the splenic white pulp. The ontogenetic development of this innervation has been found to parallel closely the development of the PALS (Ackerman *et al.*, 1987), suggesting its importance in the formation, as well as subsequent regulation, of lymphoid tissues. Felten and Olschowka (1987) have documented, at the electron microscope level, apparent synaptic contacts between TH positive nerve terminals and lymphocytes in the white pulp. These studies, along with the *in vivo* dialysis measurements of up to one uM norepinephrine in the rat spleen (Felten, Housel & Felten, 1986), provide the strongest anatomical evidence yet for the immunoregulatory role of lymphoid tissue innervation in mammals. Although subsets of teleost leukocytes can be identified on a

functional basis (e.g. Ig+ vs. Ig-, antibody secreting, or exibiting helper or accesory cell activity) (Miller, Sizemore & Clem, 1985), the lack of specific reagents for phenotypic markers of the various cell types precludes, at least for now, the type of analysis discussed above.

Although the sympathetic-parasympathetic division of the mammalian autonomic nervous system can be soundly based on anatomical (if not always functional) criteria, such a distinction cannot be made easily in teleosts (Nilsson, 1983). For this reason the functional terms 'adrenergic' (possessing catecholamines, either epinephrine or norepinephrine) and 'cholinergic' (possessing acetylcholine) are used to describe the patterns of innervation observed here. This distinction merely reflects the class of transmitter present, and does not relate to the origins of these fibers.

In mammals 6-OHDA selectively lesions adrenergic (sympathetic) nerve terminals, leaving cholinergic neurons intact (Kostrzewa & Jacobowitz, 1974). There is, however, an apparent difference in the selectivity of 6-OHDA between fish and mammals. This is evidenced by the cholinergic supersensitivity (Holmgren & Nilsson, 1976) and decreased choline acetyltransferase activity (Winberg, Holmgren & Nilsson, 1981) observed in the spleens of 6-OHDA treated atlantic cod. While these studies are certainly not conclusive, it is possible that in fish this drug affects acetycholine levels as well. Based on these findings, Holmgren and Nilsson (1982) have suggested that teleosts may possess undifferentiated autonomic neurons

containing both acetylcholine and catecholamines. There is, however, no rigorous evidence in either fish or mammals for this apparent contradiction of Dale's Principle.

The immunoenhancing effects of chemical denervation with 6-OHDA observed in this study are consistent with the results of similar studies in mammals (e.g. Besedovsky *et al.*, 1979; Williams *et al.*, 1981), which suggest a general inhibitory influence of catecholamines on cells of the immune system. The results of such manipulations in the rodents, however, vary depending on the method and timing of denervation. Surgical denervation of the spleen (Besedovsky *et al.*, 1979) or lymph nodes (Alito *et al.*, 1987), or chemical sympathectomy of neonatal animals (with immune challenge of adults) (Besedovsky *et al.*, 1979; Williams *et al.*, 1981), results in a significant enhancement of the PFC response to a T-dependent antigen (SRBC). In contrast, the antibody response to T-dependent antigens is either suppressed (Kasahara, Tanaka & Hamashima, 1977; Hall *et al.*, 1982; Felten *et al.*, 1984) or unchanged (Miles *et al.*, 1981) following chemical sympathectomy of adult animals.

Other differnces in protocol should be kept in mind in evaluating these results. Hall *et al.* (1982) were measuring serum antibody titers, rather than numbers of antibody secreting cells as in the other studies (although Kasahara *et al.* (1977) did observe suppression of both titer and PFC number), and in the study by Miles *et al.* (1981) phosphorylcholine-KLH, rather than SRBC, was used as the T-dependent antigen. Variables such as the

form of stimulating antigen, tissue examined, and immune parameter measured may all be important in determining how catecholamines exert their influence on the immune system. Also documented are altered T cell mediated responses following sympathetic denervation (Madden *et al.*, 1986; Alito *et al.*, 1987, and see Felten *et al.*, 1987 for review), and a suppression of the antibody response after parasympathetic denervation (Alito *et al.*, 1987). Miles *et al.* (1981) observed enhancement of the response to two different T-independent antigens as well. Work in progress suggests that the enhancement of PFC responses of denervated coho salmon is not due to altered kinetics, although a change in sensitivity to the antigen has not been ruled out. Preliminary *in vitro* studies demonstrate that the results obtained *in vivo* may indeed be due, at least in part, to the presence of adrenergic and cholinergic receptors on salmonid lymphocytes.

There are also many indirect ways, other than direct effects on lymphocyte function, in which altered tissue or circulating catecholamine levels could affect antibody production and other aspects of immunity. These range from effects on accessory cells (Lappin & Whaley, 1982), mast cells (Alm & Bloom, 1982), and other lymphoid cells, to effects on blood flow and lymphocyte trafficking (Ernstrom & Soder, 1975; Moore, 1984), to secondary changes in the levels or potency of other immunomodulatory hormones or lymphokines (Tuomisto & Mannisto, 1985). While the evidence for a direct effect of catecholamines on mammalian lymphocytes is certainly convincing (Sanders & Munson, 1985a; Livnat *et al.*, 1985), the picture *in*

vivo is likely to be considerably more complex and involve at least some contribution from all of these factors. Catecholamines are also not the only products of adrenergic neurons: their coexistence and cosecretion with a variety of neuropeptides has been documented for the mammalian peripheral nervous system (Holkfelt *et al.*, 1986). Since many of these peptides are known to affect immunity (Morely *et al.*, 1987), their altered levels following surgical or pharmacological denervation, or during periods of increased autonomic activity, could also affect immune capacity.

Aside from adrenergic neurons, the chromaffin tissue of the teleost pronephros is another important source of potentially immunoregulatory catecholamines. This tissue, analogous to the mammalian adrenal medulla, delivers catecholamines systemically. While epinephrine is the most prevalent catecholamine in the adrenal medulla of mammals, the ratio of epinephrine to norepinephrine in the chromaffin tissue of different teleost species varies widely (Nilsson, 1983). Preliminary studies show approximately equal levels in the pronephros of the coho salmon (data not shown). An interesting association in the teleost pronephros is the close juxtaposition of lymphoid and interrenal cells. Mammalian monocytes and lymphocytes are known to secrete, among other 'neuroendocrine' products, adrenocorticotropic hormone (ACTH) (Weigent & Blalock, 1987), which regulates glucocorticoid production in the adrenal cortex of mammals and the interrenals of fish. If fish cells also elaborate these factors, then appropriately stimulated pronephric

leukocytes may directly affect the local concentrations of these important immunomodulatory hormones.

Besides their potential for direct effects on leukocyte function, circulating catecholamines may also affect the immune system *via* their interaction with the hypothalamic-pituitary-adrenocortical (interrenal) axis. While there is still controversy over whether the effect is generally stimulatory or inhibitory (see Tuomisto & Mannisto, 1985 for review), adrenergic agents are known to play a role in the release of both CRF (corticotropin releasing factor) and ACTH in mammals. Altered levels of ACTH would then affect circulating glucocorticoid levels. As with adrenergic neurons, chromaffin cells can elaborate compounds other than catecholamines. They also secrete substantial amounts of enkephalins (Livett *et al.*, 1981), which are known to influence immunity (Morely *et al.*, 1987).

Knowledge of how immune capacity is altered by stress is of significance for not only human health, but for fisheries as well since stress-induced immunosuppression may be an important cause of disease and mortality. Normally adaptive physiological responses to stress are thought to be exacerbated, and become deleterious, by the artificial conditions and manipulations involved in intensive fish farming. The 'stress response' is a very complex phenomenon, the details of which can differ greatly depending on the stressor involved, whether it is chronic or acute, the species, the history of the animal and a host of other variables. Ellis (1981), in a review of the effects of stress on a variety of immune functions in

both fish and mammals, has made the important point that global suppression is not necessarily the rule: immune hyper-reactivity can also occur. The glucocorticoids, aside from their critical roles in secondary metabolism, are known to have significant, largely suppressive effects on a great many aspects of immunity in mammals. There is now increasing evidence in fish as well for their immunosuppressive role at physiological concentrations both *in vivo* (Pickering & Pottinger, 1985; Maule, Schreck & Kaattari, 1987) and *in vitro* (Tripp *et al.*, 1987). While these products of the adrenal cortex (and fish interrenals) are certainly the most well known and best studied of the 'stress hormones', they are not the only ones.

Increased adrenergic activity may also mediate these stress-induced alterations in the immune system. Sizable, though relatively acute increases in circulating catecholamine levels have been well documented in salmonids subjected to crowding or handling stresses (Woodward & Smith, 1985), and this study gives preliminary evidence that such increases could influence immune capacity. Whether or not this influence is of sufficient magnitude and duration to actually be of detriment to the health of the animal still needs to be determined. It is important to note that immune dysfunction is not the only potentially harmful side effect of increased adrenergic activity. General circulatory (and therefore respiratory) functions, and osmoregulatory ability, are also affected (Mazeaud & Mazeaud, 1981).

ACKNOWLEDGEMENTS

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Figure II.1. Autonomic innervation of the spleen of the coho salmon. (a) A length of the splenic artery (outlined by arrowheads) with its associated adrenergic nerve plexus just prior to entering the spleen (magnification = 400x). (b) Within the spleen adrenergic neurons (green fluorescence) are seen mainly associated with the branching vasculature. In close proximity to these blood vessels, and their affiliated nerve supply, are aggregations of melanomacrophages (arrows) and yellow autofluorescent cells. (magnification = 100x). (c) Individual adrenergic fibers with no apparent smooth muscle association (as determined from adjacent H&E stained sections) making close contact with the melanomacrophage centers and autofluorescent cells (magnification = 400x). (d) Individual adrenergic fibers (small arrows) leaving a vascular plexus (large arrow) and entering the parenchyma of the spleen (magnification = 400x).

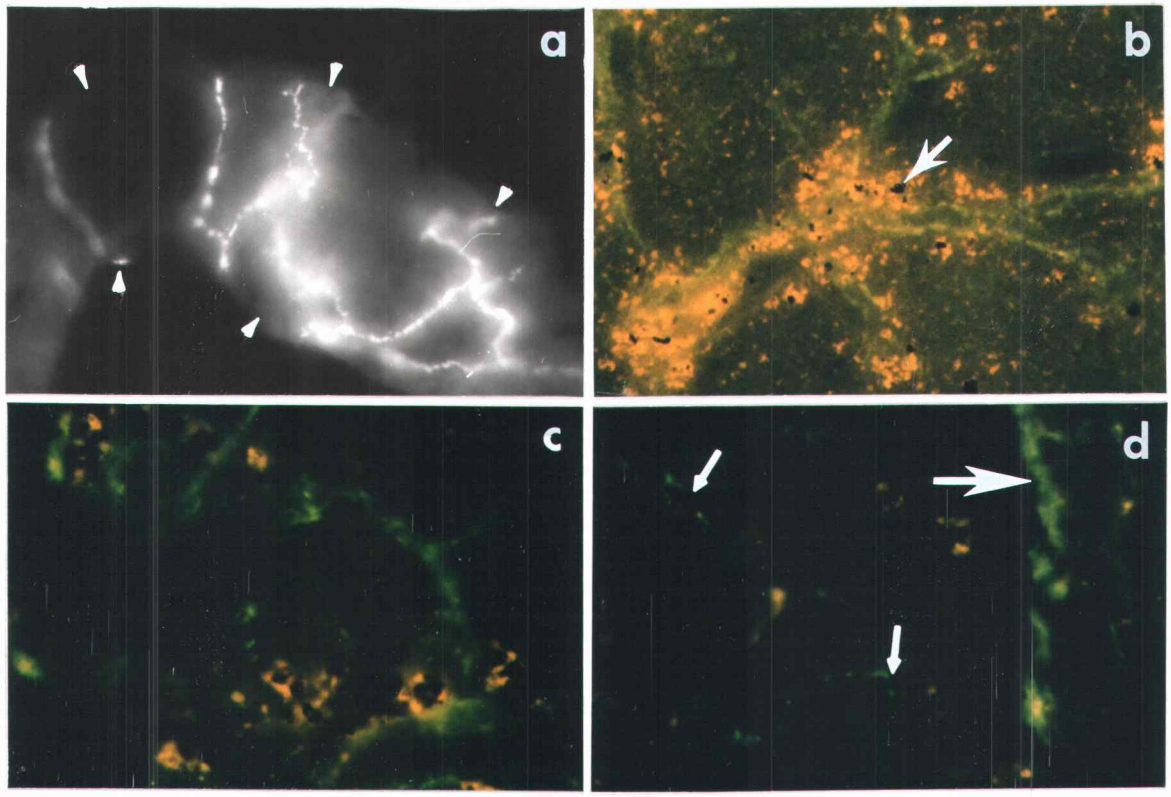

Figure II.1

Figure II.2. Histochemistry of the pronephros of the coho salmon. (a) The brightly fluorescent chromaffin tissue (CT) surrounding a branch of the posterior cardinal vein (PCV) which passes through the pronephros (magnification = 100x). (b) An adjacent, H&E stained section seen under higher magnification illustrates the large, irregular nuclei (arrows) characteristic of chromaffin cells. In the upper right can be seen erythrocytes in the lumen of the cardinal vein (magnification = 400x).

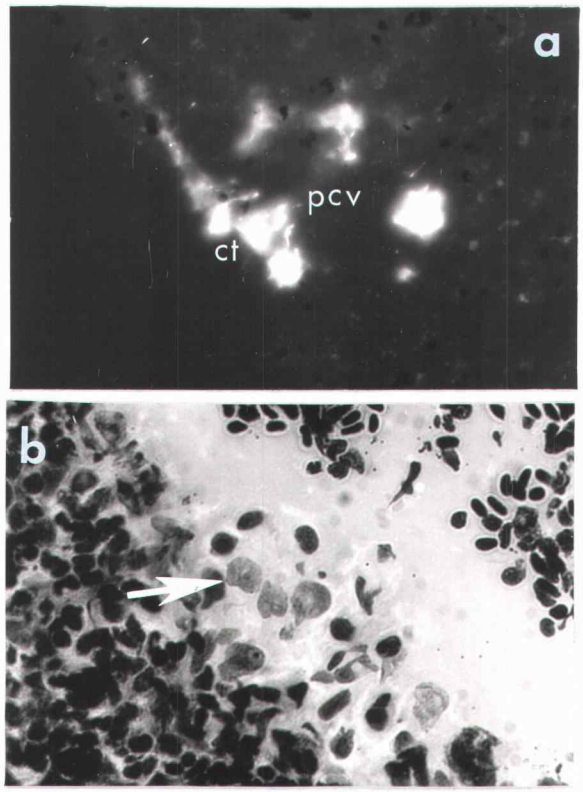

Figure II.2

Figure II.3. Effect of chemical denervation with 6-OHDA on splenic catecholamine levels in the coho salmon. Tissue samples were taken seven days after the last 6-OHDA injection. Bars represent mean +/- 1 S.D. of four fish.

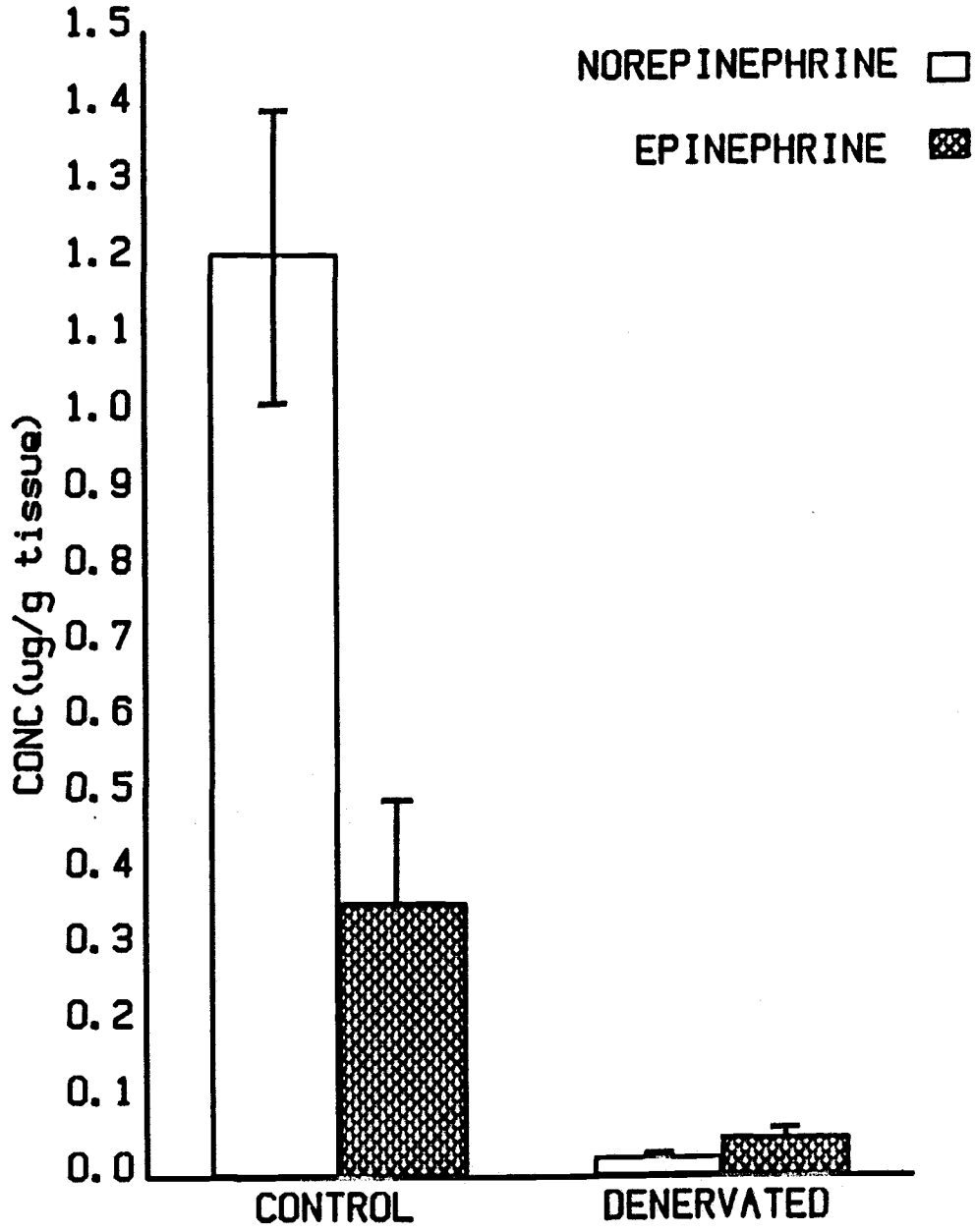
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CONC(ug/g tissue)
NOREPINEPHRINE
EPINEPHRINE
CONTROL
DENERVATED

Figure II.3

Figure II.4. Effects of denervation on the splenic plaque forming cell (PFC) response to TNP-SRBC in the coho salmon. The results are given both in terms of PFC/10^6 leukocytes (1) and total PFC/spleen (2). Open bars represent the number of PFC observed using haptenated sheep erythrocytes as target cells, and solid bars the response to unhaptenated erythrocytes (specificity control). For each graph "A" represents the response of unimmunized fish, "B" the response of immunized, non-denervated fish and "C" the response of fish immunized two days following chemical denervation with 6-OHDA. "*" between adjacent treatments denotes significant ($p<.05$) difference, for "**" $p<.01$. Student's t test. Bars represent mean +/- 1 S.D. of five fish.

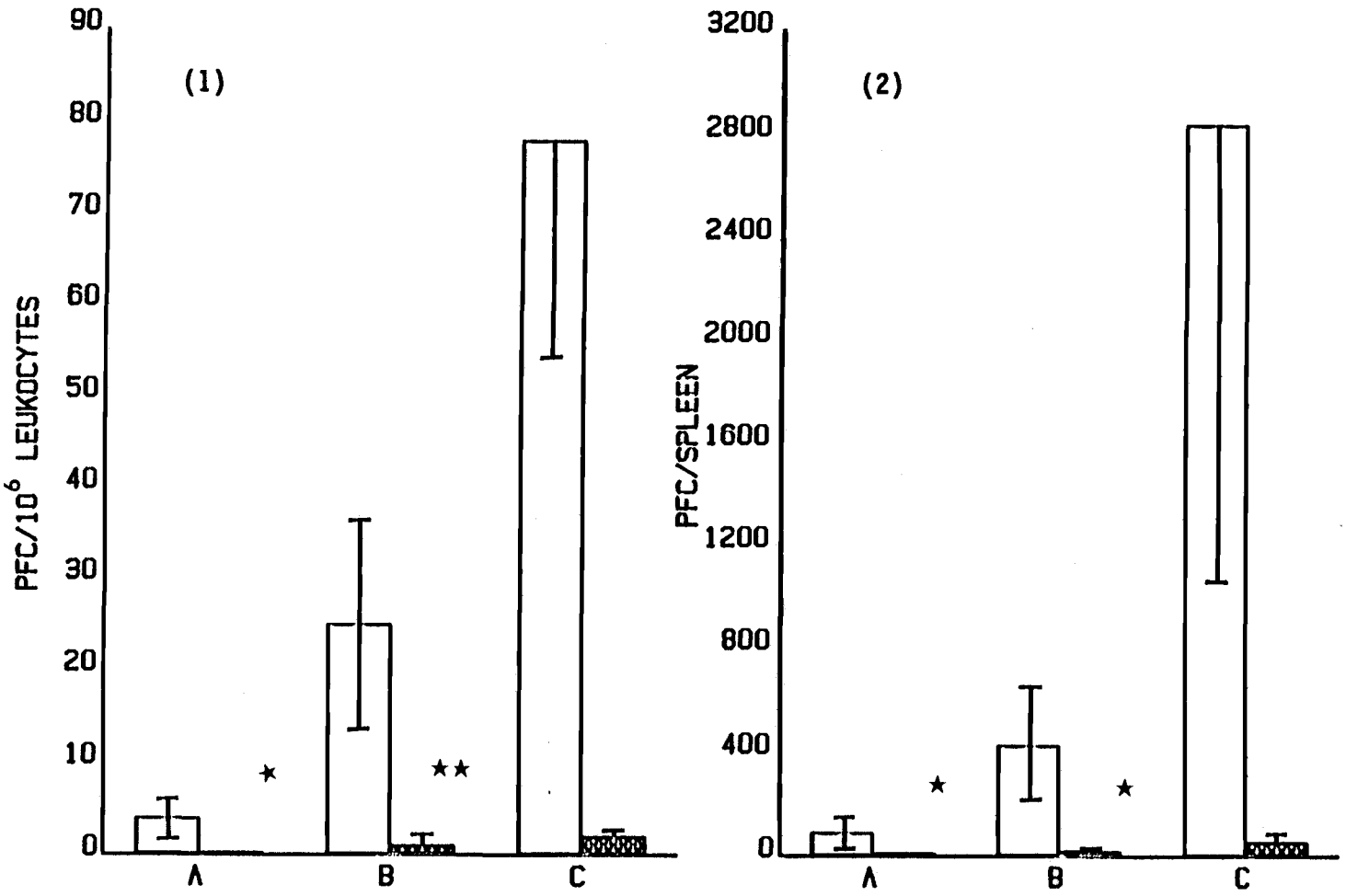
(1)
PFC/10^6 LEUKOCYTES
90
80
70
60
50
40
30
20
10
0
A
B
C
(2)
PFC/SPLEEN
3200
2800
2400
2000
1600
1200
800
400
0
A
B
C

Figure II.4

Figure II.5. Effect of timing of denervation on splenic PFC response to TNP-SRBC in the coho salmon. A) Immunized, non-denervated fish. B) Fish denervated immediately prior to immunization as before. C) Denervation begun seven days after immunization. $p < .01$. Bars represent mean +/- 1 S.D. of five fish.

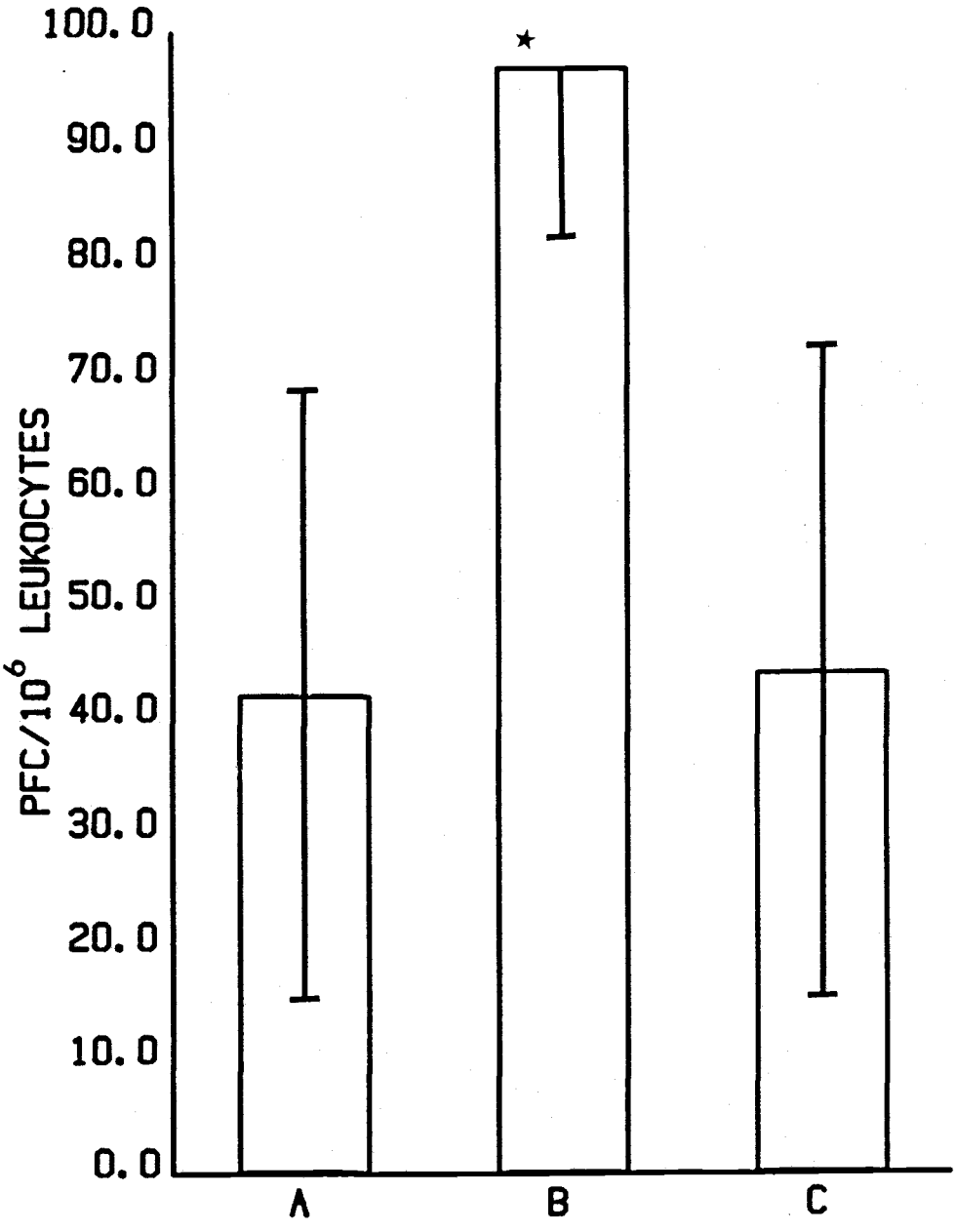
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
PFC/10^6 LEUKOCYTES
★
A
B
C

Figure II.5

Figure II.6. Effect of denervation on plasma cortisol levels in the coho salmon. Plasma samples were taken seven days after the last 6-OHDA injection. Bars represent mean +/- 1 S.D. of five fish.

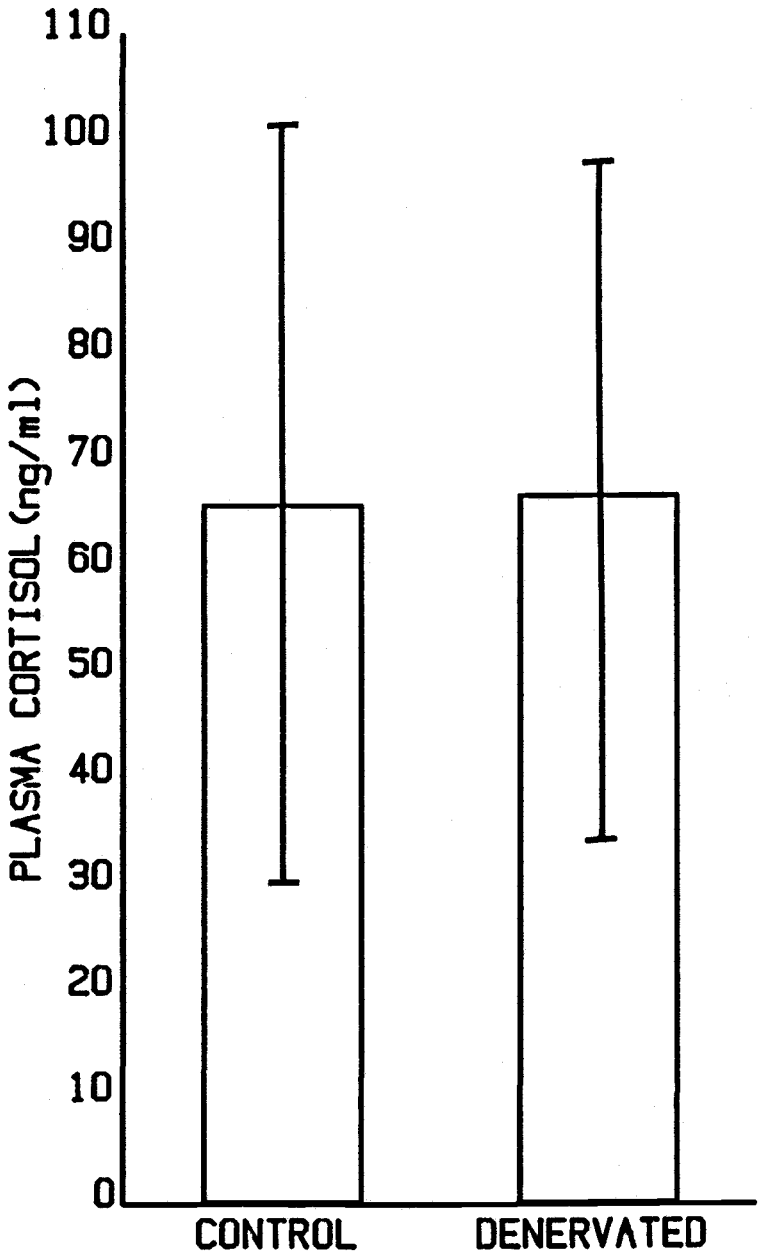
110
100
90
80
70
60
50
40
30
20
10
0
PLASMA CORTISOL (ng/ml)
CONTROL
DENERVATED

Figure II.6

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CHAPTER III

Effects of adrenergic and cholinergic agents on the *in vitro* antibody response of splenic leukocytes from the rainbow trout, *Salmo gairdneri*.

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ABSTRACT

High affinity adrenergic and cholinergic receptors are present on mammalian leukocytes, and autonomic neurotransmitters (or their analogs) can affect a variety of leukocyte functions *in vitro*. These findings constitute important evidence for the immunoregulatory role of the extensive autonomic innervation of mammalian lymphoid tissues. There are, however, few studies on how the peripheral nervous system might affect immunity in the lower vertebrates. This study demonstrates that adrenergic and cholinergic-receptor agonists significantly influence the *in vitro* induction of antibody-secreting cells in cultures of splenic leukocytes from the rainbow trout, *Salmo gairdneri*.

Beta-adrenergic agonists suppress, while alpha-adrenergic and cholinergic-receptor agonists enhance, this *in vitro* response to the T-independent antigen TNP-LPS. Specific receptor antagonists block these effects. These results, together with the previous finding that chemical sympathectomy of coho salmon results in an enhanced *in vivo* antibody response, demonstrate the ability of autonomic neurotransmitters to affect immune function in teleosts *via* leukocyte receptors for these factors.

INTRODUCTION

In mammals, the capacity of the nervous system to regulate immune function, and *vice versa*, is evidenced in part by the extensive autonomic innervation of lymphoid organs (Bulloch, 1985; Felten *et al.*, 1985), the alterations in immune function which result from either general chemical sympathectomy (Williams *et al.*, 1981) or the surgical denervation of lymphoid organs (Alito *et al.*, 1987), and the multitude of neuroendocrine changes which take place during an immune response (Besedovsky, del Rey & Sorkin, 1985). The direct innervation of lymphoid tissues, and particularly the close association of nerve terminals with specific subsets of leukocytes in secondary lymphoid organs such as the spleen (Ackerman *et al.*, 1986; Felten *et al.*, 1987; Felten & Olschowka, 1987), suggests that at least one mechanism of neural-immune system interactions is through direct effects of autonomic

neurotransmitters on leukocyte function. Specific, high affinity receptors for neurotransmitters have been identified by radioligand binding on mammalian T and B lymphocytes, macrophages, neutrophils, mast cells, and platelets (see Berczi, 1986 for review), and much is known about the effects of adrenergic and cholinergic agents on such processes as antibody production, lymphocyte proliferation, and cytotoxicity *in vitro* (see Sanders & Munson, 1985a, and Berczi, 1986, for reviews).

Few reports of this nature have dealt with the lower vertebrates. I have shown previously (Flory, 1988a) that the spleen of the coho salmon is also richly innervated by adrenergic neurons, and that chemical denervation with 6-hydroxydopamine (if applied prior to immunization with trinitrophenylated sheep erythrocytes (TNP-SRBC)) leads to a significant increase in both the overall number and percentage of anti-TNP antibody- secreting cells from the spleen. It is unknown if this enhancement is due directly to altered neurotransmitter-leukocyte interactions, or to more indirect effects of denervation such as changes in blood flow and cell trafficking, or secondary changes in the levels of other immunoregulatory hormones or lymphokines. The purpose of this study was to determine if adrenergic and cholinergic agents are able to influence the *in vitro* stimulation of antibody-secreting cells in splenic lymphocyte cultures of rainbow trout, *Salmo gairdneri*.

MATERIALS AND METHODS

Antigens

Trinitrophenylated lipopolysaccharide (TNP-LPS) was prepared by the method of Jacobs and Morrison (1975). The LPS (*E. coli* 055:B5 W) was obtained from Difco, Detroit MI. Sheep red blood cells (SRBC) were haptenated for use in the plaque assay by the method of Rittenberg and Pratt (1969). The TNP-LPS was used routinely at a final concentration of 1.0 ug/ml. Initial studies indicated that unhaptenated LPS at this concentration did not produce any polyclonal stimulation of anti-TNP-antibody-secreting cells, and that plaque development could be inhibited by TNP-Lysine.

Drugs

All drugs used in these studies were prepared immediately prior to use in complete tissue culture medium. (-)-Norepinephrine bitartrate, (-)-epinephrine bitartrate, (-)-isoproterenol bitartrate, DL-propranolol hydrochloride, L-phenylephrine hydrochloride, yohimbine hydrochloride, carbachol, and atropine were all Sigma products. The phentolamine hydrochloride was from Ciba-Geigy Corp., Summit, N.J.

Culture System

The culture system described here is modified from that of Kaattari *et al.* (1986). The complete tissue culture medium (TCM) consists of L-15 medium with L-glutamine (0.3 mg/ml) (Sigma) plus 0.2% sodium bicarbonate, 5% heat inactivated fetal calf serum, $5x10^{-5}M$ 2-mercaptoethanol, 10ug/ml each of adenosine, cytosine, guanosine and uracil, and 100ug/ml gentamycin. Single cell suspensions were prepared, rinsed twice in holding medium (TCM with 5% fetal calf serum and gentamycin only) and resuspended in TCM to a final concentration of $2.5x10^7$ cells/ml. Pooling of spleen cells from two to three fish was generally required to obtain the necessary cell numbers. Two hundred ul of the cell suspension ($5x10^6$ leukocytes) were then added with antigen, and with or without drugs, to individual wells of flat bottom, 24 well tissue culture plates (Falcon) in a final volume of 1.0ml. Controls and each treatment were run in triplicate. The culture plates were then incubated in a humidified atmosphere of 5% CO_2, 95% air, at 16^oC for 8 to 9 days before determining the numbers of anti-TNP specific antibody-secreting cells. This length of incubation results in an optimal response in control cultures.

Plaque Forming Cell Assay

The numbers of specific antibody-producing (plaque-forming) cells, or PFC, were determined using the passive hemolytic plaque

assay of Cunningham and Szenberg (1968) as modified for salmonids by Kaattari and Irwin (1985). At the end of the incubation period the cells from each well were harvested, rinsed once, and resuspended in 2.0 ml holding medium. One hundred ul of the cell suspension, 25 ul 10% TNP-SRBC in modified barbital buffer (MBB) (Kaattari & Irwin, 1985), and 25 ul steelhead (*Salmo gairdneri*) serum diluted 1:14 in MBB (complement source) were then added to individual wells of a 96 well plate, mixed, and added to the chambers. The chambers were then sealed with wax and incubated for three hours at $16^{o}C$ before counting the plaques under a dissecting microscope. Initial studies showed no difference in the results if expressed either as PFC/10^{6} recovered leukocytes, or as PFC/culture, which was subsequently used.

RESULTS

Initial experiments using L-norepinephrine and L-epinephrine, at concentrations from 10^{-4} - 10^{-9}M, gave highly variable and inconsistent results. In any one experiment the effect of each compound was similar, but whether this effect was suppression or enhancement, and which dose of drug had the greatest effect, varied with each experiment. Catechols are highly susceptible to oxidation and metabolic degradation, and this lability may explain in part the inconsistencies noted above. The use of various synthetic adrenergic and cholinergic agents in subsequent experiments avoided these

problems of stability, and gave consistent, repeatable results. Each set of data shown is a representative example of at least three replicate experiments.

Effects of beta-adrenergic agents

The beta-adrenergic receptor agonist isoproterenol significantly suppressed the *in vitro* PFC response to TNP-LPS at concentrations from 10^{-4} - 10^{-7}M (Fig. 1). Even at the highest concentrations of isoproterenol there was no effect on cell viability (as determined by trypan blue exclusion). At 10^{-5}M, propranolol, a competitive antagonist for beta-adrenergic receptors, effectively blocked the suppressive effect of 10^{-5}M isoproterenol. Propranolol alone at this concentration did not affect the PFC response.

Effects of alpha-adrenergic agents

The alpha-adrenergic receptor agonist phenylephrine significantly enhanced the PFC response at concentrations from 10^{-4} - 10^{-11}M, with the peak response occurring at approximately 10^{-9}M (Fig. 2). Phentolamine, widely used as a nonselective antagonist of alpha receptors did not block the phenylephrine-induced enhancement, and was found to have agonist activity itself in this system. At 10^{-6}M, yohimbine (an alpha-2 selective

antagonist) did not affect the response on its own and successfully blocked the enhancement due to 10^{-9}M phenylephrine.

Effects of cholinergic agents

The cholinergic agonist carbamylcholine chloride (carbachol) also significantly enhanced the anti-TNP PFC response at concentrations from 10^{-5} - 10^{-11}M (Fig. 3). The dose of carbachol which gave the greatest response was somewhat higher and less consistent than for phenylephrine, and ranged from 10^{-5} - 10^{-7}M in different runs. The muscarinic- cholinergic receptor antagonist atropine, at 10^{-5}M, blocked the effect of 10^{-5}M carbachol.

DISCUSSION

This study demonstrates that the *in vitro* stimulation of antibody-secreting cells in cultures of splenic leukocytes from the rainbow trout can be influenced, both positively and negatively, by adrenergic and cholinergic receptor agonists. While this influence appears to be receptor mediated, as evidenced by the effects of the appropriate antagonists, the actual target cell(s) for these drugs are as yet unknown. The stimulating antigen used in this study, TNP-LPS, is considered a T-independent (actually TI-1) antigen in that neither accessory cells nor T cell help is required for the

induction of an antibody response to this antigen. Although this may be the case in mammals (there is still some controversy on this subject), it has been demonstrated both in catfish (Miller, Sizemore & Clem, 1985) and in salmonids (Kaattari, personal communication) that adherent cells (macrophages) are necessary for an *in vitro* response to TNP-LPS. Therefore, these cells are also potential targets for any regulatory agents in this system. Similar studies in mammals almost invariably have used sheep erythrocytes (SRBC) as the stimulating antigen - an antigen which requires the contribution of helper T cells, as well as accessory cells, in order to generate an antibody response.

In light of the results obtained in this study, which in some instances are the reverse of those observed in the murine system, this difference in the cellular requirements of the systems should be kept in mind. The contrasting effects seen may point to a differential regulation of leukocyte subtypes by autonomic neurotransmitters, which in turn could lead - depending on the nature of the antigen - to quite different effects on the final immune response. Even within the mammals, however, there is still a good deal of conflicting evidence on how autonomic neurotransmitters affect antibody production, and there are few definitive studies using the necessary antagonist controls.

Besedovsky *et al.* (1979), in a study of the effects of adrenergic agents on the *in vitro* PFC response to SRBC in mice, also observed a highly variable response with the addition of norepinephrine. Assuming that norepinephrine would mediate its

effect through alpha-adrenergic receptors, these authors also tried the alpha-2 selective agonist clonidine, which consistently suppressed the response at concentrations from 10^{-4} - 10^{-8}M. No use of antagonists was reported. In contrast to this work, I have observed suppression of the PFC response to TNP-LPS following stimulation of beta receptors (with isoproterenol), but consistent enhancement by the nonselective alpha-agonist phenylephrine. Preliminary studies using clonidine and selective alpha-1 and alpha-2 antagonists suggest that an alpha-2 adrenergic receptor is mediating this enhancement.

Both Makino and Reed (1985) and Pearlman (1971) reported that L-epinephrine induced suppression of the murine PFC response to SRBC at concentrations of 10^{-5}M. Makino and Reed observed this suppression only when the epinephrine was present for at least the first two days of the five day culture period. At $9x10^{-5}$M L-norepinephrine, isoproterenol, and D-epinephrine were also suppressive in this study, as were propranolol and butoxamine (a nonselective beta, and a beta-2 selective antagonist, respectively), which calls into question the specificity of the effects observed here. Pearlman (1971) reported enhancement by l-norepinephrine (at 10^{-4}-10^{-7}M), and, somewhat surprisingly in light of the epinephrine-induced suppression of the PFC response, enhancement by both phenylehphrine and isoproterenol. Braun and Rega (1972) and Burchiel and Melmon (1979) also observed enhancement of this response with isoproterenol. Braun and Rega, in agreement with

Pearlman, reported a norepinephrine-induced enhancement as well, suggesting a predominantly beta effect by norepinephrine.

Sanders and Munson (1984a) observed a consistent enhancement of the anti-SRBC PFC response in mice by 10^{-5} - 10^{-7}M norepinephrine (added at the onset) on days four, five, and six of a seven day culture period. Maximal response for control cultures was on day five in this system. Norepinephrine (10^{-5}M) in the presence of the nonselective alpha antagonist phentolamine resulted in enhancement on days five and six, and this presumed beta effect of norepinephrine was mimicked by 10^{-5}M isoproterenol and by the beta-2 agonist terbutaline, but not by the beta-1 agonist dobutamine. Agonist exposure for only the first five or six hours of culture was sufficient to induce this enhancement (Sanders & Munson, 1984b), the pharmacological specificity of which has been verified by the use of selective beta-1 and beta-2 antagonists (Campbell, Sanders & Munson, 1985).

In subsequent studies it was found that 10^{-5}M norepinephrine in the presence of the beta blocker propranolol resulted in enhancement of the PFC response on day four only, and that this effect could be mimicked by the alpha-1 agonist methoxamine (at 10^{-5}M) (Sanders & Munson, 1985b). In accordance with the findings of Besedovsky *et al.* (1979), the alpha-2 agonist clonidine suppressed the response. Both the clonidine-mediated suppression and the methoxamine-mediated enhancement could be blocked by phentolamine. The nonselective alpha-adrenergic receptor agonist phenylephrine gave a biphasic response, with suppression on day five

and enhancement on days six and seven, an effect which could not, however, be antagonized by phentolamine. Supernatants from either terbutaline or clonidine-treated spleen cell cultures, with the addition of the appropriate blockers, mimicked the effects of the agents themselves (McCall *et al.*, 1985), which suggests the importance of lymphokine/cytokine induction by autonomic neurotransmitters. This effect of adrenergic agents has also been examined by Paegelow and Werner (1987).

Other mammalian studies have examined the effects of epinephrine, norepinephrine, or their analogs on total antibody production (rather than numbers of antibody secreting cells) generated *in vitro* (Sherman, Smith & Middleton 1973), on the PFC response of animals immunized *in vivo*, with the addition of drugs during the plaque assay (Melmon *et al.*, 1974), and on the PFC response of animals both treated with adrenergics and immunized *in vivo* (Pierpaoli & Maestroni, 1978; Depelchin & Letesson, 1981). Cholinergic-receptor stimulation has been shown in mammals to affect such processes as complement component synthesis in monocytes (Whaley, Lappin & Borkas, 1981), histamine release from mast cells (Fantozzi *et al.*, 1978), lysozomal enzyme release from neutrophils (Ignarro & George, 1974), and lymphocyte mediated cytotoxicity (Strom, Lane & George, 1981). With regards to lower vertebrates, there are two reports by Hodgson *et al.* (1978) and Hodgson, Clothier and Balls (1979) which document significant effects of cyclic AMP, adrenergics, and other agents which affect

cyclic nucleotide metabolism on spleen cell rosette formation in amphibia.

The effects of autonomic neurotransmitters on leukocyte function, as with other target tissues, is generally held to be mediated by alterations in cyclic nucleotide metabolism (Strom, Lundin III & Carpenter, 1977). Stimulation of beta-1 and beta-2 adrenergic receptors increases, while alpha-2 adrenergic and muscarinic(M2)-cholinergic receptor stimulation inhibits, adenyl cyclase activity (alpha-1 adrenergic receptors are involved in phosphoinositide metabolism). It has been proposed that increased intracellular levels of cAMP are generally inhibitory to the function of mature lymphocytes, while low cAMP (or high cGMP) is stimulatory (Coffey & Hadden, 1985). Although the data presented in this study conform with this simple model, the dynamic nature of cyclic nucleotide metabolism during the various phases of lymphocyte proliferation and differentiation (Plescia, Yamamoto & Shimamura, 1975; Shenker & Gray, 1979) complicates this interpretation. In mammals, early, transitory rises in cAMP appear to enhance the antibody response, while high cAMP levels later in the response are inhibitory (Teh & Paetkau, 1974; Kishimoto & Ishizaka, 1976). The end result in more complex systems (e.g. the murine anti-SRBC response) may well depend on a balance of effects on multiple cell types, and the variable and sometimes conflicting results observed in these studies could stem from differential effects of cyclic nucleotides on lymphocyte subsets. Indeed, Gilbert and Hoffman (1985) have shown that dibutyryl cAMP, if added with IL-1,

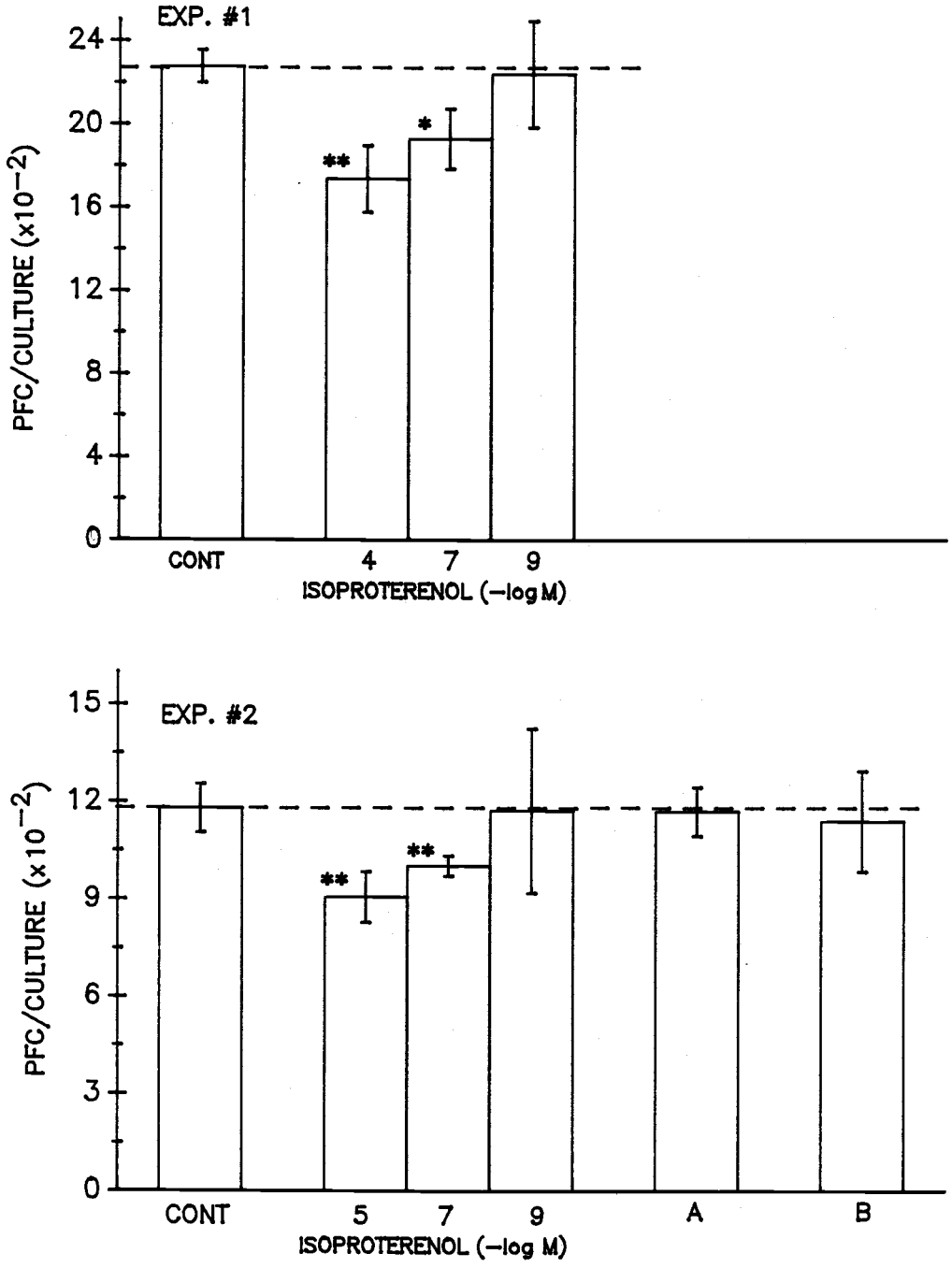
stimulates both antigen-specific and polyclonal antibody production by B cells, but inhibits the production of IL-2 and T cell replacing factor by helper T cells. There was no effect on IL-1 production by macrophages. It has also been demonstrated that activation of T lymphocytes (as measured by phosphoinositide hydrolysis and intracellular calcium levels), by either lectin or monoclonals directed against the T cell receptor, can be inhibited by isoproterenol and other agents which increase intracellular cAMP (Lerner, Jacobson & Miller, 1988).

In order to address the contribution of species differences, as opposed to the difference in stimulating antigen (and therefore the cellular requirements of the systems) to the results I have observed, more comparable systems are necessary. Good *in vitro* T-dependent responses have unfortunately been difficult to obtain in the salmonid system. Conversely, the use of a TI-2 type antigen (such as TNP-Ficoll) in the murine system would allow direct comparisons since, as with TNP-LPS in the teleost system, accessory cells are required for a response to this antigen in mice.

In conclusion, beta-adrenergic receptor agonists suppress, while alpha-adrenergic and cholinergic receptor agonists enhance, the *in vitro* stimulation by TNP-LPS of antibody-secreting cells from the spleen of the rainbow trout, and these effects are blocked by the appropriate antagonists. This research extends the finding that catecholamine depletion *in vivo* can influence the immune response (Flory, 1988a), and suggests that this influence could be due, at least in part, to direct interactions of neurotransmitters

with splenic leukocytes. We are now characterizing further the receptors mediating these effects, and determining the cell type(s) and stages of the immune response which are most sensitive to these agents.

Figure III.1. Effect of the beta-adrenergic agonist isoproterenol on the *in vitro* plaque forming cell (PFC) response to TNP-LPS in the rainbow trout. Results are shown for two separate experiments. In the second, "A" indicates 10^{-5}M isoproterenol + 10^{-5}M propranolol, and "B" indicates 10^{-5}M propranolol. "*" denotes significant difference from control ($p < .05$), "**" denotes highly significant ($p < .01$) difference. Student's t test. Each point represents mean +/- 1 S.D. of triplicate cultures.


EXP. #1
PFC/CULTURE (x10^{-2})
24
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16
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4
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ISOPROTERENOL (−log M)
EXP. #2
PFC/CULTURE (x10^{-2})
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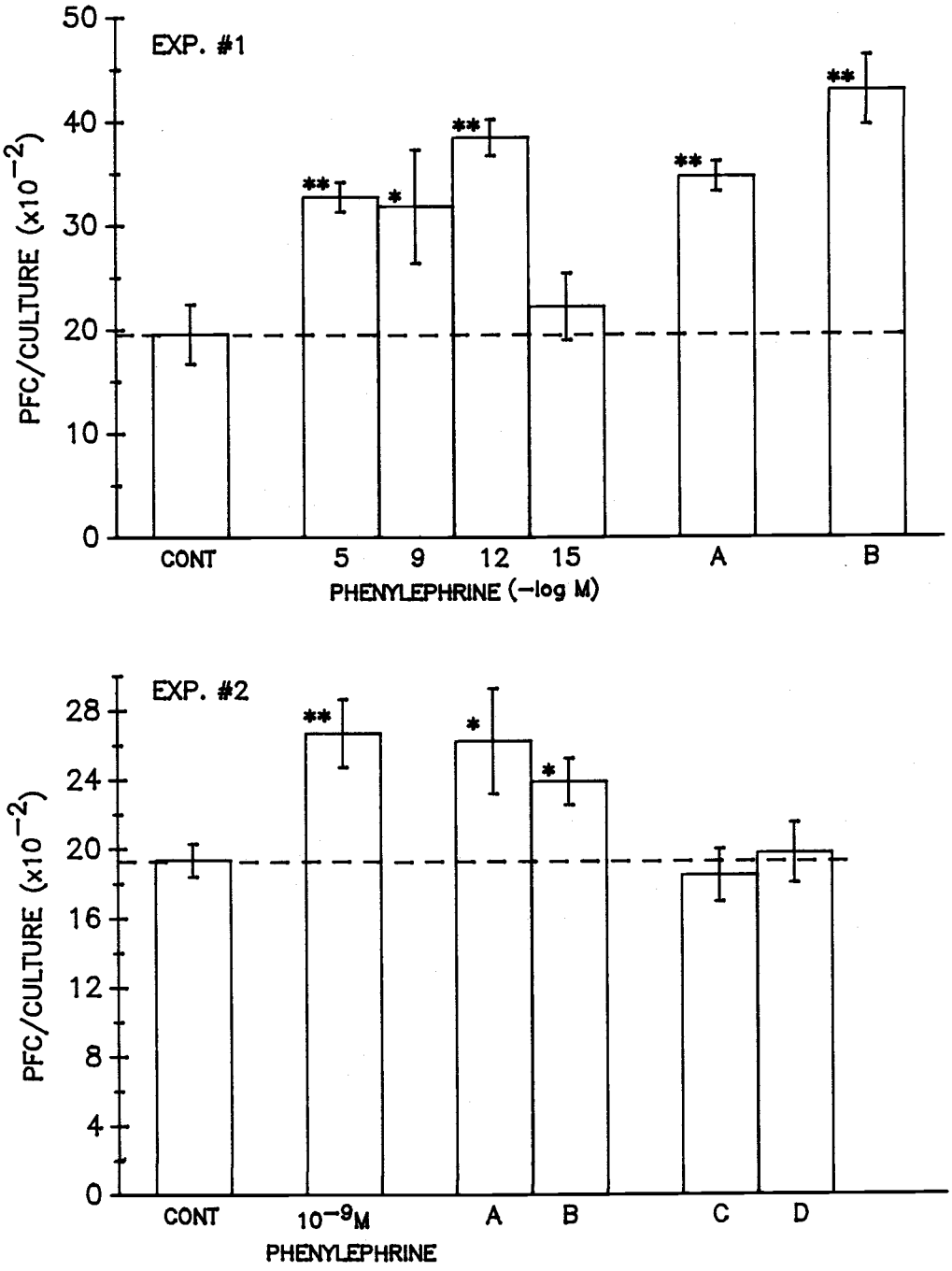
ISOPROTERENOL (−log M)

Figure III.1

Figure III.2. Effect of the alpha-adrenergic agonist phenylephrine on the *in vitro* PFC response to TNP-LPS in the rainbow trout. "A" indicates 10^{-9}M phenylephrine + 10^{-6}M phentolamine, "B" indicates 10^{-6}M phentolamine, "C" indicates 10^{-9}M phenylephrine + 10^{-6}M yohimbine, and "D" indicates 10^{-6}M yohimbine.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PHENYLEPHRINE

Figure III.2

Figure III.3. Effect of the cholinergic agonist carbachol on the *in vitro* PFC response to TNP-LPS in the rainbow trout. For experiment # 2 "A" indicates 10^{-9}M carbachol, "B" indicates 10^{-9}M carbachol + 10^{-6}M atropine, and "C" indicates 10^{-6}M atropine.

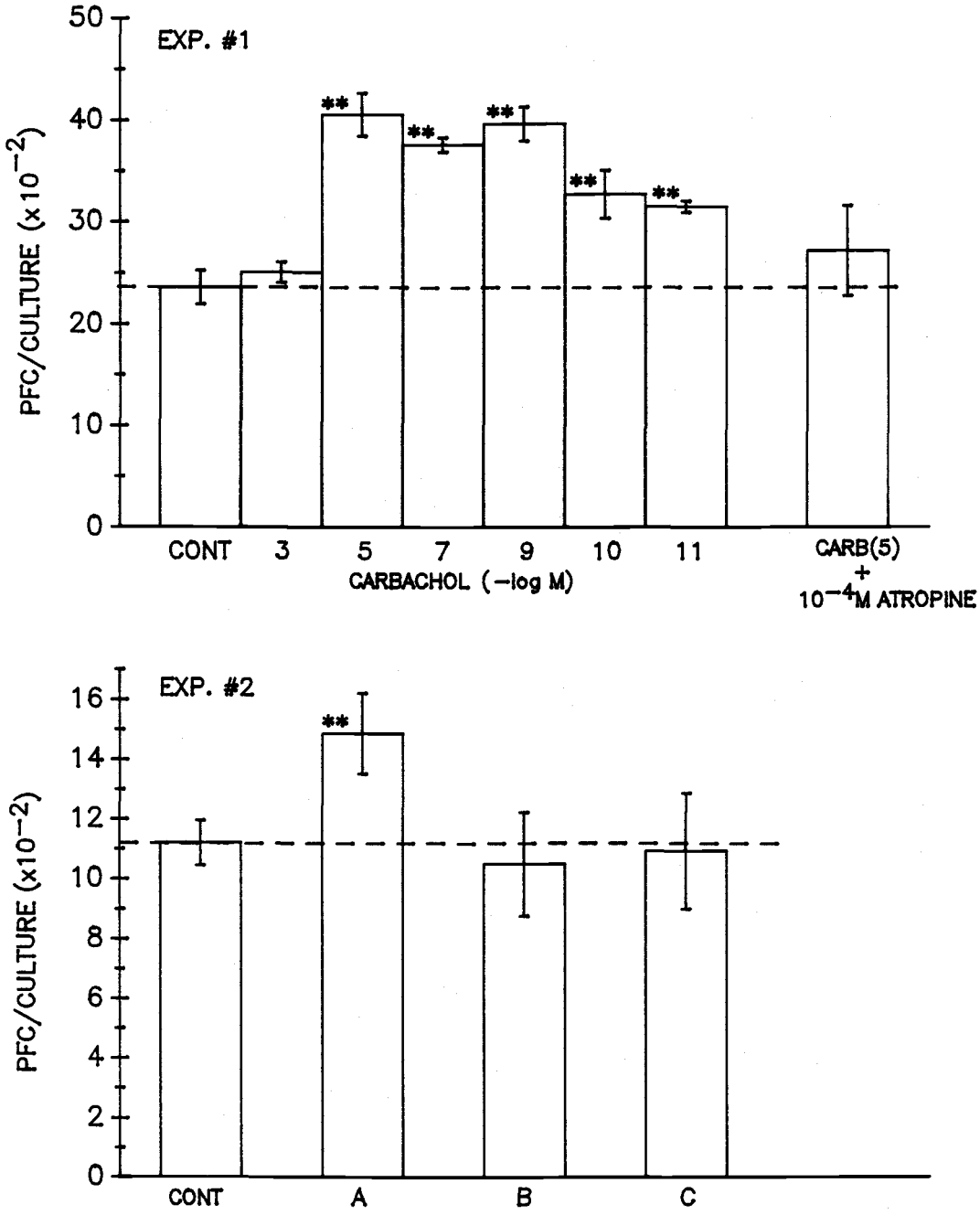
EXP. #1
PFC/CULTURE (x10^{-2})
50
40
30
20
10
0
**
CONT
3
5
7
9
10
11
CARBACHOL (−log M)
CARB(5)
+
10^{-4}M ATROPINE
EXP. #2
PFC/CULTURE (x10^{-2})
16
14
12
10
8
6
4
2
0
CONT
A
B
C

Figure III.3

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CHAPTER IV

Alpha-2 adrenoreceptor-mediated enhancement of the *in vitro* antibody response of splenic leukocytes from the rainbow trout *Salmo gairdneri*.

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ABSTRACT

Agonists of alpha-adrenergic receptors enhance the *in vitro* induction of antibody-secreting cells in cultures of splenic leukocytes from rainbow trout. The enhancement resulting from the nonselective alpha agonist phenylephrine can be mimicked by the alpha-2 selective agonist clonidine, but not by the alpha-1 agonist methoxamine. Antagonists of alpha-2, but not alpha-1, receptors are able to block the clonidine-induced enhancement. These results suggest the presence of alpha-2 adrenergic receptors on rainbow trout leukocytes.

INTRODUCTION

The capacity for neural and hormonal regulation of the immune system (Goetzl, 1985), and for lymphoid cell products to affect neuroendocrine function (Besedovsky & del Rey, 1987; Bernton *et al.*, 1987), are now well established in mammals. Mammalian leukocytes possess specific, high affinity receptors for catecholamines (see Berczi, 1986 for review), and adrenergic agents can modulate a variety of leukocyte functions *in vitro* (see Sanders & Munson, 1985a and Berczi, 1986 for reviews). These findings suggest important immunoregulatory roles for the neurotransmitters *in vivo* - a role which is supported by the extensive autonomic innervation of lymphoid tissues in mammals (Bulloch, 1985; Felton *et al.*, 1987b; Felton & Olschowka, 1987), and the alterations in immune capacity which result from either chemical lesioning of sympathetic neurons (Williams *et al.*, 1981) or surgical denervaton of lymphoid organs (Besedovsky *et al.*, 1979; Alito *et al.*, 1987).

In a previous report (Flory, 1988a) it was shown that the spleen of the coho salmon (*Onchorynchus kisutch*) is also richly innervated, and that treatment of coho with the neurotoxin 6-hydroxydopamine, which destroys sympathetic nerve terminals, results in a significant enhancement in the numbers of antibody-secreting cells in the spleens of immunized fish. Subsequent *in vitro* studies have demonstrated that alpha and beta-adrenergic and cholinergic receptor agonists can either enhance

(alpha-adrenergic and cholinergic agonists) or suppress (beta-adrenergic agonists) the *in vitro* induction of antibody-secreting cells in cultures of spleen cells from the rainbow trout, *Salmo gairdneri* (Flory, 1988b). The enhancement brought about by phenylephrine (a nonselective agonist of alpha-adrenergic receptors) could be blocked by the alpha-2 selective antagonist yohimbine, suggesting that rainbow trout leukocytes possess the alpha-2 subclass of alpha-adrenergic receptors. The purpose of the present study was to characterize further, using a panel of selective alpha-1 and alpha-2-adrenergic receptor agonists and antagonists, the receptor which mediates this phenylephrine-induced enhancement of the *in vitro* antibody response. The results of this study give further insights into the phylogeny of neuroimmunoregulation, and provide evidence that teleosts, as well as mammals, possess functional subclasses of the alpha-adrenergic receptor.

MATERIALS AND METHODS

Leukocytes from the spleen of the rainbow trout, *Salmo gairdneri*, were cultured, and the numbers of antibody-secreting cells were assayed, as described previously (Flory,1988b). The T-independent antigen trinitrophenyl-lipopolysaccharide (TNP-LPS) was used for the *in vitro* stimulation of the splenic leukocytes. The various adrenergic agents used in this study were added at the

start of the nine day culture period after which the numbers of anti-TNP specific antibody-secreting cells (or plaque forming cells (PFC)) were quantified. The clonidine hydrochloride and yohimbine hydrochloride were Sigma products, the methoxamine hydrochloride was a gift from Dr. Thomas Murray, Oregon State University, and the prazosin hydrochloride a gift from the Pfizer Corp., Groten, CT.

RESULTS

Clonidine, an agonist of alpha-2 adrenergic receptors, significantly enhanced the *in vitro* PFC response at concentrations of 10^{-7}-10^{-11}M (Fig. 1). Yohimbine, a competitive antagonist of alpha-2 receptors (which was found in a previous study to block the enhancement brought about by the non-selective alpha agonist phenylephrine) also blocked the enhancement due to 10^{-7}M clonidine. The beta-blocker propranolol had no affect on this response. In a second experiment, 10^{-7}-10^{-13}M clonidine again enhanced the *in vitro* antibody response (Fig. 2), but there was no effect of the alpha-1 agonist methoxamine at any concentration tested. While this clonidine-mediated enhancement can be blocked by yohimbine (as shown previously) it was not affected by prazosin, an antagonist of alpha-1 adrenergic receptors (Fig. 3).

DISCUSSION

This study further illustrates the ability of adrenergic receptor agonists to alter immune function in teleosts, and suggests that the alpha-agonist-induced enhancement of the *in vitro* PFC response is mediated by alpha-2 adrenergic receptors on rainbow trout leukocytes.

Although beta-adrenergic receptors on mammalian lymphocytes (Brodde *et al.*, 1981; Landmann *et al.*, 1984) and other leukocyte types (Lad *et al.*, 1984; Abrass *et al.*, 1985) have been well characterized, relatively few studies have addressed the functional significance of leukocyte alpha receptors. Alpha-adrenergic receptors have been identified on human monocytes (Lappin & Whaley, 1982) and platelets (Alexander, Cooper & Handin, 1978; Grant & Scrutton, 1979), and on splenic lymphocytes of the guinea-pig (McPherson & Summers, 1982). Interestingly, the lymphocyte receptors reported by McPherson and Summers (1982) were identified by [^{3}H]-clonidine binding, indicating their identity as alpha-2 receptors. In these studies alpha receptor binding was found to mediate platelet aggregation (Grant & Scrutton, 1979) and synthesis of complement components by monocytes (Lappin & Whaley, 1982).

In contrast to the clonidine-induced enhancement of the *in vitro* antibody response seen in the present study, Besedovsky *et al.* (1979), and Sanders and Munson (1985b), found that clonidine caused a significant suppression of the murine *in vitro* PFC

response to sheep red blood cells (SRBC). Regulatory T lymphocytes are required for the generation of immune response to SRBC, a T-dependent antigen, but not for the T-independent antigen TNP-LPS used in this study. This difference in the cellular requirements of the systems may be responsible in part for the different results (see Flory, 1988b), which nonetheless also support a functional role for alpha-adrenergic receptors on lymphoid cells.

In mammals, the subclassification of alpha and beta-adrenergic receptors into alpha-1/alpha-2 and beta-1/beta-2 types is now well established, and is based mainly on the potency of various agonists and antagonists at these sites. While there is good evidence that the lower vertebrates possess distinct alpha and beta-adrenergic receptors (Holmgren & Nillson, 1982), evidence for their further subdivision is limited to a few studies which report beta-2-like receptors (based on pharmacological evidence) in the rainbow trout (Wood, 1976; Ask, Stene-Larsen & Helle, 1980) and in several species of frog (Stene-Larsen & Helle, 1978).

Initial classification schemes for alpha receptors in mammals were based on location and function. Alpha receptors were either postsynaptic (alpha-1 type, found mainly on vascular smooth muscle) or presynaptic (alpha-2 type, found on (among other target tissues) synaptic membranes of adrenergic nerve terminals where, upon binding, they mediate inhibition of catecholamine release) (Berthelsen & Pettinger, 1977). These two receptor types are now known to be structurally distinct and to regulate different transduction and second messenger systems. Although both

postsynaptic (Holmgren & Nillson, 1974; Nillson, 1984; Nillson & Grove, 1974) and presynaptic (Nillson & Holmgren, 1976) alpha-adrenergic receptors have been reported in teleosts, it is unknown if these represent distinct types of receptor as they do in mammals. The presence of presynaptic alpha receptors in teleosts was suggested indirectly by the ability of phentolamine, an alpha-receptor antagonist, to increase norepinephrine outflow during nerve stimulation in the cod (Nillson & Holmgren, 1976).

The present study demonstrates that leukocytes from the rainbow trout possess alpha-adrenergic receptors which pharmacologically resemble mammalian alpha-2 receptors. However, without the concomitant demonstration of alpha-1-like receptors (in any tissue), one cannot be certain that two distinct types of alpha receptor actually exist. Teleosts may express only one type of receptor which happens to more closely resemble (as far as the pharmacologic agents are concerned) the alpha-2 receptor of mammals.

In conclusion, alpha-2 adrenergic receptor agonists cause a significant increase in the number of splenic lymphocytes from the rainbow trout which can be stimulated *in vitro* to produce anti-TNP antibodies. The alpha-1 agonist methoxamine has no affect in this assay, and the clonidine-induced enhancement can be blocked by alpha-2, but not alpha-1, selective antagonists. These results indicate the presence of leukocyte alpha-2 receptors. The demonstrated ability of neurotransmitter-receptor agonists to directly influence leukocyte function *in vitro* reveals one mechanism by which lymphoid tissue innervation is able to influence

immune capacity *in vivo*. Determining the target cells, the receptors involved, and the direction of this influence is important in elucidating which cell types are likely to be most affected, and what the consequences of this effect will be, during periods of increased autonomic activity *in vivo* such as occurs during stress.

Figure IV.1. Effect of the alpha-2 adrenergic agonist clonidine, and alpha and beta antagonists, on the *in vitro* plaque forming cell (PFC) response to TNP-LPS in the rainbow trout, *Salmo gairdneri*. A = 10^{-7}M clonidine + 10^{-6}M yohimbine. B = 10^{-6}M yohimbine. C = 10^{-7}M clonidine + 10^{-6}M propranolol. D = 10^{-6}M propranolol. "*" between adjacent treatments denotes significant ($p<.05$) difference, for "**" $p<.01$. Student's t test. Each point represents mean +/- 1 S.D. of three replicate cultures.

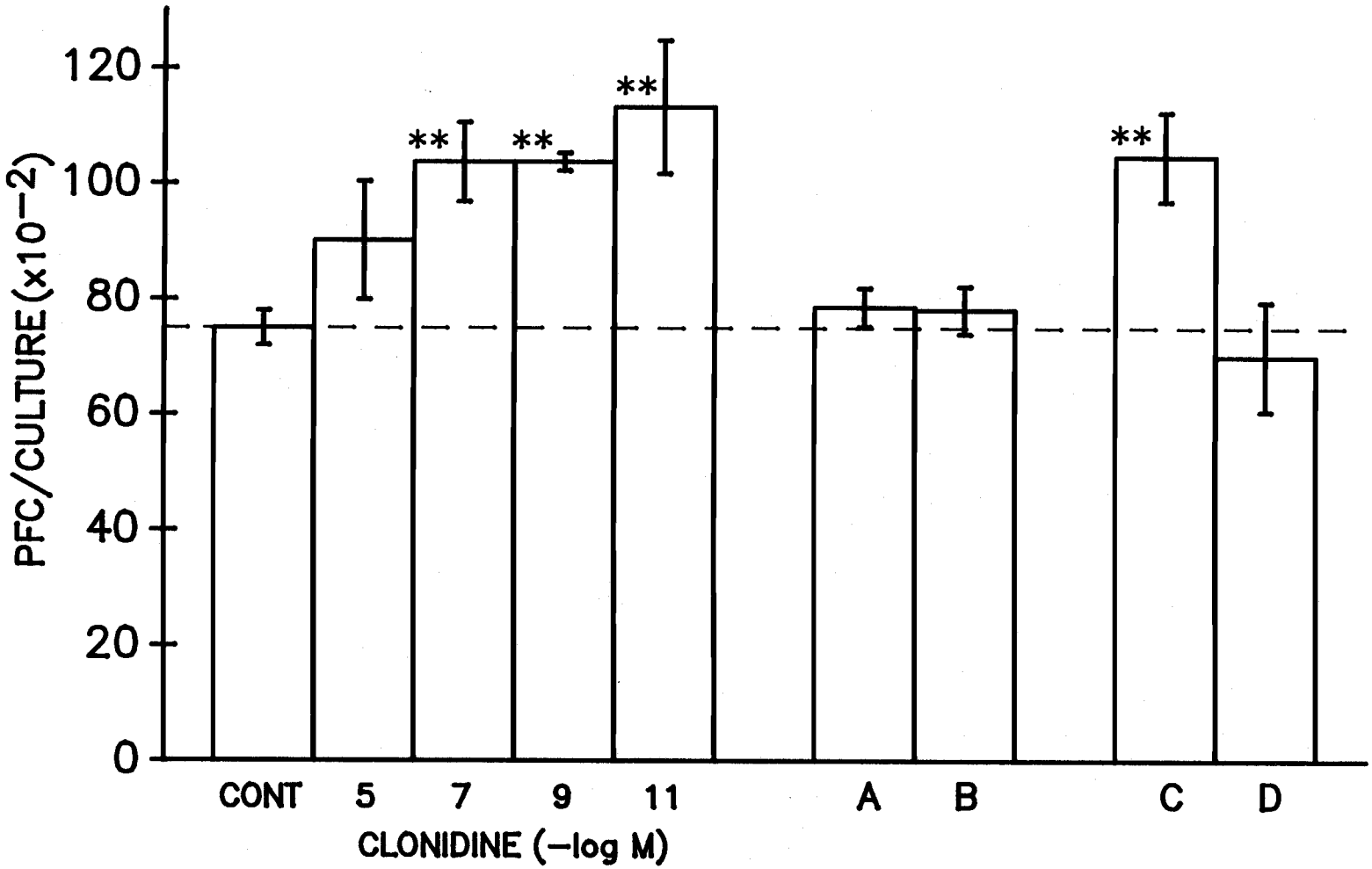
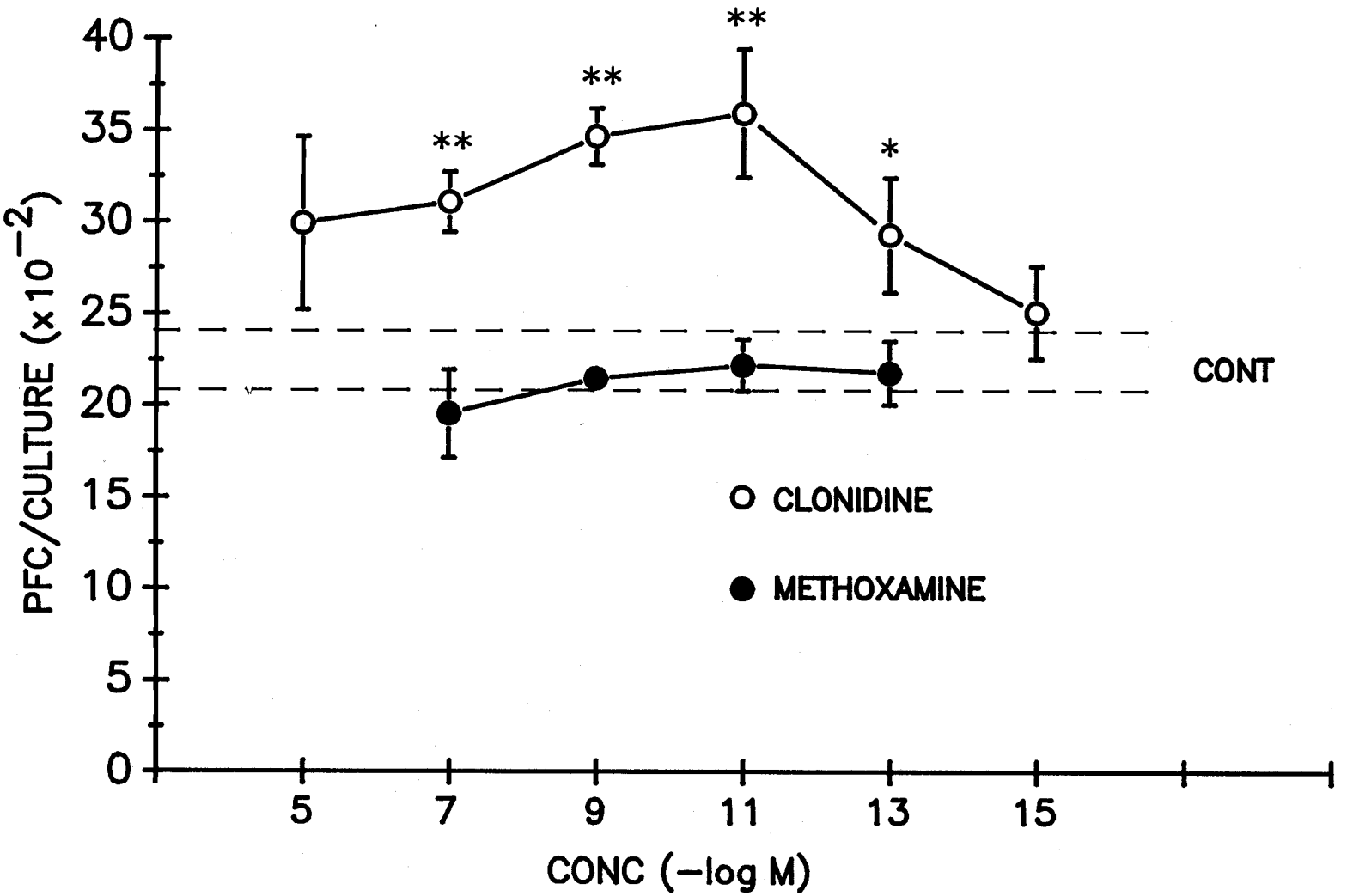

120
100
80
60
40
20
0
PFC/CULTURE (x10⁻²)
**
**
**
CONT
5
7
9
11
A
B
C
D
CLONIDINE (−log M)

Figure IV.1

Figure IV.2. Effects of alpha-2 (clonidine) and alpha-1 (methoxamine) adrenergic agonists on the *in vitro* PFC response of rainbow trout to TNP-LPS.


40
35
30
25
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15
10
5
0
PFC/CULTURE (x10^{-2})
**
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**
*
CONT
CLONIDINE
METHOXAMINE
5
7
9
11
13
15
CONC (−log M)

Figure IV.2

Figure IV.3. Effects of alpha-2 and alpha-1 adrenergic antagonists on the clonidine-induced enhancement of the *in vitro* PFC response of rainbow trout to TNP-LPS. A = 10^{-9}M clonidine. B = 10^{-9}M clonidine + 10^{-6}m yohimbine (alpha-2). C = 10^{-6}M yohimbine. D = 10^{-9}M clonidine + 10^{-6}M prazosin (alpha-1). E = 10^{-6}M prazosin.

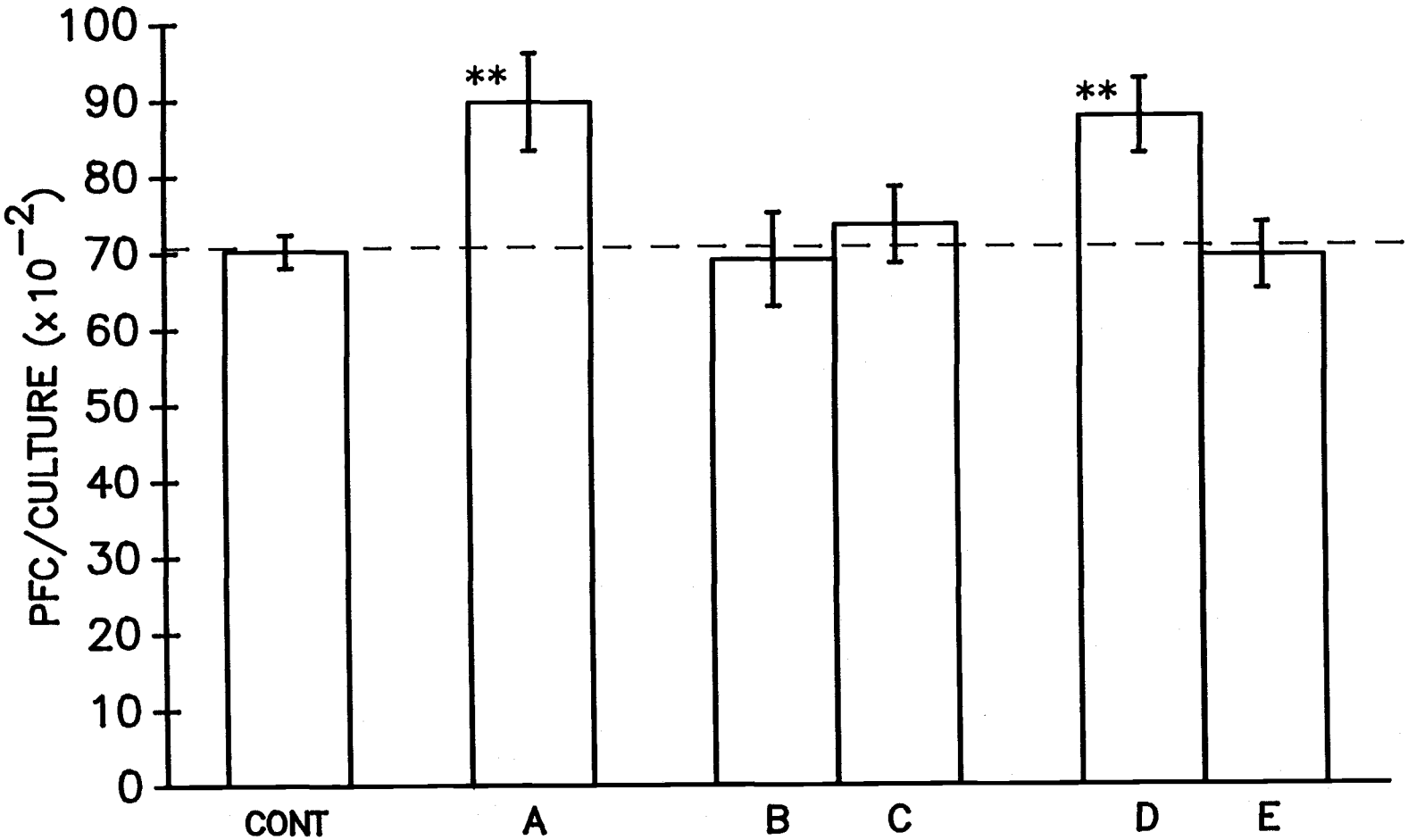

Figure IV.3

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CHAPTER V

The influence of adrenergic and cholinergic agents on the chemiluminescent and mitogenic responses of leukocytes from the rainbow trout, *Salmo gairdneri*

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ABSTRACT

In a previous report it was shown that while alpha-adrenergic and cholinergic agonists enhance, agonists of beta-adrenergic receptors suppress *in vitro* antibody responses in rainbow trout. The present study begins to address the underlying mechanisms behind these effects. Of the many potential ways in which autonomic neurotransmitters (or their analogs) could influence this antibody response, two possibilities are effects on accessory cell function, and/or effects on the clonal proliferation of antigen-stimulated lymphocytes. Epinephrine and selective beta-receptor agonists suppress, while both alpha and cholinergic-receptor agonists enhance, the ability of stimulated pronephric leukocytes (mainly macrophages and neutrophils) to produce reactive oxygen species.

Beta agonists also suppress the proliferative response of splenic leukocytes to LPS, Con A, and PHA. There was no effect, however, of alpha-adrenergic or cholinergic-receptor agonists on the mitogenic response.

INTRODUCTION

In mammals, the extensive autonomic innervation of lymphoid tissues (Bulloch, 1985; Felten *et al.*, 1985) appears to constitute an important mode of communication between the nervous and immune systems (Williams *et al.*, 1981; Alito *et al.*, 1987). While the mechanisms by which catecholamines, acetylcholine, and various neuropeptides affect mammalian immunity are beginning to come to light (Berczi, 1986; Felten *et al.*, 1987a; Morely *et al.*, 1987), only a few studies have addressed nervous-immune system interactions in the lower vertebrates (Zapata, Villena & Cooper, 1982; Hodgson *et al.*, 1978; Hodgson, Clothier & Balls, 1979).

A previous report (Flory, 1988a) demonstrated that the spleen of the coho salmon, *Oncorhynchus kisutch*, also contains a rich adrenergic innervation, and that lesioning of adrenergic nerve terminals (with the neurotoxin 6-hydroxydopamine) results in an enhanced antibody-secreting cell response in immunized fish. Subsequent studies in the rainbow trout, *Salmo gairdneri*, have established that adrenergic and cholinergic agents can directly

influence salmonid leukocyte function *in vitro* (Flory, 1988b). Beta-adrenergic receptor agonists suppress, while alpha-adrenergic and cholinergic agonists enhance, the *in vitro* stimulation of antibody-secreting cells (by trinitrophenyl-lipopolysaccharide (TNP-LPS)) in cultures of splenic leukocytes. Studies with specific receptor antagonists, and the low levels at which the agonists are active, suggest a receptor-mediated process. Pharmacologic characterization of the receptor mediating the enhancement by alpha-agonists indicates it is of the alpha-2 subclass (Flory, 1988c).

In catfish (Miller, Sizemore & Clem, 1985) and in the salmonids (Kaattari, personal communication), accessory cells (macrophages) are required for an *in vitro* antibody response to TNP-LPS. Accessory cells, as well as B lymphocytes, are therefore potential targets for the adrenergic and cholinergic agents used in this system. It is also uncertain which stage of this *in vitro* response is affected since the drugs are present throughout the nine day culture period. The present study begins to address these questions of the cellular targets for, and immune processes altered by, agonists of adrenergic and cholinergic receptors in this *in vitro* antibody response. The hypotheses specifically tested are that adrenergic and cholinergic agonists are able to directly influence phagocyte (macrophage and neutrophil) cell function in rainbow trout (as assessed by their affects on the chemiluminescent response of pronephric leukocytes), and that the alterations in the antibody response could be due also to the influence of these

agonists on antigen-induced lymphocyte proliferation (as assessed by their affects on the mitogen-induced proliferative response of splenic leukocytes).

MATERIALS AND METHODS

Mitogenesis assay

Mitogens and drugs

The lipopolysaccharide (LPS) (*E. coli* 055:B5 W) was obtained from Difco, Detroit, MI. The concanavalin A (Con A, type IV), Phytohemagglutinin (PHA-P), (-)-isoproterenol bitartrate, DL-propranolol hydrochloride, L-phenylephrine hydrochloride, and carbamylcholine chloride (carbachol) were Sigma products. All reagents were made up in complete tissue culture medium and filter sterilized, immediately prior to use. The LPS was used routinely at 100ug/ml final concentration, the Con A at 10ug/ml, and the PHA at 5ug/ml.

Culture system

The complete tissue culture medium (TCM) consisted of L-15 medium with L-glutamine (Sigma) plus 0.2% sodium bicarbonate, 5% heat inactivated fetal calf serum, $5x10^{-5}M$ 2-mercaptoethanol,

10ug/ml each of adenosine, cytosine, guanosine and uracil, and 100ug/ml gentamycin. Single cell suspensions were prepared as descibed previously (Flory, 1988a), rinsed twice in holding medium (TCM with 5% fetal calf serum and gentamycin only) and resuspended in TCM to a final concentration of $1x10^7$ leukocytes/ml. Fifty ul of the cell suspension ($5x10^5$ leukocytes) were then added with mitogen, and with or without drugs, to individual wells of flat bottom, 96 well microtiter plates (Corning) in a final volume of 100ul. Controls and each treatment were run in triplicate. The cultures were then incubated in a humidified atmosphere of 5% CO_2, 95% air, at 16^oC for four days, after which 50 ul of [^{3}H]-thymidine (NEN, s.a. = 20.0 Ci/mmol) was then added to each well. Twenty four hours later the cells were harvested, and the amount of radioactivity incorporated measured on a Beckman 460CD liquid scintillation spectrometer. The results are given in terms of the stimulation index (SI), where the SI per sample is calculated as cpm/sample vial divided by the mean cpm of the triplicate control vials. The results shown are representative of three separate experiments.

Chemiluminescent assay

Reagents

The luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and PMA (phorbol 12-myristate 13-acetate) were Sigma products. Stock

solutions were prepared by adding 17.7mg luminol to 10ml DMSO (dimethyl sulfoxide) to give a 10^{-2}M solution (stored at 4^{o}C), and 2.0mg PMA to 1.0ml DMSO (stored at -20^{o}C). Working solutions (which are diluted further in the assay) consisted of a 1:100 dilution of the stock luminol solution, and a 1:200 dilution of the stock PMA, in Cortland saline (Wolf, 1963). For use in the chemiluminescent assay, the stock solutions of isoproterenol, phenylephrine, carbachol, epinephrine bitartrate, atropine, and yohimbine hydrochloride (all Sigma products) were also diluted in Cortland saline.

Assay

Single cell suspensions of pronephric leukocytes were prepared, rinsed twice, and resuspended in L-15 medium containing 5% fetal calf serum to a final concentration of 5×10^{6} cells/ml. Five hundred ul of luminol (final concentration = $5x10^{-5}$M), 100ul of the cell suspension ($5x10^{5}$ cells), 200ul of either Cortland saline or drugs (at 5x final concentration), and 200ul of PMA (2ug/ml final), were added to the assay tubes, mixed, and placed immediately into an LKB luminometer (model 1250). For the studies with antagonists, 100ul of 10x agonist, plus 100ul of 10x antagonist, were added to the tubes. Oxygen radicals (mainly superoxide anion) generated by phagocytic leukocytes in response to PMA stimulation react with the luminol to give off light. The amount of light thus generated, which is proportional to the superoxide production (and

therefore degree of stimulation), is then measured by the luminometer. The chemiluminescent response was recorded on a Heathkit chart recorder. The results shown are representative of three separate experiments.

RESULTS

Mitogenic response of splenic leukocytes

LPS, which gave a significant stimulation index over a wide dose range (50-500 ug/ml) was used at 100 ug/ml in the following experiments. Unless stated otherwise, the Con A and PHA were used at their optimal doses (10ug/ml for Con A and 5ug/ml for PHA). At 10^{-5}M, isoproterenol, an agonist of beta-adrenergic receptors, significantly suppressed the proliferative response of rainbow trout leukocytes to LPS, Con A, and PHA (Fig. 1). The beta-blocker propranolol (which successfully antagonized the isoproterenol-induced enhancement of the antibody response) suppressed the mitogenic response on its own, and therefore could not be used as an antagonist in this system. Neither the alpha-adrenergic agonist phenylephrine, nor the cholinergic agonist carbachol, had any significant affect on the mitogenic response at concentrations from 10^{-5}-10^{-9}M. Phenylephrine and carbachol (at 10^{-5}M) were also tested using sub and supra-optimal doses of mitogen, but were still without affects (data not shown).

Chemiluminescent response of pronephric leukocytes

Initial studies with epinephrine (at 10^{-4}-10^{-6}M) demonstrated a consistent, dose-dependent suppression of the PMA-induced chemiluminescent response of pronephric leukocytes (Fig. 2). This affect of epinephrine could be mimicked by the beta agonist isoproterenol which, at concentrations greater than 10^{-6}M, also caused a dose-dependent suppression of the response (Fig. 3). This suppression could not, however, be reversed by 10^{-5}M propranolol. At 10^{-5}M, both the alpha-agonist phenylephrine, and the cholinergic agonist carbachol, enhanced the chemiluminescent response (Fig. 4). The enhancement due to phenylephrine was blocked by the alpha antagonist yohimbine (10^{-5}M), and that due to carbachol was blocked by 10^{-5}M atropine, an antagonist of muscarinic-cholinergic receptors (data not shown).

DISCUSSION

This study demonstates that the mitogenic response of splenic leukocytes from the rainbow trout, *Salmo gairdneri*, can be inhibited by an agonist of beta-adrenergic receptors, but is unaffected by either alpha-adrenergic or cholinergic-receptor agonists. Isoproterenol also suppressed the chemiluminescent response of pronephric leukocytes, and did so at concentrations similar to those required for suppression of both the *in vitro*

antibody response (Flory, 1988b), and the mitogenic response. Salmonid leukocyte cultures remain viable in the presence 10^{-5}M isoproterenol for up to two weeks, ruling out cytotoxicity as a cause for its suppressive effects in this study. Both phenylephrine and carbachol enhanced the chemiluminescent response, and this enhancement could be blocked by specific antagonists. The significance of these findings will be discussed in turn.

It was shown previously that isoproterenol inhibited, while phenylephrine and carbachol enhanced, the *in vitro* induction of antibody-secreting cells (by TNP-LPS) in cultures of rainbow trout leukocytes (Flory, 1988b). The purpose of the mitogenesis experiments in the present study was to examine the possibility that the effects of these agents on the antibody response stem from their effects directly on lymphocyte proliferation. Throughout these experiments the results were the same with either the mammalian B-cell mitogen LPS, or T-cell mitogens Con A and PHA. Whether these mitogens similarily differentiate between salmonid T and B cells has not, however, been firmly established. There was no significant affect by either phenylephrine or carbachol on the mitogenic response. This suggests that the mechanism by which these compounds enhance the antibody (PFC) response is not through any influence on cell division, but rather on other events necessary for this response such as initial leukocyte-antigen interactions, plasma cell differentiation, or antibody secretion.

The isoproterenol-induced suppression of the proliferative response would seem to explain, at least in part, the inhibition of

the antibody response by this drug. The effective doses (10^{-4}-10^{-5}M), and degree of inhibition, are similar in both cases. Propranolol, however, also suppressed mitogenesis (to an even greater extent than isoproterenol), yet had no effect on the antibody response. Given the suppressive effect of propranolol on the mitogenic response in the present study, and its lack of effect on the antibody response, the significance to the antibody response of the isoproterenol-induced suppression is unclear. The use of more selective beta agonists and antagonists would be necessary to determine if isoproterenol's action in this study is receptor mediated (as it appears to be in the antibody response), and therefore of importance for elucidating its mechanism of action in the antibody response.

Suppression of the mitogenic response by propranolol has also been reported in mice (Johnson, Ashmore & Gordon, 1981). While it is known that propranolol binds the beta-adrenergic receptor of mouse lymphocytes (Johnson & Gordon, 1980), the concentration required to suppress the mitogenic response to Con A is much greater than the average affinity of this receptor, suggesting that propranolol is acting nonspecifically. In a subsequent study Audus, Johnson and Gordon (1982) determined that 10^{-5}M propranolol significantly increased lymphocyte membrane fluidity, and at higher concentrations reduced the mobility of cell surface Con A receptors. Other beta receptor antagonists had no affect in this study. It was suggested that such nonspecific effects of propranolol may be responsible for the decreased proliferative response to Con A observed by Johnson,

Ashmor and Gordon (1981). The effects of cAMP and neurotransmitters (and other agents which influence cAMP metabolism) on mitogenesis and other aspects of leukocyte function in mammals have been reviewed by Bourne *et al.* (1974), Parker (1979), Coffey and Hadden (1985) and Berczi (1986).

Adrenergic and cholinergic agonists (at concentrations greater than 10^{-6}M) influence the chemiluminescent response of leukocytes obtained from the rainbow trout pronephros. The beta blocker propranolol (at 10^{-5}M) did not affect chemiluminescence on its own (as it did the mitogenesis assay), yet was still ineffective in blocking the isoproterenol-induced suppression of this response. This was true even if the cells were pre-incubated with propranolol for ten minutes prior to assay.

The enhancement due to 10^{-5}M phenylephrine or carbachol could be blocked by specific antagonists, suggesting that their influence is receptor-mediated. However, the dose of these compounds which is required to see significant effects in the chemiluminesent assay (10^{-5}M) is considerably higher than was necessary for enhancement of the *in vitro* antibody response (10^{-9}-10^{-11}M). Perhaps longer preincubations with agonists would result in significant affects at lower concentrations. It is also possible that whatever accessory cell function is (potentially) affected in the antibody response is much more sensitive to adrenergic and cholinergic agents than is the production of oxygen radicals. The stimulant itself may also be a factor in this regard, as there is evidence that PMA induces adrenergic receptor uncoupling in human leukocytes (Meurs

et al., 1986). A particulate stimulant such as zymosan may, therefore, give a better indication of the true sensitivity of this system.

The ability of phagocytic leukocytes (macrophages, neutrophils) from lower vertebrates to generate superoxide radicals in response to various stimuli has been demonstrated previously (Scott & Klesius, 1981; Stave, Robertson & Hetrick, 1983; Plytycz & Bayne, 1987; Plytycz *et al.*, 1988). Although this aspect of macrophage function (which is thought to be an important killing mechanism for these cells (Nathan, 1982)) is not directly related to their roles as accessory cells in an antibody response, it is a suitable assay for assessing potential regulators of macrophage function. Since whole pronephric cell suspensions were used in these experiments, neutrophils (which are abundant in the pronephros), as well as macrophages, will have contributed to the response. This may be the reason for the bimodal peaks often observed (see Fig 3). Cell separation experiments have shown that lymphocytes do not contribute to the chemiluminescent response (Plytycz *et al.*, 1988).

Both cholinergic (Lopker *et al.*, 1980) and adrenergic (Abrass *et al.*, 1985) receptors have been identified on mammalian phagocytic leukocytes. Cholinergic agonists enhanced, and epinephrine suppressed, both lysosomal enzyme secretion (Ignarro & Cech, 1976) and the mannozym-induced chemiluminescent response (Sipka *et al.*, 1985) of human neutrophils. Beta-adrenergic agonists suppressed the zymosan induced chemiluminescent response of

human monocytes (Schopf & Lemmel, 1983), an effect which could be blocked by propranolol.

In conclusion, this study illustrates that the function of phagocytic leukocytes can indeed be influenced by adrenergic and cholinergic receptor agonists, and supports the hypothesis that the affects of these agents on the antibody response could be due to their influence on accessory cell function. The mitogenesis data indicate that an affect on lymphocyte proliferation is probably not the mechanism involved in either the beta agonist-induced suppression, or alpha or cholinergic agonist-induced enhancement, of the *in vitro* antibody-secreting cell response.

Figure V.1. Effects of isoproterenol (a beta-receptor agonist), phenylephrine (alpha agonist), and carbachol (cholinergic agonist), on the mitogenic response of splenic leukocytes from the rainbow trout. **, $p < .01$; *. $p < .05$. Student's t test. Each point represents the mean +/- 1 S.D. of triplicate cultures.

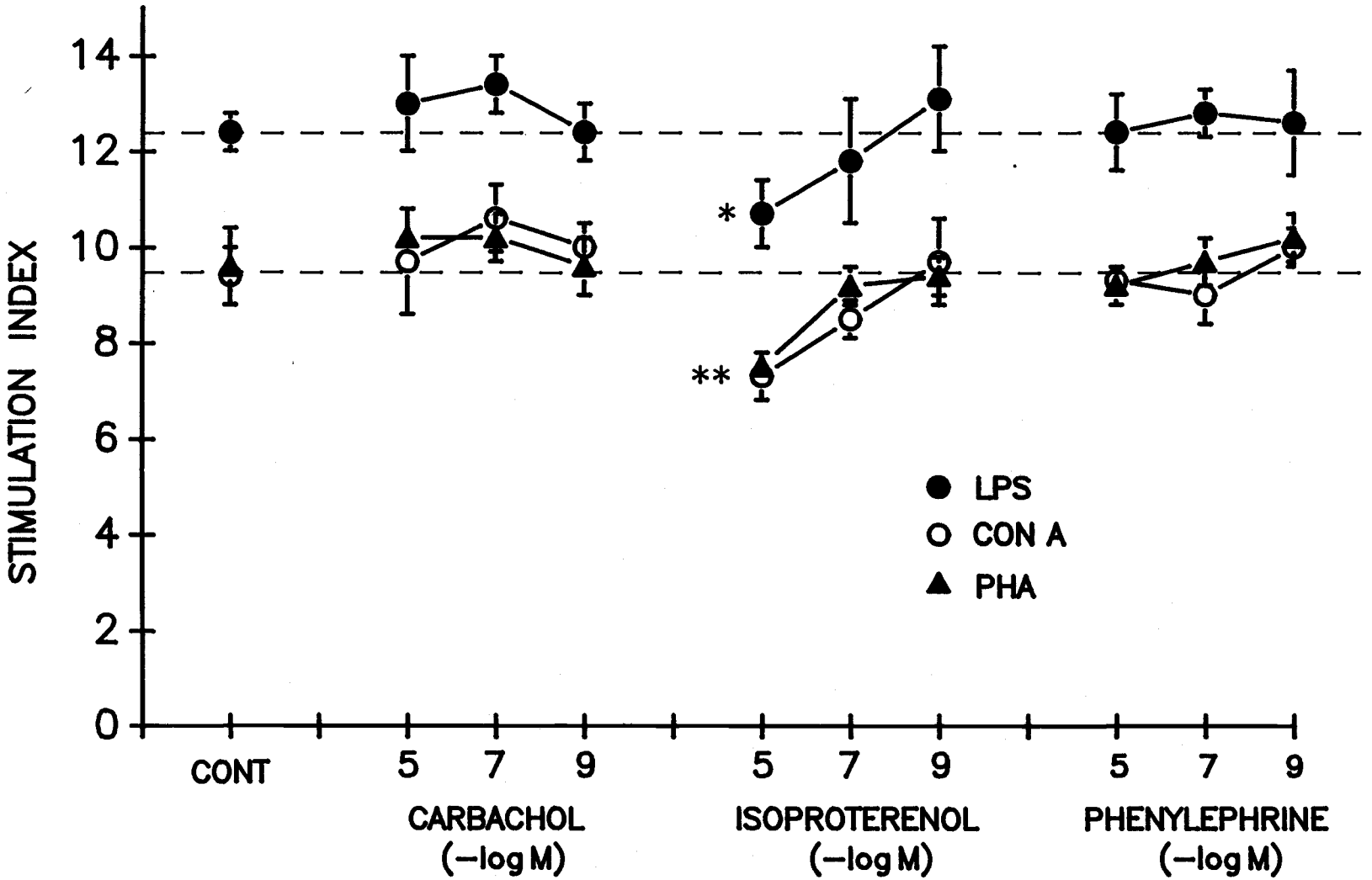
STIMULATION INDEX
14
12
10
8
6
4
2
0
*
**
LPS
CON A
PHA
CONT
5
7
9
CARBACHOL
(−log M)
ISOPROTERENOL
(−log M)
PHENYLEPHRINE
(−log M)

Figure V.1

Figure V.2. Effect of epinephrine on the chemiluminesent response of pronephric leukocytes from the rainbow trout.

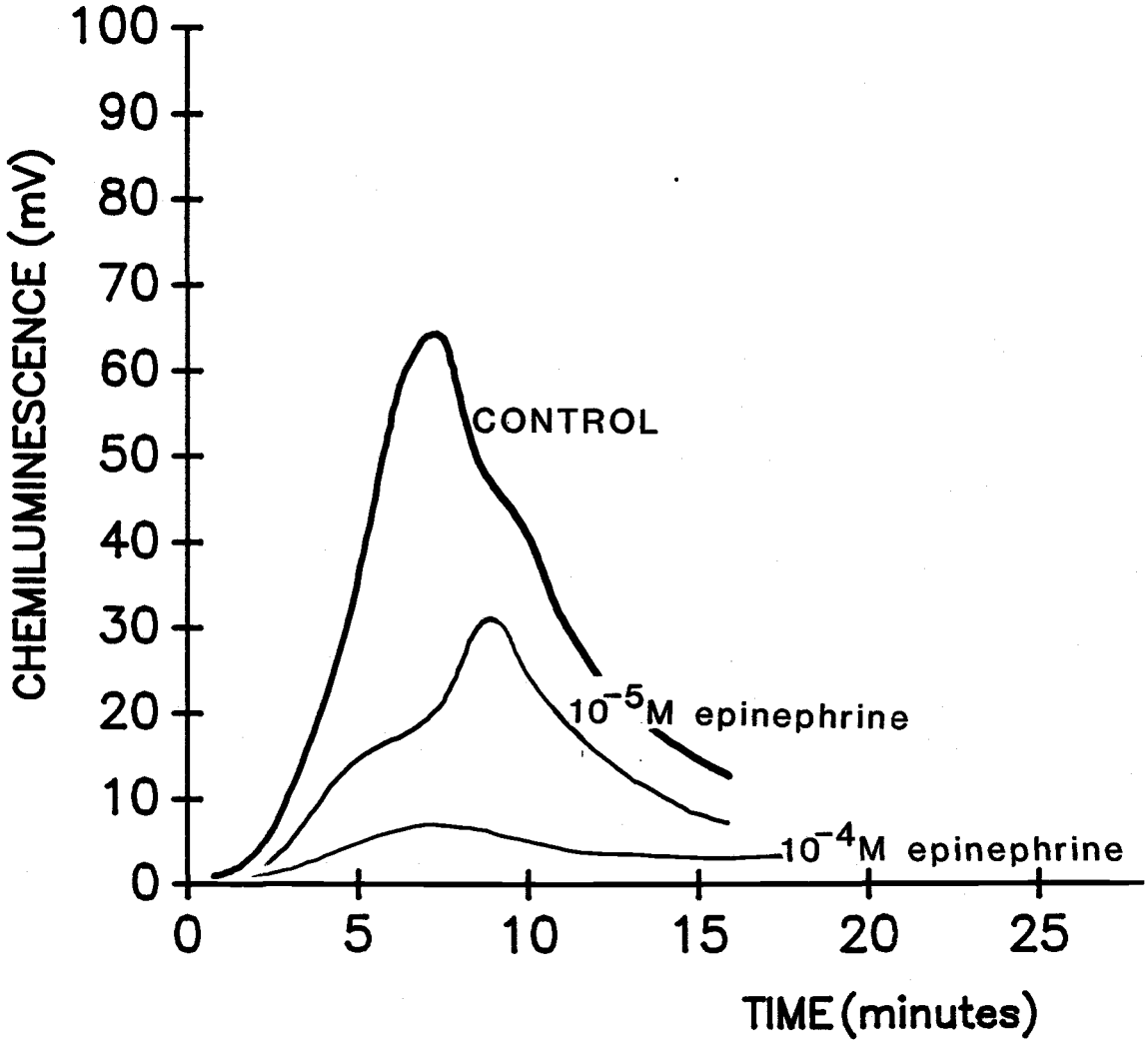
100
90
80
70
60
50
40
30
20
10
0
CHEMILUMINESCENCE (mV)
CONTROL
10^{-5}M epinephrine
10^{-4}M epinephrine
0
5
10
15
20
25
TIME (minutes)

Figure V.2

Figure V.3. Effect of the beta-agonist isoproterenol on the chemiluminesent response of pronephric leukocytes from the rainbow trout.

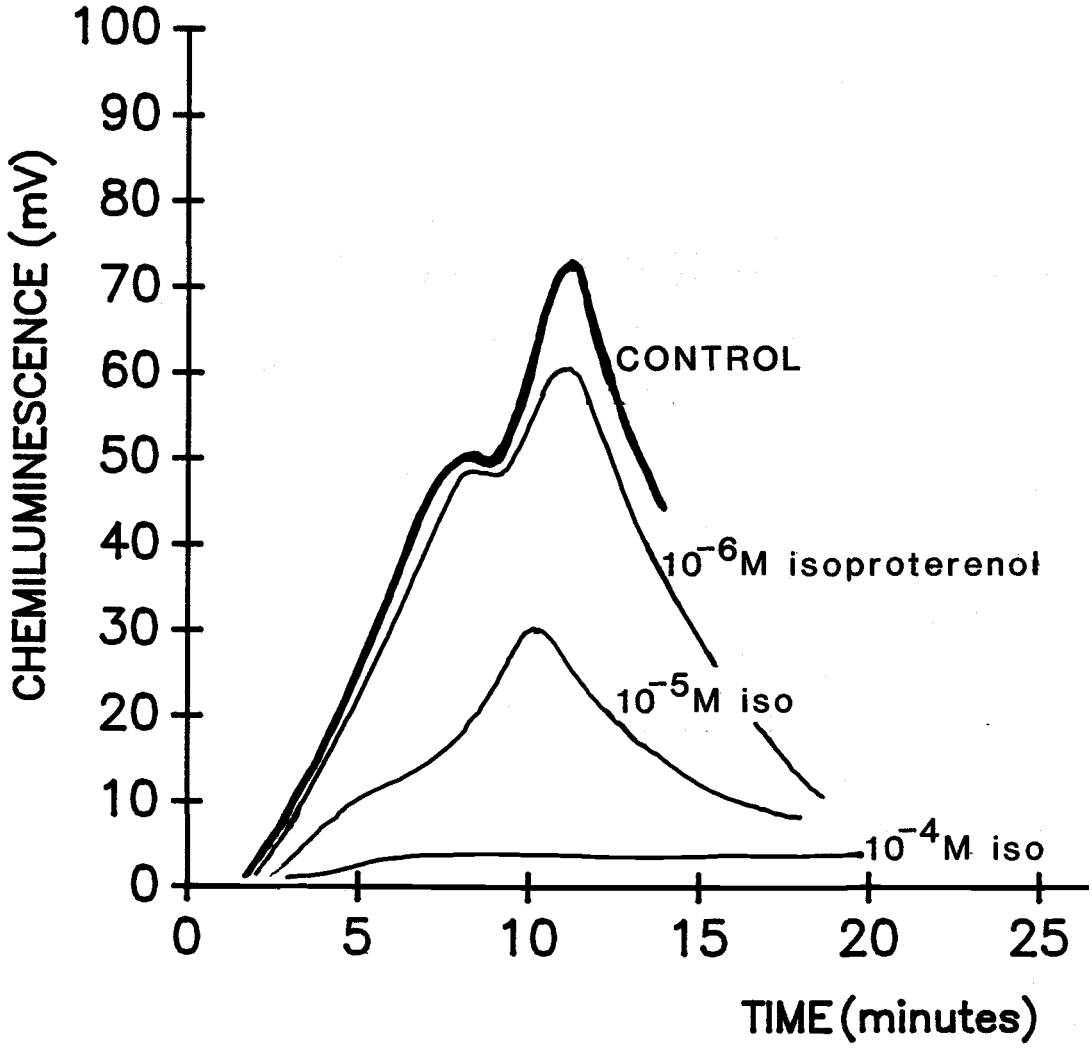
CHEMILUMINESCENCE (mV)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
TIME (minutes)
CONTROL
10^{-6}M isoproterenol
10^{-5}M iso
10^{-4}M iso

Figure V.3

Figure V.4. Effects of phenylephrine and carbachol on the chemiluminesent response of pronephric leukocytes from the rainbow trout.

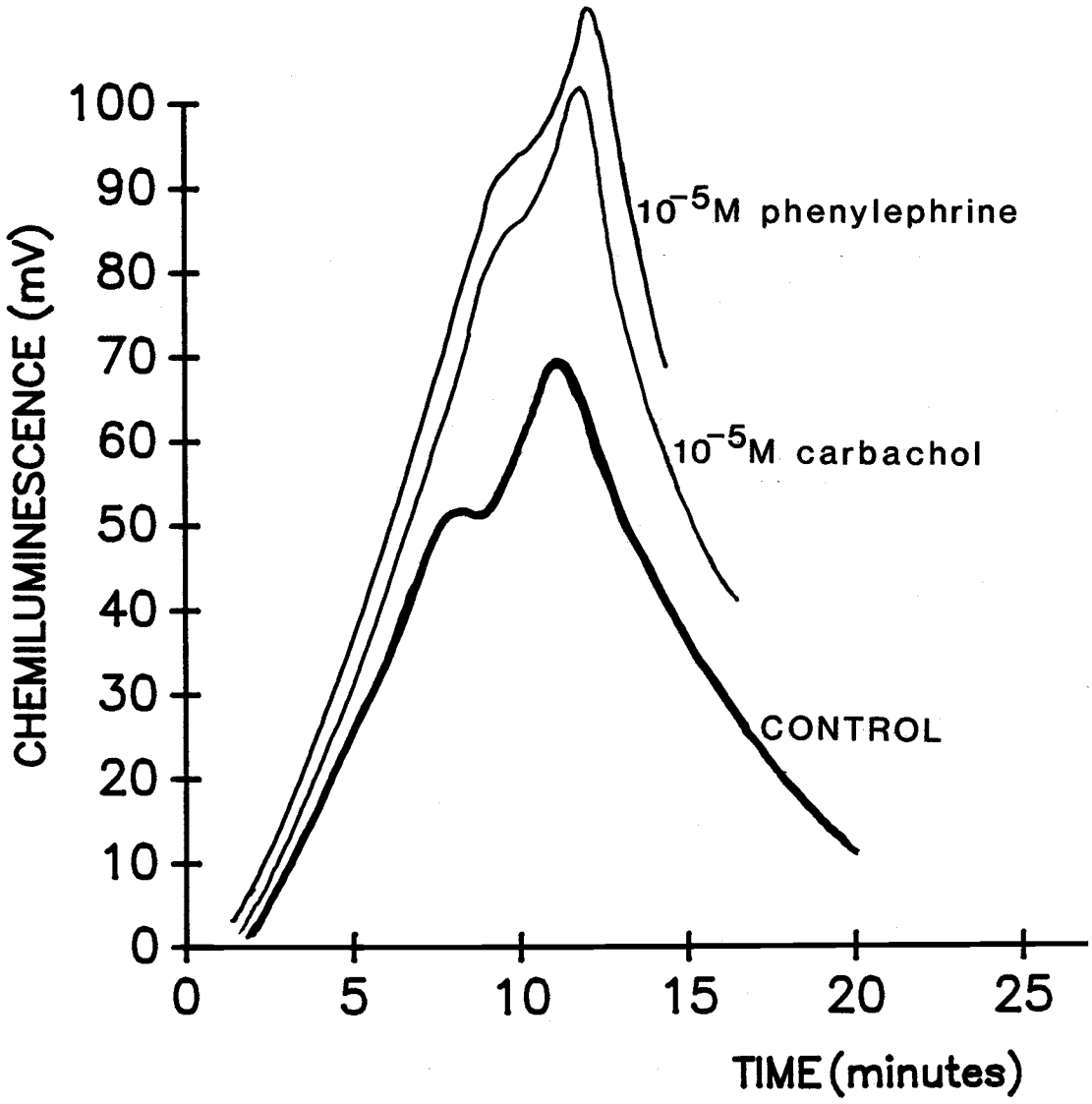
100
90
80
70
60
50
40
30
20
10
0
CHEMILUMINESCENCE (mV)
0
5
10
15
20
25
TIME (minutes)
10^{-5}M phenylephrine
10^{-5}M carbachol
CONTROL

Figure V.4

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CHAPTER VI

SUMMARY AND CONCLUSIONS

Nervous and endocrine regulation of immunity in mammals is suggested by several lines of evidence. In humans, stress and bereavement are associated with a decreased resistance to infectious diseases and cancers, and with significant alterations in immune capacity. Studies in rodents have demonstrated that while acute stress usually results in suppression of the immune response, chronic stress can actually enhance immune reactivity. The mammalian immune system is amenable to classical conditioning, and conditioned suppression of immunity has been shown to prolong the life of autoimmune mice. Both conditioned suppression and conditioned enhancement of immunity (which has also been documented) may someday be of therapeutic value in humans.

These lines of inquiry, while pointing to a dynamic relationship between the brain and immune system, do not address the underlying mechanisms of this phenomenon. The brain communicates with peripheral tissues mainly *via* hormones secreted by the pituitary (which act either directly or through the induction of other hormones) and *via* the peripheral nervous system. Mammalian leukocytes possess receptors for, and can be influenced by, a variety of peptide, protein, and steroid hormones. Several of these hormones (e.g. ACTH and endorphins) are now known to be synthesized by lymphoid cells as well as by neuroendocrine tissues.

Noradrenergic, sympathetic neurons branch extensively in the white pulp of mammalian lymphoid tissues such as the spleen, thymus, lymph nodes, and tonsils. These fibers, which are not associated with smooth muscle, have been shown to form synaptic-like contacts with lymphocytes in the spleen. Surgical or chemical lesioning of this innervation leads to significant changes in the ability of the organism to mount an immune response. While this effect may be due in part to changes in blood flow and lymphocyte trafficking, direct influences on leukocyte function must be considered as well. The autonomic neurotransmitters norepinephrine and acetylcholine (and epinephrine from the adrenal medulla) bind to specific, high affinity receptors on leukocyte membranes, and can influence a wide variety of immune phenomena both *in vitro* and *in vivo*. Depletion of peripheral catecholamine levels (or increases in adrenergic activity due to stress) would significantly alter neurotransmitter-leukocyte interactions, and therefore subsequent leukocyte activity.

The bidirectional nature of neuroendocrine-immune system interactions is evidenced by the changes in the brain which are correlated with the activity of the immune system. This feedback to the central nervous system may be vital for the coordination of neural and endocrine control of immune reactivity. The increase in plasma glucocorticoids which occurs late in some immune responses is probably a reflection of this control.

The studies reported in this thesis provide evidence that the immune system of salmonid fishes is also subject to neural

regulation. Studies employing histofluorescence techniques for catecholamines have revealed that the spleen of the coho salmon is well innervated by adrenergic neurons. This innervation first enters the spleen, and remains largely associated with, the splenic vasculature. Individual fibers extend from the vascular plexes into the parenchyma of the spleen, where they often come into close proximity to splenic leukocytes. Peripheral catecholamine stores in nerve terminals can be depleted by intramuscular injections of the neurotoxin 6-hydroxydopamine (6-OHDA). Two weeks after immunization (with TNP-SRBC) of 6-OHDA-treated fish, their spleens were found to contain significantly higher numbers of anti-TNP antibody-secreting cells. 6-OHDA did not influence the response if administered one week after, rather than two days prior to, immunization, nor did it influence plasma cortisol levels.

Subsequent experiments assessed the ability of adrenergic and cholinergic-receptor agonists to influence salmonid leukocytes *in vitro*. The beta-adrenergic receptor agonist isoproterenol, when added to cultures of splenic leukocytes, suppressed both the *in vitro* induction of antibody-secreting cells (by TNP-LPS), and the proliferative response to the mitogens LPS, Con A, and PHA. Isoproterenol also suppressed the chemiluminescent response of leukocytes from the pronephros, a lymphoid tissue which is rich in macrophages and neutrophils. The isoproterenol-induced suppression of the antibody response could be blocked by propranolol, an antagonist of beta-adrenergic receptors. Propranolol was not, however, able to block isoproterenol's influence on the

chemiluminescent response, and propranolol was itself suppressive in the mitogen assay.

Phenylephrine, an agonist of alpha-adrenergic receptors, and carbachol, a cholinergic-receptor agonist, significantly enhanced both the *in vitro* antibody response of splenic leukocytes and the chemiluminescent response of pronephric leukocytes. These effects were successfully blocked by alpha-adrenergic and muscarinic-cholinergic receptor antagonists, respectively. Neither phenylephrine nor carbachol had any influence on the mitogen-induced proliferation of leukocytes from the spleen. The alpha agonist-induced enhancement of the antibody response appears to be mediated by an alpha-2 subclass of receptor, as evidenced by the influence of selective alpha-1 and alpha-2 agonists and antagonists.

The innervation of the salmonid spleen, the effect of denervation on the *in vivo* antibody response, and the effects of adrenergic and cholinergic agents on the *in vitro* antibody response, all support an immunoregulatory role for autonomic neurotransmitters in salmonids. The results of the chemiluminescence experiments suggest that alterations of the antibody response may be due, at least in part, to effects on accessory cell function. There is little evidence that effects on cell division play a major role, at least *in vitro*, in determining antibody-secreting cell numbers.

One approach which is useful in delineating the mechanism of action of immunoregulatory agents is the use of different stimulating antigens. By using a variety of antigens, each with its

own cellular requirements for inducing a response, the cell type(s) most sensitive to the agent can often be established. As mentioned previously TNP-LPS, the antigen used in the *in vitro* experiments, requires accessory cells to induce an antibody response. Similar experiments were run using the T-dependent antigen TNP-KLH (TNP-keyhole limpet hemocyanin). None of the adrenergic and cholinergic agents influenced the PFC response to this antigen. Other evidence indicates, however, that the high concentrations of antigen required for a response (a response which was still only a few hundred PFC/culture compared to ten to twenty thousand PFC/culture obtained with TNP-LPS) was inducing proliferation, and PFCs, nonspecifically. This evidence is that the concentration of TNP-KLH required for a significant PFC response (0.5mg/ml) gave a stimulation index of 8 to 9 in the mitogen assay. Therefore, rather than assessing the effects of adrenergic and cholinergic agents on antigen-specific stimulation one is looking at their effects on nonspecific, polyclonal stimulation, which, as we have seen in the mitogenesis experiments, is not significantly influenced by these agents. TNP-LPS at 1.0ug/ml (the concentration used in the PFC assay) did not significantly stimulate mitogenesis.

Preliminary attempts were made to assess how short term pulses (rather than continuous exposure) of cells to the agonists influenced the *in vitro* antibody response. A one hour preincubation of splenic leukocytes with phenylephrine resulted in an enhancement of the PFC response which was equal in magnitude to that observed with continuous exposure to the drug. A one hour pulse

at the end of the eight day culture also significantly enhanced the response, but much less so than with continuous exposure (or one hour preincubation). Cultures preincubated for one hour with carbachol did not exhibit the enhancement attained with continuous exposure, but a one hour pulse of carbachol at the end of the culture period caused an enhancement equal to that observed with continuous exposure. The experiments need to be repeated before any firm conclusions can be drawn from these results.

In conclusion, given the substantial increase in adrenergic activity which occurs in salmonids during periods of stress, and the documented effects of adrenergic agonists on leukocyte function, it is imperative to consider the catecholamines (as well as numerous other 'stress hormones') when investigating the mechanisms by which stress affects immune competence in these and other teleosts.

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