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Characterization of anti-tumor immunity in oyster toadfish (Opsanus tau), and the effects of 7,12 -dimethylbenz(A)anthracene on this immune response

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CHARACTERIZATION OF ANTI-TUMOR IMMUNITY IN OYSTER TOADFISH (Opsanus tau), AND THE EFFECTS OF 7,12-DIMETHYLBENZ[A]ANTHRACENE ON THIS IMMUNE RESPONSE

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Kenneth R. Seeley

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DEDICATION

This work is dedicated to my parents, Kenneth and Helen Seeley. The pride and happiness I feel over completing my formal education is diminished by the fact that they did not survive long enough to see it happen.

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ABSTRACT

Cell-mediated lysis of cultured tumor target cells by nonspecific cytotoxic cells (NCC) was examined in the oyster toadfish (Opsanus tau), an estuarine teleost. NCC activity was evaluated in cells taken from the head kidney, peripheral blood, spleen and peritoneal cavity. This activity was found to be highest in peritoneal leukocytes. Binding of oyster toadfish NCC to cultured tumor target cells occurred rapidly (within 5 min), while lysis of these cells occurred over a period of several hours. NCC activity was a property of plastic nonadherent cells which lacked phagocytic activity, indicating that in terms of their functional capacity, the cells which mediate nonspecific cytoxic immune responses in oyster toadfish do not appear to be monocytes or macrophages. However, light and electron microscopic examination of these cells revealed that morphologically, they resemble monocytes and macrophages. These results indicate that oyster toadfish NCC may represent a distinct subpopulation of cells of monocytic lineage in which phagocytic and adherence properties have either been lost or never fully developed.

A new technique to assess in vitro phagocytic function of fish macrophages is described. This assay involves the spectrophotometric measurement of congo red-stained yeast cells that have been phagocytized by macrophages. The assay is simple, rapid and reproducible. Furthermore, it is less subjective than previously described methods that employ microscopic examination of cells. Using this technique with oyster toadfish, phagocytosis of yeast cells was found to increase with time, reaching a maximum between 60 and 90 minutes (as determined by absorbance at 510 nm). The efficacy of this technique as a biomarker of fish macrophage function was assessed in a field investigation using oyster toadfish taken from several stations in the highly polluted Elizabeth River, Virginia. Sampling sites selected for this field investigation had a sediment-bound polycyclic aromatic hydrocarbon (PAH) concentration gradient of 55 ppb to 96,000 ppb total PAH. Results of this study suggest that varying levels of PAH contamination did not lead to significant between-site differences in terms of simple ability of oyster toadfish macrophages to phagocytize foreign particles. However, macrophages of fish taken from the most polluted sites did exhibit a reduced phagocytic capacity, in that the total amount of foreign material they were capable

of engulfing was significantly reduced. Because this technique is a simple, rapid and accurate biomarker of macrophage function in fish, it has been used in studies designed to characterize the functioning of oyster toadfish NCC and in toxicological investigations comparing the effects of PAH on NCC and macrophage function in oyster toadfish.

Studies were undertaken to determine the effect of the chemical carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) on the function of oyster toadfish peritoneal nonspecific cytotoxic cells (NCC) and macrophages. The functioning of these two cell populations was assessed in terms of their ability to lyse cultured tumor target cells and to phagocytize yeast cells, respectively. Following intraperitoneal injection of 0, 1, 10, 50 and 100 mg DMBA/kg body weight, macrophage activity was suppressed in an essentially linear fashion, whereas NCC activity was virtually eliminated at all doses of DMBA administered.

In a time-course study, the effects of DMBA on NCC activity were found to be highly persistent. Following a single intraperitoneal injection of 10 mg/kg, NCC activity was again virtually eliminated, and did not recover throughout the 28 days of the exposure study. In the same study, macrophage activity declined gradually throughout the exposure period. This decline may have been the result of a general decline in the health of the exposed animals, rather than a direct effect of DMBA exposure. Furthermore, mortality in DMBA-exposed fish was closely correlated with the phagocytic capacity of macrophages, but not with the tumorlytic capacity of NCC.

CHARACTERIZATION OF ANTI-TUMOR IMMUNITY IN OYSTER TOADFISH (*Opsanus tau*), AND THE EFFECTS OF 7,12-DIMETHYLBENZ[A]ANTHRACENE ON THIS IMMUNE RESPONSE

CHAPTER 1

INTRODUCTION

Overview of Fish Immunotoxicology

Without a highly evolved immune system, no vertebrate would be capable of surviving the many challenges posed by our hostile environment. At any moment, an animal's survival is challenged by a vast array of threats, both external (i.e., infection by viruses, bacteria and other microorganisms) or internal (i.e., transformation of normal cells into wild, potentially cancerous cells) in nature. Normally, an animal's immune system can respond to these threats in a highly coordinated and efficient manner. At times however, the immune system is incapable of properly protecting the animal. This inability to function normally can occur for a number of reasons: the animal may suffer from a genetic disorder, the pathogen has evolved a novel system for evading the immune response, or some environmental factor has caused the animal's immune system to be impaired. This final case, environmental impairment of the immune response, has generated a considerable amount of interest in recent years, and has led to the establishment of a new subdiscipline within the field of

immunology: immunotoxicology.

Dean et al. (1986b) have given a clear rationale for studying the effects of toxic chemicals on immune system functioning:

> "It is becoming increasingly apparent that the immune system represents an important target organ for studying the toxicology of chemical exposures for the following reasons: immunocompetent cells are required for host resistance, and thus exposure to immunotoxicants can result in increased susceptibility to disease; immunocompetent cells require continued proliferation and differentiation for self-renewal and are thus sensitive to agents that affect cell proliferation; the cellular and molecular biology of the immune system is better understood than in many other target organ systems, and thus the mechanism(s) by which toxicants are immunoalterative can be determined; functional assessment or enumeration of leukocytes can be easily achieved using a small volume of blood or lymphoid tissue; and finally, observations obtained in experimental animals can be confirmed in humans using leukocytes obtained by minimally invasive methods (i.e., venipuncture)."

Our understanding of the toxic responses of the immune systems of fishes lags behind that of their mammalian counterparts. As a result, fish immunotoxicology has largely been limited to little more than anecdotes scattered throughout the vast literature of ichthyology. The need for increased emphasis on this area of research is readily apparent, however, when one considers that fish are becoming more important as a source of human food, that fish disease control measures account for approximately 30% of the cost of fish culture, that fish are frequently exposed to a wide variety of toxic agents and are capable of accumulating high levels of many of these agents, and that

tumors in fish are becoming increasingly common, presumably as a result of increased exposure to certain toxic agents (Zeeman and Brindley, 1981).

The immunosuppressive role of various environmental pollutants in the development of neoplasia in fish has begun to receive a considerable amount of attention in recent years. Numerous studies have linked tumor incidence in fish to carcinogenic compounds in aquatic ecosystems. Currently, emphasis is being placed on elucidating the toxic mechanisms associated with immunomodulatory compounds, particularly those known or presumed to be carcinogenic (Rice, 1989; Zeeman and Brindley, 1981).

Baumann et al. (1987) studied the tumor prevalence in fish populations from polluted rivers. Liver tumor prevalences in brown bullhead catfish (Ictalurus nebulosus) exhibited a strong positive association with polycyclic aromatic hydrocarbon (PAH) sediment contamination levels in the Black River, Ohio, an industrialized tributary of Lake Erie. In this study, liver tumors, primarily cholangiocarcinomas, were found to increase significantly with age and PAH levels in the brown bullhead. Frequency of external tumors, however, could only be correlated with PAH levels, and not with age. Kelly and Maddock (1985) found increased levels of unscheduled DNA synthesis, presumably an early indicator of genotoxic effects, in oyster toadfish (Opsanus tau) hepatocytes following exposure to the PAH

benzo[a]pyrene (B[a]P). Balouet (1986) has reviewed the classification, prevalence and putative etiological factors associated with a variety of tumor types in marine organisms, including wild finfish and mollusc populations, emphasizing the important role of environmental degradation in the progression of these diseases. Overstreet and Howse (1977) have also reviewed the incidence of neoplasia in finfish from polluted estuaries in Mississippi.

General Concepts of Vertebrate Immunity

This review will center mainly on the components of the immune system that have been considered to be critical to the functioning of the system as a whole. It should be noted that the immune system of fishes, with some exceptions, is remarkably similar to that found in mammals (Corbel, 1975). The immune system is a complex network of cells and tissues that function together in a remarkably coordinated fashion to protect the host animal from infection and disease. Among the immunologically active cells of mammals are granulocytes (including eosinophils, neutrophils and basophils) that function in non-specific defenses; tissue macrophages or circulating monocytes, that also play a role in non-specific defenses as well as in specific defenses; B-lymphocytes, that are responsible for specific antibody production; and T-lymphocytes, that carry out a variety of immune effector and coordinating functions

(Golub, 1987). Each of these cell types can actually carry out a number of functions, and this list is certainly not complete. All of the immunologically active cells found in mammals, or their functional analogs, are found in fish (Ellis, 1977). In mammals, the center of hematopoiesis (blood cell development) is the bone marrow, with the sites of lymphocyte maturation and storage being the thymus and spleen, respectively (Sell, 1987). Fish lack bone marrow, therefore, hematopoiesis takes place in the pronephros, or anterior kidney, with lymphocyte maturation occurring in the spleen (Anderson, 1974).

Immune responses can be broken down into two major functional categories: non-specific (constitutive) and specific (adaptive). Non-specific immune responses require no prior contact with the inducing agent. Specific immune responses do require prior contact but allow for enhanced efficiency during subsequent exposures to the inducing agent. Mononuclear phagocytes (i.e., blood monocytes and tissue macrophages) and granulocytes are phagocytic cells involved with non-specific resistance. Lymphoid cells, as well as macrophages are responsible for specific host responses.

The skin, gills (in fish) and to a lesser extent the alimentary tract usually provide the first contact with any potential pathogen. Hence, the integrity of these primary barriers will often determine whether the stressor remains

localized or becomes systemic. Epithelial surfaces with their mucous secretions form a physical barrier between a fish and its environment, and most instances of primary bacterial colonization of the body involve mucosal surfaces. Once physical barriers have been breached, the host must rely on cellular and humoral defense mechanisms. If no prior exposure to a particular antigen has occurred, then defenses would initially be non-specific in nature (Fletcher, 1982; Fletcher, 1986). In addition to external physical barriers against infection, vertebrates possess a number of innate or "natural" defense mechanisms or factors that are distinct from cell-mediated or antibody-mediated defenses. Various factors, often referred to as "natural" antibodies (since they require no prior exposure to an antigen), play an important role in maintenance of body integrity under adverse conditions. These factors include C-reactive protein (CRP), transferrin, lysozyme, chitinase, interferon and complement (Ingram, 1980).

Two groups of phagocytic leukocytes, the polymorphonuclear leukocytes (PMNs) or granulocytes, and the mononuclear phagocyte, or macrophage, are involved with non-specific host defense mechanisms. Both PMNs and macrophages are phagocytic toward foreign material and can destroy most microorganisms. PMNs represent the primary cellular line of defense against infectious agents. In the event that PMNs either cannot contain or destroy the infectious agent,

macrophages are recruited to the site of infection (Corbel, 1975; Dean et al., 1986a).

Both invertebrates and vertebrates possess the ability to distinguish between self and non-self. Invertebrates, however, lack the adaptive and highly discriminative ability to specifically recognize foreign material that can be observed in the vertebrates, and their immune responses can generally be described as non-specific. Despite this absence of adaptive, cell-mediated immune responses, the invertebrates have been quite successful in evolutionary terms (Ratcliffe, 1989). Thus, one must ask: what unique pressures on the vertebrates necessitated the further evolution of adaptive immunity? It has been theorized that the widespread incidence of malignancies in vertebrates (resulting from increased longevity, larger size and greater complexity, as well as higher susceptibility to radiation damage) made it necessary to evolve the ability to remove somatic mutations. Hence, adaptive immunity may have evolved in the vertebrates in response to internal rather than external threats (Golub, 1987).

Two forms of adaptive immunity exist. The first, cell-mediated immunity (CMI), involves specifically sensitized, thymus-derived lymphocytes, and is generally associated with delayed type hypersensitivity, graft rejection, and resistance to persistent infectious agents (i.e., certain viruses, bacteria, protozoa and fungi). The second form of

adaptive immune response is referred to as humoral immunity (HI). HI involves the production of specific antibodies (immunoglobulins) by bursal-equivalent lymphocytes (B lymphocytes or B cells) or plasma cells following sensitization to a particular antigen.

In addition to their role in non-specific immune responses, macrophages are also critical in the development of adaptive immunity. In this regard, macrophages serve as "antigen-presenting cells" (APCs). In a two-step process referred to as antigen processing and presentation, the APC phagocytizes the foreign material and "processes" it into smaller segments (i.e., proteins are broken down into short peptide sequences). These peptide sequences are then displayed or "presented" on the surface of the APC, so that other cells of the immune system can proceed to mount a response against them.

Processing and presentation of antigen by macrophages or other APCs precedes a complex sequence of events that ultimately draw lymphocytes into the response against an antigen. To summarize, following macrophage activation and presentation of the antigenic segment, the macrophage begins to release a soluble factor called Interleukin 1 (IL-1). IL-1 has been linked to a number of cellular events in mammalian systems, including thymocyte proliferation, cytokine secretion and cellular proliferation. Of particular interest in this context is the fact that IL-1

leads to the expression of receptors for another interleukin (IL-2, which, among other things, is responsible for T cell growth, Natural Killer Cell-differentiation, immunoglobulin secretion by B cells and macrophage activation) on the surface of T-cells, as well as the activation of "T helper" cells (T_h). Upon activation, the T_h cell begins to release IL-2, which will cause the proliferation of previously activated T cells expressing receptors for IL-2. In this manner, a pool of primed effector T-cells is created which can then efficiently attack and destroy invading microorganisms. The occurrence of this particular activation sequence in fish is speculative at this time, although numerous studies have supported the existence of interleukins in the piscine immune response (Sigel et al., 1986; Miller et al., 1989; Wang Yang et al., 1989).

Whether or not an antigen induces CMI, antibody production, or both depends on a multitude of factors, including the physical and chemical nature of the antigen, the mode of presentation of the antigen to lymphocytes, the localization pattern of the antigen within lymphoid tissue, and the molecular configuration of the antigen. Those antigens generally found to elicit CMI include tissue antigens present on cells, chemical agents and drugs which conjugate with autologous proteins, and antigenic determinants on persistent intracellular microorganisms. Other antigens (i.e., pneumococcal polysaccharides)

primarily elicit antibody responses. The route of antigen exposure also plays a role in the type of response generated. For example, sheep erythrocytes will elicit antibody responses when administered intravenously, but will elicit both CMI and antibodies when injected intracutaneously. Intradermal presentation favors CMI responses (Dean et al., 1986b)

Many infectious agents can be dealt with adequately by antibody-mediated (HI) or non-specific defense mechanisms. Other infectious agents have "chosen" an intracellular habitat and are thus protected from the host's humoral defenses (Kaufmann, 1988). As infected cells replicate, antigenic moieties of these pathogens appear on the surface of the host cells. These antigenic moieties can then be recognized by T-lymphocytes. In this way, infected host cells can be discriminated from uninfected cells, and appropriate responses against these infected cells can be initiated.

T-cells are capable of distinguishing between self and non-self because of the presence of identifying structures on the surface of cells known as major histocompatibility complexes (MHC). Two classes of MHC are known to exist in mammals (Class I and Class II), although little is known about MHC structure in fish. Based on the high degree of recognitive ability of fish immune systems, however, it can be assumed that the molecular biology of fish MHC is

analogous to that of mammals. The two types of MHC are expressed on different types of cells in the body (Alberts et al., 1989). Virtually all host cells express Class I MHC molecules, but antigens associated with Class I structures are generally restricted to proteins that are newly synthesized within the host cell. For example, products of viral genes that are displayed on the surface of host cells are generally associated with Class I MHC complexes. These proteins are synthesized within the host cell as it replicates, and are thus referred to as "endogenous". By contrast, antigens that are taken up into host cells via endocytosis are termed "exogenous antigens." For example, bacterial, fungal or protozoan antigens that have been phagocytized, degraded and subsequently presented on the surface of APCs are generally found in conjunction with Class II MHC complexes. Class II MHC complexes are only expressed by a limited number of host cells (i.e., B lymphocytes, dendritic cells and mononuclear phagocytes).

B and T cells utilize dramatically different mechanisms for the recognition of antigen. Antibodies produced by B cells recognize antigens in solution or on cell surfaces in their native (three-dimensional) conformation, whereas T cells can only recognize antigens when they are displayed in conjunction with MHC molecules that are present on cell surfaces. Usually antigens displayed in this manner have been degraded or processed in some way, so that the antigen

recognized by the T-cell is actually only a small fragment of the original antigen (Grey et al., 1989).

The cells on which antigen is displayed can be of two forms: specialized antigen-presenting cells (APCs) which are capable of stimulating T-cell proliferation, or any virally-infected or transformed cell which can become a target for cytotoxic T-cells. Phagocytes (among others) can serve as APCs when they go through the processes of antigen processing and presentation.

Generally, most T_h cells recognize antigen in association with Class II MHC molecules, while cytotoxic cells recognize Class I MHC molecules. Thus antigen recognition by T-cells is MHC restricted, and not all T-cells recognize the same MHC molecules. Cells which recognize Class I MHC are referred to as being Class I-restricted, while cells recognizing Class II MHC products are referred to as being Class II restricted.

T-cells are critical in the development of all cell-mediated immune reactions (Roitt et al., 1985). In this respect, T-cells serve as either modulators of the overall immune response (as is the case with T_h cells), or as the actual effector cells of the reaction (as is the case with cytotoxic T-cells). Generally, the functional differences between T-cell subpopulations are closely correlated with the different ways they recognize antigen in conjunction with MHC molecules.

Anti-Tumor Immunity in Vertebrates

It has been hypothesized that the two types of MHC exist in order to keep cell-mediated immune responses tightly regulated. Thus, cytotoxic T-cells can only recognize endogenously produced antigen when it is displayed in conjunction with Class I MHC. This mechanism requires that the T-cell be in close contact with an infected host cell before it can mount an attack against the virus. Any effort to attack a viral particle that is circulating freely would simply be wasted effort for the T-cell, as it would have no chance of destroying it. Thus, the cytotoxic T-cell responds only to infected host cells, and its response is restricted to an extremely localized area. On the other hand, Class II MHC allows T_h cells to interact with other cells involved in the immune response (i.e., B-cells, antigen presenting cells, etc.). Since the T_h cell does not act as an effector cell itself, but rather "coordinates" the actions of other immune cells, it makes sense that it can only recognize antigen that is displayed in conjunction with Class II MHC on the surface of these other immune cells (Alberts et al., 1989).

It has also been hypothesized that the highly adaptive cell-mediated immune responses of vertebrates evolved in response to internal rather than external threats (i.e., neoplasia). This hypothesis eventually led to the development of the Immune Surveillance Theory, first

proposed by Burnet (1970), which has since become the dominant paradigm in tumor immunology. In its simplest form, this theory states that tumor specific antigens are associated with the surfaces of neoplastic cells, and the immune system acts as a surveillance system to identify and destroy any cells which express these new antigens, as they are recognized as being "foreign".

An aspect of immune system functioning that has received considerable attention in recent years is the ability of certain cells to destroy transformed (neoplastic) cells. As the theory of immunosurveillance suggests, the immune system of a healthy animal will remain free of tumors. If the effector cells responsible for elimination of transformed cells are somehow compromised, the result will be that these neoplastic cells will be free to proliferate, leading to the formation of a tumor.

Lysis of tumor target cells by immune effector cells can occur via both specific and nonspecific mechanisms. When antigens on the tumor cell membranes trigger the lytic mechanism in the corresponding receptor-carrying members of the T-cell repertoire, the effectors are named cytotoxic T lymphocytes (CTLs). CTLs recognize antigen on the surface of virally-infected or transformed host cells, and thus they are Class 1 MHC-restricted. These cells carry out responses that are specific in nature and require prior exposure to a particular antigen in order to mount an attack against it.

By contrast, another type of cytoxic effector cell is not MHC-restricted, and as such, can mount non-specific responses against transformed cells to which it has had no prior exposure. In mammals, these non-specific cytotoxic effector cells are referred to as natural killer (NK) cells. NK cells can be activated to a higher level of cytotoxic potency by interferon, allowing them to destroy a wider range of target cell types. When this occurs, NK cells are referred to as interferon-activated killer (IAK) cells. NK cells can also be activated by lymphokines, particularly IL-2. When this occurs, they are referred to as lymphokine-activated killer (LAK) cells (Klein et al., 1988).

Although they utilize different recognition mechanisms, both CTL and NK cells can destroy tumor target cells (TC) by essentially the same mechanism. Once the cytotoxic effector cell has bound to the surface of its target, it releases molecules of a lethal protein (perforin) which bore into the TC's surface membrane, forming pore-like channels. The channels cause the target cell to leak, and it dies rapidly (Young and Cohn, 1988).

Prior to the actual killing event, a number of steps must first take place: 1) The effector cell must first bind to the target cell. In the case of the CTL, this is accomplished via an interaction between the T-cell receptor and the Class I MHC molecule on the surface of the TC. In the case of the NK cell, the mode of recognition is not yet

known, although it is assumed that NK cells are capable of recognizing some structure on the surface of the TC and identifying it as either self or non-self. 2) Once recognition and binding have occurred, the effector cell must be triggered or activated, so that ultimately cytoplasmic granules within the cell will be reoriented to the site of binding with the TC; 3) The contents of these granules are released at or in close proximity to the junction with the TC; 4) Cytotoxic factors released from the granules bind to the surface of the TC and cause it to lyse (Herberman, 1985).

CTL have been known to exist for quite some time (Herberman, 1981), but NK cells are a relatively new discovery, first being described in 1975 as effector cells capable of <u>in vitro</u> lysis of certain tumor targets. At this time, NK cells were postulated to have a role in the body's first line of defense against neoplasia. In recent years however, investigators have found that NK cells are capable of recognizing and destroying a wide variety of targets, including bacterial, protozoan and fungal parasites. Thus, NK cells may actually play an important protective role in a variety of infectious diseases and neoplastic conditions, and they may serve to limit the extent of dissemination during the earliest stages of infection before more specific immune responses become effective (Lanier and Phillips, 1988).

Natural cytotoxic activity seems to be carried out by a heterogenous population of effector cells, and NK cells may be best defined in terms of what they are not, rather than what they are (Herberman, 1985). NK cells in mammals are known to be lymphoid cells with azurophilic granules. They have spontaneous cytotoxic activity against tumor cells, as well as virus infected cells and some normal cells. NK cells are set apart from other lymphoid cells in that they lack some characteristic features of CTL (particularly with regard to certain surface markers displayed on CTL). NK cells also lack characteristic surface markers found on monocytes and macrophages. Mammalian NK cells are closely associated with a subpopulation of bone marrow-derived cells referred to as large granular lymphocytes (LGL). Finally, as stated before, NK cells are not MHC-restricted, as are CTL.

Both non-specific and specific cytotoxic effector cells have been described in fish and other ectothermic vertebrates (Evans and Cooper, 1990). Non-specific cytotoxic cells have been studied in bicolor damselfish, Pomacentrus partitus (McKinney and Scmale, 1988); carp, Cyprinus carpio, crucian carp, Carassius cuvieri, grass carp, Ctenopharyngodon idella, oriental weatherfish, Misgrunus anguillicandatus and northern snakehead, Channa argus (Hinuma et al., 1980; Bielak, 1988) and a hybrid tilapia (Faisal et al., 1989a).

In fish, non-specific anti-tumor activity has probably been best characterized in channel catfish (Ictalurus punctatus). It has been determined that the catfish homologues of mammalian NK cells, referred to as "nonspecific cytotoxic cells" (NCC), are capable of nonspecifically attacking and destroying a diverse array of transformed cells, i.e., human B cell lymphoma as well as various murine cell lines (Evans et al., 1984a; Graves et al., 1984). Furthermore, cells responsible for NCC activity in catfish represent a heterogenous population, being both adherent and non-adherent, while at the same time being of roughly the same density. Also, in order to demonstrate that NCC are not simply macrophages, these workers have shown that they lack phagocytic activity (Evans et al., 1984b), although the NCC of I. punctatus were found to be morphologically similar to monocytes (Evans et al., 1984c).

Effects of Environmental Chemicals on Anti-tumor Immunity

As stated earlier, a number of studies have linked tumors in fish to various aquatic pollutants. If the theory of immunosurveillance is correct, then one can assume that the functional ability of the cytotoxic effector cells of these fish has been diminished by exposure to these pollutants. Otherwise, neoplastic cells would be eliminated as they formed. A number of toxic compounds have been shown to modulate the functional ability of cytotoxic effector

cells in mammals (see discussion below), but very little research has been done thus far to link similar exposures in fish to altered cytotoxic effector cell function.

Originally, it was believed that immunosurveillance was mediated entirely by adaptive immune responses. Specifically, it was felt that T lymphocytes were the primary vectors for mounting specific immune responses against neoplastic cells. Thus, certain carcinogens such as 7,12-dimethylbenz[a]anthracene (DMBA), exerted their immunotoxic effects by impairing T-cell function, thereby impairing immunosurveillance. However, the relative importance of CTL suppression in the progression of neoplasia was called into question when it was found that the dose of carcinogen required to cause depressed T cell function was considerably higher than that required for tumor induction (Kimber et al., 1986a). These investigators have shown that DMBA is capable of transiently depressing NK function in rats at a dose of 40 mg, a dose which was also capable of inducing mammary gland tumors in Sprague-Dawley rats, while such a dose was incapable of suppressing T cell function.

As a result of this finding, more recent, studies have focused on the role played by NK cells in maintaining host resistance to malignancy, and a number of these studies have provided a link between suppression of natural cytotoxic function and tumorigenesis. To date, these studies have

been limited primarily to mammalian models, particularly rats and mice.

Erlich et al. (1983) studied suppression of murine NK following dietary exposure to DMBA. These workers focused on the period between initial DMBA exposure and tumor induction, since it is during this period that natural host resistance is critical in arresting or delaying the development of malignant tumors from transformed cells. At doses of DMBA as low as 3 mg, NK activity was suppressed, but was capable of spontaneous restoration, and tumor incidence was low. At a dose of 6 mg, NK activity could not be restored spontaneously, and tumor incidence was high. The authors concluded that DMBA was capable of inducing suppression of NK function, and with sufficiently high doses of DMBA, this suppression could last long enough to allow the development of tumors to occur.

Kimber et al. (1986a) found that suppression of NK function in DMBA-treated rats occurred without concomitant suppression of T cell function. These workers concluded that under certain circumstances, DMBA may be capable of selectively impairing NK function. This directly contradicts the findings of Dean et al. (1986a), however, who found that doses of DMBA ranging from 5 to 100 mg/kg were capable of causing general immunosuppression in mice. The immunosuppressive effects observed in this study included decreased splenic cellularity, decreased number of

peritoneal cells, decreased T and B cell mitogenic activity and lowered CTL and NK activity. Despite this generalized immunosuppression, the doses of DMBA used in this study were insufficient to reduce tumor resistance during a challenge with injected tumor cells, although reduction in CTL activity could be correlated with increased tumor frequency.

House et al. (1989) have suggested that exposure to DMBA can lead to suppression of cytotoxic T cell (CTL) function in mice. These workers have concluded that this CTL suppression was due to inhibition of T_h cell function, specifically at the point of antigen recognition by the T_h cell, with the most plausible explanation being that DMBA somehow interferes with the functioning of the T cell receptor. Because of the critical role played by this particular T cell subpopulation in virtually all aspects of immune system functioning, loss of T_h cell activity can result in a broad spectrum of immunosuppressive events, including impaired B cell activation, development of anamnestic responses, anti-tumor immunity, etc. (Dean et al., 1986a).

Cytotoxic cell function can also be altered by means other than exposure to exogenous chemical exposure. Faisal et al., (1989b) have found that social confrontation in Tilapia spp. resulted in suppression of NK activity as well as mitogen-induced proliferation of pronephric leukocytes. Interestingly, this suppression has only been seen in

subordinate fish; fish that play the role of the aggressor during confrontation do not exhibit any significant degree of immunosuppression. This suppression could be reversed by administration of naltrexone, an opioid antagonist, suggesting a link between the neuroendocrine and immune systems.

Polycyclic Aromatic Hydrocarbons

7,12-Dimethylbenz(a)anthracene is a member of a class of organic compounds referred to as "polynuclear (or polycyclic) aromatic hydrocarbons" (PAH). PAH are so named because they are composed of two or more fused benzene rings (hence the term "polycyclic") with some of the carbon atoms being doubly-bonded to other carbon atoms (hence the term "aromatic"). By definition, PAH contain only carbon and hydrogen atoms, although other atoms (i.e., nitrogen, sulfur and oxygen) can be substituted for carbon atoms in the rings, in which case the resultant compounds are referred to as "heteroaromatics" (Blumer, 1976).

PAH that are of primary environmental concern range in molecular weight from naphthalene ($C_{10}H_8$), with a molecular weight of 128.16, to coronene ($C_{24}H_{12}$), with a molecular weight of 300.36. These PAH are considered environmentally important because of their mobility. Larger PAH are relatively immobile however, because of their lower solubilities and volatilities (Neff, 1985).

According to Blumer (1976), PAH are formed whenever organic substances are exposed to high temperatures. This formation occurs via a process termed pyrolysis, in which energy is released and the resultant aromatic products are more stable than their precursors. However, high temperatures are not always necessary for aromatization to occur. In the formation of crude oil, aromatic hydrocarbons form slowly over the course of millions of years at temperatures as low as 100 to 150 °C.

The majority of PAH released into the aquatic environment are from anthropogenic sources (McElroy, et al., 1989) although a great variety of these compounds arise from natural sources, such as plant and animal pigments (Blumer, 1976). In terms of anthropogenic inputs to the environment, it appears that the primary source of PAH is from atmospheric deposition resulting from combustion of fossil fuels. This conclusion is based on the observations that PAH are ubiquitous in sediments near urban areas and their depth profiles in these sediments correspond to modern-day fossil fuel utilization (McElroy et al., 1989). In addition to combustion of fossil fuels, other important sources of PAH in the environment include spillage of crude and refined oils as well as natural combustion of organic material in forest or grass fires (Stegeman, 1981).

Neff (1985) has listed the major routes of entry of PAH into the marine and freshwater environments as including

biosynthesis, spillage and seepage of fossil fuels, discharge of domestic and industrial wastes, fallout or rainout from air, and runoff from land. Annual inputs from all of these sources into the aquatic environment is estimated to be approximately 230,000 tons. Of this amount, surface runoff from land and fallout from the air are the main sources of high molecular weight PAH, while oil spillage is the main source of total PAH.

As a group, PAH are generally hydrophobic, although they possess a wide range of solubilities. Because of their hydrophobicity, PAH rapidly bind to particles in the aquatic environment, and as a result they are ultimately deposited in sediments (Gearing et al., 1980). Once associated with sediments, PAH can go on to be redistributed via physical processes, such as local turbulence or currents, as well as through biological processes, such as bioturbation (McElroy et al., 1989). However, Neff (1985) points out that most of the environmental PAH burden remains relatively close to point sources, decreasing in an essentially logarithmic fashion away from the source. Thus, most PAH in the aquatic environment remain localized in rivers, estuaries and coastal marine areas.

The solubility of PAH appears to be only slightly affected by temperature (May et al., 1978). In natural seawater, salinity appears to have an even smaller impact on PAH solubility than does temperature (Whitehouse, 1984).

PAH do have a tendency to leave the dissolved state and bind to available solid material suspended in the water column. Because of this, dissolved organic material (DOM) has been postulated to increase the solubility of PAH in seawater (McElroy, et al., 1989). Colloids (any particle with a linear dimension between 10^{-7} cm and 10^{-4} cm) are part of the organic matter that make up DOM. Colloid organic material has a high capacity to sorb PAH (Witjayaratne et al., 1980).

The bioavailability of PAH is strongly influenced by association with colloidal or particulate material. Sorption-desorption equilibria of PAH with particulate matter indicate that some portion of even higher molecular weight compounds will be in solution (Stegeman, 1981). In interstitial waters of natural sediments, polychlorinated biphenyls (PCB), which have many physical-chemical properties in common with PAH, seem to follow a three phase partitioning model, in which equilibrium is established between the aqueous, colloid-bound and sediment-bound phases (McElroy, et al., 1989).

Once in the aquatic environment, PAH can undergo a variety of processes that lead to their transformation or degradation. Among these processes are photooxidation, chemical oxidation and biological transformation by bacteria, fungi and animals. These processes are described by Neff (1985). Despite the variety of chemical, physical and biological processes that can occur in the aquatic

environment, marine animals are nevertheless exposed to a wide variety of unaltered PAH. These unaltered compounds can then be taken up by fish via several routes, including transport across gill epithelial surfaces and skin, as well as through dietary uptake (Stegeman, 1981).

Whether or not exposure to PAH will result in immunosuppression, cancer, or a wide range of other conditions in aquatic organisms depends on a number of factors (McElroy, et al., 1989). Among these factors are the degree of exposure, ability of the fish to absorb and metabolize the PAH and to repair any resulting genetic damage. Other factors include the age of the exposed animal, genetic variation, nutritional status, water temperature, and finally, route of exposure. Route of exposure is a critical factor, particularly in terms of PAH, which will primarily be found in the environment bound to sediments. For this reason, fish that either live in direct contact with aquatic sediments or feed on these sediments represent the best models for PAH toxicity studies. For this series of experiments, the oyster toadfish (Opsanus tau) has been chosen. Oyster toadfish are bottom dwelling fish common in the Chesapeake Bay and its tributaries. Furthermore, they are carnivorous fish, feeding primarily on crabs. Thus, in nature, oyster toadfish can be exposed to sediment-bound PAH via two main routes: through direct exposure to the sediments and dietary uptake.

Overview of the Dissertation

The following chapters describe a series of experiments in which the nonspecific cytotoxic cells in oyster toadfish were examined. In Chapter Two, these cells were characterized both in terms of their morphology and function. Organ distribution, media requirements, and basic kinetics of target cell destruction by oyster toadfish NCC are described. Since teleost NCC are believed by some to be related to monocytes or macrophages, functional characteristics of oyster toadfish NCC are compared to those of macrophages. In Chapter Three, a new technique for assessing macrophage function, developed during the course of this research, is described. Finally, in Chapter Four the effects of _in vivo_ exposure to 7,12-dimethylbenz[a]anthracene on oyster toadfish NCC are described. In these final studies, including dose-response and time course experiments, the effects of DMBA on NCC are compared to its effects on macrophage function using the assay technique described in Chapter Three. The significance of the findings on toxicant-induced NCC suppression are compared to more thoroughly investigated macrophage dysfunction.

CHAPTER 2

PRELIMINARY CHARACTERIZATION OF THE NONSPECIFIC CYTOTOXIC CELLS OF THE OYSTER TOADFISH (Opsanus tau, L.)

Introduction

Lysis of virally-infected or neoplastic cells by natural killer (NK) cells has been studied extensively in mammals and has been shown to be a critical nonspecific immune function in these animals (Herberman, 1982; Herberman et al., 1986). Recently, this phenomenon has received considerable attention from fish immunologists, and has been demonstrated to occur in a wide variety of fish species (Bielek, 1988; Hinuma et al., 1980; Graves et al., 1984; Evans et al., 1984a). These studies have indicated that cytolytic activity in teleosts may be mediated by a population of leukocytes which represents a phylogenetic precursor to the mammalian NK cell. In order to distinguish these cells from their mammalian counterparts, they have been referred to as nonspecific cytotoxic cells (NCC) by Evans et al. (1988).

In this study NCC activity in the oyster toadfish (Opsanus tau), an estuarine teleost used in the past as a model for immunotoxicological investigations (Seeley and

Weeks, 1991; Rice and Weeks, 1989) was examined. The purpose of this study was to characterize NCC activity in oyster toadfish and to establish the optimal parameters for *in vitro* assessment of this activity, so that future experiments can be designed to evaluate the effects of toxic compounds on this important cellular immune response. Thus, the organ distribution of NCC activity, optimal media osmolality for destruction of cultured mammalian tumor target cells, basic kinetics of target cell lysis, and whether the leukocytes responsible for this activity are functionally related to lymphocytes or monocytes and macrophages have been determined.

Oyster toadfish NCC were also characterized in terms of their morphology. Previous studies have found that nonspecific cytotoxic function in teleosts and other fish appears to be carried out by a wide variety of cell types (Table 1). In this study, oyster toadfish NCC were examined using light and electron microscopy.

Materials and Methods

Adult oyster toadfish (300-600 g) were captured by 5-minute net trawls from the relatively non-polluted York River, Virginia. Following capture, fish were held for up to 6 months in 250 gallon holding tanks with mechanically and biologically filtered, UV-irradiated, recirculating seawater with constant salinity (20 ppt.) and temperature

(21°C). Fish were fed bi-weekly a protein and vitamin rich diet that has been described elsewhere (Van Veld et al., 1988).

Prior to dissection, fish were anesthetized in seawater containing 200 mg l^{-1} MS-222 (Sigma, St. Louis, Missouri). Blood samples were taken from the caudal vein using heparinized Vacutainer® syringes (Becton-Dickinson, Rutherford, New Jersey). Peritoneal lavages were performed by injecting 5 ml phosphate buffered saline (PBS, Sigma) containing 5 IU ml^{-1} heparin (Sigma) into the peritoneal cavity. This fluid was subsequently removed by suction through a small ventral incision. Head kidneys and spleens were aseptically removed and leukocytes were obtained by gently forcing these tissues over a stainless steel mesh in petri dishes containing 5 ml RPMI-1640, supplemented with 10% heat inactivated fetal bovine serum (FBS, Sigma). Hypotonic shock was employed to remove erythrocytes from all samples, using a method described by Ghoneum, et al. (1990), and in all samples, leukocyte viability was determined to be 90% or greater by trypan blue exclusion. Cells were suspended in complete RPMI-1640 (RPMI-C, containing 10% FBS, 2 mM L-glutamine, 50,000 IU l^{-1} penicillin, 50 mg l^{-1} streptomycin, 100 mg l^{-1} kanamycin, 2.0 g l^{-1} sodium bicarbonate and 25 mM HEPES), and incubated overnight at 23°C.

The mammalian cell lines K-562 (a human erythroleukemia cell line), P-815 (a mastocytoma cell line derived from a DBA/2 mouse) and YAC-1 (a lymphoblastoid line derived from an A/Sn mouse) were maintained as suspensions in RPMI-C in a 37°C, 95% air/5% CO_2 atmosphere. Mammalian cell lines were generously provided by Dr. C.D. Rice of the Medical College of Virginia.

Cytotoxic activity was determined using a standard ^{51}Cr-release assay (Evans et al., 1984b). Briefly, $1x10^7$ target cells suspended in 1 ml RPMI-C were labeled with 100 μCi of ^{51}Cr-Na_2CrO_4 (DuPont-NEN, Boston, Massachusetts) for 1 hour at 37°C. Excess label was removed by washing the cells once in PBS, followed by a 30 min incubation of the cells at 37°C in 50 ml PBS. The cells were then centrifuged for 10 min at 250xg and the resulting pellet was resuspended in 10 ml RPMI-C. After another centrifugation, the cells were resuspended in fresh RPMI-C at a concentration of $1x10^5$ cells/ml. An aliquot (100 μl) of this suspension was added to each well of a 96 round-bottomed well tissue culture plate (Corning, Corning, New York). Fish (effector) cells were added to these wells at various concentrations needed to give the appropriate effector:target cell ratios. Plates were incubated for 4 hr at 23°C. Following this, plates were centrifuged at 250xg for 10 min, and 100 μl of supernatant was harvested from each well. Radioactivity was measured with a Beckman LS-5000 liquid scintillation

counter. Percent specific release of ^{51}Cr was calculated using the following formula:

$$\%\text{Specific release} = [(\text{E.R.}-\text{S.R.})/(\text{M.R.}-\text{S.R.})] \times 100$$

Where E.R. represents experimental release, M.R. represents maximum release, measured by adding 0.1 ml Triton X-100 (Sigma) to designated wells, and S.R. represents spontaneous release, measured by adding 0.1 ml RPMI-C to designated wells.

Since media osmolality requirements differ between oyster toadfish leukocytes and mammalian target cell lines, it was necessary to determine the optimal media osmolality for target cell lysis. Briefly, RPMI-C was adjusted with either H_2O or NaCl to 280, 300, 320 and 340 mOsm/kg·H_2O with the aid of a freezing point osmometer (Osmette A, Precision Instruments, Natick, Massachussetts). Head kidneys were aseptically removed, cut into four equally sized sections and one section was placed into each of the media preparations and mascerated. Following overnight incubation at 23°C, the leukocytes were co-incubated with YAC-1 target cells and cytotoxic activity was measured at each of the osmolalities.

Peritoneal and head kidney leukocytes were obtained and cell suspensions were prepared as described above. Approximately 2 x 10^6 cells of each cell suspension (in 2 ml

RPMI-C) were placed in one well of a 24-well plate (Corning). Following a six hour incubation at 23°C, nonadherent cells were removed and resuspended in RPMI-C. Cytotoxic activity was then measured using the ^{51}Cr-release assay described above.

Phagocytic activity was determined spectrophotometrically, using a previously described technique (Seeley et al., 1990). Briefly, $1x10^6$ leukocytes in 1 ml RPMI-C were incubated with $4x10^7$ congo red-stained yeast cells (Saccharomyces cerevisiae, Fleischmann's, New Jersey) for 0 and 120 min. Following incubation, 5 ml of ice-cold PBS was added to the samples to prevent further phagocytic activity, and 3 ml of Percoll® (Pharmacia, Uppsala, Sweden), adjusted to a density of 1.055 g ml^{-1} with PBS, was injected into the bottom of each tube. The samples were then centrifuged at 850 x g for 3 min in order to separate leukocytes from any remaining free yeast cells. Following centrifugation, leukocytes located at the Percoll®-PBS interface were removed with a pipet and washed several times in PBS. The cells were then pelleted and supernatants were carefully removed. To each tube, 1.2 ml of a trypsin-EDTA solution (1.5 g l^{-1} trypsin, 0.4 g l^{-1} EDTA in PBS; Sigma) was added in order to solubilize the cells and release any congo red stain. Following an overnight incubation at 37°C, absorbance at 510 nm was determined against a blank containing only the trypsin-EDTA solution.

Target cell binding and destruction was determined using a method described by Grimm and Bonavida (1979). Briefly, $1x10^5$ target cells (YAC-1) were suspended with an equal number of effector cells in 1 ml RPMI-C and incubated for 5 min at 23°C. Following a low speed centrifugation for 10 min, 0.5 ml of the supernatant was removed, and the pellet was gently resuspended in the remaining medium. Next, the cells were resuspended in 3.5 ml of 0.5% agarose in RPMI-C (maintained at 42°C) and 0.5 ml aliquots of this suspension were immediately poured into plastic petri dishes. One ml of complete medium was gently placed on the agarose, and the plates were incubated at 23°C for 0, 0.5, 1 and 2 hours. Control plates containing only target cells were also prepared for each time point. Following the appropriate incubation period the gels were stained with 0.2% trypan blue for 5 min. Gels were then examined with a light microscope, and the percentage of effector-target cell conjugates resulting in target cell lysis was determined in approximately 100 conjugates.

All data were shown to be normally distributed using the Chi-square test for normality (Horning and Weber, 1985) and homogeneity of variance was established using Bartlett's test (Zar, 1984). Analysis of variance was performed on all treatment groups, and significantly different groups were identified using the Tukey method of multiple comparisons (Zar, 1984). T-tests were employed for comparison of

cytotoxic and phagocytic responses of peritoneal and anterior kidney leukocytes before and after removal of plastic adherent cells (Zar, 1984).

For ultrastructural analysis, oyster toadfish leukocytes from kidney, blood, and peritoneal cavity were obtained as described above. These cells were co-incubated with either YAC-1, P815, or K562 target cells for various times, ranging from 5 to 120 minutes. Following co-incubation, the cells were fixed in 0.1 M sodium cacodylate buffer (NaCac) with 3% glutaraldehyde and 0.05% ruthenium red stain for a minimum of 24 hours. Following fixation, cells were washed several times in NaCac. Post-fixation was performed in NaCac with 1% osmium tetroxide for one hour at room temperature. Following post-fixation, samples were again washed several times in NaCac.

Once fixed, samples were enrobed in agar (2% in 0.1 M NaCac) at 58°C for one hour, followed by staining with uranyl acetate (1% in NaCac) for one hour at room temperature. The samples were then dehydrated in a graded series of ethanol, and embedded in Epon 812®. Ultra-thin sections were then prepared and stained using routine methods. For light microscopy, smears were made from the cell suspensions described above, and stained with Wright's stain. In some cases, semi-thin sections of the fixed samples, stained with toluidine blue, were used.

Results

Cytotoxic activity was evaluated in leukocytes from the spleen, anterior kidney, peritoneal cavity and peripheral blood. Using K562 cells as targets, peritoneal leukocytes possessed the highest cytotoxic activity, followed by leukocytes of the anterior kidney, peripheral blood and spleen (Figure 1). Furthermore, the cytotoxic activity of peritoneal leukocytes remained at relatively high levels when effector:target cell ratios were reduced from 50:1 to either 25:1 or 10:1, indicating that these cells possess a greater lytic potential vs K562 than do leukocytes from the other tissues examined.

Cytotoxic activity of anterior kidney leukocytes against the target cell YAC-1 was evaluated at various medium osmolalities (Figure 2). Cytotoxic activity was highest at 280 mOsm/kg·H_2O. Increasing osmolality beyond this range resulted in a substantial reduction of cytotoxic activity. Thus, for the purposes of our assay system, medium osmolality was optimized for the mammalian target cells (280 mOsm/kg·H_2O), rather than the oyster toadfish leukocytes.

The effect of removing plastic adherent cells on the phagocytic activity of oyster toadfish anterior kidney and peritoneal leukocytes is shown in Figure 3a. Phagocytic activity in peritoneal cells was substantially diminished in the nonadherent fraction of leukocytes compared to

unfractionated cells, indicating that the majority of phagocytically active cells are plastic adherent. Conversely, removal of plastic adherent cells from the anterior kidney had no significant effect on phagocytic activity, which was significantly lower than the activity of peritoneal cells in all cases.

The effect of removing plastic adherent cells on NCC activity in peritoneal and anterior kidney leukocytes is shown in Figure 3b. With peritoneal cells, removal of plastic adherent cells resulted in a substantial increase in cytotoxic activity, indicating that NCC activity is concentrated in the plastic nonadherent fraction. Removal of plastic adherent cells from the anterior kidney also resulted in increased cytotoxic activity, although this increase was not statistically significant ($p<0.05$).

The kinetics of target cell destruction following conjugate formation by peritoneal, anterior kidney and peripheral blood leukocytes are shown in Figure 4. Although NCC activity varies between these tissues, analysis of rates of target cell destruction indicates that once effector:target conjugates have formed, lysis of target cells occurs at essentially the same rate. Since cells are immobilized in agarose for this assay, it is not possible to determine recycling capacity of oyster toadfish NCC using this technique.

The results of light and electron microscopic analyses are shown in Figures 5-7. These figures follow the general sequence of events involved in the destruction of target cells by oyster toadfish NCC. Figure 5 shows the initial steps in target cell lysis, including recognition and binding of the target cell by NCC. Figure 6 shows target cells which are in the process of being lysed by NCC, and finally, in Figure 7, destroyed target cells are engulfed and degraded by phagocytic cells.

Electron and light microscopic analyses showed that conjugate formation between oyster toadfish NCC and YAK-1 or P815 target cells occurred rapidly, within minutes following initial contact. In cases where cell suspensions of target and effector cells were fixed following a five minute co-incubation period, conjugates composed of effector cells and generally intact target cells were observed (Figures 5 and 6). However, when co-incubation periods were extended to one or more hours, viable target cells were rarely observed in conjugates. In fact, when these extended co-incubation periods were used, target cells occuring in conjugates were generally undergoing phagocytosis by macrophages (Figure 7). In these cases, it could not be determined if the oyster toadfish leukocytes phagocytizing the dead target cells or lysed target cell debris were the same cells that had originally killed the target cells.

Ultrastructurally, the cells responsible for nonspecific cytotoxic activity in oyster toadfish generally had the morphologic characteristics of monocytes or macrophages. That is, these cells possessed a low nucleus/cytoplasm ratio, long microvillous projections, and large, irregularly shaped nuclei. The size of these cells varied considerably because of their irregular shape, but typically ranged between 10-20 μm, depending on the degree of cytoplasmic spreading involved. The cytoplasm of these cells had numerous organelles and in some cases, particularly with peritoneal leukocytes, numerous lysosomes containing degraded cellular debris. Presumably, this degraded material was phagocytized debris from lysed target cells.

In certain cases, cells resembling lymphocytes were found in close proximity to conjugates, although these cells were never found in direct contact with target cells. On the other hand, light microscopic analysis of conjugates seemed to show the presence of lymphocyte-like cells in conjugate pairs. Since the presence of these cells could not be confirmed through ultrastructural analysis, it is reasonable to assume that these cells were in fact monocytes, or possibly even cellular "blebbings" on the target cell surfaces that appeared to be lymphocytes.

Figure 1. Oyster toadfish NCC activity against the target cell K562 in leukocytes from various tissues. Bars represent sample means (N=5) $\pm$ S.E.M. *, samples which are significantly different from all others at the same E:T ratio ($P<0.05$).

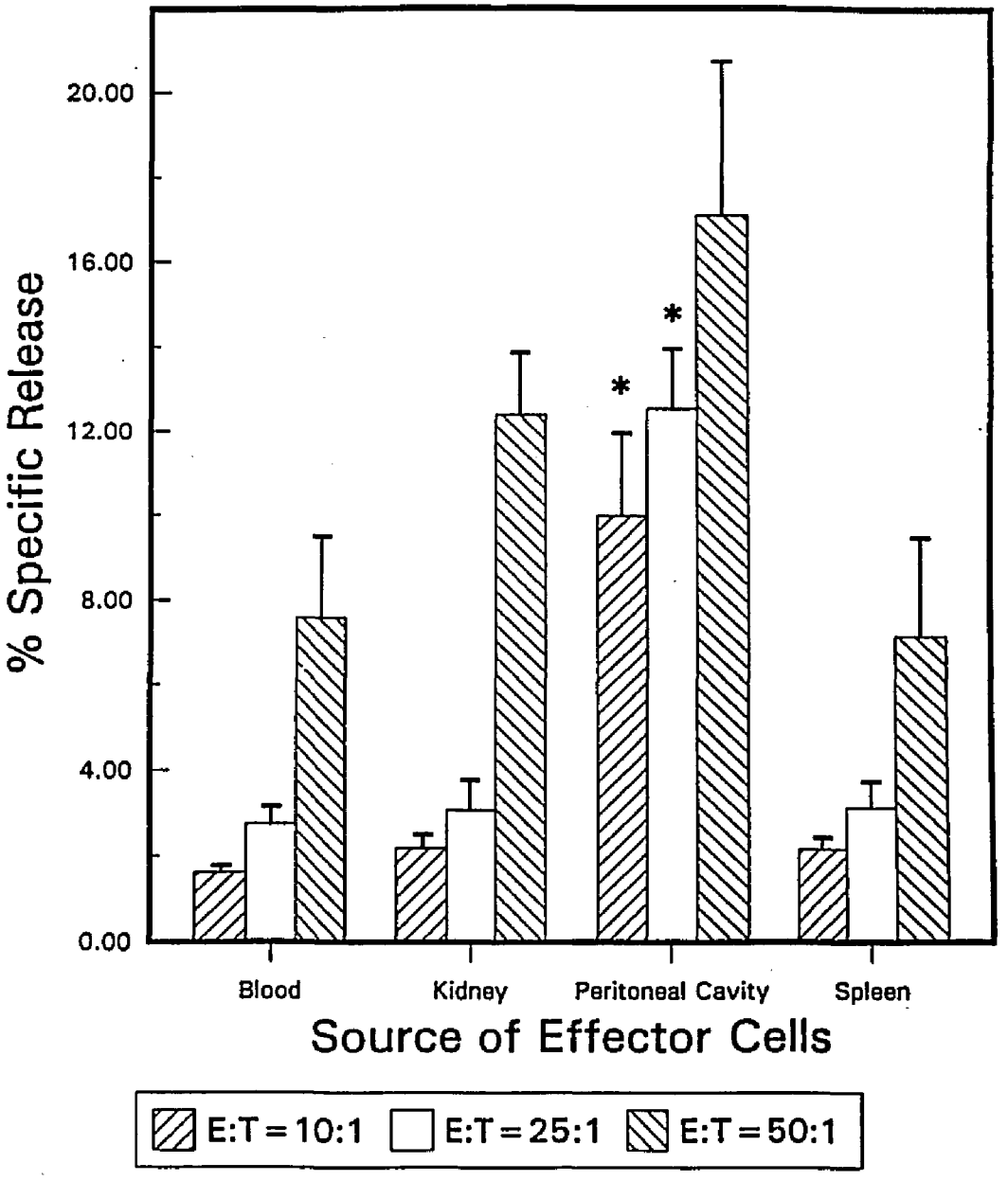

20.00
16.00
12.00
8.00
4.00
0.00
% Specific Release
*
*
Blood
Kidney
Peritoneal Cavity
Spleen
Source of Effector Cells
E:T = 10:1
E:T = 25:1
E:T = 50:1

Figure 2. Effect of medium osmolality on oyster toadfish NCC activity. Responses at 280 mOsm/kg·H_2O were significantly higher for all E:T ratios than at the other osmolalities tested ($P<0.05$). Bars represent sample means (N=3) $\pm$ S.E.M. Anterior kidney NCC activity was measured against the mammalian cell line YAC-1.

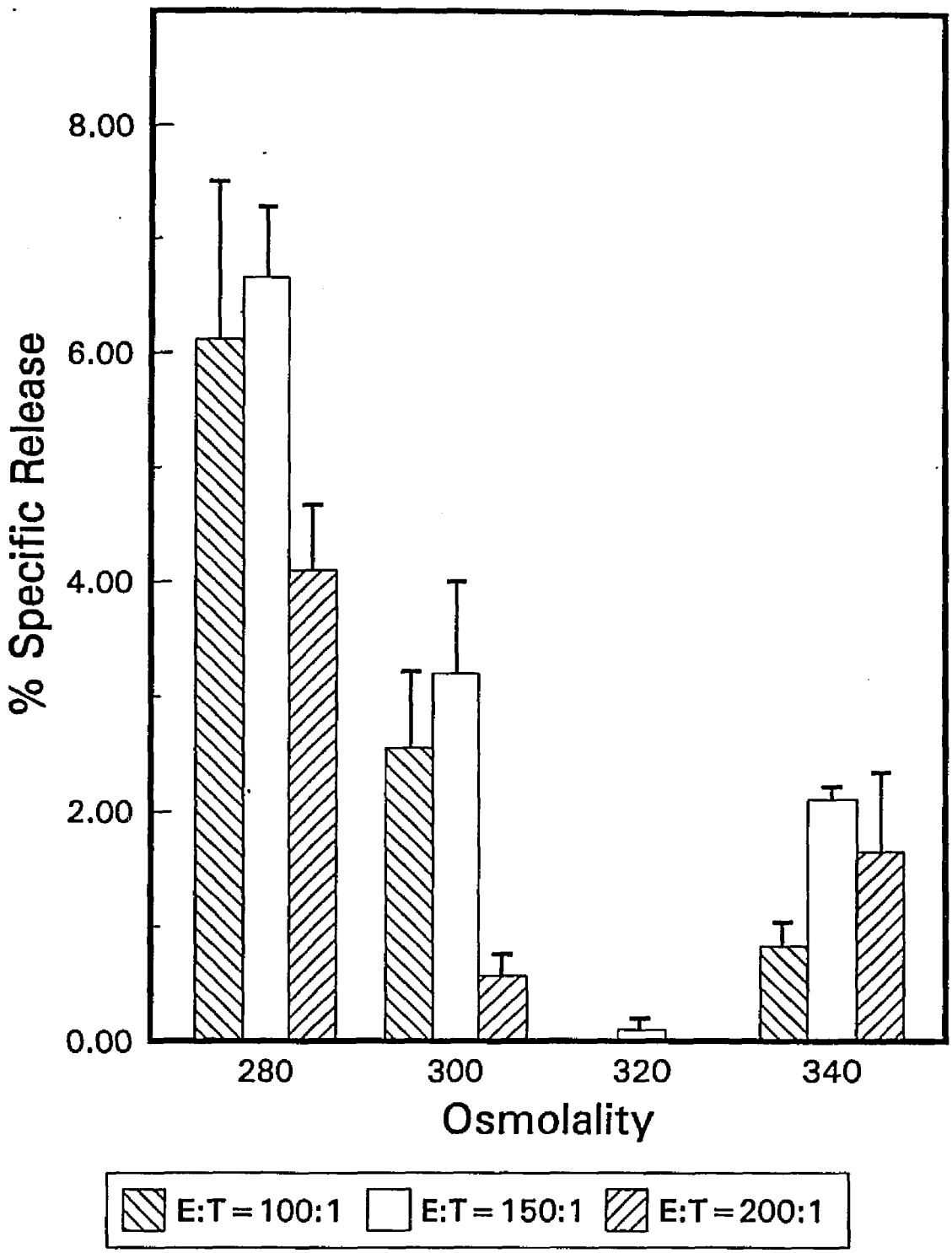
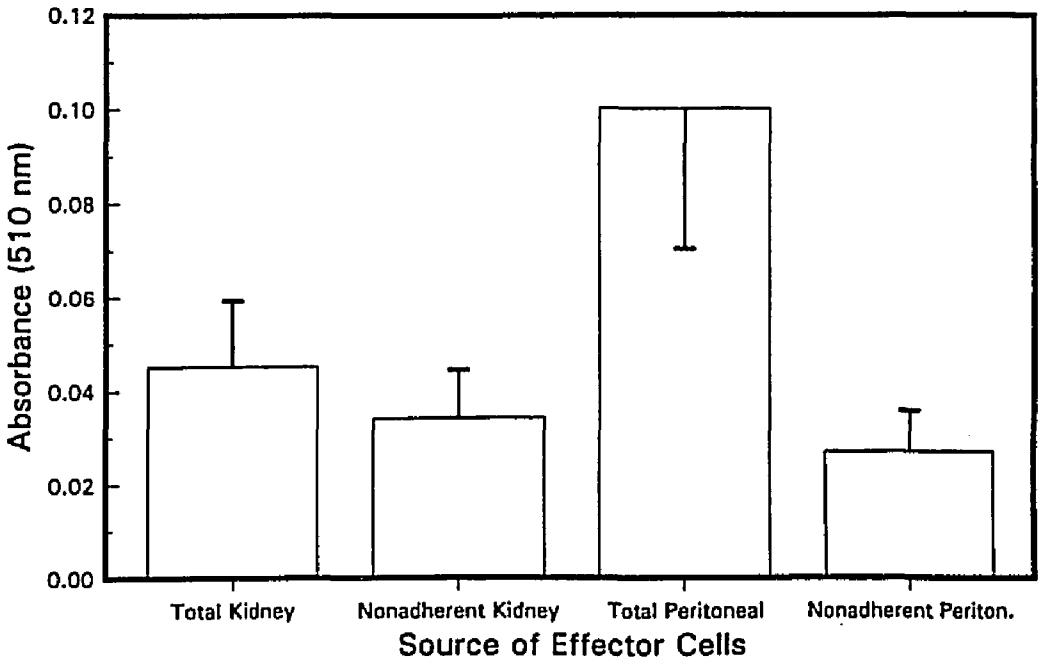

8.00
6.00
4.00
2.00
0.00
% Specific Release
280
300
320
340
Osmolality
E:T = 100:1
E:T = 150:1
E:T = 200:1

Figure 3. Effect of removing plastic adherent peritoneal and anterior kidney leukocytes on phagocytic (a) and NCC (b) activity. Bars represent sample means (N=4) ± S.E.M. NCC activity was measured against the mammalian cell line YAC-1 at an E:T ratio of 50:1. Removal of plastic adherent cells had no significant effect on either phagocytic or NCC activity of anterior kidney leukocytes, although in peritoneal leukocytes, phagocytic activity was significantly reduced and NCC activity was significantly increased following removal of these cells ($P<0.05$).

(a)


0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance (510 nm)
Total Kidney
Nonadherent Kidney
Total Peritoneal
Nonadherent Periton.
Source of Effector Cells

(b)

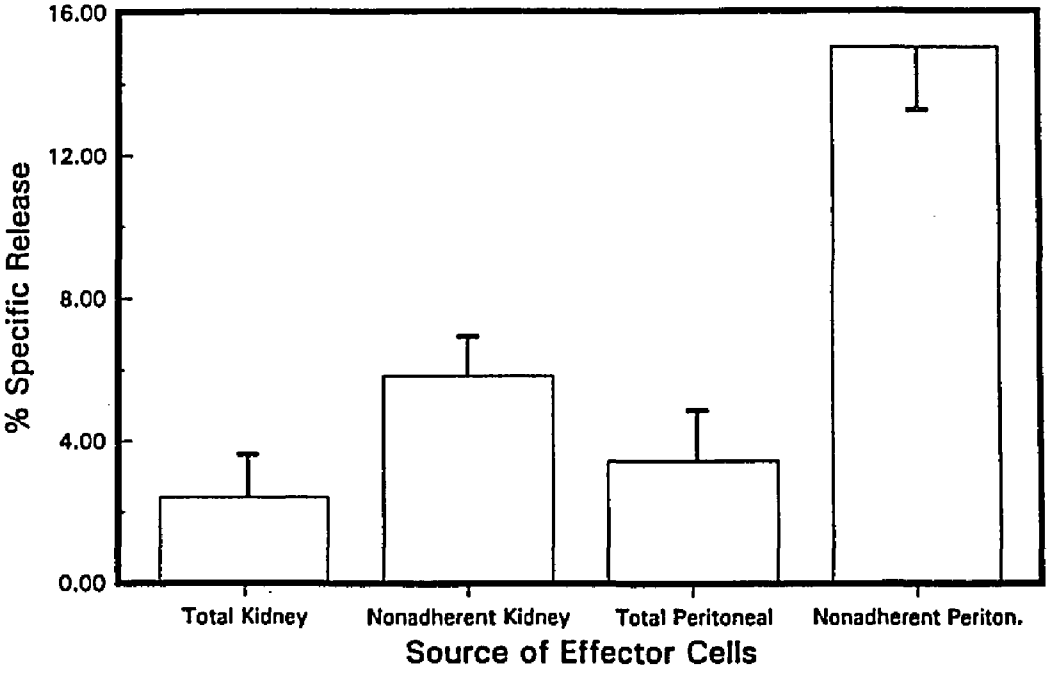

16.00
12.00
8.00
4.00
0.00
% Specific Release
Total Kidney
Nonadherent Kidney
Total Peritoneal
Nonadherent Periton.
Source of Effector Cells

Figure 4. Kinetics of target cell destruction following conjugate formation with oyster toadfish NCC. Effector and target cells were co-incubated at an effector:target ratio of 1:1 for 5 minutes, then resuspended in agarose for the indicated incubation times. Values shown are the percentages of conjugates formed which resulted in target cell death. Bars represent sample means (N=3) $\pm$ S.E.M. No significant differences were detectable in the rates of target cell destruction by leukocytes from any of the tissues examined ($P<0.05$).

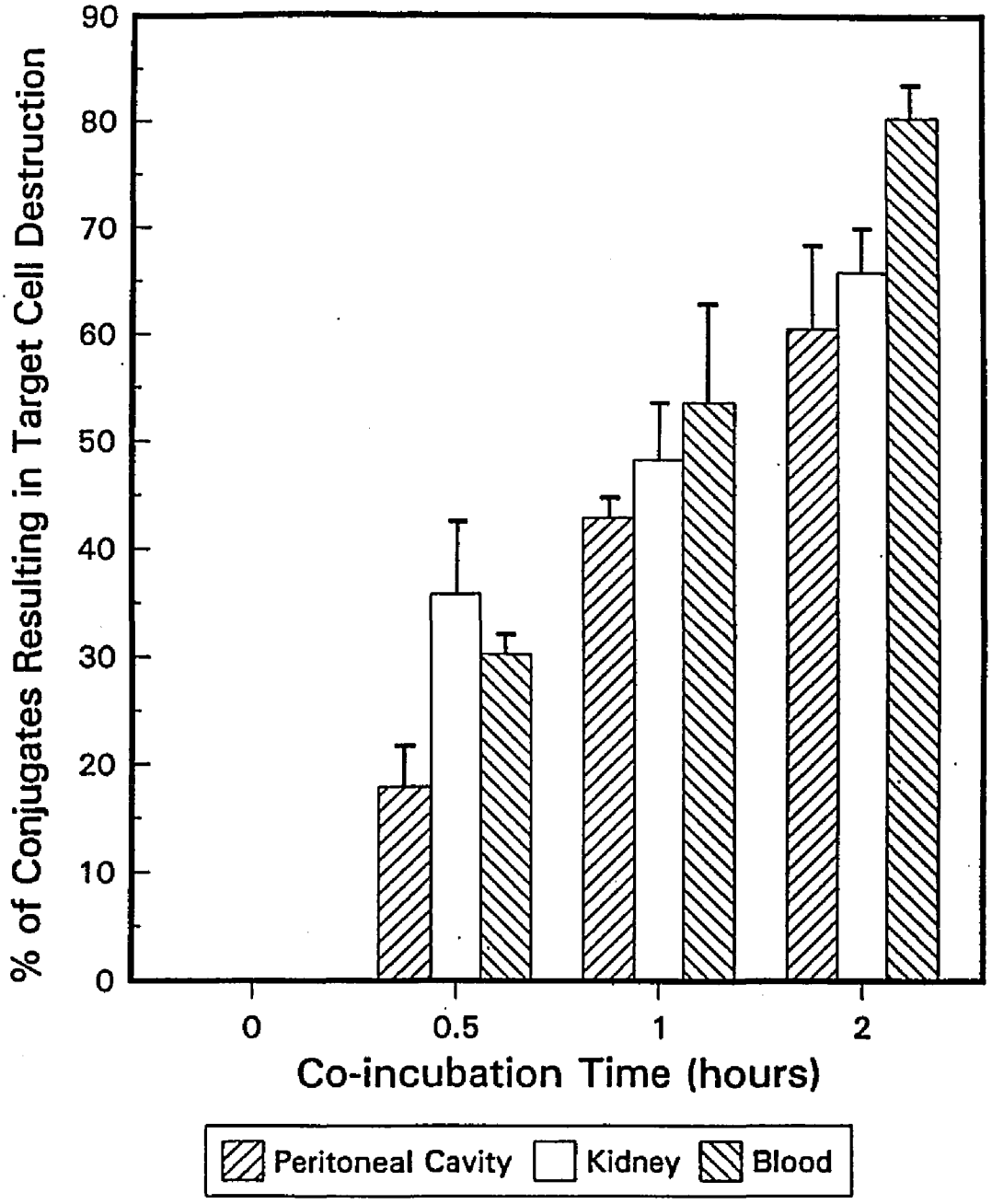

% of Conjugates Resulting in Target Cell Destruction
90
80
70
60
50
40
30
20
10
0
0
0.5
1
2
Co-incubation Time (hours)
Peritoneal Cavity
Kidney
Blood

Figure 5. Early events in the lysis of tumor target cells by oyster toadfish nonspecific cytotoxic cells. a). A single macrophage-like effector cells approaches a tumor target cell. Several cytoplasmic extensions project out from the effector cell to the target cell. b). Two macrophage-like effector cells begin binding to the surface of a tumor target cell. Both figures show semi-thin sections of anterior leukocytes (effector cells) and mammalian P815 cells (target cells), stained with toluidine blue (1000x). c). Macrophage-like blood leukocyte (E) binding to the surface of a YAC-1 target cell (T) at several points (arrows). Bar = 5 μm.

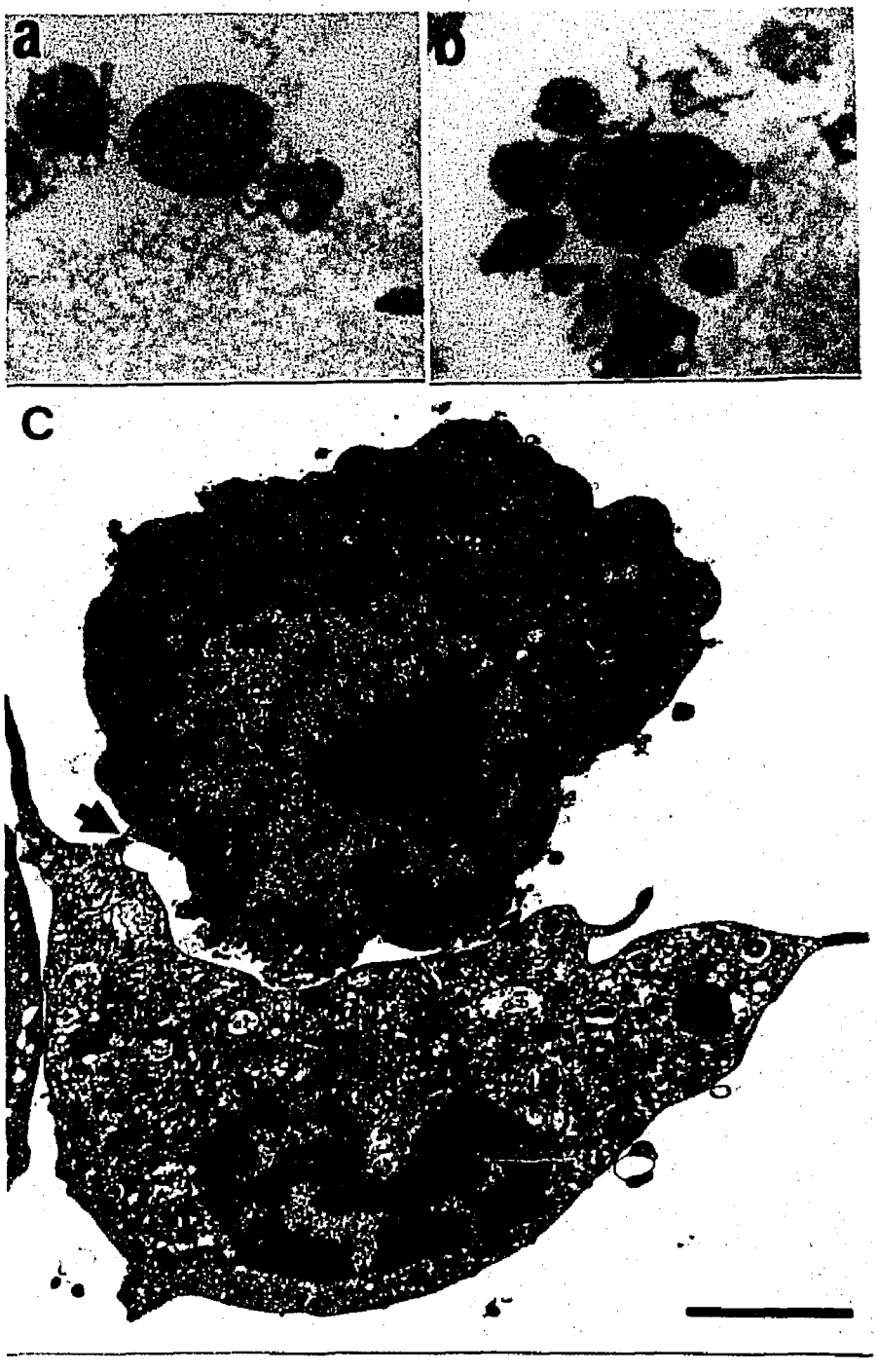

a
b
c

Figure 6. Intermediate steps in the destruction of tumor target cells by oyster toadfish nonspecific cytotoxic cells. a). Semi-thin section showing two anterior kidney macrophage-like effector cells attacking a P815 target cell following one hour of co-incubation (1000x; toluidine blue staining). b). YAC-1 target cell (T) contacted by a peritoneal macrophage following a five-minute co-incubation. At one contact point (open arrow), the membranes of the two cells remain distinct, while at another contact point (closed arrow), the membranes of the two cells appear to have become fused. 12,000x.

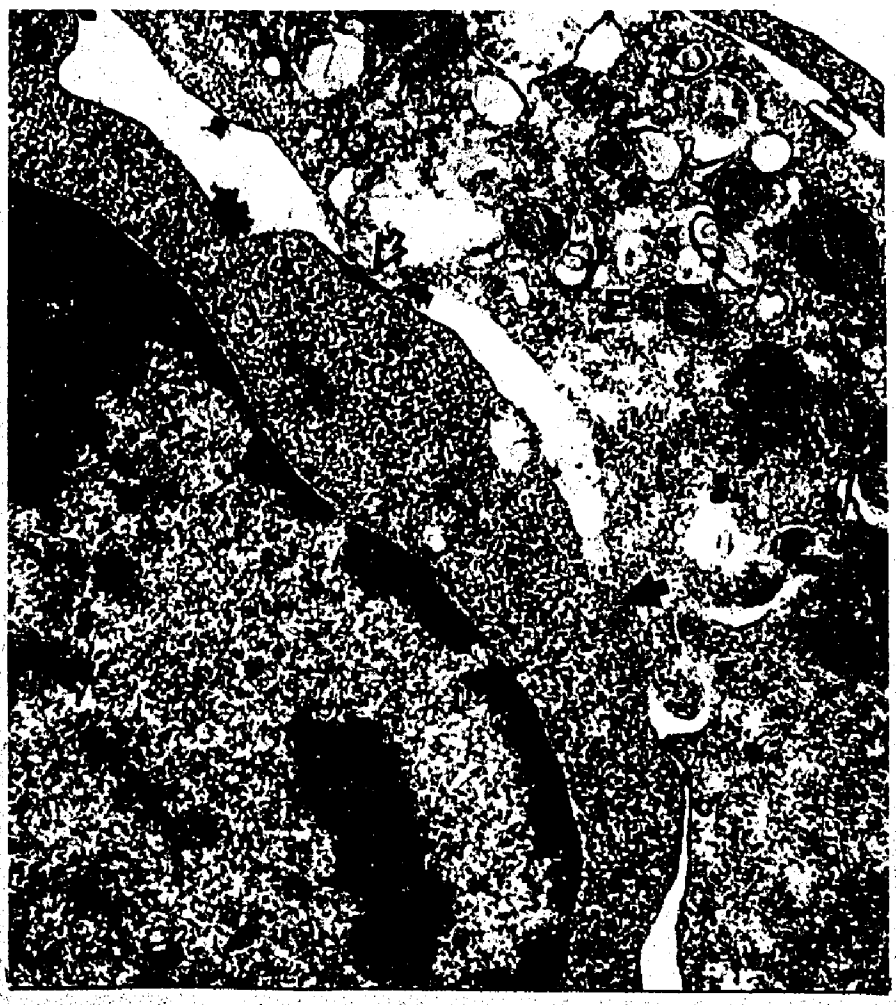
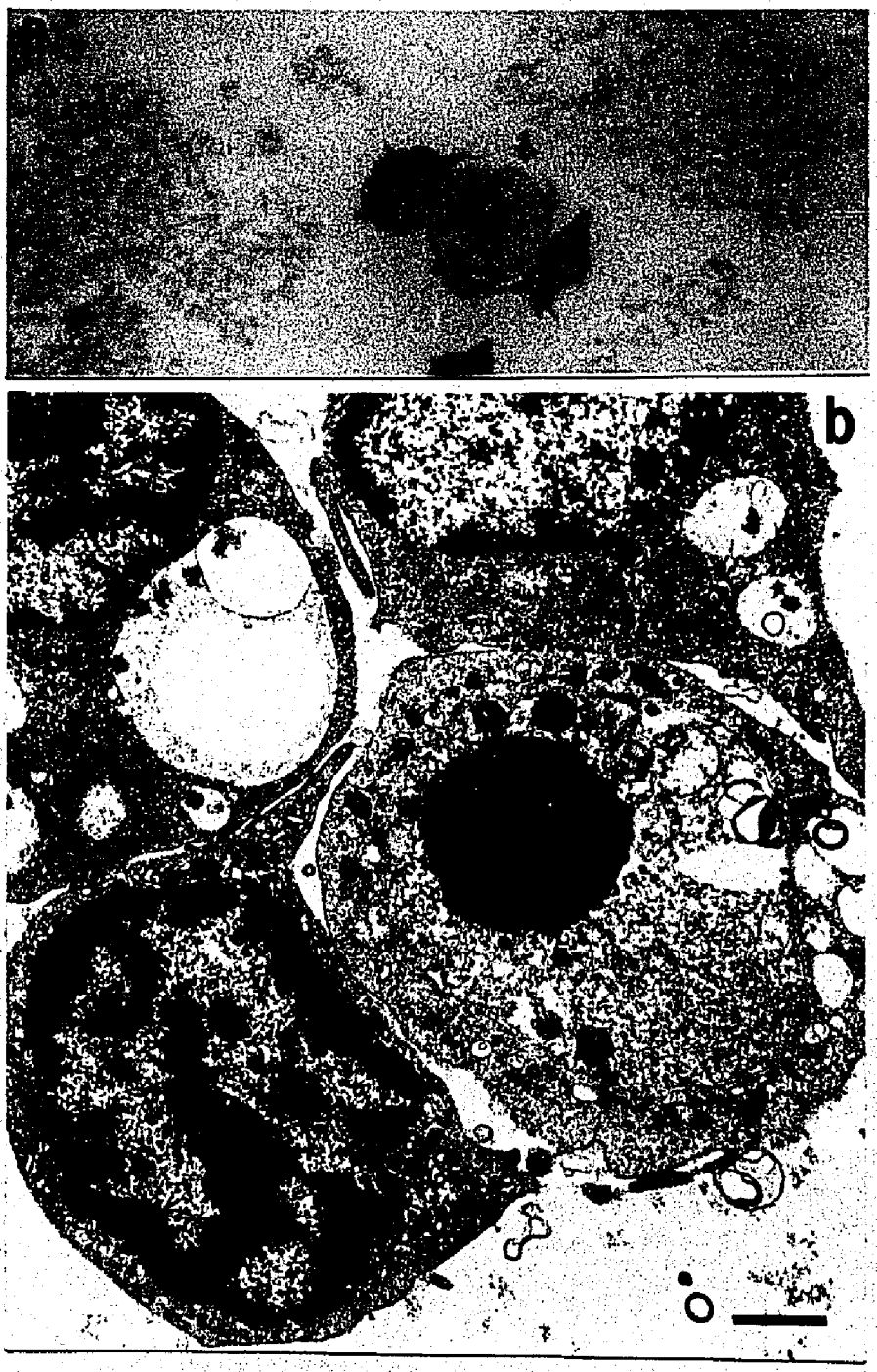


Figure 7. Final stages in the destruction of tumor target cells. Macrophage-like cells begin engulfing dead target cells following a two hour co-incubation. a) 1000x; toluidine blue staining. b). Bar = 2 μm.


b

DISCUSSION

Nonspecific cytotoxic activity has been shown to exist in a number of fish species (Bielek, 1988; Evans et al., 1984a; Greenlee et al., 1991; Hinuma et al., 1980 & Mckinney et al., 1986). Data presented in this paper indicate that a functionally similar cell type exists in the oyster toadfish. We have found that NCC activity in oyster toadfish is present in the peripheral blood, spleen, anterior kidney and peritoneal cavity, with activity being highest in the peritoneal leukocytes. To our knowledge, no other workers have attempted to examine NCC activity in peritoneal leukocytes of fish, although Klempau and Cooper (1984) have demonstrated that peritoneal exudate cells of anuran amphibians possess relatively high cytotoxic potential.

Oyster toadfish NCC activity against mammalian target cells was highest at a medium osmolality of 280 mOsm/kg·H_2O. This osmolality is essentially optimal for mammalian cell cultures. In preliminary studies on oyster toadfish leukocytes, we found that these cells remained viable over a relatively wide range of osmolalities, although viability was reduced at osmolalities below 280 mOsm/kg·H_2O (unpublished data). Other workers have achieved different results in this respect, however. Hinuma et al. (1980) found that cytotoxic activity in saltwater fish (against a human amniotic epithelial cell line) remained stable between

260 and 425 mOsm/kg·H_2O. Conversely, Greenlee et al. (1991) adjusted medium osmolality to 320 mOsm/kg·H_2O when studying NCC activity in rainbow trout, while Evans et al. (1984a) adjusted medium osmolality to 250 mOsm/kg·H_2O when working with channel catfish.

Variability in NCC activity between experiments in this study was substantial. This was not unexpected. Numerous studies have shown that stress associated with the handling of fish under artificial or laboratory conditions can alter their immune responses (Peters et al., 1988; Ellsaesser and Clem, 1986). This has also been shown to be true for NCC activity (Faisal et al., 1989b). Since this current study was performed on feral fish which had been captured by trawling, it was impossible to completely match holding conditions from one experiment to another. For this reason, comparison of results obtained from different experiments is difficult, although it is possible to observe basic trends in the data.

Our results show that NCC activity in the oyster toadfish, an estuarine fish, is relatively low compared to NCC activity observed by other workers working with freshwater species (Etchberger et al., 1987; Evans et al., 1984a, 1984b; Moody et al., 1985). This lower activity could be an artifact of our assay procedures, or it could indicate that NCC activity is naturally lower in oyster toadfish compared to other species. In support of the

Table 1. Putative leukocyte types responsible for nonspecific cytotoxic activity in various species of fish.

Species	Proposed Cell Type	Reference
Rainbow Trout (*Oncorhynchus mykiss*)	Agranular Mononuclear Cells	Greenlee et al (1991)
Catfish (*Ictalurus punctatus*)	Lymphocytes	Evans et al. (1984c)
Common Carp (*Cyprinus carpio*)	Lymphocytes & Monocytes	Bielak (1988)
Nurse Shark (*Ginglymostoma cirratum*)	Macrophages	McKinney etal. (1986)
Crucian Carp (*Carassius cuvieri*) Common Carp (*Cyprinus carpio*) Northern Snakehead (*Channa argus*) Grass Carp (*Ctenpharyngodon idella*) Oriental Weatherfish (*Misgrunus anguillicandatus*)	Activated Macrophages or Natural Killer Cells	Hinuma et al. (1980)
Tilapia (*Messambica honorum*)	Lymphocytes, Monocytes, Macrophages, and Polymorphonuclear Leukocytes	Faisal et al. (1989a)

latter possibility, Hinuma et al. (1980) have found NCC activity in several species of marine fish to be extremely low (with specific release ranging from 0 to 0.6%). Etchberger et al. (1987) have shown that NCC activity in marine fish is highly variable, and efforts to measure this activity may be hampered by difficulty in establishing an optimal medium osmolality for both fish leukocytes and mammalian cell lines.

Mammalian NK cells are neither plastic adherent nor phagocytic (Warner et al., 1982; Kiessling et al., 1975). Evans et al. (1984b) have shown that this is also true of catfish NCC. By removing plastic adherent cells from total leukocyte populations of the anterior kidney and peritoneal cavity, we have demonstrated here that oyster toadfish NCC lack these macrophage characteristics as well. Optimally, it would have been desirable to determine conclusively whether or not NCC activity exists in the adherent fractions of these leukocyte populations as well. However, the procedures involved in dislodging adherent cells from plastic surfaces (i.e., trypsinization or physical scraping with a rubber policeman) probably would have served to reduce the functional capacity of these cells themselves, confounding any attempt to compare NCC activity between adherent and nonadherent fractions of leukocytes.

By examining target cell binding and lysis with the method of Grimm and Bonavida (1979), we have found that

oyster toadfish NCC bind to target cells rapidly (within minutes of initial contact), while lysis can take up to several hours. Although the lytic potential of peritoneal leukocytes was greater than that of leukocytes from the other tissues we examined (based on chromium release data), it appears that once an NCC has bound to a target cell, lysis occurs at essentially the same rate, regardless of the leukocyte source. This could mean that the increased lytic potential observed in peritoneal leukocytes is the result of either greater capacity for target cell recognition and binding, and/or enhanced recycling capacity (i.e., the ability to attack and lyse multiple target cells). In this assay however, cells were immobilized in agarose and it was not possible to evaluate recycling capacity using this technique.

As can be seen in Table 1, a wide range of leukocyte types have been shown to be involved in nonspecific cytotoxic activity in different fish species. With the exception of Hinuma et al. (1980), no previous investigations into this immune response in fish have identified the involvement of a cell type with the morphological characteristics of mammalian NK cells. In this current study, cells possessing the morphological charateristics of monocytes or macrophages appear to be responsible for nonspecific cytotoxic activity in oyster toadfish. While a number of workers have attempted to find

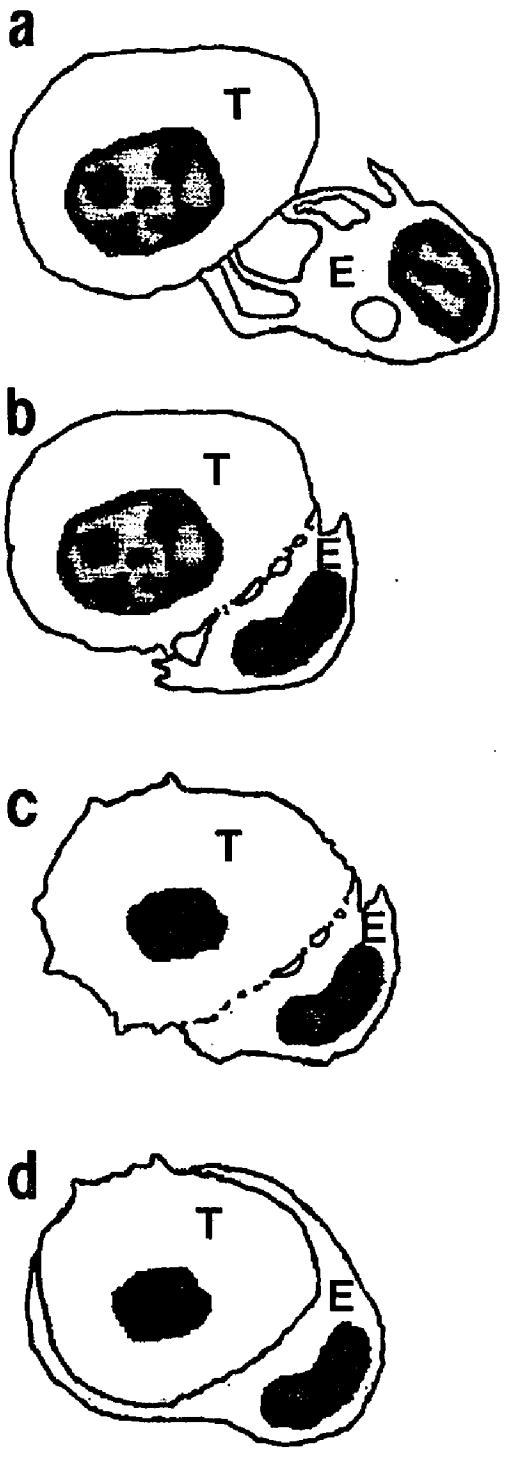
a specific population of cells in fish that could be considered the equivalent of the mammalian NK cell, a more plausible hypothesis is that nonspecific cytotoxic activity in fish is mediated by a more heterogeneous group of target binding cells. Bielak (1988) has proposed that an unspecialized ancestor to the mammalian NK cell may have emerged early in the course of fish evolution from a monocyte/macrophage-like precursor.

Functionally, oyster toadfish NCC lack certain characteristics commonly associated with macrophages. For example, nonspecific cytotoxic activity was concentrated in a population of cells that did not adhere to plastic and were not phagocytic. On the other hand, oyster toadfish NCC were morphologically similar to monocytes or macrophages. This apparent inconsistency has also been noted by other investigators. Bielak (1988), has stated that while adherence to plastic is an accepted characteristic of macrophages, it is by no means unequivocal. Moody et al. (1985) have also concluded that teleost macrophages are heterogeneous in terms of their ability to adhere to plastic, and that a non-adherent, non-phagocytic subpopulation of macrophages may actually mediate nonspecific cytotoxic activity in teleosts.

When the results of these functional and morphologic characterizations are viewed as a whole, a putative sequence of events in the destruction of tumor target cells by oyster

toadfish NCC may be postulated (Figure 8). The first two steps in target cell destruction involves recognition and binding of the target cell by the effector cell. From the target cell binding data presented in this study, these two steps occur as rapidly as within five minutes of initial contact. Following binding, effector cells rapidly destroy target cells. The results presented in this study show that this step begins to occur immediately following binding and continues for up to several hours. Differences in time required to destroy targets following binding could be explained by differential activation states of effector cells, or by the fact that two different mechanisms may be involved in the destruction process. Greenlee et al. (1991) have found that rainbow trout NCC are capable of killing target cells via rapid, necrotic mechanisms (i.e., membrane disruption), as well as through slower, apoptic mechanisms (i.e., programmed cell death caused by DNA fragmentation). Immediately following destruction of the target cell, resultant cellular debris is engulfed and destroyed by phagocytic cells.

Figure 8. Putative sequence of events in the destruction of tumor target cells by oyster toadfish nonspecific cytotoxic cells (NCC). a) Following initial contact, the NCC begins to recognize the target cell. b) Within minutes, the NCC binds tightly to the target cell. c) Over the course of minutes to hours, the target cell is killed. d) Finally, the dead target cell (or its debris) is phagocytized and subsequently degraded. E, effector cell (NCC). T, tumor target cell.


a
T
E
b
T
E
c
T
E
d
T
E

CHAPTER 3

DEVELOPMENT AND APPLICATION OF A SIMPLE TECHNIQUE FOR THE RAPID SPECTROPHOTOMETRIC DETERMINATION OF PHAGOCYTOSIS BY FISH MACROPHAGES

Introduction

The ability of fish macrophages to phagocytize foreign material has been shown to be highly sensitive to the effects of immunotoxic compounds (Weeks et al. 1986; Warinner et al., 1988). A variety of techniques have been developed to assess this ability, although problems are associated with many of these techniques that could limit their applicability as biomarkers of contaminant exposure in fish. For example, microscopic analysis of macrophages following uptake of bacteria or other foreign material can be used to obtain direct numerical data on the phagocytic capacity of macrophages. However, this technique is highly subjective, and the process of examining and counting stained slides can be extremely time consuming. Other techniques, such as the measurement of macrophage chemiluminescence or chemotaxis, are relatively complicated to perform, and although they may yield important information on the functional ability of macrophages, they

may be difficult to apply in large-scale biomonitoring programs.

For these reasons, it was necessary to develop a method that combined the relative simplicity of microscopic analysis while eliminating the inherent subjectivity associated with that technique. One such method had been described previously, utilizing congo red-stained yeast cells as phagocytic targets (Kaminski et al., 1985). Endpoint measurement in this assay is performed spectrophotometrically, eliminating the need for time-consuming and highly subjective microscopic quantification. Originally, this method was developed for use with adherent macrophages of mammalian origin. However, Fisher et al. (1982) found that certain environmental contaminants, particularly polynuclear aromatic hydrocarbons (PAH), are capable of inhibiting or preventing macrophage adherence. Thus, in order to adapt this assay for use as a biomonitoring technique, it has been substantially modified for use with phagocytic cell suspensions.

In this chapter, two series of experiments are described. In the first, the spectrophotometric technique and the modifications made to it are described. Results obtained using this technique are compared to more commonly used techniques involving microscopic analysis. In the second series of experiments, this technique is applied in a field investigation in which the functional capacity of

oyster toadfish (Opsanus tau) macrophages, taken from several sites in the Elizabeth River, Virginia, is examined.

The Elizabeth River has been reported to be the most polluted subestuary in the lower Chesapeake Bay region, having been heavily contaminated with industrial, agricultural and domestic wastes (Huggett et al., 1987; Hargis and Zwerner, 1988). This is particularly evident in the Southern Branch of the river, where for the major part of this century, run-off from various wood treatment facilities has resulted in extremely high accumulations of PAH in the sediments (Huggett et al.,1987; Lu, 1982; Van Veld et al.,1990).

Fin erosion, cataract formation, and other external lesions in a number of fish species have been attributed to the high PAH levels in the sediments and water column of the Elizabeth River, with these lesions being most prevalent in the vicinity of the wood treatment facilities (Huggett et al., 1987; Hargis and Colvocoresses, 1986; Hargis and Zwerner, 1988). Over the past several years, a number of studies have also linked the high concentrations of toxic substances in Elizabeth River sediments to decreases in the immunocompetence of naturally occurring finfish populations in that area. These studies have demonstrated a significant degree of immunomodulation in fish taken from the Elizabeth River compared to those taken from the relatively non-polluted York River, as determined by macrophage chemotaxis

(Weeks et al., 1986), chemiluminescence (Warinner et al., 1988), phagocytosis (Weeks and Warinner, 1984) and pinocytosis (Weeks et al., 1987). Similar effects have been found in fish from clean environments exposed to Elizabeth River sediments in laboratory experiments (Roberts et al., 1989).

Materials and Methods

For the first series of experiments, oyster toadfish (Opsanus tau) were obtained from the relatively nonpolluted York River by 5-minute bottom trawls. Following capture, fish were returned to the laboratory and held in tanks with flowing ambient York River water for a minimum of two weeks prior to sacrifice.

In the second series of experiments, fish were sampled from several sites on the Elizabeth River. The sampling sites used in this investigation are shown in Figure 10. These sites were selected because they have been shown previously to represent a gradient of sediment-bound PAH concentrations, ranging from 55 ppb total PAH at Station I to 96,000 ppb total PAHs at Station IV (Van Veld et al., 1988). Station I is located near the Hampton Roads Bridge-Tunnel, and was originally considered to be a reference site for this study, as total PAH levels here were previously determined to be 55 ppb. However, several fish taken from this site were observed to be heavily infected with internal

parasites, and it was necessary to exclude this area as a reference site, although data on the fish captured at this station have been kept in this report. Station II is located off Craney Island, an artificial land mass which has been built-up over the past 36 years with sediments that have accumulated during various dredging projects within the Elizabeth River. Station II is considered to have relatively low levels of PAH contamination (3,100 ppb), compared to Stations III and IV. Station III is located off downtown Norfolk, Virginia, and is considered to have an intermediate level of PAH contamination for the purposes of this study (16,000 ppb). Station IV is located near an operating wood treatment plant, and sediments in this area have been heavily contaminated over several decades with creosote. In the field investigation, adult oyster toadfish (300-600 g) were captured by 5-minute bottom trawls and sacrificed immediately.

In all experiments, macrophages were collected by peritoneal lavage from fish anesthetized with Tricaine (MS-222, Sigma). Ten ml of Hank's Balanced Salt Solution (HBSS, supplemented with 5 U/ml heparin) was injected intraperitoneally and removed by suction through a small ventral incision. Immediately following lavage, cells were washed several times in HBSS and resuspended in Minimum Essential Medium (MEM), supplemented with 10% fetal bovine serum (FBS, Gibco), at a concentration of 2×10^6 cells/ml.

Yeast cells (Saccharomyces cerevisceae, Fleischmann's, East Hanover, NJ) were stained with congo red and stored as described by Kaminski et al. (1985). Three ml of a congo red solution, 0.87% w/v in phosphate-buffered saline (PBS), was added to 1.5 g of yeast cells, and this mixture was allowed to stand for fifteen minutes at room temperature. Seven ml of distilled water was added and the resulting solution was thoroughly mixed, then autoclaved for fifteen minutes to kill and fix the yeast. The cells were then washed several times in HBSS in order to remove any excess stain, and stored at 4° C until needed. Prior to use, cells were resuspended at 4 x 10^7 cells/ml in HBSS.

The method used for spectrophotometric analysis of macrophage phagocytic activity has been described previously (Seeley et al., 1990), and was a modification of a technique originally described by Kaminski et al. (1985). Briefly, one ml of the macrophage suspension was mixed with two ml of the stained yeast cell suspension. The final suspension contained a yeast cell:macrophage ratio of 40:1. Cell suspensions were incubated at room temperature for 0 and 90 minutes in duplicate. Following incubation, 5 ml of ice-cold HBSS was added to slow any further phagocytic activity, and 3 ml of Percoll® (adjusted to a density of 1.055 g/ml with HBSS) was gently injected into the bottom of each tube. The samples were centrifuged at 850xg for 3 min to separate macrophages from any remaining free yeast cells.

Macrophages, which were located at the Percoll®-media interface were then harvested and washed in HBSS. A small portion of the pelleted cells (5 μl) was removed and used to prepare smears for microscopic examination. The remainder of the pellet was resuspended in 1.2 ml trypsin-EDTA solution (1.5 g\L trypsin 0.4 g\L EDTA in PBS; Gibco) to solubilize the macrophages and the yeast cells contained within them. Following an overnight incubation at 37° C, absorbance at 510 nm was determined against a blank containing only the trypsin-EDTA solution.

Microscopic examination of the smears was performed to determine phagocytic indices. Briefly, 100 macrophages were randomly examined per slide and the percentage of macrophages containing at least one phagocytized yeast cell was determined. The average number of yeast cells per macrophage (from approximately 100 randomly selected macrophages) was also determined.

Statistical analysis consisted of analysis of variance for significant differences in values for phagocytic index, absorbance due to phagocytosis of stained yeast cells and average number of phagocytized yeast cells per macrophage between sites. Post mortem analyses of differences among means were carried out using Scheffe's multiple comparisons test (Scheffe, 1953).

Results

Results of the spectrophotometric and microscopic determinations of phagocytic activity as well as the average number of yeast cell per macrophage are shown in Figure 9. Values shown represent the mean ± standard deviation of four fish. Phagocytic activity reached a maximum value within 60 minutes, regardless of the method of analysis employed. Thus, all three methods of analysis yielded comparable results, although spectrophotometric determination substantially reduced the amount of time required to analyze the samples.

Results of determinations of percent phagocytosis, average number of yeast cells per macrophage and absorbance due to phagocytosis of congo red-stained yeast cells for the field investigation are shown in Figure 10. As seen in Figure 10, there was no significant differences between stations in terms of percent phagocytosis (i.e., the percentage of macrophages containing yeast cells). On the other hand, the average number of yeast cells and absorbance due to phagocytized yeast cells do differ dramatically from station to station, with stations II and IV having statistically significant differences in terms of these assays ($p=0.01$ for average number of yeast cells and $p=0.05$ for absorbance due to phagocytosis of congo red-stained yeast cells).

Figure 9. a) Phagocytosis of congo red-stained yeast cells by toadfish macrophages as determined spectrophotmetrically by measuring absorbance at 510 nm (n=4). b) Percent of macrophages containing phagocytized yeast cells as determined by microscopic examination (n=4). c) Average number of phagocytized yeast cells per macrophage as determined by microscopic examination (n=4).

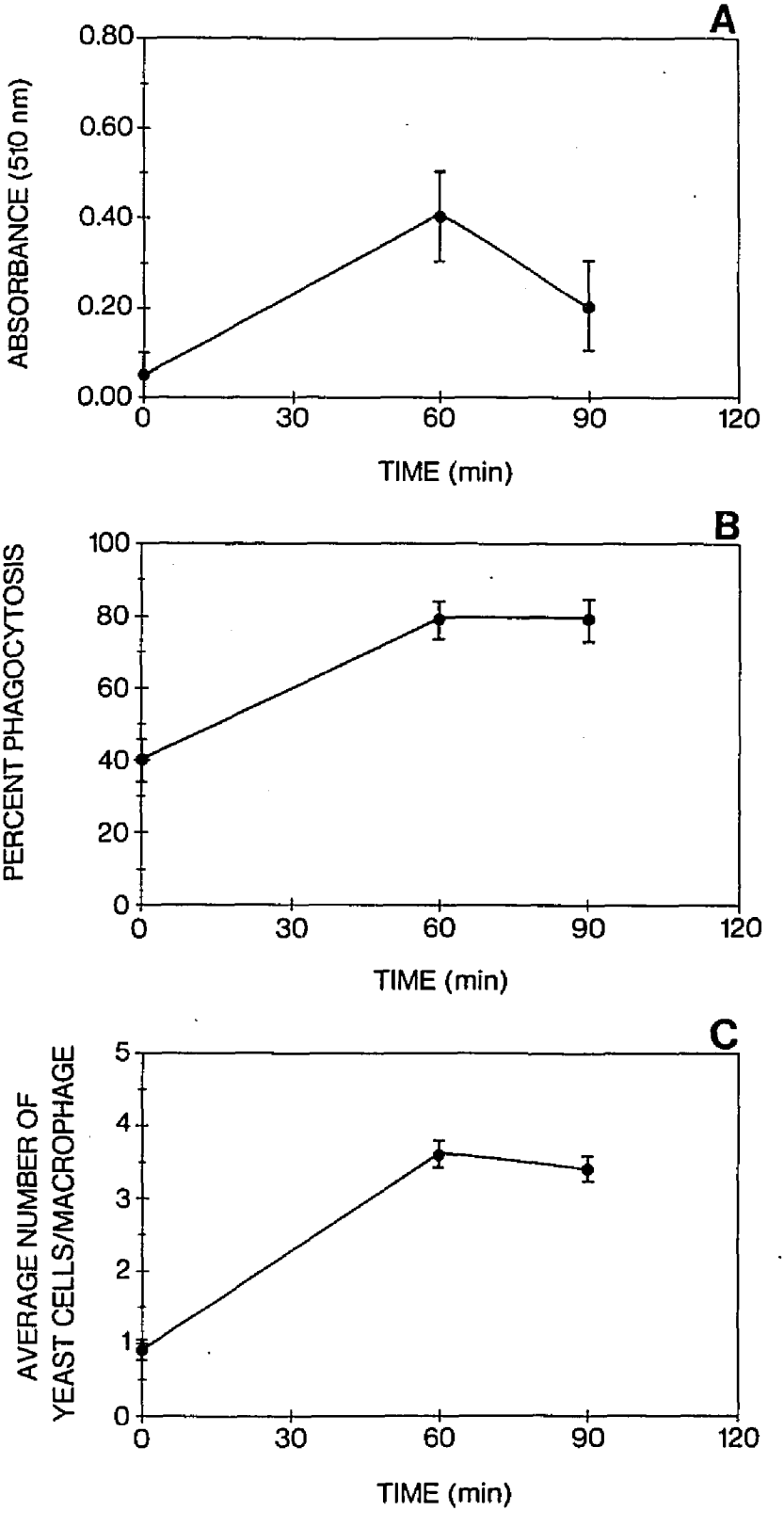

A
ABSORBANCE (510 nm)
0.80
0.60
0.40
0.20
0.00
0
30
60
90
120
TIME (min)
B
PERCENT PHAGOCYTOSIS
100
80
60
40
20
0
0
30
60
90
120
TIME (min)
C
AVERAGE NUMBER OF YEAST CELLS/MACROPHAGE
5
4
3
2
1
0
0
30
60
90
120
TIME (min)

Figure 10. Sampling stations in the Elizabeth River, Virginia used in determining the efficacy of the spectrophotometric technique for measuring phagocytosis as a biomarker of cantaminant exposure (inset: Chesapeake Bay Region). Total unresolved PAH levels (in parts per billion, ppb) at the various stations have been previously shown to increase in an upriver gradient as follows:

Station I: 55 ppb
Station II: 3,100 ppb
Station III: 16,000 ppb
Station IV: 96,000 ppb

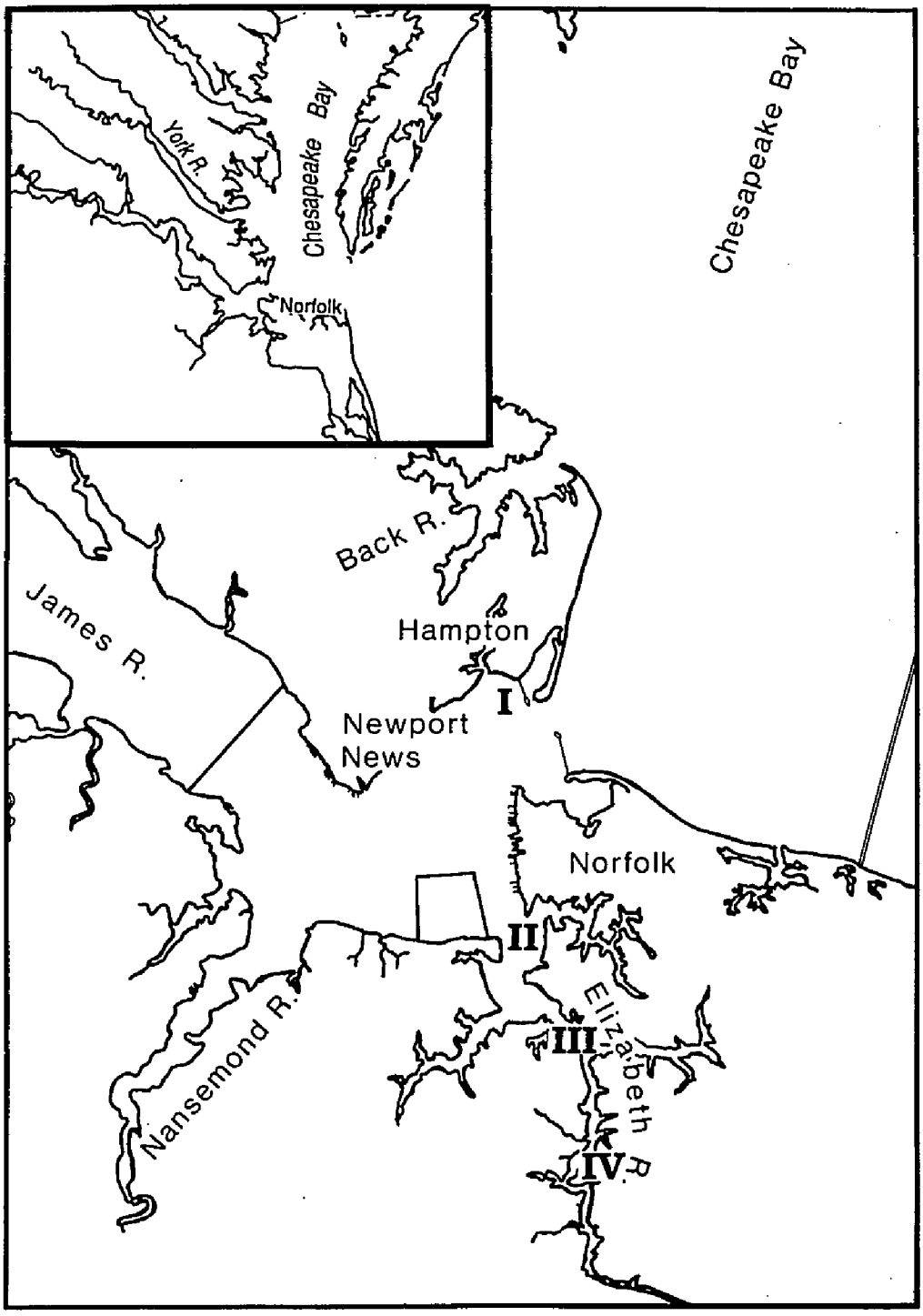
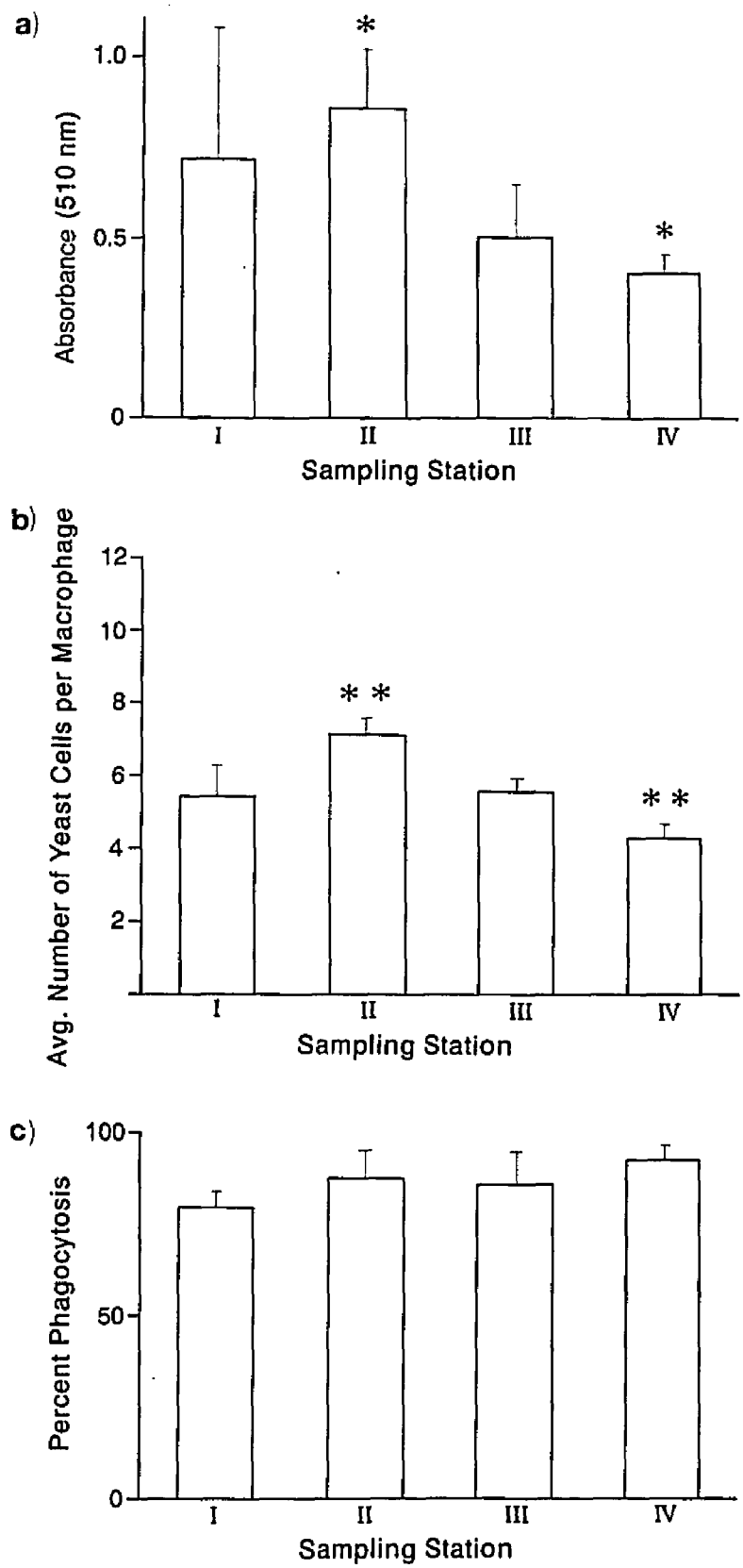

York R.
Chesapeake Bay
Norfolk
Chesapeake Bay
Back R.
James R.
Hampton
I
Newport
News
Norfolk
II
III
Elizabeth R.
Nansemond R.
IV

Figure 11. Phagocytic capacity of of macrophages from Elizabeth River oyster toadfish as determined by spectrophotometric and microscopic analyses. a) Spectrophotometric determination of phagocytosis of congo red-stained yeast cells. b) Average number of phagocytized yeast cells per macrophage as determined by microscopic examination. c) Percent of macrophages containing phagocytized yeast cells, as determined by microscopic examination. Bars represent mean values $\pm$ SEM (n=5). Asterisks indicate stations which exhibit significantly different responses (* $p<0.05$, ** $p<0.01$).


a)
Absorbance (510 nm)
1.0
0.5
0
*
*
I
II
III
IV
Sampling Station
b)
Avg. Number of Yeast Cells per Macrophage
12
10
8
6
4
2
* *
* *
I
II
III
IV
Sampling Station
c)
Percent Phagocytosis
100
50
0
I
II
III
IV
Sampling Station

Discussion

Phagocytic function of macrophages can be determined using a variety of methods. However, in order for any particular method to be cost-effective and widely applicable as a biomonitoring tool it should be relatively simple to perform and should produce results that are easily evaluated. The microscopic and spectrophotometric techniques for quantification of phagocytosis described in this paper meet these criteria. However, the considerable amount of time required to examine cells microscopically can be substantially reduced by using the spectrophometric method. Furthermore, spectrophotometric analysis eliminates the inherent subjectivity of the techniques involved in microscopic examination.

As originally described, the spectophotometric technique was developed for use with adherent populations of macrophages. However, the additional steps involved in the isolation of adherent macrophages would tend to make this technique too cumbersome for use in any large-scale biomonitoring program. Furthermore, the differential adherence of macrophages from pollutant-exposed animals could be the source of considerable error in assay results. Thus, this technique was adapted for use with suspensions of macrophages. In order to accomplish this, it was necessary to introduce a rapid (3 min) Percoll® separation step following incubation of the macrophage-yeast cell mixture.

Preliminary investigations revealed that yeast cells were denser than macrophages, and that optimal separation of the two cell types could be achieved by layering the entire cell suspension over Percoll® followed by centrifugation. It was also determined that this centrifugation step could be performed in as little as 3 minutes. Reducing this step to a minimum amount of time was critical, as excessive time spent on cell separations can result in a substantial alteration of the baseline response at time zero.

The results of the Elizabeth River experiments indicated that the ability of oyster toadfish macrophages to engulf foreign material was not significantly altered from station to station in the Elizabeth River. However, the capacity of these cells to engulf foreign material was dramatically reduced in conjunction with increasing levels of PAHs. Thus, if one were to simply examine the ability of macrophages to phagocytize yeast cells (using a simple indicator such as percent phagocytosis, in which cells are examined for the mere presence or absence of phagocytized material) the assumption could be made that differing levels of toxicants in the river have no significant impact on macrophage function in fish. However, by examining the functional capacity of these cell types, that is, by determining the amount of foreign material that individual macrophages can phagocytize within a given period of time, differences from station to station become more apparent.

Results obtained using the spectrophotometric technique described in this paper are closely correlated with results obtained by counting the number of yeast cells in individual macrophages.

Interestingly, fish taken from station I exhibited low macrophage responses in this study. Recorded PAH levels and preliminary immunological investigations (unpublished data) had indicated that this area could serve as a reference site for Elizabeth River biomonitoring. However, as mentioned earlier, fish taken from this area exhibited a significant degree of internal infection as well as external lesions. These conditions may have been linked to intensive human activity in this area (i.e., fishing and boating), and its associated stress on the fish there, since numerous reports in the immunological literature have shown that "physiological stress" can result in a state of immunosuppression which is quite similar to that caused by certain toxicants. Thus, in this study, we were unable to differentiate between physiological and chemical stressors using immunological biomarkers alone.

CHAPTER 4

SUPPRESSION OF NATURAL CYTOTOXIC AND PHAGOCYTIC FUNCTION IN OYSTER TOADFISH EXPOSED TO THE POLYCYCLIC AROMATIC HYDROCARBON 7,12-DIMETHYLBENZ[A]ANTHRACENE.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are a class of compounds which have become widely distributed in the environment as a result of both natural processes, such as forest fires, as well as human activities, such as the burning of fossil fuels (White, 1986). Many PAH have been shown to be carcinogenic following metabolic activation by mixed function oxidase enzyme systems (Guengerich et al, 1985). This is particularly true of those PAH which are derived from an angular benz[a]anthracene skeleton, such as dibenz[a,h]anthracene or benzo[a]pyrene. Substitutions of methyl groups at certain carbons tend to increase the carcinogenicity of PAH, as is the case with 7,12-dimethylbenz[a]anthracene (DMBA), one of the most carcinogenic PAH (Williams and Weisberger, 1986).

The carcinogenicity of various PAH have been correlated with immunotoxic effects (White, 1986). In explaining this correlation, some workers have hypothesized that in addition

to their direct carcinogenic effects, PAH may suppress the immunosurveillance mechanisms which are normally responsible for arresting or delaying the development of malignant tumors from transformed cells (Erlich et al., 1983).

In mammals, natural killer (NK) cells are believed to play a critical role in immunosurveillance against neoplasia and in controlling the spread of tumor cells (Herberman and Ortaldo, 1981). These cells carry out their immunosurveillance function by recognizing transformed cells and lysing them as they develop. Destruction of transformed cells requires a sequence of events, including recognition of the transformed (target) cell by a NK (effector) cell, binding of the effector cell to the target cell, and finally, disruption of the target cell membrane by a pore-forming protein referred to as perforin (Young and Cohn, 1988). A specific antigenic determinant on the surface of the target cell which is recognizable by NK has yet to be determined.

In fish, nonspecific cytotoxic cells (NCC) are believed to be functionally similar to mammalian NK (Evans et al., 1988). We have recently described a similar cell type in oyster toadfish (Opsanus tau), an estuarine teleost (Seeley and Weeks-Perkins, 1993).

NK function has been shown to be affected by to a wide range of toxic compounds, including tributyltin (Ghoneum et al., 1990) and 2-acetylaminofluorene (Kimber et al., 1986a).

Carcinogenic PAH, particularly 7,12-dimethylbenz[a]anthracene (DMBA), have been shown to be extremely toxic to NK (Dean et al., 1986a; Erlich et al., 1983; Kimber et al., 1986b; White, 1986), and this toxicity has been correlated with increased tumor susceptibility and incidence (Erlich et al., 1983).

The purpose of this study was to examine the effects of DMBA exposure on NCC function in oyster toadfish. Cytotoxic function of NCC was studied over time following a single intraperitoneal exposure to DMBA, as well as at various doses of DMBA. Patterns of DMBA-induced suppression of NCC activity were compared to those of macrophage phagocytic activity, an immune response which has been more thoroughly investigated in oyster toadfish (Rice and Weeks, 1989; Seeley and Weeks, 1991).

Materials and Methods

Adult oyster toadfish (300-500 g) were captured in crab pots from the relatively non-polluted York River, Virginia. Prior to beginning experiments, fish were maintained for a minimum of two weeks in 250 gallon tanks with mechanically and biologically filtered, UV-irradiated, recirculating seawater with constant salinity (20 ppt.) and temperature (21°C). Fish were fed bi-weekly a protein and vitamin rich diet that has been described elsewhere (Van Veld et al., 1988).

For the dose-response experiments, 25 fish were randomly assigned to five 55 gallon aquaria. Each aquarium (containing five fish) was randomly assigned to a DMBA dosage group (described below). All aquaria were covered with black plastic, and received a constant influx of sand-filtered York River water.

For the time course experiment, 35 fish were randomly separated into two groups. Twenty of these fish were assigned to a control group, and the remaining 15 were assigned to a DMBA-exposure group. The fish were held in two 250 gallon tanks which received a continuous influx of sand-filtered, UV-irradiated York River water. The tanks were covered with black plastic, and fish were allowed to acclimate for two weeks prior to initiation of dosing experiments.

7,12-Dimethylbenz[A]anthracene (Sigma, St. Louis, Missouri, USA) was dissolved in sterile corn oil. For the dose response experiment, fish were injected intraperitoneally with 0 (corn oil only), 1, 10, 50 and 100 mg DMBA/kg body weight. Concentrations of DMBA in corn oil were adjusted so that fish in all treatment groups were injected with the same volumes of corn oil (on a volume/body weight basis). All treatment groups received injections on the same day and were sacrificed 7 days later. Prior to injection and sacrifice, fish were anesthetized in 200 mg/l tricaine methanosulfate (MS-222, Sigma).

In the time course experiment, control fish (Days 0, 7, 14, and 28) were injected with corn oil only. DMBA-exposed fish (Days 7, 14, and 28) were injected with a sufficient volume of DMBA, suspended in corn oil to provide a dose of 10 mg DMBA/kg body weight. Day 0 (control) fish were sacrificed immediately.

Peritoneal lavages were performed by injecting 5 ml phosphate buffered saline (PBS, Sigma) containing 5 IU ml/l heparin (Sigma) into the peritoneal cavity. This fluid was subsequently removed by suction through a small ventral incision. Hypotonic shock was employed to remove erythrocytes from all samples, using a method described by Ghoneum, et al. (1990), and in all samples, leukocyte viability was determined to be 90% or greater by trypan blue exclusion. Cells were suspended in complete RPMI-1640 (RPMI-C, containing 10% FBS, 2 mM L-glutamine, 50,000 IU/l penicillin, 50 mg/l streptomycin, 100 mg/l kanamycin, 2.0 g/l sodium bicarbonate and 25 mM HEPES), and incubated overnight at 23°C.

Prior to sacrifice, fish were weighed and measured. Body condition factor was determined according to a formula described by Esch and Hazen (1980), in which body condition, $K = 10^5 * W/L^3$, where W = body weight (g), and L = length (mm). Blood samples were taken, and hematocrits and leucocrits were determined as described by Wedemeyer and Yasutake (1977). Spleens and livers were removed and weighed for

determination of spleen-somatic indices (SSI) and liver-somatic indices (LSI). These organ-somatic indices were determined by dividing the weight of the organ (g) by total body weight (g).

The capacity of oyster toadfish NCC to lyse tumor cells was determined using the murine lymphoblastoid cell line YAC-1 as a model target cell. NCC activity was determined using a standard ^{51}Cr-release assay (Evans et al., 1984). Briefly, $1x10^7$ target cells suspended in 1 ml RPMI-C were labeled with 100 μCi of ^{51}Cr-Na_2CrO_4 (DuPont-NEN, Boston, Massachusetts) for 1 hour at 37°C. Excess label was removed by washing the cells once in PBS, followed by a 30 min incubation of the cells at 37°C in 50 ml PBS. The cells were then centrifuged for 10 min at 250xg and the resulting pellet was resuspended in 10 ml RPMI-C. After another centrifugation, the cells were resuspended in fresh RPMI-C at a concentration of $1x10^5$ cells/ml. An aliquot (100 μl) of this suspension was added to each well of a 96 round-bottomed well tissue culture plate (Corning, Corning, New York). Fish (effector) cells were suspended in RPMI-C at a concentration of $1x10^7$ cells/ml, and 100 μl of this suspension were added to each well to give an effector:target cell ratio of 100:1. Plates were incubated for 4 hr at 23°C. Following this, plates were centrifuged at 250 x g for 10 min, and 100 μl of supernatant was harvested from each well. Radioactivity was measured with a

Beckman LS-5000 liquid scintillation counter. Percent specific release of ^{51}Cr was calculated using the following formula:

$$\%\text{Specific release} = [(\text{E.R.}-\text{S.R.})/(\text{M.R.}-\text{S.R.})] \times 100$$

Where E.R. represents experimental release, M.R. represents maximum release (measured by adding 0.1 ml Triton X-100 (Sigma) to designated wells), and S.R. represents spontaneous release (measured by adding 0.1 ml RPMI-C to designated wells).

Phagocytic activity was determined spectrophotometrically, using a previously described technique (Seeley et al., 1990). Briefly, $1x10^6$ leukocytes in 1 ml RPMI-C were incubated with $4x10^7$ congo red-stained yeast cells (Saccharomyces cerevisiae, Fleischmann's, New Jersey) for 0 and 120 min. Following incubation, 5 ml of ice-cold PBS was added to the samples to prevent further phagocytic activity, and 3 ml of Percoll® (Pharmacia, Uppsala, Sweden), adjusted to a density of 1.055 g/ml with PBS, was injected into the bottom of each tube. The samples were then centrifuged at 850 x g for 3 min in order to separate leukocytes from any remaining free yeast cells. Following centrifugation, leukocytes, located at the Percoll®-PBS interface, were removed with a pipet and washed several times in PBS. The cells were then pelleted and

supernatants were carefully removed. To each tube, 1.2 ml of a trypsin-EDTA solution (1.5 g/l trypsin, 0.4 g/l EDTA in PBS; Sigma) was added in order to solubilize the cells and release any congo red stain. Following an overnight incubation at 37°C, absorbance at 510 nm was determined against a blank containing only the trypsin-EDTA solution.

Data were shown to be normally distributed using the Chi-square test for normality (Horning and Weber, 1985). Homogeneity of variance in the data was established using Bartlett's test (Zar, 1984). Analysis of variance was employed to compare responses of control and exposed groups of fish, and when appropriate, the Tukey method of multiple comparisons was used to identify significantly different responses among treatment groups (Zar, 1984).

Results

As stated earlier, phagocytic activity was used in this study as a general indicator of immunomodulation. Phagocytic activity, as determined by uptake of congo red-stained yeast cells, was suppressed in an essentially linear fashion across the range of DMBA doses used in this study (Figure 12a). At DMBA doses of 1 and 10 mg/kg, phagocytosis of yeast cells was reduced by 50% and 49%, respectively, compared to control values. At DMBA-doses of 50 and 100 mg/kg, phagocytic activity was reduced to 26% of control values.

In comparison to phagocytic activity, NCC activity was suppressed at all doses of DMBA used in this study, compared to NCC activity in the control fish (Figure 12b). No statistically significant differences could be observed in the NCC activity of DMBA-exposed fish. Interestingly, mean NCC activity in the exposed fish was never suppressed to below a level of 1.2% specific release in any of the treatment groups.

In the time-course experiment, mean values for phagocytic activity increased over time in control fish (Figure 13a), reaching a maximum at day 28. This trend was not statistically significant, however. Phagocytic activity in the exposed fish dropped gradually during the exposure period, rather than experiencing maximum suppression immediately following exposure to DMBA. Fourteen days after exposure to DMBA, mean phagocytic activity had dropped to 29% of day 0 control values. Beyond this time point, mortality began to increase in the DMBA-exposed fish, so that by 28 days after exposure, all of the exposed fish were dead.

NCC activity followed a different pattern during the time-course experiment (Figure 13b). Seven days after exposure, NCC activity in control and DMBA-exposed fish was 9% and 6% of day 0 levels, respectively. NCC activity gradually increased in the control fish following this initial period of suppression, so that by day 28, NCC

activity had been restored to 74% of day 0 control values. This activity was never restored in the DMBA-exposed fish.

Data on body condition factors (K), spleen-somatic indices (SSI), liver-somatic indices (LSI), hematocrits (Hct) and leucocrits (Lct) are shown in Table 2. No statistically significant trends can be observed with respect to these general stress indicators, although mean values for K dropped slightly in the DMBA-exposed fish over the course of the exposure period. Although the general stress indicators used in this study failed to detect any significant differences between control and DMBA-exposed fish, mortality in the exposed fish increased significantly throughout the 28-day exposure period (Table 3). This increased mortality was more closely correlated with reduced phagocytic function in the exposed fish ($r^2 = 0.85$) than with NCC activity ($r^2 = 0.29$).

Discussion

In this study, the effects of DMBA on NCC and macrophage function in oyster toadfish were examined. While functional suppression of both of these cell types was observed, natural cytotoxic activity was considerably more sensitive to DMBA than was macrophage phagocytic activity. This differential sensitivity could be observed in both the dose-response and time-course experiments. Conversely, DMBA

Figure 12. Effect of varying doses of DMBA on macrophage phagocytic activity (a) and NCC tumorolytic activity (b). Fish were injected intraperitoneally with DMBA dissolved in corn oil. Control fish were injected with corn oil only. NCC activity was determined at an effector:target cell ratio of 100:1. Bars represent mean responses (± s.e.) of treatment groups consisting of 5 fish each. Responses that were significantly different ($p < 0.05$) from control values are indicated by *.

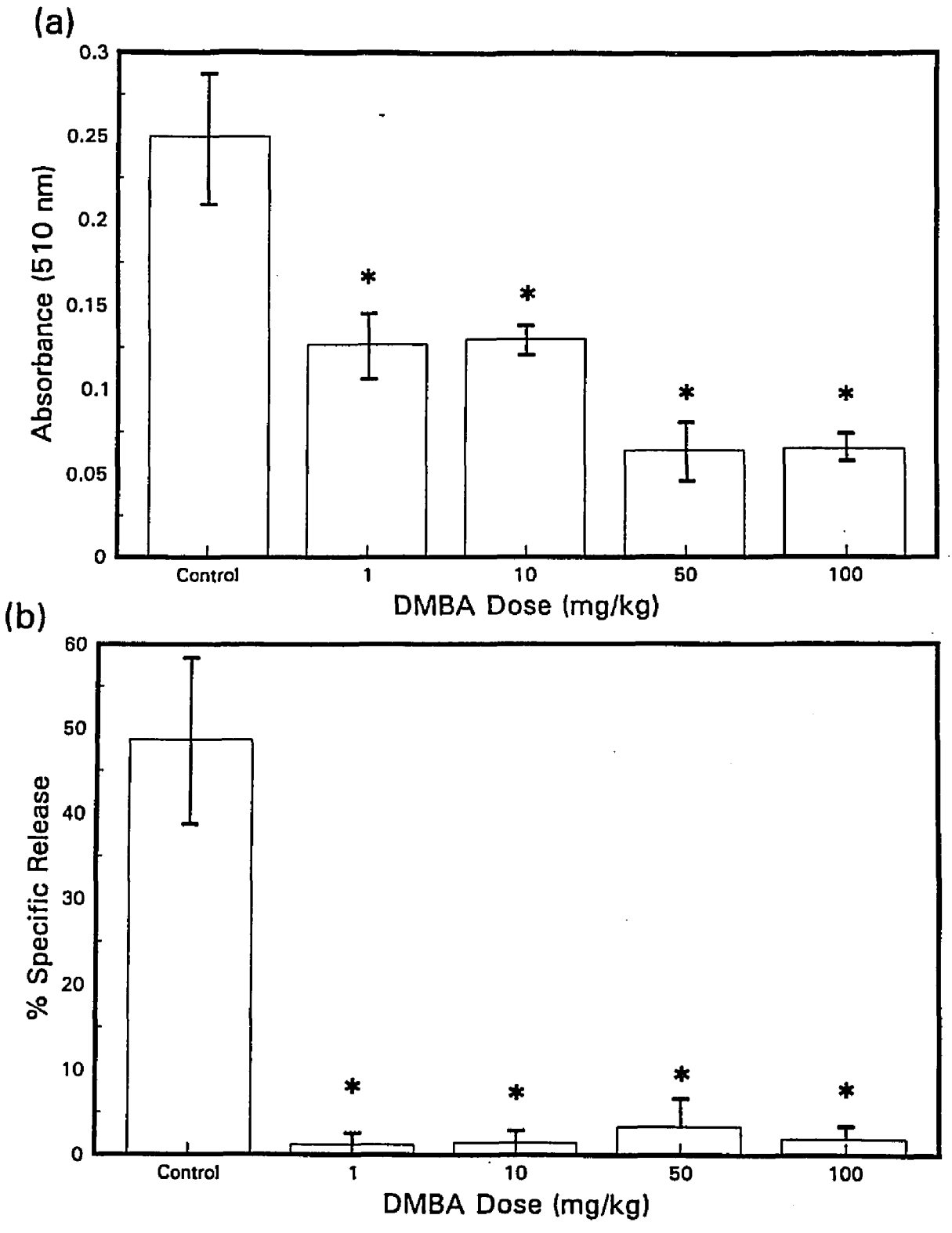
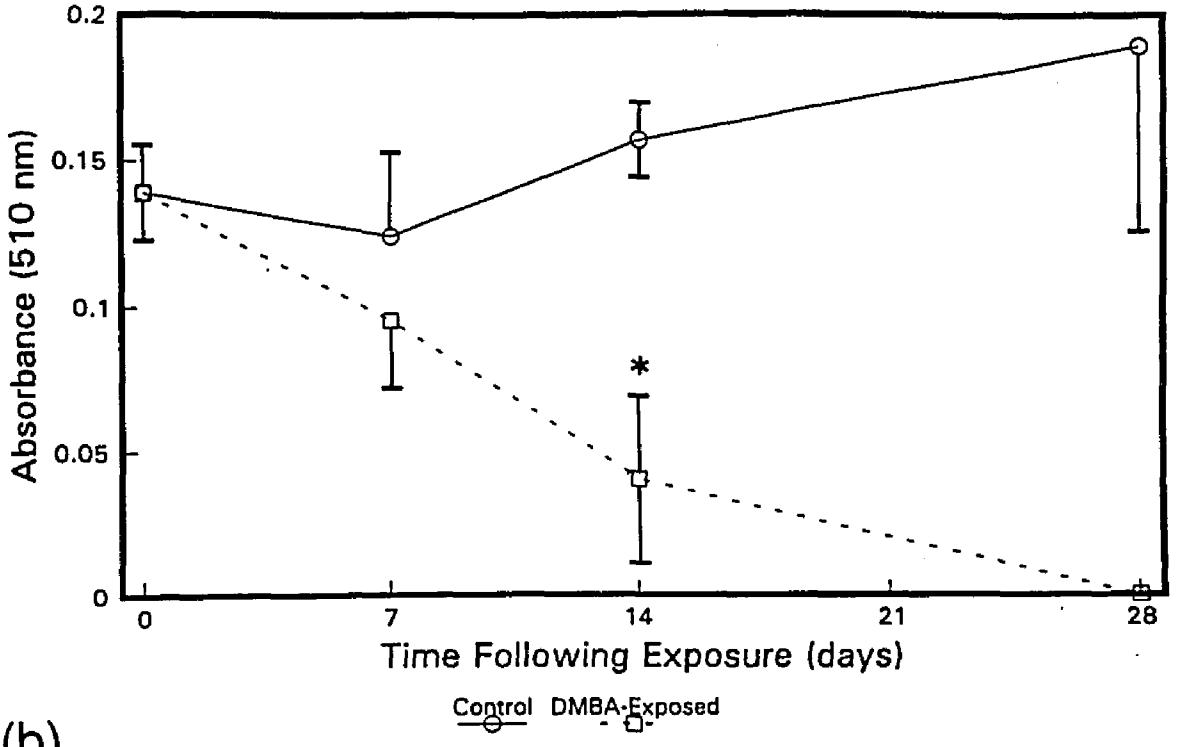

(a)
Absorbance (510 nm)
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
*
*
Control
1
10
50
100
DMBA Dose (mg/kg)
(b)
% Specific Release
60
50
40
30
20
10
0
*
*
*
*
Control
1
10
50
100
DMBA Dose (mg/kg)

Figure 13. Suppression of macrophage phagocytic activity (a) and NCC tumorilytic activity (b) over time following a single intraperitoneal injection of 10 mg DMBA/kg body weight. Bars represent mean repsonses (± s.e.) of 3-5 fish. *, responses which were significantly different ($p < 0.05$) from day 0 control values. £, responses in DMBA-exposed fish which were significantly different ($p < 0.05$) from control values for the same sampling period. §, mortality in the DMBA-exposed fish had reached 100% by day 28 of the exposure period.

(a)


0.2
0.15
0.1
0.05
0
Absorbance (510 nm)
*
0
7
14
21
28
Time Following Exposure (days)
Control
DMBA-Exposed

(b)

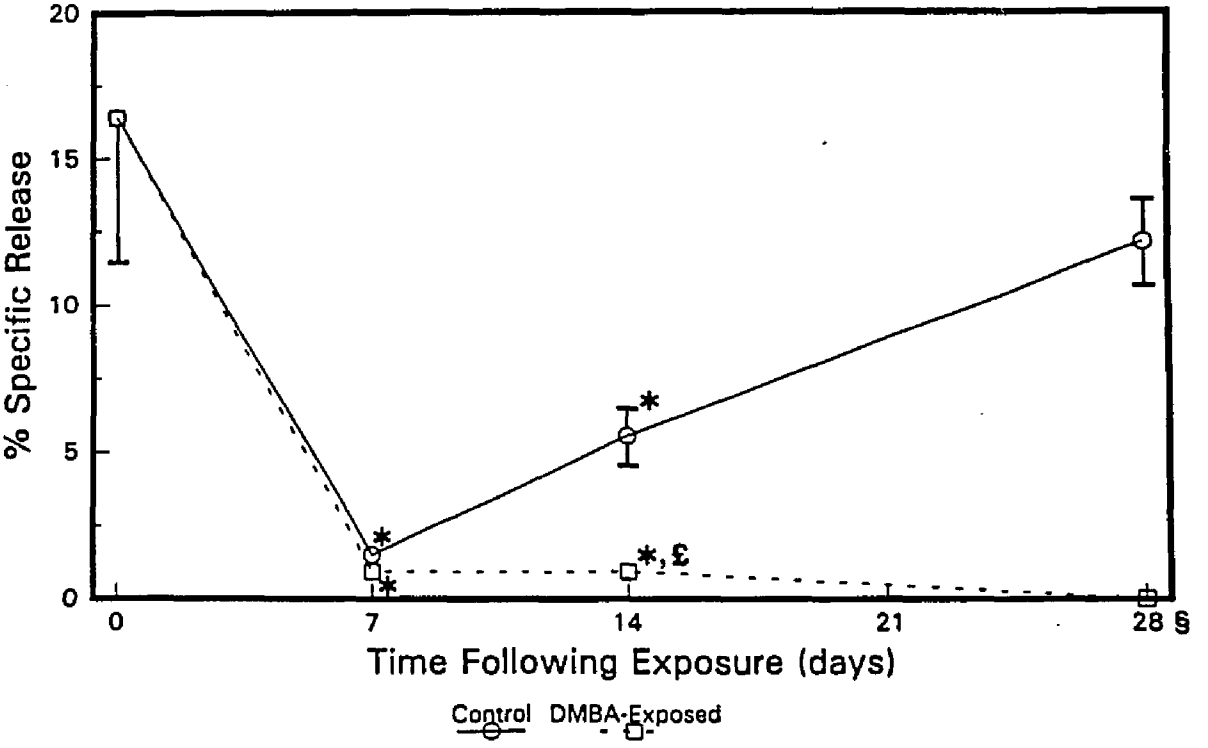

20
15
10
5
0
% Specific Release
*
*
*
*, £
0
7
14
21
28 §
Time Following Exposure (days)
Control
DMBA-Exposed

Table 2. Oyster toadfish body condition factor (K), spleen somatic-index (SSI), liver-somatic index (LSI), hematocrit (Hct) and leucocrit (Lct) obtained during the time-course exposure experiment. No statistically significant differences were observed between control (C) and exposed (E) groups of fish.

	Day 0		Day 7		Day 14		Day 28	
	C	E	C	E	C	E	C	E
SSI[a]	0.001 ±0.001	ND	0.002 ±0.001	0.001 ±0.001	0.002 ±0.001	0.002 ±0.001	0.002 ±0.001	ND
LSI[b]	0.028 ±0.004	ND	0.018 ±0.004	0.020 ±0.002	0.019 ±0.002	0.016 ±0.001	0.019 ±0.003	ND
HCT[c]	21.0 ±1.14	ND	23.1 ±2.3	25.5 ±3.5	19.2 ±1.36	18.0 ±1.50	18.4 ±0.7	ND
LCT[d]	1.4 ±0.24	ND	1.5 ±0.32	1.2 ±0.20	1.2 ±0.2	1.5 ±0.5	1.3 ±0.3	ND
K[e]	1.62 ±0.09	ND	1.63 ±0.06	1.64 ±0.09	1.69 ±0.06	1.48 ±0.09	1.61 ±0.11	1.50 ±0.03

[a] SSI = Spleen Weight(g)/Total Body Weight(g)
[b] LSI = Liver Weight(g)/Total Body(g)
[c] HCT = Hematocrit; the percentage of total blood volume occupied by erythrocytes
[d] LCT = Leucocrit; the percentage of total blood volume occupied by leukocytes
[e] K = Body Condition Factor = 105*W/L3

Table 3. Mortality in control and DMBA-exposed fish during the time-course experiment.

	Total Deaths[a] / % Mortality[b]	
	Control	DMBA-exposed
Week 1	0/0	0/0
Week 2	0/0	2/20
Week 3	0/0	3/43
Week 4	1/20	2/100

[a] Number of fish dying during the specified week
[b] Mortality expressed as a percentage of the remaining fish in the specified treatment group

appeared to have no effect on the general stress indicators examined.

Although DMBA-induced suppression of NCC was expected, based on the results of studies such as those described above, the extreme sensitivity of oyster toadfish NCC to DMBA observed in this study was unexpected. Exposure studies using mice required doses of DMBA as high as 50 mg/kg in order to induce significant suppression of NK function (Dean et al., 1986a). Similarly, Kimber et al. (1986a) found that a dose of 40 mg was sufficient to suppress NK function in rats. The sensitivity of peritoneal NCC in this study could be a result of the direct exposure of these cells to DMBA via intraperitoneal injection, while exposures in the studies described above were carried out by subcutaneous injection and gavage, respectively. As stated earlier, however, Erlich et al. (1983) found that dietary exposure to doses of DMBA as low as 3 mg (doses in this case were not normalized to body weight of experimental animals) could result in significant, but reversible suppression of murine NK function.

Following the dose-response experiment, a time-course experiment was conducted in order to determine the duration of immunosuppresion following administration of a single dose of DMBA. Although NCC activity was virtually obliterated at all doses of DMBA administered during the dose-response experiment, it was assumed that at a dose of

10 mg/kg NCC activity would be at least partially restored over time. However, this turned out not to be the case, as NCC activity was not spontaneously restored throughout the duration of the time-course experiment.

In contrast to the results obtained during the dose-response experiment, the NCC activity of control fish in the time-course experiment was initially suppressed to the same degree as in the DMBA-exposed fish. This suppression lasted through day seven of the study, and beyond that time point, NCC activity in control fish was gradually restored to control levels. This transient suppression of NCC activity in control fish may be attributable to differences in how fish were handled between the dose-response and time-course experiments. In the dose-response experiment, fish receiving different doses were held in separate 55 gallon aquaria, with five fish being placed into each aquarium. Further, it was possible to inject fish in the same laboratory in which they were held, thereby minimizing stress associated with handling, and access to this room by other workers was limited. During the time-course experiment, however, it was necessary to maintain fish in large holding tanks, and fish had to be transported to a different room in order to be injected. Access to this holding area by other workers was not restricted. While efforts were made to minimize any extraneous stressors, it appears as though stress associated with handling may have

resulted in a transient suppression of NCC activity in the control fish. This finding provides additional evidence for the extreme sensitivity of NCC. Other investigators have also found that a variety of non-chemical stressors, such as social aggression, could suppress NCC function in fish (Faisal et al., 1989b).

Despite the fact that NCC activity was nearly obliterated in DMBA-exposed fish throughout the 28 days of the time-course experiment, it is possible that the increased mortality seen in the exposed fish over time was actually due to the gradual suppression of macrophage function. Mortality in the exposed fish correlated more closely with decreased macrophage function than with NCC activity (r^2 = 0.85 and 0.29, respectively). Nathan (1986) has concluded that macrophage phagocytic activity, in its role in defense against infection by opportunistic microbial organisms, may be more critical to an animal's survival than tumoricidal activity. This conclusion was based on the fact that in naturally occurring populations of animals, it is uncommon for an individual animal to survive long enough to develop tumors, whereas exposure to infectious agents is a constant threat to an animal's survival. In this current study, where both macrophage phagocytic activity and NCC cytotoxic activity were significantly suppressed, the immediate threat to the survival of the experimental animals would have been from infection, since it was not possible to

totally eliminate opportunistic pathogens from the fish holding tanks, rather than from the long term threat posed by tumor formation.

Furthermore, it is possible that suppression of macrophage function in exposed fish may not have been caused directly by DMBA. Whereas NCC activity was suppressed immediately following exposure to DMBA, phagocytic function exhibited a gradual decline. This gradual loss of phagocytic function was coupled with a number of other observable effects which included cessation of feeding activity, lethargy, and a drastic change in the animals' coloration from dark brown to pale yellow. None of these effects were observed in the control fish.

Thus, while NCC activity in the DMBA-exposed fish experienced a pronounced, long term suppression which can be attributed directly to exposure to DMBA, reduced macrophage function with time may have been linked to a general decline in the health of the experimental animals as evidenced by the observed changes described above. Unfortunately, it was not possible to determine the presence of infection in experimental animals in this study. Such information would have been helpful in dermining whether the ultimate cause of mortality in the exposed fish was caused by infection or poisoning by DMBA.

CHAPTER 5

SUMMARY AND CONCLUSIONS

In terms of their functional characteristics, oyster toadfish nonspecific cytotoxic cells (NCC) are similar to those found in other fish species. These cells are capable of attacking and destroying a variety of cultured tumor cell lines without prior exposure. Oyster toadfish NCC also lack certain characteristics that are commonly associated with macrophages, another critical cellular component of nonspecific immunity. For example, NCC are nonphagocytic, and they lack the adherence properties of macrophages.

NCC activity in oyster toadfish was found to occur in the spleen, anterior kidney, peritoneal cavity and peripheral blood, although this activity varies considerably between these organs. In these studies, the highest NCC activity was found to occur in peritoneal leukocytes. This increased activity may be due to the fact that peritoneal NCC are activated <u>in situ</u> by a wide range of antigenic substances.

Although NCC activity varies from organ to organ, analysis of the kinetics of target cell destruction revealed

that once an NCC binds to a target cell, lysis of that cell will proceed at essentially the same rate, regardless of the organ source of the NCC. These results suggest that the rate-limiting step in the process of target cell destruction may be related to the ability of NCC to recognize and bind to target cells, with more highly activated NCC having a greater capacity to carry out these initial steps in target cell lysis.

Morphologically, oyster toadfish NCC resemble monocytes or macrophages. While this observation would seem to contradict the functional evaluation of oyster toadfish NCC, which indicated that NCC are different from monocytes or macrophages, these results are consistent with those of other investigators. Previous studies have suggested that fish NCC may actually represent a non-adherent, non-phagocytic subpopulation of macrophages. Some investigators have also suggested that nonspecific cytotoxic activity may actually be found in a wide range of immunologically active cell types in fish, and as such, this activity may be mediated by a heterogeneous population of cells, rather than a single cell type. While the cells involved in target cells destruction in these studies appeared to be monocytes or macrophages, conclusive identification of fish immune cells is hindered by a lack of specific cell surface markers.

In order to characterize NCC in oyster toadfish, it was

necessary to compare their function to macrophages. While a wide range of techniques have been developed to assess the phagocytic function of macrophages, many of these are cumbersome and yield highly subjective results. As part of this research, a new technique has been developed to determine the phagocytic capacity of fish macrophages. In a field investigation, it was found that exposure to sediment-bound polycyclic aromatic hydrocarbons (PAH) was capable of reducing the phagocytic capacity of macrophages (i.e., the total number of yeast cells an individual macrophage could phagocytize) in a dose response fashion.

Oyster toadfish NCC were found to be extremely sensitive to the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA). In a dose-response experiment, fish received intraperitoneal injections of DMBA in doses of 0, 1, 10, 50, and 100 mg/kg body weight. While suppression of peritoneal macrophage activity increased linearly with increasing doses of DMBA, peritoneal NCC activity was virtually eliminated at all doses.

While many immunotoxic compounds have been found to exert transient effects on immune system function, DMBA has been shown to have relatively long lasting effects. In a time-course experiment, oyster toadfish were given a single intraperitoneal injection 10 mg DMBA/kg body weight. Following this single exposure, NCC activity was completely suppressed for the 28 day duration of the study. Macrophage

activity dropped gradually during the 28 day exposure period. Mortality in the DMBA-exposed fish began to rise significantly fourteen days after the initial exposure to DMBA. This increased mortality was closely correlated with reduced macrophage activity, but not NCC activity. Interestingly, NCC activity in control fish was also significantly suppressed following injection with corn-oil, although by day 28, this activity had been restored. This suppression may have been the result of handling stress on the fish during injections. Macrophage activity in control fish was not suppressed to any significant degree during the time-course experiment.

APPENDIX

GLOSSARY OF ABBREVIATIONS

APC	Antigen Presenting Cell
B Cell	B Lymphocyte
CMI	Cell Mediated Immunity
CRP	C Reactive Protein
CTL	Cytotoxic T Lymphocyte
DMBA	7,12-Dimethylbenz[a]anthracene
EC	Effector Cell
FBS	Fetal Bovine Serum
HBSS	Hank's Balanced Salt Solution
HCT	Hematocrit
HI	Humoral Immunity
IAK	Interferon Activated Killer Cell
IL-1	Interleukin 1
IL-2	Interleukin 2
K	Body Condition Factor
LAK	Lymphokine Activated Killer Cell
LCT	Leukocrit
LSI	Liver Somatic Index
MHC	Major Histocompatibility Complex
NCC	Nonspecific Cytotoxic Cell
NK	Natural Killer Cell
PAH	Polycyclic (or Polynuclear) Aromatic Hydrocarbon
PBS	Phosphate Buffered Saline
PCB	Polychlorinated Biphenyl
PMN	Polymorphonuclear Leukocyte
RPMI-1640	Roswell Park Memorial Institute Cell Culture Media
RPMI-C	Complete RPMI-1640 Cell Culture Media
SSI	Spleen-Somatic Index
TC	Target Cell
T Cell	T Lymphocyte
T_h	T Helper Cell

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