COMPARATIVE IMMUNOTOXICOLOGY - A NEW DIRECTION

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Abstract

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Increasing concentrations of xenobiotics in the environment, food and water supplies has direct or non-direct effects on the immunocompetent cell activity, cellular and humoral defence mechanisms and protection from diseases in animals and human. At this moment we must develop comparative aspects of immunotoxicology, justifying the use of invertebrates, amphibians and especially fish and reptiles, as models. The defence mechanism indices have been used in various laboratory and field experiments to analyze the influence of xenobiotics on the defence mechanisms and disease resistance of mammals, birds, amphibians and fish. Many of the standard immunological techniques have been modified for optimal results. In this paper we present the basic immunological techniques for study of the effects of xenobiotics on the cellular and humoral defence mechanisms in fish.

Xenobiotics, fish, defence mechanisms

Understanding the human and animal health risk associated with environmental exposures involves defining the cascades of events between exposure to an environmental agent and the resulting effects on health. To cause health impairment, a pollutant must be absorbed into the body and result in biological change. For most environmental pollutants, little is known about the flow of events between exposure and health effect. Biological markers are powerful tools that can be used to address many different issues confronting environmental health scientists. Effects of markers are indicators of biological responses. They provide information concerning the likely health outcomes associated with different target doses of environmental pollutants or their metabolites. Overall, biological markers offer the possibility of using human data to make health risk assessment more meaningful, realistic and host-effective. Risk assessment attempts to determine the consequence of toxicant exposure at the human population level. Biomarkers are frequently used to assess the responses and effects of toxicants on laboratory animals. Therefore, there is a need to extrapolate from animal data to human health effects, not only because most of the uncertainties associated with human epidemiological studies. The immune and host defences are highly conserved across species such that immunocompetent cells and some products of the immune system in humans, mice, rats and also in teleost fish are similar. Also, in instances where controlled human studies have been possible, results of immune function studies have been accurate predictors of effects in humans (Huggett et al. 1989).

Increasing concentrations of xenobiotics in the environment, industry, food and water

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supplies, has become a problem of significant proportions; today, the danger is greater than ever before. Many environmental pollutants are toxic to animals and humans, resulting in overt clinical disease and even death. All of the pollutants have direct or indirect effects on the immunocompetent cells and nonspecific defence mechanisms. At this moment we must start open discussion about comparative aspects of immunotoxicology, justifying the use of invertebrates, amphibians and especially fish, and reptiles as animal models. First, they are numerous, highly visible and ubiquitous members of the biosphere, and we should understand how xenobiotics affect their immune systems. Second, they are important to essential food chains and are therefore potential candidates for vector of diseases. Third, they can be used as surrogates, sentinels, to ascertain the effects of immunotoxicants on humans for legal and ethical reasons. Surely, an appropriate animal, not necessarily a mammal, may be chosen since lower vertebrate animals (especially fish) possess immune systems that often mirror those of humans. Finally, by analyzing the effects of xenobiotics as potentially deleterious to the immune system of all organisms, the comparative approach can assume an evolutionary flavor that should be of deep concern for all of us. For the biological consequences of such pollution (as a source of environmental stress) may range beyond that which is immediately apparent. Nevertheless we must exert caution in extrapolating causes and effects from invertebrates and lower vertebrates to humans (Huggett et al. 1989; Vos 1977; Vos 1980).

A broad spectrum of chemicals alter immune functions allowing for the immune mechanisms potential as a target organ system for use in assessing the toxicity of exposure to chemicals. Because considerable information on the nonspecific defense mechanisms, cell-mediated immunity and molecular components of the immune system exists, immune responses are especially well suited for comparative analysis that emphasize mechanisms of toxicity. Cellular or subcellular and organismal immunological indicators of either exposure to or effects of chemicals can be used as assessment tools. Such immunological indicators fall within the category of measurement endpoints defined as biomarkers (Huggett et al. 1989).

The immune system is a highly evolved system that functions to provide organisms with ability to resist pathogenic agents, destroy neoplastic cells, and reject nonself components. The immune system has increased in complexity as animals have evolved. According to the present level of knowledge, differentiated lymphoid organs first appear in the fish, with an develop in structural definition through amphibians and reptiles to birds and mammals. Higher mammals possess five immunoglobulin classes, birds three, reptiles and amphibians two, and fish only one class. No immunoglobulins are found in invertebrates, but functionally analogous proteins, mostly agglutinins and inducible humoral factors (apidaecins and abaecins) are present (Glinski and Jarosz 1995a; Glinski and Jarosz 1995b). Although all vertebrate species synthesize immunoglobulins, selection of humoral immunoglobulin assays as biomarkers will be constrained by the sophistication of the immune system of the species under investigation. Similar assays have been developed in invertebrates using agglutinins.

Several aspects of defence mechanisms amenable for use as specific biomarkers are conserved phylogenetically. Phagocytosis and cell-mediated responses are very important part of immunological mechanisms and perform a key role in the regulation of immune response. Assays of these responses can provide useful measures of immunosuppression. The fundamental units of the defence mechanisms are the leukocytes, which develop from pluripotent stem cells and undergo differentiation, maturation, and proliferation into morphologically and functionally distinct cell population: polymorphonuclear (PMN) cells, MN phagocytes, and lymphocytes in higher vertebrates (Huggett et al. 1989).

Generally, defense mechanisms characterize two pathways: nonspecific immune

responses mediated by MN phagocytes (blood monocytes, tissue macrophages) and granulocytes, which can recognize foreign material nonspecifically and specific immune responses mediated by several effector lymphocytes, which are directed and specific for an eliciting antigen.

The nonspecific defence mechanisms comprise two types of responses: phagocytosis and inflammation. The phagocytic and inflammatory responses can be enhanced by immunocompetent cell products (cytokines). Other important cells involved in immune surveillance in vertebrates are the natural killer (NK) cells. These cells can recognize changes in surface markers that occur on virally infected cells and on some tumour cells. They can be activated by cytokines such as IL-2.

The specific immune responses have two major components: cell-mediated immunity (CMI) and humoral-mediated immunity (HMI). The CMI response is characterized by the sensitization of T-cells through antigen presentation and by the differentiation and proliferation of T-cells into effector, regulatory and memory cells. Sensitization is assisted by macrophages and other accessory cells that concentrate antigenic determinants on their surfaces for presentation to T-cells. Macrophages also provide soluble factors called cytokines (IL-1). Macrophages are necessary for T-cells to function, especially for T helper and T suppressor cells (T_h, T_s). Memory cells are produced for each T-cell type and are stimulated by antigen. The HMI response is characterized by production of antibody molecules that react specifically with antigen. Mammals possess five Ig classes, birds three, reptiles and amphibians two, and fish only one class. The major role of antibodies is to protect the host from infectious disease and can involve the following functions: opsonization, complement-mediated lysis, antibody-dependent cell cytotoxicity (ADCC) and virus neutralization.

The defence mechanism parameters have been used in various laboratory and field experiments to analyze the influences of xenobiotics on the nonspecific cellular and humoral defence mechanisms, specific immune responses and disease resistance of mammals, birds, amphibians and fish. In the following examples, immune assays are broadly grouped according to the specificity of responses. Some nonspecific assays such as the determination of lysozyme, C-reactive protein (CRP) or complement levels have been used extensively in the past for analyzing the effects of environmental contamination on the health of fish, birds and mammals. Lysozyme and CRP are important in the initial destruction of invasive agents and in some cases can serve as an early biomarker indicating deterioration of some protective mechanisms (Blaxhall 1972; Rice 1986; Siwicki and Studnicka 1987). Phagocytosis is an important parameter of nonspecific defense mechanisms as it is well conserved throughout phylogeny and important in immune surveillance in vertebrates as well as invertebrates. Therefore, assays of phagocytosis and intracellular killing activity have potential as bioindicators of environmental contamination among several animal phyla (Jabs et al. 1980; Rook et al. 1985). Also macrophage functions have been proposed as sensitive indicators for the effects of xenobiotics present in the aquatic environment (Dunier and Siwicki 1994; Subasinghe and Arthur 1990; Zeeman and Brindley 1981).

The humoral-mediated immunity (HMI) assays have been used in many studies to determine altered immune capacity which is caused by xenobiotics. Various laboratories demonstrated immunosuppression of humoral antibody levels in fish and birds exposed to heavy metals. A reduction in antibody-secreting cells following bath immunization was observed in fish that had been exposed to phenol, formalin, detergent solutions and antibiotics (Siwicki et al. 1993). HMI has also been evaluated in chickens and fish following exposure to pentachlorophenol (PCP), polychlorinated biphenyl (PCB) and pesticides (Dunier and Siwicki 1994).

The scientific community should adopt an integrated ecological approach to risk analysis that identifies direct and indirect health risk of human activities ranging from chemical contaminants development practices (Epstein 1995).

Several immunotoxicological laboratories have organized immunological assays applicable to several classes of animals. Zeeman and Brindley (1981) and Siwicki and Anderson (1993) previously presented the protocols and the immunological assays applicable to control the influence of xenobiotics on the nonspecific cellular and humoral defence mechanisms in fish.

In this paper we present the rationale for development of biomarkers of fish host defence mechanisms as targets for study immunotoxicity of xenobiotics and discuss them in terms of sensitivity to toxicants known to affect high vertebrate immunity.

Simplified assays for study nonspecific defence mechanisms in fish

In presenting the following assays, we discuss some of the advantages and disadvantages of each. Without a doubt, as biologists, pathologists, comparative immunologists, and research personnel work with assays, new and better modifications will be found to make the tests more reliable and easier to do. We hope the following protocols can be benefit for control effects of pollutants or xenobiotics on defence mechanisms in fish.

Leukocrit samples assay

The percent volume of leukocytes in blood gives clues to the health of the animal, and can be helpful in determining abnormalities arising from polluted aquatic environments. The leukocrit levels in normal fish are usually between 1-2%. In our experience, this assay is not very dependable and often does not correlate well with the leukocyte counts by hemocytometer. In some cases the top of the haematocrit (RBC) pack is difficult to distinguish from the infiltrating leukocytes (Blaxhall 1972; Hesser 1970).

Adherence/NBT assay

Adherence to glass is an important early indicator of activated neutrophils and monocytes. The following assay is designed to show that activation in addition to the increases in oxygen radical products. Activated neutrophils should adhere to glass and show a segmented nucleus. A blue-staining halo will surround the cell. We usually count 2 of the most dense fields at $400 \times$ magnification. An unstimulated animal may give about 20-40 cells per field; a strongly stimulated one 200-400, and a suppressed animal only 5-10 cells (Anderson 1992; Anderson et al. 1992; Siwicki and Anderson 1993).

Phagocytic activity of PMN cells by spectrophotometric assay

These simple and rapid spectrophotometric assay should be quite adaptable for general field use. The NBT is reduced to formazan in the reaction with the oxygen radicals from neutrophils and can be analyzed by spectrophotometer. This assay is particularly helpful when many fish are sampled (Anderson 1992; Siwicki et al. 1985; Siwicki 1989; Siwicki et al. 1993).

Respiratory burst activity assay

Phagocytes possess a unique membrane enzyme, NADPH oxidase, capable of the one electron reduction of molecular oxygen into superoxide anion (O_2^-) during a process named the respiratory burst. The reducing equivalents are provided by NADPH, which is generated via the hexose monophosphate (HMP) shunt, and are independent of the mitochondrial cytochrome system. Following membrane stimulation, as occurs when a particle adheres to the phagocyte, both the respiratory burst and HMP shunt are activated

and these two processes are generally agreed to be linked together. Most of the O_{2^-} produced is dismutated to H_2O_2, and this occurrence either spontaneously or catalyzed by the enzyme superoxide dismutase (SOD). The two main methods used to measure this processes are: the reduction of ferricytochrome C and the reduction of the redox dye nitroblue tetrazolium (NBT). The first method detects extracellular O2 whereas the second detects intracellular O_{2^-}. Before the respiratory burst will occur the macrophage and neutrophil surface membrane must be stimulated. This can be achieved with particulate stimuli such as bacteria, zymosan or phorbol myristate acetate (PMA) (Jabs et al. 1980; Rook et al. 1985; Siwicki and Anderson 1993). In our study, we used NBT reduction assay for the study of respiratory burst activity of phagocytes stimulated by PMA.

Potential killing activity of phagocytic cells assay

Fish phagocytic cells (neutrophils, monocytes/macrophages) have a vital role in many aspects of the nonspecific defence mechanisms and specific immune response. As well as being accessory cells important in initiating immune responses they are also potent effector cells capable of killing a wide range of pathogens. The following methods were used for analysis of blood neutrophils and monocytes/macrophages killing activity (Jabs et al. 1980; Rook et al. 1985; Siwicki and Anderson 1993).

Myeloperoxidase (MPO) activity assay

MPO is a very important intracellular enzyme that participates in intracellular killing of pathogens. The production of myeloperoxidase in neutrophils is detected by overlays of substrates. Those cells having the MPO will appear as having dark granules. The total activity of MPO activity in peripheral blood granulocytes should range between 95-110 points in normal animals and in polluted aquatic environments this value may be reduced to 50-70 points (Sigma Chemical Company 1991; Siwicki 1989; Siwicki and Anderson 1993).

Lysozyme activity assay in serum and organs

Lysozyme is an important enzyme in blood involved in active lysis of bacteria. This enzyme attacks specifically structures containing muramic acid and has also been reported to have antiviral and antiparasitic properties. The presented assay is based upon the lysis of the lysozyme-sensitive Gram-positive bacterium, *Micrococcus lysodeikticus,* which is obtained freeze-dried from major chemical suppliers. Lysis can be measured either turbidimetrically or in agarose plates. For convenience, accuracy and sensitivity of the turbidimetric assay is preferable in comparative immunotoxicology (Siwicki and Studnicka 1987; Siwicki et al. 1993).

Ceruloplasmin and CRP activity assay in serum

Ceruloplasmin is a blue alpha-2-globulin and C-reactive protein are enzymes of "acute-phase protein". The acute phase response is the reaction of the animal to disturbances in its homeostasis caused by infection, tissue injury, neoplastic growth or immunological disorders. These responses are in turn associated with production of cytokines and other immunological mediators. The mediators activate receptors on different target cells leading to a systemic reaction. Many quantitative methods for ceruloplasmin and CRP in plasma or serum have been proposed. The ceruloplasmin (p-phenylenediamine: PPD oxidase) assay procedure described herein has been so evaluated, and a very strong positive linear correlation between the total copper content standard serum and the PPD oxidase activity exist (Rice 1986).

Total protein and immunoglobulin (Ig) levels assay in serum

The study of fish immunoglobulin has provided the foundation of numerous investigations involving the phylogeny and ontogeny of the fish immune system and detailed characterization of the fish immune response. More recently, fish immunoglobulin has been used in assessing the specificity of the immune response in disease resistance, studying the mechanisms of antigen uptake and processing and evaluating the effects of toxicants and immunomodulators on the humoral immune response. Isolation and characterization of immunoglobulin from fish has been the subject of a number of investigations. A variety of techniques for separation and purification of fish immunoglobulin have been used and have included precipitation in saturated ammonium sulfate, separation by molecular sieving, and ion exchange chromatography. All applied techniques for classification of the class of immunoglobulin showed that in fish only one class, IgM exists.

The technique described here utilizes a spectrophotometric technique previously developed for the study of total immunoglobulin levels in serum or plasma. Analysis of total Ig level in plasma is based on the biuret colorimetric method. However, by following methods, first the immunoglobulins are separated from plasma by precipitation with polyethylenic glycol 10,000 (Sigma) and remaining supernatant is read. That number is subtracted from the total protein to give total immunoglobulin. Protein readings from supernatant will give the amount of protein taken out by absorption to polyethylene glycol. To calculate total immunoglobulin, subtract these readings from total protein on individual samples. Compare with standards for calculation of protein (Siwicki and Anderson 1993; Siwicki et al. 1993; Siwicki et al. 1994).

Other methods

Other techniques for analyzing the effects of xenobiotics on the defence mechanism were used in our immunotoxicological study: ELISPOT assay for quantification of antibody secreting cells and lymphocyte proliferation (LP) assay for study the lymphocyte activity (Siwicki and Dunier 1990; Siwicki and Dunier 1993; Siwicki et al. 1993). In our experimental study, we observed that xenobiotics and other aquatic pollutants increased the lymphocyte T and lymphocyte B activity, and the antibody secreting cells levels in fish (Dunier and Siwicki 1994).

Nové směry ve srovnávací imunotoxikologii

Zvýšené koncentrace xenobiotik v prostředí, potravě i vodě mají přímý i nepřímý vliv na aktivitu imunokompetentních buněk, buněčné a humorální obranné mechanismy a odolnost proti nemocem u zvířat i lidí. Proto je nutné rozvíjet srovnávací aspekty imunotoxikologie při využití bezobratlých, obojživelníků i plazů jako modelů. Parametry obranných mechanismů mohou být využity při různých laboratorních i terénních experimentech k analýze vlivu xenobiotik na obranné mechanismy a odolnost vůči nemocem u savců, ptáků, obojživelníků a u ryb. Řada standardních imunologických technik (metod) byla modifikována pro dosažení optimálních výsledků. V předkládané práci jsou prezentovány základní imunologické techniky a metody pro studium působení xenobiotik na buněčné a humorální obranné mechanismy u ryb.

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