IN VITRO IMMUNOTOXICOLOGY - PRACTICAL APPLICATION

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Received July, 1998
Accepted September, 1998

Abstract

Siwicki A. K., M. Morand, M. Studnicka: *In vitro Immunotoxicology - Practical Application.* Acta vet. Brno 1998, 67: 303-307.

The *in vitro* immunology is one of the most important directions in immunotoxicology. At present, many *in vitro* methods are used in control drug discovery, agents for chemotherapeutics and for monitoring of environmental contamination. In this paper, we present various methods which could by used for examination of the influence of different environmental chemicals and other xenobiotics on the immunocompetent cell activity in fish. The chemiluminescence response assay is important for study the phagocyte activity and the proliferative response assay of lymphocytes stimulated by mitogens is very important method for study the effects of xenobiotics on the lymphocyte T and B activities. We also demonstrate that *in vitro* immune response assay analyzed by PFC and ELISPOT are very sensitive techniques for studies of the effects of xenobiotics on the antibody secreting cells.

Fish, immunotoxicology, in vitro techniques

Information available on the role of the immunocompetent cells and immune mechanisms in the manifestation of toxicity by various chemicals is inadequate. More experimental studies need to be performed, particularly when the chemicals can produce allergic sensitization or may lead to autoimmune diseases. Immune suppression is sufficiently important to toxicology work and more studies relating dose and effect need to be performed by *in vitro* and *in vivo* examinations. The immunosuppressive potential of chemicals, especially environmental chemicals, are of great importance if the effects occur not only directly on the health. Predicting the immunotoxicological effects of chemicals requires more information. Defining the mechanisms of action, the structure-activity relationships and influence on the immunocompetent cells await additional data (Dunier and Siwicki 1993ab; Siwicki et al. 1990).

One of the most important directions in toxicology is *in vitro* immunotoxicological studies. Today many *in vitro* methods are very important in development of product, drug discovery, testing of chemotherapeutic agents and natural or synthetic immunomodulators and for monitoring of environmental contamination (Anderson et al. 1989; Anderson et al. 1986; Chan et al. 1991; Cossarini-Dunier et al. 1990; Dean 1972; Dunier et al. 1991; Elsasser et al. 1986; Fisher and Mueller 1981; Holsapple et al. 1986; Roux et al. 1979; Siwicki et al. 1989; Siwicki and Dunier 1994; Urban and Jarstrand 1986)

This article will review briefly the application of various *in vitro* methods to study the influence of different environmental chemicals and xenobiotics on the immunocompetent

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cells in fish - a very important aquatic bioindicator and model for experimental immunotoxicological investigation.

Chemiluminescence assay (CL)

The phenomenon of light emission or chemiluminescence (CL) during phagocytosis was originally described by Allen et al. (1976). Bacteria and latex bead particles that were opsonized elicited CL when they were phagocytosed by polymorphonuclear (PMN) cells. Chemiluminescence, measured in a liquid scintillation counter operated in an out-of-coincidence mode, was correlated with the number of cells, the concentration of the stimulus and the HMPS activity. Modification of the CL assay by addition of luminol, a cyclic hydrazide that can be oxidized to emit light, greatly amplified the sensitivity of the assay. Because the CL response represents the bactericidal mechanisms during the respiratory burst, it is widely used for studying phagocytosis. A correlation between the bactericidal activity and the CL response was shown by Welch (1980). Chemiluminescence has been demonstrated from phagocytes of different animals including humans, bovine, dogs, mice, rabbits, rats, pigs, fish and oyster.

Many substances elicit a CL response from phagocytes (Van Dyke 1987; Welch 1980; Wishkowsky et al. 1987; Zimmer and Jones 1990). The most common stimuli are zymosan, a yeast extract, latex beats, PMA, a soluble tumour-promoting compound, many bacteria and bacterial particles, and other soluble and insoluble activators (Van Dyke 1987). The CL assay provides information regarding the health of an organism because the assay measures changes in phagocytosis, which affects the susceptibility of an individual to pathogenic agents. The most attractive features of the CL assay are objectivity, sensitivity, simplicity and rapidity.

Experiments to study the effect of environmental contaminants, chemicals and drugs on fish phagocytes have been carried out by applying the CL response. Wishkowsky et al. (1987) showed suppression of CL responses of phagocytes exposed to tetracycline *in vitro*. The oxytetracycline dosage recommended for aquaculture suppressed the CL by approximately 50 %. Elsasser et al. (1986) employed for the first time CL responses assay for *in vitro* study of the influence of heavy metals on phagocytosis in fish. Copper caused a decrease in the emission of the light to the baseline level. Aluminum also caused a partial suppression, whereas cadmium enhanced the CL response when added within 1 h prior to the assay or caused variable results after a 24 h exposure. Preliminary studies to compare the CL response of cells obtained from normal and pollutant-exposed fish exhibited a decrease in the response of the exposed fish (Warinner et al. 1988).

The CL responses assay were used for *in vitro* and *in vivo* studies of the effects of aquatic environmental contaminants on the phagocytic activity of polymorphonuclear (PMN) and mononuclear (MN) cells in fish (Dunier et al. 1994; Dunier et al. 1995).

Lymphocyte proliferation assay (LP)

The mechanisms for the immunotoxicity of heavy metals, pesticides and other xenobiotics are still debated owing to the fact that the numerous papers that previously dealt with this issue, frequently reported on *in vitro* examinations conducted with irrelevantly high concentrations. Therefore, future research efforts are still warranted to ascertain the *in vivo* relevance of these findings from a immunotoxicological point of view, with levels of exposure closer to those of environmental contamination. Because fish is one of the important bioindicators and frequently exposed to many pollutants in their aquatic environment, their immunocompetent cells may also become compromised by environmental contaminants.

The influence of chemicals and other xenobiotics on the lymphocyte activity have been investigated in an *in vitro* study. The mitogen responses of lymphoid cells subpopulations demonstrated according to their selective responsiveness to different mitogens i.e. LPS (B lymphocyte mitogen) and ConA or PHA (T lymphocyte mitogens). Most proliferation assays were modified and adapted for *in vitro* studies. Dunier et al. (1991) prepared *in vitro* protocol for study the effects of xenobiotics on proliferative response of lymphocytes in fish.

In vitro immune response assays

The *in vitro* techniques have many advantages for testing the effects of chemicals, pollutants and drugs on the cell-mediated immunity and specific immune response before more extensive *in vivo* examination is initiated.

The purpose of our studies was to investigate the possibilities of using *in vitro* assays to demonstrate the influence of environmental contamination on the nonspecific and specific immune responses. Many promising drugs have yet to be approved for use in humans and animals, and preliminary investigations might be used for screening *in vitro*. In addition, this is a promising techniques for studying the effects of pollutants and aquatic contaminants on the immune response in fish.

In immunotoxicological studies, two methods for determination of antibody secreting cells (ASC) are used: Passive Hemolytic Plaque assay (PHPA) and ELISPOT assay.

The passive haemolytic plaque assay is the most widely used test to assess antibody-secreting cells and to analyze the regulatory mechanisms of antibody production by B lymphocyte in fish. Jerne and Nordin (1963), the first to use the direct hemolytic plaque assays in mice injected with SRBC, were awarded the Nobel prize for developing this assay. In the test, the antibody-producing cells are held suspended in agar and centered in plaques or clear areas where the released antibody and complement have lysed the surrounding antigen-labeled red blood cells. Based upon these characteristics, the antibody-producing cells (APC) are plaque-forming cells (PFC), and both those abbreviations are used interchangeably in the literature. This techniques has been used after *in vitro* immunization to compare bacterin doses (Anderson et al. 1986; Anderson et al. 1983; Siwicki et al. 1990) and to analyze the effects of chemicals, drugs and xenobiotics on the specific immune response mediated by several effector cells (Anderson et al. 1989; Anderson et al. 1989; Siwicki et al. 1989).

Recently, a new assay has been developed which utilizes solid-phase immunoenzyme technology and is named the ELISPOT assay (Siwicki and Dunier 1993; Siwicki et al. 1994). This assay can now be done in 96 well microtiter plates (25) enabling a large number of replicates to be performed and has recently been applied to *in vitro* and *in vivo* studies for quantification of antibody secreting cells after contamination by chemicals, drugs or xenobiotics. Dunier and Siwicki (1994) applied ELISPOT assay for immuno-toxicological studies to quantify the total and specific ASC. The quantification of cells secreting specific antibody to pathogenic agents after incubation a cell suspension or organs (spleen, head kidney) with contaminants is possible with the ELISPOT assay. The use of the monoclonal anti-trout Ig or anti-carp Ig is well suitable and yields countable blue spots (Siwicki and Dunier 1993).

The development of the *in vitro* immunotoxicological assays is a very important part of monitoring of aquatic environmental contamination. Application of these methods has marked advantages over using *in vivo* models:

- fish requiring low water temperature are often more difficult to hold for long periods for testing effects of pollutants or drugs; the *in vitro* immunization models can be held optimally at 14 °C or at room temperatures,
- most experimental fish are not from inbred populations, therefore the immune response in highly variable among individuals. Replicate sections from the immunopoietic organs of the

same animal, however, show more homogenous responses *in vitro* and differences due to immunomodulators can be indicated more accurately,
- timing of when the immune assay is done can be regulated in tissues from poikilothermic animals by temperature regulation. For instance, the assays could be delayed by holding the tissues at colder temperatures,
- fewer fish are used as the immunopoietic organs can be divided.

The comparative immunotoxicologists expect the *in vitro* immunization and other techniques for fish to become important in future fish toxicology studies as they are being developed for mammalian models. Holsapple et al. (1986) used immunopoietic cell suspension to study immunosuppression by dioxins; we have had less success using suspensions and have continued to use the organ section method. Differences in timing and doses of immunomodulators in relation to immunogens will require many experimental runs before these drugs and contaminants can be predictive of duplicating field situations, however, the applications and benefits of *in vitro* immunization assays are numerous.

Imunotoxikologie in vitro - praktické využití

Imunotoxikologie *in vitro* je jedním z nejdůležitějších směrů v imunotoxikologii. V současné době řada metod *in vitro* je používána při posuzování nových léčiv, chemoterapeutik a při monitoringu zatížení životního prostředí. V předkládané práci jsou prezentovány různé metody, které mohou být využity při sledování vlivu různých chemikálií a dalších xenobiotik na aktivitu imunokompetentních buněk u ryb. Chemoluminiscenční metoda je důležitá pro studium fagocytární aktivity a metoda využívající proliferaci lymfocytů stimulovaných mitogeny je velmi důležitá pro studium působení xenobiotik na T a B lymfocyty. Také je demonstrováno, že *in vitro* imunologické metody analyzované pomocí PFC a ELISPOT jsou velmi citlivými metodami pro studium působení xenobiotik na buňky produkující protilátky.

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