Phagocytosis as a Biomarker of Immunotoxicity in Wildlife Species Exposed to Environmental Xenobiotics

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SYNOPSIS. In the present paper, we are reviewing experimental evidence demonstrating that phagocytic cells, such as macrophages, may be used as a biomarker of immunotoxicity in wildlife studies. We will first present data obtained after exposure in vitro with selected chemicals showing the comparative sensitivity of phagocytic cells from different species. These results demonstrate that, at least for metals, each species produce a similar shaped dose-response curve, although considerable interspecies sensitivity is evident. These results also demonstrate the sensitivity of the phagocytic activity, suggesting indeed that this function could be used to monitor exposure to chemicals. The similar shaped dose-response curves imply that mechanisms of action may also be similar. Furthermore, based on the relative species sensitivity, sentinel species could be selected for field monitoring. Such an approach may also be useful to establish correction factors required to extrapolate results between species. This sensitivity of the phagocytic activity of macrophages will be further under controlled conditions in laboratory animal models. Finally, the reliability of this approach will be demonstrated using case studies with wildlife species.

INTRODUCTION

In the past 20 years, there has been a growing awareness in industrial, academic, regulatory and public sectors concerning the health risks associated with the exposure to persistent toxic chemicals, especially those affecting the endocrine system. These pollutants, collectively referred to as endocrine disruptors, include compounds from various chemical classes (heavy metals, polychlorinated biphenyls, dioxins, bisphenols, etc.) (Colborn and Clement 1992). There is an impressive body of evidence demonstrating that these chemicals can severely impact on health when exposures occur, even at low doses, during sensitive periods of embryonic development (Bernier et al., 1995; EPA, 1995; Foster, 1995; Johnson et al., 1997). There is a general consensus that these compounds may cause, directly or indirectly, detrimental effects on four main physiological systems including the endocrine, reproductive, nervous and immune systems (Allen et al., 1994).

Exposure of adult animals to toxic environmental contaminants such as organochlorine compounds or heavy metals can lead to immunosuppression. Using laboratory models such as rodents, this chemically-induced immunosuppression has been associated with decreased humoral and cellular responses, by compromised lymphocyte proliferation, and by decreased thymic weight (Bernier et al., 1995; Kerkvliet, 1995; Krzysztyniak et al., 1995; Tryphonas, 1995; Wong et al., 1992). In addition, a number of in vitro tests, in which the toxic substances were in direct contacts with the cells, have shown that phagocytosis, mixed lymphocyte culture, lymphocyte prolifera-
tion in response to mitogens (Omara et al., 1997, 1998), plaque forming cell and cytokine receptors were severely reduced (Payette et al., 1995), indicating that both the afferent and the efferent phases of the immune response can be affected. For many chemical exposures, immunosuppression was correlated with decreased resistance to infections using parasite, bacterial, viral or tumoral models (reviewed by Fournier et al., 2000). Indeed, this issue about the correlation between immunomodulation and the capacity of the exposed animals to develop an appropriate immunity is under scrutiny. Indeed, there is a general consensus among scientists involved in immunotoxicology that following the observation of an immunomodulation, host resistance models should be performed as a routine assay.

The immune system is integrated into the organism and interacts intimately with the function of many organs and organ systems. Moreover, this system is extremely vulnerable to injury by xenobiotics. Although major changes in the immune system are rapidly expressed in significant morbidity and even mortality of the organisms involved, they are often preceded by subtle changes in some of the components of the immune system, which could be used as early indicators of immunotoxicity or as biomarkers (Fournier et al., 2000; Brousseau et al., 1997; Dean and Murray, 1990). This aspect has stimulated great interest because these effects generally occur at levels that are lower than those associated with acute toxicity (Brousseau et al., 1997; Koller and Exon, 1985). In terms of indicators, the use of one of the key function of the phagocytic cells, the phagocytosis, seems a priori, very promising for an in situ application. Indeed, monitoring immune competence, although more sensitive, usually requires the use of protocols that are not extensively adapted presently to the contingencies of field work (i.e., exposure to a specific antigen at least one week before the biomarker is measured). Moreover, phagocytic activity is a well conserved function maintained throughout the evolution and therefore is present in all living species. In more evolved species this function is also central in the development of more complex immune responses such as humoral and cellular mediated immunity.

For the majority of the species of concern, phagocytic cells can be collected from peripheral blood or circulatory fluid using non-invasive techniques. The main activities of these cells are phagocytosis of foreign particles or production of biocide molecules, and these functions can be assessed using standardized methodologies in a wide variety of species (Brousseau et al., 1999).

In the present paper we will review experimental indications showing that phagocytic cells, such as macrophages, may be used as biomarker of immunotoxicity in wildlife studies. We will first present data obtained after in vitro exposure with selected chemicals showing the comparative sensitivity of phagocytic cells from different species. This sensitivity will be further evaluated using controlled exposure in laboratory animal models. Finally, reliability of the approach will be demonstrated using case studies with wildlife species.

Comparing the sensitivity of the immune system of different species to environmental contaminants is an important consideration in order to identify species that may be the most at risk. The complexity of the immune system of different species also represents an important consideration as certain species rely more heavily on phagocytic cells than humoral components for their resistance to pathogens. In the course of our studies we have used both in vitro and in vivo approaches to study differential sensitivity of immune functions to environmental contaminants. These have allowed us to predict and identify species and classes of both vertebrates and invertebrates that may be at risk following exposure to immunotoxicants.

**Controlled In Vitro Exposure**

Phagocytic cells are highly conserved throughout evolution and by using the phagocytic activity of these cells it has been possible to address the comparative sensitivity of these cells to environmental contaminants in a variety of aquatic animal species. As a mean of comparison we have decided to use three species of mollusks,
three species of fish and one amphibian species.

In order to assess the comparative sensitivity of phagocytosis from different aquatic species, phagocytic cells were collected from different individuals and exposed in vitro to various chemicals including a selection of heavy metals. Species used in the study were the clam (Mya arenaria), the blue mussel (Mytilus edulis), the American plaice (Hypoglossoides platessoides), the mummichog (Fundulus heteroclitus), the rainbow trout (Oncorhynchus mykiss) and one amphibian (Xenopus laevis). For bivalves, phagocytic cells or hemocytes were collected from hemolymph. For fish, pronephric cell suspensions were prepared and for amphibian splenocytes were used.

The metals tested were: mercuric chloride, methylmercury chloride, cadmium chloride, zinc chloride, and silver nitrate. Each metal compound was prepared as a 0.1 M solution in distilled water with the exception of methylmercury chloride which was first dissolved in ethanol and diluted in distilled water. The purity of the metal compounds was ≥99.0%. Working solutions for each metal compound ranged from 10^{-2} to 10^{-8} M allowing an exposure range from 10^{-3} to 10^{-9} M.

Aliquots of each cell suspension were prepared at a concentration of 1 × 10^6 cells/ml. Cells were exposed to various concentrations of metals for 18 hr, the optimum duration of the phagocytic activity monitoring assay. As antigen, yellow-green latex FluoSpheres were added to the cell suspensions, containing various dilutions of the metals (as described above), at a ratio of 100:1 (beads: cell). The mixtures were incubated in optimal conditions for each species. Afterward, an aliquot was removed from each of the mixtures, layered over a 3% bovine serum albumine (BSA) gradient and centrifuged at 150 × g for 8 min at 4°C to remove the free beads. The cell pellets were resuspended in 0.5 ml of 0.5% formalin in hematall. A FACSscan cytometer (Becton Dickinson, San Jose, CA) with an air cooled argon laser providing excitation at 488 nm was used to determine the fluorescence of the engulfed beads by the phagocytes. Fluorescent emissions were measured at 520 nm. Phagocytic populations were defined based on their forward and right angle scatter properties. A total of 10,000 events were acquired for each sample which were stored in the list mode data format and analysed, once displayed as two-parameters, complexity and cell size, in order to define a gate for fluorescence analysis performed as a frequency distribution histogram. Data collection and analysis were performed with a Consort 30 system and LYSIS-II program (Becton Dickinson). Results are normally expressed as percentage of phagocytic cells or percentage of normal response, the latter obtained with cells exposed to vehicle only. In all of these studies we have used sodium azide treated cells as a negative control. This allows us to differentiate between beads that have been actively phagocytized and those beads adhered to the external plasma membrane.

Figure 1 presents an example of a dose-response curve of phagocytic activity and viability obtained with Xenopus laevis leukocytes exposed to various concentrations of methylmercury chloride. These data indicate that there was no significant difference in cell viability for splenocytes cultured in the presence of methyl mercury at concentrations ranging from 10^{-9} M to 10^{-5} M. However, metal-related cytotoxicity, expressed as a significant decrease in splenocyte viability was noted at 10^{-4} M and 10^{-3} M. The phagocytic activity was not affected by methyl mercury for concentrations ranging from 10^{-9} M up to 10^{-7} M. With increasing metal concentrations phagocytosis was progressively and significantly suppressed.

When several metals are considered, it is possible to compare the sensitivity of exposed cells, as illustrated in Figure 2 with surfclams (Spisula polynyma). At low concentrations (10^{-9} to 10^{-8} M) we observed that all metals, with the exception of silver nitrate seem to have the potential to stimulate phagocytosis. Although this increase is not significant, it is interesting to note that stimulation of immune function by very low metal concentrations is commonly observed (Bernier et al., 1995; Blakley et al., 1980, 1981, 1986, 1978; Cheng and...
Sullivan, 1984; Exon et al., 1984; Otsuka and Ohsawa, 1991). As metal concentrations increase the trend is toward immunosuppression. Indeed at $10^{-5}$ M and higher, both speciations of mercury drastically impaired the phagocytic activity as well as silver nitrate at $10^{-4}$ M and $10^{-3}$ M. These results are similar to those obtained with another bivalve, Mya arenaria (Brousseau et al., 2000).

As shown by these dose-response studies, the sensitivity of surfclam hemocytes to different metals varies considerably. Therefore, the sensitivity for metals was quantified by graphical determination of the concentration for each metal which induced 50% suppression of the phagocytosis (IC50) using dose-response curves as presented in Figure 2. These data are presented in Table 1. Methylmercury chloride was the most potent inhibitor of phagocytosis with lowest IC50 followed by silver nitrate and mercuric chloride. Cadmium chloride and zinc chloride were the least immunotoxic with respect to phagocytosis and showed similar IC50.

When the same protocol was applied to different species, it became possible to compare species sensitivity to various chemicals. As an example results with methylmercury chloride are presented in Table 2. These data showed interestingly that the...
two species of fish and *Xenopus* are the most sensitive species with methylmercury with similar IC50.

**Controlled in vivo Exposure**

In order to validate the use of phagocytosis as biomarker, a series of experiments was undertaken in which animals were exposed to chemicals or mixtures of chemicals under laboratory controlled conditions or in their natural environment.

For rodents, phagocytic activity was found to be quite sensitive to most of the known toxicants (Wong *et al.*, 1992; Fournier *et al.*, 2000). In wildlife species such as the trout, suppression of the phagocytosis was in direct correlation with the known toxicity of chemicals whether it was heavy metals (Sanchez-Dardon., 1999), pulp mill effluents (Voccia *et al.*, 1997) or more complex chemical mixtures (Omara *et al.*, 1997, 1998).

Similar sensitivity to chemicals was obtained when test animals were free ranging. For instance, clams, surfclams and American plaice were exposed for a period of three weeks to contaminated marine sediments collected from St.Lawrence River. The contamination level and the basic toxicity of these sediments have been well characterized by our group (Lee *et al.*, 1999). After the exposure, hemocytes collected from the hemolymph or head kidney macrophages were assessed for their phagocytic competence. The results expressed as percentage of controls (animals exposed to clean sand) are presented in Figure 3. The data shown that the contaminants present in the sediments suppressed at 90% and 40% respectively the phagocytic capabilities of the surfclam and the plaice.

With mollusks, *in vivo* exposure of *Mya arenaria* to methylmercury sampled every week for up to 28 days, has significantly impaired the phagocytic capabilities of hemocytes collected from these animals. Quite interestingly, we observed a good correlation ($R^2 = 0.8226$) between the accumulation of methylmercury, as determined by total mercury body burdens, and lower hemocyte phagocytic activity. (Fig. 4). These data allow us to assume that it is the accumulation of methylmercury which is responsible for the depressed immune function and not simply the result of an increased stress response of the organism.

**Field Monitoring**

Observations of suppression of phagocytic activity in relationship to exposure to chemicals obtained with free ranging aquatic species are still quite limited. An exception is the case of the mummichogs exposed to pulp mill effluents for which significant suppression of phagocytic competence of head kidney macrophages was noted. In this...
study male and female mummichogs were sampled along the Miramichi River in New Brunswick (Fournier et al., 1998). The river contains large pulp mill which discharges its treated effluent into the river. We have observed that pronephric macrophages from mummichogs sampled near the effluent discharge have significantly lower activity than those sampled either further downstream or in a fish sampled in the Boucto-uche River which is located at the same latitude of the Miramichi River but does not receive pulp mill discharge. Interestingly, we observed a sexual dimorphism in the response of mummichogs exposed to the pulp mill effluent in the Miramichi River. Indeed, we observed that immune cells from female mummichogs were much more impaired with respect to phagocytic activity than their male counterparts (Fournier et al., 1998). This highlights an important consideration for field studies which is to establish whether or not gender differences can bias data collected in wildlife species.

Field monitoring studies using clams as sentinel species have also been done. An example of these studies are those involving the collection of soft shell clams along the Saguenay River (Blaise et al., 1999). These studies were able to demonstrate that cadmium accumulation in these bivalves from the river could result in significant immunotoxicity. Furthermore, these studies indicated that seasonal differences played an important role in modifying the immunotoxicity of contaminants from the Saguenay River. Together these examples demonstrate not only the important of gender differences but also seasonal factors which can contribute to modifying the immunotoxicity of chemicals and the immune response of organisms exposed to these toxicants.

**DISCUSSION**

Using phagocytosis with the *in vitro* approach we could observe that phagocytic cells from *Xenopus* showed at least two different patterns of the metal-specific and concentration-related toxicity within the range of concentrations selected. A first pattern could be observed for $10^{-6}$ M and $10^{-5}$ M; while mercury did not show any sign of toxicity, the phagocytosis was impaired and quite severely at $10^{-5}$ M. The second pattern was observed at a higher concentration at which increased toxicity, expressed as decreased cell viability, was noted in parallel with an impairment of splenocyte phagocytic activity.

Moreover, amongst the metal compounds studied, our results clearly show that the sensitivity of lymphoid cells varies considerably with the metals and also between species. Quantification of the sensitivity by using IC50 indicates that methylmercury chloride was the most potent inhibitor of phagocytosis. So far, there is no evident pattern to the variation in sensitivity among species. However, at least for heavy metals, there are some indications that aquatic species are more sensitive than terrestrial species and among those, the fish and the frog are the most sensitive. Differential sensitivity in manifestation of immunotoxicity towards heavy metals has been already demonstrated (Lawrence, 1985; Fugère et al., 1996). However, the immunotoxicity mechanism remains unexplained and further work is required.

Using phagocytosis with the *in vivo* approach following single or multiple exposures through sediments, water column or effluents, our results revealed that variation in the sensitivity of species and among sexes could be detected also. Another key issue in the field of immunotoxicology today remains in the establishment of the relationship between the amplitude of the immunomodulation obtained and the body bur-
den. In the present study using a bivalve model, our assay was sensitive enough in order to establish a very good correlation between the suppression of the phagocytosis and the body burden of mercury.

The responses of immunological biomarkers are sometimes contradictory since, for a given contaminant, the effects can range from almost total immunodepression to immunostimulation, including no effect, depending on the species studied, the mode and duration of exposure, the test protocol used, etc. (Sharma, 1981; Koller and Exon, 1985). This variability in responses is the result of parameters characteristic of each species as well as the complexity of the immune system, even though the standardization of protocols and the consideration of different forms of stress limits it to a certain extent (Weeks et al., 1992). Despite an apparent diversity, there are a certain number of analogies (immunocyte involvement, for example) between the immune systems of different groups of animals, including invertebrates and vertebrates. Regardless of the species considered, and the route of exposure used, it is logical to think that an efficient immunological biomarker should be accurate enough to detect immunomodulation whatever the protocol used and moreover should reveal harmful effects for exposure levels that are clearly below those that produce acute toxicity.

Moreover, the use of nonspecific immunity biomarkers (phagocytic cells, NK activity, etc.) seems, a priori, more promising for an in situ application because these assays could be adapted quite easily to the contingencies of field work and more importantly they do not require any sensitization of animals which is a critical issue when we work with wildlife species. Functional aspects of macrophages have already been proposed to be used as biomarkers to follow immunotoxic chemicals (Weeks and Warinner, 1984). Weeks et al., (1986; 1987a, b) have shown that exposure of fish to PAHs in their natural environment, as well as in the laboratory, have resulted in significant but reversible changes in various functions of the macrophages. Among these functions we can cite microorganism phagocytosis, chemotaxis, pinocytosis and melanin accumulation. These functions were either inhibited or stimulated, depending upon the species considered. Cossarini-Dunier et al., (1988) have reported that exposures of carp to manganese have stimulated the phagocytosis, while inhibition of this function has been reported in rainbow trout (Oncorhyncus mykiss) exposed to lindane (Dunier et al., 1994; Siwicki and Dunan 1994a).

So far, these results have confirmed that phagocytosis should be considered as a very good immunological biomarker at least for heavy metals for its capability to demonstrate immunomodulation in a variety of circumstances. Moreover, if we use the calculation of the IC50 it gives us the possibility of classifying the relative toxicity of xenobiotics as well as the relative sensitivity of species, allowing us to identify sentinel species that could be selected for field monitoring and species that are at risk because of their high sensitivity towards xenobiotics.

REFERENCES


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