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Cell-Mediated Immunity and Its Application in Toxicology

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A variety of *in vivo* and more recently *in vitro* assays have been described to assess cell mediated immunity (CMI). Two methods routinely employed in our laboratory to assess CMI following exposure to chemicals in rodents include delayed hypersensitivity and *in vitro* lymphoproliferation. Preliminary studies indicate that depressed delayed hypersensitivity responses, as performed by a radiometric assay, correlates with altered susceptibility to infectious agents and tumor cell challenge following exposure to immunotoxic chemicals. Furthermore, suppression of T-cell lymphoproliferative responses to at least 50% below control values correlated with depressed delayed hypersensitivity responses and altered host susceptibility. On the other hand, when suppression of T-cell lymphoproliferative responses are within 50% of control values, delayed hypersensitivity and host susceptibility parameters are not affected. Assuming adequate technical expertise and accurate data interpretation, CMI assays of these types can provide a valuable data base for toxicology studies and immunotoxicity assessment.

Introduction

Reports indicating that exposure to various environmental pollutants leads to immunological alterations in laboratory animals and even man has raised concerns regarding sensitive methodology for routine assessment of immunotoxicity (*1*, *2*). One concern in assay battery development is the use of appropriate models to examine cell-mediated immunity (CMI). In recent years, large numbers of *in vivo* and more recently *in vitro* assays have been developed to assess CMI. To insure accurate evaluation an *in vivo* assay, which represents a holistic approach, and a more defined *in vitro* assay, for CMI should be employed. Routinely, CMI is assessed in our laboratory by quantitating *in vivo* delayed hypersensitivity responses using a radiometric technique and *in vitro* lymphoproliferative responses following chemical exposure (*3*). These techniques and their relationship to other aspects of immunity (i.e., host susceptibility, humoral immunity and the reticuloendothelial system) are examined. Considerations in selecting appropriate CMI assays include sensitivity, simplicity and extrapolation of dose-response curves from effect to no-effect levels as well as from rodent model systems to humans.

Assays for Cell-Mediated Immunity

CMI is evoked by T-lymphocytes, although macrophages and to a lesser extent polymorphonuclear leukocytes (PMNs) play an active role in these reactions (*4*). CMI is responsible for classical delayed hypersensitivity reactions (DHRs), rejec-

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Table 1. Assays commonly employed to assess cell mediated immunity.

In vivo
Delayed hypersensitivity
Skin grafts
Graft vs. host
In vitro
Lymphocyte proliferation
Mitogens
Specific antigens
Mixed lymphocytes
Enumeration of lymphocyte subpopulations
Antigenic determinants on surface membrane
Rosette formation
Complement and Fc receptors
Mediator production by lymphocytes
Migration inhibitory factors
Lymphocytotoxicity
Immune interferon
Assays for polymorphonuclear leukocytes and macrophages
Phagocytosis
Chemotaxis
Cytostasis
Chemiluminescence

tion of transplants, tumor immunity, some autoimmune diseases and resistance to facultative intracellular parasites as well as many recurrent infections. In general, *in vivo* assays predominantly measure the effector mechanism of CMI, while *in vitro* assays may evaluate cellular components. A number of these assays appear to represent a sensitive and reliable indicator of immunotoxicity and will be discussed in some detail.

Table 1 lists some of the more common techniques employed to assess CMI in both humans and laboratory animals and have been discussed in detail elsewhere (*5*). These assays are, in general, fairly quantitative and capable of detecting either depression or augmentation. Despite the recent development of *in vitro* techniques, delayed hypersensitivity testing remains the most widely accepted means of assessing CMI. Other *in vivo* measures that have been used to assess CMI in laboratory animals, include graft vs host and skin graft assays (*2*). More recently, a number of *in vitro* assays have been developed to assess CMI (*5*). The microculture lymphocyte proliferation (LP) assay utilizes T- and B-cell mitogens (usually plant lectins or bacterial products), specific antigens or alloantigens in a one-way mixed leukocyte culture (MLC) to examine proliferative function in lymphocyte populations (*6*). The assay provides a relatively simple, reproducible and semiquantative *in vitro* correlate for CMI. Various methods have been used to enumerate lymphocyte subpopulations in peripheral blood of humans and spleens of mice (*7*). Enumeration, alone, however, does not measure functional capabilities. B-lymphocytes possess readily detectable membrane immunoglobulins (mIgs) on the cell surface which can be detected using fluorescein-conjugated antiserum to the Igs. In addition, B-cells possess receptors for complement and the Fc region of IgG although other cell subpopulations also possess these receptors. T-lymphocytes, on the other hand, can be recognized in some species, excluding rodents, by spontaneous formation of rosettes with sheep erythrocytes (E-rosettes). Additionally, T-lymphocytes can be identified by distinct antigenic markers on the surface membrane (e.g., Thy 1). T-lymphocytes that are antigenically or nonspecifically stimulated, elaborate soluble mediators referred to as lymphokines. The biological function of these lymphokines are measured by a variety of *in vitro* techniques. Migration inhibitory factors retard the migration of macrophages or PMNs. Lymphocytoxicity assays quantitate cytotoxic or cytostatic activity elaborated when sensitized T-cells are cultured with specific soluble or particulate antigen. The end point in this type of assay is quantitation of target cell destruction. Immune interferon, another type of lymphokine, is active against a wide range of viruses. Interferon production is readily quantitated by determining reduction of viral plaques in an appropriate tissue culture system.

There are also a number of *in vitro* techniques that specifically assess macrophage and PMN functions. Those assays routinely employed in our laboratory include chemotaxis, phagocytosis, cytostasis, and chemiluminescence (*8*). Chemotaxis reflects the ability of various cells including PMNs, monocytes and/or lymphocytes to preferentially migrate to an increasing chemical gradient. These chemical attractants include components of complement and bacterial products. Phagocytosis of ^{51}Cr-radiolabeled SRBCs is determined on resident and induced adherent peritoneal cells. Adherent cells are also examined for their ability to inhibit the growth of various tumor cells in a microculture growth inhibition assay referred to as cytostasis and is presumably a measure of activation.

Delayed Hypersensitivity Responses

Delayed hypersensitivity responses (DHRs) remain the most widely accepted means of clinical or experimental assessment of CMI. The reaction is initiated by actively sensitized T-lymphocytes which respond specifically to the antigen through the

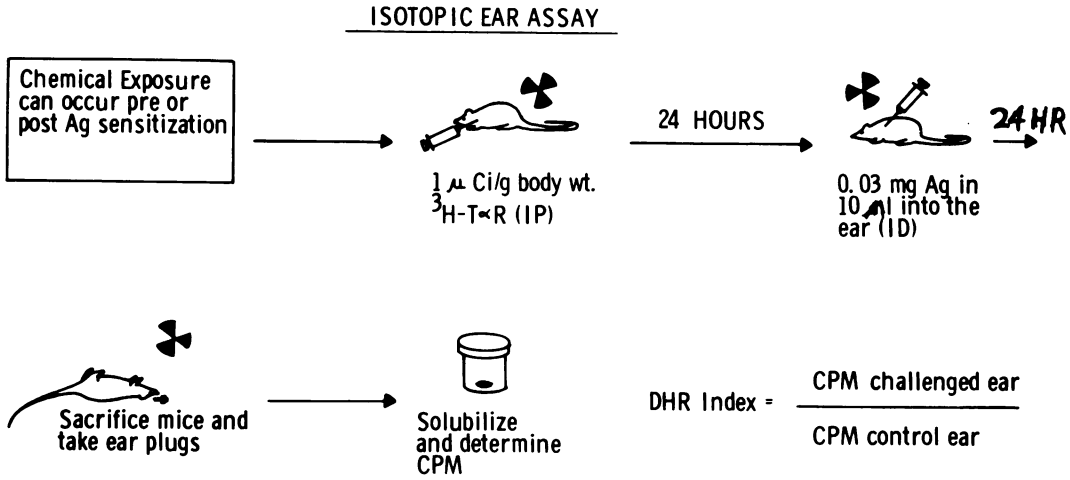

FIGURE 1. Radiometric ear assay for determining DHRs in chemically exposed mice. Mice are sensitized to an antigen, (e.g., 100 μg keyhole lymphet hemocyanin in Freund's adjuvant). Ten or more days following the last sensitization, mice received 1 μCi/g body weight of H-TdR; 24 hr later, animals are given 30 μg KLH in a volume of 10 μl into the pinna of one ear. The other ear serves as a control and is injected with saline. After 24 hr the mice are sacrificed, ear plugs taken, solubilized and radioactivity determined. The data are expressed as an index (CPM Challenged Ear/CPM Control Ear).

release of lymphokines and/or development of specific cytotoxicity without the participation of antibody (*9*). Locally it is manifested by cellular infiltration at the site of antigen administration. In humans, DHRs are measured by the intradermal administration of a panel of antigens to which previous exposure is likely (*10*). The response is determined at the challenge site by measuring the diameter of erythema and induration at 24 and 48 hr. Anergy (reduced DHRs) can be interpreted as positive response to one or less antigens and/or reduced skin reactions. Depressed DHRs, as most recently demonstrated by MacLean (*11*), correlate with decreased host resistance to infectious agents. In these studies 178 patients were serially skin tested following surgery. Of the 135 patients in this study who displayed normal DHRs only 21% developed sepsis and 2% deaths. In contrast, 65% sepsis and 74% mortality occurred in patients demonstrating anergy.

Radiometric assays, which offer greater sensitivity than skin tests, are often employed to assess DHRs in rodents (*12*, *13*). We routinely employ a modification (*3*) of a radiometric ear assay originally described by Lefford (*13*). This method, as performed in our laboratory, is depicted in Figure 1. Administration of tritiated thymidine (^{3}H-TdR) prior to antigenic challenge radiolabels monocyte precursors in the bone marrow. The response is presumably represented by mononuclear infiltration around the reaction site.

In Table 2 the effects of chemical exposure on DHRs, as measured by the radiometric ear assay, are compared to alterations in antibody responses and host resistance. The panel of host resistance assays represented in Table 2 are considered under the influence of CMI and are discussed in detail elsewhere (*14*). Although preliminary, the data in Table 2 indicate that decreased DHRs correlate with increased host susceptibility to artificial infection. DHRs did not correlate with antibody production, since exposure to benzo(a)-pyrene induced severe depression of antibody production without concomitant effects on DHRs. As would be expected, benzo(a)pyrene failed to alter hose resistance. In mice, we routinely employ keyhold lymphet hemocyanin (Pacific Biomarine, Pacific Grove, Calif.) in Freund's adjuvant as an antigen although a variety of sensitizing antigens are suitable including extracts of *T. spiralis*. Good responses can be obtained in rats sensitized with complete Freund's adjuvant (e.g., H37Ra; Difco) and subsequently challenged with purified protein derivative (PPD; Parke-Davis) (*15*). While a variety of antigens can be used to study DHRs, one needs to select an antigen and sensitization schedule that favors CMI and not antibody synthesis. Furthermore, consideration must be given to the time of sensitization in relationship to chemical exposure. Sensitization prior to chemical exposure may be considered an assay for memory cell function, while short-term immunological alterations

Table 2. Relationship of DHRs, antibody responses and host susceptibility in mice following exposure to various chemicals.

Species	Chemical treatments[a]	Total dosage, mg/kg	Antigen[b]	DHR index	Antibody responses[c]	Host resistance[c]
Mice	Mpme	–	KLH	2.93 ± 0.28	–	–
	DES (pre-sens)	8	KLH	2.94 ± 0.33	↓	↓ ↓
	DES (post-sens)	8	KLH	1.88 ± 0.15[d]	↓	↓ ↓
	Cyclophosphamide	180	KLH	1.54 ± 0.11[d]	↓ ↓	↓ ↓
	TCPP	100	KLH	3.13 ± 0.26	–	–
	Benzo(a)pyrene	400	KLH	2.73 ± 0.19	↓ ↓	–
	None	–	*T. spiralis*	3.23 ± 0.28	–	–
	DES (post)	8	*T. spiralis*	1.67 ± 0.23[d]	↓	↓ ↓
Rats	None	–	PPD	5.64 ± 0.65	–	–
	TCDD	0.02	PPD	3.17 ± 0.44[d]	–	↓

[a]All mice were exposed to the various chemicals subchronically at 6-8 weeks of age. Rats were treated pre/postnatally (via maternal exposure) as described previously (*15*).
[b]Abbreviations: DES = diethylstilbestrol; TCPP = tris(1,3-dichloropropyl) phosphate; TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; KLH = keyhole lymphet hemocyanin; *T. spiralis* = an antigen extract.
[c]Code: — = no effect; ↓ = slight to moderate; ↓ ↓ = marked effect.
[d]Significant decreased from control values ($p < 0.01$).

may be missed if sensitization is not initiated until chemical exposure has been completed. Of course, subchronic 30 to 90 day toxicity studies will tend to minimize these types of problems.

Lymphoproliferative Responses

Lymphoproliferative (LP) responses are a widely used correlate of CMI and can be defective in the absence of lymphopenia. In the microculture LP assay, general mitogens (e.g., plant lectins, bacterial products), specific antigens or alloantigens (i.e., mixed lymphocytes) are used to stimulate selectively the cell proliferation of splenic or peripheral lymphocytes (*6*). Proliferation is measured by ^{3}H-TdR incorporation into DNA, represented as CPM. Unlike specific antigens, mitogens are polyclonal activators some of which are capable of tions (see Table 3). In mice, phytohemagglutinin (PHA) and concanavalin A (Con A) are often used to activate T-lymphocytes, while lipopolysaccharide (LPS), isolated from gram-negative bacteria, is used to transform B-lymphocytes (*3*). Depressed LP responses occur in humans with inherited immunodeficiency (primary) diseases as well as various pathological or physiological conditions, including malignancies (Hodgkin's disease, lung cancer), intestinal lymphangiectasia, aging, chronic lymphatic leukemia, nutritional deprivation and in severe stress (e.g., post-surgery) (*6*, *17*).

Depressed LP response in experimental animals with normal numbers of lymphocytes has usually been interpreted as failure of cell activation. Recent studies have indicated that this may occur through tolerance or suppressor substances produced by regulatory subpopulations of macrophages and T-lymphocytes (*6*, *7*). Other factors, however,

Table 3. Lymphocyte activators (mitogens) and their characteristics.[a]

Source	Common examples	Cells transformed
Plant extracts	Phytohemagglutinin	T
	Concanavalin A	T
	Pokeweed mitogen	T or B
Bacterial products	Lipopolysaccharide (Lipid A)	B
	Staph. enterotoxin	T
	Purified protein derivative	B
Antibody reagents	Anti-immunoglobulin	B
	CHO antibodies	T or B
Miscellaneous chemicals	Phorbol ester	T
	Dextran sulfate	B
	Metal ions (zinc, mercury, lead, etc.)	T or B

[a]Modified from Cunningham et al. (*16*).

Table 4. Relationship of lymphoproliferative responses to DHRs, antibody PFCs and host susceptibility, following chemical exposure in mice.

Chemical treatment[a]	Total dosage, mg/kg	Deviation from control, %				Host resistance[b]
		T-Cell mitogen	B-Cell mitogen	DHR	Antibody PFC's	
DES	8	70 ↓	29 ↓	33 ↓	23 ↓	↓ ↓
Cyclophosphamide	180	97 ↓	97 ↓	47 ↓	99 ↓	↓ ↓
TCPP	100	25 ↓	32 ↓	6 ↑	13 ↑	–/ ↓
Benzo(a)pyrene	400	32 ↓	54 ↓	4 ↓	75 ↓	–
TCDD	0.02	53 ↓	18 ↓	60 ↓	ND	↓
PBB	1220	39 ↓	35 ↓	12 ↑	5 ↑	–
o-Phenylphenol	2000	7 ↓	11 ↓	13 ↑	13 ↑	–

[a]TCDD was administered in mice pre/postnatally (via maternal exposure) as described previously (*18*). PBB was given chronically (*19*). All other chemicals were administered as described in Table 2.

[b]Code:— = no effect; ↓ = slight to moderate; ↓ ↓ = marked effect: ND = not done.

should be considered as possibly causing depression of LP responses. Some of these factors include: a chemically induced lymphocytotoxicity as may occur with TCDD (*1*); redistribution of lymphocyte subpopulations (i.e., B-, T- or null cells) as may occur in chronic lymphatic leukemia; and/or an early maturational defect in lymphocyte development since mature lymphocytes respond differently to mitogens than less mature lymphocytes. This is not to say that LP assays are not a reliable predictor of immune alteration but, rather, may prove to be an extremely sensitive indicator of immunotoxicity, if appropriately interpreted.

Table 4 summarizes some recent studies in our laboratories in which LP responses were compared to a variety of immunological and host susceptibility assays in mice following exposure to therapeutic and environmental chemicals. All data reported were obtained in animals administered dosage levels that did not induce overt signs of toxicity. As in Table 2, resistance to the biological agents used to assess altered host susceptibility in these studies are considered primarily under the influence of CMI and not humoral mediated immunity (HMI). While preliminary, certain profiles emerged following examination of these data. As would be expected a large number of chemicals examined (e.g., *o*-phenylphenol) did not effect any host resistance or immune function assays. Other chemicals, such as cyclophosphamide, diethylstilbestrol and 2,3,7,8-TCCD, were clearly immunotoxic, causing greater than 50% depression of LP responses, suppressed DHRs, increased host susceptibility and in most cases depressed antibody PFC responses. In earlier studies these chemicals have been shown to be selectively toxic to the immune system (*1, 8*). Another group of chemicals, such as tris(1,3-dichloropropyl) phosphate (TCPP) and polybrominated biphenyls (PBB), displayed minimal suppression of LP responses, ranging from 25 to 50% below control values, without concomitant effects on other immune parameters or host susceptibility. Although presently speculative, this immune profile may be representative of a general toxicity rather than a selective effect on the immune system. Support for this hypothesis has been provided in collaborative studies with Boorman (*20*). In these studies, mice with depressed LP responses following exposure to TCPP or PBB also revealed decreased hematopoietic stem cell proliferation using *in vitro* spleen and bone marrow colony forming assays. Since these chemicals are biologically active, probably capable of binding to DNA, it would be expected that the more proliferative cell populations (i.e., stem and lymphoid cells) would have a greater predilection to chemical injury. Benzo(a)pyrene appeared selectively to affect B-lymphocytes while sparing CMI, although T-cell LP responses were somewhat affected. This is not a unique phenomenon in immunotoxicology, since exposure to heavy metals under many circumstances induces similar effects (*21*).

In summary, many assays are available to assess CMI following chemical exposure. Since, in general, these assays represent "biological phenomena" one should employ both *in vivo* and *in vitro* assays for proper assessment. Assay selection should be based upon sensitivity, simplicity and extrapolation of dose response curves from effect to no-effect levels as well as from rodent systems to humans. Preliminary studies reported here, which should be interpreted with caution, suggest that a depressed DHR corresponds with increased host susceptibility. On the other hand, while marked suppression of T-cell LP responses correlates with suppressed DHRs, less severe depression does not correlate with other immune or host resistance parameters, but rather may be an early indicator of general toxicity.

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