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Predictive Immunotoxicological Test Systems: Suitability of the Popliteal Lymph Node Assay in Mice and Rats

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ABSTRACT: This article reviews results obtained with popliteal lymph node assays (PLNAs) in rodents and discusses their ability to detect and analyze immunotoxic effects of drugs and other low molecular weight (LMW) chemicals. In its basic form, the PLNA measures activation of the draining lymph node of the hind paw (i.e., the PLN) after injection of a test chemical into the hind foot pad. The assay appears to be appropriate to recognize sensitizing, that is, allergenic and autoimmunogenic, chemicals, as well as nonsensitizing immunostimulatory chemicals. With modifications, PLNAs can detect immunosuppressive chemicals and distinguish sensitizing from nonsensitizing chemicals. Furthermore, modified PLNAs enable detection of known as well as unknown sensitizing metabolites, and may assist in the identification of the self-molecules that act as carriers for chemical sensitization or as targets of chemical-induced autoimmune disease.

Experience with PLNAs shows that they are rapid, reproducible, and objective tests for recognition of sensitizing or otherwise immunomodulating chemicals. Because current protocols of toxicity testing are insensitive in predicting a chemical's potential to result in immunomodulation, PLNAs, when further validated, may provide welcome supplements to routine toxicity screening of chemicals, thus enhancing chemical safety.

KEY WORDS: adverse immune reactions, allergy, autoimmunity, drug metabolites, hypersensitivity reaction, immunotoxicity, local lymph node assays.

I. INTRODUCTION

Immunotoxicity caused by low molecular weight (LMW) chemicals generally results from one of two pathogenically different modes of action:

1. Chemicals or their metabolites exert toxic effects *on* the immune system as the target organ. One or more cell type may be affected or depleted, such as granulocytes, natural killer cells, and lymphocytes. From the point of view of the immunologist, this type of immunotoxicity is nonspecific because it is not mediated by the specific antigen receptors of T or B lymphocytes. Therefore, secondary (anamnestic) responses to the etiologic agent fail to occur. Immunotoxic effects of this type cause a general dysfunction of immune responsiveness, most often immunosuppression, but nonspecific immunostimulation also may ensue.
2. Chemicals or their metabolites elicit specific reactions *by* the immune system. By definition, these reactions are mediated by the specific antigen receptors of B and/or T cells. Only this mode of action leads to sensitization, that is, the expansion of chemical-spe-

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cific memory lymphocytes, which mediate secondary responses upon reexposure to the chemical. These adverse immune reactions, sometimes termed hypersensitivity reactions, may lead to allergy and/or autoimmunity.

Both types of immunotoxicity have been reviewed extensively elsewhere.[18,23,27,29,39,51,53,75,80,114] This review emphasizes the second above-defined category of immunotoxic effects. We discuss the properties and detection of sensitizing LMW chemicals because such chemicals, with the exception of contact sensitizers, are not yet reliably recognized by the current toxicity testing procedures, despite their being among the most frequent causative agents of side-effects of medication, occupational exposure, and other types of exposure.

Adverse immune reactions to drugs and other LMW chemicals may either be confined to specific target organs or affect many organs and tissues, thus inducing systemic immunopathological alterations. Within a given target tissue, the type of immunopathological alteration may vary. For instance, within the lympho-hemopoietic system, adverse immune reactions to chemicals encompass a spectrum of pathological alterations that range from immunostimulatory alterations, such as lymphoid hyperplasia, hypergammaglobulinemia, and systemic lupus erythematosus (SLE), to immunosuppressive alterations, such as hypogammaglobulinemia and cytopenias.[23,29,32,38,47,51,105,114,115]

Except for contact sensitizers, which are dealt with in more detail in Section V, recognition of the potential of chemicals to cause adverse immune reactions is problematic. This is not surprising if we consider that in humans with manifest immune-mediated disease, demonstration of the involvement of a particular chemical can be difficult. Usually, only a certain percentage of the total population exposed to a given drug or chemical develops adverse immune reactions. Moreover, if they become manifest, the reactions often run a transient course, show a putative independence of dose and duration of exposure, and may vary considerably as to their severity and the type of immune effector mechanism involved. The last point is illustrated by animal experiments with mercuric chloride ($HgCl_2$). In rats and mice, this simple chemical was found capable of inducing either contact dermatitis, a self-limiting systemic autoimmune disease with increased IgG and IgE levels, or T cell-mediated nonspecific immunosuppression, depending on the genetic make-up of the rodent strain studied and, to a lesser extent, the dose, frequency, duration, and route of $HgCl_2$ administration.[8,30,47,86]

Considering the variability and the often complex pathogenesis of adverse immune reactions, it is not surprising that most attempts to reproduce in animals the immunological side-effects of drugs and other chemicals observed in man have been unsuccessful.[52,55] Hence, attempts to detect the allergenic and autoimmunogenic potential of chemicals in routine toxicology by using endpoints of the immune response, such as a full-blown allergy or autoimmune disease, would underestimate the hazard, in addition to being expensive.

From an immunological point of view, there can be no doubt that $CD4^+$ T lymphocytes, also designated T helper (Th) cells, initiate and sustain the vast majority of adverse immune reactions to sensitizing LMW chemicals, just as in the case of conventional antigens (Figure 1). The central role of $CD4^+$ T cells in virtually all immune reactions mounted against foreign or self-antigens is irrespective of the ultimate type of effector mechanism, being classified as type I (IgE-mediated), type II (antibody-mediated cytotoxicity), type III (immune complex-mediated), and type IV (cell-mediated) by Gell and Coombs.[37] Actually, it is the $CD4^+$ T cell that largely directs the type of effector response. Its activation by antigen causes the cell to differentiate into one of two functionally different subsets, i.e., Th1 cells that, by producing a particular set of cytokines, favor development of cell-mediated immunity and Th2 cells whose cytokines promote production of IgE and other antibody isotypes.[78] This dichotomy of the Th cell response has been shown to be at play in responses not only to conventional antigens and pathogens, but also sensitizing LMW chemicals, as exemplified by experiments in rodents treated with $HgCl_2$,[8,31,47,63,108] and supported by studies on contact dermatitis and IgE-mediated allergy in humans.[54,96]

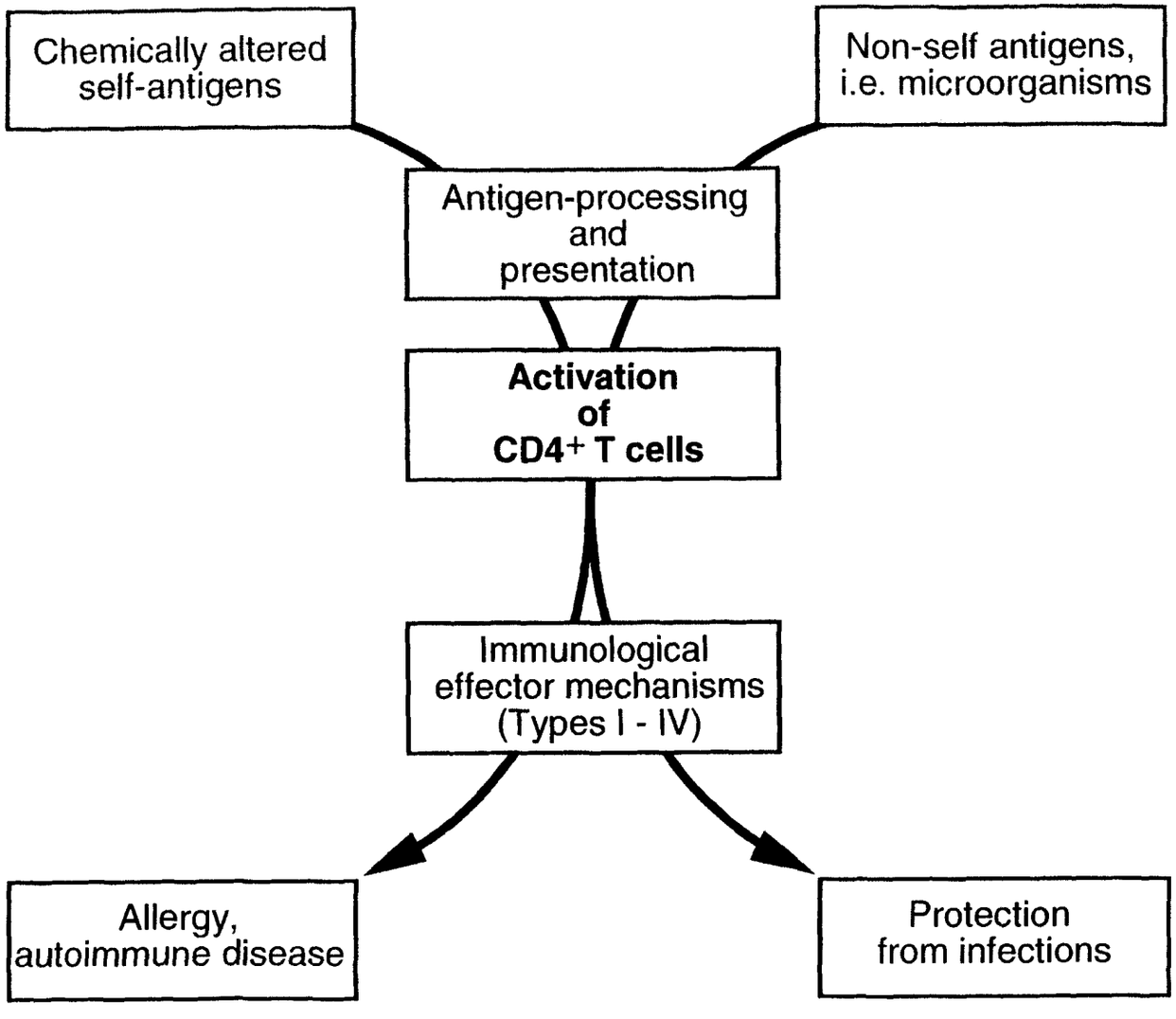


FIGURE 1. Scheme depicting the central role of $CD4^+$ T cells in specific immune responses toward T-cell-dependent *foreign antigens, irrespective of the eventual* effector mechanism involved (types I–IV according to Gell and Coombs). Specific, clonal activation of $CD4^+$ T cells by antigen on antigen-presenting cells is required not only for desired immune reactions, such as against pathogens, but also for adverse immune reactions, such as allergies and autoimmunity induced by chemical-modified self-proteins.

With respect to the safety assessment of LMW chemicals, drugs in particular, it is essential that tests be available that are capable of predicting a chemical's capacity, either as such or as a metabolite, to elicit the pivotal T-cell reactions. The popliteal lymph node assay (PLNA) in rodents is capable of doing so. Because the initiating events, as opposed to endpoints of immune responses, are measured by this assay, it indicates both the allergenic and autoimmunogenic potentials of xenobiotics without distinguishing between them.

II. THE DIRECT PLNA

In the direct PLNA, a solution or fine suspension of the test chemical without adjuvant is injected subcutaneously (s.c.) into one hind foot pad of a rat or a mouse (Figure 2, left part). Hypodermic needles of at least 27 gauge and, preferably, a maximum volume of 50 µl in the mouse and 100 µl in the rat is used. The contralateral foot pad usually is left untreated when a physiologic solvent, such as saline, can be used; however, it is used as an internal control.[41,42] In the case of hydrophobic chemicals, solvents such as dimethyl sulfoxide (DMSO) are used and in these instances the effect of the solvent must be assessed in separate control animals.[49,50,52]

After injection into the foot pad on day 0, the test chemical is transported via the afferent lymphatics to the nearest draining lymph node, the PLN, being situated in the hollow of the knee, the poplitea. In the node, the immune response is generated by a complex process of cell-cell communication and activation. On a specified day, usually day 6 or 7, the left and right PLN are isolated, as depicted in Figure 3. The isolation is a simple and reliable procedure because, at least in mice, there is no anatomic variation in either localization or number of PLN. In rats, two adjacent PLN in one hind leg may occur. In that case, both nodes are removed and analyzed. An immune response to the test compound manifests itself by quantitative and qualitative changes of lymphoid cells in the draining PLN, and all of these can be measured in an objective way. Routinely, the PLN weight is determined.[41,42,48,49,52,98] Other, more sensitive parameters are the PLN cell count[63–66,92,93,101–103], [^{3}H]thymidine incorporation into the PLN[42,48], and measurement of Ig production in the PLN.[42,101–103] The day of PLN isolation appears not very critical as kinetic studies with various chemicals have shown that PLN weight and cell number rapidly increase till day 4 or 5 and either remain at that significantly increased level at least till day 10 or reach a clear maximum in that period.[26,41,48,60,93,98,100] Results can be expressed as a PLN index, that is, the ratio of values obtained from the PLN of experimental control side. Then the PLN indexes of the experimental animals are statistically compared with those of untreated controls. When separate solvent controls are used, either the chemical- and solvent-induced indexes or the absolute PLN reactions can be compared. The latter is preferred because some chemicals, among others $HgCl_2$, may cause minor reactions in the PLN of the untreated paw as well.

In most published studies, five to six animals have been used both in the control and the treatment groups. When not dealing with false-negative chemicals, this appears sufficient to detect a significant effect of sensitizing chemicals because weight and cell number of PLN of control and treatment groups show little variation, that is, the standard deviations are generally <20%. These are small when compared with the size of PLN reactions to sensitizing chemicals, usually giving PLN indexes >2.

In mice, PLN weight indexes >10 are rarely observed, irrespective of whether the injected antigen is a LMW chemical or a potent conventional antigen, such as endotoxin or sheep red blood cells.[26,65,103] In rats, as far as tested, relative PLN enlargement in response to chemicals seems comparable to that of mice.[85,109] Soluble foreign proteins usually fail to induce PLN enlargement unless they were mixed with adjuvant, or adsorbed to small particles such as nitrocellulose particles, which enhance their uptake and presentation by antigen-presenting cells.[1,3,66] The primary PLN response to most immunogenic chemicals peaks within the first 10 days after injection and returns back to normal by week 3 to 4. Notable exceptions from these rules were seen with undegradable materials, such as quartz (SiO_2) crystals.[97,113]

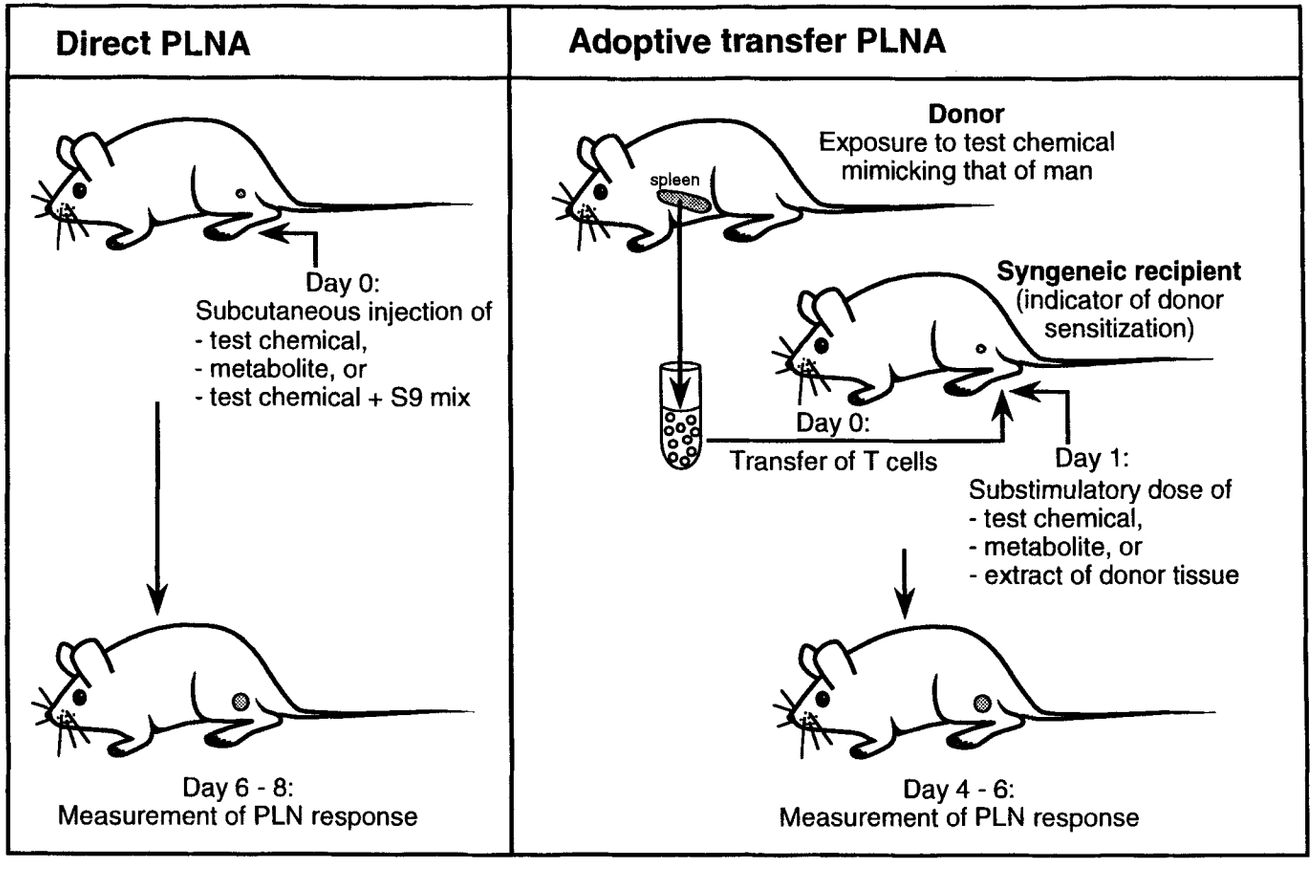


FIGURE 2. Schemes of the direct and the adoptive transfer PLNAs. The direct PLNA measures primary immune reactions to chemicals or their metabolites, usually reaching an optimum between days 6 and 8, as judged by an increase in weight or cell number. A significant response as compared to untreated or solvent-treated controls indicates, but does not formally prove, specific sensitization of T cells. The adoptive transfer PLNA assesses specific sensitization of donor animals to a chemical. After exposure of donors to the chemical according to any desired protocol, their T cells from spleen (and lymph nodes) are transferred to the hind paw of syngeneic recipients, using recipients of T cells from unexposed or solvent-treated donors as controls. One day later, the recipients are challenged at the same site with a presumed nonstimulatory dose of the same chemical, its metabolite, or a tissue extract of donors exposed to that chemical. Sensitization is proven when recipients display significant PLN enlargement within 3 to 5 d after challenge, when compared with the controls, which received T cells from donor animals not exposed to the test chemical (see text for further explanation).

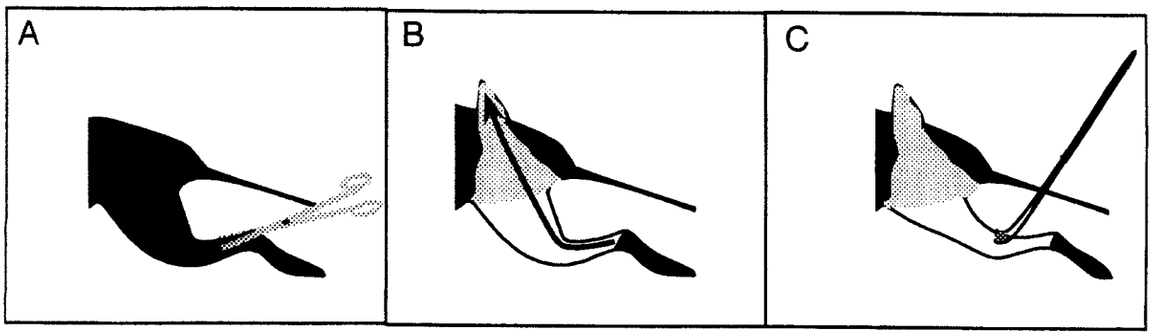


FIGURE 3. Procedure for fast and reproducible isolation of a PLN. Euthanize the animal and moisten the fur of the hind paw to prevent spreading of dust and hairs. (A) Take the hind foot between thumb and forefinger and make a V-shaped incision of a few millimeters in the heel skin keeping the scissors almost parallel to the calcaneal tendon to prevent damage to it. (B) Grasp the skin flap between the (nails of the) thumb and forefinger of the other hand and strip off the paw skin. (C) Take the hind leg at the lateral sides between thumb and forefinger while stretching the knee joint. Frequently, the PLN pops out spontaneously from the hollow of the knee and can be easily removed with a pair of curved micro-dissecting forceps. If not, the PLN can be removed after having made a small sagittal incision alongside the PLN with one leg of the micro-dissecting forceps. Clean of adherent fatty tissue prior to weight determination.

Whereas the increase of PLN weight or cell count is a rapid and reliable parameter of PLN responsiveness, many other events take place in the draining PLN in response to sensitizing chemicals. These events include cytokine-mediated and cell surface molecule (integrin)-mediated interactions of T and B lymphocytes and antigen-presenting cells, cell recruitment, and migration, proliferation, and differentiation of various cell types with the aim to eliminate the antigen by effector cells and molecules. Therefore, further information on the PLN response can be obtained by other methods, such as histopathology and immunohistochemistry.[26,42,97,100,113] For instance, by using the BrdU DNA-labeling technique, it can be shown that proliferation in T- as well as B-cell compartments contribute to the chemical-induced PLN enlargement as illustrated in Figures 4A and B, while immunochemical staining for B cells (Figures 4C and D) or other cells shows their contribution to the induced response. B-cell staining also facilitates detection of germinal center development (Figure 4D), which, among others, indicates T-cell-dependence of the induced response.[71,81,82] The contribution of particular cell types to the response also can be measured in suspensions of immunochemically stained PLN cells by flow cytometry.[28,62,72] This technique, although leading to a loss of *in situ* information, is preferred for fast and objective enumeration of particular PLN cell types after staining for one or more markers, including BrdU. Functional evidence of B-cell activation is obtained when the chemical induces antibody formation and, when IgG production is prominent, this also indicates T-cell-dependence of the reaction.[15,16,42,101–103] Another advantage of measurement of chemical-induced IgG production in the PLN is that, as judged by PLN indexes, it is a much more sensitive parameter than increase of weight, cell number, or proliferation.[15,16,42,101–103] The T-cell subset involved in a given PLN response can be inferred from antibody isotype profiles[16] because antigen-activated Th1 and Th2 cells through the production of different cytokines promote the production of different isotypes.[78] Chemical-induced cytokine profiles have not been measured in the PLNA, but have been measured in another local lymph node assay, the auricular lymph node assay (ALNA), in response to a contact allergen.[106]

Compounds studied in some depth in the direct PLNA and the results obtained with them are listed in Table 1. In these and other published studies,[85,103,109] a total of more than 60 different chemicals, most of them drugs, has been tested. As judged by weight or cell count indexes, com-

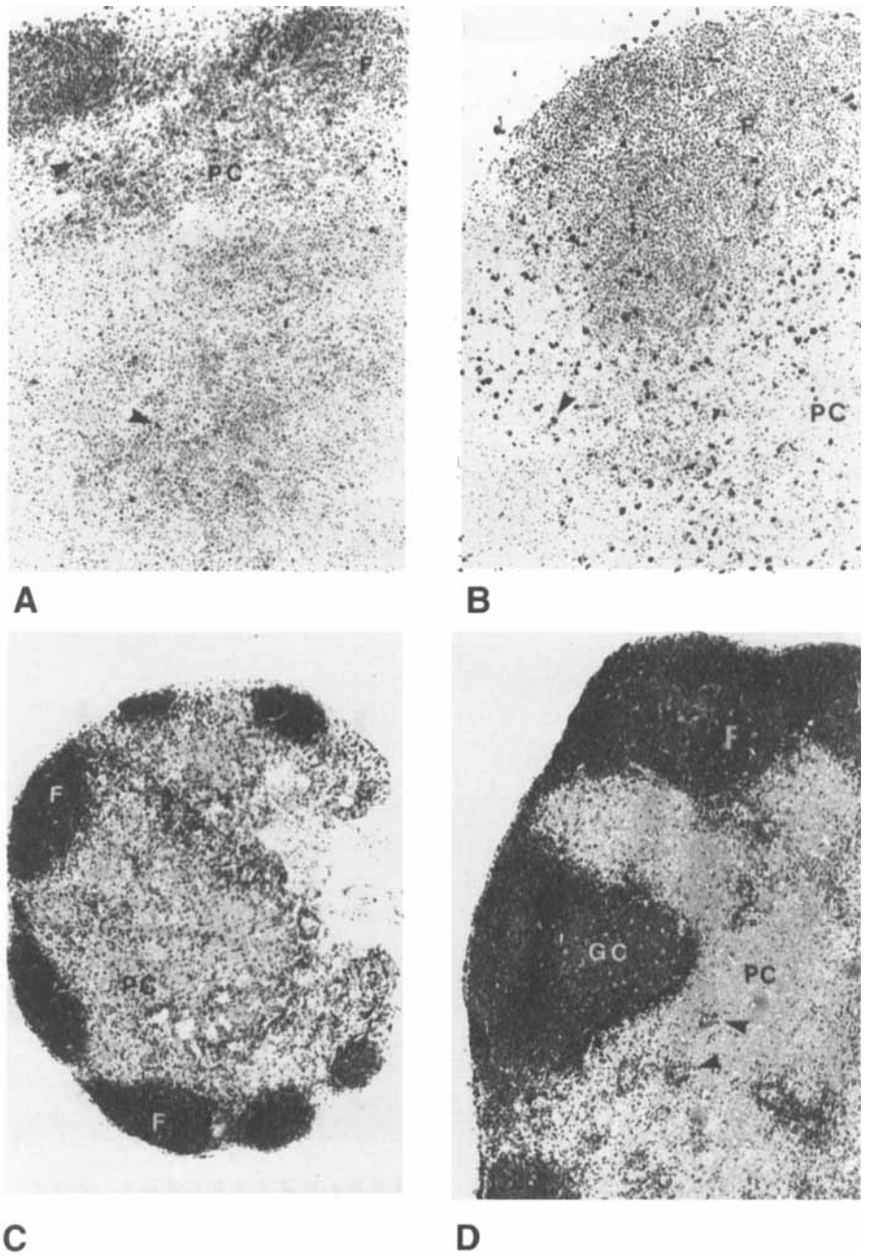


FIGURE 4. Immunohistological characterization of PLN responses to the contact allergen 2,4-dinitro-1-chlorobenzene (DNCB). On day 0, BALB/c mice received a s.c. injection of 10 μmol DNCB in 10 μl DMSO (B and D) or DMSO only (A and C) into the foot pad on day 0. (A and B) Sections from PLN isolated on day 4 of treatment, 2 h after *in vivo* labeling of dividing cells by i.p. injection of the thymidine analog, bromodeoxyuridine (BrdU). Sections were immunochemically stained for BrdU with anti-BrdU antibodies and slightly counterstained with hematoxylin. Compared with the control (A), DNCB (B) caused a prominent increase of dividing cells (arrow heads) in the T-cell-*rich paracortex* (PC), *and* to a lesser extent in the B-cell-rich follicles (F), indicating that local proliferation contributes to lymph node enlargement. (Magnification ×140.) (C and D) Sections from PLN isolated on day 7 of treatment. (Magnification ×55.) Sections were stained with an antibody to the B220 molecule, being present on virtually all B cells, and slightly counterstained with hematoxylin to enable easy distinction of B- and T-cell areas. The marked DNCB-induced PLN enlargement (D) can be attributed to an expansion both of T- and B-*cell compartments. The* latter contain germinal centers, indicating the capacity of DNCB to induce memory B cells and antibody class switch, both being T-cell-dependent phenomena. Increase of high endothelial venules (arrowheads) is another marker of lymph node activation. The high endothelium facilitates the migration of lymphocytes from the blood into the lymph node tissue.

TABLE 1
Overview of the Drugs and Chemicals Studied in Some Detail in the Direct PLNA

Compound studied	Main human exposure	Adverse immune effects in humans	Animal species studied	Effects observed in the direct PLNA[a]								Comment	Ref.
				Primary response	Strain differences	T-cell dependence	B-cell activation	Secondary response	Dose dependence	Structure dependence	Metabolites studied		
Diphenylhydantoin (phenytoin)	Antiepileptic drug	LLS, lymphomas, skin rash, hepatitis	Several mouse strains	Yes	Yes	Yes	Yes	Yes[b]	Yes	Yes	NT		15, 26, 41, 42, 49, 50
			One rat strain	Yes	NT	NT	NT	NT	NT	NT	NT		109
D-Penicillamine	Antirheumatic drug	LLS, IGN	Several mouse strains	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NT	Can bind to protein via its –SH group	48
$HgCl_2$	Medical, occupational, environmental	Contact dermatitis, IGN	Many mouse strains	Yes	Yes	Yes	Yes	NT	Yes	Yes	Yes	Can bind to –SH and $–NH_2$ groups of proteins and form coordination complexes with up to four groups	3, 63, 98
Streptozotocin	Antineoplastic agent	Skin rash	Several mouse strains	Yes	No	Yes	Yes	NT	Yes	NT	NT		60, 62
			One rat strain	Yes	NT	NT	NT	NT	NT	NT	NT		109
Zimeldine	Antidepressant	Guillain-Barré syndrome	Several mouse strains	Yes	Yes	Partially	Yes	No	Yes	Yes	Yes		100–102
Gold(I) salts	Antirheumatic drug	Dermatitis, HGG, IGN	Several mouse strains	No	No	—	—	No	No	Yes	Yes: see below	Can be oxidized to gold(III) by myeloperoxidase	45, 64, 92
Gold(III) salts	Via exposure to gold(I) drugs	Contact dermatitis (and see above)	Several mouse strains	Yes	No	Yes	NT	Yes	Yes	Yes	—	Can oxidize, thus denature, proteins	45, 64, 92
Hexachloroplatinate	Occupational, environmental	Allergic asthma, dermatitis	Several mouse strains	Yes	Yes	Yes	NT	Yes	Yes	Yes	NT		93
Procainamide (PA)	Antiarrhythmic drug	LLS	Two mouse strains	No	No	—	No	NT	No	Yes	Yes: see below	Cannot bind to proteins, unless metabolized	52, 56, 64, 65
			One rat strain	No	NT	—	NT	NT	NT	NT	Yes[c]		85, 109
N-Hydroxyl-PA	Medical use of PA	Same as PA	Two mouse strains	Yes	No	NT	Yes	NT	Yes	Yes	—	Protein-reactive PA metabolite made by S9 mix and phagocytes	64, 65

N-Acetyl-PA	Medical use of PA	Not reported	Two mouse strains	No	No	—	—	NT	No	Yes	—	*N*-Acetylation prevents binding to protein	64, 65
Propyl-thiouracil (PTU)	Anti-thyroid drug	LLS	Several mouse strains	No[d]	No	—	—	NT	No	Yes	Yes	Cannot bind to protein, unless metabolized	49, 50
Propyl-uracil sulfonate ($PU\text{-}SO_3$)	Medical use of PTU		Two mouse strains	Yes	NT	Yes	NT	NT	Yes	Yes	—	Protein-reactive PTU metabolite made by myelo-peroxidase	111

Note: HGG = hypergammaglobulinemia: IGN = immune glomerulonephritis; LLS = lupus-like syndrome; NT = not tested; — = not relevant here.

[a] Yes and No indicate the presence and absence, respectively, of a statistically significant effect as compared to the appropriate controls.
[b] Secondary response manifested itself as a specific suppression of the PLN response.
[c] Procainamide that had been preincubated with S9 mix *in vitro* was tested (see Section II.C).
[d] In one mouse strain (B10.PL), PTU caused a moderate PLN enlargement when compared with the T-cell-deficient littermates (B10.PL nu/nu).[49]

pounds known to induce adverse immune effects in man generally induced positive reactions in the PLNA, whereas those not known to do so were negative in the PLNA. Actually, when all possible immune side effects of the chemicals observed in man were included, no false-positive reactions were noticed. However, false-negative reactions are relatively frequent in the direct PLNA (see references in Table 1; and References 85 and 109). The false-negative reactions to three drugs, that is, gold(I) disodium thiomalate, procainamide, and propylthiouracil, were further examined. In all three cases, the false-negative reactions were shown to be caused by deficient metabolism of the drugs under the conditions of the direct PLNA (see Sections III.A, IV.A, and Table 2).

Experience so far with the PLNA shows that there is good intra- and interlaboratory concordance of results. Conflicting results have been obtained with one chemical only, hydralazine, which was negative in the PLNA according to one group[43] and positive according to another group.[52] However, the mouse strains used by both groups were not identical.

A. Primary Responses to Sensitizing Chemicals

By definition, an immune response to a given antigen is T-cell-dependent, that is, requires $CD4^+$ T cells, if T cell-competent animals react to the antigen, whereas T-cell-deficient animals of the same strain fail to do so. In this way, PLN responses to various LMW chemicals were shown to be T-cell-dependent (Table 1). *In vitro,* T-cell-dependence can be demonstrated by the lymphocyte proliferation (or transformation) test. This test usually requires T lymphocytes from already sensitized donors. When conventional antigens, such as foreign proteins and cells, are used, this test gives high responses. Specific T-cell responses to LMW chemicals, by contrast, have scarcely been detected using this *in vitro* test. These false-negative results in the lymphocyte proliferation test pose a well-recognized problem[25] that hampers easy detection of sensitizing chemicals and is inherent to the inability of most LMW chemicals to act as an antigen per se, as addressed in the next section.

B. The Mechanisms of Primary Responses to Sensitizing Chemicals

The difficulties in generating T-cell reactions to LMW chemicals, especially *in vitro,* can be understood on the basis of the current knowledge of T-cell physiology. $CD4^+$ T cells can be specifically activated only upon recognition of molecules presented to them by MHC class II molecules on antigen-presenting cells. As the antigen-binding site of MHC molecules almost exclusively accommodates short peptides, the antigen receptor of the T cell is confronted with MHC-embedded peptides. The peptides are generated from protein antigens by intracellular protein cleavage (Figure 5A). Subsequently, MHC class II molecule/peptide complexes are formed and transported to the cell membrane where the peptide can be specifically recognized by $CD4^+$ T cells. The complex formation is defined by various factors, such as the proteolytic activity in the cell, the proteolytic cleavage sites in the protein, and the make-up of the genetically highly polymorphic binding site of the MHC molecules, which determines the binding affinity of a given peptide. Moreover, the peptides have to assume an extended, stick-like conformation in which the hydrophobic portion, the agretope, competitively binds to the MHC molecule and the polar or charged portion, the epitope, faces the antigen receptor of the T cell.[20] Because of the many constraints to peptide presentation, only few peptide stretches of a given protein antigen are presented to the T-cell repertoire, namely, those that have the highest affinity to the MHC-binding site and are present at a sufficient concentration. These peptides are the so-called dominant antigenic determinants. It is important to realize that, because antigen-presenting cells cannot distinguish self- and nonself-proteins, foreign and self-peptides are presented indiscriminately. The subsequent immune responses, however, are diametrically opposed to each other. Whereas foreign peptide sequences, in general, induce "stimulatory" T-cell responses, the dominantly presented self-sequences induce "inhibitory" T-cell responses, such as peptide-specific cell deletion in the thymus during generation of the T-cell repertoire and induction of specific tolerance or anergy in the established peripheral T-cell repertoire. The nondominant (i.e.,

TABLE 2
Overview of the Results Obtained with Compounds Tested in the Adoptive Transfer PLNA in Inbred Mice

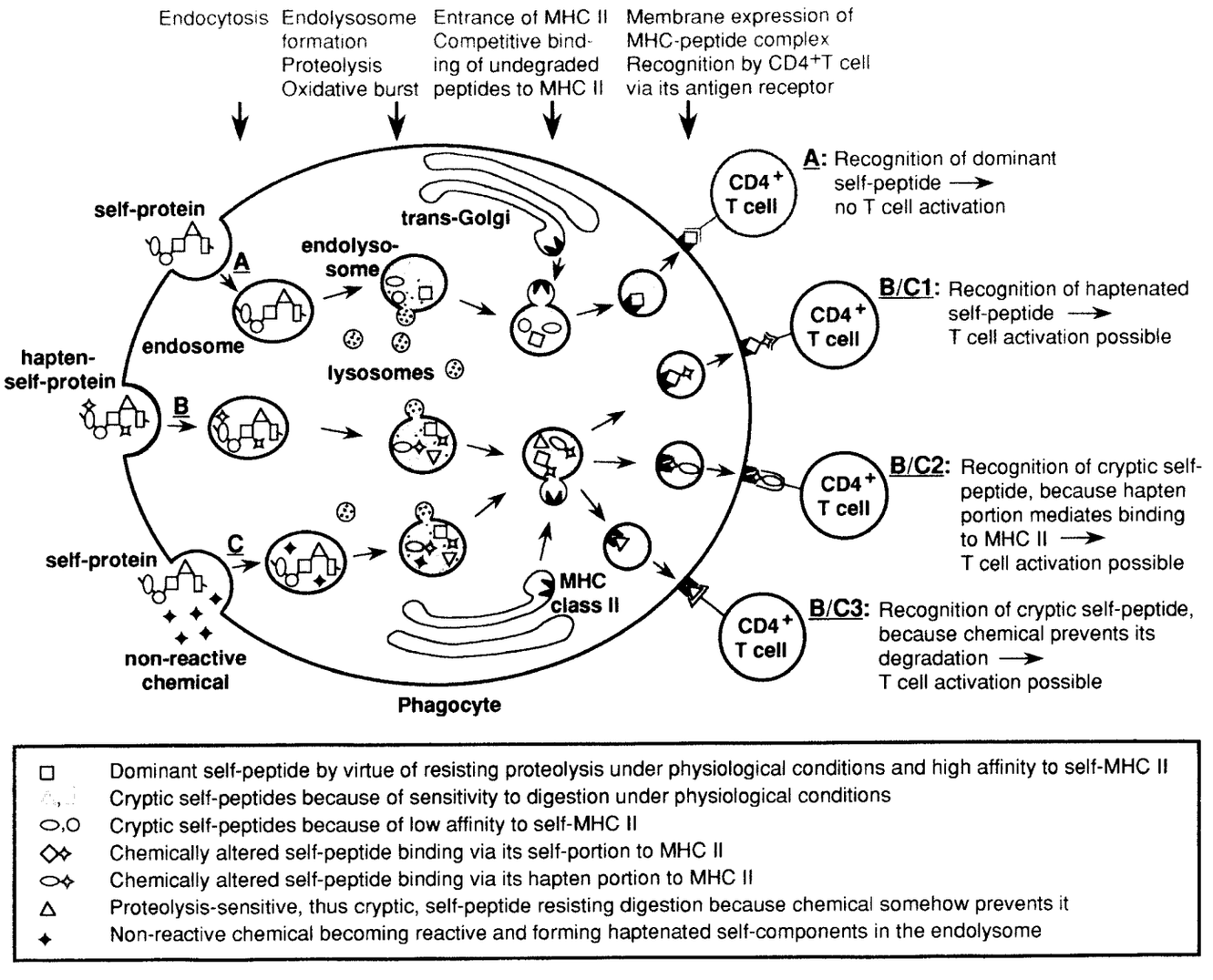
Compound systemically given to T-cell donors	Secondary response of donor T-cell recipients[a]				Ref.
	To compound	To metabolite(s)	To donor phagocytes	B-cell activation	
Streptozotocin	Yes	NT	NT	NT	60
$HgCl_2$	Yes	NT	Yes	Yes	63, 64
Gold(I) disodium thiomalate	No	Yes: gold(III)	Yes	Yes	92
Gold(III) (given as $HAu[III]Cl_4$)	Yes	—	—	No	45, 92
Procainamide (PA)	No	Yes: *N*-hydroxy-PA No: *N*-acetyl-PA	Yes	NT	64, 65
N-Hydroxy-PA	Yes	—	—	NT	65
Hexachloroplatinate	Yes	NT	NT	NT	94
Propylthiouracil (PTU)	No	Yes: PU-SO_3	NT	NT	111
Propyluracil-2-sulfonate (PU-SO_3)	Yes	—	NT	NT	111

Note: NT = not tested; — = not relevant here.

[a] *Yes* and *No* indicate the presence and absence, respectively, of a significant secondary response as compared to recipients of T cells from untreated donors.

cryptic self-determinants) fail to do so because they are not sufficiently presented under physiological conditions in order to have an impact on T cells.[2,14,70,79,83,89,95] The presentation of cryptic self-peptides, however, can be upregulated under certain circumstances.[70] In such cases, T cells can mount autoreactive responses because they have not been tolerized to these peptides. For instance, expression of cryptic self-peptides of nucleolar proteins appears to be a decisive step in the pathogenesis of $HgCl_2$-induced formation of antinucleolar autoantibodies in mice.[67]

Because many drugs and other chemicals are amphipathic molecules that are likely to be internalized into endosomes, they may bind to the MHC molecules by hydrophobic interactions. Actually, the latter has been shown for L-tyrosine-*p*-azo-benzenearsonate, a well-studied immunogenic chemical per se that induces arsonate-specific memory T cells.[44,87] To bind to MHC molecules, however, chemicals have to compete with many, comparatively large self-peptides. Because at least the dominant self-peptides will have larger hydrophobic contact sides than the average chemical, peptides will be selected by the MHC molecules in preference to the majority of chemicals. Hence, due to their supposedly relatively low MHC affinity, most chemicals will not be immunogenic per se. Therefore, terms such as "T cell reactions to chemical X" actually are misleading, but are used for the sake of brevity. Chemicals may become immunogenic, however, when they bind to self-proteins and when processing of the altered proteins ultimately results in presentation to the T cells of foreign epitopes, as depicted in Figure 5B/C1. Presumably, this is the most common pathway of chemically induced adverse immune reactions. However, processing of chemically altered self-proteins may also result in the presentation of cryptic, thus potentially T-cell-activating, self-peptides by the following mechanisms, namely, the creation of new agretopes (Figure 5B/C2) or the modification of the physiological intracellular protein degradation (Figure 5B/C3). Immune responses generated in this way are highly defined by the peptide sequences to which the chemical has bound. Therefore, a chemical's failure to trigger secondary T-cell reactions *in vitro* may well be attributed to the absence of the crucial protein or to insufficient chemical binding



□	Dominant self-peptide by virtue of resisting proteolysis under physiological conditions and high affinity to self-MHC II
⬭,⬭	Cryptic self-peptides because of sensitivity to digestion under physiological conditions
⬭,O	Cryptic self-peptides because of low affinity to self-MHC II
◇✧	Chemically altered self-peptide binding via its self-portion to MHC II
⬭✧	Chemically altered self-peptide binding via its hapten portion to MHC II
△	Proteolysis-sensitive, thus cryptic, self-peptide resisting digestion because chemical somehow prevents it
✦	Non-reactive chemical becoming reactive and forming haptenated self-components in the endolysosome

FIGURE 5. Schematic representation of events leading to MHC-restricted self-peptide presentation by phagocytes to $CD4^+$ T cells under physiological conditions (A), and some potential changes after exposure to reactive (B), and nonreactive chemicals (C). (A) Under physiological conditions, self-proteins are continuously internalized by phagocytes, for instance, by phagocytosis of senescent cells or through receptor-mediated or fluid-phase endocytosis. The resulting endosomes thus contain self-proteins from the cell's microenvironment and from its own plasma membrane. Subsequently, lysosomes fuse with the endosome to form an endolysosome in which the self-proteins are degraded by lysosome-derived proteinases. However, particular peptide sequences will be relatively resistant to the proteolytic attack and will compete for capture by self-MHC class II molecules that enter the endolysosome later on in the pathway. The whole process ultimately leads to membrane expression of proteolysis-resistant self-peptides, with the highest affinity to self-MHC molecules. Immune reactions to these so-called dominant self-peptides, however, have been silenced through physical or functional deletion of the $CD4^+$ T cells that recognize the peptides. (B) Exposure to reactive chemicals will most likely lead to conjugation of external or membrane-bound self-proteins. Their processing on endocytosis may result in the presentation of other or altered self-peptides, for example, the presentation of a haptenated-self-peptide enabling activation of T cells with specificity to the hapten portion (B/C1) or the presentation of physiologically cryptic self-peptides that have not silenced CD^+ T cell reactivity and thus can activate T cells with specificity to the cryptic self-peptides. They can be presented when the hapten generates a site of high affinity to self-MHC class II molecules (B/C2) or when the hapten changes proteolytic digestion, resulting in sparing of self-peptides that are usually degraded (B/C3). (C) Exposure to nonreactive chemicals will require their bioactivation in order to get reactive intermediates that bind to self-molecules. This may occur in the endolysosome when the chemical is present in this compartment through endocytosis, diffusion, or otherwise. Bioactivation may be the result of triggering the oxidative burst in phagocytic cells. It among others leads to the activation of the NADPH-oxidase and myeloperoxidase and hence the presence of reactive oxygen species in the endolysosome. Reactive chemical intermediates formed in the oxidative environment[105] will bind to self-peptides, in this instance including lysosomal peptides as well. The potential consequences for $CD4^+$ T-cell activation are the same as described under (B).

under the conditions of the *in vitro* assay. One of the reasons accounting for the latter could be the limited metabolic capacity of leukocyte suspensions, namely, most drugs and many other chemicals inducing adverse immune effects are only poorly reactive as such and have to be metabolized to chemically reactive intermediates in order to create immunostimulating hapten–self-protein complexes.[18,29,105]

C. Primary Responses to Sensitizing Metabolites

As already mentioned, the direct PLNA failed to indicate an immune response toward the drugs gold(I) disodium thiomalate, procainamide, and propylthiouracil, all of which are notorious inducers of adverse immune reactions in man. In marked contrast, their reactive metabolites, gold(III), *N*-hydroxyprocainamide, and propyluracil-2-sulfonate, respectively, proved to be capable of inducing primary responses in the direct PLNA (Table 1). These findings indicate that the parent drugs genuinely lack immunogenicity and that the direct PLNA per se provides insufficient opportunity for generation of and subsequent responses to sensitizing metabolites. The latter obviously is related to the limited metabolic capacity of the paw and the PLN. This is evidenced by the observation that procainamide induced a positive PLN response in mice when injected shortly after *in vitro* metabolism by incubation with rat-derived S9 mix and subsequent removal of foreign rat macromolecules to eliminate unwanted immune reactions to the latter.[56] Using a similar procedure in the rat, the drug isoniazid, which likewise gave negative results in the direct PLNA, was shown to induce a positive PLN response. In contrast, preincubation of procainamide with S9 mix failed to induce a positive PLN reaction in the rat,[85] probably because of the low dose of procainamide used (cf. Reference 56). At least with some chemicals,[105] the lack of metabolic conversion in the PLNA should also be overcome by incubation of the test chemical with syngeneic monocytes or macrophages prior to testing in the direct PLNA. This was formally demonstrated for gold(I) disodium thiomalate, which is converted to the immunogenic gold(III) intermediate by preincubation with phagocytes.[45,64] Presumably, other drugs found to be negative in the direct PLNA likewise will induce positive results after prior exposure to metabolizing enzyme systems or cells (cf. Section IV.A).

D. Structure-Activity Relationships

Adverse immune reactions to LMW chemicals, as detected in the direct PLNA, generally seem to require binding of the chemical, or a metabolite, to self-proteins. As discussed in Section II.B, the chemical binding can result in T-cell activation by foreign epitopes or cryptic self-epitopes (Figure 5B1–3). Other qualities of a chemical, however, may contribute to immunogenicity as well. For instance, by using closely related analogs of different groups of chemicals, it was shown that immune responsiveness increased with increasing lipophilicity.[49,50,101,102] This may reflect increased delivery of the more lipophilic compounds, either as such or bound to self-proteins, to the T-cell compartments of the lymph node and, hence, more efficient T-cell sensitization.[21] In the case of lipophilic substitution, such a mode of action may be potentiated by the introduction of a suitable agretope on cryptic self-peptide sequences, which, as already discussed, may promote their presentation by antigen-presenting cells (Figure 5; Section II.B).

For zimeldine and congeners, besides lipophilicity, the molecular conformation was shown to be at play. The capacity of this drug to elicit PLN reactions was found to be favored by a conformation in which lipophilic and hydrophilic portions line up at a suitable distance on opposite sides of the molecule. Because of these conformational constraints and the structural resemblance with L-tyrosine-azobenzene arsonate, it has been suggested that, like the latter molecule (see Section II.B), zimeldine might act as an immunogen per se.[101,102] The high discriminatory capacity of the direct PLNA as to subtle changes in the chemical structure has also been demonstrated for other groups of chemicals. With penicillamine and zimeldine, stereo-selectivity of the PLN response has been observed,[48,101] and alter-

ations of chemical charge due to changes in solvent pH values were found to modulate the immunogenicity of D-penicillamine.[48] Neither acid nor alkaline pH values of solvent per se induce PLN reactions,[41,48,92] unless the ionic strength of the solvent is unusually high. In other words, nonspecific PLN reactions may occur when the material injected causes overt irritation at the injection site.

E. Secondary Responses after Local Priming

The PLN reaction that follows a single injection of a test compound does not tell whether the reaction is immunologically specific, that is, whether or not it is mediated by T lymphocytes with specific antigen receptors. Immunologically nonspecific reactions can be distinguished from specific ones, such as those involved in allergy and autoimmunity, in that the former do not generate specific clones of memory T cells, whereas the latter do. Because of the increased number of specific memory T cells on sensitization and the increased capacity of these T cells to interact with antigen-presenting cells, secondary responses are featured by faster kinetics and lower elicitation doses than that required for priming. Based on these principles, the PLNA has been adapted in three different ways for detection of secondary responses. The three approaches differ as to how T-cell priming to the test compound is achieved. In the first, most straightforward approach, priming is accomplished by treating the animals as for the direct PLNA. Upon complete resolution of the primary PLN response, usually after 4 to 6 weeks, the animals are challenged in the same paw with a, as far as priming is concerned, substimulatory dose of the same chemical to measure PLN enlargement prior to day 6. Secondary reactions to chemicals found in this way, that is, priming and challenge into the same foot pad, have been compiled in Table 1. It is likely that similar results would have been obtained after challenge of the contralateral paw inasmuch as secondary PLN reactions can also be measured in systemically primed mice, as addressed in the next section. The third variation of PLNA used for detection of secondary T-cell responses to chemicals is described in Section III.

F. Secondary Responses after Systemic Priming

Sensitization to antigens, chemicals included, is highly influenced by the route of first exposure to antigen. For example, exposure by the s.c. route is known to favor sensitization in general, whereas exposure via mucosal routes tends to induce unresponsiveness. Thus, for safety assessment of chemicals, it is essential to know whether likely routes of human exposure can cause sensitization. The direct PLNA can be used to this end, as indicated by preliminary studies in which mice were intranasally treated with minute doses of hexachloroplatinate for several months and then left untreated for 3 weeks. These mice showed a specific secondary PLN response after subsequent challenge with a small dose of this agent injected into the hind paw.[94] This approach, if validated, could be conveniently applied as an adjunct to routine toxicity screening of chemicals.

G. Responses to Nonsensitizing chemicals

Among the compounds tested for T-cell-dependence of their effects in the direct PLNA only quartz (SiO_2) crystals[97,113] and, to some extent, the antidepressant drug zimeldine[100] were found to induce T-cell-independent PLN reactions. Although zimeldine was capable of inducing a moderate PLN enlargement in T-cell-deficient mice, T-cell involvement in the reactions to this drug was apparent from (1) the significantly higher PLN enlargement in T-cell-competent mice when compared with T-cell-deficient mice and (2) the prominent induction of germinal centers and IgG production.[100,101] In the case of quartz crystals, T-cell-independence of the PLN response is perhaps not surprising. The crystals can neither be degraded nor eliminated from the body, and they are powerful and persistent stimulators of macrophages.[76] The macrophages show an enhanced cytokine production and this, in turn, seems suf-

ficient to induce a persistent stimulation and accumulation of several cell types in the PLN.[113]

Solvents, such as DMSO, used to inject water-insoluble chemicals, also cause PLN enlargement, albeit a moderate one. This reaction is probably caused by nonspecific irritation because it was not attended by qualitative morphological changes in the PLN[26] or significant antibody formation.[103] Similarly, solvents with a high ionic strength, for instance as a consequence of pH adjustment, might cause nonspecific PLN enlargement by causing tissue damage at the site of injection.

H. Detection of Immunosuppressive Chemicals

Although there are numerous assays capable of measuring decreased immune responsiveness, it is worth noting that the PLNA is also capable of doing so. This is illustrated by the effects of cyclosporin A, a well-known inhibitor of T-cell functions, on the direct PLN response of mice to the immunogenic drug, streptozotocin (Figure 6). Systemic injection as well as oral administration of the immunosuppressant caused a dose-dependent decrease of the PLN response. Virtually identical findings have been reported for the rat.[99] Likewise, immunosuppressive action of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was demonstrated by using ovalbumin in Freund's complete adjuvant as the PLN stimulant.[72]

III. THE ADOPTIVE TRANSFER PLNA

A. Secondary Responses to Sensitizing Chemicals

As already outlined in Section II.E, a chemical's capacity to sensitize T cells can be assessed by measurement of secondary immune responses to that chemical. These data are needed for hazard identification, but more accurate knowledge requires assessment of a chemical's sensitizing potential under expected or actual conditions of human exposure. Such conditions can be met by (1) the recently developed approach described in Section II.F, which uses the direct PLNA, and (2) the adoptive transfer PLNA developed by Klinkhammer et al.[60] The latter assay measures secondary responses of T lymphocytes from a chemical-exposed "donor" animal after transfer of its T cells to a syngeneic recipient (Figure 2, right part). The donor animals can be treated with a test chemical under conditions of exposure as to dose, route, duration, frequency, etc. that mimic the human situation. At the desired time of exposure of the donor animals, their spleen cells or splenic T cells are transferred s.c. into one hind foot pad of recipient animals, using cells of unexposed donors as controls. One day later, the recipients are challenged at the same site by s.c. injection of the chemical at a dose that is too low to induce a significant primary PLN response in the controls. When the recipients show significant PLN enlargement another 3 to 6 d later, it can be concluded that exposure of the donor animals had resulted in T-cell sensitization and subsequent generation of memory T cells. This is based on the knowledge that memory, but not inexperienced, T cells can react to low doses of antigen within a short period of time.

A prerequisite for performing the adoptive transfer PLNA is the use of syngeneic, specific pathogen-free donors and recipients in order to avoid interference by T-cell reactions against allogeneic and pathogen-derived determinants following cell transfer. A different kind of interference is caused by nonspecific recipient PLN reactions to the transferred cells. Such nonspecific reactions seem to occur especially when the T cells were activated by antigen shortly before the transfer. These background reactions can be reduced by irradiation of the transferred cells,[63,65,66,92] but this is not obligatory.[60]

Using the adoptive transfer PLNA, the T cell-sensitizing capacity of various chemicals has been assessed (Table 2). For two of these chemicals, streptozotocin and hexachloroplatinate, specimens of spleens and lymph nodes, demonstrated to harbor memory T cells, were examined by conventional histopathology and found to look inconspicuous. This suggests that T-cell sensitization cannot be reliably detected by conventional histopathology, an impression that is supported by the experience with the drugs nomifensine and zimeldine, both of which had to be withdrawn from the market shortly after their introduction because of unexpected immunological side-ef-

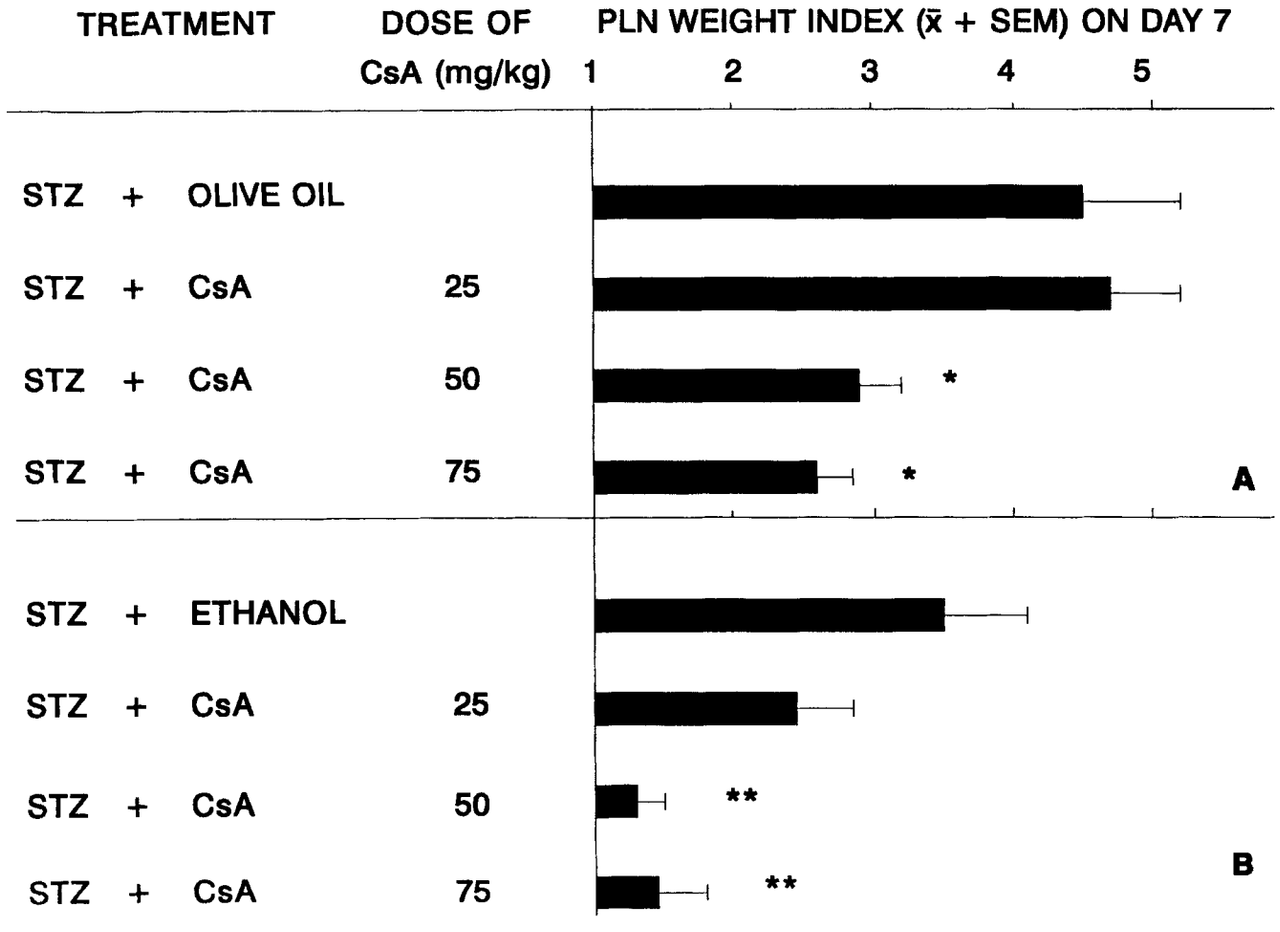


FIGURE 6. Suppression of the PLN response to streptozotocin by cyclosporin A (CsA). On day 0, groups of ten male BAL/c mice received a single s.c. injection of 0.5 mg streptozotocin as the antigen and daily i.p. (A) or oral (B) treatment with CsA (25, 50, or 75 mg/kg body weight) from day 0 till 7, using the solvents olive oil and ethanol as the respective controls. Horizontal bars indicate PLN weight indexes (means + SE) as determined on day 7.*,**: Significantly different from the control at $p \leq 0.05$ and $p \leq 0.01$, respectively (two-sided Student *t*-test).

fects.[6,61] Apparently, the extensive histopathology required for safety assessment had failed to demonstrate their immunomodulating capacity. In contrast, both drugs were found to cause PLN enlargement and antibody formation in the direct PLNA,[100,103] and in the case of zimeldine these were demonstrated to be partially T-cell-dependent.

B. Secondary Responses to Sensitizing Metabolites

As reviewed in the preceding section and Table 2, streptozotocin, $HgCl_2$, and hexachloroplatinate apparently do not need to be metabolized in order to evoke specific secondary reactions by adoptively transferred T cells. In contrast, adoptive transfer PLNAs performed with lymphoid cells from donors repeatedly treated with gold(I) disodium thiomalate, procainamide, or propylthiouracil failed to give PLN responses in recipients when the original drugs were used for challenge. Significant secondary responses ensued, however, when their active intermediates gold(III), N-hydroprocainamide, and propyluracil sulfonate, respectively, were used for challenge.[45,64,65,92,111] Thus, data show that repeated administration of the parent compounds resulted in sensitization to their metabolites. Data also stress the insufficient metabolic capacity in the hind paw as the secondary responses could be elicited with small amounts of the metabolites, apparently not generated after a single local injection of the parent drugs. Prompted by the results obtained with gold(I) and gold(III) in mice,[92] peripheral blood lymphocytes of rheumatoid arthritis patients who had developed dermatitis under treatment with gold(I) disodium thiomalate were studied by means of the lymphocyte proliferation assay *in vitro*. Human data thus obtained[40,110] confirmed the results of the murine experiments that T cells ignore the original gold(I) drug but become sensitized to the reactive intermediate gold(III).

IV. PLNAS AS A RESEARCH TOOL

A. Study of Metabolic Events Underlying a Chemical's Immunogenicity

According to Uetrecht,[105] most drugs are not chemically reactive but can be metabolized to chemically reactive intermediates. Translated to immunology, most drugs will not cause immune reactions unless metabolized to reactive intermediates. Only the latter, after binding to self-proteins, may initiate immune reactions. Because self-proteins located in the immediate vicinity of the origin of short-lived reactive intermediates are the most likely carriers, potentially resulting immune responses should become manifest at the same site. Therefore, although the liver is the most important site of biotransformation, immune-mediated side-effects of chemicals in organs other than the liver presumably involve locally generated metabolites. In these instances, phagocytes, that is, neutrophilic granulocytes, monocytes, and macrophages, may be at play. These ubiquitous cells not only phagocytose foreign matter, but they also dispose of various oxidizing enzymes that generate oxidants such as hydrogen peroxide, superoxide, and hypochlorous acid. Whereas the physiological function of the oxidants is to kill infectious agents, they may oxidize chemicals with readily oxidizable functional groups as well and thus generate highly reactive intermediates.[105] In addition, monocytes and macrophages may act as antigen-presenting cells inasmuch as they constitutively express MHC class II molecules. *In vitro*, the generation of reactive intermediates by activated phagocytes (neutrophils and monocytes) or their isolated enzyme systems has been formally demonstrated for several drugs, including gold(I) disodium thiomalate[13] and procainamide.[69,90,112]

The immunological relevance of these findings was demonstrated for the first time by means of the adoptive transfer PLNA, using T lymphocytes from donor mice that had been repeatedly treated with these drugs. As mentioned in Section III.A and summarized in Table 2, these T cells were refractory to challenge with the parent drugs, but mounted secondary responses to the reactive metabolites, gold(III), and *N*-hydroxyprocainamide, respectively.[65,92] Instead of using the immunogenic synthetic drug metabolites, homogenates of macrophage-rich peritoneal cells from syngeneic mice that had undergone long-term treatment with the parent drugs gold(I) disodium thiomalate and procainamide, respectively, could be used as well.[45,65] Obviously, these cells harbored the immunogenic metabolites. An interesting observation in this context is that the

immunogenic procainamide metabolite, as judged by adoptive transfer PLNAs, was present in peritoneal cells from slow-acetylator but not fast-acetylator mice.[65] Data are well in line with the autoimmune effects of procainamide in man, which are known to develop faster in individuals with the slow-acetylator phenotype (see References 90 and 105). Apparently, slow *N*-acetylation of procainamide leaves more substrate for *N*-hydroxylation of the drug and, hence, for generation of the immunogenic metabolite *N*-hydroxyprocainamide. The crucial role of oxidation in the adverse immune effects of procainamide was further evidenced by PLNA transfer experiments with fast-acetylator mice treated with procainamide for several months. Homogenized peritoneal cells of these mice failed to elicit secondary T-cell responses and thus had failed to sufficiently generate the immunogenic metabolite, but they stimulated a secondary response when the mice were treated not only with procainamide, but also with phorbol myristate acetate, a stimulant of the oxidative metabolism of phagocytes.[65]

The above-described studies demonstrated the presence of immunogenic metabolites in peritoneal cells, but did not indicate whether the cells themselves, mononuclear phagocytes in particular, actually generated the immunogenic metabolites. Indications of this have been obtained in the gold model using bone marrow-derived monocytes or peritoneal macrophages from untreated mice. When these cells were incubated with the gold(I) drug *in vitro,* then homogenized and used as antigenic material to challenge recipients of gold(III)-sensitized T cells, a secondary response ensued.[45] These results provide firm evidence for the capacity of mononuclear phagocytes to convert an immunologically harmless chemical into immunogenic molecules. In all likelihood, the conversion involves biotransformation of the chemical into a reactive intermediate, its conjugation to self-proteins, and, subsequently, presentation of an MHC-embedded immunogenic peptide at the membrane. These events enable recognition by T cells (Figure 5). It is noteworthy, however, that activation of the T cells requires accessory signals from the antigen-presenting cell, among others cytokines, and molecules that promote cell-cell adhesion. These signals can be delivered by activated monocytes and macrophages as well.[12,68,91]

B. Detection of Chemically Altered Self-Proteins

As addressed in Section II.B, chemicals or their metabolites are usually not the immunogens per se, but conjugates of these with particular self-proteins, the so-called hapten-carrier complexes. The adoptive transfer PLNA may assist in their detection irrespective of whether the chemical binds to self-proteins as such or as a reactive metabolite. This is illustrated by studies with $HgCl_2$ and mice that display T-cell-dependent B-cell stimulation and autoantibody formation after repeated treatment with this metal salt. After transfer, their T cells mounted secondary responses not only to $HgCl_2$ itself but also to erythrocytes and homogenates of peritoneal macrophages, whole spleens, and all electrophoretically separated fractions thereof.[63,66] Presumably, unknown conjugates of Hg^{2+} with a variety of different self-proteins cause the specific reaction. This $HgCl_2$-induced "multireactivity" is presently the subject of further study using the adoptive transfer PLNA to guide purification of the self-proteins that form immunogenic conjugates by reacting with Hg^{2+}.

V. COMPARING THE PLNA WITH OTHER ANIMAL TEST SYSTEMS FOR RECOGNITION OF SENSITIZING CHEMICALS

With respect to predictive immunotoxicity assessment of chemicals, current regulatory toxicology guidelines only include a variety of guinea pig tests to identify skin sensitizers.[4,17,73] All these tests measure secondary immune responses in the form of challenge-induced skin erythema. Although the guinea pig tests are well validated and of proven practical importance, the prediction of sensitizing potential is based on visual assessment of erythema, a subjective endpoint that, moreover, can be obscured in the case of dyes and irritating chemicals.[9] Furthermore, guinea pig tests are relatively costly and time consuming, and the use of Freund's complete adjuvant in some of the

protocols increasingly raises objections by animal welfare committees. More recently developed tests for identification of contact allergens use mice, instead of guinea pigs, and are based on objective measurement of challenge-induced ear swelling after topical sensitization on the abdomen.[17,35,36] These tests are considered to be relatively fast and inexpensive and permit accurate assessment of sensitizing dyes but may include additional treatment with Freund's adjuvant to increase sensitivity.

Another mouse test introduced for screening contact sensitizers is, like the PLNA, a local lymph node assay, but confusingly is referred to as "the local lymph node assay".[57] Following topical application of the test compound to the ear skin, this assay, like the direct PLNA, measures primary responses of the draining lymph node; the latter is referred to in the literature as auricular[33] or submaxillary[84] lymph node in the mouse and facial lymph node[104] in the rat. To avoid confusion with the PLNA, the local lymph node assay henceforth will be called auricular lymph node assay (ALNA). It is based on determination of proliferative activity in the ALN, as determined by [^{3}H] thymidine incorporation. The test chemical is applied to the dorsal ear skin daily for 3 consecutive days, and 24 to 72 h after the last administration [^{3}H] thymidine is injected intravenously. Five hours later, the draining ALN are isolated, and incorporated radioactivity is counted. The test chemical is considered a contact sensitizer when resulting proliferative activity in the ALN is at least three times that found in ALN from vehicle-treated controls.[9,10] Although the ALNA assesses the induction phase of primary responses only, it is considered to have the same advantages as the ear swell test of Gad et al.[35] It, however, produces considerable radioactive waste. Additional advantages of the ALNA that are mentioned are no requirement of adjuvants and no need for prior determination of maximal nonirritant concentrations because nonsensitizing irritants would not influence the ALN response[57] or by exception only.[10] The latter, however, has been disputed by the finding that various irritants induced false-positive proliferative reactions in the ALNA.[77,88] Hence, as concluded on the basis of weight or cell increase in the direct PLNA, proliferative responses in the ALNA suggest, but do not prove, specific activation of CD4$^+$ T cells. Demonstration of specific T-cell involvement requires measurement of secondary responses, for instance by measurement of secondary responses in preexposed animals by the ALNA[59] or an ear swell test.[35,36,74] Another similarity between the direct PLNA and ALNA is the relatively high incidence of false-negatives. Actually, four out of ten chemicals classified as moderate sensitizers in the guinea pig maximization test appeared negative in the ALNA according to current criteria.[10]

The apparent lack of sensitivity of the ALNA may be related to the parameter used, that is, proliferation expressed as dpm/ALN. Induction of antibody formation, expressed as ng IgG1/ALN, appears to be a more sensitive parameter than cell proliferation as indicated by indexes of test to control ALN. Notably, proliferation measurements yielded indexes of 31, 45, and 51 after 3 applications of 2.5, 5, and 10% TMA, respectively,[10] whereas similar applications of 1, 3, and 10% TMA yielded indexes of 56, 383, and 1369, respectively, when IgG1 was measured.[107] In addition to providing higher sensitivity in the ALNA, as earlier noted in the PLNA (Section II.A), the use of antibody production as the parameter also may indicate T-cell involvement and the type of immune effector mechanism involved. For example, the observation that application of 1 and 3% TMA induced IgG1 and some IgE, but no other isotypes, is suggestive of preferential activation of Th2 cells by TMA. This observation is in agreement with the major immunotoxicity of TMA, namely, allergic asthma, but disagrees with the paradigm that the epicutaneous route favors induction of Th1-cell-mediated contact sensitization. However, other studies on antibody induction suggest that besides the route, the dose may be a decisive factor because application of 50% TMA or 1% 2,4-dinitro-1-chloro-benzene (DNCB), concentrations inducing about the same degree of contact sensitization,[24] caused production of the Th1-cell-dependent IgG2a isotype in the ALN.[16] Interestingly, s.c. injection of the same compounds (10 µmol; about 2 mg) failed to induce IgG2a in the PLN, but caused more prominent induction of typical Th2-dependent isotypes by both compounds when compared with the ALNA. In both assays, however, TMA caused much higher overall antibody production, espe-

cially with respect to IgG1, than DNCB; this agrees with the preferential induction of antibody- and cell-mediated allergies by TMA and DNCB, respectively.

Together these data suggest that the immunotoxic potential of sensitizing chemicals and its relationship to dose and route of exposure may be estimated on the basis of induced antibody isotype profiles in the ALNA and PLNA. However, assessment of the validity of this approach must await comparative studies with a large number of chemicals because up until now only very few chemicals have been tested in both assays. Results of these studies also may show the relative strength and weakness of the assays as to recognition of sensitizing chemicals as well as their preferentially induced immune side-effects.

VI. SUMMARIZING DISCUSSION, CONCLUSIONS, AND OUTLOOK

A. PLNAs As a Screening Tool

A chemical that is able to sensitize T cells constitutes a health hazard because of its potential to cause a wide variety of allergic and autoimmune side-effects. Whether one or more of the possible side-effects of a chemical becomes manifest depends on conditions of exposure in interaction with genetically determined intrinsic factors, as well as other extrinsic factors. This has been formally demonstrated, for instance, in animal experiments with mercuric chloride (Section I), and many observations from medical practice are consistent with it. For instance, drugs such as penicillins, promethazine hydrochloride, chlorpromazine, isoniazid, and sulfonamides are capable of provoking a wide range of immunological diseases, including allergic contact dermatitis after topical treatment[5,22] and SLE after systemic treatment.[115] In terms of hazard identification, the latter example shows that T-cell-sensitizing chemicals, as manifested by their capacity to cause contact allergy, can cause a quite different immune disease when given by a different route. Consequently, the guinea pig models used in routine toxicology and the ALNA are not confined to identification of chemicals inducing contact allergy (type IV reactions) but detect chemicals that may induce other types of allergy and autoimmune disease as well. Skin testing, however, restricts identification of sensitizing chemicals to those that can penetrate the epidermis. How many potentially sensitizing chemicals will be negative in skin tests because of this is not known. The s.c. route of exposure used in the PLNA does not have this restriction. Therefore, the PLNA is likely to favor detection of sensitizing chemicals when compared with the ALNA. A systematic comparison of both assays, however, is needed to resolve these issues.

In any case, since the introduction of the direct PLNA into immunotoxicology over 12 years ago,[41] several research groups have shown that the assay is capable of recognizing a wide variety of sensitizing chemicals, including autoimmunogenic chemicals that despite many different attempts failed to produce manifest disease in the very mouse strains that showed clearly positive PLN responses. Moreover, it has been shown that the direct PLNA detects T-cell-independent immunostimulating chemicals. Together these data demonstrate the robustness of the direct PLNA. In addition, this assay is fast, objective, relatively inexpensive, and simple to perform. Albeit proficiency tests for reproducibility and variability of the results in a given strain of mouse or rat have not been performed as yet, results obtained with the direct PLNA showed intra- and interlaboratory as well as interspecies concordance. A problem, however, is the existence of false-negative results in the direct PLNA. Whether these, as demonstrated for a few compounds, may be generally overcome by *in vitro* incubation of the test compound with S9 mix or phagocytes prior to assay awaits future studies, including those using S9 mix or phagocytes from human P450-transgenic animals when they become available. If so, it would obviate the major weakness of the direct PLNA. If not, it can be stated that for the time being there are no other reliable *in vivo* assays and certainly no reliable *in vitro* assays (cf. Section II.A and B) that can be offered as an alternative to the direct PLNA for the screening of chemicals that cause human sensitization by routes other than the skin. Therefore, the recommendation of an International Workshop on Immunotoxicology to use the direct PLNA for monitoring chemicals with respect to their capacity to cause immune

activation[7] seems justified, especially because there are no false-positive compounds as far as is known. Based on the available experimental evidence, we consider the direct PLNA appropriate for the immunotoxicological screening of unknown chemicals that are likely to expose the human population by routes other than the skin. However, chemicals that cause inflammatory reactions with extensive necrosis at the site of injection should not be tested in this way for ethical reasons. Specifically, during drug development, the direct PLNA may be of great value since structure-activity studies may assist in the selection of the least immunogenic analogs or analogs with the least immunogenic metabolites. Because only small quantities of analogs and metabolites may be available at a point in the development of a chemical, it is worth mentioning that usually a dose of ~1 mg per mouse suffices to elicit positive responses.

It should be realized, however, that T-cell-dependent and -independent immune-activating chemicals provide different hazards with respect to adverse immune effects and that a positive response, as judged by weight or cell increase in the direct PLNA, cannot give definite proof of specific T-cell involvement. To obtain formal evidence of the latter, direct PLNAs with T-cell-deficient animals need to be performed or secondary responses to the chemical need to be measured. Presumably, studies in the near future will show that more sophisticated parameters in the direct PLNA, such as antibody isotype profiles (Section V) and particular phenotypic changes in PLN cell composition, predict T-cell involvement and, ideally, the predominant T-cell type, that is, Th1 vs. Th2 cells. A quite different potential application of the direct PLNA is the rapid screening for immunosuppressive chemicals by assessing their effects on the PLN response to a known antigen (Section II.H).

The adoptive transfer PLNA (Figure 2) by measuring secondary responses is suitable to assess specific T-cell sensitization toward a chemical or its metabolites, as recognized by the International Workshop on Immunotoxicology.[7] Another advantage over the direct PLNA is that immunogenicity of a chemical can be checked under conditions of likely human exposure. When guidelines allow the use of inbred strains, the adoptive transfer PLNA can be performed in adjunct to routine toxicity testing. Obvious disadvantages are that cell transfer procedures are laborious and prone to error and that additional animals are required as recipients. These disadvantages may be overcome by assessment of secondary PLN responses in the very animals that had been exposed earlier to the chemical as required by regulatory authorities (Section II.F).

For the time being, PLNAs are the only reliable assays for the screening of chemicals that cause human sensitization by routes other than the skin. PLNAs, however, have their limitations. They indicate hazards, not risks of immunotoxicity. A chemical's ability to sensitize animal T cells by the subcutaneous and other routes of exposure, as demonstrated with PLNAs, merely suggests that the chemical may cause one or more of numerous kinds of immune side-effects in an unknown portion of the human population exposed to it. The underlying reasons have been extensively addressed in this review, a main reason being that sensitization is much more frequent than manifestation of overt immune disease. Another issue concerning the significance of PLNAs is when to accept a negative result as negative, assuming proper performance of the PLNAs. This means the use of sufficient animals in treatment and control groups, the use of at least two genetically different strains to exclude the rare possibility of a nonresponder strain, and the use of a sufficient dose (up to 2 mg in the mouse when not contraindicated by severe reactions at the injection site). When results of guinea pig tests for dermal sensitization, frequently obtained at a point in the development of a chemical, were negative as well, it cannot be excluded yet that the negative result is caused by insufficient or inappropriate metabolic conversion of the chemical under conditions of the PLNA. This, as shown by studies with a few false-negative compounds, can be obviated by additional measures (Sections II.C, II.F, and IV.A). It is unknown, however, whether this is true for all false-negative chemicals, and warrants further studies.

B. PLNAs As a Research Tool

Immunotoxicology as an interdisciplinary science benefits from and contributes to its com-

posing sciences, immunology and toxicology. This is exemplified by the history and scope of application of the PLNA. Originally, the direct PLNA was applied to study the specificity of graft-vs.-host reactions.[34] Later, when it was realized that chemical-induced autoimmune disease could be the result of graft-vs.-host-like T-cell reactions to chemicals or their metabolites, the PLNA was introduced into immunotoxicology, as reviewed by Gleichmann et al.[38] As apparent from the present review, studies with this assay, and its modifications and extensions since then, have substantially furthered the insight into the mechanisms of chemical-induced immune diseases and probably will continue to do so. Thus, progress is to be expected as to the identification of immunogenic drug metabolites responsible for the immune side-effects of the nonimmunogenic parent compounds (Sections II.C. and III.A). This can aid diagnosis of adverse drug reactions in man by assessment of *in vitro* lymphocyte reaction toward the relevant metabolites, as has already been demonstrated in the case of gold(I) drugs. Furthermore, PLNAs can be instrumental in the delineation of the cell types and their metabolic systems that actually are responsible for the generation of immunogenic metabolites *in situ* (Section IV.A). In both respects, immunotoxicology should exploit the extensive knowledge on adduct formation elaborated in the field of genetic toxicology. Whereas genetic toxicologists study the formation of DNA adducts as a cause of carcinogenicity, immunotoxicologists should study protein adducts, that is, the chemically altered self-proteins or hapten-carrier complexes, that may trigger specific immune reactions to the chemical as well as native self-proteins (Section II.B).

Future PLNA experiments probing into the metabolic requirements of chemical immunogenicity also will advance the identification of risk factors. This is illustrated by the study with procainamide, which showed that both genetic polymorphisms of drug metabolism and external stimuli, namely, a stimulant of oxidative metabolism, determined the formation of immunogenic metabolites (Sections III.A and IV.A). Moreover, further insight into the role of the route of exposure in sensitization to chemicals can be obtained by using PLNAs for measurement of secondary responses (Section II.F) as well as by comparison of responses in the direct PLNA and the ALNA. Finally, detailed analysis of chemical-induced functional and phenotypic changes in local lymph nodes may yield not only more sensitive parameters for screening a chemical's immunotoxicity, but also more insight into the mechanisms of sensitization.

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