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The popliteal lymph node assay in mice: Screening of drugs and other chemicals for immunotoxic hazard

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Abstract. The popliteal lymph node assay (PLNA) in mice represents a predictive test for assessing the sensitizing (allergenic and autoimmunogenic) potential of drugs and low molecular weight chemicals. Measuring activation of the draining lymph node of the hind paw, the PLNA facilitates the detection and analysis of immunotoxic effects in a rapid and reproducible manner. An attractive feature of the PLNA is that it can be performed in combination with the routine toxicity testing required for new drugs. Thus, it is possible to investigate whether animals exposed by the oral, intravenous, or inhalative route have been sensitized to the test compound or a reactive metabolite of the test compound generated in vivo. PLNAs may be appropriate supplements to routine toxicity screening of chemicals, thereby enhancing chemical safety.

Key words: Assessment of immunotoxicity – Popliteal lymph node assay – Adverse immune reactions – Sensitization

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Introduction

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 side-effects of a chemical become manifest depends on conditions of exposure together with genetically determined intrinsic factors. This has been formally demonstrated, for instance, in animal experiments with mercuric chloride [1–7] and is supported by many observations from medical practice For instance, drugs such as penicillins and sulfonamides are capable of provoking a wide range of immunological diseases, including allergic contact dermatitis after topical treatment and autoantibody formation after systemic treatment [8, 9]. In terms of hazard identification, 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.

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Testing of chemicals by circumventing the skin barrier

Testing for sensitizing potential

Use of the popliteal lymph node assay (PLNA) as a predictive immunotoxicological test system in mice and rats has recently been reviewed [10, 11]. In the direct PLNA, a single injection of the test chemical, without adjuvant, is administered subcutaneously into one hind footpad on day 0. The contralateral footpad serves as an internal control and is usually left untreated Upon injection, the test chemical is transported via the afferent lymphatics to the nearest draining lymph node, the popliteal lymph node (PLN), where a primary immune response may take place. On a specified day after injection, usually day six, the left and right PLN are isolated and analyzed. Routinely, PLN weight is determined. Other, more sensitive parameters of PLNs are the cell count, cell proliferation measured as [^{3}H] thymidine incorporation, expression of cell surface markers determined by flow cytometry, and total immunoglobulin production. Results usually are expressed as a PLN index, which is the ratio of values obtained from the experimental and control side. A primary PLN response to most immunogenic chemicals peaks within the first 4 to 10 days after injection and returns back to normal by week 3 to 5, unless undegradable materials, such as quartz crystals (silica), are injected.

Applications. Since the introduction of the direct PLNA into immunotoxicology over 12 years ago (12), several research groups have shown that the assay is capable of recognizing a wide variety of sensitizing chemicals, including auto-immunogenic chemicals which, despite many different attempts, failed to produce manifest disease in the very mouse strains that clearly showed positive PLN responses. Moreover, it has been shown

Table 1. Examples of drugs and chemicals studied in PLNAs

Compound	Exposure	Adverse immune effects	Primary response in the direct PLNA in						Secondary PLN response to compound or reactive metabolite	Oxidative metabolism required for protein reactivity	References
			inbred mice				inbred athymic mice				
			compound only	compound preincubated		preformed reactive metabolite	compound only	preformed reactive metabolite			
				with MΨ[1]	with S9-Mix						
Aniline	environmental, occupational	TOS[2]	no	yes	nt	yes nitrosobenzene	no	no	yes	yes	[27] manuscript in preparation
Benzene	environmental, occupational	bone marrow toxicity	no	nt	nt	yes, benzoquinone	no	no	yes	yes	manuscript in preparation
Chloroplatinate	occupational	allergic asthma, dermatitis	yes	–	–	–	no	–	yes	no	[21, 46]
Diphenylhydantoin	antiepileptic drug	LLS, skin rash, lymphoma	yes	nt	nt	nt	no	nt	nt	no	[12, 47]
Gold(I) salts	antirheumatic drug	dermatitis HGG, IGN	no	yes	nt	yes, gold (III) salts	nt	no	yes	yes	[19, 28]
Mercury (II) chloride	occupational	contact dermatitis, IGN	yes	yes	–	–	nt	–	yes	no	[3, 7]
Procainamide	antiarrhythmic drug	LLS	no	yes	yes	yes, HAPA	nt	nt	yes	yes	[18, 29]
Propylthiouracil	antithyroid drug	LLS	no	yes	nt	yes, PTU-SO_3^-	no	no	yes	yes	[26]
Streptozotocin	antineoplastic agent	skin rash	yes	–	–	–	no	–	yes	no	[48–50]

[1] HAPA = N-hydroxyprocainamide, HGG = hypergammaglobulinemia, IGN = immune glomerulonephritis, LLS = lupus-like syndrome, MΨ = macrophages, PTU-SO_3 = propyluracil 2-sulfonate, TOS = toxic oil syndrome
[2] Caused by the contamination of cooking oil with aniline.
nt = not tested
– = not relevant

that the direct PLNA also can detect T-cell-independent immunostimulating chemicals, e.g. quartz dust (silica) 13, 14]. Together, these data demonstrate the robustness of the direct PLNA. In addition, this assay is fast, objective, relatively inexpensive, and simple to perform. Although 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 [15–17]. A problem, however, is the existence of false-negative results in the direct PLNA. Whether these, as demonstrated for a few compounds such as gold(I), procainamide, propylthiouracil, aniline, and benzene (Table 1), may be generally overcome by in vitro incubation of the test compound with metabolic systems such as S9 mix [18] or phagocytes [19] prior to assay awaits future studies, including those using metabolic systems such as 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 [16, 20] 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 [21] to use the direct PLNA for monitoring chemicals with respect to their capacity to cause immune activation seems justified, especially because there are no false-positive compounds as far as it 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 be exposed to 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 nearly 1 mg per mouse suffices to elicit positive responses.

It should be realized, however, that T-cell-dependent and T-cell-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 and particular phenotypic changes in PLN cell composition, predict T-cell involvement and, ideally, the predominant T helper 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 [22, 23].

Testing for specificity of the immune response

The PLN reaction that follows a single injection of a test compound does not tell whether the reaction is immunologically specific, i.e., mediated by *T cells with specificity for antigen.* T cells sensitized by antigen will differentiate into memory cells and, hence, may be identified by their capacity to *mount a secondary response*, which is featured by faster kinetics and lower elicitation doses than those required for primary reaction. In order to test for a secondary response by means of the PLNA, three different ways for T-cell priming may be used. Firstly, priming can be accomplished by treating the animals as for the direct PLNA. Upon complete resolution of the primary PLN response, usually after four to six weeks, the animals are challenged in the same paw with a lower dose of the same chemical, which is substimulatory in the direct PLNA [24]. Secondly, animals can be primed not via the hindfoot, but via a different route that is more relevant for human exposure, e.g. intranasally [25], orally [26], or intravenously [27]. The animals then are challenged by injection of the chemical into a hindfoot pad, using a dose that is too low to induce a primary PLN response Thirdly, the *adoptive transfer PLNA* may be used. This test measures secondary responses of T lymphocytes from a chemically-exposed donor animal following their transfer to a syngeneic recipient animal [19, 28, 29]. The donor animals can be treated with the test chemical under conditions of exposure as to dose, route, frequency, and duration that mimic the human situation. After a desired time of exposure, spleen cells or splenic T cells of the donor animals are transferred by subcutaneous injection into one hind footpad of recipient animals, using T cells of unexposed or solvent-exposed donors as negative control. One day later, the recipients are challenged at the same site by subcutaneous injection of the chemical at a substimulatory dose. The secondary T cell response is assessed three to six days after the challenge using the same read-out parameters as in the direct PLNA (see above). A positive response indicates that exposure of the donor animals had resulted in T cell sensitization and subsequent generation of memory T cells. While the adoptive transfer PLNA is more laborious than the direct PLNA, it has the advantage of directly proving the T-cell dependence of the response to the chemical.

Applications. The adoptive transfer PLNA for measuring secondary responses is suitable to assess specific T-cell sensitization towards a chemical or its metabolites, as recognized by the International Workshop on Immunotoxicology [21]. Using the adoptive transfer PLNA, specific T cell responses to chemicals not requiring metabolism [7] and to reactive intermediate metabolites formed in vivo [19, 28, 29] and in phagocytes in vitro [19, 30] have been detected (Table 1). When guidelines allow the use of inbred strains, the adoptive transfer PLNA can be performed as an 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 animals that had been exposed earlier to the chemical, as required by regulatory authorities.

Limitations of the PLNA

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 the numerous kinds of immune side-effects in an unknown portion of the human population exposed to it. A main reason for this is 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 correct performance of the PLNAs. This is addressed by 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 during the development of a chemical, are negative as well, it cannot be excluded at this point 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 [11, 18, 31]. It is unknown, however, whether this holds true for all false-negative chemicals, and warrants further studies.

Skin penetrating chemicals

Testing for the skin-sensitizing potential

The *auricular lymph node assay (ALNA)* is the test of choice when the *skin-sensitizing potential of chemicals* is to be assessed. Following topical application of a test compound to the ear skin of mice, the auricular lymph node assay, just like the direct PLNA, measures primary responses of the draining auricular lymph node usually by determination of cell proliferation by [^{3}H] thymidine incorporation. 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. [32].

An advantage of the ALNA is that, like the PLNA, adjuvants are not required. Moreover, it has been stated that there is no need for prior determination of maximal non-irritant concentrations because non-sensitizing irritants would not influence the ALN response [33]. Other investigators, however, have reported that some irritants do induce proliferative reactions [34, 35]. As concluded, on the basis of weight or cell increase, for primary immune responses in the direct PLNA, proliferative responses in the ALNA suggest, but do not prove, specific T-cell involvement.

Testing for specificity of the immune response against skin-penetrating chemicals

With regard to predictive assessment of potentially sensitizing chemicals, current regulatory toxicology guidelines only include a variety of guinea pig tests to identify skin sensitizers [36, 37]. All these tests measure secondary immune responses, which probably are Th1 cell-dependent, in the form of challenge-induced skin erythema and edema. 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 end-point which, moreover, can be obscured in the case of dyes and irritating chemicals [32]. 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 [36, 38].

Limitations of the ALNA

Skin testing, in general, 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. A similarity between the direct PLNA and ALNA is the relatively high incidence of false-negatives. Actually, four of ten chemicals classified as moderate sensitizers in the guinea pig maximization test appeared negative in the ALNA according to current criteria [39].

PLNAs as a research tool

The PLNA was introduced into immunotoxicology 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, as reviewed previously [11, 15, 40, 41]. 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 on the identification of immunogenic drug metabolites responsible for the immune side-effects of the non-immunogenic parent compounds. This can aid diagnosis of adverse drug reactions in man by assessment of in vitro lymphocyte reactions toward the relevant metabolites, as has already been demonstrated in the case of gold(I) drugs [11, 42]. 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 [19, 29]. 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 [43-45]

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 [29], which showed that both genetic polymorphisms of drug metabolism and external stimuli, namely, a stimulant of oxidative metabolism, determined the formation of immunogenic metabolites. 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 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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