Screening Tests for Autoimmune-related Immunotoxicity
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A large number of chemicals induce or exacerbate autoimmune-like diseases in man. Because of the complexity of processes involved, these adverse effects are often if not always missed in standard toxicity testing. To date no validated and generally applicable predictive animal model exists and only a few chemicals have actually been shown to induce adverse autoimmune effects in certain animals. The popliteal lymph node assay (PLNA) is a very promising animal test to (pre)screen for systemic immunosensitizing, including autoimmuneenetic potential. This review describes the essentials of the various PLNAs against the background of current understanding of chemically induced systemic immunostimulation. The most simple primary PLNA measures enlargement of the popliteal lymph node 6-8 days after subcutaneous injection of a chemical into the footpad. The primary PLNA can distinguish between immunostimulating (both sensitizers and irritants) and innocent chemicals but does not assess the involvement of T cells or immunosensitization. For this, but also for elucidation of relevant mechanisms, detection of anamnestic responses in secondary PLNAs or responses to reporter antigens in the modified PLNA are suitable. To date over 100 compounds (drugs and environmental pollutants) have been tested, and results show a good correlation with reported immunostimulating (both autoimmuneenetic and allergic) potential. Importantly, no false-negative chemicals were detected if metabolism was considered. The various types of the PLNA, but in particular the secondary and modified PLNAs, await extensive validation before they can be recommended as a standard test for autoimmuneenetic potential. Key words: autoimmunity, chemicals, popliteal lymph node assay, reporter antigens. — Environ Health Perspect 107(suppl 5):673-677 (1999).
http://ehpnet1.niehs.nih.gov/docs/1999/suppl-5/673-677/pieters.abstract.html

Various drugs and environmental chemicals or metabolites thereof collectively referred to as low molecular weight compounds (LMWCs) generally less than 1,000 Da can provoke the onset of autoimmune diseases (AID) (1-4). The pathogenesis of AID is a multifactorial and complex process, involving a large number of predisposing factors (major histocompatibility complex (MHC) haplotype, gender, metabolism, concurrent infections). Not surprisingly, it has proven very difficult to develop animal models that mimic certain AID, let alone LMWC-induced AID. Consequently, no generally applicable predictive animal models exist using symptomatic AID as an end point and a chemical's capacity to induce or exacerbate AID in man is hardly ever recognized in standard toxicity testing (1). Reasonably, predictive test models for autoimmuneenetic potential of xenobiotics should rely on detection of initiating immune responses rather than on symptomatic AID. The presently available popliteal lymph node assays (PLNAs) are very promising test systems that focus largely on initiating immunostimulatory effects of chemicals.

Autoimmune diseases are the ultimate clinical consequence of destructive immune responses triggered by unregulated adaptive responses to autoantigens (1,2,5). These unregulated adaptive immune responses are the consequence of breakthrough of immunologic tolerance and subsequent activation of autoreactive lymphocytes. According to current understanding in immunology, lymphocytes require two signals to become activated. Signal 1 is provided by receptors upon recognition of a specific antigen and signal 2 is antigen-non-specific and provided by costimulatory molecules and cytokines. Peripheral and probably also central tolerance is accomplished by recognition of an adequate antigen or reception of signal 1 in the absence of a nonspecific costimulatory or "helping" signal 2 (5,6). Consistently, induction of self-tolerance involves specific recognition of autoantigen leading to selective inactivation or destruction of autoreactive lymphocytes, and tolerance only exists for dominant autoantigens/epitopes. Signal 2 alone does not elicit an immune reaction (7).

To fully understand the strengths but also the limitations of the PLNA, we need to consider how the generation of signal 1 and/or 2 is incorporated into our present understanding of chemical-induced immunostimulation.

Generation of Neoepeptltes or Signal 1
Generally, LMWGs are too small to function as antigen by themselves, but provided that they are chemically reactive, they may bind to (self)-proteins, thus forming a hapten-carrier complex or chemically alter a (self)-protein otherwise. The self-protein can, for example, be a peptide-presenting MHC molecule to which the reactive chemicals can bind directly. Alternatively, LMWGs can influence antigen presentation so that previously cryptic epitopes are presented by the MHC molecules on antigen-presenting cells (APCs). These hapten-carrier complexes and cryptic epitopes are recognized as new, therefore collectively designated as neoantigens or neoeptiotes, and they can elicit signal 1 in neospecific T and B cells (7). Provided a costimulatory or second signal is induced by the chemical, immunostimulation and immunosensitization may follow.

Generation of Costimulation or Signal 2
Many if not all autoimmunogenic LMWCs can directly elicit immune responses without requiring additional stimulators (see "Poptileal Lymph Node Assays"). This indicates that these LMWCs have intrinsic adjuvant activity, which is also obvious because they induce an inflammation upon subcutaneous injection in the paw. This proinflammatory property of chemicals may in certain cases suffice to activate antigen-presenting dendritic cells and neoantigen-specific and autoreactive lymphocytes. In the underlying processes, costimulatory molecules on the surface of dendritic cells as well as immunomodulating cytokines also designated as second signals, are thought to be of pivotal importance.

The B cell as a costimulatory APC is considered to play a crucial role in eliciting autoantigen-specific immune reactions upon initial neoantigen-specific immunostimulation. A B-cell that presents an altered MHC molecule (a neoantigen) may receive a helping signal 2 from the respective neoantigen-specific T cell. In case the B cell recognizes an unaltered autoantigen with repetitive epitopes that crosslinks B-cell receptors (a so-called T-cell-independent type 2 antigen), the B cell will start to produce antigen-specific antibodies with a switched isotype. Among these T-cell-independent type 2 antigens are autoantigens such as DNA molecules or collagen. Notably, this type of immune response resembles noncognate stimulation provided by graft T-helper (Th) cells activated by allogeneic MHC molecules on host B cells during graft
versus host reactions. Clinical symptoms observed in chemical-induced systemic autoimmune derangements often resemble those seen in graft versus host disease (8).

The B cell may also directly recognize a neoepitope or a self-epitope of the neoantigen with its antigen-specific receptor. This B cell will then present a mixture of altered and unmodified autoantigens complexed to distinct MHC-II molecules on its surface. Initially, only neoantigen-specific Th cells will provide a helping signal 2 to the B cell. As a result, the B cell will start producing either neoantigen- or autoantigen-specific antibodies. More relevant in this case, the B cell also becomes an efficient APC expressing an effective level of costimulatory molecules. As a result of the latter, the B cell can provide a signal 2 that activates previously silent autoreactive Th cells. This process of changing specificity of a neoantigen-specific immune response to include autoantigens is an example of epitope or determinant spreading (9). As a result of this process, neoantigen- and autoantigen-specific T cells are concurrently present in immunosensitized animals. This would imply that the distinction between allergic and autoimmune responses induced by LMWCs can only be gradual, reflecting the relative antigenicity of the neoantigens and autoantigens involved.

In conclusion, reactive LMWCs are able to give rise to either signal 1 or 2 or both, but as signal 1 by itself leads to inactivation of specific lymphocytes, induction of costimulation (i.e., signal 2) may be regarded as more decisive in the initiation of (auto)immune responses than the nature of the neoantigens involved (5).

It is important to realize that induction of an autoimmune response alone is not sufficient to get an AID and usually autoimmune responses are downregulated to fade away. Obviously, other factors are involved as well. For example, number of LMWCs modulate not only initiating events but also the production of regulatory cytokines [for example, HgCl2 stimulates interleukin-4 production by mast cells (10)] or effector mechanisms [for example, procainamide and D-penicillamine inhibit complement factor C4 (11)].

### Popliteal Lymph Node Assays

At present, basically three variations of the PLNA can be recognized (Figure 1): the primary or simple PLNA, the secondary PLNA, and the modified PLNA.

**The Primary PLNA**

The primary PLNA is the most simple variation and is recommended in the screening of immunostimulating potential of, for instance, newly synthesized drugs. In the primary PLNA, the cell number in the popliteal lymph node (PLN) or the weight of the PLN is measured usually 1 week (6–8 days) after the test chemical (without additional adjuvant) is subcutaneously injected into one hind footpad of the test animal, either a mouse or a rat. Control animals are treated similarly with the respective vehicle only and the ratio (PLN index) of the response in treated versus control animals is a measure for immunostimulation.

Although the primary PLNA is capable of distinguishing immunostimulating from innocent chemicals, this assay in its most simple form fails to distinguish haptons or neoantigen-forming chemicals from mere inflammatory irritants. This deficit in discriminating these various types of chemicals may hamper proper hazard assessment and mechanism-based risk assessment and can be circumvented by direct measurement of T-cell–dependent immune reactions in the primary PLNA or the involvement of T cells in either the secondary or modified PLNA.

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**Figure 1.** Simplified representation of various popliteal lymph node assays. Of the secondary PLNAS, only the adoptive transfer PLNA is depicted. ELISPOT assay, enzyme-linked spot assay; PLNA, popliteal lymph node assay. See text for further details.

**Figure 2.** In the modified PLNA, either TNP–Ficoll or TNP–OVA is injected together with the chemical. Abbreviations: OVA, ovalbumin; PLNA, popliteal lymph node assay; TNP, 2,4,6-trinitrophenyl. As a T-cell–independent type 2 antigen that cannot be recognized by T cells, TNP–Ficoll delivers a strong signal 1 to the B cell so that this cell will produce TNP-specific IgM antibodies. However, to form switched isotypes (IgG1, IgG2a, or IgE), the TNP-specific B cell requires help from T cells, which in the case of injection of low molecular weight compounds are neoantigen specific. The T-cell–dependent antigen TNP–OVA can be recognized by B cells as well as by T cells, but the dose of TNP–OVA is too low to elicit a measurable immune response. To get an optimal antibody production toward TNP–OVA, adjuvant signals such as inflammatory cytokines are needed.
The Secondary PLNA

Secondary responses in previously sensitized animals. In the secondary PLNA, a previously sensitized animal is challenged with a suboptimal dose of the same chemical. Sensitization can be done by subcutaneous injection into the footpad as in the primary PLNA. Assessment of the specific anamnestic immune response, being faster in kinetics than a primary immune response, is determined as a PLN index within 4–6 days.

The adoptive transfer PLNA. The adoptive transfer PLNA demonstrates the involvement of T cells in a straightforward manner. Briefly, splenic T cells from previously sensitized animals are injected into the footpad of naive syngeneic recipient mice 1 day before a nonsensitizing dose of the same LMWC or a reactive metabolite is injected into the same footpad. The PLN index is again measured 4–6 days later. This PLNA appears suitable to combine with sensitization via a relevant route of exposure, which has been done for example, intramuscularly with Au(I) salts [reviewed in Bloksma et al. (22)].

The adoptive transfer PLNA has been used extensively by Gleichmann and colleagues, and in addition to proving the involvement of T cells for some chemicals they were also able to eliminate a number of false-negative autoimmunogenic chemicals (procainamide, propylthiouracil [PTU], and Au+) by detecting memory T-cell responses to their metabolites (n-hydroxyl-amino-procainamide, PTU-SO3-, Au3+) (13–15). These false-negative LMWC appeared to require bioactivation, which apparently is not sufficiently available in the uninflamed paw. Bioactivation, however, can be easily introduced into the PLNA by coinjection of an S9 mix or activated phagocytes.

Results obtained with the adoptive PLNA have also shown that mice immunized with the untreated non-self-model antigen bovine RNase in adjuvant respond exclusively to dominant epitopes, whereas injection of RNase preincubated with Au(III) elicited additional T-cell responses to cryptic epitopes (16). Using the adoptive transfer PLNA, it was also demonstrated that T cells, isolated shortly after initiating an autoimmune response with HgCl2, responded exclusively to unidentified mercury-modified self-proteins; later in the response, T cells responding to unmodified self-proteins were detectable as well (17). The first study shows that autoimmunogenic chemicals can provide signal 1 by releasing cryptic epitopes. The second study demonstrates that the immune response can spread from a hapten-or neoantigen-specific to a autoantigen-specific immune response. Together, they illustrate the usefulness of the adoptive transfer PLNA in increasing the fundamental knowledge of

Figure 3. Comparison of the immune response to reporter antigens TNP–Ficoll and TNP–OVA induced by a number of nonsensitizing irritants, allergens, and autoimmunogenic chemicals. Abbreviations: AFC, antibody-forming cells; FIA, incomplete Freund’s adjuvant; OVA, ovalbumin; TNP, 2,4,6-trinitrophenyl. Results are expressed as the number of antibody-forming cells per 106 popliteal lymph node cells, measured by enzyme-linked SPOT assay. Of note, streptozotocin stimulates a Th1-driven immune response, hence the occurrence of IgG2a instead of IgG1 isotype.

Table 1. List of chemicals tested in the popliteal lymph node assay.

| Category | Chemicals | No. | Literature |
|----------|-----------|-----|------------|
| Vehicles | Dimethyl sulfide, ethanol, acetone, saline | 4 | (18,22,23); Figure 3 |
| Antiarrhythmic | PA and metabolites n-acetyl-PA and n-hydroxylamino-PA | 3 | (14,18,23) |
| Antihypertensive | Hydralazine | 1 | (23,35) |
| Antituberculous | Isoniazid, p-aminosalicylic acid | 2 | (23) |
| Antipsychotic | Chlorpromazine | 1 | (23) |
| Anticonvulsant | Diphenylhydantoin + 43 structural derivatives | 50 | (22,23) |
| Antineuritic | p-Penicillamine | 1 | (18,34) |
| Antithyroid | Propylthiouracil and its metabolite propylthiouracil-sulfonate | 2 | (15) |
| Anti-inflammatory | Phenylbutazone, monophenylbutazone, 1-phenyl-3-pyrazolidone, ibuprofen, diclofenac | 5 | (23,35) |
| Antibacterial | Nitrofurantoin | 1 | (18) |
| Analgesic | Glaefine | 1 | (23) |
| Antihelminthic | Quinacrine, levamisole | 2 | (23) |
| Antischistosomal | Nisidazole | 1 | (23) |
| Alcohol deterrent | Disulfiram | 1 | (23) |
| Allergic, contact sensitizers | Dinitorhlorobenzene; oxazoline; trimellitic anhydride; α-methylene-γ-butyrolactone (tulipaline); p-phenylenediamine; 5-nitro-2-furaldehyde semicarbazone; mercaptopenithiazole; carbanilide; diethydithiocarbamate sodium; 1,3-dimethyl-2-thiourea; 1,3-diethyl-2-thiourea; 1,3-dibutyl-2-thiourea; 1-phenyl-3-(2-thiazolyl)-2-thiourea; hexachloroplatinate | 15 | (23,36); Figure 3 |
| Antidepressants | Zimelidine + 18 structural homologs, paroxetine, fluvoxamine, imipramine, maprotiline, nortriptilne, clomipramine, LICI, amitriptyline, nomifensine, ritanserin, serotonin | 30 | (24–27) |
| Metals | HgCl2, Au+*, Au3+ (metabolite of Au+*), NiCl2, CuCl2 | 5 | (13,16,18, 19,32,33) |
| Antineoplastic | Streptozotocin | 1 | (18); Figure 3 |
| Environmental pollutants | 2,3,7,8-TCDD, HCB, silica, diesel exhaust particles | 5 | (29–31) |
| Miscellaneous | LPS, IFN, CFA, glutamic anhydride, SDS, dichlorotioobenzene (innocent homolog of DNCB) | 4 | (28–31) |

Total | 131 |

Abbreviations: CFA, complete Freund’s adjuvant; DNCB, dinitorhlorobenzene; HCB, hexachlorobenzene; IFN, incomplete Freund’s adjuvant; LPS, lipopolysaccharide; PA, procainamide; SDS, sodium dodecyl sulfate; 2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenz-p-dioxin.

*False-positive (underlined) and false-negative (dotted) chemicals are underlined. Compounds tested in the modified PLNA are shown in italics, and chemicals tested in one of the secondary PLNAs are in bold. No. of tested chemicals without vehicles. *Positive in pure concentrations, but when used in concentrations lower than 20% (v/v) diluted in saline, they are negative.
The Modified PLNA

In the recently introduced modified PLNA, the local 2,4,6-trinitrophenyl (TNP)-specific antibody response to well-defined antigens TNP-ovalbumin (TNP-OVA) or TNP-Ficoll is analyzed after coinjection with a given LMWC (18). These antigens are chosen because they need different and (in relation to LMWC-induced autoimmune responses) relevant conditions to elicit a TNP-specific antibody response. A TNP-specific IgG response to the T-cell–independent antigen TNP-Ficoll can only occur in the presence of T-cell help, which is noncognate. Hence, in case of co-injection with an LMWC, a TNP-specific IgG response to TNP-Ficoll indicates soluble help from haptens of neoantigen-specific T cells (Figure 2) (26).

The T-cell–dependent antigen TNP-OVA, which requires cognate T–B cell interaction, is used in such a low dose (10 μg/mouse) that it does not cause a measurable immune response by itself. To get a TNP-specific antibody response to this low concentration of TNP-OVA, costimulatory adjuvant signals are needed, which may arise from inflammatory responses induced by a reactive chemical. So any reactive LMWC that induces an inflammation, including nonsensitizing irritant chemicals or adjuvants, will induce an IgG response to TNP-OVA.

The IgG response to these two reporter antigens may be used to discriminate between compounds that create neoantigens and thereby activate T cells (positive IgG response to both TNP–Ficoll and TNP-OVA, e.g., HgCl₂ [Figure 3]) and compounds that only cause an inflammatory response and do not sensitize neoantigen-specific T cells [positive response to TNP-OVA but not to TNP-Ficoll, e.g., the irritant glutaric anhydride or the macrophage stimulator silica (Figure 3)] (18). Surprisingly, the classic irritant sodium dodecyl sulfate induced an IgG1 response to TNP–Ficoll, suggesting that this compound induces formation of neoantigens upon subcutaneous exposure (Figure 3).

The modified PLNA with TNP-OVA has a number of additional advantages:

- In comparison to the number of lymph node cells, which ranges from 1 × 10⁶ cells/node in controls to 20 × 10⁶ cells/node in treated mice, the increase of the IgG response to this reporter antigen, which is measured as the number of TNP-specific antibody-forming cells (AFC), is considerably higher and ranges from 10 to 1,000 AFC/10⁶ PLN cells. This improves discrimination and ranking of a chemical's immunostimulating capacity.
- The type of immune response (cellular or Th1-dependent vs Th2-dependent) stimulated by a chemical can be analyzed easily by measuring the isotype specificity of the antibody response to TNP-OVA (IgG2a for Th1 and IgG1 and IgE for Th2). By doing so, the diabetogen streptozotocin stimulates an IgG2a response to TNP-OVA in BALB/c mice, whereas HgCl₂ induces IgG1 and also IgE-AFCs to the same antigen in the same mouse strain (19).
- By using congenic strains of mice, the type of the immune response to TNP-OVA can be linked to strain-specific susceptibility to adverse immune effects induced by a given LMWC. For example, HgCl₂ stimulates a higher IgG1 response to TNP-OVA in glomerulonephritis-sensitive B10s (H2s) mice than in resistant B10dd (H2d) mice (20).
- The modified PLNA with TNP-OVA also allows measuring of chemical-induced secondary responses (delayed-type hypersensitivity reactions; antibody-forming cells in bone marrow, spleen, and lymph nodes; and serum titers of TNP-specific isotypes) (19,20).

Thus, the use of well-defined antigens in the PLNA allows better hazard identification of a chemical's immunostimulating potential and in addition offers a relatively easy opportunity to perform mechanistic studies related to chemical-induced autoimmunity.

Overall Results Obtained with the PLNA

Presently, over 100 compounds have been tested, predominantly in the primary PLNA and mostly in the mouse but some also in the rat (21). The list of compounds (Table 1) includes many drugs of various categories (e.g., antiarrhythmics, anticonvulsants (22,23), antidepressants (24–27), and structural homologs of some drugs, but also a...
number of environmental pollutants, i.e., hexachlorobenzene (28), 2,3,7,8-tetra-chlorodibenzo-p-dioxin (29), diesel exhaust particles (30), and silica (31). Allergic compounds (e.g., dinitrochlorobenzene) (23) also induce a positive PLN index; the response in the PLNA appears to be dose dependent (23) and to vary with genetic background (both MHC and non-MHC genes) of the animals (31–34).

PLN responses show a good correlation with the reported ability of a compound to induce AID or contact dermatitis in man. If metabolism is considered, no false-negative LMWC are found. Among the false positives are strong irritants like ethanol, aceton, dimethyl sulfoxide (22), and sodium dodecyl sulfate (Table 1, Figure 3) unpublished observations as well as a few drugs such as quinacrine and niridazol (23).

Concluding Remarks
To date, no generally applicable predictive animal models for autoimmune potential of LMWC exist or are expected in the near future. However, the immunostimulating potential of LMWC can be screened in the primary PLNA, a simple, rapid, and objective assay to test an array of xenobiotics such as newly synthesized and structurally homologous drugs. Distinction of the involvement of T cells and discrimination between sensitizing and irritant LMWC can be accomplished in one of the secondary PLNAs; however, the adoptive transfer PLNA cannot be regarded as an early screening assay. The modified PLNA appears to be a very promising extension of the primary PLNA and a simple and fast assay to indicate the involvement of T cells. A possible strateg- ie scheme that may help to decide which PLNA is most useful in a certain occasion is depicted in Figure 4.

Positive responses in any of the PLNAs indicate the potential of a chemical to stimulate the immune system, suggesting that the chemical may induce AID in genetically predisposed individuals. However, the PLNAs and in particular the secondary and modified PLNAs await extensive further validation before they can be recommended as standard tests to identify immunostimulatory agents, including autoimmunogenic chemicals.