

The Reporter Antigen Popliteal Lymph Node Assay

UNIT 18.12

BASIC PROTOCOL

A large number of chemicals, including environmental pollutants and drugs, have the capacity to induce or exacerbate hypersensitivity reactions that may result in allergic or autoimmune phenomena in susceptible subjects. The causal mechanisms of these reactions are poorly understood, and there is evidence that etiology of the pathologies is multifactorial, i.e., they are dependent on the host (e.g., genetics), chemical, and additional environmental factors. As a result, these reactions are rarely, if ever, recognized in standard toxicity testing.

The popliteal lymph node assay (PLNA) provides a suitable tool to assess the immunomodulating potential of chemicals, and the PLNA might be a potential candidate as a screening tool in immunotoxicological hazard identification. The use of reporter antigens (RA) in this assay (so called because they report a certain potential) additionally enables differentiation between immunosensitizing chemicals (sensitizers), immunostimulating chemicals (irritants), and innocent chemicals.

By means of measuring the secretion of RA-specific antibodies in the draining lymph node 7 days after the initial footpad exposure, the RA-PLNA provides mechanistically relevant immunological information to study the immunomodulating capacity of chemicals.

Chemicals that are injected in the hind footpad of mice may provoke immunological changes in the draining lymph node located in the popliteal space, the PLN. Irritant chemicals, by acting as adjuvants, may have the capacity to enhance antigen-specific lymphocyte proliferation in the PLN, whereas immunosensitizing chemicals induce direct sensitization of antigen-specific T and B lymphocytes, producing cytokines and antibodies, respectively.

In the RA-PLNA, lymphocyte proliferation of PLN cells can be measured 6 to 7 days after a single footpad injection with a chemical. Coinjection of the chemical together with RA enables the determination of RA-specific immune responses in the PLN that are representative of the immunosensitizing, or merely immunostimulating, capacity of the compound of interest.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Materials

- Female mice (weighing ~20 g), 6 to 8 weeks old at time of arrival
- Standard laboratory mouse food pellets
- Test compound(s) and vehicle(s)
- T-dependent reporter antigen (e.g., TNP-OVA) and/or T-independent reporter antigen [e.g. TNP-Ficoll: available from Biosearch Technologies (<http://www.biosearchtech.com/>); prepare solutions according to manufacturer's instructions, test for immunogenicity in PLNA against positive control; store in aliquots; Inman, 1975]
- 70% ethanol
- Solid-bottom mouse cages with wood-chip bedding
- 1-ml syringes

Immunotoxicology

18.12.1

Contributed by Stefan Nierkens and Raymond Pieters

Current Protocols in Toxicology (2006) 18.12.1-18.12.5

Copyright © 2006 by John Wiley & Sons, Inc.

Supplement 29

25-G, 5/8-in. or 26-G, 1/2-in. needles

Dissecting equipment: forceps and dissecting scissors

Additional reagents and equipment for footpad injection of the mouse (Donovan and Brown, 2006a) and euthanasia of the mouse (Donovan and Brown, 2006b)

NOTE: All reagents should be screened for endotoxin.

Prepare the animals

1. Acclimate mice for ≥ 1 week in solid-bottom cages with wood-chip bedding. Provide drinking water and standard laboratory food pellets ad libitum.

Animals should be housed according to the guidelines of The Association for Assessment and Accreditation of Laboratory Animal Care (<http://www.aaalac.org/>).

2. Divide the mice randomly into groups of five to eight mice per group, one group for each treatment.

Use three concentrations of test compound and the appropriate vehicle as a control. Vehicle is selected based on physicochemical properties of the test compound.

Setup for a typical experiment would include:

A. Vehicle plus RA.

B, C, D. Test compound at three different concentrations, plus RA.

E. Positive control: e.g., 1 mg/mouse d-(–)penicillamine hydrochloride (Sigma).

IMPORTANT NOTE: *Do not use the contralateral PLN of the chemical-treated mice as a control, because they sometimes respond to the injection at the other side as well, but use a separate group of mice as a control group (receiving treatment with vehicle in combination with the RA only).*

Administer the test compound and the reporter antigen

3. Prepare compound of interest as a solution or fine suspension in a volume of vehicle equal to the volume that will finally be needed minus the volume of RA stock solution that will have to be added. After obtaining the test compound in complete solution or fine suspension, add the appropriate volume of RA stock solution.

The concentration of the RA should be sufficiently low that the RA itself does not stimulate the formation of antibody-forming cells. This has to be checked each time a new stock of the RA is prepared. RA stocks, usually in mg/ml concentrations, are stored in aliquots of 50 to 100 μ l at -20°C . Usually, the amount of RA (for TNP-OVA as well as TNP-Ficoll) used is 10 μ g/mouse.

The vehicle to use depends on the solubility properties of the compound, but, if possible, saline is the preferred vehicle. Other vehicles should be carefully tested for their reactivity in the RA-PLNA. If, for instance, DMSO, ethanol, or acetone is used, the maximum volume injected should not be >10 μ l. Alternatively, these vehicles can be diluted in saline, to a maximum concentration of 20% (v/v), so that 50 μ l of the solution can be injected.

The dosage of the compound depends on its general toxicity, but, mostly, dosages ranging from 0.1 to 2 mg/mouse are used. Occasionally, dosage should be lower, for instance HgCl_2 should not be used at >50 μ g/mouse.

4. Inject the solution subcutaneously (Donovan and Brown, 2006a) into the hind footpad of the mouse in either a heel-to-toe or toe-to-heel direction in a total volume of 50 μ l using a 1-ml syringe with (preferably) a 25-G, 5/8-in. or 26-G, 1/2-in. needle attached.

For injection of volumes <50 μ l, a gas-chromatograph syringe with small volume should be used, preferably with needles of the abovementioned sizes.

5. Check mice daily for discomfort, irritation of the paw, or other general signs of illness.

Dissect the PLN

6. Sacrifice the animals 6 or 7 days after footpad injections by cervical dislocation or CO₂ asphyxiation (Donovan and Brown, 2006b).
7. Moisten the hind leg with 70% ethanol to avoid contamination. Hold the paw between the thumb and forefinger of one hand and make a small incision in the skin covering the heel, without cutting the tendon. Use the other hand, or forceps, to grab the skin.
8. Slowly strip the skin from the leg, far enough to access the knee joint. Stretch the paw and use tweezers to dissect the PLN. Separate the lymph node from adherent fatty tissue.

When dissecting the PLN, do not squeeze it, but gently lift it from the surrounding tissue.

Test PLN for response to reporter antigen

9. Use the dissected lymph node for preparation of single-cell suspensions to be used in ELISpot assays (or to determine other parameters).

For instance, for assessment of responses to RA, cells can be used in an enzyme-linked immunosorbent spot (ELISpot) assay to determine the formation of RA-specific antibody-forming cells (AFC; Schielen et al., 1995). In addition, cells can also be restimulated ex vivo with RA, T cell mitogen (e.g. concanavalin A), or other stimuli (LPS, anti-CD3) for detection of cytokines (Nierkens et al., 2002). In addition, cell surface markers can be assessed by flow cytometry (UNIT 18.8) to identify shifts in cell subtypes (e.g. CD4⁺ and CD8⁺ T cells, B cells, macrophages, and dendritic cells) or in activation status (e.g. CD25, and CD40L on T cells, CD80, CD86 on dendritic cells).

COMMENTARY

Background Information

The PLNA focuses on the initiation of lymphocyte proliferation in the draining lymph node of a mouse upon footpad injection of the test compound. The immunostimulatory potential of tested chemicals (currently over 130) as determined with the PLNA correlates well with the known immunostimulatory effects of these compounds in man (Bloksma et al., 1995). This test has, therefore, been recognized as a potential screen for identifying the immunotoxicological hazard of immunostimulation.

The PLNA was originally developed to investigate the immunostimulating effects of autoimmunogenic drugs. The parameters used—PLN size, weight, cellularity, and lymphocyte proliferation—are indicative for immunostimulation. The introduction of reporter antigen (RA) additionally makes it possible to differentiate between sensitizing drugs, nonsensitizing drugs with adjuvant activity, and “innocent” drugs (drugs with no detectable immunomodulatory activity; Albers et al., 1997; Gutting et al., 1999, 2002) by means of the specific immunological requirements for obtaining an IgG antibody response to the RA.

Two well-defined antigens, trinitrophenyl-ovalbumin (TNP-OVA; a “regular” T cell-dependent antigen, or type 1 antigen) and

TNP-Ficoll (a so-called T cell-independent, or type 2 antigen), are used in the RA approach for the PLNA. The RA and chemical of interest are injected in a mixture. When the coadministered chemical has adjuvant-like properties, an increased immune response to the RA TNP-OVA will occur (formation of TNP-specific IgM and IgG AFC, determined by ELISpot). The RA TNP-Ficoll, on the other hand, is a non-protein-sugar type antigen unable to activate T cells by itself but able to stimulate IgM production by B cells. B cells that are triggered by TNP-Ficoll are susceptible to T cell help and will, with that help, produce IgG antibodies. It is unknown what antigenic determinants are actually recognized by these chemically induced T cells, but they may include either conjugates of the chemical with host proteins (so-called hapten-carrier complexes) or other neoantigens (parts of an autoantigen). Thus, increased production of IgG in case of coinjection of chemical and TNP-Ficoll indicates chemical-induced T cell help. After prolonged and systemic exposure to TNP-Ficoll there is a possibility that the IgG response simply results from inflammatory enhancement leading to nonspecific increases in stimulatory cytokines. IgG1 response to TNP-Ficoll is highly susceptible to noncognate or neoantigen-specific help.

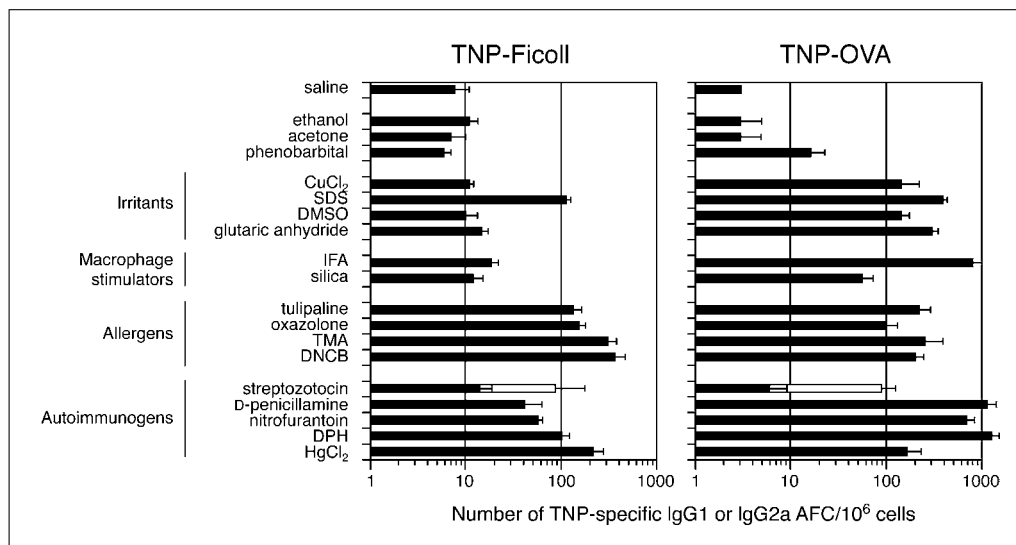


Figure 18.12.1 Typical results of a series of compounds tested with either TNP-Ficoll or TNP-OVA. Number of TNP-specific antibody-forming cells (AFC), expressed per 10^6 PLN cells, were determined by ELISpot assay. Black bars represent IgG₁; the white stippled bars represent IgG_{2a}, in case of streptozotocin. Abbreviations: DMSO, dimethylsulfoxide; DNCB, dinitrochlorobenzene; DPH, diphenylhydantoin; IFA, incomplete Freund's adjuvant; SDS, sodium dodecyl sulfate; TMA, trimellitic anhydride.

With the use of TNP-OVA and TNP-Ficoll, the RA-PLNA indicates whether a chemical can stimulate an immune response in a non-specific manner (through adjuvant or proinflammatory activity, i.e., IgG response to TNP-OVA but not to TNP-Ficoll), or whether a chemical can specifically sensitize T cells (IgG response to TNP-Ficoll).

In summary, the specific requirements for obtaining an IgG antibody response to TNP-OVA or TNP-Ficoll make it possible to differentiate between sensitizing chemicals, chemicals that are nonsensitizing but which possess adjuvant activity, and innocent chemicals (Albers et al., 1997; Gutting et al., 1999, 2002).

An important additional advantage of the RA approach is that it circumvents the need to know the exact specificity of these chemical-induced responses and provides a standardized parameter irrespective of the chemical that is investigated. The assay also has an important disadvantage in that the chemicals are injected subcutaneously in the footpad, whereas most chemicals are administered orally or applied topically. As a result, the PLNA may produce false negatives when the drug requires (hepatic) metabolism to induce reactive metabolites responsible for the adverse immunological effects (e.g., procainamide, sulfamethoxazole).

Critical Parameters and Troubleshooting

Vehicles must be carefully selected. It is recommended to use sterile saline or PBS. For hydrophobic compounds, organic vehicles may be considered. Some of these, such as DMSO, may elicit positive immune responses in the RA-PLNA with TNP-OVA and should not be used. It is always imperative to include a control group that is injected with the RA alone, diluted in the vehicle used.

Differences in pH of the solutions may result in different PLNA outcomes. For instance the antirheumatic drug D-penicillamine induces a positive PLN response when injected at low pH (≤ 2), but a negative response when injected in a solution with a pH of 5 or higher.

If no IgG is detected after injection of the positive control, it may be due to too low a ratio of TNP bound to OVA or Ficoll (< 3 TNP per molecule). If this occurs, prepare new RA with a higher ratio of TNP per molecule (preferably > 10).

Anticipated Results

The cell numbers of the PLNs indicate whether a chemical is able to stimulate the immune system or not, but responses against RA also make it possible to discriminate chemical-induced sensitization from mere adjuvant activity. Numbers of AFC are determined by the

ELISPOT assay, and, in control (saline-treated) mice, are <10 per 10^6 cells. For stimulated lymph nodes, >1000 spots per well can be found. Variation between mice can be quite extensive, with standard errors of the mean (SEM) of 10% to 30%.

Figure 18.12.1 show the responses of several compounds in the RA-PLNA.

Time Considerations

Mice must be acclimatized to their environment ≥ 1 week prior to footpad injections. Generally optimal PLN responses are measured at day 6 to 8.

Literature Cited

- Albers, R., Broeders, A., van der Pijl, A., Seinen, W., and Pieters, R. 1997. The use of reporter antigens in the popliteal lymph node assay to assess immunomodulation by chemicals. *Toxicol. Appl. Pharmacol.* 143:102-109.
- Bloksma, N., Kubicka-Muranyi, M., Schuppe, H.C., Gleichmann, E., and Gleichmann, H. 1995. Predictive immunotoxicological test systems: Suitability of the popliteal lymph node assay in mice and rats. *Crit. Rev. Toxicol.* 25:369-396.
- Donovan, J. and Brown, P. 2006a. Parenteral injections. In *Current Protocols in Immunology* (J.E. Coligan, B.E. Bierer, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 1.6.1-1.6.9. John Wiley & Sons, Hoboken, N.J.
- Donovan, J. and Brown, P. 2006b. Euthanasia. In *Current Protocols in Immunology* (J.E. Coligan, B.E. Bierer, D.H. Margulies, E.M. Shevach, and W. Strober, eds.) pp. 1.8.1-1.8.4. John Wiley & Sons, Hoboken, N.J.
- Gutting, B.W., Schomaker, S.J., Kaplan, A.H., and Amacher, D.E. 1999. A comparison of the direct and reporter antigen popliteal lymph node assay for the detection of immunomodulation by low molecular weight compounds. *Toxicol. Sci.* 51:71-79.
- Gutting, B.W., Updyke, L.W., and Amacher, D.E. 2002. Investigating the TNP-OVA and direct popliteal lymph node assays for the detection of immunostimulation by drugs associated with anaphylaxis in humans. *J. Appl. Toxicol.* 22:177-183.
- Inman, J.K. 1975. Thymus-independent antigens: The preparation of covalent, hapten-Ficoll conjugates. *J. Immunol.* 114:704-709.
- Nierkens, S., van Helden, P., Bol, M., Bleumink, R., van Kooten, P., Ramdien-Murli, S., Boon, L., and Pieters, R. 2002. Selective requirement for CD40-CD154 in drug-induced type 1 versus type 2 responses to trinitrophenyl-ovalbumin. *J. Immunol.* 168:3747-3754.
- Schielen, P., van Rodijnen, W., Tekstra, J., Albers, R., and Seinen, W. 1995. Quantification of natural antibody producing B cells in rats by an improved ELISPOT technique using the polyvinylidene difluoride membrane as the solid support. *J. Immunol. Methods* 188:33-41.

Contributed by Stefan Nierkens and
Raymond Pieters
Institute for Risk Assessment Sciences
Utrecht University
Utrecht, The Netherlands