

Popliteal lymph node (PLN) assay to study adjuvant effects on respiratory allergy

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Abstract

Different variants of the popliteal lymph node (PLN) assay have been published. Here we describe the adjuvant popliteal lymph node assay, an immune response assay to study the adjuvant activity of soluble substances as well as particulate matter. The substance to be studied for adjuvant activity is injected into the hind footpad of mice or rats together with an antigen. Adjuvant activity is determined as the increase in PLN weight and cell numbers in animals receiving antigen together with the substance under study, compared with PLN weight and cell numbers in animals given the antigen without the substance in question, and animals given the putative adjuvant alone. Because lymph node weight and cell numbers are immunologically non-specific parameters, specific immune response assays like serum antibody responses or antibody-forming cell numbers should additionally be performed. Different antigens and immune response assays may be used, depending on the research question asked. In relation to respiratory (or food) allergy, the assays should as a minimum include determination of specific IgE in serum, and preferably also IgG1 (mouse). Serum specific IgG2a antibody determination may be added to get an indication of the Th1–Th2-balance of the response. The adjuvant PLN assay, with cellular response assays performed in the draining popliteal lymph node and antibody determinations in serum, requires small amounts of test material. The assay offers a practical, sensitive and reproducible method to determine the adjuvant activity of soluble substances as well as particulate material, with the possibility to also perform mechanistic studies.

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1. Introduction

1.1. Anatomic and functional basis for the PLN assays

Mice and rats have a large sole, the footpad, under their hind legs (Fig. 1). The footpad contains enough loose tissue to allow the injection of sufficient volumes of fluid for experimental purposes. The lymphatic drainage from the footpad appears to be exclusively into the popliteal lymph node (PLN), behind the knee, both in the mouse [1] and the

rat [2]. Some investigators have suggested that a small fraction of the lymphatic drainage from the footpad is to the exterior inguinal lymph node [3]. This observation may, however, be due to further dissemination of injected material, as discussed by Closs [4]. Anyway, material injected into the footpad will, if not retained there, predominantly if not exclusively drain to the popliteal lymph node. The immune response to foreign material that enters the body takes place in the lymph node draining the portal of entry [5], and the popliteal lymph node therefore will be the exclusive site of primary immune responses to material injected into the footpad. This makes the footpad–PLN model particularly well suited for cellular response studies. Additionally comes the apparently very high sensitivity of

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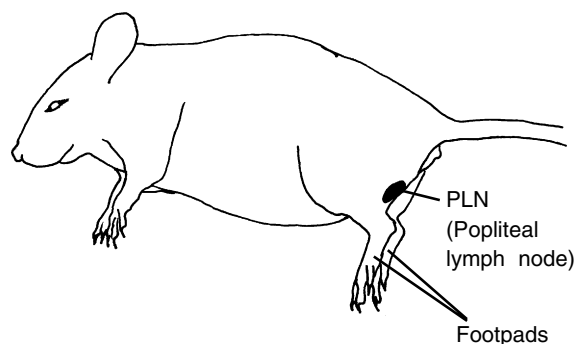


Fig. 1. Footpads and PLN (popliteal lymph nodes) in a small rodent (redrawn after [1]).

the footpad in terms of supporting the initiation of an immune response (the footpad is a “highly immunogenic site”) (see below). Further, the popliteal lymph node is relatively large (see below), and is so easy to find that it is possible to locate and remove even in live, anaesthetized animals (Løvik, unpublished material). All of this contributes to making the popliteal lymph node a unique and sensitive site for studying immune responses to material injected into the footpad.

1.2. Historical background

Because of the anatomical advantages, the footpad injection–PLN response approach has a long history of use for the study of immune responses. It has been used to address research questions in relation to infectious diseases, cancer, transplantation immunity, toxicology and allergology.

The footpad injection route in the mouse was taken into use to establish a mouse model of human leprosy, or to at least find a way to grow the non-cultivable *Mycobacterium leprae*. The reasoning was that the lower temperature of the peripheral part of the leg would offer better opportunities for bacterial growth. Indeed, limited bacterial growth in the footpad was achieved [3,6]. Closs [4] used the footpad injection route in a model of *Mycobacterium lepraemurium* infection, with determination of bacterial growth both in the footpad and the popliteal lymph node as well as histological examination of both sites. This model was further developed by Løvik [7,8]. The footpad–PLN model has been used also to study microbial growth and the immune response to other microorganisms, e.g. *Leishmania* and BCG [9–11].

Similarly, the PLN has been used to study the cellular reaction to lymphoma cells and other malignant cells injected into the footpad [12].

In relation to transplantation immunity, the PLN assay has been used to study graft-versus-host reactions (GvH reactions) after injection, e.g. of parental lymphocytes into the footpad of F1 hybrid rats which resulted in an enlarged PLN node because of the GvH reaction [13–15]. Extensive immunological studies of the popliteal lymph node cells were performed in this type of experiment, with lymph node enlargement as the basic parameter for the response.

Gleichmann [16] reported that the injection of the anti-convulsant drug diphenylhydantoin (DPH) into the footpad of mice induced a weight increase in the homolateral PLN similar to that seen after injection of heterologous cells causing a GvH reaction. Further studies demonstrated that many drugs and chemicals known to induce systemic autoimmune reactions in humans caused PLN reactions after injection into the footpad [17–20]. Results could be reproduced blindly between laboratories [21]. The driving force behind the development of the PLN assays has been the need to develop assays to predict the autoimmunity-inducing [18,20,22,23] capacity, and to a lesser degree the allergy sensitizing capacity of low-molecular weight chemicals and drugs [24–27]. In addition to the original direct PLN assay, secondary and adoptive PLN assays have been described (see below) [26], and the modified PLN assay with reporter antigens was introduced by the group of Pieters [28]. We introduced the PLN assay as a tool to screen for adjuvant activity of particles and substances in relation to IgE-mediated allergy against common environmental (and food) protein allergens (adjuvant PLN assay) [29].

1.3. Overview of PLN assay modifications

1. *Direct PLN assay*: Day 0: The test compound is injected subcutaneously into one hind footpad. Day 7: Harvesting of popliteal lymph node, determination of weight and cell numbers. Supplementary studies on lymph node cells may be performed (histology, flow cytometry, etc.).
2. *Secondary PLN assay*: Pre-sensitization (weeks to months): the animals are sensitized by immunization with the test compound via a relevant route. If footpad immunization, sufficient time must be allowed for the PLN to normalize after the immune response. Day 0: Footpad challenge with low (suboptimal) dose of test compound. Day 4–6: Harvest of popliteal lymph node, determination of weight and cell numbers.
3. *Adoptive PLN assay*: Donor animals are sensitized with the test compound. Day-1: Spleen cells suspensions are prepared and cells are irradiated, and injected into recipient animal's footpad. Day 0: Footpad challenge with low (suboptimal) dose of test compound. Day 4–6: Harvest of popliteal lymph node, determination of weight and cell numbers.
4. *Modified PLN assays with reporter antigens*: Day 0: The test compound is injected subcutaneously into one hind footpad together with the T-cell dependent reporter antigen TNP-ovalbumin and the T-cell independent reporter antigen TNP-Ficoll. Day 7: Harvesting of popliteal lymph nodes, and determination of weight, cell numbers and numbers of anti-TNP-Ficoll and anti-TNP-ovalbumin antibody producing cells.
5. *Adjuvant PLN assay*: Day 0: Putative adjuvant test substance and antigen/allergen are mixed and injected into one hind footpad of two parallel test groups. Day 6:

Harvest of PLN from one of the parallel groups (PLN group), weight and cell number determinations, supplementary cellular studies. Day 21: Antigen/allergen booster dose of second parallel animal group (serum group). Day 26: Exsanguination of the second parallel animal group, serum preparation for antibody measurements.

2. Description of methods: adjuvant PLN assay

The protocol is based on the paper by Løvik et al. [29] and unpublished preparatory work for that study, together with later studies.

2.1. The principle of the PLN assay used to study adjuvant activity

The substance to be studied for adjuvant activity is injected into the hind footpad of mice or rats together with an antigen. The adjuvant activity is determined as an increased immune response in animals receiving antigen together with the substance under study, compared with the immune response in animals given the antigen without the substance in question, and the putative adjuvant substance alone. Different antigens and immune response assays may be used, depending on the research question asked. Ideally, the antigen should be one that is relevant for the putative adjuvant substance under study (e.g. cat allergen in the study on endotoxin adjuvant effects by Ormstad et al. [30]). However, the antigen must be one for which the laboratory has available the necessary immune response assays, in relation to respiratory allergy in particular an assay to measure specific IgE production, and preferably also specific IgG1 and IgG2a (mouse). In our laboratory, we have made extensive use of ovalbumin as a standard experimental allergen, but also other allergens like the cat allergen Fel d 1, [30] have been used. Depending on the immune response assays used, the adjuvant PLN assay can be used to measure *quantitative* adjuvant activity (e.g. increase in IgE production), and adjuvant activity causing *qualitative* changes in the immune response (e.g. an increase in Th2-dependent IgE and IgG1 antibody production without a concomitant increase of Th1-dependent IgG2a antibodies), as well as mechanistic studies.

2.2. Animals

The mouse as well as the rat [19–21,24,27,31–34] can be used for the PLN assays, and there are many publications with each species in the literature. Generally, there is no definitive evidence that one specific species, mouse or rat, is more suitable than the other [35]. The size of the PLN is about ten times larger in the rat than in the mouse. Consequently, the rat will give more material for cellular studies, and the larger size of the rat PLN

may also reduce experimental variation because it is easier to handle and to trim free of fat. However, immune response properties of different strains and availability of natural and man-made genetic variants, availability of reagents for cellular and serological studies, as well as practical considerations in relation to cost and housing, will influence upon the choice of species. Our own experience with the PLN assay used to study adjuvant activity of substances and particles is limited to the mouse.

Genetically determined responder properties in relation to the antigen must be considered, and there is evidence that genetic factors may play a role also in relation to the adjuvant effect [36,37]. Eight animals per group has been found suitable in many of the published studies, but statistical considerations together with the need for sufficient experimental material must determine the size of the experimental groups. Our experience is that antibody responses are much stronger when antigen is given by footpad injection, compared to intranasal instillation (Samuelsen et al., manuscript in preparation). When serum antibody measurements are planned in addition to the PLN assay, parallel groups are necessary. Antibody-forming cell determinations (plaque assay) in the PLN may to some degree substitute for serum antibody determinations (analogous to the modified PLN with reporter antigens, cfr. above) and thereby reduce the number of animals needed and the duration of the experiment [28], but even in a secondary response the number of IgE-producing cells may be low [38].

In the PLN adjuvant assay it is necessary to distinguish between the effects caused by the increased response to the antigen and the effects caused by the adjuvant directly (e.g. inflammation), by comparing the response to the antigen given with and without adjuvant as well as the response to the adjuvant alone. Control groups must be injected with adjuvant alone, allergen alone, and vehicle alone, in addition to the test groups receiving the adjuvant-antigen combination.

2.3. Allergen

The selection of allergen/antigen will depend on the assays for antibody determination available in the laboratory. In relation to respiratory (and food) allergy, an assay for specific IgE to the antigen is obligatory, and determination of IgG1 is often done, and also IgG2a measurements. The relevance of the putative adjuvant–allergen combination may be considered. The antigen dose will depend on the immunogenicity of the antigen. Contamination by substances with adjuvant activity, in particular endotoxin, must be checked. To optimally detect adjuvant effects, an antigen dose should be selected that will give a low but detectable response in the most important read-out parameter without adjuvant. With ovalbumin, doses from 10 to 100 µg have been successfully used [29,39,40], and with Fel d 1 a dose of 5 µg [30].

2.4. Adjuvant

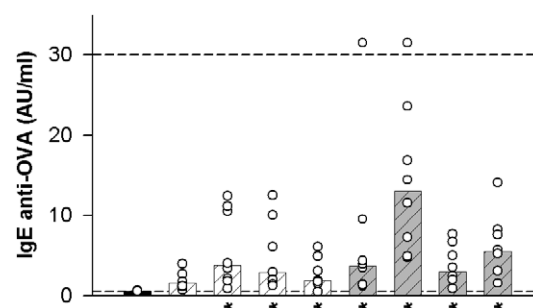
Soluble substances as well as particulate matter can be tested [29,30]. Practical problems with sedimentation and solubility may be encountered. Particle preparations may need agitation or sonication to obtain a homogenous suspension. If the test substance is poorly soluble, DMSO or similar compounds may be used to improve solubility, but possible adjuvant or inhibitory effects of such compounds must be controlled for. Hydrophobic particles may be more easily brought into a homogenous suspension if additional protein (homologous serum should be used) is added to the medium. Some protein coating of the particles before entering the tissues may be considered physiological, as mucosal surfaces are covered by fluid layers containing proteins and other high-molecular weight substances that presumably are adsorbed to particles before the particles interact with cells. Proteins may potentially influence the response to the particles. We have in some studies used up to 8% homologous mouse serum with diesel exhaust particles [29]. An even higher serum concentration may help slow down the precipitation of the particles in the suspension to be injected [22]. Various methods for suspension and mixing of particles have been found to give little difference in experimental outcome (Samuelsen et al., manuscript in preparation).

Regarding the dose of adjuvant, one must consider toxic and irritant properties, and what doses can be considered relevant in relation to the research question asked. Dose-response studies indicated 100 µg of diesel exhaust particles to be an optimal dose in the original publication [29], but a dose of 50 µg has successfully been used in later experiments [40,41]. Ambient air particles have been used in doses of 100–200 µg [40,41], and polystyrene particles in doses from 40 to 200 µg [42]. Practical limitations may be encountered with particle preparations. Different doses must be tested, as a too low (Fig. 2) or too high dose may fail to reveal differences between adjuvant preparations.

2.5. The injection procedure

The maximum volume that can be injected into the footpad of a mouse is about 50 µl and for a rat 100 µl [35]. However, although injected fluid is rapidly removed from the footpad, our experience is that with a high volume the risk of injected material leaking out is considerably increased. Also, there is the possibility that a larger volume may cause discomfort in the animal (see below). We have therefore routinely used 20 µl as the injected volume. For precision delivery and to cause as little trauma to the tissues as possible, a 100 µl Hamilton syringe (Hamilton Bonaduz AG, Switzerland) with a 30 G needle (Becton, Dickinson and Co., Dublin, Ireland) should be used. It is practical to insert the mouse head first into a 50-ml tube with breathing holes in the bottom end (Fig. 3). Rats may be rolled up in a towel. The dose should be injected

A 200 µg single dose



B 50 µg single dose

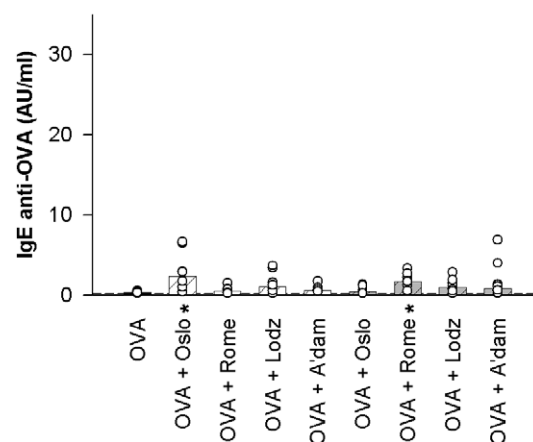


Fig. 2. The levels of IgE anti-OVA in BALB/cA mice 25 days after footpad sensitization with OVA and European cities ambient particles followed by a challenge with OVA into the same footpad 20 days later, are shown. Ambient particles were given as 200 or 50 µg single doses shown in (A) and (B) respectively. Individual values (points) and medians (columns) for groups of eight mice are shown. The dotted lines indicate the upper and lower detection limits of the ELISA assay. Coarse and fine ambient particle columns in the presence of OVA are shown in hatched white and hatched grey, respectively. The stars indicate a statistically significant stronger response compared to OVA ($p \leq 0.05$). The data were generated under RAIAP, a European Commission 5th Framework Programme shared-cost research project QLK-CT-2000-00792.

subcutaneously without the use of anaesthesia, with the needle inserted from the heel in the direction of the toes (Fig. 4). A long subcutaneous needle passage helps prevent fluid leakage. Intracutaneous injection must be avoided.

The fine needle quickly gets dull from penetrating the skin. The needle should be changed at frequent intervals, because it is essential for a good result that the needle is sharp.

2.6. Time schedules

In preparatory experiments for the original study [29], it was established that with 100 µg diesel exhaust particles as adjuvant and 100 µg ovalbumin as allergen, the reaction in the PLN peaked 5–6 days after footpad injection (thymidine incorporation tended to peak somewhat earlier). Six days after inoculation was therefore chosen as the standard

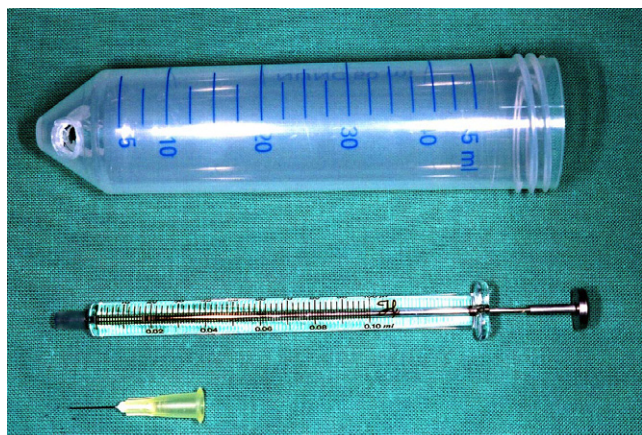


Fig. 3. The picture shows necessary equipment for footpad injection: a 50 ml tube with breathing holes, a 100 µl Hamilton syringe, and a 30 G needle.



Fig. 4. The picture shows how the mouse is held in a 50 ml tube during footpad injection.

time for harvesting the PLN. Without a booster dose, IgE levels started to rise late, from week 4 to peak at week 9 [29], but this presumably will depend on the adjuvant and the dose of allergen. We therefore found it useful to give a booster dose with allergen alone in the footpad on day 21, which accelerated the kinetics of the IgE response. Further, day 5 after the booster was found optimal for serum IgE determination. Other antibody isotypes must be expected to show different kinetics [29]. For cellular responses, we have observed a peak about two days after the booster dose (unpublished data).

2.7. PLN harvest and cell preparation

As described above, the PLN is located in the *fossa poplitea* on the back side of the knee (Fig. 1). However, in particular in control animals with small nodes, it is essential not to disturb the tissues when looking for the node. A slit is made in the skin below the knee with iris scissors, and the skin is thereafter pulled upwards towards the

thigh, leaving muscle and fat tissue in place. Careful dissection can then be done. If the knee is broken backwards, the PLN if enlarged often will pop out. The PLN has a very constant location in the fork of a large branching artery. The grey colour and firm consistence of the lymph node contrast well with the white transparent colour of the soft fat tissue. When placed in medium, the lymph node will sink while fat tissue will float.

Single cell preparations can be made in different ways. What is best, will in part depend on the size of the lymph nodes. One way, if the nodes are large, is to crush the lymph nodes against a 200-µm stainless steel grid placed in a petri dish with medium, using the butt of a disposable syringe [35]. Another way, particularly if the nodes are small, is to crush them between the frosted ends of two microscopy glass slides, and flush the cells into a Petri dish with medium. A third method, the one most used in our laboratory, is to tease the nodes apart with two 18G injection needles in medium droplet in a Petri dish. With all methods, aspiration of the cells in and out of a narrow pipette or syringe fitted with a needle to break up clumps is advisable. The cells may be counted in a microscope, or on a cell counting instrument.

2.8. Popliteal lymph node assay endpoints

The traditional way of expressing the PLN response is as a response index, generated by dividing the measured value of the PLN on the side injected with test substance with the value of the PLN on the contralateral side injected with vehicle alone (or even un-injected in screening studies in case the vehicle alone has been documented to give no significant response by itself). An index of one indicates no reaction. However, we have observed that in some cases the contralateral lymph node appears to be not entirely unaffected by the injection of the other side. Instead of using the traditional indices described below, comparison of absolute values (weight, cell numbers, etc) between the groups (allergen + adjuvant, allergen alone, adjuvant alone, vehicle alone, untreated animals) may in many cases be preferable. Below the indices are described, together with information that is relevant also if absolute values are used.

2.8.1. Weight index

The original endpoint in the popliteal lymph node assay was lymph node weight, expressed as the weight index, which is the weight of the PLN of the homolateral (treated) side divided by the weight of the PLN of the contralateral (untreated) side:

Weight index

$$= \text{Weight PLN treated side} / \text{Weight PLN untreated side}$$

To get a correct measurement of the PLN weight, it is essential to carefully trim the PLN free of adherent fat tissue. Because a normal mouse PLN weighs about 1 mg or

less and a rat PLN 10 mg or less, precision scales must be used. The lymph nodes must be kept in isotonic medium, and be placed on filter paper absorbing all free fluid before weighing.

2.8.2. Cell index

The addition of cellularity as a measure of the PLN response increased the accuracy of the PLN assay [43], and cell indices are by some groups considered to be more sensitive than weight indices [44].

$$\text{Cell index} = \frac{\text{Cell numbers PLN treated side}}{\text{cell numbers PLN untreated side}}$$

2.8.3. Cell proliferation index

PLN weight and cellularity as endpoints are relatively crude, and show somewhat limited amplitudes of their responses. In studies on the IgE adjuvant activity of diesel exhaust particles, we therefore added lymph node cellular proliferation as an additional parameter [29]. We exposed cells *ex vivo* to [³H]thymidine without further antigen stimulation, to measure ongoing proliferative activity at the time of lymph node excision. Results with proliferation indices were very parallel to those obtained with weight and cellularity indices. Some tendency for a larger amplitude of responses was seen with proliferation compared to weight and cellularity, but experimental variation also tended to be larger. The kinetics of proliferative activity may be different from weight and cellularity, with an earlier and shorter peak (Løvik, unpublished data), so that the time point optimal for the determination of proliferative activity may differ from that of weight and cellularity. Because of little added value, we included proliferative activity as an outcome parameter in the early but not later scientific publications in large screening studies on the adjuvant activity of ambient air particulate matter [41,45]. Our experience is in agreement with the findings of Ruat et al. [44], who compared [³H]thymidine incorporation indices after *in vivo* administration with weight indices, and found that incorporation of tritiated thymidine did not improve the sensitivity of the PLN assay. Thymidine incorporation as an endpoint does not rule out false-positive results due to primary irritation [44].

$$\text{Proliferation index} = \frac{\text{Thymidine incorporation treated side}}{\text{thymidine incorporation untreated side}}$$

It should be noted that in the local lymph node assay (LLNA) used for the detection of contact sensitizers [46], in which the retroauricular lymph node is used, the response is measured as lymphocyte proliferation expressed as incorporation of radioactive thymidine administered *in vivo*.

Proliferation determined by thymidine incorporation or non-radioactive techniques will not require additional groups of animals.

2.8.4. Histology

Lymph node histology requires animals in addition to those used for the cell index and other assays employing single-cell preparations. Conventional histology as well as cytochemistry and immunohistochemistry may be used to get additional information in relation to specific research questions. Histology may be useful to study mechanisms of the reaction in rats [47] as well as mice [17,48], including the elimination of reactions caused by irritants [47].

Histology can be performed also to study if there is a local reaction in the footpad. The soft tissues of the footpad can be separated from the bones of the foot by placing the foot on a solid surface with the footpad facing up. A pair of sharp, straight small scissors are then held in the longitudinal direction (toe-to-heel), opened slightly and pressed down on each side of the footpad, so that the soft tissues down to the bone can be cut off.

2.8.5. Flow cytometry

Flow cytometry can be used to characterize the cells in the lymph node [48,49], including immunophenotyping and measurement of intracellular mediator substances like cytokines. However, a limiting factor is the small amount of cells available, in particularly in the mouse (a normal mouse PLN may give $0.7(\pm 0.3) \times 10^6$ cells [35]. To increase the number of cells available and limit the number of experimental animals, it may therefore be advisable to inject the test suspension into both hind footpads [36], and then to base weight and cellularity results if needed on comparison with an untreated group of animals. Flow cytometry may also be used to evaluate cell proliferation.

2.8.6. Cytokine release

Cytokine release from PLN cells as an endpoint has been reported to be disappointing in the traditional PLN assay with low-molecular weight compounds [50]. However, in the adjuvant PLN, cytokine release *ex vivo* after mitogen or antigen stimulation can give useful mechanistic information and contribute to the characterization of the adjuvant [36]. Cytokine fingerprinting has been found to be helpful in ruling out a false-positive PLN response due to primary irritation [51].

2.8.7. Antibody producing cells

Antibody producing cells can be determined by the ELISPOT assay, based on the *in vitro* culture of PLN cells on an antibody-binding surface. Isotype-specific enumeration of antibody producing cells can then be made by counting spots after staining of the surface for bound antibodies [28,38].

2.8.8. Serum antibody determinations

For serum antibody determinations, we refer to the general literature. A parallel group of mice in addition to the PLN group will be needed. It is essential to perform serum antibody determination at a time point optimal for the prioritized antibody isotype. In relation to allergy, specific

IgE to the antigen as determined with ELISA or PCA (passive cutaneous anaphylaxis) must be included and will be the isotype that determines the time point for serum collection. Additionally, determination of IgG1 is often done (this Th2-dependent antibody isotype is anaphylactic in the mouse, and apparently does not always parallel the IgE response), and also IgG2a (Th1-dependent antibody isotype, to judge the Th1–Th2 balance of the response). The specific IgE assay described in [29] has been found reliable, and has by another group been reported to give results almost identical to those obtained by the PCA assay [52].

3. Technical considerations and discussion

3.1. False positive and false negative results

For obvious reasons, there hardly will be a technically false positive response in terms of a specific IgE response. However, in terms of lymph node parameters, both primary irritants and immune responses not leading to allergy may contribute to a response. Weight and cellularity will be combined results of an irritative response and an immune response (allergic and/or non-allergic). In addition to a group receiving antigen without adjuvant, it is necessary to include a control group receiving the adjuvant preparation only, to get an indication of how much of the effect on the PLN response in the test group is due to irritation (or even to an immune response against the adjuvant if it contains immunogenic components), and how much is caused by an immune response against the antigen, and to determine whether these contributions are additive or synergistic. Cytokine fingerprinting has been found to be helpful in ruling out a false-positive PLN response due to primary irritation [51]. Specific IgE measurements (or enumeration of IgE-producing cells) are necessary to verify that a lymph node response is relevant in relation to allergy.

False negative results can be due to a need for metabolic activation, if chemical substances are tested for adjuvant activity. In this case, the use of metabolic activators may be helpful [34]. Also, species differences and genetically determined differences must be considered in case of negative results.

3.2. Relevance of the footpad injection route

Injection into the footpad is not a normal route of exposure. Further, sensitivity in terms of producing an immune response may be significantly greater for the footpad route than for more physiological routes of administration. Therefore, the PLN adjuvant assay should primarily be used as a screening assay for hazard identification.

3.3. Ethical issues

The footpad is a sensitive organ, and trauma and pathological processes in the footpad may cause discomfort to

the animal. Further, historically, the footpad route was used in tumour and infectious disease experiments that caused swelling, ulceration and sometimes destruction of the footpad. This brought methods based on footpad injection into some disreputation. It is therefore essential to provide animal experiment ethical committees with proper information about the nature of the adjuvant PLN assay, to carefully consider toxic and inflammatory properties of the material used, and to limit the amount of injected material and the volume of the inoculate. Further, it is important to inform the ethical committees about the advantages of the footpad route in terms of sensitivity and reproducibility and the resulting reduction of the number of animals needed to answer a research question. For example, the footpad route appears to be much more sensitive in terms of eliciting an antibody response than the intranasal route for immunization (Samuelsen, manuscript in preparation).

In relation to a study of the adjuvant effect of diesel exhaust particles [29], we did experiments in which the material was inoculated on the dorsum of the foot instead of the footpad. The rationale was the supposedly lower pain sensitivity of the dorsum of the foot compared to the footpad, and the fact that the animal will not step on the upper side of the foot. However, inoculation into the dorsum of the foot did not consistently produce a PLN response, and the route could not be used for the PLN assay (Lovik, unpublished data).

In connection with the same study, animals were carefully observed after footpad inoculation of 100 µg diesel exhaust particles and 100 µg of ovalbumin in 20 µl of Hank's Balanced Salt Solution with 8% homologous mouse serum. The animals used the foot as normally immediately after the inoculation and the following days, and no swelling or redness of the footpad could be observed. Therefore, properly performed, footpad inoculation for the PLN assay appears to cause little or no discomfort to the animal. However, as a precautionary measure it might be considered to provide additional bedding material to offer a soft surface for the animals.

3.4. The adjuvant PLN assay should be supplemented with antibody measurements

Most of the PLN literature deals with identifying chemicals and drugs causing autoimmunity. One special problem in relation to chemicals inducing autoimmunity and autoimmune-like disease is the absence of reliable antibody assays that can be used as an outcome. The conclusion with regard to autoimmunity-inducing potential therefore more or less has to be based on the findings in the PLN assay. The application of the PLN assay that we have established, namely to measure adjuvant activity of substances and particles in relation to IgE-mediated allergy (that is respiratory and food allergy), should not depend on the PLN results alone as the final outcome [41]. The main outcome should be the specific IgE response in serum, whereas the PLN

data should serve screening purposes and will give mechanistic information.

4. Conclusions

The great value of the PLN assay in relation to the study of adjuvant activity, lies in the following:

The high “immunogenicity” of the footpad as an injection site giving high sensitivity in terms of producing a measurable immune response;
the small amount of material that is needed because all injected material that is not retained in the footpad will go to the popliteal lymph node;
the advantage of having the immune response focused to one single well-defined and easily detectable lymph node;
the possibility to combine screening and mechanistic studies in the popliteal lymph nodes with a definitive allergy-relevant outcome in the form of specific serum IgE;
the high reproducibility of the results compared to less well-defined procedures and less “immunogenic” sites for immunization (e.g. intranasal instillation);
the reduced number of experimental animals needed because of the high sensitivity, the high reproducibility, and the possibility to combine with the same immunization protocol measurements of definite allergy outcomes like specific IgE with mechanistic studies in the local lymph node.

In predictive testing, the adjuvant PLN assay should serve as a screening and hazard identification assay. Based on the PLN assay results, selected substances can then be tested in appropriate airway or peroral exposure models for mucosal adjuvant effects.

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