

IN VITRO TESTS WITH SENSITIZED LYMPHOCYTES—RELEVANCE FOR PREDICTIVE ALLERGENICITY TESTING

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Abstract—The main obstacle to routine *in vitro* tests to identify contact sensitizers is the inappropriate application of the allergens *in vitro*, even when sufficient antigen-presenting cells are available. Some allergens may need the skin tissue for adequate conjugation with carrier molecules, whereas others may be poorly solubilized or toxic for the cultures. Methods, therefore, which make use of the *in vivo* system for allergen binding and presentation, but which assess *in vitro* the subsequent lymphocyte activation are most promising. For this reason, the local lymph node assay deserves more attention as a predictive assay in allergenicity testing and should be validated in more laboratories in the near future. The value of allergen-stimulated *in vitro* tests for predictive allergenicity screening will remain limited. This type of *in vitro* test is, however, invaluable for mechanistic studies on contact sensitivity in well defined allergen models. Also for clinical diagnosis of allergic contact sensitivity to selected allergens, the lymphocyte transformation test and macrophage migration inhibition test may be useful, particularly when skin tests fail to be conclusive.

Introduction

Various *in vivo* methods have been developed to screen chemicals for potential allergenicity. Most protocols include repeated application of compounds onto or into the skin in the presence of adjuvants. Hypersensitivity is usually assessed some weeks later by challenging the skin with non-irritating doses of the chemical. For this purpose guinea-pigs are adequate, not only because contact hypersensitivity is long-lasting in these animals, as in man, but also because the guinea-pig back allows dose-response studies and simultaneous testing of potential cross-reacting substances (Polak, 1980).

Positive skin tests are, however, not always the result of sensitization. Irritant reactions are sometimes difficult to discriminate from true allergic reactions (von Blomberg *et al.*, 1990). Moreover, the irritation threshold of the skin may vary from time to time, so that previous non-irritating doses may become irritant (Bruynzeel *et al.*, 1983).

As not only the degree of sensitivity, but also the frequency of sensitization is considered an important parameter for the potential allergenicity of a substance, smaller animals, like mice, might provide better models. It is not surprising, therefore, that the mouse is now being explored as an experimental animal for contact hypersensitivity studies (Robinson, 1989).

A serious disadvantage of the mouse model for screening the allergenic potential of chemicals is the fact that only two skin tests, that is on each ear, can

be performed at the same time. In addition, contact sensitivity in the mouse is highly susceptible to down-regulation by suppressor mechanisms. The risk of 'false' negative responses caused by testing with the wrong dose at the wrong time point, or by overdosing during sensitization is, therefore, much higher than in the guinea-pig.

Since it became clear, some twenty years ago, that contact sensitivity is the result of increased numbers of specifically sensitized T lymphocytes in the circulation, attempts have been made to study the contact hypersensitivity reaction in lymphocyte cultures *in vitro*. Much progress has been made in lymphocyte culture technology ever since, and knowledge on the process of T cell activation by allergens has increased greatly (Polak, 1980; von Blomberg *et al.*, 1990). Although much experience has been gained with the use of *in vitro* tests in diagnostic procedures, implementation of this technology in allergenicity testing has received little attention (Kimber *et al.*, 1986; Parish, 1986; Robinson, 1989).

The potential role of *in vitro* tests with sensitized cells in predictive allergenicity testing will be discussed as follows: first a summary will be given of the processes occurring *in vivo*, in the context of (a) sensitization and (b) challenge, and secondly the potential role of the *in vitro* correlates of (a) sensitization and (b) challenge will be discussed.

Contact hypersensitivity *in vivo*

Sensitization to contact allergens. Most contact allergens are small, chemically reactive molecules. Upon penetrating the epidermis they readily bind to skin components forming 'hapten-carrier' complexes. The ability to associate with carrier molecules, either covalently or through weak interaction bonds, is a prerequisite for contact sensitization. Whereas most allergens bind spontaneously, some need enzyme- or

Abbreviations: APC = antigen-presenting cells; IL-1 = interleukin-1; IL-2 = interleukin-2; LLNA = local lymph node assay; LTT = lymphocyte transformation test; MIF = migration inhibition factor; TCR = T cell receptor for antigen.

ultraviolet-induced activation before binding can take place.

In the skin not only soluble amino acids or macromolecules but also cell-membrane-bound structures may serve as carriers. Nevertheless, only those allergen molecules eventually associated with MHC-class II molecules on antigen-presenting cells (APC) will stimulate specific T cells and cause sensitization. That is, the T cell receptor for antigen (TCR) recognizes allergens only in MHC-class II context. Whether T cell activation results from allergens that bind directly to Ia molecules or to nearby cell-membrane constituents is still unclear. Both pathways would seem feasible with chemically reactive allergens. A third pathway, probably less relevant to contact sensitivity, is provided by allergen conjugated to soluble proteins. Such hapten-protein complexes would require degradation by APC to small fragments before being presented on the cell membrane. Here again close association could occur through peptide-binding sites present on Ia molecules. It would appear that the immune response in contact sensitivity is directed to a large variety of hapten-carrier determinants.

In healthy skin, the epidermal dendritic Langerhans cell, bearing many Ia molecules on its cell membrane, is the primary antigen-presenting cell. The degree of sensitization was found to be related to the density of LC at the site of allergen application. Interestingly, *in vitro* experiments with Langerhans cells, isolated from the skin, showed that the allergen-presenting capacity of these cells has not yet been fully developed in the skin. Just culturing the cells or incubating them with allergen has been reported to increase Ia expression and APC-capacity of Langerhans cells (Robinson, 1989; Schuler and Steinman, 1985; von Blomberg *et al.*, 1990). This may partly explain why sensitization of unprimed T cells, a process that requires optimal allergen-presenting capacity, does not take place until the Langerhans cells have reached the draining lymph nodes.

It is now well accepted that allergen-bearing Langerhans cells travel from the skin as 'veiled cells' through the lymphatics to the lymph nodes. Here, the intricate tissue structure of the paracortical areas provides optimal conditions for cell contact between lymphocyte and APC (in lymph nodes known as 'interdigitating cells'). A close APC-lymphocyte contact, as required for T cell triggering, is achieved by several sets of adhesion molecules (Fig. 1).

As resting, unprimed lymphocytes express such adhesion molecules in relatively low density on their cell membranes, other qualities of the APC, such as the characteristic membrane ruffling of dendritic antigen-presenting cells, are crucial for inducing primary sensitization of T lymphocytes.

Thus, as soon as allergen-specific T cells (present in very low numbers in the circulation of non-sensitized individuals) pass the lymph nodes, triggering and activation by allergen-bearing APC may occur. This activation of naïve specific T cells is characterized by a delayed, but high interleukin-2 (IL-2) production by the cells as well as by IL-2 receptor expression on their membranes, which results in strong proliferation (Fig. 2). When, some days later, these recently activated allergen-specific cells spread over the body, to

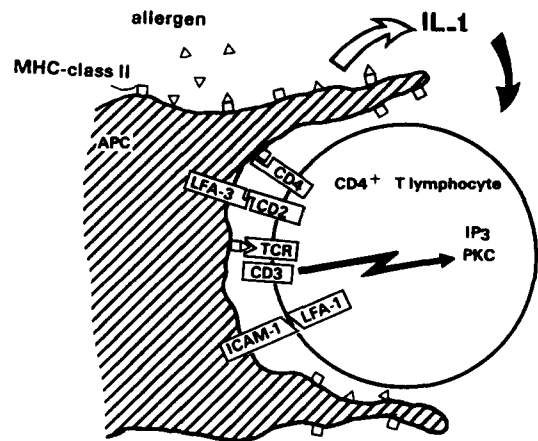


Fig. 1. T cell triggering by antigen-presenting cells and allergen. Specific 'helper' T lymphocytes (bearing the CD4 determinant) are triggered by allergen associated with MHC-class II molecules on the membrane of APC. IL-1, produced by APC, macrophages or keratinocytes provides an essential growth factor for T lymphocytes in this phase. A close lymphocyte-APC contact is maintained by several sets of molecules: CD4-MHC class II; CD2-LFA-3; LFA-1-ICAM-1. The actual function of these molecules is, however, not yet clear.

give an increased frequency of specific cells in the circulation, a state of systemic hypersensitivity has been reached (Fig. 3).

Challenge reaction to contact allergens. When allergen is being applied on the skin of contact-sensitized individuals, as is the case in skin testing, again Langerhans cells will bind and present the allergen. However, T cell activation now takes place within the skin, which results in a local inflammatory reaction.

The fact that recently sensitized T lymphocytes express adhesion molecules in high density on their surface and secrete upon activation a slightly different set of lymphokines than do unprimed T cells, might explain this local reaction. The presence of increased numbers of adhesion molecules facilitates migration through the tissues and at the same time renders the cells more susceptible to triggering by APC. In addition, the immediate production of high levels of both IL-2 and interferon- γ by sensitized T cells upon triggering may initiate an inflammatory reaction at the site of allergen application. Further development of the contact allergic reaction is supported by a number of other lymphokines affecting endothelial cells, attracting cells from the circulation or inhibiting cell movement.

***In vitro* correlates in contact hypersensitivity**

In vitro correlates of sensitization. As the protein-binding potential of a compound is considered a prerequisite for allergenicity, various attempts have been made to predict allergenicity from chemical binding studies. Both proteins and macrophages served as carriers in these assays (Levkovits, 1985). Despite much effort, however, these assays did not lead to new methods to identify allergens. Moreover, these studies do not really concern allergen-specific cells and are therefore beyond the scope of this survey.

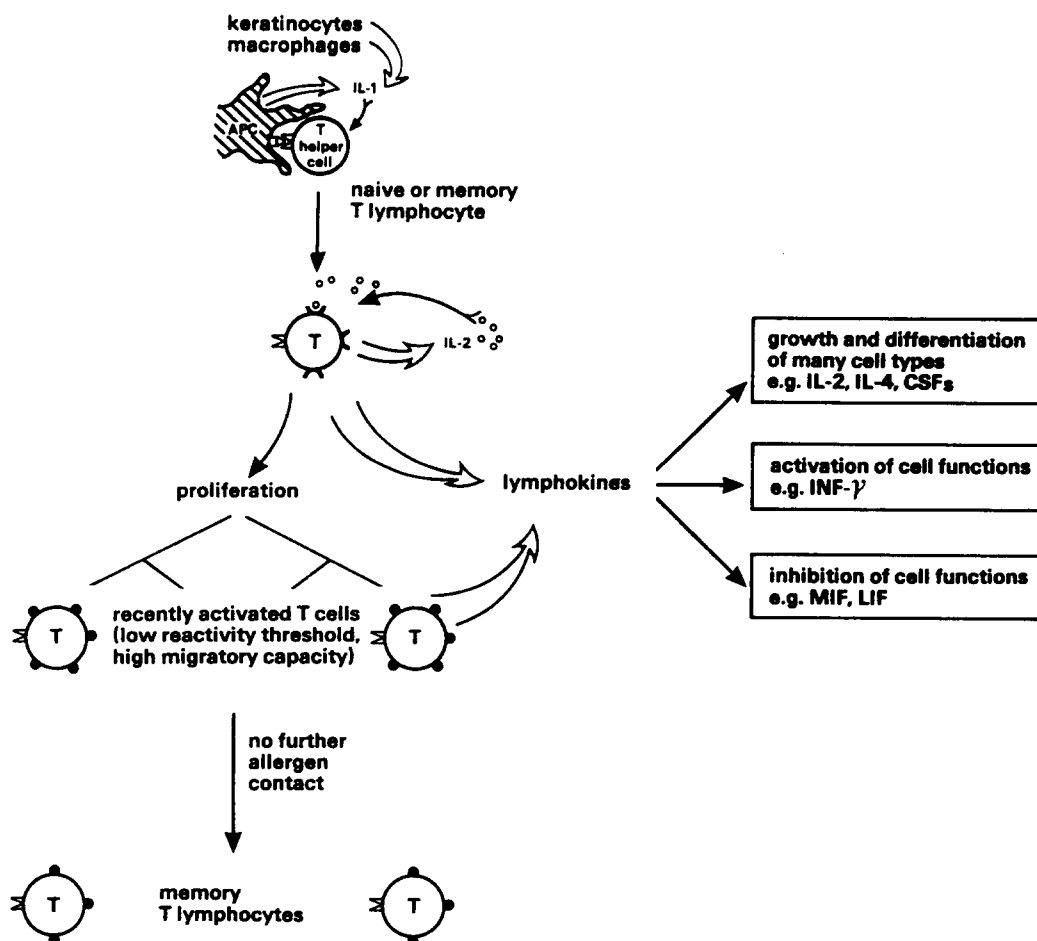


Fig. 2. T cell activation in contact sensitization. Upon triggering, T lymphocytes produce their own growth factor (IL-2) and start to proliferate. The activated T lymphocytes now start to express increased numbers of cell-surface molecules, such as transferrine receptors, adhesion molecules and MHC class II molecules. Activated T cells have a low reactivity threshold and a high migratory capacity. At the same time they produce an array of lymphokines with various biological activities. In the absence of further allergen contact activated T lymphocytes gradually lose their 'activation markers', and become memory cells. These memory cells, however, will continue to express high numbers of several surface glycoproteins as compared with naive T lymphocytes. Δ , allergen; $\square$, MHC class II molecule; Σ , TCR; $\cup$, IL-2R; $\circ$, IL-2; $\bullet$, surface glycoproteins associated with activation; $\Rightarrow$, producing; $\rightarrow$, exerting influence on; $\rightarrow$, becoming, leading to.

It is generally agreed that the degree of contact sensitization is directly related to the degree of lymphocyte proliferation in the lymph nodes draining the site of allergen application.

Based on this idea, Kimber *et al.* (Kimber *et al.*, 1985; Kimber and Weisenberger, 1989) introduced the local lymph node assay (LLNA) for identifying contact allergens in mice. Measurement of T lymphocyte proliferation proved to be the most sensitive marker for local immune activation. To evaluate this proliferation, lymphocytes, isolated from the draining lymph nodes at 3–5 days after allergen application, were cultured with [^{3} H]thymidine for 24 hr. To further increase *in vitro* proliferation of activated cells, exogenous mouse IL-2 was added. Promising results were also obtained by a modification of this technique in which the [^{3} H]thymidine had been injected *iv* and isotope incorporation was evaluated directly in the pooled lymph nodes.

The results of the LLNA show a good correlation with data obtained *in vivo* in guinea-pigs with the maximization test. Irritants and non-sensitizing chemicals failed to induce lymphocyte proliferation, whereas virtually all sensitizers gave positive *in vitro* responses. As compared with *in vivo* screening methods, the LLNA has a number of advantages: adjuvants are not needed, results can be obtained after one week, the test is cost effective and irritant or coloured compounds do not influence the readings. As compared with the allergen-driven *in vitro* tests for T cell immunity, described below, one very important advantage should be mentioned: the LLNA makes use of the intact skin and lymphatics for the process of conjugation and allergen presentation, thereby avoiding a major obstacle limiting the use of conventional *in vitro* tests in contact hypersensitivity.

In conclusion, lymph node assays, such as the LLNA, offer an attractive alternative to the currently

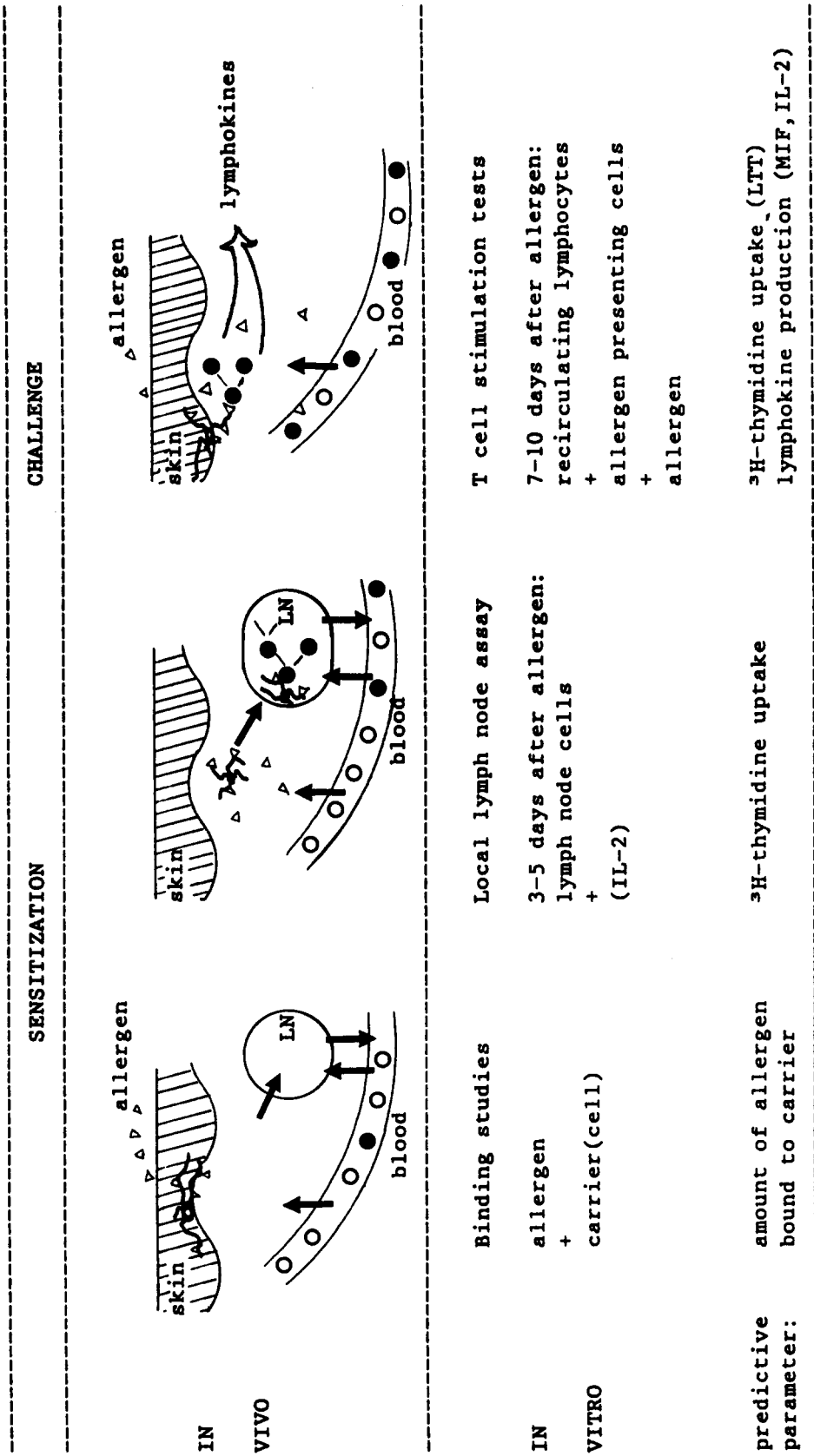


Fig. 3. *In vitro* correlates of contact sensitization and challenge. Contact sensitization is effected by proliferation of allergen-specific cells (●) in the draining lymph nodes (LN). In hypersensitive individuals increased numbers of specific lymphocytes are present in the circulation. These sensitized lymphocytes readily migrate into the skin. Upon renewed allergen contact (challenge) these lymphocytes proliferate and produce a number of inflammatory mediators, thus causing a local hypersensitivity reaction. → indicate the routes of cell migration between the compartments blood, skin and lymph nodes.

available *in vivo* techniques. Although as yet only a few laboratories seem to be involved in this type of predictive testing, the LLNA deserves a place in the routine screening of new compounds in the near future.

In vitro correlates of the challenge reaction. As the degree of contact sensitivity is determined by the frequency of allergen-specific cells in the circulation, *in vitro* tests should ideally provide parameters reflecting the number of sensitized cells per ml of blood. Unfortunately, direct identification of allergen-specific T lymphocytes, for example with labelled allergen, has not been successful so that bioassays are still required.

Such functional assays are always based on lymphocyte cultures: lymphocytes, obtained from blood, lymph nodes or spleen, are challenged *in vitro* with allergen, just as *in vivo* in the skin test. When challenging *in vitro*, however, a substitute for the skin Langerhans cells is needed to present the allergen to the T lymphocytes. The source of APC to be used for *in vitro* lymphocyte stimulation in contact sensitivity has been the subject of a large number of studies (von Blomberg *et al.*, 1990).

The presence of Ia molecules on the APC is a prerequisite for effective allergen presentation. Generally, cells from the dendritic family (Langerhans cells, dendritic cells) are more effective in allergen presentation than monocytes and macrophages.

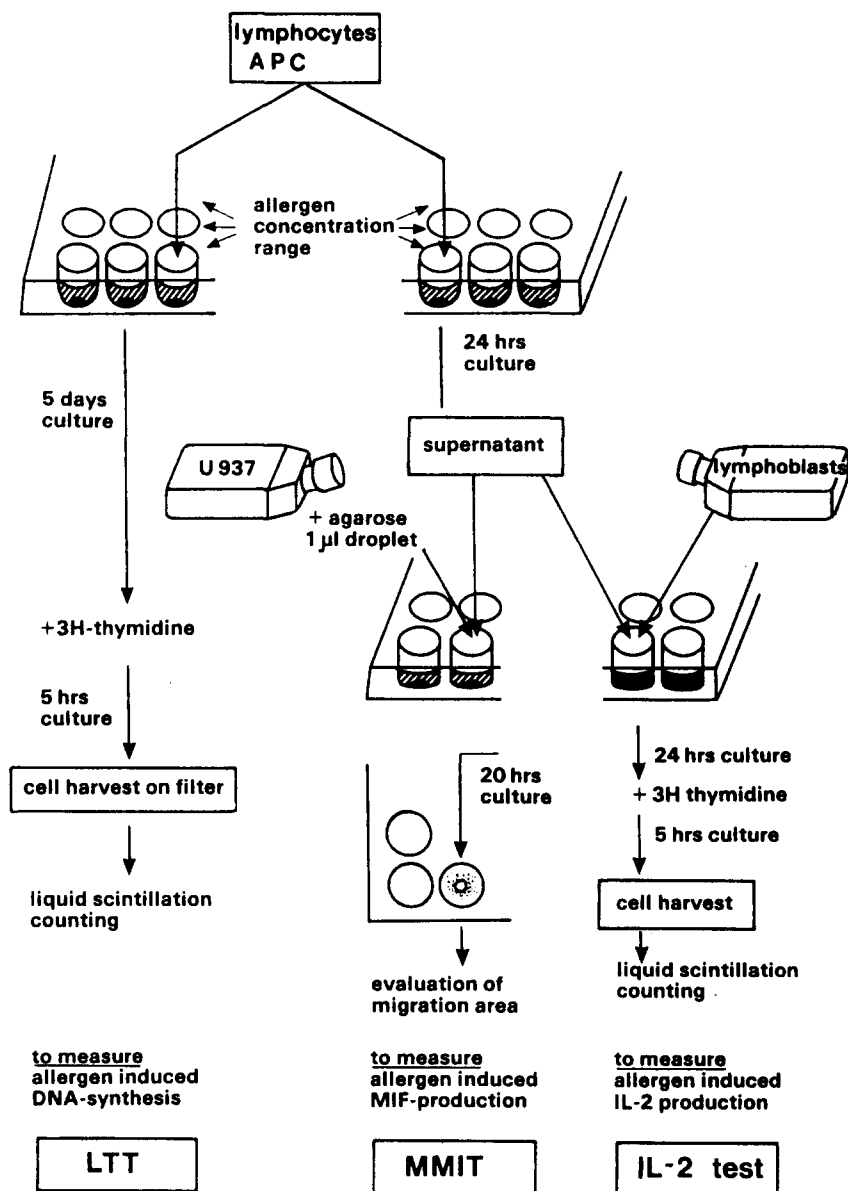


Fig. 4. *In vitro* techniques for evaluating sensitized T cells. All techniques are based on stimulating lymphocytes *in vitro* with APC and allergen. T cell activation is then evaluated with: (a) LTT; (b) the macrophage migration inhibition factor test (MMIT) and (c) the IL-2 production test.

It should be realized, however, that recently activated T lymphocytes are less demanding with respect to the allergen-presenting capacity of the presenting cell. In addition, monocytes have been shown to acquire APC features when kept in culture. Therefore, members of the monocyte/macrophage family should suffice as allergen-presenting cells in active contact hypersensitivity.

Nevertheless, allergen application *in vitro* is still felt to be one of the main bottlenecks in large-scale performance of diagnostic *in vitro* tests in contact sensitivity (Robinson, 1989). Poor water-solubility of test allergens as well as their frequent toxic effects on the lymphocytes often hamper adequate *in vitro* testing. However, for some allergens, like nickel sulphate, which is water-soluble and well tolerated by lymphocytes, *in vitro* studies provide important data on the process of T cell activation in contact sensitivity (von Blomberg *et al.*, 1987 and 1990).

Methods to evaluate *in vitro* T cell activation have been well studied. A classic parameter of T cell activation is provided by the transformation of lymphocytes into blastoid cells. As such morphological evaluation is time-consuming and not really sensitive, allergen stimulated lymphocyte transformation tests (LTT) are now generally evaluated by assessment of [³H]thymidine uptake. Although several alternatives for the use of a radiolabel are now being developed, the sensitivity of the thymidine uptake as a parameter for T cell activation has still not been improved.

In vitro T cell activation may also be evaluated by quantitative assessment of the lymphokines released in the culture supernatant. As only small quantities of lymphokine may exert clear biological effects on target cells, bioassays are indicated for lymphokine measurement. The availability of cell lines as targets has largely contributed to the development of reproducible bioassays for a number of these lymphokines: for example macrophage migration inhibition factor (MIF) and IL-2 (Fig. 4). As most lymphokines show a certain species restriction, target cell lines should be carefully chosen (e.g. the U937 line is sensitive for both human and guinea-pig MIF, whereas the Wehi3 line should be used as target for rat and mouse MIF).

For diagnosis of contact allergy, the LTT and MIF tests have most frequently been used. When com-

paring these tests, MIF production appeared to be less sensitive, but slightly more specific than lymphocyte transformation (von Blomberg *et al.*, 1987). IL-2 production, on the other hand, has up to now mainly been determined in mechanistic studies; as a parameter for contact sensitization it is probably less sensitive than the MIF test.

REFERENCES

- Bruynzeel D. P., von Blomberg-van der Flier B. M. E., Van Keter W. G. and Scheper R. J. (1983) Depression or enhancement of skin reactivity by inflammatory processes in the guinea pig. *Int. Archs Allergy appl. Immun.* **72**, 67.
- Kimber I., Mitchell J. A. and Griffin A. C. (1986) Development of a murine local lymph node assay for the determination of sensitizing potential. *Fd Chem. Toxic.* **24**, 585.
- Kimber I. and Weisenberger C. (1989) A modified murine local lymph node assay for identification of contact allergens. In *Current Topics in Contact Dermatitis*. Edited by P. J. Frosch, A. Dooms-Goossens, J. M. Lachapelle, R. J. G. Rycroft and R. J. Scheper. p. 592. Springer-Verlag, Heidelberg.
- Levkovits H. (1985) Hapten binding assay using guinea pig macrophages. *Expl Cell Biol.* **53**, 189.
- Oliver G. J. A., Botham P. A. and Kimber I. (1986) Models for contact sensitization—novel approaches and future developments. *Br. J. Derm.* **115**, 53.
- Parish W. E. (1986) Evaluation of *in vitro* predictive tests for irritation and allergic sensitization. *Fd Chem. Toxic.* **24**, 481.
- Polak L. (1980) Immunological aspects of contact sensitivity. An experimental study. *Monogr. Allergy* **15**, 1–170.
- Robinson M. K. (1989) Optimization of an *in vitro* blastogenesis assay for predictive assessment of immunologic responsiveness to contact sensitizers. *J. invest. Derm.* **92**, 860.
- Schuler G. and Steinman R. M. (1985) Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells *in vitro*. *J. exp. Med.* **161**, 526.
- Von Blomberg B. M. E., Bruynzeel D. P. and Scheper R. J. (1990) Recent advances in contact allergy research. In *Dermatotoxicology*. 4th Ed. Edited by F. N. Marzulli and H. I. Maibach. Hemisphere Publishing Corporation, Washington.
- Von Blomberg B. M. E., Van Der Burg C. K. H., Pos O., Van De Plassche-Boers E. M., Bruynzeel D. P. and Sheper R. J. (1987) *In vitro* studies in nickel allergy; the diagnostic value of a dual parameter analysis. *J. invest. Derm.* **88**, 355.