Clin. exp. Immunol. (1987) **67**, 383–390

Immunohistological evidence of lymphokine production and lymphocyte activation antigens in tuberculin reactions

M. A. FULLMER, J.-Y. SHEN, R. L. MODLIN & T. H. REA *Section of Dermatology, Department of Medicine, University of Southern California School of Medicine Los Angeles, California*

(Accepted for publication 28 August 1986)

SUMMARY

Evidence of lymphokine elaboration and lymphocyte activation was sought in tuberculin skin test reactions at 24 and 48, or 48 and 96 h in patients with active, culture-proven, pulmonary tuberculosis. Through the use of frozen sections, immunoperoxidase techniques and monoclonal antibodies, anti-interleukin 2 positive cells were found to constitute 0·4% to 0·6% of the dermal infiltrate, and keratinocyte Ia expression at 96 h was consistent with a marker for interferon-gamma production. Cells bearing the interleukin 2 receptor more than doubled in prevalence from 24 to 48 or 96 h but cells staining with Ta_1, an antibody identifying activated lymphocytes, were 10% of the cells of the infiltrate at all three times. One-half of the cells of the infiltrate were OKM1-positive, presumably macrophages, perhaps reflecting the presence of active tuberculosis.

Keywords tberculin reactions interleukin 2 antigen Ta_1 keratinocyte Ia

INTRODUCTION

Cutaneous tuberculin reactions are one of the fundamental expressions of delayed-type hypersensitivity in man (Turk, 1980). As such, it is not suprising that at least seven immunohistological studies of tuberculin reactions utilizing monoclonal antibodies have already been published (Scheynius, Klareskog & Forsum, 1982; Poulter *et al.*, 1982; Platt *et al.*, 1983; Konttinen *et al.*, 1983; Scheynius & Tjernlund, 1984; Gibbs *et al.*, 1984; Kaplan *et al.*, 1986). Although these studies have not been uniform with regard to antibodies used, specificities sought, events looked for, methods of analysis or patients studied, in broad outlines a consistent pattern has emerged. Perivascular areas are characterized by a relatively dense lymphocytic infiltration having by a predominance of the helper/inducer phenotype, a definite portion of macrophages, and a smaller number of OKT6-positive cells (putatively dermal Langerhans cells). The epidermis and interstitial areas of the dermis are characterized by a comparatively sparse lymphocytic infiltration. Apart from these areas of agreement, there are some unresolved conflicts, for example, epidermal Langerhans cells have been observed to be increased (Poulter *et al.*, 1982; Scheynius & Tjernlund, 1984), unchanged (Platt *et al.*, 1983) or decreased (Kaplan *et al.* 1986). Also, potentially important details such as evidence of T cell activation or lymphokine production have received little attention, particularly when compared with that given T lymphocyte phenotypes.

In the present study we have examined tuberculin reactions in patients with active pulmonary tuberculosis and have emphasized staining reactions which may help to identify activated lymphocytes and lymphokine production.

Correspondence: Dr Thomas H. Rea, Section of Dermatology, USC School of Medicine, 2025 Zonal Avenue, Los Angeles, CA 90033, USA.

MATERIALS AND METHODS

Patients chosen for study had culture-proven, pulmonary tuberculosis, were on chemotherapy for less than 3 months, and had been demonstrated to be tuberculin-positive at the time of their initial medical evaluation. For tuberculin testing, five tuberculin units of purified protein derivative (Aplisol, Parke-Davis) were used. Tests were applied so that all biopsies were obtained in a 3 h period and processed in a uniform manner. Six millimeter punch biopsies were bisected; one portion processed for routine paraffin embedding and the other placed in OCT medium (Ames), snap frozen in liquid nitrogen and stored at −70°C until sectioned.

The details of the methods used to section and stain tissues have been published in detail elsewhere (Modlin *et al.*, 1984; Hsu, Raine & Fanger, 1981). Briefly, 6 μm sections were air-dried overnight and fixed in fresh, reagent-grade acetone before staining. Application of the primary mouse monoclonal antibody was followed sequentially by either peroxidase-conjugated goat anti-mouse and a chromogenic substrate or by biotinylated horse anti-mouse, a complex of avidin, biotin and peroxidase, and the chromogenic substrate. Haematoxylin was used as a counterstain.

Antibody titres were predetermined by checkerboard titration. The antibodies used, specificities sought and dilutions employed are listed in Table 1.

To monitor specificity of the antibody directed against interleukin 2 (IL-2), peripheral blood lymphocytes were stained in the manner described by Steinman *et al.* (1983) (data not shown). Specificity of anti-Tac (anti-IL-2 receptor) was monitored by its use upon fetal and adult lymphoid tissues (Hofman *et al.*, 1985). The specificities of the remaining antibodies were monitored by their

Table 1. Mean percent ± s.d. of the cells of the dermal infiltrate staining positively for each specificity tested.

Antibody	Specificity	Dilution	24 h	48 h	96 h
Leu 4	Pan T cells	1/60	38±2·9	47±9·0	52±11·5
Leu 2a	T suppressor/ cytotoxic	1/60	9±5·4	15±6·8	19±8·2
Leu 3a	T helper/inducer	1/60	36±2·5	49±12·7	57±12·0
(Leu 3a/Leu 2a)			4·0	3·3	3·0
OKM1	Macrophages, monocytes, neutrophils	1/100	60±10·0	47±10·3	55±14·7
Anti-IL-2	Interleukin 2*	1/10	0·4±0·15	0·6±2·2	0·6±0·30
Anti-Tac	Interleukin 2 receptor (Tac)†	1/1000	3·3±1·6	6·7±2·5	8·2±3·0
H4 (Dr. Ron Billing, UCLA)	Ia (HLA-DR)	1/1000	Virtually every cell in the infiltrate was positive		
OKT6	Langerhans cells,‡§ indeterminant cells	1/50	Positive cells in the infiltrate were usually in the upper dermis. The distribution was irregular		
Ta_1	Activated T cells‖	1/50	11·5±6·3	10·8±4·5	9·2±2·8

* Steinman *et al.* (1983).
† Leonard *et al.* (1982).
‡ Fithian *et al.* (1981).
§ Murphy *et al.* (1983).
‖ *Fox et al.* (1984).

staining patterns in normal adult tonsils (data not shown). Normal skin controls consisted of the clinically normal skin included in routine surgical excisions.

Positively staining cells were semi-quantified by one of three techniques. If greater than 5% of the infiltrate, the percentage of positive cells was directly estimated by two independent observers, who were almost always within 5 per cent points of each other. If fewer than 5%, each positive cell was counted, the area of the section occupied by infiltration estimated with a calibrated reticule, the number of cells per mm^2 of infiltrate determined by averaging the counts in three randomly selected fields, and the per cent of the infiltrate staining positively calculated. As previously described (Modlin *et al.*, 1984), intraepidermal Langerhans cells were graded by density, i.e. if greater than 10% of cells exhibited cell–cell contact of dendritic extensions, the Langerhans cells were judged to be increased.

For staining controls in tuberculin reactions, the primary antibody was omitted, and a primary antibody of irrelevant specificity was used.

RESULTS

Tuberculin reactions in five patients were biopsied at 24 and 48 h and in four at 48 and 96 h. All responses biopsied were positive reactions, i.e. 10 mm or greater of induration at 24, 48, and 96 h. Generally, 48 h reactions were larger than 24 h responses, but no constant kinetic relationship was discernible between 48 and 96 h test sizes. Conventionally processed tissues showed the anticipated patchy perivascular infiltration of mononuclear cells (Turk, Rudner & Heather, 1966).

Dermal responses. The patchy nature of the infiltrate was present at 24, 48 and 96 h. None of these three time points could be distinguished from one another by either the density of cells infiltrating a given area or by the overall percentage of the dermis infiltrated. A sparse interstitial infiltrate of lymphocytes was also present.

The mean percentage of the cells of the dermal infiltrate staining positively for each phenotype used is summarized in Table 1. Fewer T lymphocytes and more macrophages appear to be the case at 24 h, with near parity at 48 and 96 h. The sum of cells positive for Leu 2a and Leu 3a always exceeded those positive for Leu 4; this is consistent with the error of the method, but a double-labelled population cannot be excluded. The ratio of Leu 3a to Leu 2a was greatest at 24 h, 4·0, but was always close to 3. Lymphocytes and macrophages were always admixed with one another. A few OKT6-positive cells, putatively Langerhans cells (Murphy *et al.*, 1983), were present in the perivascular infiltrate particularly in the upper portion of the reticular dermis.

Interleukin 2 positive cells were present throughout the infiltrate (see Fig. 1) and ranged from 0·4% to 0·6% of the cells of the infiltrate. The positive cells showed thick, cytoplasmic staining consistent with IL-2 production (Modlin *et al.*, 1984). Cells staining for Tac, the IL- 2 receptor, constituted 3% of the infiltrate at 24 h but doubled in prevalence by 48 and 96 h (see Fig. 2). Ta_1-positive cells were approximately 10% of the infiltrate, ranging from 2% to 20% (see Fig. 3).

Virtually every cell in the infiltrate appeared to bear the Ia antigen.

Epithelial changes. A few lymphocytes bearing Leu4, Leu2a, or Leu3a phenotypes were found infiltrating the epidermis in all specimens. This was focal in nature and often not evident in the paraffin-embedded tissues. Some Tac-positive, Ta_1 positive and, rarely, IL-2 positive cells were found in the epidermis.

At 24 h the dendrites of the Langerhans cells stained readily with OKT6, appearing as a well-developed syncitium, and the numbers of cell bodies were perhaps increased. At 48 and 96 h the cell bodies were definitely increased. These changes were present in both epidermis and hair follicles (see Fig. 4).

In both epidermis and hair follicles at 24 and 48 h, the cells staining for the Ia antigen were usually readily identifiable as Langerhans cells or as infiltrating mononuclear cells. At 96 h virtually every follicular or epidermal nucleated keratinocyte appeared to express the Ia antigen, giving a net-like or reticular staining pattern (see Fig. 5). Such staining was not present at 24 and 48 h. With anti-Ia staining at 96 h, Langerhans cell cell-bodies were less readily discerned than at 48 h, but were present in abundance as judged by OKT6 staining (see Fig. 4).

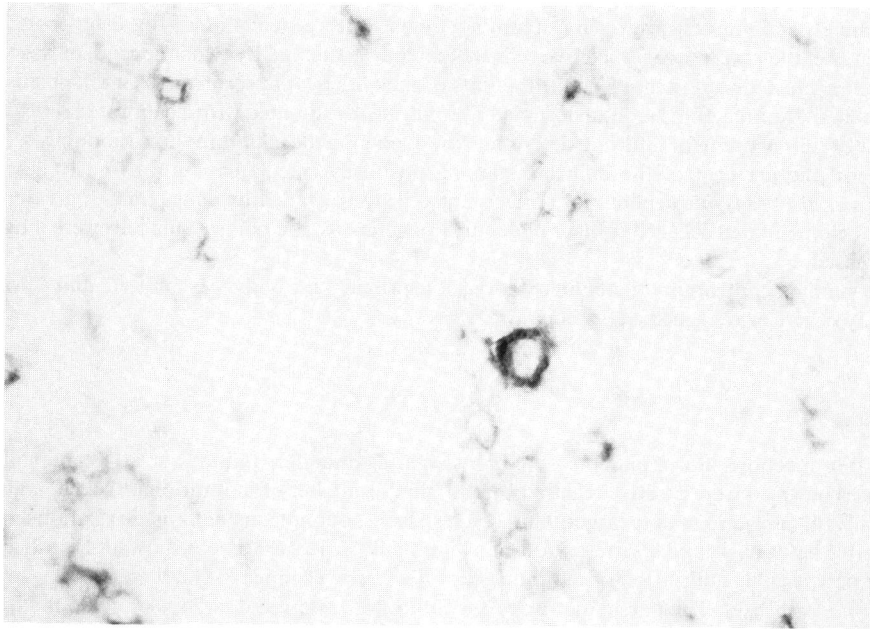

Fig. 1. A 48 h reaction. An IL-2-positive cell adjacent to an eccrine sweat duct shows thick, cytoplasmic staining Anti-IL-2, haematoxylin counterstain ×40 objective.

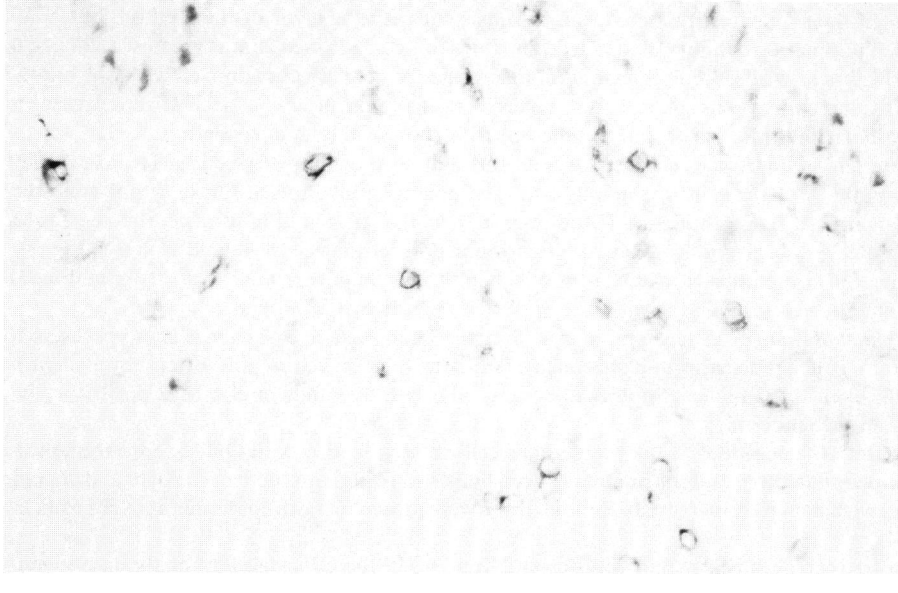

Fig. 2. A 48 h reaction. Tac positive cells in the upper dermis. Epidermal rete are in both upper corners. Anti-Tac, haematoxylin counterstain, ×25 objective.

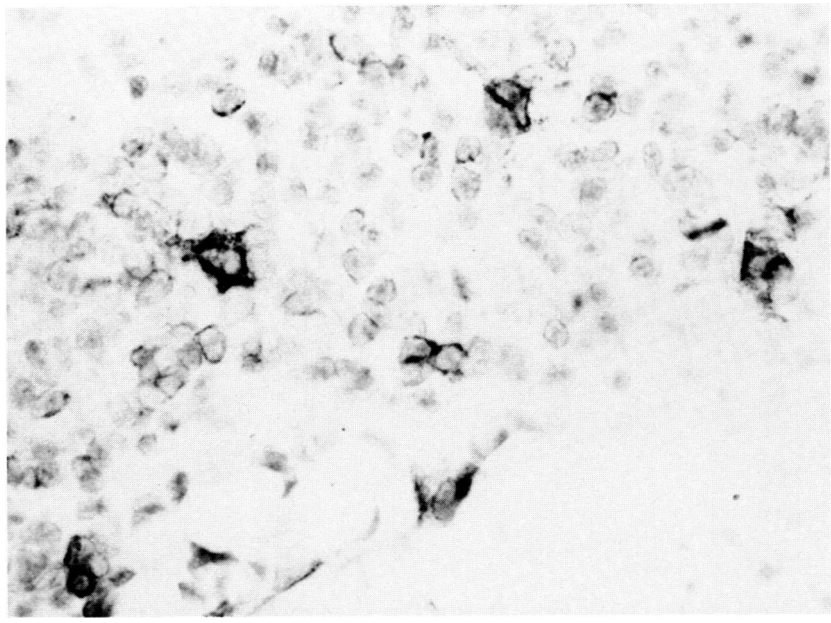

Fig. 3. A 48 h reaction. Ta_1 positive cells in an area of lymphocytic infiltration. Anti-Ta_1, haematoxylin counterstain, $\times 40$ objective.

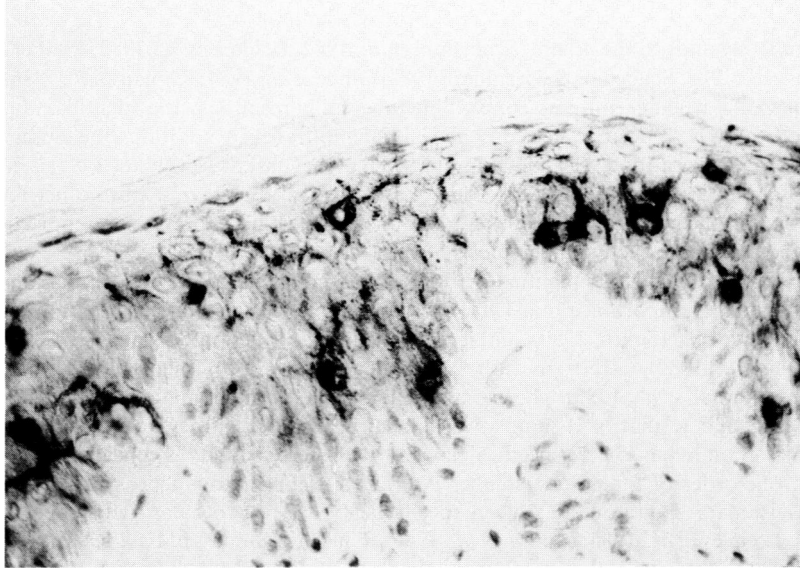

Fig. 4. A 96 h reaction. Langerhans cells are increased. The cell bodies are readily apparent, the thickness of the staining reaction is highly variable, a dendritic configuration is evident and staining between basal cells is not a common event. OKT6, haematoxylin counterstain, $\times 25$ objective.

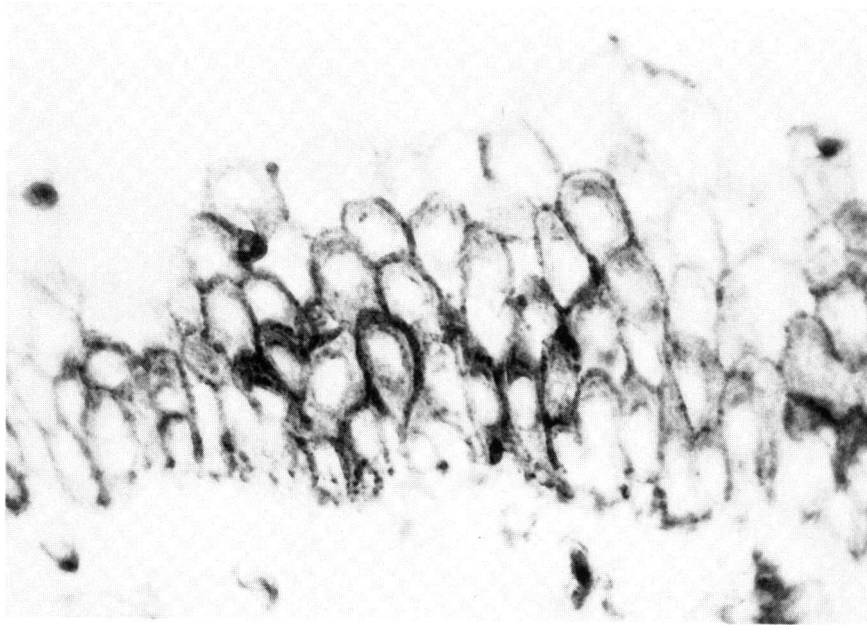

Fig. 5. A serial section of the tissue in Fig. 4. The keratinocytes appear to bear the Ia antigen, giving a net-like pattern. The staining tends to be of uniform thickness, is often found between the basal cells extending to the basement membrane region and Langerhans cell bodies are not easily discerned. H4, haematoxylin counterstain ×40 objective.

DISCUSSION

The present study adds to the others (Scheynius *et al.*, 1982; Poulter *et al.*, 1982; Platt *et al.*, 1983; Konttinen *et al.*, 1983; Scheynius & Tjernlund, 1984; Gibbs *et al.*, 1984; Kaplan *et al.*, 1986) two new observations of particular interest concerning tuberculin reactions: the identification of Ta_1-positive cells and IL-2-positive cells in the dermal infiltrate. In addition, the observation of expression of Ia by keratinocytes at 96 but not at 48 h (Scheynius & Tjernlund, 1984) is confirmed, as is that of an increased number of epidermal Langerhans cells. In contrast to other reports, we found one-half of the infiltrating cells to be macrophages, the highest prevalence reported, perhaps reflecting the presence of active tuberculosis.

Approximately one in 200 inflammatory cells appeared to synthesize IL-2. Functionally, IL-2 has two activities of importance in a DTH reaction; it is necessary for proliferation of antigen-specific T-effector cells (Smith, 1980), and it is a potent inducer of γ-interferon (γ-INF) production (Kasahara *et al.*, 1982); γ-INF in turn is a potent activator of macrophage microbiocidal mechanisms (Nathan *et al.*, 1983). The prevalance of one in 200 inflammatory cells as putative IL-2 producers is similar to that of IL-2 positive cells in tuberculoid leprosy and localized cutaneous leishmaniasis granulomas, where specific delayed-type hypersensitivity is considered strong; but a prevalence one-order of magnitude smaller is found in lepromatous leprosy and disseminated cutaneous leishmaniasis granulomas, where specific delayed-type hypersensitivity is absent (Modlin *et al.*, 1984; Longley *et al.*, 1985; Modlin *et al.*, 1985). Thus the numbers of IL-2 positive cells appear to be closely associated with the presence or absence of a delayed-type hypersensitivity response.

In the context of lymphokines, keratinocyte Ia expression is a putative marker for γ-INF production: (1) keratinocytes having been proven to synthesize Ia (Volc-Platzer *et al.*, 1984); (2) keratinocyte Ia having been expressed in graft-versus-host reaction (Lampert, Smitters & Chrisholm, 1981), and allergic contact dermatitis (Suitters & Lampert, 1982; Mackie & Turbitt, 1983), as well as tuberculin reactions (Platt *et al.*, 1983; Scheynius & Tjernlund, 1984; Kaplan *et al.*, 1986) and, finally, (3) keratinocyte Ia being induced *in vitro* (Basham *et al.*, 1984; Volc-Platzer *et al.*,

1985) and *in vivo* (Nathan *et al.*, 1986) by γ-INF. Thus the appearance of keratinocyte Ia is a likely footprint, so to speak, of γ-INF production, an opinion shared by others (Scheynius & Tjernlund, 1984; Kaplan *et al.*, 1986).

Two antibodies identified putative activation antigens, Tac or the IL-2 receptor, and Ta_1. Tac-positive cells increased in prevalence from 24 to 48 or 96 h in accordance with the finding of Platt *et al.* (1983) and this increase appeared to be significant, $P < 0{\cdot}05$. At all three times Ta_1 positivity was present in a mean of 10% of infiltrating cells, a figure similar to that found in tuberculosis lymph nodes (unpublished data).

This immunohistological study provides new morphological evidence for the role of lymphocytes and their products in the pathogenesis of tuberculin reactions. Lymphocytes appear to be activated by 24 h, bearing Tac, Ta_1 and HLA-DR antigens. Both macrophages and Langerhans cells are numerous and may participate in the immune response by presenting antigen to these lymphocytes (Sontheimer, 1985). Lymphocyte products such as IL-2 and perhaps γ-INF are elaborated and reflect the occurence of a delayed-type hypersensitivity reactions. These results may provide a standard for comparing other skin reactions and diseases in order to determine whether delayed-type hypersensitivity is present.

This work was supported by grant AI22187 from the National Institutes of Health, a grant from the Heiser Program for Research in Leprosy, by a gift from the Military and Hospitaller Order of St Lazarus of Jerusalem and a grant from the National Hansen's Disease Center.

REFERENCES

BASHAM, T.Y., NICKOLOFF, B.J., MERIGAN, T.C. & MORHENN, V.B. (1984) Recombinant gamma interferon induces HLA-DR expression on cultured human keratinocytes. *J. invest. Dermatol.* **83,** 88.

FITHIAN, E., KUNG, S., GOLDSTEIN, G., RUBERFELD, M., FENOGLIO, C. & EDELSON, R. (1981) Reactivity of Langerhan's cells with hybridoma antibody. *Proc. natn Acad. Sci. USA.* **78,** 2541.

FOX, D.A., HUSSEY, R.F., FITZGERALD, K.A., ACUTO, O., POOLE, C., PALLEY, L., DALEY, J.F., SCHLOSSMAN, S.F. & REINHERZ, E.L. (1984) Ta_1, a novel 105 KD human T cell activation antigen defined by a monoclonal antibody. *J. Immunol.* **133,** 1250.

GIBBS, J.H., FERGUSON, J., BROWN, R.A., KENICER, K.J.A., POTTS, R.C., COGHILL, G. & BECK J.S. (1984) Histometric study of the localization of lymphocyte subsets and accessory cells in human mantoux reactions. *J. Clin. Pathol.* **37,** 1227.

HOFMAN, F.M., MODLIN, R.L., BHOOPAT, L. & TAYLOR, C.R. (1985) Distribution of cells bearing the Tac antigen during ontogeny of human lymphoid tissue *J. Immunol.* **134,** 3751.

HSU, S.M., RAINE, L. & FANGER, H. (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J. Histochem. Cytochem.* **29,** 577.

KAPLAN, G., WITMER, M.D., NATH, I., STEINMAN, R.M. LAAL, S., PRASAD, H.K., SARNO, E.N., ELVERS, U. & COHN, Z.A. (1986) Influence of delayed immune reactions on human epidermal keratinocytes. *Proc. natn Acad. Sci.* **83,** 3469.

KASAHARA, T., HOOKS, J.J., DOUGHERTY, S.F. & OPPENHEIM, J.J. (1982) Interleukin 2-mediated immune interferon (IFN-gamma) production by human cells and T cell subsets. *J. Immunol.* **130,** 1784.

KONTTINEN, Y.T., BERGROTH, V., VISA-TOLRANEN, K., REITAMO, S. & FORSTROM, L. (1983) Cellular infiltrate *in situ* and response kinetics of human intradermal and epicutaneous tuberculin reactions. *Clin. Immunol. Immunopathol.* **28,** 441.

LAMPERT, I.A., SUITTERS, A.J. & CHRISHOLM, P.M. (1981) Expression of Ia antigen on epidermal keratinocytes in graft vs host disease. *Nature* **293,** 149.

LEONARD, W.J., DEPPER, J.M., UCHIYAMA, T., SMITH, K.T., WALDMAN, T.A. & GREEN, W.C. (1982) A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor; partial characterization of the receptor. *Nature* **300,** 267.

LONGLEY, J., HARGEWOIN, A., YEMANEBERHAN, T., VAN DIEPEN, T.W., NSIBAMI, J., KNOWLES, D., SMITH, K.A. & GODAL, T. (1985) *In vivo* responses to *mycobacterium leprae*: antigen presentation, interleukin-2 production, and immune cell phenotypes in naturally occuring leprosy lesions. *Int. J. Lepr.* **53,** 385.

MACKIE, R.M. & TURBITT, M.L. (1983) Quantitation of dendritic cells in normal and abnormal human epidermis using monoclonal antibodies directed against Ia and HTA antigens. *J. invest. Dermatol.* **81,** 216.

MODLIN, R.L., HOFMAN, F.M., HORWITZ, D.A., HUSMANN, L.A., GILLIS, S., TAYLOR, C.R. & REA, T.H. (1984) *In situ* identification of cells in human leprosy granulomas with monoclonal antibodies to interleukin-2 and its receptor. *J. Immunol.* **132,** 3085.

MODLIN, R.L., TAPIA, F.J., BLOOM, B.R., GALLINOTO, M.E., CASTES, M., RONDON, A.J., REA, T.H. & CONVIT, J. (1985) *In situ* characterization of the cellular immune response in American cutaneous leishmaniasis. *Clin. exp. Immunol.* **60,** 241.

MURPHY, G.F., BHAN, A.K., HARRIST, T.J. & MIHM, M.C. (1983) *In situ* identification of T6-positive cells in normal human dermis by immunoelectron microscopy. *Br. J. Dermatol.* **108,** 423.

NATHAN, C.F., KAPLAN, G., LEVIS, W.R., NUSRAT, A., WITMER, M.D., SHERWIN, S.A., JOB, C.K., HOROWITZ, C.R., STEINMAN, R.M. & COHN Z.A. (1986) Local and systemic effects of low doses of recombinant interferon-γ after intradermal injection in new patients with lepromatous leprosy. *New Eng. J. Med.* **315,** 6.

NATHAN, C.F., MURRY, H.W., WIEBE, M.E. & RUBIN, B.Y. (1983) Identification of interferon as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. exp. Med.* **158,** 670.

PLATT, J.L., GRANT, B.W., EDDY, A.A. & MICHAEL, A.F. (1983) Immune cell populations in cutaneous delayed-type hypersensitivity. *J. exp. Med.* **158,** 1227.

POULTER, L.W., SEYMOUR, G.J., DUKE, O., JANOSSY, G. & PANAYI, G. (1982) Immunohistological analysis of delayed-type hypersensitivity in man. *Cell. Immunol.* **74,** 358.

SCHEYNIUS, A., KLARESKOG, L. & FORSUM, U. (1982) *In situ* identification of T lymphocyte subsets and HLA-DR expressing cells in the human skin tuberculin reaction. *Clin. exp. Immunol.* **49,** 325.

SCHEYNIUS, A. & TJERNLUND, U. (1984) Human keratinocytes express HLA-DR antigens in the tuberculin reaction. *Scand J. Immunol.* **19,** 141.

SMITH, K.A. (1980) T-cell growth factor. *Immunol. Rev.* **51,** 337.

SONTHEIMER, R.D. (1985) The mixed epidermal cell-lymphocyte reaction. II. Epidermal Langerhans cells are responsible for the enhanced allogeneic lymphocyte-stimulating capacity of normal human epidermal suspensions. *J. Invest. Dermatol.* **85,** 21s.

STEINMANN, G., CONLON, P., HEFENEIDER, S. & GILLIS, S. (1983) Serologic visualization of interleukin-2. *Science* **220,** 1188.

SUITTERS, A.J. & LAMPERT, I.A. (1982) Expression of Ia antigen on epidermal keratinocytes is a consequence of cellular immunity. *Br. J. exp. Pathol.* **63,** 207.

TURK, J.L. (1980) *Delayed Hypersensitivity. Research Monographs in Immunology*. Vol. 1. 3rd edn. Elsevier/North Holland Biomedical Press, Amsterdam.

TURK, J.L., RUDNER, E.J. & HEATHER, C.J. (1966) A histochemical analysis of mononuclear cell infiltrates of the skin. II. Delayed hypersensitivity in the human. *Int. Arch. Allerg.* **30,** 248.

VÖLC-PLATZER, B., MAJDIC, O., KNAPP, W., WOLFF, K., HINTERBERGER, W., LECHNER, K. & STINGL, G. (1984) Evidence of HLA-DR antigen biosynthesis by human keratinocytes in disease. *J. exp. Med.* **159,** 1984.

VÖLC-PLATZER, B., LEIBL, H., LUGER, T., ZAHN, G. & STINGL, G. (1985) Human epidermal cells synthesis HLA-DR alloantigens *in vitro* upon stimulation with γ-interferon. *J. invest. Dermatol.* **85,** 16.