# IMMUNE CELL POPULATIONS IN CUTANEOUS DELAYED-TYPE HYPERSENSITIVITY*

BY JEFFREY L. PLATT,‡ BARBARA W. GRANT, ALLISON A. EDDY, AND ALFRED F. MICHAEL

*From the Departments of Pediatrics, Laboratory Medicine and Pathology and Internal Medicine, University of Minnesota Medical School, Minneapolis, Minnesota 55455*

Participation of immune cells in graft rejection, tumor surveillance, resistance to infection and autoimmunity was demonstrated by classical observations relating adoptive immunity to cell transfer (1–4). T lymphocytes are thought to mediate these processes either by direct cytotoxicity as in allograft rejection, or by cell-mediated, lymphokine-generated inflammation, as observed in delayed-type hypersensitivity (DTH)[1] (5). Although distinct subpopulations of T lymphocytes may effect these two responses (6) other investigations have suggested that cytotoxicity may have a role in DTH and that DTH may contribute to allograft rejection (7–10).

Monoclonal antibodies have been used to correlate lymphocyte membrane antigen expression with human T lymphocyte differentiation (11). While initial studies appeared to support a functional classification based upon surface phenotype (12), later investigations using cloned progeny of single human and animal cells also suggested that either cytotoxicity and DTH are not distinct phenomena, or available monoclonal antibody probes may not distinguish functionally disparate subpopulations of T lymphocytes (13–15). Still, in situ immunohistochemical studies of allograft rejection in kidney (16) and skin (17, 18), sarcoidosis (19), leprosy (20), and graft vs. host disease (21) suggest that under certain circumstances, T lymphocyte populations are enriched with one or another identifiable subpopulation.

To further explore the correlation of surface phenotype and cell-mediated immunity, we studied skin tissues obtained from individuals who manifest DTH to tuberculin, using monoclonal antibodies reactive with various populations of mononuclear cells. This prototypic immune response was characterized by early infiltration of lymphocytes bearing a helper cell marker and monocytes and subsequently by the expression of cellular antigens associated with activation.

## Materials and Methods

*Source of Tissue.* 12 adult individuals (10 male, 2 female) known to exhibit a delayed cutaneous response to tuberculin antigens (responders) and 6 unsensitized controls (5

[1] *Abbreviations used in this paper:* DTH, delayed-type hypersensitivity; E-rosette, sheep erythrocyte rosette; FITC, fluorescein isothiocyanate; IL-2, interleukin 2; PBS, phosphate-buffered saline, PMN, polymorphonuclear leukocyte; TRIC, tetraethylrhodamine isothiocyanate.

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males, 1 female) were studied. All subjects gave informed consent to the protocol that had been approved by the Committee on the Use of Human Subjects in Research, University of Minnesota.

Tuberculin-purified protein derivative (Connaught Laboratories, Ontario, Canada) (0.1 ml) was administered intracutaneously to the 12 responders and 3 controls and a 4-mm punch biopsy of the innoculation site was performed 6–48 h later. Tuberculin was administered to one control subject at two sites that were separately biopsied 24 and 48 h later. Three normal skin tissues obtained without prior innoculation of tuberculin were used as additional controls. Biopsy tissue was snap frozen in precooled isopentane and stored at −70°C until used.

Peripheral venous blood was obtained from nine responders and four controls at the time of tuberculin administration and at the time of skin biopsy 6–48 hours later.

*Monoclonal Antibodies.* Mononuclear cells were identified using the following monoclonal antibodies: T11 (mouse $IgG_1$) (T lymphocytes, Coulter Immunology, Hialeah, FL); OKT3 ($IgG_{2a}$) (T lymphocytes, Ortho Pharmaceutical, Raritan, NJ); OKT4 ($IgG_{2b}$) (helper T cells, Ortho); OKT8 ($IgG_{2a}$) (cytotoxic/suppressor T cells, Ortho); OKM1 ($IgG_{2b}$) (monocytes and null cells, Ortho); Mo2 (IgM) (monocytes, Coulter); 63D3 ($IgG_1$) (monocytes, Bethesda Research Laboratories, Inc., Gaithersburg, MD); anti-B1 ($IgG_{2a}$) (B cells, Coulter); Leu-7 (IgM) (large granular lymphocytes, Becton-Dickinson, Mountain View, CA); OKT6 ($IgG_1$) (thymocytes and Langerhans cells, Ortho) OKIa1 ($IgG_2$) (HLA-DR, Ortho); OKT9 ($IgG_1$) (transferrin receptor, Ortho); OKT10 ($IgG_1$) (activated T cells, Ortho); and anti-Tac ($IgG_{2a}$) (interleukin 2 [IL-2] receptor, kindly provided by T. Waldmann, National Institutes of Health, Bethesda, MD).

In addition, MBM4, a monoclonal antibody ($IgG_1$) that reacts with Type 4 collagen (22), was used in concert with certain of the above antibodies as a morphologic probe to evaluate the relationship of reactive cells to vascular basement membranes. Poly C9-MA, a monoclonal antibody ($IgG_1$) that reacts with a neoantigen on polymerized C9 of the membrane attack complex of complement (23) and fluorescein isothiocyanate (FITC)-labeled goat anti-human C3 were used to search for evidence of complement activation.

*Preparation and Staining of Tissue Sections.* Frozen tissue sections (4 μm) were prepared in a Lipshaw cryostat under conditions of constant temperature (22°C) and humidity (30%), air dried, acetone fixed and washed with phosphate-buffered saline (PBS) pH 7.4. Sections were then sequentially reacted with a monoclonal antibody, human plasma absorbed affinity-isolated FITC F $(ab^1)_2$ rabbit anti-mouse IgG (heavy and light chains) (Zymed Laboratories, Inc., San Francisco, CA), affinity-isolated FITC $F(ab^1)_2$ goat anti-rabbit IgG (heavy and light chains) (Cappel Laboratories, Cochranville PA), ethidium bromide to stain nuclei, and *p*-phenylenediamine to retard fluorescence fading (16, 24).

Some sections were sequentially reacted with two different monoclonal antibodies followed by the double fluorochrome layer described above or by human plasma-absorbed FITC $F(ab')_2$ goat antimouse IgM (u) (Cappel) (to detect Leu-7) and human plasma-absorbed tetraethylrhodamine isothiocyanate (TRIC)-conjugated $F(ab^1)_2$ goat anti-mouse IgG (Fc) (Cappel Laboratories, Cochranville, PA) (to detect T11, OKT8, or OKM1). In other studies, tissues were sequentially reacted with mixtures of FITC-labeled T11 (Coulter) plus anti-Tac or FITC T11 plus OKIa1; followed by rabbit anti-mouse IgG2a (which binds to anti-Tac or OKIa1 but not T11) and then $F(ab^1)_2$ TRIC goat anti-rabbit IgG (heavy and light chains) (Cappel) that had been immunoabsorbed with human plasma and mouse serum.

Additionally, sections of each tissue were reacted with a mixture of FITC $F(ab')_2$ goat anti-human IgM (u) and FITC $F(ab')_2$ goat anti-human IgD(δ) (Tago, Inc., Burlingame, CA).

*Standardization of Monoclonal Antibodies and Controls.* Monoclonal antibodies (except anti-Tac) were standardized by immunofluorescence using frozen sections of normal human lymphoid tissue (25–28). Anti-Tac was standardized as follows: the maximal dilution without diminished fluorescence of anti-Tac ascites (1:40,000) was ascertained by indirect immunofluorescence on frozen tissue sections. Anti-Tac (1:20,000 and 1:40,000) was then absorbed for 1 h with T lymphocytes that had been isolated by sheep erythrocyte

binding (E rosette) and maintained for 4 d in 1% phytohemagglutinin-leukocyte conditioned medium (29); as a control anti-Tac was absorbed in a similar manner with unstimulated E rosette-isolated lymphocytes. Frozen sections of three tissues known to contain numerous anti-Tac binding cells were then reacted with the absorbed anti-Tac antibodies followed by the double fluorochrome layer as described above. Immunofluorescence was assessed independently by three observers, each of whom was unaware of which absorbed anti-Tac had been applied to the section.

Control sections of each tissue were prepared as described above, omitting application of monoclonal antibodies. Other sections were reacted with mouse myeloma proteins of the same immunoglobulin subclasses as the monoclonal antibodies used in the study followed by a double fluorochrome layer (16). Additional control sections were reacted with FITC anti-mouse IgM or TRIC anti-mouse IgG with and without prior applications of appropriate mouse monoclonal IgG and IgM antibodies (to exclude cross-reactivity of antisera). Finally, sections of NZB/NZW mouse kidney tissue were sequentially reacted with rabbit anti-mouse $IgG_{2a}$, mouse serum absorbed TRIC $F(ab')_2$ goat anti-rabbit and FITC-labeled T11 to exclude binding of T11 by the heteroantisera.

*Tissue Analysis.* Sections were examined at 500× with a Zeiss Universal microscope equipped for epifluorescence and with a 10 mm × 10 mm indexing grid. Reactive cells (apple-green fluorescent plasma membrane surrounding an ethidium bromide–labeled orange-red nucleus) and total cells were enumerated in two distinct dermal loci within each field; (*a*) mononuclear cell aggregates surrounding vessels; and (*b*) interstitial dermal cells apart from aggregates. These data were expressed as the proportion of cells in aggregates or the proportion of cells in the dermal interstitium reactive with a given monoclonal antibody. Excluded from cell counts were cells lacking an orange-red nucleus, fragmented cells and polymorphonuclear cells (PMN) reactive with OKM1 or 63D3. Epidermal Langerhans cells reactive with OKT6 were enumerated in a similar fashion and expressed as reactive cells per 100 basilar epidermal cells.

*Blood Cell Analysis.* Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient reacted with monoclonal antibodies followed by FITC goat anti-mouse Ig (IgG, IgA, IgM) (Cappel) and quantitated individually by indirect immunofluorescence as previously described (16). Control suspensions were reacted with mouse ascites in lieu of monoclonal antibodies or with the fluorochrome alone.

## Results

Tissues from responders sensitive to tuberculin contained focal aggregates of 20–400 or more mononuclear cells which surrounded small blood vessels (capillaries or venules) and less numerous infiltrating cells scattered in the interstitial dermis and epidermis. Within each tissue, the size of aggregates and the number of scattered infiltrating cells varied with time after injection of the antigen and with the intensity of the inflammatory response. Epidermal mononuclear cell infiltration increased with time from inoculation. Numerous PMN (identified by autofluorescent cytoplasmic granules and characteristic nuclear morphology) were present in tissues obtained 6 and 12 h after tuberculin administration while these cells were less frequently observed at later times. Weak granular reactivity with antihuman C3 and Poly C9-MA was present along the dermal-epidermal junction of skin and skin appendages and associated with occasional blood vessels in the dermis.

*Composition of Perivascular Mononuclear Cell Aggregates.* Overall 75–90% of mononuclear cells in perivascular aggregates were identifiable as T lymphocytes or monocytes, this proportion being greatest in tissues containing the largest aggregations. There was no distinct relationship between the cellular composition of perivascular aggregates and time from inoculation of tuberculin or intensity

of inflammation except that the proportion of T lymphocytes reactive with OKT4 was greatest at 6 h. The proportion of aggregated mononuclear cells reactive with each of the monoclonal antibodies is indicated in Table I.

Large aggregates (containing >100 cells) consisted mainly of T lymphocytes (T11+, OKT3+) most of which reacted with OKT4 (Fig. 1). OKT8-reactive cells were less numerous and located in small clusters or scattered within the aggregate (Fig. 1). These aggregates contained variable proportions of monocytes (OKM1+, 63D3+, Mo2+) located adjacent to capillary walls, at the periphery or scattered among more numerous T lymphocytes. The proportions and locations of cells reactive with OKM1, 63D3, and Mo2 were generally similar (Tables I and II, Fig. 2).

Occasional cells were reactive with anti-B1, with anti-IgD/IgM or with Leu-7. Clusters of cells reactive with these antibodies were not seen.

*Mononuclear Cell Composition of Dermal Interstitium and Epidermis.* Infiltration of the dermis by T lymphocytes (T11+, OKT3+) and monocytes (OKM1+, 63D3+, Mo2+) increased between 12 and 24 h after tuberculin administration (Table II). Interstitial cells reactive with OKT4 were more numerous than those reactive with OKT8 especially at 6 h PPD injection. Cells binding anti-B1, anti-IgD/IgM, and Leu-7 were infrequently observed.

Epidermal infiltration by T lymphocytes and monocytes increased with duration of exposure to tuberculin. Cells reactive with OKT8 and OKT4 were

TABLE I

*COMPOSITION OF CELLS IN PERIVASCULAR AGGREGATES IN DTH*

*Mononuclear Cells Reactive with Monoclonal Antibodies by Indirect Immunofluorescence*

*Percent of Cells in Aggregates*

| Time after tuberculin | T11 | OKT3 | OKT4 | OKT8 | OKM1 | 63D3 | $Mo_2$ | Leu-7 | Anti-B1 | Anti-IgD/M* |
|---|---|---|---|---|---|---|---|---|---|---|
| *6 h* | | | | | | | | | | |
| Tissue | | | | | | | | | | |
| 1 | 46 | 40 | 41 | 11 | 46 | 51 | 43 | 1.2 | 1.1 | 1.9 |
| 2 | 63 | 60 | 48 | 15 | 27 | 30 | 30 | 2.4 | 2.3 | 2.5 |
| 3 | 63 | 63 | 52 | 11 | 26 | 28 | 31 | 0.61 | 2.2 | 0.96 |
| 4‡ | 20 | 15 | 14 | 4.1 | 24 | 15 | 27 | 2.2 | 2.9 | 3.8 |
| *15–19 h*§ | | | | | | | | | | |
| Tissue | | | | | | | | | | |
| 5 | 58 | 57 | 44 | 25 | 16 | 17 | 12 | 3.5 | 0.46 | 1.1 |
| 6 | 26 | 23 | 19 | 11 | 31 | 27 | 26 | 2.3 | 1.0 | 0.40 |
| 7 | 49 | 27 | 36 | 8.2 | 37 | 54 | 31 | 3.0 | 2.7 | 1.1 |
| 8‡ | 32 | 21 | 9.5 | 10 | 27 | 28 | 23 | 0.9 | 0.48 | 0.84 |
| *24 h* | | | | | | | | | | |
| Tissue | | | | | | | | | | |
| 9 | 59 | 59 | 29 | 15 | 27 | 30 | | 0.72 | 1.8 | 1.1 |
| 10 | 52 | 53 | 38 | 13 | 40 | 22 | 44 | 2.6 | 0.50 | 1.1 |
| 11 | 61 | 60 | 44 | 14 | 21 | 20 | 15 | 1.2 | 1.3 | 0.83 |
| 12‡ | 30 | 20 | 21 | 10 | 9.9 | 15 | 18 | 0.42 | 1.0 | 0.78 |
| *48 h* | | | | | | | | | | |
| Tissue | | | | | | | | | | |
| 13 | 71 | 55 | 51 | 19 | 28 | 29 | | 6.3 | 1.9 | 2.5 |
| 14 | 58 | 60 | 37 | 25 | 29 | 20 | 26 | 3.3 | 0.67 | 0.92 |
| 15 | 58 | 65 | 46 | 21 | 26 | 25 | 19 | 0.63 | 1.8 | 2.5 |
| 16‡ | 25 | 15 | 20 | 3.2 | 21 | 22 | 15 | 1.3 | 1.9 | 0.86 |

* Affinity-purified $F(ab')_2$ FITC goat anti-human IgD + IgM.

‡ Control tissues from subjects not sensitized to tuberculin.

§ Tissue 5 obtained 15 h after tuberculin administration; tissue 6, 16 h; tissue 7, 19 h; and tissue 8, 15 h.

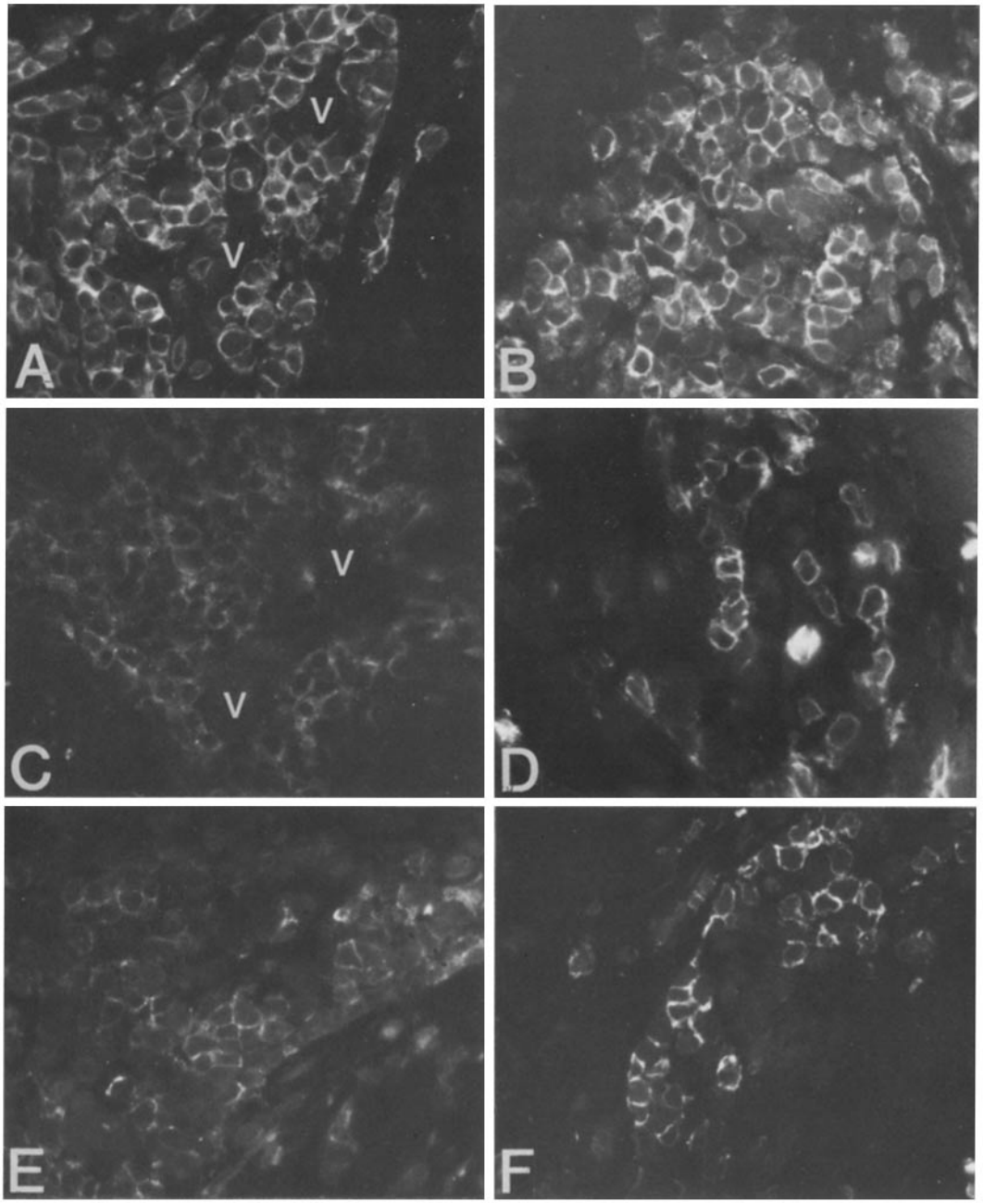


FIGURE 1. T lymphocyte populations in cutaneous delayed hypersensitivity, identified using monoclonal antibodies by indirect immunofluorescence in tissues obtained from known responders to tuberculin. (*A*) T11-reactive cells form an aggregate around a small blood vessel (*V*) in tissue obtained 48 h after tuberculin administration (× 400); (*B*) OKT3-reactive cells in a serial section of the same aggregate (× 400); (*C*) a large cluster of OKT4-reactive cells surrounds a blood vessel (*V*) in tissue obtained at 6 h (× 500); (*D*) OKT8-reactive cells scattered in a perivascular aggregate in tissue obtained at 6 h (most aggregates in 6-h tissues contained fewer OKT8-reactive cells). Several autofluorescent cells (brightly fluorescent cytoplasm) are seen (× 500); (*E*) OKT4-reactive cells in an aggregate from tissue obtained at 48 h (× 400); (*F*) OKT8-reactive cells in an aggregate from tissue obtained at 48 h (× 400).

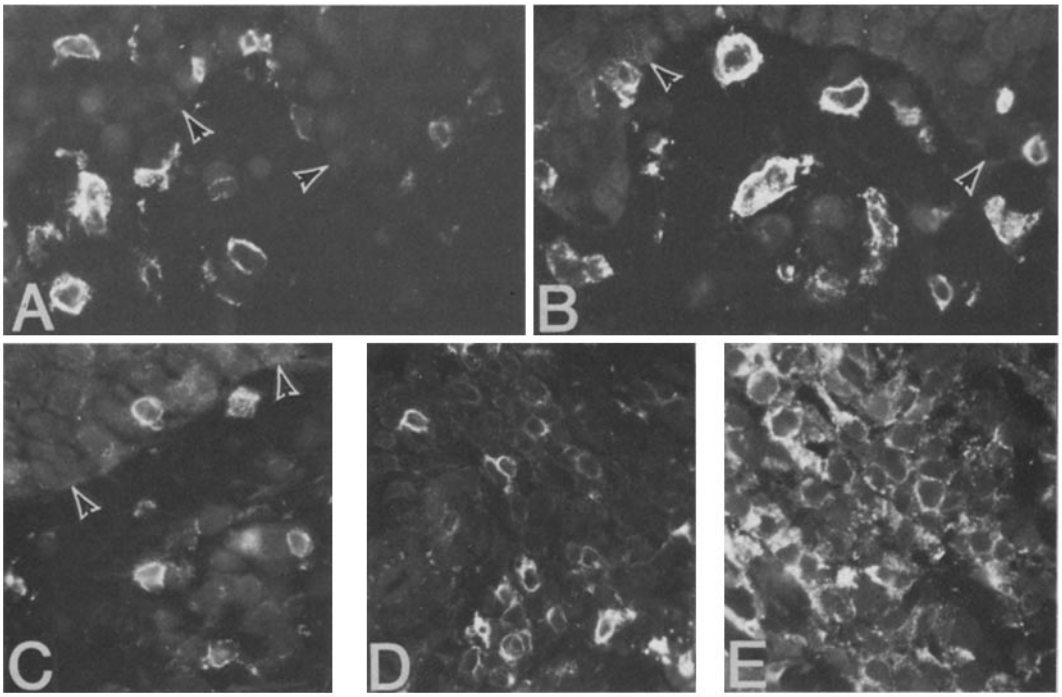


FIGURE 2. Monocytes (*A* and *B*) and activated T lymphocytes (*C–E*) identified in cutaneous delayed hypersensitivity using monoclonal antibodies by indirect immunofluorescence. Tissues were obtained at 48 h after tuberculin administration to known responders. (*A*) OKM1-reactive cells infiltrating papillary dermis and epidermis (× 450); (*B*) 63D3-reactive cells in papillary dermis and epidermis (× 450); (*C*) mononuclear cells reactive with anti-Tac in a small dermal perivascular aggregate. A single reactive cell is present in epidermis (× 390); (*D*) A large perivascular cluster contains some mononuclear cells strongly reactive with anti-Tac. A population of weakly reactive cells is also seen (× 350); (*E*) Granular OKT9 fluorescence of many cells forming a perivascular aggregate (× 400). The patterns of reactivity in *C–D* were not observed in 6-h tissues or in control tissues. Arrowheads denote dermal-epidermal junction in *A–C*.

present in this site in approximately equal numbers. Cells binding Leu-7 were occasionally observed.

*Peripheral Blood Cell Populations.* Peripheral blood cell populations in nine responders and four controls were studied using monoclonal antibodies OKT3, OKT4, OKT8, OKM1, 63D3, and BA-1 6–48 h after tuberculin administration. The emergence of delayed hypersensitivity was not characterized by specific changes in the relative proportions of mononuclear cells in peripheral blood. On the other hand analysis of ratios of T lymphocyte subpopulations in tissue compared with blood revealed a relative concentration of OKT4 reactive cells in tissue from responders during the early course of DTH (Fig. 3).

*T Lymphocyte Activation.* Evidence of T lymphocyte activation in DTH was sought using monoclonal antibodies directed against the transferrin receptor (OKT9), interleukin 2 receptor (anti-Tac), T10 antigen, and HLA-DR antigen (OKIa1). In responders the proportion of dermal cells reactive with OKT9, OKT10, and anti-Tac increased with duration of exposure to tuberculin and

TABLE II

*COMPOSITION OF DERMAL INTERSTITIAL CELLS IN DTH*

*Mononuclear Cells Reactive with Monoclonal Antibodies by Indirect Immunofluorescence*

*Percent of Cells in Interstitium*

| Time after tuberculin | T11 | T3 | T4 | T8 | M1 | 63D3 | Mo2 | Leu-7 | Anti-B1 | Anti-IgD/M* | T11 + OKM1 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *6 h* | | | | | | | | | | | |
| Tissue | | | | | | | | | | | |
| 1 | 9.5 | 8.5 | 7.0 | 2.5 | 24 | 19 | 17 | 0.25 | 0.47 | 1.2 | 34 |
| 2 | 6.8 | 8.3 | 7.7 | 0.76 | 18 | 18 | 14 | 0.39 | 0.89 | 0.68 | 25 |
| 3 | 10 | 10 | 13 | 1.6 | 19 | 14 | 18 | 0.44 | 0.51 | 0.42 | 29 |
| 4‡ | 4.3 | 6.9 | 5.4 | 3.2 | 23 | 20 | 20 | 0.68 | 2.5 | 1.4 | 27 |
| *15–19 h*§ | | | | | | | | | | | |
| Tissue | | | | | | | | | | | |
| 5 | 6.5 | 8.9 | 5.8 | 4.6 | 16 | 14 | 13 | 0.20 | 0.35 | 0.84 | 23 |
| 6 | 4.3 | 4.2 | 3.0 | 3.1 | 18 | 13 | 13 | 0.17 | 0.63 | 1.3 | 22 |
| 7 | 8.8 | 9.1 | 7.5 | 3.5 | 20 | 38 | 16 | 0.72 | 0.68 | 0.32 | 29 |
| 8‡ | 3.2 | 11 | 4.5 | 2.4 | 14 | 11 | 13 | 1.0 | 0.74 | 0.50 | 17 |
| *24 h* | | | | | | | | | | | |
| Tissue | | | | | | | | | | | |
| 9 | 26 | 22 | 17 | 9.3 | 18 | 22 | | 0.23 | 0.21 | 0.29 | 44 |
| 10 | 11 | 13 | 11 | 7.1 | 29 | 15 | 24 | 0.50 | 0.51 | 0.38 | 40 |
| 11 | 21 | 16 | 13 | 7.5 | 20 | 20 | 16 | 0.40 | 1.0 | 0.60 | 41 |
| 12‡ | 3.9 | 2.2 | 2.4 | 1.4 | 7.1 | 7.1 | 9.1 | 0.40 | 0.12 | 0.30 | 11 |
| *48 h* | | | | | | | | | | | |
| Tissue | | | | | | | | | | | |
| 13 | 22 | 19 | 7.7 | 6.4 | 14 | 19 | | 1.0 | 1.0 | 0.91 | 36 |
| 14 | 29 | 19 | 11 | 10 | 25 | 21 | 18 | 1.5 | 0.81 | 0.60 | 54 |
| 15 | 35 | 23 | 19 | 8.3 | 20 | 21 | 16 | 0.11 | 1.4 | 1.0 | 55 |
| 16‡ | 3.8 | 4.0 | 3.5 | 1.9 | 8.7 | 8.8 | 6.5 | 0.89 | 0.42 | 0.40 | 13 |

* Affinity-purified $F(ab')_2$ FITC goat anti-human IgD + IgM.

‡ Control tissues from subjects not sensitized to tuberculin.

§ Tissue 5 obtained 15 h after tuberculin administration; tissue 6, 16 h; tissue 7, 19 h; and tissue 8, 15 h.

reactivity was particularly noted 48 h after administration of tuberculin (Figs. 2 and 4). Reactive cells were of variable size and were located in the interstitium and epidermis as well as in aggregates. In contrast, OKIa1 reacted with few infiltrating T lymphocytes as described below. Weak granular reactivity of endothelium with OKT9 accounted for many of the reactive cells observed at 6 and 12 h. The proportion of T lymphocytes in the epidermis reactive with anti-Tac appeared to exceed that observed in dermal aggregates. By epifluorescence phase-contrast microscopy the thin plasma membrane of cells infiltrating the epidermis and reactive with OKT9, OKT10, and anti-Tac were clearly distinguished from keratinocytes and Langerhans cells. Basilar keratinocytes of both responders and controls reacted weakly with OKT9 at 15, 24, and 48 h. The reactivity of peripheral blood lymphocytes with OKT10 (nine subjects, two controls 6–48 h) and with anti-Tac (two subjects, 48 h) was studied by indirect immunofluorescence as described above. No change in the small proportion (<2%) of OKT10 reactive and no anti-Tac reactive peripheral blood lymphocytes

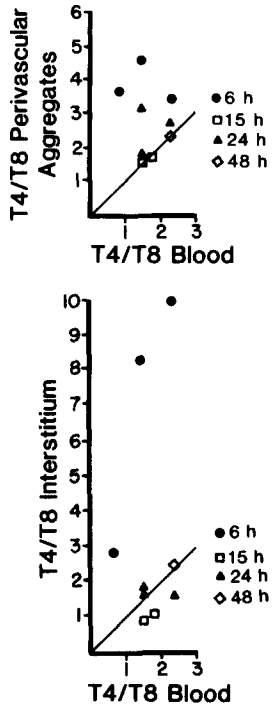


FIGURE 3. Comparison of T lymphocyte subpopulations identified using monoclonal antibodies by indirect immunofluorescence in tissue and blood during course of cutaneous delayed hypersensitivity to tuberculin. (*A*) OKT4/OKT8 ratio of cells in perivascular aggregates vs. OKT4/OKT8 ratio of peripheral blood at 6, 15–19, 24, and 48 h after tuberculin administration to presensitized subjects. (*B*) OKT4/OKT8 ratio of cells in interstitial dermis apart from aggregates vs. OKT4/OKT8 ratio in blood. The OKT4/OKT8 ratio is increased in all tissues at 6 h but not at 15–48 h.

were observed.

The specificity of anti-Tac for activated T lymphocytes in tissue was suggested by the following studies: (*a*) reaction of tissue sections with a mixture of FITC T11 and anti-Tac (the latter recognized by rabbit anti-mouse $IgG_{2a}$ and mouse serum absorbed TRIC goat anti-rabbit IgG as described above) revealed that cells reactive with anti-Tac also reacted with T11; (*b*) tissue binding of anti-Tac was abrogated by immunoabsorption with activated T lymphocytes but not with unstimulated T lymphocytes.

*Cutaneous Response of Controls.* Control tissues contained perivascular aggregates of mononuclear cells—generally containing 10–50 cells although at least 1 large cluster of >100 cells was observed in each tissue. Substantial infiltration of the dermal interstitium and epidermis was not seen. The number of infiltrating PMN in control tissues appeared similar to that in responder tissues; however the proportion of PMN among aggregated cells was greater in controls than in responders. Weak reactivity with anti-human C3 and Poly C9-MA was present in a pattern similar to that observed in responders.

The relative proportions of T lymphocytes, T lymphocyte subpopulations and monocytes were generally similar to those observed in responders (Tables I and

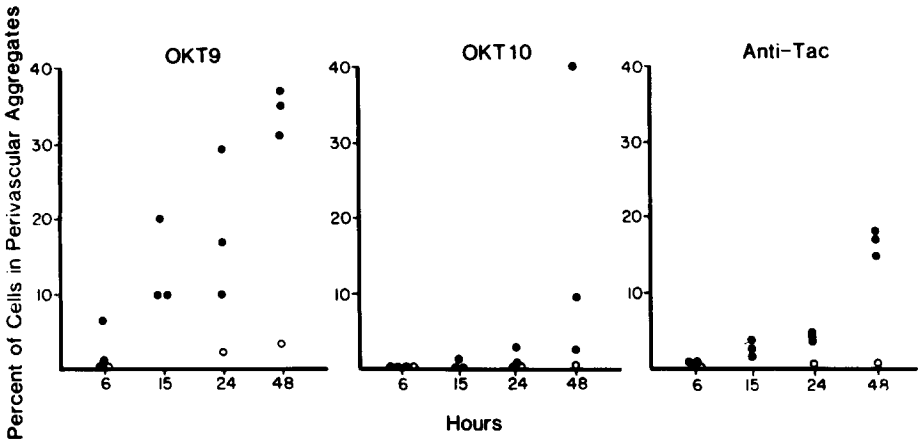


FIGURE 4. Expression of T lymphocyte activation antigens identified using monoclonal antibodies by indirect immunofluorescence during course of cutaneous delayed hypersensitivity. Proportion of cells in dermal perivascular aggregates reactive with OKT9, OKT10, and anti-Tac in presensitized subjects (●) and nonresponding controls (○) was determined at 6, 15–19, 24, and 48 h after tuberculin administration.

II). However, 6 h after administration of tuberculin, OKT4- and OKT8-reactive cells were present in almost equal numbers in controls while OKT4-reactive cells notably exceeded OKT8-reactive cells in responders as described above. Additionally, the proportion of cells in the dermal interstitium identifiable as T lymphocytes and monocytes decreased with time after tuberculin injection, whereas in responders this proportion tended to increase. Study of control tissues using OKT9, OKT10, and anti-Tac revealed few reactive cells (Fig. 4).

*Reactivity of Tissues with OKT6 and OKIa1.* Tissues obtained from responders and controls were reacted with OKT6, which identifies epidermal Langerhans cells (30). These cells appeared larger and more reticulated 15–24 h after tuberculin administration; however, the number of reactive epidermal Langerhans cells was similar in responders, controls, and normal skin tissues (Fig. 5). Occasional cells in mononuclear cell aggregates and in the interstitial dermis reacted with OKT6.

In all tissues OKIa1 reacted with fewer Langerhans cells than did OKT6. In responders and controls up to one-half of the mononuclear cells in perivascular aggregates reacted with this antibody. Most of the cells appeared to be mononuclear phagocytes or endothelium. OKIa1 also reacted with basilar keratinocytes of responders but not control tissues at 48 h.

Reaction of tissue with T11 FITC and OKIa1 (the latter recognized by rabbit anti-mouse IgG2a and mouse serum and human plasma absorbed $F(ab^1)_2$ TRIC goat anti-rabbit) revealed with rare exceptions that the reactive populations did not overlap.

*Identity of Leu-7 Reactive Cells.* Leu-7 reacts with a population of peripheral blood mononuclear cells that has the capacity to mediate natural killing (31). Other cell surface antigens expressed by Leu-7–reactive cells were sought using FITC anti-mouse IgM to identify Leu-7 reactivity and TRIC anti-mouse IgG to detect binding of T11, OKT8, and OKM1. ~50% of Leu-7–reactive cells were

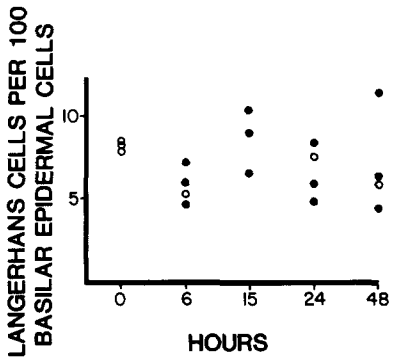


FIGURE 5. Epidermal Langerhans cells identified with monoclonal antibody OKT6 by indirect immunofluorescence in cutaneous delayed hypersensitivity to tuberculin expressed as reactive cells per 100 basilar epidermal cells. Tissues were obtained from presensitized subjects (●) and nonresponding controls (○) at 6, 15–19, 24, and 48 h. Results obtained from study of three skin tissues from normal subjects to whom tuberculin was not administered is shown at 0 h.

reactive with T11, while fewer Leu-7 cells reacted with OKT8 and OKM1. For example, in tissue 13, these proportions were 53%, 19%, and 37% respectively.

*Immunofluorescence Controls.* Tissue sections reacted with a double fluorochrome layer alone or with mouse myeloma antibodies in lieu of hybridoma-derived antibodies did not reveal cell membrane fluorescence. Sections reacted with Leu-7 (IgM) and TRIC ($F(ab')_2$ goat anti-mouse IgG, or with T11 (IgG) and FITC $F(ab')_2$ goat anti-mouse IgM were likewise negative. Sections sequentially reacted with FITC T11, rabbit anti-mouse $IgG_{2a}$, and $F(ab')_2$ TRIC goat anti-rabbit IgG (human plasma and mouse serum absorbed) revealed only background rhodamine fluorescence. NZB/NZW mouse kidney tissue controls were likewise negative. Of peripheral blood lymphocytes treated with PBS or NS-1 ascites in lieu of monoclonal antibodies 0–1.5% were weakly reactive.

## Discussion

Delayed-type hypersensitivity is a slowly emerging response by presensitized subjects to locally administered antigen (32). This study demonstrates that the cutaneous DTH response to tuberculin is characterized by (*a*) initial accumulation of aggregates of T lymphocytes and monocytes in the vicinity of small blood vessels; (*b*) early (6 h) enrichment of OKT4-reactive T lymphocyte populations in skin in comparison to blood; (*c*) later (15–48 h) infiltration of the interstitial dermis and epidermis by T lymphocytes and monocytes; and (*d*) progressive expression by infiltrating T lymphocytes of activation antigens recognized by OKT9, OKT10, and anti-Tac, but not by OKIa1.

Cellular mediation of DTH was first demonstrated by transfer of sensitivity using unfractionated cells from peritoneal exudates (33, 34) and more recently by transfer by a single T lymphocyte (15, 35). Since the majority of cells participating in the reaction are not specifically sensitized (5, 32, 36) these studies and others (35, 37) suggest an important role for nonspecific inflammation in initiation of DTH. Furthermore, the development of localized edema which

distinguishes an antigen-specific response from nonspecific inflammation is likewise generated by nonspecific effector cells such as macrophages and mast cells (38). Thus in situ analysis may not identify unique antigen-specific cellular components of DTH. However, prominence of perivascular clusters, early enrichment of OKT4-reactive lymphocytes, and evidence of T cell activation are potential morphologic concomitants of antigen-specific recognition.

Antigen recognition and mitogen stimulation are associated with T lymphocyte proliferation and expression of cell surface activation antigens (39). Recent studies have demonstrated that the transferrin (OKT9) and IL-2 (anti-Tac) receptors are expressed shortly after (6 h) in vitro stimulation of T lymphocytes, while expression of T10 and HLA-DR occur later (40). Analysis of cell activation in a process such as DTH is somewhat more difficult since there are potentially numerous stimuli and varied durations of activation. Still, our data demonstrate that the transferrin and IL-2 receptors are elaborated early in the course of DTH compared with the delayed and more variable expression of T10 antigen, and that the display of HLA-DR as manifested by reactivity with OKIa1 is not a requisite feature of this phenomenon.

The mononuclear cell populations that participate in skin graft rejection (17), cutaneous leprosy (20), and graft vs. host disease (21) have been investigated using of monoclonal antibodies for analysis of frozen tissue sections. In skin graft rejection and tuberculoid leprosy, most infiltrating T lymphocytes were reactive with OKT4, while in lepromatous leprosy and graft vs. host disease most reacted with OKT8. It is of interest that in allograft rejection both the graft and graft bed were infiltrated by similar populations of cells (17), and in leprosy and graft versus host disease monocytes and macrophages were present in abundance (20, 21). These findings suggest either that nonspecific inflammation plays a role in immune disorders other than DTH or that these disparate processes are mediated in part at least by DTH.

An analysis of the DTH response to tuberculin using monoclonal antibodies to identify T lymphocytes, T cell subsets, and HLA-DR-bearing cells was recently reported by Scheynius et al. (41). In contrast to our findings a preponderance of Leu-3a–reactive cells (analogous to OKT4+) was found at 48 h but not at 6 h, as in the present report. On the other hand, their observations of balanced proportions of Leu-2– and Leu-3a–reactive cells at 6 h are consistent with our findings in small perivascular cell clusters. Thus our study patients may have exhibited a more rapid or intense DTH response. Poulter et al. (42) studied the cutaneous DTH response in a similar fashion. They observed dermal infiltration by T lymphocytes and macrophages, and enumeration of OKT4- and OKT8-reactive cells yielded results similar to those reported herein. In contrast to our observations however, OKT8-reactive cells were not seen in the epidermis.

That occasionally substantial numbers of dermal cells reacted with OKT8 was not anticipated and their function is not known. They may delimit specific activity of T4 + lymphocytes, exert cytotoxicity, or mark the contribution of nonspecific inflammation to DTH. Similarity of proportions of T lymphocyte populations in established DTH, control tissues, and blood should not imply functional similarity of cells in each locale. Rather, these observations underscore the limitations of in situ analysis of T cell subsets in established DTH.

Dermal infiltration by monocytes and macrophages was studied using three monoclonal antibodies. In general these antibodies identified similar proportions of cells in similar locations in a given tissue. Perivascular aggregates generally contained fewer cells reactive with these antibodies than with anti–T cell antibodies, while in the dermal interstitium the number of monocytes often equaled or exceeded that of T lymphocytes. The presence of substantial numbers of mononuclear phagocytes in DTH are in accord with some prior reports and at odds with others (32). It may reflect an inflammatory stimulus unrelated to the antigen-specific response (35, 37).

Langerhans cells of the epidermis are reactive with OKT6 (30) and are reported to increase in number within 12 h of tuberculin challenge in presensitized individuals (42). However, analysis of this cell population in responder and control tissues revealed no specific changes associated with emerging cutaneous DTH. On the other hand enlargement and reticulation of Langerhans cells was observed in some tissues 12–48 h after inoculation. The apparent discrepancy may result from methodological differences since only cells sectioned through the ethidium-bromide stained nucleus were enumerated in our study.

Using a monoclonal antibody directed against the B1 antigen and heteroantisera directed against cell surface immunoglobulins we detected only occasional B lymphocytes in the tissues studied. While this finding does not exclude participation of humoral immunity it is consistent with the differential homing properties of B and T lymphocytes (43). The paucity of infiltrating cells bearing surface IgM has importance in another context. Despite substantial evidence of T cell activation, we observed that rare T lymphocytes expressed recognizable HLA-DR determinants. Since IgM serum antibodies capable of blocking recognition of class II determinants have been detected in tuberculin-challenged responders (44) our studies could not exclude the possibility that HLA-DR expression by infiltrating T cells was hidden. However, the absence of significant numbers of cells with surface IgM, the relatively late (48 h) appearance of HLA-DR blocking activity in serum (44) and of surface HLA-DR after stimulation (40) provides substantial evidence that the expression of HLA-DR antigen by T lymphocytes was not overlooked.

OKIa1 reacted with basilar keratinocytes in tissues obtained 48 h after tuberculin administration in responders but not controls. This pattern of reactivity is similar to that previously observed in graft vs. host disease in humans (21) and contact sensitivity in the rat (45). It is of further interest that OKT9 reacted with basilar keratinocytes as early as 15 h after tuberculin administration, before notable infiltration of the epidermis occurred. These findings suggest that DTH influences keratinocyte antigen expression by means other than direct T lymphocyte contact.

Prior studies have not suggested a role for complement activation in DTH (38) yet some deposition of complement components often accompanies inflammation (23). We examined the study tissues using a monoclonal antibody that identifies the poly C9 component of the membrane attack complex and with heteroantisera directed against human C3, the presence of which have been shown to be a highly sensitive indication of humoral immune injury (23). In these studies we observed scanty granular reactivity at the dermal epidermal junction

(including hair follicles) of all tissues and about eccrine glands. However only focal endothelial deposits were observed in DTH lesions and the absence of reactivity amidst large collections of mononuclear cells was striking.

Control tissues contained small perivascular mononuclear cell clusters that morphologically resembled the smaller clusters in tissues from presensitized subjects. However, responder tissues obtained 24 and 48 h after tuberculin administration contained more exuberant infiltration of the dermal interstitium and epidermis than was observed in any control tissue. Furthermore, early enrichment of OKT4-reactive cells and activation of substantial numbers of infiltrating cells was not observed in controls. Despite these differences the similarity of cell populations in responders and controls is striking and is consistent with prior studies performed by light microscopy (38). This finding inferentially supports aforementioned studies, which have emphasized the role of nonspecific inflammation in the initiation of DTH.

## Summary

Delayed-type hypersensitivity (DTH) is a prototypic T lymphocyte-mediated response to antigenic challenge. In this study, mononuclear cells infiltrating the skin during cutaneous response to tuberculin in presensitized human subjects (responders) and nonimmune controls were identified using monoclonal antibodies by indirect immunofluorescence. In both responders and controls the infiltrate consisted mainly of T lymphocytes (T11+ and OKT3+) and monocytes (OKM1+, 63D3+, Mo2+) which initially accumulated in proximity to small blood vessels and later infiltrated the interstitial dermis and epidermis. More T lymphocytes reacted with OKT4 than with OKT8. 6 h after tuberculin the ratio of OKT4/OKT8 in tissue from responders exceeded that in blood, whereas in tissues studied at 15–48 h and in all control tissues those ratios in blood and tissue were similar. Evidence of T lymphocyte activation was sought using monoclonal antibodies anti-Tac, OKT9, and OKT10. In responders but not in controls the proportion of infiltrating cells reactive with these antibodies increased during the course of DTH. The presence of activated T lymphocytes in tissue was not associated with a comparable increase in peripheral blood cell populations identified by anti-Tac and OKT10. Studies using anti-B1, Leu-7, and anti-IgD/IgM revealed comparatively few reactive cells. Dual-labeling studies demonstrated that most Leu-7–reactive cells also bound T11 while fewer bound OKM1 or OKT8 and that cells reactive with OKIa1 and T11 constituted largely nonoverlapping populations. Specific patterns of reactivity were not observed when tissues were stained with anti-human C3, or poly C9-MA, a monoclonal antibody reactive with a neoantigen on polymerized C9 of the membrane attack complex of complement. The number of epidermal Langerhans cells identified by OKT6 was similar in responders and controls. Thus, the cutaneous response to tuberculin in sensitized individuals is characterized by early enrichment of the OKT4 subpopulation of T lymphocytes in tissue infiltrates and subsequent (15–48 h) evidence of T lymphocyte activation.

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