Histometric study of the localisation of lymphocyte subsets and accessory cells in human Mantoux reactions

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SUMMARY Intradermal injection of purified protein derivative produced typical delayed type hypersensitivity reactions in five healthy human subjects. The major subpopulations of lymphocytes and certain accessory cells were located in frozen sections of biopsies of the lesions with monoclonal antibodies and immunohistochemical staining. The densities (expressed as number/unit area for comparison) of the different types of cells were counted at various microanatomical locations in the tissue. The inflammatory cells were concentrated in narrow zones, initially (24 h) only surrounding small blood vessels but later (48–96 h) also around sweat ducts. Lymphocytes were the predominant cell type at these sites with T4 and T8 cells randomly intermixed at a ratio similar to that in the mononuclear cell fraction of the peripheral blood samples removed at the time of biopsy. There was also a scanty diffuse infiltrate in the intervening dermis, but here the T4:T8 ratio was significantly lower than that in the peripheral blood or perivascular cuffs. There was considerable intersubject variation in the relative preponderance of T8 cells in the diffuse infiltrate.

The results suggest that there is no subset selection in the initial emigration of lymphocytes through vascular endothelium in the delayed hypersensitivity reaction, but that the subsets behave differently during the subsequent migration through the tissues. It remains to be determined whether the extent to which T8 cells migrate more rapidly than T4 cells through the tissues may influence the reaction at the site of entry of organisms or antigens into the body by altering the balance of the immunoregulatory lymphocyte subsets. This may underlie some of the differences in susceptibility to infection between subjects and determine the type of granuloma that develops in a particular patient.

Delayed hypersensitivity is a frequent component of chronic inflammatory disease, but it is often difficult to interpret the lesions of spontaneous disease since the current cellular infiltrate is superimposed on the scars and residues of previous reactions. Skin tests, such as the Mantoux reaction to intradermal injection of purified protein derivative, have proved useful for elucidation of the effector arm of the immune response since the changes occur in otherwise normal dermis. Focal perivascular infiltrate of many lymphocytes and few macrophages is the most prominent feature of a delayed hypersensitivity reaction.[1] The introduction of monoclonal antibodies to vari-

ous subsets of lymphocytes[2] and accessory cells[3] has recently made possible the localisation of cells bearing phenotypic markers that possibly indicate their functional role in the Mantoux reaction.[3-5] The broad picture is now clear: most of the lymphocytes are T cells, T4 cells are more numerous than T8 cells in the perivascular foci, macrophages are diffusely scattered in such foci, and B cells are very infrequent. These reports,[3-5] however, differ in detail in their conclusions on the localisation and density of the various cell types, and the supposition[5] that the subjects from the UK differed in speed of development or ultimate intensity of delayed hypersensitivity reaction, or both, from those in USA has been presented without consideration of the differences

in methods of measurement used in the two centres. The present study was undertaken with rigorous histometric methods to evaluate intersubject variation in the localisation of the various subpopulations of lymphocytes and accessory cells in the Mantoux reactions in healthy tuberculin positive individuals.

Material and methods

Five male hospital workers (aged 31, 31, 35, 39, and 55 years) were studied. The two older subjects had had pulmonary tuberculosis (10 and 30 years previously) and the lung lesions were now healed; the three younger subjects had been immunised with BCG previously. The Mantoux test was performed on the dorsum of the forearm by intradermal injection of 0·1 ml tuberculin purified protein derivative BP (Evans Medical Ltd, Middlesex, England) solution 1/10 000. Several intradermal injections of purified protein derivative were given at different sites on both forearms of the 55 year old subject for biopsy at daily intervals for the first four days to determine the time course of the reaction. Some months later all subjects were given a single injection to study subject variation in response at 96 h. Biopsy was performed (under local anaesthesia with 0·5 ml 2% plain lignocaine) with a 4 mm disposable skin biopsy punch (Stiefel Laboratories Ltd, Slough, England). Each specimen was divided in two: one half was snap frozen for immunocytochemistry and the other half was fixed in 4% neutral buffered formaldehyde for resin embedded histopathological study.

Frozen sections (6 μm) were cut in a cryostat (immediately after biopsy, or on the following day), fixed briefly in acetone, rehydrated, and treated with commercial monoclonal antibodies: OKT3, OKT4, OKT8, OKT6, OKM1, and OKIa1 (Ortho Diagnostics, Raritan, NJ, USA); anti-Leu 1, anti-Leu 3a, anti-Leu 2b, and anti-Leu M3 (Becton Dickinson, Sunnyvale, CA, USA); or B1 Coulterclone (Coulter Immunology, Hialeah, FL, USA). The Ortho and Coulterclone reagents were followed by peroxidase labelled antimouse immunoglobulin (Serotec, Bicester, England) and the Becton Dickinson reagents were followed by biotinylated antimouse immunoglobulin (Becton Dickinson) and peroxidase labelled avidin (Becton Dickinson). After histochemical development with dimethylaminoazo-benzene, the sections were dehydrated and mounted in DPX. Because of the difficulties in histometry of immunocytochemical staining by direct viewing in the microscope, monochrome photographic prints (20 × 16 inches) covering the whole area of each section at a magnification of × 250 were prepared. The margins of the perivascular foci were delineated on the prints with a felt pen and the areas of these foci and of the intervening dermis were measured by cursor planimetry in a semiautomated apparatus (Imagan, Graphic Information Systems, Blairgowrie, Scotland). The number of cells in each of these areas was then counted.

The relative numbers of T4 and T8 lymphocytes were measured in the mononuclear fractions of heparinised venous blood prepared by density gradient centrifugation over Ficoll/Hypaque.[6] The cells were treated with the appropriate monoclonal antibody (Ortho Diagnostics), washed in RPMI 1640 containing 5% fetal calf serum (Gibco-Biocult, Paisley, Scotland), and stained in fluorescein isothiocyanate sheep antimouse immunoglobulin (Meloy Laboratories Inc, Springfield, VA, USA). After washing and staining with propidium iodide (0·5 g/l) in 0·1% sodium citrate,[7] the proportion of fluorescein isothiocyanate stained and unstained cells was determined in a flow cytofluorimeter (Model 50H, Ortho Diagnostics, Westwood, MA, USA) with the gate set to include only nucleated particles.

Results

DEVELOPMENT OF DERMAL INFLAMMATION
This was studied in one subject with biopsy samples taken at 24, 48, 72, and 96 h after injection of purified protein derivative. All biopsies showed a dermal chronic inflammatory infiltrate which became progressively more intense during the period of study and had a distribution characteristic of a delayed hypersensitivity reaction (Fig. 1a). The inflammatory cells and exudate were concentrated in spaces where the dermal collagen bundles had been stripped away from the adventitia of the vessel or the skin appendage (Fig. 1b and c), but there was also a less intense diffuse inflammatory infiltrate between the dermal collagen bundles, which was more prominent in the upper dermis. Most of the infiltrating cells were small mononuclear cells with the appearance in haematoxylin and eosin stained sections of "small" and "large" lymphocytes, but no germinal centres were seen. Few of the cells stained for acid phosphatase or other enzyme markers of macrophages, and neutrophil polymorphonuclear granulocytes were infrequent. Arteritis (vasculitis) was never seen. At 24 h the endothelium of capillaries and venules was normal, but at 48 h and later the endothelial cells were swollen. Small masses of amorphous eosinophil debris (precipitated proteinaceous exudate) were scattered between the inflammatory cells, mostly in the perivascular foci. In the more intense reactions of the later biopsies, there was patchy separation of the epidermis from
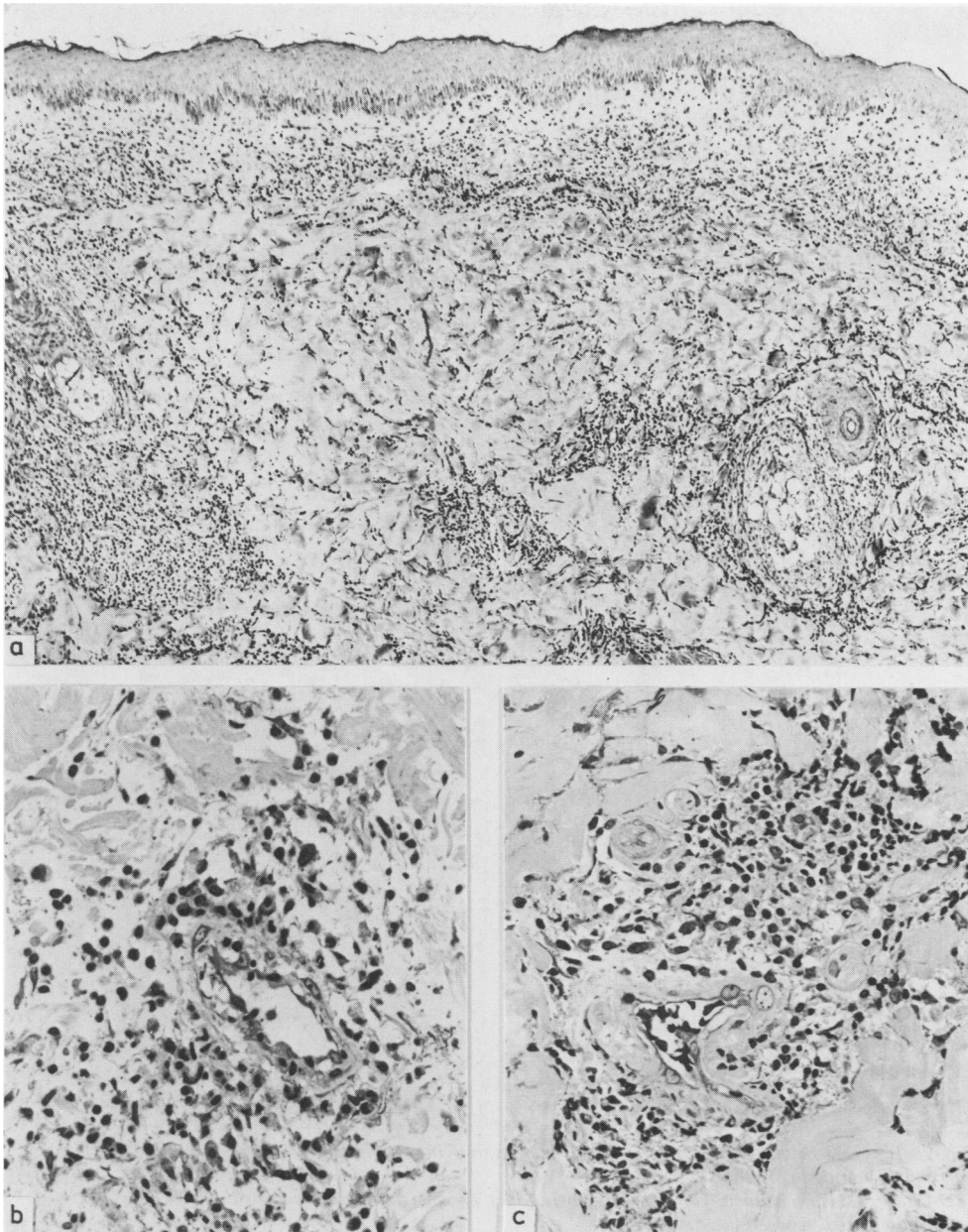
Fig. 1 *(a) Low power photomicrographs of a three day Mantoux reaction stained with haematoxylin and eosin. There is obvious cuffing of mononuclear cells in the dermis around blood vessels and epidermal appendages. The diffuse infiltrate is more intense in the upper dermis. × 70.*
(b) One day Mantoux reaction showing loose mononuclear cell infiltrate in the perivascular space; a lymphocyte is migrating through the vascular endothelium. × 240.
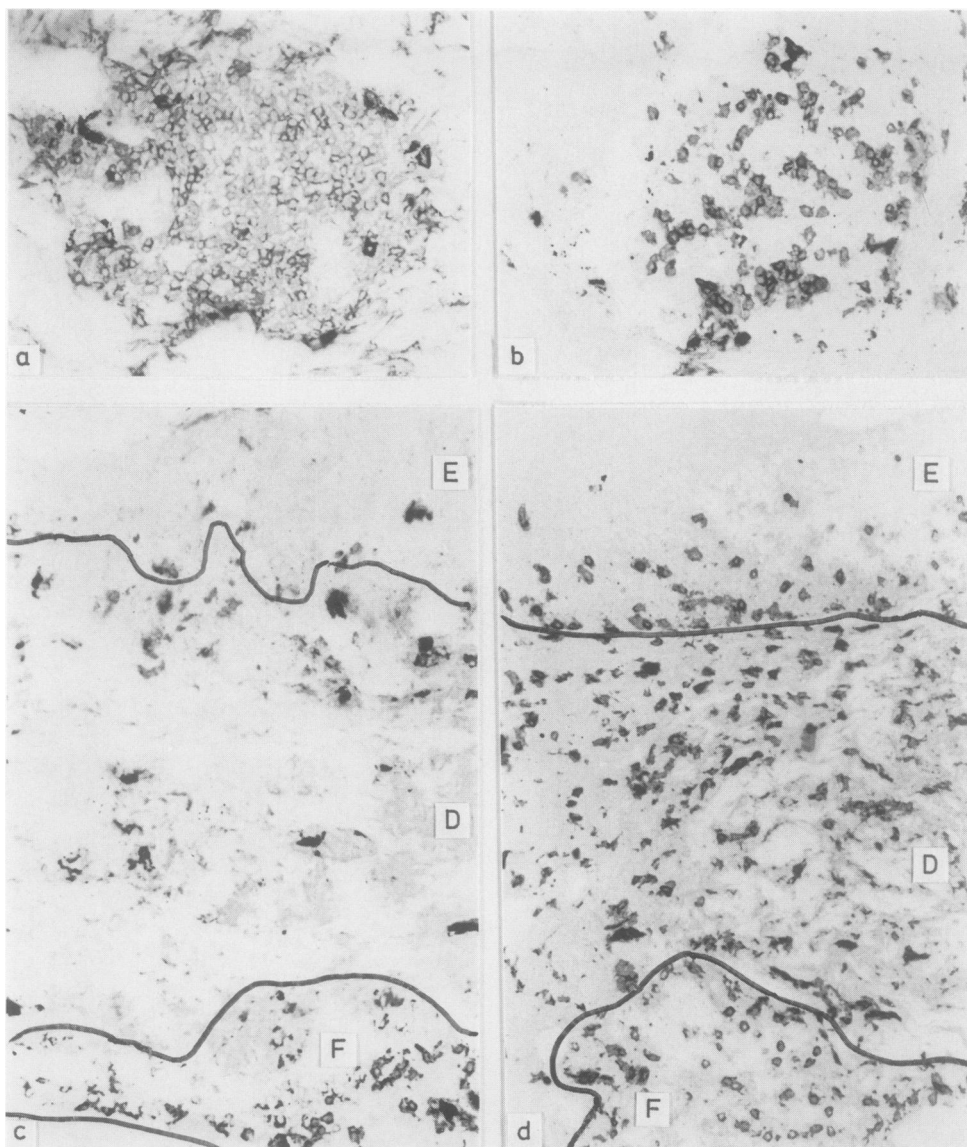*(c) Four day Mantoux reaction showing infiltrate more densely packed than that in (b). × 240.*

Fig. 2 *(a) OKT4 stained four day Mantoux reaction showing that most cells in the perivascular infiltrate carry the T4 antigen. × 240.*
(b) Adjacent section stained with OKT8 showing that T8 cells are less numerous than T4 cells, but randomly mixed between them. × 240.
(c) Section from subject 4 stained with OKT8 showing very few T8 cells outside the perivascular cuff (F); biopsy taken after 96 h. × 240.
(d) Section from subject 1 stained with OKT8 showing many more T8 cells infiltrating diffusely through the dermis (D) and entering the epidermis (E); biopsy taken after 96 h. × 240.

the dermis with bulla formation; eosinophilic debris and sparse mononuclear cells were present in the bulla fluid.

Most cells in the focal infiltrates stained for T3 and T4 markers. T8 cells were much less frequent and had a random distribution within each focus (Fig. 2a and b). In contrast, within the diffuse dermal infiltrate, T8 cells were relatively more numer-

Table 1 *Distribution of lymphocyte subsets in the dermis during the first four days of the development of the Mantoux reaction*

Time after injection of purified protein derivative (h)	Monoclonal antibody	Area of section (mm²)	Perivascular infiltrate area (mm²) (and % of section)	No of cells	Density (cells/mm²) in total infiltrate	T4:T8 ratio in infiltrate*
48†	OKT3	3·95	1·24 (32)	2361	598	
	OKT4	4·51	0·61 (14)	1848	410	
	OKT8	4·92	1·55 (32)	979	199	2·06
72‡	OKT3	8·75	1·67 (19)	6283	718	
	OKT4	8·32	1·46 (18)	4938	594	
	OKT8	8·54	3·19 (37)	2407	282	2·11
96†	OKT3	3·02	0·89 (29)	4942	1636	
	OKT4	2·61	0·45 (17)	2879	1103	
	OKT8	3·16	0·93 (29)	1700	538	2·05

*Standardised to constant area of whole section.
†One section counted.
‡Two sections counted.

ous and were more abundant on the epidermal side of the perivascular foci than on the deep aspect. The general pattern of perivascular concentration of T4 cells and more extensive diffuse infiltration of T8 cells persisted in the 96 h biopsy, where the infiltrate was becoming confluent. The results of cell counts are summarised in Table 1. The proportion of the dermis occupied by perivascular infiltrate remained relatively unchanged during the study period, but the density of cells increased progressively due to close packing in the perivascular foci (Fig. 1b and c). The T4:T8 ratio remained stable, suggesting unselective accumulation of T cell subsets in the inflammatory exudate.

INTERSUBJECT VARIATION IN CELL CONTENT OF MANTOUX REACTION
The results of counts of T lymphocyte subsets in biopsies from five volunteers are summarised in Table 2. There was considerable variation in the percentage of the dermis occupied by perivascular infiltrate, most severe in subject 4 (19 and 21% of dermis) and least in subject 5 (9 and 12% of dermis). The packing density of lymphocytes was remarkably constant in the perivascular foci (range 16 235 to 20 907 cells/mm²), but relatively inconstant in the diffuse infiltrate in the dermis (range 468 to 1169 cells/mm²). The T4:T8 ratio in the perivascular infiltrate was not significantly different from

Table 2 *Intersubject variation in perivascular focal accumulation and in diffuse dermal infiltration of lymphocyte subsets in Mantoux reactions in men biopsied at 96 h*

| Subject	Age (yr)	Dimensions of induration (mm)	Monoclonal antibody	Perivascular infiltrate				Intervening dermis				% of infiltrating cells in perivascular foci	T4:T8 ratio in peripheral blood mononuclear cells
				Area (mm²) (% of dermis)	No of cells	Density (cells/mm²)	T4:T8 ratio	Area (mm²)	No of cells	Density (cell/mm²)	T4:T8 ratio		
1	31	100 × 100	Anti-Leu 3a (anti T4)	0·62 (16)	9670	15597	2·94	3·28	2368	722	1·80	80	2·20
			Anti-Leu 2a (anti-T8)	0·51 (14)	2708	5310		3·21	1286	401		68	
2	35	20 × 20	Anti-Leu 3a	0·37 (13)	4641	12543	2·51	2·40	1158	482	1·97	80	3·58
			Anti-Leu 2a	0·39 (16)	1948	4995		2·06	504	245		80	
3	39	25 × 20	Anti-Leu 3a	0·38 (16)	4830	12711	1·88	1·97	1052	534	1·77	82	1·96
			Anti-Leu 2a	0·33 (11)	2232	6764		2·71	820	303		73	
4	31	250 × 150	Anti-Leu 3a	1·01 (19)	12396	12273	2·47	4·30	1086	253	1·18	92	2·66
			Anti-Leu 2a	1·14 (21)	5666	4970		4·28	919	215		86	
5	55	50 × 50	Anti-Leu 3a	0·12 (9)	1437	11975	2·81	1·28	807	630	1·17	64	2·18
			Anti-Leu 2a	0·15 (12)	639	4260		1·14	615	539		51	
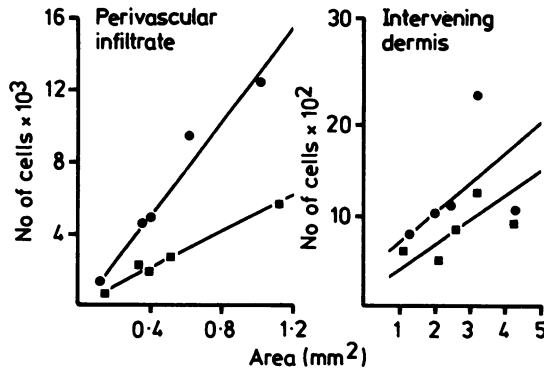
Fig. 3 *Density of both T4 (•) and T8 (▪) cells in the perivascular cuff shows little scatter about the fitted lines (left), suggesting little intersubject variation. There is a much poorer relation between cell number and area of diffuse infiltrate in the intervening dermis (right) owing to significant differences between subjects in the relative reduction of T4 cells in this part of the infiltrate.*

that in the peripheral blood mononuclear cells in samples taken at the time of biopsy. In all cases there were proportionately many more T8 cells in the intervening dermis than in the perivascular cuffs (p < 0·001). The individual subjects, however, differed significantly (p < 0·001) both in the numbers and density of T8 cells (Fig. 2c and d) and in the extent of lowering of the T4:T8 ratio in the diffuse infiltrate (Fig. 3). The T4:T8 ratios in the various parts of the sections were unrelated to the clinical measurements of the size of the Mantoux reactions, to the total amount of infiltrate, or to its distribution between perivascular cuffs and the intervening dermis.

Cells stained with B1 monoclonal antibody (putative B cells) were infrequent in all biopsies. Cells stained with OKM1 or Leu M3 monoclonal antibodies (putative monocytes/macrophages) were more numerous in the perivascular foci (density ranged from 279 to 1407 cells/mm²) than in the diffuse dermal infiltrate (88 to 389 cells/mm²), but the density and relative distribution of those cells were unrelated either to the clinical appearances of the reactions or to the measured features of the lymphocyte infiltrate. Dendritic cells bearing the T6 marker (putative Langerhans' cells) were readily identified in the epidermis (37 to 87/mm²) and infrequent in the dermis (4 to 19/mm²). The OKIa1 monoclonal antibody stained many components in the sections intensely, including dendritic cells and endothelial cells, and gave weak staining of many of the infiltrating lymphocytes; this reagent did not show any substantial difference between the reactions in the individual subjects and no cell counts were made.

Discussion

The introduction of monoclonal antibodies for localisation of antigens in tissue sections has been an enormous advance and has enabled much more discriminating analysis of the cell types concerned in pathological lesions. There are situations, however, where the full potential of these reagents will not be exploited until the immunocytochemical staining can be quantified either in terms of antigen content of individual cells or as precise measurements of the relative numbers of cells bearing particular markers in the microanatomical compartments of the lesion. In this paper we report a simple histometric method for analysis of chronic inflammatory infiltrates. The immunoperoxidase method was selected for visualisation of monoclonal antibody localisation since it gives a relatively permanent image; this is clearly an advantage for histometry over the immunofluorescence method used previously[3] since that image fades relatively rapidly during examination. Moreover, counts from large photographic prints allow accurate delineation of the microanatomical regions in each section and have greater precision than those made with an eyepiece graticule.[5]

The nature of the response to intradermal injection of purified protein derivatives depends on the current immune response of the subject; in most subjects it is a delayed hypersensitivity reaction, but in some patients with active tuberculosis it may be an Arthus reaction.[8 9] Conventional histological sections have indicated that the Mantoux reactions studied for this paper are typical delayed hypersensitivity reactions. It is clear that most of the cells in the chronic inflammatory infiltrate are T lymphocytes and that most of the remainder are monocytes/macrophages. The T cells are first seen in a space around small blood vessels in the 24 and 46 h biopsies; the density of cells in this situation increases with time and cuffing of sweat ducts becomes prominent at 72 and 96 h. Lymphocytes "pavemented" on the endothelium were seen in vessels with perivascular infiltrate (Fig. 1b) but not in the uninvolved dermal capillaries; moreover, the infiltrate was most abundant in the perivascular space in the earlier biopsies, while the diffuse interstitial infiltrate became prominent in the later biopsies. We therefore conclude that the most likely site of primary emigration is through the endothelium of the cuffed capillaries and postcapillary venules with subsequent infiltration through the dermis. Since the T4:T8 ratio in the perivascular infiltrate is not significantly different from that in the lymphocytes in the peripheral blood, we deduce that the emigration of lymphocytes through the endothelium is not selective for the major subsets of T cells and provide

quantitative data to confirm the suggestion made by Poulter *et al.*[3] The relative proportion of T8 cells is greater in the diffusely infiltrating cells than in either the perivascular cuffs or the peripheral blood. Since none of the lesions was more than 96 h old it is likely that the relative preponderance of T8 cells has arisen from selective outward migration of T8 cells from the perivascular cuffs rather than from selective removal of T4 cells into the lymphatic system.

Compared with the granuloctyes, relatively little is known of factors controlling the movement of lymphocytes; chemotactic and chemokinetic responses have not been clearly distinguished. There is some evidence from animal experiments that chemoattractants may be extracted from thymus[10] and skin granuloma,[11] but it is not known whether the chemoattractant is derived from lymphocytes or monocytes.[12] It is now clear that mitogen stimulated human lymphocytes release not only a lymphocyte chemoattractant[13] but also a lymphocyte migration inhibitor.[14] Human T cells migrate more rapidly towards casein than non-T-cells (B cells and "null" cells).[15] Moreover, human peripheral blood Tμ lymphocytes (a subpopulation containing helper cells) migrate well, whereas the Tγ cells (a subpopulation containing suppressor cells) do not respond to casein.[16] Thus, although the Tμ cells migrate actively in vitro, the T4 bearing cells (which also contain the helper/inducer cells) do not appear to migrate as freely in the Mantoux reaction and remain localised in the perivascular cuffs: this apparent contradiction remains to be explained, but it is tempting to speculate whether the lymphocyte migration inhibitor[14] has a dominant role in vivo.

In the granulomas of various human diseases the T4 and T8 lymphocytes have been shown to have distinctive histological localisation,[17-21] but it is not possible to draw firm conclusions on their pathogenesis from the distribution of cells at the time of biopsy because the lesions have evolved over a long time with changing distribution of antigens, waxing and waning immunological effector mechanisms, and progressive scarring. Nevertheless, the microanatomical segregation of modulating cells would be expected to exert a major control on the activity of the responding cells. It has been suggested that the peripheral segregation of T8 cells at the edge of the tuberculoid leprosy granuloma may restrict the extent of tissue damage and that diffuse admixture of T4 and T8 cells throughout the lepromatous leprosy granuloma may prevent epithelioid cell differentiation[18-21]; moreover, the individual patient's response is not fixed, since peripheral localisation of T8 cells appears during reversal reaction.[19]

The experiments reported here show that normal subjects vary greatly in the way in which their T8 cells (a population containing the important regulatory suppressor cells as well as cytotoxic cells) migrate in the tissues after a standardised antigen exposure, but it is possible that this variable is an important factor in explaining the observed differences in response to purified protein derivative skin testing.[8 9] It remains to be established whether the relative velocity of migration of regulatory lymphocytes determines the immediate response of the previously primed subject to the introduction of antigen into the tissues; if the suppressor influence is dominant, the patient may be more susceptible to infection and if the helper influence predominates, then hypersensitivity may result.

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