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Diversity in migration of CD4 and CD8 lymphocytes in different microanatomical compartments of the skin in the tuberculin reaction in man

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Summary. The lymphocytes in the perivascular foci of tuberculin skin tests have a similar CD4:CD8 ratio to those in the peripheral blood, suggesting that these subsets do not show bias in their initial emigration. By contrast, the diffusely infiltrating lymphocytes show a relative preponderance of CD4 cells which is progressively greater in successive 250 μm layers into the dermis. A generally similar pattern is seen in healthy controls and in patients with untreated pulmonary tuberculosis, treated leprosy, haemophilia A and chronic obstructive lung disease (COLD) patients treated with prednisolone, but the gradient of increasing CD4:CD8 ratio with depth into the dermis is significantly less steep in patients with tuberculosis, haemophilia and prednisolone-treated COLD than in the healthy controls. Selective migration results in a relative preponderance of CD4 cells in the diffuse infiltrate and it is suggested that this is a mechanism likely to potentiate defensive reaction to *Mycobacterium tuberculosis:* any deficiency in selective migration may make immunological defences less effective and so contribute to the chronicity of the lesions of tuberculosis.

Keywords: delayed hypersensitivity, tuberculin, CD4 and CD8 lymphocytes

During the development of a delayed hypersensitivity (DHS) reaction (e.g., in the dermis at the site of a tuberculin reaction) lymphocytes and monocytes emigrate from the microcirculation into the tissue (Turk 1980). Most of the inflammatory cells accumulate around the adventitia of the vessels, pushing aside the adjacent dermal connective tissue to form a perivascular cuff, while a minority of cells migrate further into the dermis (Gibbs *et al.* 1984): the associated fluid exudate diffuses widely through the dermis. The T lymphocytes in the peripheral blood mainly belong to the CD4 and CD8 subsets (with a ratio of about 2:1 in the normal human subject). Previous studies of biopsies taken from tuberculin reactions at 48–72 h have suggested that the infiltrating lymphocytes in these reactions also have a CD4:CD8 ratio of about 2:1 (Poulter *et al.* 1982; Konittinen

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et al. 1983; Platt *et al.* 1983). In the DHS reaction to DNCB there was a substantial preponderance of CD4 cells in the early (2 h) phase with a later (48 h) preponderance of CD8 cells (Carr *et al.* 1984). None of these workers attempted to count the cells in the perivascular cuffs separately from those in the diffuse infiltrate, nor did they take into account the gradient of cell densities seen at different depths into the dermis.

Accordingly, this histometric study was undertaken to measure the CD4:CD8 ratios in various regions of the dermal infiltrate of tuberculin tests to ascertain whether or not there was selectivity in the migration of the lymphocyte subpopulations participating in human DHS skin reaction. Subjects with a range of different clinical conditions were studied to determine whether the relative densities achieved may be related to different manifestations of the effector arm of the cellular immune response or whether there is a common pattern independent of disease.

Materials and methods

The subjects comprised (*a*) 29 healthy controls, 44 patients with untreated pulmonary tuberculosis, and four multidrug-treated leprosy patients (two borderline lepromatous and two borderline tuberculoid) from Surabaya, Indonesia, (*b*) 18 patients with chronic obstructive lung disease (currently under treatment with prednisolone) and six normal controls from Dundee and (*c*) nine patients with haemophilia A (all of whom were free of HIV infection) from Glasgow. All subjects had given informed consent to participate in the study: the projects had been approved by the Local Ethics Committees in Surabaya, Indonesia, and in Glasgow and Dundee, Scotland. All the participants in the study were skin tested on the volar aspect of the forearm by intradermal injection of 0.1 ml New Tuberculin prepared from *Mycobacterium tuberculosis* by the method of Stanford and Lema (1983). After measurement of the extent of induration in two axes with a ruler at 48 h, a 4 mm punch biopsy was removed from the injection site under local anaesthesia with 1% lignocaine without adrenalin and snap-frozen with dry ice (Gibbs *et al.* 1984). Immediately before sectioning, the tissue was cryopreserved in glycerol (Coghill *et al.* 1985): 5 μm sections were stained immunocytochemically for CD4 and CD8 markers with Leu 3a and Leu 2a murine monoclonal antibodies (Becton Dickinson, Sunnyvale, CA, USA) and developed with the peroxidase avidin–biotin complex (ABC) kit (Vectastain, Sera-Lab Ltd, Crawley Down, Sussex) using diaminobenzidene (Sigma Chemical Company, Dorset) as a substrate (Gibbs *et al.* 1984). The histometric counts were performed on monochrome photographic prints; the outline of the perivascular and periappendicular foci of chronic inflammatory infiltrate and the limits of three successive 250 μm layers of the dermis from the dermo-epidermal junction inwards were delineated with a felt pen (Beck *et al.* 1986). Within these three layers, the areas of the focal infiltrate and of the intervening dermis was measured by planimetry (Cree *et al.* 1985) and the number of cells in the two compartments in each layer was counted: to facilitate comparisons, the results were expressed as density (number/mm^2). Counting the number of cells in the foci was very time-consuming (*c.* 4 h per section) because the cells were very closely crowded and confident identification of individual cells required frequent comparison between the photograph and the image under direct microscopy: consequently, counts of the cell density in the foci were made on all the patients with leprosy, haemophilia A and the chronic obstructive airways disease patients under treatment with prednisolone, but on only four of the 44 tuberculosis patients and four of the 29 healthy Indonesian controls.

Mononuclear cells were isolated from the peripheral blood by density gradient centrifugation over Ficoll Hypaque. After treatment with anti-CD4 and anti-CD8 monoclonal antibodies (Becton Dickinson), the cells were stained in suspension with FITC anti-mouse immunoglobulin for FACS

analysis on the Dundee and Glasgow patients and for fluorescent microscopy on the Indonesian patients and controls.

Statistical analysis. The comparisons between CD4:CD8 ratios in the peripheral blood with those in the perivascular cuffs and the diffuse infiltrate were performed using the Statistical Package for the Social Sciences (Nie *et al.* 1975) by *t*-test analysis of mean value for CD4:CD8 ratios. The comparisons of CD4:CD8 ratios between the three successive layers into the dermis were made by analysis of variance using the GLIM package (Baker & Nelder 1978): the subsequent comparison between subject groups was made using the Newman–Kreuls procedure (Miller 1966).

Results

Lymphocyte content of inflammatory foci

The densities of CD4 and CD8 cells in foci at the three successive 250 μm levels into the dermis in the five subject groups are summarized in Table 1. Within each subject group the densities were similar in all three levels, but there were considerable differences between groups. The density of cells was greater in the controls and tuberculosis patients than in the patients with leprosy, haemophilia and steroid-treated chronic obstructive lung disease.

For each subject group (Table 1), the CD4:CD8 ratio in foci were closely similar at all three levels (Fig. 1). These ratios were not significantly different from each other nor from those of lymphocytes in the corresponding samples of peripheral blood mononuclear cells in each subject group (Table 1) or the series as a whole (Table 2).

Lymphocyte content of diffuse inflammatory infiltrate

In biopsies from the healthy Indonesian controls there is a progressive fall in density of CD4 and CD8 cells from the epidermal junction inwards: the density of CD4 cells is substantially greater than that of CD8 cells at all levels (Fig. 2). The gradient of decreasing density is much greater for CD8 cells than for CD4 cells and so the CD4:CD8 ratio increases with increasing depth into the dermis. The CD4:CD8 ratios in the diffuse infiltrate are significantly greater than in the peripheral blood lymphocytes at all levels.

The other subject groups showed generally similar trends in CD4:CD8 ratios with a relatively shallow gradient in density of CD4 cells and a much steeper slope of CD8 cells (Table 1): there was, however, a great deal of variation between subjects in all the groups. The mean values for CD4:CD8 ratios in the three successive layers for each subject group are shown in Fig. 3 and the findings in the whole series are summarized in Table 2, confirming a common pattern of emigration. Analysis of variance showed that there were significant differences between the subject groups in the slopes of CD4:CD8 ratio against depth into the dermis ($VR = 7.915$; 4·9 DF; $P < 0.005$). The Newman–Kreuls analysis showed that the fitted slope for the controls (gradient 2.225) was not significantly different from that for the leprosy patients (gradient 1.715): the slopes for the tuberculosis (gradient 1.05), haemophilia (gradient 0.61) and steroid-treated chronic obstructive lung disease patients (gradient 0.025) were not significantly different from each other, but they were all significantly ($P < 0.05$) less steep than the slope for the healthy controls.

The findings on CD4:CD8 ratios in the various dermal compartments were not related to the extent of dermal induration measured clinically.

Discussion

The antigen used in this study was a single batch prepared by ultrasonication of killed *M. tuberculosis* (New Tuberculin). This preparation appears to attract more lymphocytes into the perivascular foci than the more

Table 1. Mean density (and SD) of CD4 and CD8 lymphocytes (per mm^2) and CD4 : CD8 ratios in tuberculin skin tests and in peripheral blood in various groups of human subjects

Region	Lymphocyte subsets	Level	Subject groups				
			Controls	Tuberculosis	Leprosy	Haemophilia	Steroid-treated COLD
Skin test biopsy			$n=4$	$n=4$	$n=4$	$n=9$	$n=16$
Focal infiltrate		1	6316 (980)	5545 (1636)	3784 (1306)	1527 (637)	2146 (757)
	CD4	2	7894 (1070)	6372 (1536)	3250 (1177)	1701 (919)	3202 (1285)
		3	7674 (1341)	6378 (1172)	2839 (1083)	1450 (828)	1836 (857)
	CD8	1	3658 (2756)	1937 (653)	1374 (486)	1771 (089)	1835 (1156)
		2	3472 (1027)	2013 (913)	1408 (513)	1542 (748)	2069 (1344)
		3	3158 (1137)	1982 (813)	1733 (590)	1288 (825)	1696 (858)
	CD4:CD8	1	2.54 (1.73)	3.01 (0.93)	2.76 (0.38)	1.03 (0.50)	1.48 (0.75)
	ratio	2	2.35 (0.36)	3.44 (1.03)	2.43 (0.78)	1.17 (0.38)	1.29 (0.59)
		3	2.77 (1.78)	3.49 (0.90)	1.68 (0.66)	1.24 (0.43)	1.04 (0.31)
			$n=29$	$n=44$	$n=4$	$n=9$	$n=16$
Diffuse infiltrate	CD4	1	351 (156)	362 (144)	246 (125)	181 (145)	174 (181)
		2	270 (144)	269 (140)	1325 (90)	127 (182)	118 (138)
		3	22 (107)	207 (97)	99 (64)	111 (153)	90 (118)
	CD8	1	140 (79)	136 (62)	80 (54)	85 (104)	123 (143)
		2	74 (58)	96 (68)	23 (11)	41 (37)	48 (61)
		3	45 (34)	66 (59)	17 (10)	29 (19)	32 (33)
	CD4:CD8	1	2.89 (1.35)	2.99 (1.47)	4.01 (2.64)	4.73 (7.41)	3.73 (6.27)
	ratio	2	5.74 (5.94)	4.07 (4.59)	5.62 (2.62)	4.04 (4.82)	3.32 (2.28)
		3	7.65 (7.00)	5.08 (7.14)	7.48 (6.93)	5.46 (7.16)	3.74 (2.49)
Peripheral blood	CD4:CD8 Ratio		2.27(0.58)	1.85(0.70)	1.74(0.37)	1.25(0.37)	1.75(0.36)

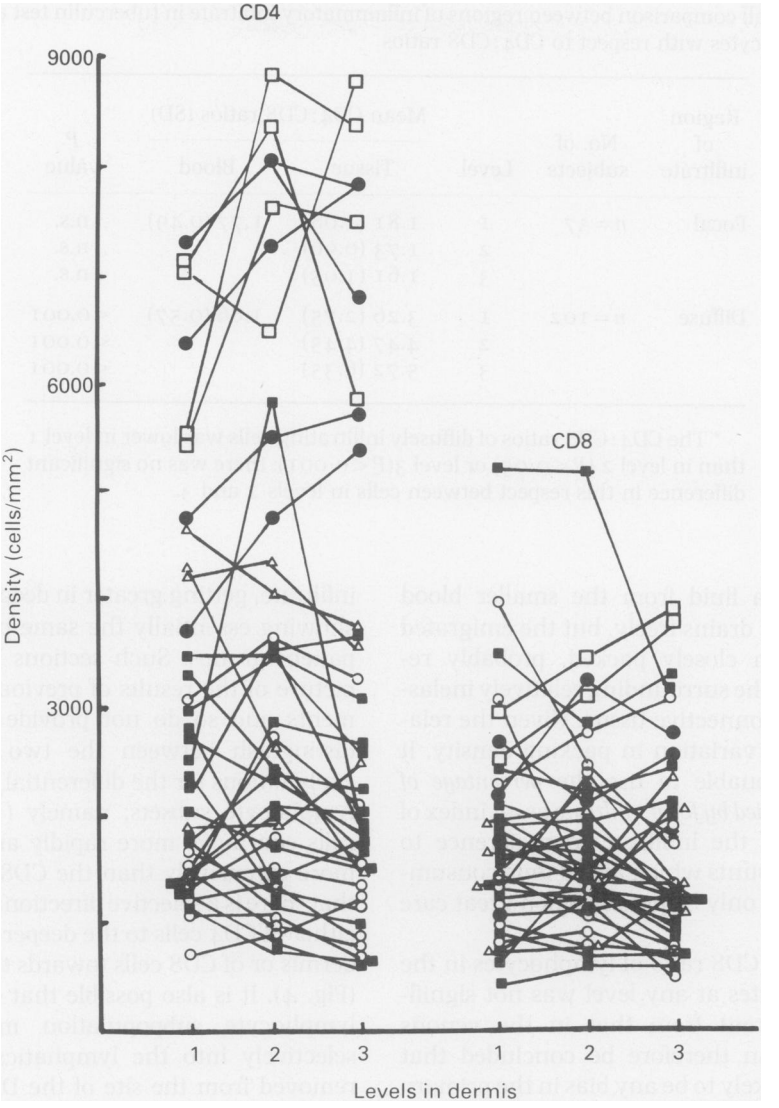

Fig. 1. The density of CD4 (left) and CD8 (right) lymphocytes in perivascular foci at successive 250 μm layers of the dermis 48 h after skin testing with New Tuberculin. The density of CD4 cells is greater than that of the CD8 cells, with no significant variation between levels. □, Controls; △, leprosy patients; ○, haemophilia patients; ●, tuberculosis patients; ■ COLD patients.

conventional PPD preparation (Morley *et al.* 1987).

The density of lymphocytes (CD4 plus CD8) in the perivascular and periappendicular foci showed much less variability than that in the diffuse infiltrate. There was little change in lymphocyte density with level into the dermis and the differences between the subject groups must be considered relatively minor, bearing in mind the technical difficulties in distinguishing the outlines of individual cells when closely packed together. Concurrent with the emigration of mononuclear cells there is exudation of protein-

Table 2. Overall comparison between regions of inflammatory infiltrate in tuberculin test and peripheral blood lymphocytes with respect to CD4:CD8 ratios

Region of infiltrate	No. of subjects	Level	Mean CD4:CD8 ratios (SD)		*P* value
			Tissue	Blood	
Focal	$n=37$	1	1.81 (1.08)	1.77 (0.49)	n.s.
		2	1.73 (0.96)		n.s.
		3	1.61 (1.05)		n.s.
Diffuse	$n=102$	1	3.26 (2.75)	1.86 (0.57)	<0.001
		2	4.47 (4.45)		<0.001
		3	5.72 (6.35)		<0.001

* The CD4:CD8 ratios of diffusely infiltrating cells was lower in level 1 than in level 2 ($P<0.05$) or level 3($P<0.001$): there was no significant difference in this respect between cells in levels 2 and 3.

rich oedema fluid from the smaller blood vessels. This drains freely, but the emigrated cells remain closely packed, probably restrained by the surrounding relatively inelastic dermal connective tissue. Given the relatively small variation in packing density, it seems reasonable to use the *percentage of dermis occupied by focal infiltrate* as an index of this part of the infiltrate in preference to direct cell counts which are so time consuming and will only be precise when great care is taken.

The CD4:CD8 ratio of lymphocytes in the focal infiltrates at any level was not significantly different from that in the venous blood. It can therefore be concluded that there is unlikely to be any bias in the primary emigration of lymphocytes from blood circulating through the walls of the exchange vessels into the perivascular space. This observation does not, however, give any indication of the likely site in the microcirculation (dermal papillary capillary loops, venules in the superficial or deep dermal plexuses and/or the communicating vessels) of the preponderant emigration.

The density of lymphocytes in the diffuse infiltrate is always at least tenfold less than that in the focal infiltrate, but the CD4:CD8 ratio is consistently raised in the diffuse infiltrate, getting greater in deeper levels and showing essentially the same pattern in all patient groups. Such sections give a static picture of the results of previous cell movements and so do not provide evidence to distinguish between the two most likely explanations for the differential migration of lymphocyte subsets; namely (1) that CD4 cells can move more rapidly and so spread more extensively than the CD8 cells, or (2) that there is a selective directional migration, either of CD4 cells to the deeper layers of the dermis or of CD8 cells towards the epidermis (Fig. 4). It is also possible that one or other lymphocyte subpopulation may migrate selectively into the lymphatics and so be removed from the site of the DHS reaction. Whatever the cause of differential mobility, it is likely that the divergence in subset density will increase the larger the distance from the original point of emigration from the blood vessel. It would therefore appear that the most likely site of greatest emigration has been in level 1 which will correspond approximately in microanatomical terms with the papillary dermis (Fig. 4).

It would be unwise to draw conclusions from the observation that the gradient of CD4:CD8 ratios in the leprosy patients was not significantly different from that in the

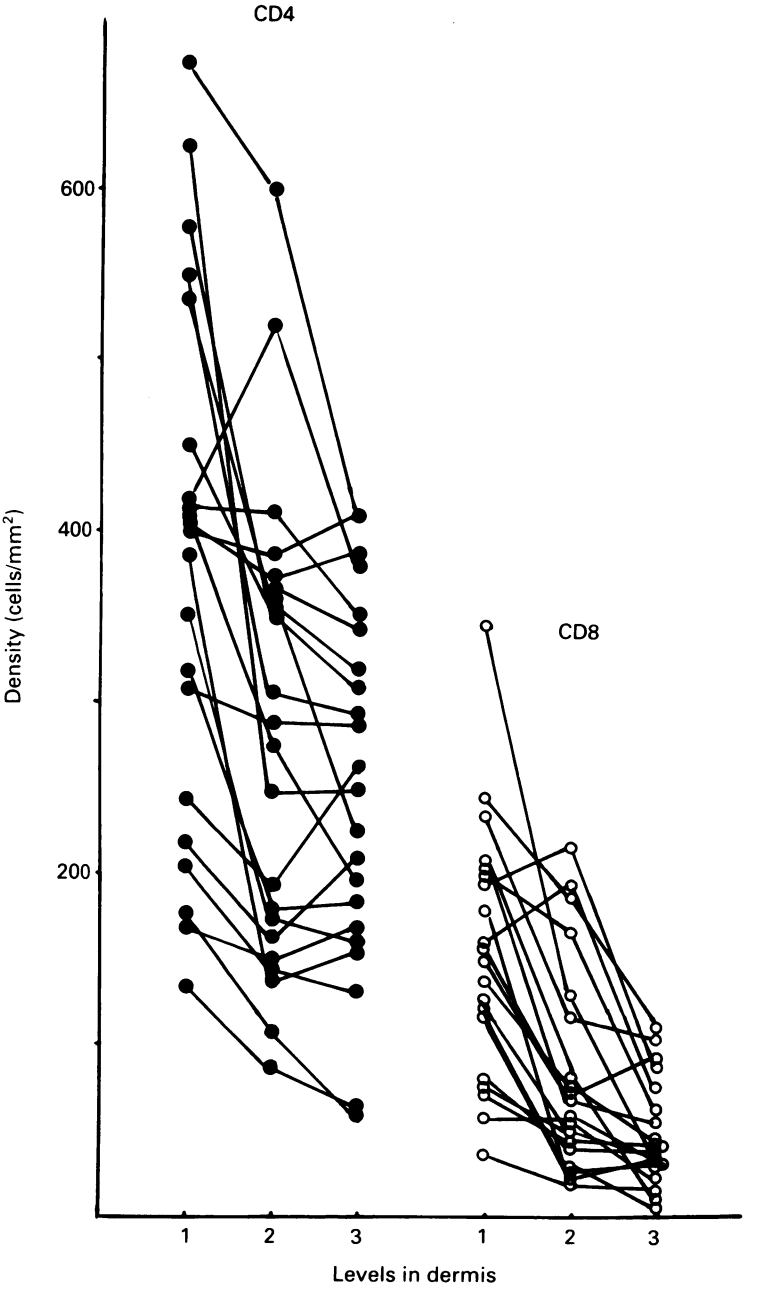

Fig. 2. Density of CD4 (left) and CD8 (right) lymphocytes in successive 250 μm layers in the dermis in skin tests to New Tuberculin in healthy controls. The CD4 cells are more numerous than the CD8 cells and both show reduction in density with increasing depth into the dermis, but the gradient is greater for the CD8 cells.

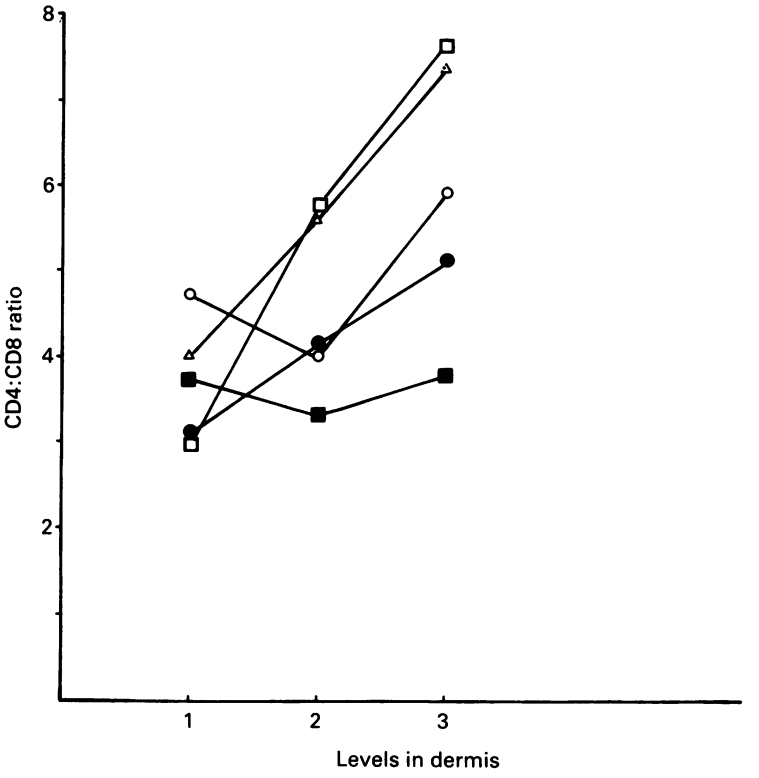

Fig. 3. Comparison between subject groups of mean CD4:CD8 ratios for lymphocytes in the intervening dermis at three levels in skin test reactions to New Tuberculin. □, Controls; △ leprosy patients; ○, haemophilia patients; ●, tuberculosis patients; ■, COLD patients.

controls, since the number of leprosy patients studied was small. The finding of much smaller numbers of infiltrating cells in the leprosy patients confirms our previous report of lower cell density in skin tests on patients with this disease (Beck *et al.* 1986).

It was, however, noteworthy that the CD4:CD8 gradients were significantly less steep in the other groups of patients. The lowest slopes were seen in the prednisolone-treated chronic obstructive lung disease patients: differential migration resulting in an exaggerated preponderence of CD4 lymphocytes in the diffuse infiltrate should therefore he added to the long list of glucocorticoid effects in the immunologically mediated inflammatory reaction (Fauci 1978–9).

The lowered slope in biopsies from haemophilia patients who are not infected with HIV implies that there is some impairment of the effector mechanisms of cellular immunity in these patients. Such patients may also show a minor CD4 lymphopenia (Madhok *et al.* 1986), but they have not lost the capacity for 'recall' immunity (Sharp *et al.* 1987).

The finding that there is a lesser relative preponderance of CD4 cells in the diffuse part of the infiltrate in the tuberculosis patients than in the controls has not yet been explained. The CD4 subset contains most of the 'helper' cells and the CD8 subset most of the 'suppressor' cells: it is likely that the intensity of the local immunological effects in tissue will be strongly influenced by the CD4:CD8 ratio of the lymphocytes in the immediate vicinity. It is therefore possible that the raised CD4:CD8 ratio in the diffusely infiltrating lymphocytes in tuberculin skin

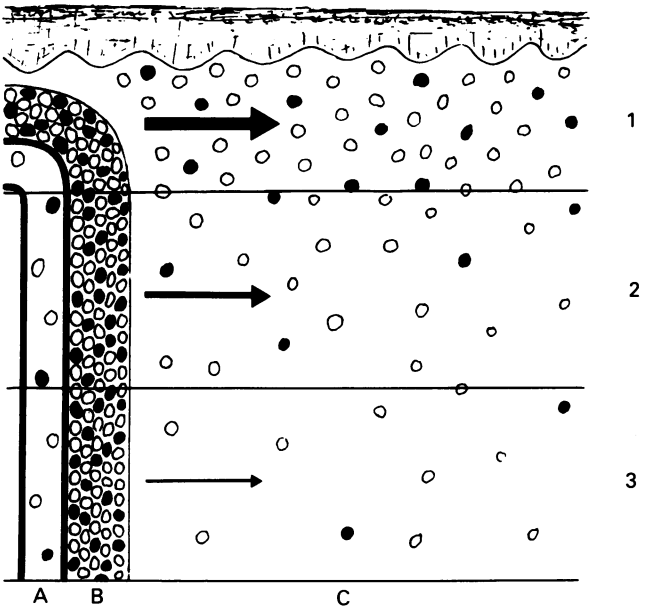

Fig. 4. The distribution of CD4 (○) and CD8 (●) cells in the tuberculin reaction. A, the CD4:CD8 ratio in the capillary is approximately 2:1. B, the perivascular space contains tightly packed cells with a CD4:CD8 ratio of approximately 2:1. C, the intervening dermis contains a less dense infiltrate of cells. The density of cells decreases and the CD4:CD8 ratio increases in the succeeding 250 μm depths of the dermis (1–3). This could be due to variations in migration of the cells into, or clearance from, the dermis at the different depths or to differential migration of the cell types towards the epidermis.

tests of healthy Indonesian controls will contribute to their successful defences againt *M. tuberculosis* infection, even after repeated exposure. By contrast, the tuberculosis patients show much lower CD4:CD8 ratio in the diffusely infiltrating lymphocytes of the tuberculin skin test: if a similar cellular selection occurs in the pulmonary lesions, then it is likely that the defensive reaction will be reduced, so contributing to the chronicity of the disease. The frequent presence of T4 (CD4) lymphopenia in untreated pulmonary tuberculosis patients may further compromise protective immunity (Beck *et al.* 1985).

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