Histometric studies on cellular infiltrates of tuberculin tests in patients with haemophilia

J G LOWE,* J SWANSON BECK,* R MADHOK, A GRACIE, J H GIBBS,* R C POTTS,* G D O LOWE, C D FORBES

From the *Department of Pathology, University of Dundee, and Department of Medicine, Royal Infirmary, University of Glasgow, Scotland

SUMMARY The number and microanatomical location of CD4 and CD8 lymphocytes, of cells bearing receptors for I12 and transferrin, and of monocyte/macrophages in the dermis at the site of a tuberculin test were measured in 13 patients with haemophilia (10 seronegative and three seropositive for human immunodeficiency virus (HIV)). The overall density of lymphocytes in the perivascular and diffuse parts of the infiltrate was similar to that reported in other groups of subjects without evidence of immunosuppression. The CD4:CD8 ratio of the infiltrating lymphocytes throughout the section showed an inverse relation with clotting factor consumption. There was no significant change in the CD4:CD8 ratio in the diffuse infiltrate at various levels into the dermis in tuberculin reactions in patients with haemophilia, unlike healthy controls and other groups with no evidence of immunosuppression, who have previously been shown to have increasing CD4:CD8 ratio with increasing depth into the dermis. The number of cells bearing receptors for I12 and transferrin and of monocyte/macrophages was related to total lymphocyte density in the infiltrate. There was no evidence of serious impairment of the cell mediated response to a long term recall antigen, but the relatively low preponderance of CD4 lymphocytes in the diffuse infiltrate, particularly in the deeper dermis, may be the earliest indicator of impending immunosuppression.

Patients with haemophilia treated with injections of factor VIII concentrate are at risk of infection with the human immunodeficiency virus (HIV) through contamination of the donor plasma pool. The extent of the risk is related to the amount, type, and source of factor VIII concentrate used.^{1,2} Infected patients pass through a latent period and the development of AIDS is usually heralded by a progressive CD4 (T4) lymphopenia with reversed CD4:CD8 ratio in the peripheral blood lymphocytes. Patients with AIDS are vulnerable to opportunistic infection and often cutaneous anergy.

Skin test reactivity to DNCB is similar in HIV seropositive and seronegative haemophiliacs³; both groups show an inverse relation between the amount of clotting factor consumed by the patient and the intensity of the DNCB skin test response, suggesting that repeated parenteral administration of factor VIII concentrate is immunosuppressive (in the sense of reducing the response to DNCB immunisation), even in patients apparently free from HIV infection. Furthermore, it has been shown that the proliferative response of lymphocytes to phytohaemagglutination (PHA) in vitro is reduced by added factor VIII concentrate.⁴

Gibbs *et al* have devised a simple histometric method for measurement of subsets of immunologically competent cells in tuberculin skin test reactions⁵; this method can detect differences not readily distinguishable on direct microscopy.^{6,7} This technique was used to analyse skin test responses in haemophiliacs without AIDS with and without anti-HIV to determine whether the effector mechanisms of cell mediated immunity to a recall antigen are defective in these high risk patients.

Material and methods

Thirteen patients with clinically severe haemophilia were studied: three had serum antibody to HIV and the remaining 10 patients remained seronegative for a follow up of eight months. All had participated in a previously reported study on responses to experimental immunisation with DNCB.³ The mean annual consumption of clotting factor concentrate for the four years before the investigation was calculated. All subjects had volunteered to participate in the present study, which had been approved by the local ethics committee.

The subjects were skin tested by intradermal injection

Fig 1 Comparison between clinical appearance of tuberculin skin tests in haemophiliacs and (a) DNCB response; (b) percentage of dermis occupied by focal infiltrate; (c) total density of lymphocytes (CD4 + CD8) in diffuse infiltrate; (d) overall macrophage density in dermis. ● = seronegative for HIV; ○ = seropositive for HIV.

tion of 0.1 ml of "New Tuberculin", an ultrasonicate skin test antigen prepared from *Mycobacterium tuberculosis*.⁸ The injection site was examined 48 hours later and the response was recorded as "negative" if no change was observed, "+" if there was localised erythema, "++" if the erythema was accompanied by induration and "+++" if the epidermis was blistered over the indurated area. The absolute counts of CD4 and CD8 lymphocytes were measured on venous samples treated with heparin,³ before biopsy of the skin test site (1% plain lignocaine local anaesthetic) with a 4 mm skin punch under full aseptic precautions⁶; the biopsy procedure was covered by additional clotting factors and local pressure was maintained until a stable haematoma was formed. All biopsy sites healed normally and none was complicated by infection.

Cryostat sections were stained immunocytochemically for localisation of the major T lymphocyte subsets (with anti-CD4 and anti-CD8), monocyte/macrophages (Leu-M3) and "activated" lymphocytes (bearing I12 and transferrin receptors) with murine monoclonal antibodies (Becton Dickinson, Sunnyvale, California, USA). The slides were stained with the peroxidase avidin-biotin complex (ABC kit, Vectastain, Sera-Lab Ltd, Crawley Down, Sussex)

using diaminobenzidine as substrate.⁵ The micro-anatomical distribution of cellular infiltrate in the dermis at the site of the tuberculin test in the haemophiliacs was similar to that seen previously in normal subjects.⁶ The density (number/mm²) of each cell type in the two compartments (the perivascular foci and the intervening dermis) was counted in the whole of the section and in three successive 250 µm layers into the dermis.⁹ The results of histometric studies were compared with those obtained previously in normal subjects with the same methods.^{6,9}

Results

The intensity of the changes seen on clinical examination of the tuberculin tests (a long term recall response) was clearly correlated with that observed with DNCB (a short term recall response) in the previous investigation³ (fig 1a), but it was not correlated with the clotting factor consumption or the HIV antibody state. Two of the three seropositive patients were negative to both tests, but the other patient gave a relatively strong reaction in both tests.

The extent of focal infiltrate ranged from 2.14% to 26.96% of the dermis (similar to that measured previously in other subject groups⁹) and this was not

Fig 2 CD4:CD8 ratios in diffuse infiltrate in three successive 250 µm layers into dermis (level 1 is most superficial) of tuberculin skin test of haemophiliacs. Findings from a previous study on normal subjects⁹ are shown with mean (SD) plots.

related closely to the clinical appearance (fig 1b), clotting factor consumption, or HIV antibody state.

The density of lymphocytes (CD4 + CD8) in the diffuse infiltrate ranged from 20.2 to 137.6 cells/mm², except for one outlier with 674.6 cell/mm²; thus 12 of the 13 patients had diffusely infiltrating lymphocyte densities similar to that seen previously in other patient groups.⁹ These measurements were unrelated to clinical appearances (fig 1c), clotting factor consumption, or HIV antibody state. There was a lesser preponderance of CD4 lymphocytes than was seen previously in other subject groups.⁹

The CD4:CD8 ratio of peripheral blood lymphocytes of the patients was within the normal range, but it was generally at the lower end in the HIV seropositive subjects. The CD4:CD8 ratio in the

dermal focal inflammatory infiltrate was not significantly different from that of cells in the blood. The CD4:CD8 ratio in the diffusely infiltrating lymphocytes in tuberculin tests was greater than that in either the blood or focal lymphocytes, but this ratio did not show any significant change with increasing depth into the dermis (fig 2). In this respect the haemophiliacs differed considerably from normal volunteers or patients with leprosy, pulmonary tuberculosis, or chronic obstructive airways disease receiving prednisolone (analysis of variance, variance ratio 7.92, $p < 0.005$); the Newman-Kreuls analysis¹⁰ showed that the fitted slope for healthy controls was 2.23 and that for haemophiliacs was 0.025 ($p = 0.05$).⁹ The CD4:CD8 ratio of lymphocytes throughout the section showed an inverse relation with clotting factor consumption.

The number of "activated" lymphocytes was positively correlated with lymphocyte density throughout the section. It was noticeable that some of the clinically "negative" reactions had substantial numbers of "activated" cells in their infiltrate (fig 3a).

The density of monocyte/macrophages throughout the section was 42.2 to 167.9 cells/mm² (similar to that found in normal subjects⁹) and was not correlated with the clinical appearance (fig 1d) nor with clotting factor consumption or HIV antibody state. The overall density of macrophages was clearly related to that of the lymphocytes (fig 3b).

Discussion

The microanatomical distribution of lymphocytes and macrophages and their density in the dermal compartments was generally similar in the tuberculin reactions

Fig 3 Comparison between density of lymphocytes over the whole section and (a) number of activated cells and (b) density of macrophages.

of haemophiliacs to that seen in healthy controls and patients where there has been active antigenic stimulation with mycobacterial antigens.⁶ Moreover, the number of "activated" lymphocytes in the reactions was clearly related to the total density of lymphocytes in the infiltrate. These histometric measurements provide strong evidence that there is no major disturbance in the basic effector arm of CMI in the haemophiliacs studied in this investigation.

Fig 1b shows that three haemophiliacs, one of whom was seropositive for HIV, had substantial cellular infiltrate at the site of antigen injection despite the absence of erythema or induration of the skin. This disparity between the external and histological appearances cannot necessarily be taken as evidence of defective immunity, as a similar "pseudo-nergy" has been seen in normal subjects and in patients with sarcoidosis,¹¹ mycobacterial disease,⁶ and chronic obstructive airways disease receiving prednisolone.¹²

The diffuse infiltrate in healthy subjects has a relative preponderance of CD4 cells (three to five times that seen in the blood^{6,9}): the haemophiliacs (none had clinical evidence of immunosuppression) showed a lesser preponderance of CD4 cells in the diffuse infiltrate. Moreover, their tuberculin tests did not show a gradient of increasing CD4:CD8 ratio of diffusely infiltrating lymphocytes with depth into the dermis: this contrasts with the strong gradient seen previously in healthy controls and patient groups without evidence of immunosuppression.^{6,9} This subtle disorder in T cell subset migration through tissues may be the earliest indication of impending immunosuppression: as it is seen in seronegative subjects it may help to explain the anecdotal evidence of immunosuppression reported in haemophiliacs apparently without HIV.¹³⁻¹⁶

We are grateful to The Scottish Hospital Endowments Research Trust for financial support. Dr J L Stanford of the Middlesex Hospital Medical School generously supplied the "New Tuberculin" used for skin testing. Mr R S Fawkes and Mrs Sheila Gibbs prepared the diagrams and Mrs Rosalind Mitchell gave valuable secretarial assistance.

References

- 1 Melbye M, Froebel KS, Madhok R, *et al*. HTLV3 seropositivity in European haemophiliacs exposed to factor VIII concentrate imported from the USA. *Lancet* 1984;ii:1444-6.
- 2 Ludlam CA, Tucker J, Steel CM, *et al*. Human T lymphotropic virus type III (HTLV3) infection in seronegative haemophiliacs after transfusion of factor VIII. *Lancet* 1985;ii:233-6.
- 3 Madhok R, Gracie A, Lowe GDO, *et al*. Impaired cell mediated immunity in haemophilia in the absence of infection with human immunodeficiency virus. *Br Med J* 1986;293:978-80.
- 4 Froebel KS, Madhok R, Forbes CD, Lennie SE, Lowe GDO, Sturrock RD. Immunological abnormalities in haemophilia: are they caused by American factor VIII concentrates? *Br Med J* 1983;287:1091-3.
- 5 Gibbs JH, Ferguson J, Brown RA, *et al*. Histometric study of the localisation of lymphocyte subsets and accessory cells in human Mantoux reactions. *J Clin Pathol* 1984;37:1227-34.
- 6 Beck JS, Morley SM, Gibbs JH, *et al*. The cellular responses of tuberculosis and leprosy patients and of healthy controls in skin tests to "New Tuberculin" and Leprosin A. *Clin Exp Immunol* 1986;64:484-94.
- 7 Morley SM, Beck JS, Grange JM, Brown RA, Kardito T. The method of preparation of an antigen may influence the cellular reaction to it in skin tests for delayed hypersensitivity: comparison between responses to two different reagents prepared from *Mycobacterium tuberculosis*. *Clin Exp Immunol* 1987;69:584-90.
- 8 Stanford JL, Lema E. The use of a sonicate preparation of *Mycobacterium tuberculosis* (new tuberculin) in the assessment of BCG vaccination. *Tubercle* 1983;64:275-82.
- 9 Beck JS, Morley SM, Lowe JG, *et al*. Diversity in migration of CD4 and CD8 lymphocytes in different microanatomical compartments of the skin in the tuberculin reaction in man. *Br J Exp Pathol* 1988;(in press).
- 10 Miller RG. *Simultaneous statistical inference*. New York: McGraw Hill, 1966;82.
- 11 Mishra BB, Poulter LW, Janossy G, James DG. The distribution of lymphoid and macrophage-like cell subsets of sarcoid and Kveim granulomata: possible mechanism of negative PPD reaction in sarcoidosis. *Clin Exp Immunol* 1983;54:705-15.
- 12 Lowe JG, Beck JS, Gibbs JH, *et al*. Skin test responsiveness to four new tuberculin in patients with chronic obstructive airways disease receiving short term high doses of, or long term maintenance treatment with, prednisolone: clinical appearances and histometric studies. *J Clin Pathol* 1987;40:42-9.
- 13 Aronson DL. Pneumonia deaths in haemophiliacs. *Lancet* 1983;ii:1023.
- 14 Beddall AC, Hill FGH, George RH. Haemophilia and tuberculosis. *Lancet* 1983;i:1226.
- 15 Madhok R, Lowe GDO, Forbes CD, Stewart CJR, Lee F. Extranodal lymphoma in a haemophiliac negative for antibody to HIV. *Br Med J* 1987;294:679-80.
- 16 Mannucci PM, Quattrone P, Matturri L. Kaposi's sarcoma without human immunodeficiency virus antibody in a haemophiliac. *Ann Intern Med* 1986;105:466.

Requests for reprints to: Professor J Swanson Beck, Department of Pathology, Ninewells Hospital and Medical School, PO Box 120, Dundee DD1 9SY, Scotland.