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CD4 lymphocyte subset abnormalities associated with impaired delayed cutaneous hypersensitivity reactions in patients with X-linked agammaglobulinaemia

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SUMMARY

Circulating CD4 lymphocyte subset (CD45RA; CD45RO; CD29; Leu8) levels were determined in nine patients with X-linked agammaglobulinaemia (XLA), nine patients with common variable immunodeficiency (CVI) and in 18 age- and sex-matched controls. CD4CD45RO and CD4CD29 cells were significantly lower ($P<0{\cdot}01$) in the XLA patient group (CD45RO, $15{\cdot}7 \pm 10{\cdot}2\%$; CD4CD29, $32{\cdot}1 \pm 14{\cdot}6\%$) compared with CVI patients ($61{\cdot}8 \pm 25{\cdot}4\%$; $60{\cdot}1 \pm 11{\cdot}2\%$) and normal controls ($43{\cdot}7 \pm 22{\cdot}3\%$, $54{\cdot}5 \pm 22{\cdot}0\%$). The levels of CD4CD45RA and CD4Leu8 cells were not abnormal in the XLA patient group. No selective reduction in CD4 subsets was observed in the CVI patient group. Delayed cutaneous hypersensitivity testing of five XLA and five CVI patients revealed a significantly reduced response to recall antigens in patients with XLA. This may relate to the deficiency of circulating memory T cells observed in these patients.

Keywords CD4 lymphocytes X-linked agammaglobulinaemia common variable immunodeficiency delayed hypersensitivity reactions

INTRODUCTION

The immunopathogenic mechanisms responsible for primary hypogammaglobulinaemia remain incompletely understood. However, for the two main types, X-linked agammaglobulinaemia (XLA) and common variable immunodeficiency (CVI), well-defined differences have been identified.

In XLA, a defect in B cell maturation at the pre-pre-B cell/pre-B cell level leads to an absence of mature B cells in the circulation [1]. T cell numbers and responses are generally normal, although occasionally increased T cell suppressor activity has been observed [2].

In CVI, the acquired immunodeficiency is a consequence of variable B cell defects that may be associated with T cell and accessory cell abnormalities [3]. Immunophenotypically mature B cells are present in the circulation of most CVI patients [4,5], but these cells fail to differentiate further [6,7] or to produce immunoglobulin [8,9]. Functional B cell studies have identified subgroups of the disease [10]; however, it is uncertain whether these represent a spectrum of severity of a single defect or reflect distinctive molecular defects [3]. Increased T suppressor cell activity [11,12] and reduced T helper function [2,13], have been reported but these are not consistent findings in all patients [3]. Consequently, the role of T cells, whether in the initiation, perpetuation or in response to the disease, remains to be clearly defined.

Immunophenotypic analyses combined with *in vitro* functional assays have allowed further characterization of cells of the CD4 and CD8 lineages [14–16]. Using MoAbs against the 205/220-kD (CD45RA) [17] and 180-kD (CD45RO) [18] determinants of the leucocyte common antigen on CD4 cells it is possible to identify naive (CD4CD45RA) and memory (CD4CD45RO) T helper cells. CD4CD45RA cells respond poorly to recall antigens and are believed to represent unprimed CD4 cells [14]. In addition, these cells fail to promote immunoglobulin production in a pokeweed mitogen (PWM) system but do induce suppressor cell function [19]. These cells have been termed suppressor–inducer lymphocytes [19].

The reciprocal population of CD4CD45RO cells are responsive to recall antigens and can provide help for immunoglobulin production in PWM assays [18], but fail to induce suppressor activity [19]. These cells have been described as inducers of help [15].

Two further MoAb reagents, anti-CD29, which binds the VLA β chain [20], and anti-Leu 8 [21] which identifies leucocyte adhesion molecule-1 [22] also define CD4 subpopulations with helper–inducer (CD4CD29) [20] and suppressor–inducer (CD4Leu8) [23] activities.

Although CD4CD29 and CD4Leu8 lymphocytes share broadly overlapping *in vitro* functional characteristics with

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Table 1. Circulating lymphocyte counts and percentages of T, T subsets and B cells in patients with X-linked agammaglobulinaemia (XLA), patients with common variable immunodeficiency (CVI), and in the control subject group

Group	Patient number	Sex	Age (years)	Peripheral lymphocyte count ($\times 10^9/l$)	Percentage			
					CD3	CD4	CD8	CD19
XLA	1	M	32	2·11	74	56	19	<1
	2	M	28	1·39	57	53	10	<1
	3	M	21	1·74	79	40	15	<1
	4	M	19	2·39	70	50	23	<1
	5	M	20	2·40	89	52	31	<1
	6	M	5	2·25	51	43	17	25
	7	M	6	2·18	95	80	13	<1
	8	M	6 months	6·12	77	60	15	<1
	9	M	9	0·90	82	74	10	11
CVI	10	M	54	2·60	70	30	33	12
	11	M	30	0·68	58	33	13	3
	12	F	73	2·11	65	34	29	17
	13	F	48	1·45	59	23	19	10
	14	M	18	2·11	80	36	36	9
	15	F	52	0·96	70	54	28	8
	16	M	42	2·07	80	39	45	6
	17	M	37	1·37	71	25	ND	12
	18	F	51	0·71	70	53	17	7
Controls ($n=18$) (mean $\pm$ s.e.m.)		14M 4F	1–71 (range)	2·67 $\pm$1·23	69·5 $\pm$10·2	44·1 $\pm$10·6	24·8 $\pm$6·8	8·3 $\pm$3·6

ND, Not determined.

CD4CD45RO and CD4CD45RA cells respectively, they are not identical, and probably represent subsets within the CD4CD45RA subpopulations [15].

Given the importance of these cells in immune responses, determination of the roles of CD4 lymphocyte subsets may contribute to our understanding of the primary immunodeficiency disease states. We have investigated the composition of circulating CD4 lymphocyte subpopulations in patients with XLA and CVI and have related the findings to an evaluation of the patients' cell-mediated immune status.

SUBJECTS AND METHODS

Patients

Eighteen patients with primary hypogammaglobulinaemia attending the Immunology Clinic Royal Victoria Hospital, Belfast, were studied (Table 1). Nine patients had XLA and nine had CVI.

Patients with XLA had an age range of 6 months–32 years (mean 16 years). All patients were receiving i.v. gammaglobulin replacement therapy every 3 weeks. The patients with CVI (five men, four women) had an age range of 18–73 years (mean 45). Six of these patients were receiving regular i.v. replacement therapy, one patient (patient 16) intramuscular gammaglobulin and two patients (patients 10 and 14) were not receiving treatment at the time of study. Blood samples were obtained from patients prior to gammaglobulin replacement therapy. None of the patients had evidence of intercurrent infection at the time of study.

Eighteen age- and sex-matched normal subjects were the control group for lymphocyte subset analyses.

MoAbs

The following MoAbs were used, either in direct or indirect immunofluorescence labelling procedures, to identify the respective lymphocyte subpopulations:

Indirect labelling: OKT3 (CD3), OKT8 (CD8) (Orthomune) anti-CD19 (CD19) (Dako) goat anti-mouse FITC (Orthomune).

Direct labelling: Leu3a + b–FITC (CD4), Leu8–PE, Leu3a–FITC/Leu8–PE (Simultest) (CD4/Leu8) (Becton Dickinson); UCHL1–FITC (CD45RO), CD4–PE (CD4) (Dako); T4–FITC/2H4–RD1 (CD4/CD45RA), T4–FITC/4B4–RD1 (CD4/CD29), mouse IgG1–FITC/IgG1–RD1 (CD4/CD29), mouse IgG1–FITC/IgG1–RD1 (Coulter Immunology).

Immunofluorescence labelling and flow cytometry

Lymphocyte subpopulations were determined in EDTA anticoagulated peripheral blood samples using a whole blood staining procedure and flow cytometry.

For indirect labelling, 100-μl aliquots of blood were incubated with 10 μl of the respective MoAbs for 15 min at 4°C. After two washes in PBS, blood samples were incubated with 100 μl of goat anti-mouse FITC (1 : 20 dilution) for 15 min at 4°C and washed twice in PBS. For direct labelling, 10 μl of the respective conjugated antibodies were incubated with the blood samples for 30 min at 4°C, then washed twice in PBS. Appropriate control antisera were included in each experiment.

Erythrocytes were lysed using Whole Blood Quick Stain Lysing Reagent (Coulter Immunology) and the remaining leucocytes resuspended in 1% paraformaldehyde–PBS prior to immediate flow cytometric analysis.

Analyses were performed on the EPICS 541 flow cytometer (Coulter Electronics). Lymphocytes were identified on the basis of forward and 90° light scatter signals and gated appropriately. Single parameter log integral green or red signals were obtained from the gated populations.

Ten-thousand lymphocytes were counted in each sample and the percentages of positive cells obtained using the instrument's Immuno programme. Dual labelled cells were determined following appropriate electronic subtraction of overlapping red and green signals and the percentages of these cells obtained using the instrument's Quadstat programme. The cut-off point for positive/negative cells was determined by reference to the profiles obtained using the respective antibodies for single-colour labelling and from negative control histograms using the mouse IgG1–FITC/IgG1–PE reagent.

In vivo assessment of cell-mediated immunity

In vivo assessment of cell-mediated immunity was performed in 10 adult patients (five with XLA and five with CVI) using the Multitest CMI system (Institut Merieux, Lyon, France) according to the manufacturer's instructions. Responses were scored according to the number of positive reactions and degree of induration 48 h after application as previously described by our group [24]. Skin testing and reading of the results were performed by the same investigator (NAMB) to minimize observer error. Results obtained for the XLA patients were compared with a population of 50 age- and sex-matched volunteers. For the CVI group, the control population consisted of 125 age- and sex-matched healthy subjects.

Statistical analysis

Statistical analyses were performed using the Mann–Whitney *U*-test.

RESULTS

Lymphocyte subpopulations

Circulating lymphocyte numbers and the relative proportion of T cells (CD3), T cell subpopulations (CD4, CD8) and B cells (CD19) which were determined for each patient are shown in Table 1.

In two patients with XLA (patients 6 and 9), B cells were repeatedly detected in peripheral blood samples. Patient 6 was diagnosed at 18 months and has a family history of X-linked disease. Patient 9 has X-linked disease with high serum IgM.

CD4 lymphocyte subsets

The distributions of CD4 subsets identified by reactivity with anti-CD45RA, Leu8, CD29 and CD45RO reagents in the respective patient and control groups are shown in Fig. 1.

CD4CD45RA: A broad distribution of CD4CD45RA$^+$ cells was observed in the patient and control subject groups (Fig. 1a). Overall, the percentage of CD4CD45RA$^+$ cells was decreased in the CVI patient group (16·8 ± 22·7%) compared with the XLA patients (22·9 ± 24·6%) and control subjects (25·6 ± 17·7%), although these values did not reach statistical significance. The levels of CD4CD45RA cells in patients with XLA were within the normal range.

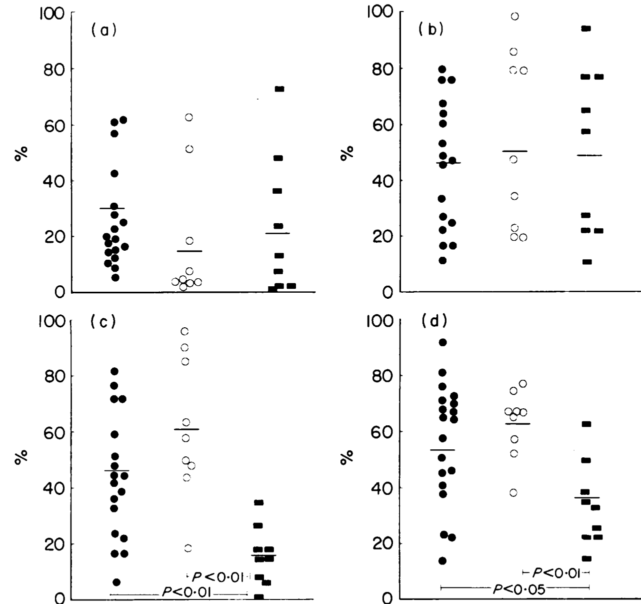

Fig. 1. Percentages of circulating CD4 lymphocytes expressing CD45RA (a), Leu8 (b), CD45RO (c) and CD29 (d) reactivity in patients with X-linked agammaglobulinaemia (■), in patients with common variable immunodeficiency (○) and in normal subjects (●).

CD4Leu8: Wide variations in Leu8 positivity were observed in CD4 cells from patients and control subjects (Fig. 1b). However, no statistically significant differences were observed on comparisons of CD4Leu8 levels in the respective patient and control subject groups.

CD4CD45RO: The values obtained for CD4CD45RO cells in patients and control subjects are shown in Fig. 1c. A highly significant reduction ($P<0·01$) in CD4 cells displaying CD45RO positivity was observed in patients with XLA (XLA patients, 15·7 ± 10·2%; CVI patients, 61·8 ± 25·4%; normal subjects, 43·7 ± 22·3%). Consequently, a relatively clear distinction between XLA and CVI patients was apparent on the basis of CD4CD45RO positivity with only one CVI patient having a CD4CD45RO percentage within the range observed for XLA patients.

CD4CD29: The percentages of CD4CD29 lymphocytes determined in patients and controls is shown in Fig. 1d. A significant reduction ($P<0·01$) in CD4CD29 cells was apparent in the XLA patient group (32·1 ± 14·6%) compared with the CVI patient group (60·1 ± 11·2%) or normal subjects (54·5 ± 22·0%).

CD4CD45RA : CD4CD45RA ratios: Determination of CD4CD45RA : CD4CD45RO ratios for the patient and control subject groups revealed a depressed ratio (0·3 : 1) in the CVI patient group compared with the control subject group (0·6 : 1). This reflected the lower levels of CD4CD45RA cells observed in the CVI patient group relative to the normal control population. The ratio of CD4CD45RA : CD4CD45RO cells calculated for the XLA patient group was 1·5 : 1, which reflected the significantly reduced numbers of CD4CD45RO cells evident in these patients.

Table 2. Responses to test antigens in patients with X-linked agammaglobulinaemia (XLA) and patients with common variable immunodeficiency (CVI)

	Positive responses to test antigens (mean ± 1 s.d.)		Area of total induration (mm) (mean ± 1 s.d.)	
XLA	1·4 ± 1·9	$P<0·05$	4·4 ± 2·8	$P<0·05$
Control subjects	3·6 ± 0·1		15·2 ± 0·1	
CVI	2·8 ± 1·9	NS	15·8 ± 1·9	NS
Control subjects	2·9 ± 0·7		12·0 ± 4·1	

NS, Not significant.

Cell-mediated immune responses

The results of the *in vivo* assessment of CMI using the Multitest CMI system are summarized in Table 2.

Patients with XLA showed fewer positive reactions to test antigens than was observed in 50 age- and sex-matched healthy subjects (XLA patients, 1·4 ± 0·9, control subjects, 3·6 ± 0·1; $P<0·05$). This reduction in cell-mediated immune responses was also reflected by decreased inflammatory reactions in the XLA patient group (total induration: XLA patients, 4·4 ± 2·8 mm; control subjects; 15·2 ± 0·1 mm; $P<0·05$).

The mean number of positive reactions to test antigens and the mean area of total induration observed in the CVI patient group (2·8 ± 1·9; 15·8 ± 1·9 mm) were not significantly different to those recorded for 125 age- and sex-matched control subjects (2·9 ± 0·7; 12·0 ± 4·0 mm) ($P>0·9$; $P>0·3$, respectively).

DISCUSSION

We have demonstrated that the composition of circulating CD4 lymphocyte subsets in patients with XLA and CVI are different. Most notably we observed a significant reduction in CD4CD45RO and CD4CD29 cells in peripheral blood of patients with XLA compared with CVI patients and with an age- and sex-matched control group.

The CD45RO population contains cells that have differentiated in response to antigen and represent the T cell memory pool [14, 15]. Similarly, the CD29 determinant is preferentially expressed on previously activated T cells [15]. Our results indicate that both of these subpopulations are reduced in patients with XLA. The explanation for this is unclear, although there are several possibilities. For instance, circulating CD4CD45RO and CD4CD29 cell levels are lower in infancy than in adulthood [25,26]. Consequently the inclusion of four children in our XLA patient groups could have resulted in a lowering of the overall CD4CD45RO and CD4CD29 levels in the patient group. However, we would argue against an age-related phenomenon being responsible, as we observed low CD4CD45RO and CD4CD29 levels in adult as well as in paediatric XLA patients. Secondly, when compared with a carefully age-matched control population, the proportions of CD4CD45RO and CD4CD29 cells were significantly reduced in the XLA patient group.

Another possible explanation for the observed subset alterations is that they are a consequence of regular gammaglobulin infusion. Several reports (reviewed by Ballow *et al.* [27]) have described altered immune responses in patients receiving i.v. immunoglobulin (IVIg). Furthermore, Macey and Newland [28] have described a fall in CD4CD29 cells with resultant reduction in T-dependent B cell activation in patients with autoimmune thrombocytopenia purpura undergoing high-dose IVIg therapy. However, it is unlikely that the selective depletion in CD4CD45RO and CD4CD29 cells in our XLA patient group is a result of gammaglobulin replacement therapy, as most of the CVI patients were also undergoing a similar treatment regime and did not demonstrate reduced levels of CD4CD45RO and CD4CD29 cells.

In contrast to the depletion of CD4 memory subsets, the distributions of CD4CD45RA$^+$ and CD4Leu8$^+$ lymphocyte subpopulations were not abnormal in the XLA patient group. Thus it would appear that, on the basis of expression of these characteristic membrane determinants, the transition from naive to memory cell is defective with XLA. Corroborative evidence for this comes from previous studies which have reported an immature T cell phenotype in XLA patients by virtue of enzymatic profiles [29] or reactivity with monoclonal antibody HB10 [30]. A possible explanation for the apparent failure in differentiation of naive to memory cells is that T cell activation is dependent on bi-directional communication between B and T cells [31]. Thus in the absence of mature B cells or at the subnormal levels observed in XLA, the normal process of T cell differentiation does not occur. However, as reduced CD4CD45RO and CD4CD29 lymphocyte levels were observed in two of our XLA patients in whom circulating B cells were identified, additional factors must pertain. It is becoming clear that during T cell activation, synergistic interactions between naive and memory T cell populations occur and the responses of the respective subpopulations are influenced by cytokine production of each subset [16]. Consequently it would be interesting to determine whether cytokine production by naive CD4 cells is defective in XLA patients, as this could conceivably result in a failure to generate CD4 memory cells.

Functional differences between naive and memory T cells have been described [14, 16]. In particular, *in vitro* functional analyses have established that T cell help for immunoglobulin production is mediated by CD4CD29 [20] and CD4CD45RO [18] cells, whereas induction of suppressor cell activity is a feature of the CD4CD45RA [19] and CD4Leu8 [23] populations. Consequently, depletion of CD4CD45RO and CD4CD29 cells in patients with XLA may further reduce the capability for Ig production from a limited B cell population. Interestingly, Rozynska *et al.* [2] have reported defective T cell help for IgG and IgM production in XLA patients which, in view of our findings, may be a feature of the imbalance of the helper/inducer, memory CD4 lymphocyte population.

In contrast to our findings in XLA, normal levels of CD4CD45RO and CD4CD29 cells were observed in our CVI patients. Similar observations have been made by Lebranchu *et al.* [32] who, in addition, reported a reduction in circulating CD4CD45RA cells in patients with CVI. Similarly, a deficiency in CD4CD45RA cells has been reported by Wright *et al.* [33] in a sub-group of patients with reduced CD4:CD8 ratios. A trend towards reduced CD4CD45RA cell numbers, resulting in a decreased CD4CD45RA:CD4CD45RO ratio (0·3:1), was also observed in our patients. Although various functional T cell defects have been described in CVI (reviewed by Spickett *et al.*

[3]), the contribution played by the respective CD4 subsets, and in particular CD4CD45RA cells, to these functional abnormalities remains to be elucidated.

In skin testing for delayed hypersensitivity responses to a group of common recall antigens we have attempted to add a functional dimension to the enumeration of T cell subsets. Responses to skin-test antigens without biopsy definition are open to misinterpretation and obviously a reaction to a panel of antigens depends on an individual's previous history of exposure to various antigens. However, at face value, the skin test results indicate a degree of anergy in the XLA patient group relative to normal controls and patients with CVI. As the ability to respond to recall antigens lies within the memory T cell pool [16,19] the relatively poor delayed hypersensitivity responses observed in the XLA patient group could be consistent with the reduced levels of CD4CD45RO and CD4CD29 cells present in these patients.

Our findings differ from two previous reports [34,35] in which depressed delayed responses were observed in a subgroup of CVI patients but not in patients with XLA. The reasons for these discrepancies are not clear, although the skin test system used was different from that employed in our study. Furthermore, data from a control population were not included in either of the earlier studies.

The finding of decreased delayed hypersensitivity responses in XLA is surprising, since such patients are usually free of the types of infection traditionally associated with defects of cell-mediated immunity. IVIg therapy is another complicating factor, although in our study the XLA patients had received a lower total dose compared with CVI patients and this is unlikely, therefore, to have resulted in the observed abnormality. Clearly it would be unwise to draw any firm conclusions from the available data; however, we suggest that the T cell abnormalities observed in XLA require further investigation as this may help to elucidate the pathophysiology of this form of primary immunodeficiency.

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