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Inhibition of non-MHC-restricted cytotoxicity by CD45 but not CD3 monoclonal antibodies in patients with large granular lymphoproliferative disease

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SUMMARY

Nine patients with a lymphoproliferative disorder characterized by a persistent expansion of large granular lymphocytes (LGL) and an increased proportion of cells labelling with natural killer (NK) and T cell markers were identified. The six patients with an expansion of $\alpha\beta$ CD3/TcR positive cells were shown to have rearranged T cell receptor (TcR) genes whereas three patients whose LGL lacked CD3/$\alpha\beta$ TcR on the surface had no β TcR rearrangement detected. Eight of the nine patients were shown to exhibit non-MHC-restricted cytotoxic activity against K562; this activity was inhibited by CD45 and CD45-associated monoclonal antibodies known to inhibit normal non-MHC-restricted cytotoxicity but not specific MHC-restricted cytotoxic T cell activity. In contrast, the CD3 monoclonal antibody OKT3 did not inhibit but redirected LGL non-MHC-restricted cytotoxicity against K562. Following modulation of the CD3 molecule, the LGL were still capable of cytolysis of K562 targets, but additional OKT3 could no longer redirect cytoloysis. The data indicate that the CD3/TcR complex on the LGL clones in patients with large granular lymphoproliferative disease is not the receptor for antigen on K562 cells, although it retains functional capabilities. Thus the CD3/TcR positive subset appears to have bipotential cytotoxic characteristics involving additional unique receptors for non-MHC-restricted cytotoxicity.

Keywords non-MHC-restricted cytotoxicity large granular lymphoproliferative disease leucocyte common antigen T cell receptor

INTRODUCTION

The description of patients with expanded populations of large granular lymphocytes (LGL) in their peripheral blood (Reynolds & Foon, 1984) has led to speculation concerning the lineage of these cells. In many patients, the lymphocytes exhibit non-MHC-restricted cytotoxicity against a range of target cells. Cytotoxic activity is a function of at least four separate effector cell types (Lanier *et al.*, 1986; Hersey & Bolhuis, 1987) that are classified by their distinctive surface marker and functional characteristics. The first type is the MHC-restricted cytotoxic T lymphocyte (CTL), which recognizes antigen bound to the MHC antigens on the target cell. Specific CTL activity is mediated mainly via the $\alpha\beta$ form of the T cell receptor (TcR). A second form of CTL also rearranges its β TcR genes, but is not MHC restricted in its recognition of target cell antigen. The third CTL type expresses the $\alpha\beta$ TcR chains in association with the CD3 molecule, and this receptor complex recognizes targets in a non-MHC-restricted manner (Borst *et al.*, 1987; Moingeon *et al.*, 1987) and in some instances an MHC-restricted manner (Matis, Cron & Bluestone, 1987). However, the majority of non-MHC-restricted cytotoxicity in normal blood is attributed to a population of cells that do not rearrange genes for or express a TcR/CD3 complex. This population of cells are known as natural killer (NK) cells. NK cells are also capable of antibody dependent cellular cytotoxicity, mediated through the CD16 (FcR10) molecule.

Most NK cells that exhibit non-MHC-restricted cytotoxicity have the morphology of LGL (Timonen, Ortaldo & Herberman, 1981). Although numerous molecules have been postulated to be involved in NK-cell-mediated cytolysis, including CD2 (Jondal, 1987), CD16 (van de Griend *et al.*, 1987), CD18 (Hildreth *et al.*, 1983) and CD45 (Newman, 1982; Burns, Werkmeister & Triglia, 1984; Starling *et al.*, 1987), the NK cell receptor for target antigen remains elusive (Burns *et al.*, 1984; Starling *et al.*, 1987, Targan & Newman, 1983). CD45 antibodies block NK cell activity at a post-conjugate stage of cytolysis analogous to the stage at which OKT3 inhibits MHC-restricted CTL activity. They do not, however, inhibit MHC-restricted cytolysis mediated by $CD8^+$ CTLs through the TcR complex (Targan & Newman, 1983; Starling *et al.*, 1987).

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Table 1. Presentation details of patients with proliferation of large granular lymphocytes (LGL)

					Peripheral blood counts ($\times 10^9/l$)		
Patient	Age	Sex	Presentation	Splenomegaly	Neutrophils	Lymphocytes	LGL
1	38	F	Infection	−	0·4	12·1	9·6
2	67	F	Arthritis		1·1	2·4	1·6
3	74	F	RA, neutropenia	+	0·2	3·1	2·6
4	51	F	Anaemia	+	3·3	1·0	1·0
5	72	F	RA, neutropenia	−	0·6	1·6	0·8
6	75	F	Osteoarthritis	−	0·6	2·3	1·0
7	64	M	Vascular disease	−	3·6	4·1	1·8
8	69	M	Lymphocytosis	−	3·2	3·1	2·6
9	48	M	Lymphocytosis	+	3·9	1·6	1·0

RA, rheumatoid arthritis.

We have identified nine patients with sustained proliferation of LGL which have the surface marker and functional characteristics of non-MHC restricted lymphocytes. In order to characterize these cells in more detail, we have examined the inhibitory effect of CD45 and CD3 monoclonal antibodies on the cytotoxic activity of these LGL against K562. The results indicate that the non-MHC-restricted cytotoxicity by cells from these patients against K562 is inhibited by certain CD45 antibodies and is mediated by a receptor other than a known TcR/CD3 complex.

MATERIALS AND METHODS

Patients

Nine patients who gave informed consent for blood sampling were studied and the clinical details are summarized in Table 1. The case of patient 1 has been published elsewhere (Hart *et al.*, 1987). Blood was taken into heparin or EDTA, spun over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradients, and peripheral blood mononuclear cells (PBMC) were harvested for the tests described below.

Monoclonal antibodies

The following monoclonal antibodies were used in this study to phenotype the patients peripheral blood lymphocytes by immunofluorescence and flow cytometry on a FACS IV (Becton Dickinson, Mountain View, CA): WT31 recognizing the $\alpha\beta$ TcR heterodimer (Spits *et al.*, 1985a); the antibodies OKT3 (CD3), OKT4 (CD4), OKT8 (CD8), OKT11 (CD2) and HNK1 were produced in our laboratory from hybridomas obtained from the American Type Culture Collection (Rockville, MD); Leu 11b and phycoerythrin-conjugated Leu 11c (CD16) from Becton Dickinson; NKH1 (Coulter, Hialeah, FA) and OKM1 (Ortho Pharmaceutical Corp, Raritan, NJ). Fluorescein isothiocyanate–goat anti-mouse (FITC–GAM, Tago, Burlingame, CA) was used as the second antibody to label antibody-reactive cells.

Southern blot analysis

Genomic DNA was extracted according to standard methods. DNA samples were digested with the restriction endonucleases *Eco*RI, *Hin*dIII and *Bam*H1 under conditions recommended by the manufacturer (New England Biolabs, Beverley, MA). The restriction digests were separated and blotted according to the Gene Screen protocol supplied by New England Nuclear (Boston, MA). Membranes were probed using a β TcR chain probe provided by Dr T. Mak (Toronto, Canada).

Cytotoxicity assays

Cytotoxicity against the erythroleukaemia cell line K562 was determined using a standard ^{51}Cr release assay (Starling *et al.*, 1987) in 96-well microtitre plates (Flow Laboratories, McLean, VA).

ADCC assays were performed using ^{51}Cr labelled group O Rh-positive erythrocytes labelled with a mix of the monoclonal antibodies (CMRF-4 (IgG2a), CMRF-10 (IgG1) and CMRF-17 (IgM) which react with different erythrocyte antigens (Hart *et al.*, 1987). Normal PBMC were used as a positive control in both assays.

Inhibition studies

The ability of several monoclonal antibodies to inhibit cytotoxic activity against K562 was examined. OKT3 (CD3) was produced as described above; the monoclonal antibody mix CMRF-11+12+26 (CD45 and CD45R) was produced in our laboratory (Starling *et al.*, 1987); 13.3 (Newman, Fast & Rose, 1983) was obtained from the leucocyte common antibody panel of the 3rd Leucocyte Differentiation Antigen Workshop; 9.1C3, which recognizes molecules associated with CD45 (Burns *et al.*, 1984) was a gift from Dr G. F. Burns (Adelaide, Australia); and MHM23 (CD18) which recognizes LFA-1 (Hildreth *et al.*, 1983) was a gift from Professor A. J. McMichael (Oxford, UK). These antibodies were used at known saturating quantities and serially diluted in 10% fetal calf serum/RPMI 1640 (Gibco, Auckland, New Zealand) hereafter referred to as medium. Antibody dilutions were added to 5×10^5 lymphocytes/microtitre well 30 min prior to adding 1×10^4 ^{51}Cr labelled K562 targets, i.e. 50:1, effector-to-target (E:T) ratio, as previously described (Starling *et al.*, 1987). A normal control was included to confirm the ability of the monoclonal antibodies to inhibit activity of normal NK cells. The percentage of inhibition/enhancement obtained with each antibody was calculated in relation to the cytotoxicity observed in the absence of antibody.

Table 2. The surface phenotype β T cell receptor (β TcR) gene rearrangement status and cytotoxic activity of lymphocytes from patients with proliferation of large granular lymphocytes (LGL)

Patient	CD2	CD3	WT31	CD4	CD8	HNK1	CD16	NKH1	CD11b	TcR	NK*	ADCC†
1	92	82	81	12	74	59	81	80	15	R	Yes	No
2	89	89	90	9	70	67	77	0	7	R	No	Yes
3	95	91	95	30	62	32	0	0	5	R	Yes	No
4	90	91	86	2	88	34	79	1	8	R	Yes	—
5	95	92	91	11	7	81	52	1	3	R	Yes	Yes
6	84	79‡	78	28	42	55	23‡	41	6	NR§	Yes	Yes
7	94	30	31	12	17	71	69	7	3	NR	Yes	Yes
8	70	23	19	16	71	36	3	72	1	NR	Yes	Yes
9	85	45¶	40	29	30	18	45¶	47	8	NR	Yes	Yes

R, rearranged; NR, not rearranged
* > 20% specific lysis at E:T ratio 50:1.
† > 20% lysis at E:T ratio 10:1.
‡ Double labelling studies showed a population of $CD3^+$ $CD16^+$ (10%) cells.
§ Faint band indicated rearrangement of approximately 10% of cells.
¶ Double-labelling studies showed CD3 and CD16 to be mutually exclusive on lymphocytes.

Modulation experiments

The role of the CD3 molecule in cytotoxicity against K562 was further examined by incubating non-adherent lymphocytes with OKT3 antibody for 20–24 h at 37°C. Following this, cells incubated with antibody or medium alone were then used in the ^{51}Cr release assay at a 20:1 or 25:1 E:T ratio using K562 targets. A cell sample was relabelled with OKT3 and analysed on the FACS IV to confirm modulation of the CD3 antigen had occurred.

RESULTS

Surface markers and gene rearrangements

The expanded large granular lymphoid populations expressed a heterogeneous array of T cell and NK cell antigens (Table 2). The lymphocytes from patients 1–5 expressed the CD3 antigen which was in each case associated with the surface expression of αβ TcR chains as judged by the fact that a similar percentage of cells labelled with both the OKT3 and WT31 antibodies. Rearrangement of β TcR genes was clearly seen in each of these cases on Southern blots probed with the β TcR probe. Patient 6, unlike the other cases, did not have an obvious predominant clonal expansion but double labelling studies identified a small population (approximately 10%) of cells expressing the CD3 and CD16 antigens. Gene rearrangement studies showed that a minor population of cells from patient 6 had rearranged β TcR genes. The cells reacting with NK cell markers from the remaining three patients (numbers 7, 8 and 9) were OKT3 and WT31 negative, i.e. CD3/αβ TcR negative, and had a cell surface phenotype consistent with the normal NK cell subset. The β TcR genes in the cells from these patients showed a germ line configuration.

Cytotoxicity assays

Non-MHC-restricted cytotoxic activity against K562 was readily demonstrated in all but one case (patient 2). This individual, with arthritis, was taking prednisone which is known to affect NK activity (Dupont, Vandercruys & Wybran, 1984).

All but patients 1 and 3 exhibited ADCC activity against antibody-coated erythrocytes. The lack of ADCC activity in patient 3 is consistent with the absence of CD16 (FcR10) positive cells, which mediate ADCC activity.

Inhibition studies

The effect of a range of monoclonal antibodies on LGL-mediated cytotoxicity against K562 was tested to determine which cell membrane structures were involved in the non specific cytotoxic process (Fig. 1). The CD3 reagent OKT3 did not inhibit cytotoxicity, even in the patients with CD3-positive LGL. Instead, most patients' cells showed some increase in cytotoxicity with addition of OKT3 to the ^{51}Cr release assay. The limited increase in killing by CD3 negative cells from patient 9 in the presence of OKT3 was probably due to redirected killing by residual $CD3^+$ T cells (Spits *et al.*, 1985b).

In contrast, the mixture of anti-leucocyte common antibodies (CMRF-11+12+26) inhibited the killing of K562 by all patients whose LGL had cytotoxic activity, although only limited inhibition was seen in patient 8. The anti-leucocyte common reagent, 13.3, inhibited cytotoxicity in five of the patients, and 9.1C3 also inhibited cytolysis but with more variable results. The CD18 reagent, MHM23, was used as a positive control antibody, and predictably blocked the cytotoxicity of all patients' lymphocytes by 50% or more.

Modulation experiments

In view of the fact that the CD3 antibody, OKT3, not only failed to inhibit but actually increased the LGL-mediated lysis of K562, further antigen modulation experiments were performed where possible. Overnight incubation with OKT3 antibody removed CD3 antigen from the LGL cell membrane (Fig. 2) but did not abrogate cytotoxicity against K562 (Fig. 3) indicating that membrane CD3 was not a prerequisite for antigen recognition of K562 targets. Modulation of the CD3 molecule did, however, prevent the ability of additional OKT3 antibody to increase cytotoxicity against K562. Cells from patient 4 showed a marked increase in cytotoxicity (from 9% to 57% specific lysis) after overnight incubation with OKT3.

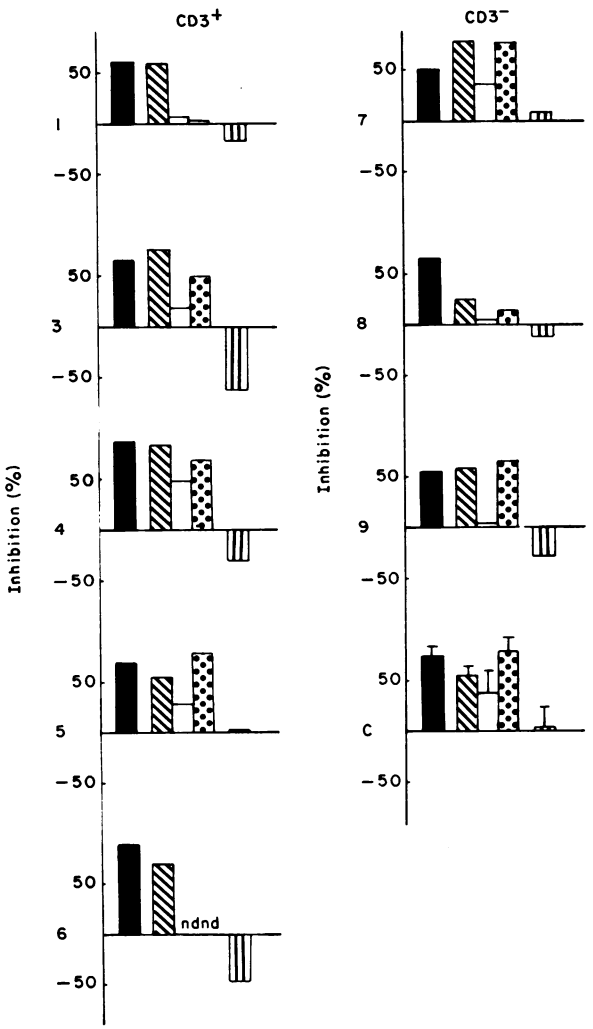

Fig. 1. Inhibition or enhancement of non-MHC-restricted cytotoxic activity by monoclonal antibodies. Patients 1, and 3–9 were designated $CD3^+$ or $CD3^-$ by expression of CD3 on the surface of their large granule lymphocytes (LGL). Mean percentage inhibition of cytotoxicity from representative experiments are shown. Normal controls (C) were tested concurrently with the patients' peripheral blood mononuclear cells. Patient 2 had insufficient cytotoxic activity to test for blocking. Antibodies used for blocking studies included MHM 23 (CD18) (■); CMRF-11+12+26 (CD45+CD45R) (▧); 9.1C3 (leukocyte common associated) (□); 13.3 (leukocyte common) (⊠); and OKT3 (CD3) (■).

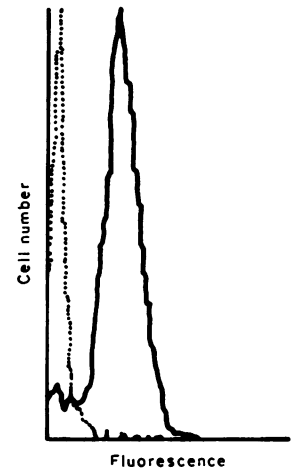

Fig. 2. Modulation of the CD3 molecule using OKT3. The FACS profile of the cells from patient 6 labelled with OKT3 after overnight incubation in medium (solid line) and after overnight incubation with OKT3 and then relabelled with OKT3 (dotted line). Similar complete modulation of the CD3 molecule was observed on cells from patients 1 and 4.

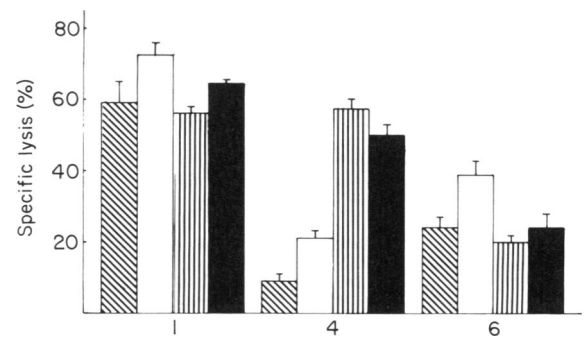

Fig. 3. Modulation of the CD3 molecule fails to abrogate cytotoxicity against K562. Cells from patients 1, 4 and 6 were incubated overnight in medium (▧, □) or OKT3 antibody (■, ■), and their cytotoxic activity against K562 was measured in the absence (■, ▧) or presence (■, □) of further OKT3 antibody. The cytotoxic activity is expressed as % specific lysis ±1 s.d.

DISCUSSION

The use of monoclonal antibodies enabled us to examine the roles of the CD3, CD18 and CD45 molecules in the non-MHC-restricted killing by cells from patients with large granular lymphoproliferative disease. To investigate the cytotoxic mechanism involved we used anti-leucocyte common (CD45) monoclonal antibodies which inhibit the cytotoxic activity of normal $CD3^-$ NK cells but do not inhibit MHC-restricted CTL (TcR/CD3) mediated killing (Newman *et al.*, 1983; Starling *et al.*, 1987). These anti-leucocyte common antibodies were shown to inhibit the cytotoxic activity of both the $CD3^-$ and $CD3^+$ non-MHC-restricted cytotoxic cells. However, the CD3 antibody, OKT3, did not inhibit LGL-mediated cytotoxicity against K562 from the patients with $CD3^+$ LGL and enhanced cytotoxicity in the majority of cases. Furthermore, modulation of the CD3 antigen by overnight incubation of OKT3 with non-adherent lymphocytes from the patients did not inhibit cytotoxicity against K562, but removed the ability of additional OKT3 to increase the cytotoxicity as observed prior to modulation. We conclude that the $\alpha\beta$ TcR/CD3 on these LGL does not function as the receptor for K562 target cell antigen and that both the $CD3^+$ and $CD3^-$ examples of lymphoproliferative disease mediate non-specific killing via an alternative recognition system.

The non-specific killing mechanism used by these cells appears to involve the leucocyte common antigen in some way. The exact function of the leucocyte common antigen with its broad cellular distribution has yet to be defined, although it is interesting to note that the anti-leucocyte common monoclonal

antibodies may inhibit at a comparable stage of non-MHC-restricted cytolysis by normal NK cells (Targan & Newman, 1983; Burns *et al.*, 1984; Starling *et al.*, 1987) to that which OKT3 inhibits in MHC restricted by $CD3^+$ T cells (Lanzavecchia, 1986). The CD45 antibodies inhibit killing at a post-conjugation step; either at the 'trigger' phase of the cytolytic reaction (Targan & Newman, 1983) or at a later stage of cytolysis (Burns *et al.*, 1984; Starling *et al.*, 1987). However, certain CD45 antibodies do not induce Ca^{++} fluxes (Anasetti *et al.*, 1987) or phosphoinositide turnover (Seaman *et al.*, 1987) in non-MHC-restricted cytotoxic cells in the manner that OKT3 does with $CD3^+$ cells (Weiss & Imboden, 1987). This may relate to the relative functional importance of different epitopes on the leucocyte common molecule (Newman *et al.*, 1983; Starling *et al.*, 1987) or be a consequence of the immunoglobulin subclass of CD45 antibodies used in the activation studies. Other data suggest that the lymphocyte molecule may be involved in the initial stages of cytolysis (Sparrow & McKenzie, 1983; Gilbert, Zaroukian & Esselman, 1988). Whether or not the leucocyte common molecule is a receptor for a target cell antigen or involved at a post-recognition stage in non-MHC-restricted killing is uncertain.

Our results provide the first indication that the $\alpha\beta$ TcR/CD3 on the surface of the $CD3^+$ LGL in patients with large granular lymphoproliferative disease does not necessarily act as a recognition structure for non-MHC-restricted cytotoxicity. This conclusion is in accord with reports that other non-MHC-restricted CTL remain cytotoxic after CD3 modulation (Phillips *et al.*, 1988; Thiele, Patel & Lipsky, 1988). Nonetheless, it is clear that the $\alpha\beta$ TcR/CD3 complex retains functional properties on these cells, as judged by the ability of OKT3 antibody to enhance killing after short (30 min) or long (20–24 h) incubations with the cytotoxic lymphocytes. The former phenomenon, described as redirected cytotoxicity (Spits *et al.*, 1985b), results from OKT3 antibody binding to the cytotoxic cell CD3 complex via its F(ab′) portions, and simultaneously to Fc receptors on K562 (van Seventer *et al.*, 1987). This permits surrogate recognition of K562 by the CD3 molecule to occur, thereby activating the cytolytic process of the CTL. Activation of cellular cytotoxicity via the CD3 molecule also occurred independently of redirected cytotoxicity as demonstrated by the substantial increase in cytotoxic activity of patient 4 after modulation of the CD3 antigen by overnight incubation with OKT3. Loughran *et al.* (1987), when describing the ability of long-term incubation with CD3 antibody to induce cytotoxicity in large granular lymphoproliferative disease patients, were probably looking at a similar phenomenon. We have extended this observation by showing that the CD3 molecule is not involved in the target recognition of OKT3-activated LGL.

Many questions are yet to be resolved as to the cytolytic mechanisms of these non-MHC-restricted cytotoxic cells. Firstly, from what differentiation pathways are the $CD3^+$ and $CD3^-$ non-MHC-restricted cytolytic LGL derived, and what, if any relationships are there between them and the MHC-restricted cytotoxic T cell? Secondly, by what receptor(s) do these cells recognize different targets? It would be interesting to speculate that the $CD3^+$ LGL may have at least bipotential cytotoxic capabilities, recognizing different targets via different receptors for antigen. Those cells expressing the CD16 (FcR10) molecule will of course have the additional potential to kill via ADCC. It is possible that non-MHC-restricted killing can be mediated by one of several different receptors on the cytotoxic cell (possibly including the $\alpha\beta$ TcR/CD3 complex), recruited in a target-dependent manner.

Further studies as to the LGL receptors and their specificity for different targets will be possible only when the as yet undefined non-MHC-restricted recognition structure(s) is characterized. This may also assist in purifying the native LGL clones as current technology cannot guarantee that limited contamination with other small cell populations will not interfere with functional results. Likewise culturing or cloning these cells alters their function, thereby limiting attempts to characterize the cells on the basis of their *in vivo* functional properties; it was for that reason that we chose to investigate the unmodified LGL proliferations.

Our analysis of large granular lymphoproliferative disease raises interesting questions as to whether there are differences in the prognosis or other features, e.g. neutropenia or arthritis, between the patients with $CD3^+$ and $CD3^-$ LGL proliferations which may be relevant to the specificity of the TcR. The β TcR gene rearrangement studies in our patients indicated that each $CD3^+$ LGL proliferation was clonal, an observation that confirms previous studies (Rambaldi *et al.*, 1985). However, although the $CD3^-$ LGL proliferations we have described are sustained it has not proved possible to determine the clonal nature of the proliferations in the absence of cytogenetic markers. It will be interesting to study the TcR in the $CD3^+$ patients, and to determine whether these expansions involve a restricted number of or a range of TcR idiotypes; it may be possible subsequently to establish whether the LGL $\alpha\beta$ TcR/CD3 have any specificity for self-antigens, particularly on myeloid precursors (Minato *et al.*, 1988). Identification of the appropriate TcR target would make it possible to test whether LGL-mediated TcR recognition is MHC restricted. Finally, although it has yet to be established whether an infectious agent is involved, there is some serological evidence that an HTLV-1-like retrovirus may be involved (Starkebaum *et al.*, 1987) and it is possible that these receptors may have specificity for a viral antigen.

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