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Differential response of human thymus cells to CD2 antibodies: fragmentation of DNA of CD45RO$^+$ and proliferation of CD45RO$^-$ subsets

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SUMMARY

Human thymocytes bearing the CD45RO 'memory' cell phenotype do not proliferate in concanavalin A (Con A)-stimulated cultures and may be destined for intrathymic death. To determine whether this subset would exhibit characteristics of programmed cell death (apoptosis), we examined the integrity of the nuclear DNA by gel electrophoresis. DNA fragmentation was restricted to the CD45RO$^+$ subset of human thymocytes following exposure to stimulating concentrations of anti-CD2 antibodies. Both CD45RO$^-$ and CD45RO$^+$ subsets mobilized cytoplasmic Ca^{2+} following cell-surface CD2 ligation, but entry into the cell cycle and vigorous thymidine uptake were restricted to the CD45RO$^-$ subset. Our results provide a mechanism which may account for the failure of thymic CD45RO$^+$ cells to respond to stimuli which elicit proliferation by the reciprocal CD45RA$^+$ subset.

INTRODUCTION

Only a minority of the thymocytes generated by cell division within the thymus are thought to survive the positive and negative selection steps which precede their maturation to the CD3$^+$CD4$^+$CD8$^-$ or CD3$^+$CD4$^-$CD8$^+$ single-positive phenotypes. Some or all of the cells which do not satisfy the selection criteria are thought to undergo a programmed cell death, characterized by the fragmentation of nuclear DNA and resulting from the activation of an endogenous endonuclease,[1,2] which is known as apoptosis. Apoptosis can be induced *in vitro* in thymocyte and some other T-cell populations by ligation of cell-surface CD3.[3,4]

Most thymocytes have cell-surface CD1 and CD2, CD4 and CD8, and express varying amounts of CD3 and T-cell receptor (TcR) depending on their selection status. The common leucocyte antigen (CD45) is present on mammalian cells in multiple isoforms.[5] Amongst human thymocytes, cells expressing high molecular weight isoforms (p190, p205 and/or p220) are located mainly in the thymic medulla[6] and they subsume the entire clonogenic potential of the thymocyte population.[7] The 180 kDa form identified by the CD45RO antibody, UCHL1, appears to be expressed on a subset of thymocytes which has no potential to proliferate in response to Con A.[8] This lack of response may indicate that CD45RO$^+$ thymocytes are in a developmental blind alley, and that they may be candidates for apoptosis.

Thymocytes express high levels of CD2 from an early stage of their differentiation.[9] The finding that combinations of anti-CD2 antibodies trigger thymocyte proliferation has prompted speculation that CD2 may have a natural receptor function *in vivo*.[10] A recent report that anti-CD2 antibodies trigger calcium flux in up to 85% of thymocytes is consistent with this possibility.[11] The data of Merkenschlager and Fisher[12] showing that anti-CD2 antibodies induce a characteristic phenotypic change in the leucocyte common antigen family (CD45) that is followed by cellular DNA fragmentation of the CD45RO$^+$ thymus cell subset in chimaeric organ culture system give further support to our speculation. Because of the potential importance of the CD2 activation pathway for thymocytes,[10] we determined whether the otherwise unresponsive population of CD45RO$^+$ cells could be driven to proliferate by stimulatory combinations of anti-CD2 antibodies.

The studies reported here indicate that the CD45RO$^-$ cells proliferated more than the CD45RO$^+$ subset in response to anti-CD2, so we compared the integrity of DNA from thymic CD45RO$^-$ and CD45RO$^+$ subsets. Our cell cycle studies indicated that many of the CD45RO$^+$ subset cells were in the G2 or M phase when analysed up to 48 hr after isolation. Exposure of this subset to stimulating combinations of anti-CD2 antibodies elicited a Ca^{2+} flux and DNA fragmentation, without evidence for entry of additional cells into cell cycle.

MATERIALS AND METHODS

Thymus samples

Thymus was obtained from children undergoing surgery for the repair of congenital cardiac defects only when necessary for surgical access. The tissue was teased in cold Hanks' balanced salt solution (HBSS) and filtered through nylon mesh to obtain a

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single-cell suspension. Cells were centrifuged on a Ficoll–Hypaque (sp. gr. 1·077) gradient to deplete dead cells and red blood cells where indicated. Cell viability was >95% as assessed by trypan blue exclusion.

Cell separation

Thymus cell suspensions (2×10^8 in 2 ml of HBSS) were mixed with 200 μl of ascites of the CD45RO antibody, UCHL1, or the CD45RA antibody, 2H4, and rocked for 30 min at 4° and then washed three times with HBSS. The cells were then mixed with a 10-fold numeric excess of goat anti-mouse IgG magnetic beads (Advanced Magnetics, Cambridge, MA) which had been washed and resuspended in 10 ml of HBSS and were rocked at 4° for 30 min. The magnetic beads, together with adherent cells, were then attracted to the side of the tube with neodynium magnets. The cells remaining in suspension were depleted with goat anti-mouse IgG magnetic beads once more, as described above. Samples of the final depleted cell preparations were stained with a fluorescein-conjugated anti-mouse second-stage antibody, analysed by flow cytometry, and found to contain less than 1% mouse immunoglobulin $(Ig)^+$ contaminants. Use of directly conjugated antibodies specific for the markers involved in the depletion also indicated less than 1% contaminants.

Certain experiments made use of the cells which had adhered to the magnetic beads. These cells were recovered by incubating the material which had been attracted to the magnet at 37° in medium containing 20% mouse serum (as a source of competing mouse IgG) overnight in flat-bottom wells. After 18 hr the magnetic beads were separated from the cells by re-exposing to magnets. Over 80% of the cells were viable as judged by trypan blue exclusion.

Detection of DNA fragments by agarose gel electrophoresis

Two methods were used. One used DNA extracted from 1×10^7 unseparated thymus cells, thymus cells depleted of $CD45RO^+$ cells or thymus cells depleted of $CD45RA^+$ cells. The cells which had been incubated for 24 hr in RPMI-1640 supplemented with 10% foetal calf serum (FCS), with or without anti-CD2 antibodies, were lysed in digestion buffer [100 mM NaCl, 10 mM Tris, 25 mM ethylenediaminetetra-acetic acid (EDTA), 0·5% sodium dodecyl sulphate (SDS) and 0·1 mg/ml proteinase K, buffered to pH 8 with HCl]. The mixture was shaken at 50° for 12–18 hr and the resulting solution was extracted sequentially with phenol, phenol–chloroform and chloroform. DNA, recovered in the aqueous phase was dialysed extensively against 10 mM Tris and 1 mM EDTA, pH 7·4. Samples were incubated with 180 μg/ml RNase A (Sigma, St Louis, MO) at 37° for 1 hr and DNA was precipitated by adding 7·5 M ammonium acetate and ethanol and centrifuged, then washed in 70% ethanol before dying. The DNA was redissolved in 10 mM Tris–1 mM EDTA buffer at 37° and quantitated by ultraviolet (UV) absorption at 260 nm, with additional readings at 280 and 230 nm to exclude protein and phenol contamination.

The method of Cohen and Duke[13] was also applied to detect the fragmented DNA. Cells were harvested by centrifugation at 200 *g* for 10 min and the pellet was lysed with 0·5 ml of hypotonic lysing buffer (10 mM Tris, 1 mM EDTA, pH 7·5) containing 0·2% Triton X-100. The lysates were centrifuged at 15,000 *g* for 10 min to separate intact from fragmented chromatin. The pellet and supernatant fractions were precipitated overnight at −20° in 0·4 M NaCl and 52% isopropanol in microfuge tubes. The precipitates were sedimented at 15,000 *g* for 10 min and resuspended in 30 μl of 10 mM Tris–1 mM EDTA buffer. Twenty micrograms of extracted DNA or 30 μl of dissolved precipitates from isolated supernatant was added to each slot of an 0·8% horizontal agarose gel and electrophoresed at 30 V and 21 mA for 15 hr. The gels were washed, stained with 0·5 μg/ml ethidium bromide and viewed under UV light.

Proliferative response of thymocyte subpopulations

Thymocytes, unseparated or as subpopulations, were cultured at $0{\cdot}5 \times 10^6$ cells/ml in RPMI-1640 medium supplemented with 10% FCS in 96-well round-bottom plates. Anti-CD2 antibodies (1:500 dilution of anti-$T11_2$ and anti-$T11_3$ ascites fluid), $0{\cdot}5 \times 10^6$ 3000-rad-irradiated mononuclear cells (MNC) or 15 U/ml interleukin-2 (rIL-2; Amgen, Thousand Oaks, CA) were added where indicated. Eighteen hours before harvest, 0·2 μCi of tritiated thymidine ([^{3}H]TdR; ICN) was added to each well, and thymocytes were harvested and counted using a liquid scintillation counter.

Flow cytometry and cell cycle analysis

Unseparated thymocytes were incubated under the same conditions as those for measuring thymidine uptake. After various incubation times the cells were centrifuged on Ficoll–Hypaque (to remove dead cells) and washed twice. The cells were surface stained with fluorescein isothiocyanate (FITC)-conjugated antibodies on ice for 20 min, washed twice in HBSS and fixed with 70% methanol. After washing the fixed cells were treated with 180 μg/ml protease-free RNase A at 37° for 30 min. The cells were washed with phosphate-buffered saline (PBS) and stained with propidium iodide before two-colour analysis on an EPICS C using Quadstat software. Cells were bitmapped and their surface fluorescence quantitated on a 3 log scale using cells defined by their forward and right-angle scatter: DNA was quantitated as red staining on a linear scale. Controls were included to ensure that RNA digestion was adequate to abolish green staining of RNA by the propidium iodide.

Ca^{2+} flux of thymocytes

Thymocytes were loaded with 5 μl of Fluo3 (Molecular Probes, Eugene, OR) at 37° in 1 ml of RPMI-1640 adjusted to pH 7·2 with HEPES as previously described.[14] After 30 min incubation, 1 ml of RPMI-1640 containing 5% FCS at pH 7·4 was added and incubation continued for a further 30 min. The cells were then spun down and resuspended in 20 μl of PE-conjugated UCHL1 antibodies for 10 min at room temperature. The cells were washed twice with RPMI-1640 containing 5% FCS and maintained at room temperature in the dark until examined. Immediately prior to viewing, the cells were warmed to 37°. Ca^{2+} flux was measured on an EPICS C using forward and right-angle light scatter parameters to identify thymocytes. The red fluorescence channel was used to identify CD45RO-positive and-negative cells, and intracellular Ca^{2+} levels were measured as green fluorescence. The percentage of positive cells was determined in sequential 10-second periods with a flow rate of 10^3 cells/sec. Baseline readings were obtained for 1 min prior to adding the antibody.

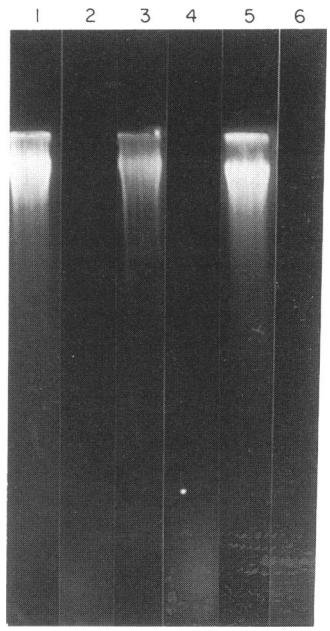

Figure 1. Electrophoresis of extracted DNA or lysate supernatants from human thymus cells which were not treated with anti-CD2 antibodies. Lanes 1, 3 and 5 are DNA extracted from unseparated, CD45RO⁻ and CD45RA⁻ thymus cells, respectively. Lanes 2, 4 and 6 are supernatants isolated from lysed unseparated, CD45RO⁻ and CD45RA⁻ thymus cells, respectively.

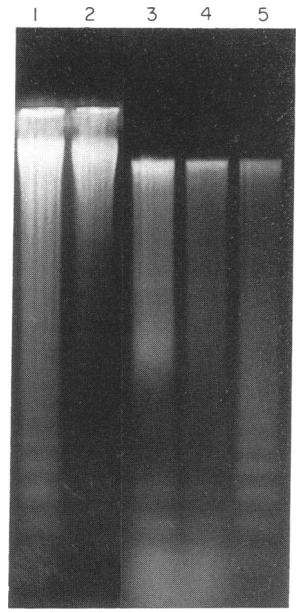

Figure 2. Electrophoresis of DNA in lysate supernatants or extracted from human thymus cells which had been treated with anti-CD2 antibodies for 24 hr. Lanes 1 and 2 are DNA extracted from CD45RA⁻ and CD45RO⁻ thymus cells, respectively. Lanes 3, 4 and 5 are supernatants isolated from lysed unseparated, CD45RO⁻ and CD45RA⁻ thymus cells, respectively.

RESULTS

DNA integrity of thymus cells separated with CD45RA and CD45RO antibodies

Thymus cells were separated into CD45RO⁻ and CD45RA⁻ subsets by depleting with UCHL1 and 2H4 antibodies respectively. The fraction of CD45RO⁻ thymus cells was 10%. Of these, 72±3% were CD4⁻CD8⁻. The fraction of CD45RA⁻ thymus cells was 93%: 70±5% of these cells were CD4⁺CD8⁺ and all of them were CD45RO⁺. These percentages are similar to those reported by Pilarski *et al.*[8] and Serra *et al.*[15] The integrity of DNA present in cell lysates or extracted from thymus cells, either unseparated or separated into CD45RA⁻ and CD45RO⁻ subsets, is shown in Fig. 1. There is some increased entry of ethidium bromide-staining material into the gel from the extracted DNA of unseparated (lane 1) and CD45RA⁻ (Lane 5) cell subsets but no suggestion of a ladder pattern. No DNA fragments were detected in the lysates of the cells (Lanes 2, 4 and 6).

Since CD45RA and CD45RO identified largely reciprocal subsets of cells in the thymus, the effects of anti-CD2 antibodies were investigated on cells negatively selected with these antibodies. Figure 2 shows that the amount of DNA fragmentation in the CD45RO⁻ subset is increased by adding CD2 antibodies, and that this is detected as the appearance of a ladder pattern in the cell lysate fraction (Lane 5). The changes in the lysate are accompanied by the appearance of a ladder pattern in DNA extracted from the cells (Lane 1). The results seem to show some low molecular weight of ethidium bromide-staining material in the supernatant isolated from anti-CD2 antibody-treated CD45RO⁻ thymus cells (Lane 4), though it did not show the typical ladder pattern seen in CD45RA⁻ (Lane 5) and unseparated (lane 3) cells. No DNA fragments were detected when the DNA excreted from anti-CD2 antibody treated CD45RO⁻ thymus cells was used (Lane 2). The results in Figs 1 and 2 are derived from two thymus samples: identical results were obtained from four other samples.

Proliferative response and cell cycling of thymocyte subpopulations stimulated by anti-CD2 antibodies

The occurrence of DNA breaks amongst the CD45RO⁺ subset when the cells were stimulated with anti-CD2 antibodies raised the question of whether these cells would be able to enter and complete a proliferative cycle. To investigate this possibility we stimulated thymus cells with the combinations of anti-CD2 antibodies which are reported to stimulate thymus cells.[9,16] The results (Table 1) show that after 48 hr incubation most of the proliferative response to anti-CD2 stimulation is by CD45RO⁻ cells. This difference was more marked after 72 hr of incubation. As expected from previous reports,[9] accessory cells as well as IL-2 were required for a proliferative response to anti-CD2.

The proliferative response of cells separated after positive adherence to the magnetic beads might have been affected by the separation process itself. To exclude this, we stimulated thymocytes with anti-CD2 antibodies in the presence of IL-2 and followed the entry of CD45RO⁺ and CD45RO⁻ cells into the cell cycle by propidium iodide (PI) staining. The results (Table 2 shows a representative experiment and Fig. 3 a representative cytofluorograph profiles) indicate that 42% of the CD45RO⁺ cells were in cycle after 12 hr of culture without stimulus, compared with 3% of the CD45RO⁻ cells. The percentages of cells in cycle in unstimulated cultures did not change over 48 hr of culture. The percentage of CD45RO⁻ cells in cycle increased

Table 1. Thymidine uptake of thymocytes stimulated by anti-CD2*

Hours culture	Added to culture			Counts per min of		
	CD2	MNC	IL-2	Unseparated	CD45RO$^-$	CD45RO$^+$
48	0	0	0	2250±168	1503±91	1552±484
	0	+	+	2199±256	2784±463	1844±348
	+	+	0	7817±1602	10567±616	4803±1046
	+	+	+	9080±1018	10880±1000	5621±1915
72	+	0	0	543±123	1490±500	633±142
	+	+	+	11704±4492	19691±2370	1726±1576

* Thymocytes separated into CD45RO$^+$, CD45RO$^-$ subsets were stimulated as shown and their thymidine uptake determined. Additions to culture were: CD2 = 1:500 diluted $T11_2$ and $T11_3$ antibodies; IL-2 = 15 u/ml of recombinant IL-2; MNC = irradiated mononuclear cells as a source of accessory cells. Results are the mean ± 1 SEM for three experiments.

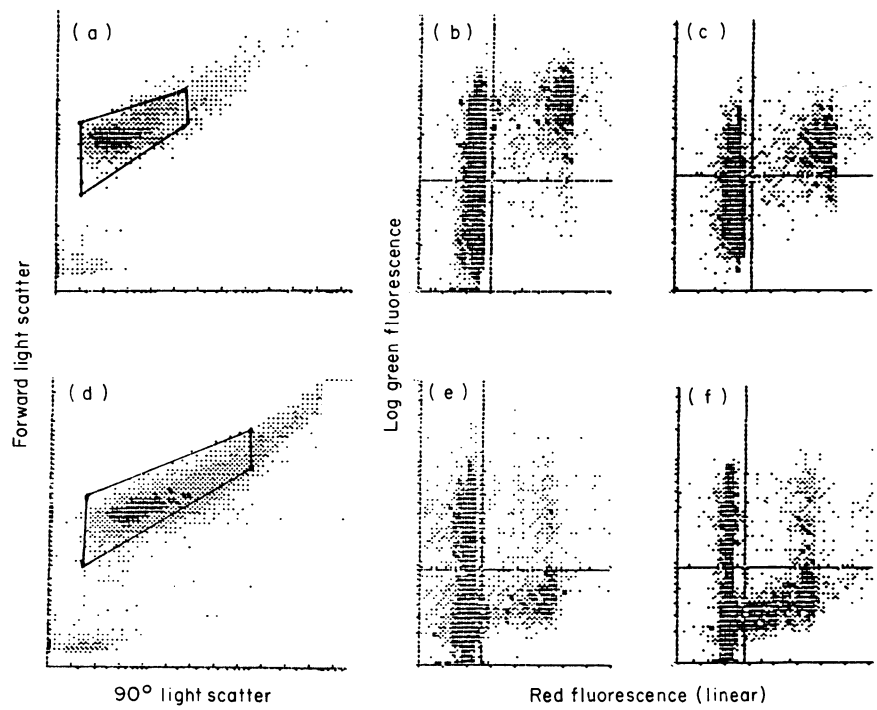

Figure 3. Cell cycle analysis of cultured human thymus cells. Upper series, unstimulated; lower series, stimulated with the anti-CD2 antibodies, $T11_2$ and $T11_3$ in the presence of 10 U/ml IL-2 and irradiated blood MNC. (a) and (d) show forward and right-angle light scatter parameters of cells after 48 hr incubation, with the population selected for analysis enclosed within the line. (b) and (e) show the propidium iodide profiles of thymus cells after 24 hr culture, (c) and (f) after 48 hr culture. The vertical axis separates cells in G0 (left) from those which have entered cells cycle (right). The horizontal axis separates cells staining positively (above) or negatively (below) with FITC conjugated CD45RO.

in the anti-CD2-stimulated cultures from 3 to 15%, and similar changes were obtained with two further thymuses. By pooling data from the 24- and 48-hr time points for three thymuses, the increase in the percentage of cells in cycle is statistically significant ($P < 0.05$ by Mann & Whitney paired-ranks test) compared with control. Anti-CD2 did not increase the percentage of CD45RO$^+$ cells in cycle, but effected a statistically significant reduction in the percentage of CD45RO$^+$ cells surviving in culture (from 40 to 15% after 48 hr culture).

Ca^{2+} flux following anti-CD2 stimulation

The lack of thymidine uptake by the CD45RO$^+$ subset could have arisen if the cells had not experienced any triggering

Table 2. Cell cycle analysis of thymocyte subsets*

Subset analysed	Stimulus	Measurement (%)	Hours of culture 12	24	36	48
CD45RO⁻	0	Cells	53	62	67	60
		G2SM	3	4	4	4
	CD2+IL-2	Cells	75	86	80	85
		G2SM	15	11	14	15†
CD45RO⁺	0	Cells	47	39	33	40
		G2SM	42	57	57	72
	CD2+IL-2	Cells	24	16	20	15†
		G2SM	58	42	51	42

* Thymus cells were cultured with or without anti-CD2, IL2 and irradiated blood MNC for the times indicated, surface stained for CD45RO and fixed for PI staining.

† Results differing from unstimulated controls with $P<0{\cdot}05$ by Mann-Whitney test.

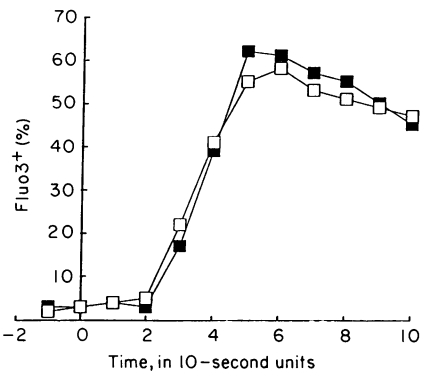

Figure 4. Calcium flux amongst CD45RO⁺ (■) and CD45RO⁻ (□) thymus subsets following addition of anti-CD2 antibodies at time 0.

stimulus as a result of the anti-CD2 stimulation. To exclude this possibility, we analysed the subsets which fluxed Ca^{2+} following anti-CD2 stimulation. The results (Fig. 4) confirm that both CD45RO⁻ and CD45RO⁺ subsets are triggered by anti-CD2.

DISCUSSION

The majority of thymocytes die intrathymically by a programmed cell death (PCD) or apoptotic process which is presumed to result from the elimination of cells expressing self-reactive or irrelevant receptors for self-major histocompatibility complex (MHC)+antigen. One of the main characteristics of apoptosis is DNA fragmentation.[17,18] Observations on transgenic mice suggest that negative selection occurs at a point when thymus cells express low levels of CD3, both CD4 and CD8, CD1 and the T-cell receptor.[19] Human thymus cells with this phenotype can be subdivided according to the splice variant of the T200 common leucocyte antigen (CD45) which they express. Cells with the 180 kDa variant, CD45RO, do not proliferate in cultures stimulated by mitogens even in the presence of accessory factors, and Pilarski *et al.*[8] suggested that this population is destined for intrathymic death. The reciprocal subset, expressing CD45RA, proliferates in response to mitogens in the presence of co-stimulator factors and may have precursor function.

Our present studies were undertaken to determine whether the CD45RO⁺ subset of thymocytes would fail to proliferate in response to anti-CD2 antibodies, because previous observations suggested that anti-CD2 is a potential ligand for thymocyte stimulation.[10] We found that the reciprocal, CD45RO⁻, subset both fluxed calcium and incorporated thymidine when stimulated by combinations of anti-CD2 antibodies, provided that IL-2 and irradiated MNC were added as a source of co-stimulator factors. Amongst the CD45RO⁺ subset , the occurrence of a calcium flux indicated that the anti-CD2 antibodies delivered a triggering signal, even though thymidine uptake was not increased. Possible explanations for the lack of proliferation by the CD45RO⁺ subset include the lack of an essential co-stimulator factor and a pre-existing commitment to apoptosis.

The propidium iodide staining of CD45RO⁺ cells gave no evidence that these cells moved into the cell cycle as a result of anti-CD2 stimulation. If lack of proliferation by these cells were indeed because of the absence of an unidentified co-stimulator factor, then the factor(s) must differ from those required by CD45RO⁻ cells and must be required for cell cycle entry. Interleukins can certainly act at this point, as illustrated by the failure of mitogen-stimulated cells deprived of IL-2 to enter the S phase of the cell cycle.[20] The CD4⁺CD8⁺ subset of mouse thymocytes is known not to make IL-2 in response to phorbol 12-myristate 13-acetate (PMA) stimulation.[21] An alternative to the view that interleukins limit the response of the CD45RO⁺ thymocyte subset is the view that these cells cannot enter the cell cycle because they are already irreversibly committed to opoptosis. The finding that exposure to anti-CD2 antibodies for 24 hr of culture elicited DNA fragmentation by the CD45RA⁻ cell subset, but not by the CD45RO⁻ subset, is consistent with this view. The clearest evidence for DNA fragmentation in the cultures came from the analysis of extracted DNA and isolated supernatants, which showed a typical ladder pattern. The presence of some low molecular weight ethidium bromide-staining material in supernatants isolated from CD45RO⁻ thymus cells incubated with anti-CD2 antibodies may relate to the anti-CD2-induced conversion of CD45RA⁺ into CD45RO⁺ thymocytes as described by Merkenschlager and Fisher.[12] Our own data (not shown) confirm that a subset of CD45RO⁻ thymocytes switches to become CD45RO⁺ when incubated in medium containing IL-2, irradiated MNC and anti-CD2 antibodies. The *in vivo* fate of cells making this switch remains to be investigated.

Several stimuli are known to induce DNA fragmentation in mouse thymus cells, ranging from corticosteroids[13] to antibodies to the CD3 complex.[3] Our own data and those of Merkenschlager and Fisher[12] add anti-CD2 to this list and also show that, at least amongst human thymocytes, the DNA fragmentation is restricted to the CD45RO⁺ subset. Our finding that anti-CD2 stimulation reduced the numbers of CD45RO⁺ cells in culture even in the presence of IL-2 and irradiated MNC as helper factors provides no support for the view of McConkey *et al.*[22] that the alternative outcomes of proliferation or DNA fragmentation by stimulated thymocytes are dictated by the availability of accessory factors. Our data favour the view that these are opposing responses by different subsets, the

CD45RO$^-$ cells having the potential to proliferate in the presence of accessory factors and the CD45RO$^+$ cells being commited to apoptosis.

The events which lead to the phenotypic change in developing thymocytes from CD45RO$^-$ to CD45RO$^+$ are unresolved. Amongst peripheral blood T cells, the change follows a cycle of cell division.[23] Our own results and those of Egerton *et al.*[24] show CD45RO$^+$ thymocytes in the G2, S and M phases of the cell cycle at the time the cells are isolated. Whether this cycle was responsible for the phenotypic switch in CD45 isotype, and the relationship the switch may have to negative selection, remains to be determined.

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