Immunology 1990 **71** 467–472

CD45RA is detected in all thymocyte subsets defined by CD4 and CD8 by using three-colour flow cytometry

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Accepted for publication 22 August 1990

SUMMARY

In the mouse, using three-colour flow cytometry, the presence of CD45RA$^+$ cells is demonstrated amongst all of the thymocyte subsets defined by expression of CD4 and CD8, i.e. amongst the double negatives, immature CD8 single positives, double-positive blasts and CD4 and CD8 single positives. This evidence is compatible with the existence of a continuous lineage of T cells expressing CD45RA which would develop from double-negative to mature single-positive T cells.

INTRODUCTION

Subsets of leucocytes can be differentiated by various proteins expressed on their surface membrane. These proteins are referred to as CD (cluster differentiation) antigens. CD4 and CD8 are accessory molecules which interact with major histocompatibility (MHC) class II or MHC class I, respectively. Mature T cells express on their surface either CD4 or CD8, whilst thymocytes, depending on their maturation stage, express neither (least mature), both or only one of them (most mature). Peripheral T cells recognize antigen only in the context of MHC molecules and tend not to react against self-antigens. This is the result of two selection mechanisms occurring during T-cell ontogeny in the thymus: negative selection (deletion of self-antigen reactive T cells; Kappler *et al.*, 1988, Macdonald *et al.*, 1988) and positive selection (need of self-MHC recognition by T cells; Kisielow *et al.*, 1988). However, it is not clear at which stage of thymic T-cell maturation these events occur and exactly which surface molecules are involved in such selective processes. Moreover, a paradox in the development of T cells in the thymus is that the least mature cells (CD4$^-$CD8$^-$ double negatives) often share with the most mature (CD4 single positives and CD8 single positives) markers, which are poorly expressed in intermediate cells (CD4$^+$CD8$^+$ double positives); this is true of pgp-1 (Budd *et al.*, 1987a; Lynch & Ceredig, 1988), CD45R in man (Pilarski *et al.*, 1989), rat (Law *et al.*, 1990) and mouse (Lefrancois & Goodman, 1987; Fisher *et al.*, 1989), and STB1 (Fisher *et al.*, 1989; Andrew & Jayakumar, 1990). This might represent two successive shifts in phenotype, from positive to negative and then back to positive, and that is what studies based solely on double-fluorescence have tended to conclude (Law *et al.*, 1990). Alternatively the intermediate population might contain a minor subpopulation, possibly a very small one, which would retain the markers in question and which might identify a continuous lineage of cells. Since most thymocytes die at the CD4$^+$CD8$^+$ double-positive stage (Scollay *et al.*, 1988), it is critical to assess whether all are subjected to selection processes or if only small populations are, since in the latter case the interpretation of signalling effects in these cells would be radically altered. Such studies (Smith *et al.*, 1989) would then be telling us more about the special properties of a doomed cell than about signals for negative selection. It would be technically difficult but not impossible to test this alternative hypothesis in full, but in the meanwhile valuable information can be obtained simply by examining expression of the markers within the intermediate population. This we do here for the CD45RA marker, within all subsets defined by CD4 and CD8 in the mouse thymus, by means of three-colour fluorescence.

All peripheral leucocytes express the heavy molecular weight glycoprotein CD45. However, isoforms of this molecule are differentially expressed on subsets of both peripheral CD4 single-positive and CD8 single-positive T cells. These single-positive T cells can be split into reciprocal CD45RA$^+$ and CD45RA$^-$ subsets using anti-CD45RA monoclonal antibodies. Recognition by these antibodies depends on the expression of exon A at the 5′ end of the CD45 gene. CD45RA isoforms can be immunoprecipitated as two heavy molecular weight (MW) bands of 220,000 and 205,000, respectively (Marvel, Poirier & Lightstone, 1989). In humans CD4 and CD8 CD45RA$^+$ cells are 'naive', whilst the negative (CD45RO) subset, which expresses the lower MW isoform (180,000), comprises memory cells (Merkenschlager *et al.*, 1988). The subsets are not believed

Abbreviations: CD4$^+$, CD4$^+$CD8$^-$ single positive; CD8$^+$, CD4$^-$CD8$^+$ single positive; CTL, cytotoxic lymphocyte; DN, CD4$^-$CD8$^-$, double negative; DP, CD4$^+$CD8$^+$, double positive; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; HSA, heat-stable antigen; IL-2R, interleukin-2 receptor; mAb, monoclonal antibody; MEM, modified Eagle's medium; PBS, phosphate-buffered saline; PE, phycoerythrin; SP, single positive.

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Table 1. Summary of antibodies used in this study

Antibody	Specificity	Reference
RA3-2C2	Rat IgM, anti-CD45RA	Coffman & Weissman (1981a); Marvel *et al.* (1989)
GK1.5	Rat IgG2b, anti-CD4	Dialynas *et al.* (1983)
53-6.7	Rat IgG2a, anti-CD8	Ledbetter & Herzenberg (1979)
YBM5.10	Rat IgM, anti-HSA	Watt *et al.* (1987); Alterman *et al.* (1990)

to be the product of separate lineages since activation of $CD45RA^+$ cells has been shown to lead to irreversible loss of expression of CD45RA *in vitro* in humans (Merkenschlager *et al.*, 1988; Akbar, Timms & Janossy, 1989) and *in vivo* in rats (Powrie & Mason, 1989). From these data it might be expected that thymocytes would express CD45RA but, surprisingly, in humans (Pilarski *et al.*, 1989) and in the mouse (Lefrancois & Goodman, 1987) unseparated thymocytes show a predominance of the CD45RO phenotype (p180), while the CD45RA isoforms are only expressed on 10–30% of human thymocytes, many of which also express CD45RO (p180) (Serra *et al.*, 1988). The $CD45RO^-CD45RA^+$ subset is enriched in single positives and double negatives and contains most of the clonogenic precursors found in the human thymus (Pilarski *et al.*, 1989). In mice CD45RA was shown, by immunoprecipitation, to be expressed on CD4 and CD8 single-positive thymocytes but not amongst the double positives or double negatives (Lefrancois *et al.*, 1987). Using the monoclonal antibody RA3-6B2, which immunoprecipitates the 220,000 MW band of the CD45 but which does not recognize the isoform requiring expression of exon A (Johnson *et al.*, 1989), conflicting data on the presence of $CD45R^+$ cells amongst the double-negative subset have been obtained (Gause, Mountz & Steinberg, 1988; Wilson *et al.*, 1988). So although CD45RA is clearly expressed on some of the single-positive thymocytes in mice, little is known about the two other thymocyte subsets, the double negatives and double positives. In this paper we have used three-colour flow cytometry to analyse expression of CD45RA amongst the separate thymocyte subsets delineated by CD4 and CD8 expression. We report here that CD45RA is expressed mainly amongst the $CD4^-CD8^-$ double-negative and CD8 single-positive subsets but that it is also unambiguously present amongst the $CD4^+CD8^+$ double-positive and CD4 single-positive subsets.

MATERIALS AND METHODS

Mice

Male and female (the latter used only for embryos) B10.A mice were obtained from the ICRF breeding facilities.

Antibodies

Antibodies and their specificities are listed in Table 1. Phycoerythrin (PE)-conjugated GK1.5 and FITC-conjugated 53-6.7 were purchased from Becton-Dickinson (Mountain View, CA). RA3-2C2 (ATCC, Rockville, MD) and YBM5.10 (a gift from C. Kinnon) were coupled to biotin in our laboratory using standard methods. RA3-2C2 was used in preference to the other two anti-CD45RA antibodies available, RA3-3A1 (Coffman & Weissman, 1981b) and 14.8 (Kincade *et al.*, 1981), as we have previously shown that it gives the brightest staining profile on murine T cells (Marvel *et al.*, 1989). Antibodies were used at their optimal concentrations as determined by prior titration. The second layer used was streptavidin conjugated to a phycoerythrin texas red conjugate ('Duochrome') purchased from Becton-Dickinson.

Fluorescent antibody staining

Single-cell suspensions were obtained using a glass plunger homogenizer (Jencons, Beds, U.K.), which gave good (>98%) viability. Aliquots of 10^6 cells were suspended in MEM-HEPES 5% foetal calf serum (FCS) and mixed with antibodies in phosphate-buffered saline (PBS), 1% FCS, 0·1% azide in U-bottomed tubes (Falcon, Becton-Dickinson, Oxford, U.K.). The first layer was incubated for 45 min at 4°, followed by a large volume wash (4 ml). The second layer (Duochrome) was then incubated for 30 min at 4° followed by a further large volume wash and resuspended in filtered PBS + 0·1% azide and kept on ice whilst being analysed. In each experiment controls of second layer only, single antibody and dual antibody were run as well as the triple stain. For the latter 4×10^6 cells (120 μl) were stained in each aliquot with an appropriate increase in the amount of reagents used (380 μl). Foetal thymocytes were treated in an identical fashion except that several lobes were pooled in order to obtain adequate cell numbers. For the triple stain the negative control values for the percentage of positive anti-CD45RA staining were obtained by incubating one aliquot with all three labelled antibodies together with excess unlabelled RA3-2C2 in order to inhibit specific anti-CD45RA binding.

Analysis of surface fluorescence

All samples were analysed on a FACScan flow cytometer (Becton-Dickinson) using a single argon ion laser and logarithmic intensity scales. Dead cells and debris were excluded by gating on the basis of forward and side scatter. To assess the percentage of $CD45RA^+$ cells in each subset, gates were set at the acquisition level on the following populations: $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$ (hereafter referred to as double negatives, double positives, CD4 single positives and CD8 single positives, respectively). Further acquisition gates used were (i) blasts only, based on forward scatter, and (ii) gating on all $CD45RA^+$ cells. In most instances 10^4 cells were analysed per sample. Analysis markers were critically set according to the negative controls described above.

RESULTS

CD45RA expression amongst thymocyte subsets in young and adult B10.A mice

Triple-colour analysis was used to define the percentage of CD45RA-positive cells in each of the four major subsets defined by expression of CD4 and CD8, i.e. double negatives, double positives, CD4 and CD8 single positives. Single-cell suspensions from the thymuses of either 4- or 7-week-old mice were stained with directly conjugated anti-L3T4 and anti-Ly-2 and biotinylated anti-CD45RA. The specific anti-CD45RA binding was calculated by subtracting the value obtained with a control comprising all three labelled antibodies together with excess unlabelled anti-CD45RA. Table 2a summarizes the data for 4-week-old mice and Table 2b the data for 7-week mice, and typical FACS profiles for a 4-week mouse are shown in Fig. 1.

Table 2. Expression of CD45RA (%) amongst thymocyte subsets defined by staining with anti-CD4 and anti-CD8

Exp.	1	2	3	4	5	6	7	8	9	Mean (SD)
(a) 4 weeks										
DN	10	14	13·5	11	10	9·3	5	5·2	7	9·4 (3·2)
DP	0·5	0·3	0·4	0·3	0·3	0·1	0·1	0·1	0·2	0·25 (0·1)
$CD4^+$	0·5	0·7	0·4	0·5	0·6	0·4	0·1	0·1	0·3	0·4 (0·2)
$CD8^+$	13·5	3	6	3·3	4·2	2·1	1·1	2·3	10	5·0 (4·0)
(b) 7 weeks										
DN	4·8	8	20*	9	5	4				6·2 (2)
DP	0·7	0·5	0·5	0·4	0·7	0·2				0·5 (0·3)
$CD4^+$	0·1	0·5	1	0·5	0·7	0·2				0·5 (0·3)
$CD8^+$	2	2·3	5·2	6·3	1·6	1·7				3·2 (2)
(c) 4 weeks										
Exp.	4	5	6	7	8	9				
DP	0·5	2·5	1	0·7	0·7	1				1·1 (0·7)
$CD4^+$	6	1·2	5	1	2	0·7				2·7 (2·3)
7 weeks										
Exp.	4	5	6							
DP	1·5	1·2	1·3							1·3 (0·2)
$CD4^+$	2	ND	ND							

* Point omitted from SD calculation.

Cells were stained with FITC-conjugated anti-CD8, PE-conjugated anti-CD4 and biotinylated anti-CD45RA revealed with a second layer of PE-Texas Red conjugated with streptavidin.

Gates were set at the acquisition level on each of the four subsets defined by CD4 and CD8 expression and the percentage of $CD45RA^+$ cells amongst the 10^4 cells acquired is shown. For each experiment the mean and standard deviation (SD) for each group of mice is also given.

(a) Four-week-old mice; (b) 7-week-old mice; (c) a further gate was placed at the acquisition level based on forward scatter so that only blast cells were analysed. The data shown are for the age described and the experiments are numbered as in (a) and (b). Subsets gated on: DN, double negative; DP, double positive; $CD8^+$, CD8 single positive; $CD4^+$, CD4 single positive.

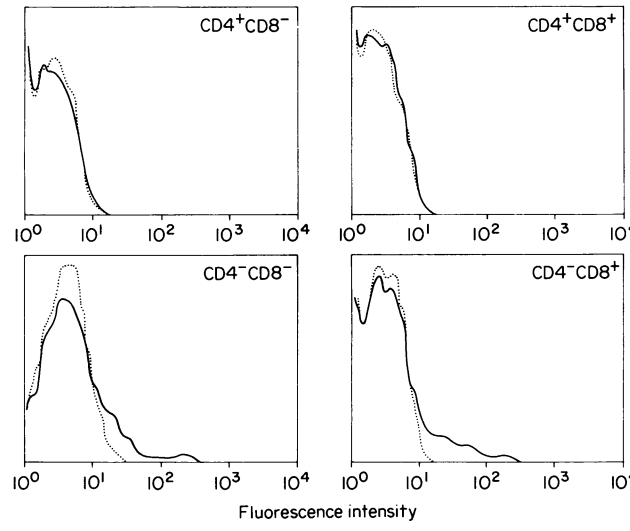

Figure 1. Staining of the thymocyte subsets of a 4-week-old B10A mouse with anti-CD45RA monoclonal antibody (data given in Table 2a, experiment 9). The cells were stained with FITC-conjugated anti-CD8, PE-conjugated anti-CD4 and biotinylated anti-CD45RA revealed with a second layer of PE-Texas Red conjugated with streptavidin. Gates were set at the acquisition level on each of the four populations defined by expression of CD4 and CD8 and 10^4 cells were acquired and analysed for expression of CD45RA (——). The negative control (· · · · ·) was stained in an identical manner but with the addition of 20 μl of 10 × concentrated unlabelled anti-CD45RA supernatant to inhibit specific anti-CD45RA binding.

A definite subset of $CD45RA^+$ cells can be seen within the double-negative and CD8 single-positive subsets. The percentage of $CD45RA^+$ cells found amongst the two other subsets was very low (Fig. 1).

Each population had a distinctive size profile (based on forward scatter) that was highly reproducible from one experiment to another, with double-negative cells the largest and single positives somewhat smaller. In contrast, double-positive cells show two clear populations (data not shown): one (the majority) the size of small lymphocytes, the other of much larger cells (Fig. 2).

If a gate was placed so that only the blast population is examined then small but distinct populations of $CD45RA^+$ cells could be seen within both the double-positive and CD4 single-positive subsets (Table 2c).

Thymocyte subset distribution amongst $CD45RA^+$ cells

To confirm the existence of a subset of $CD45RA^+$ cells amongst CD4 single-positive and double-positive thymocytes, we examined the CD4/CD8 phenotype of the $CD45RA^+$ cells. Since $CD45RA^+$ cells only represent 1–2% of the total thymic population, gating on that subset would allow us to detect $CD45RA^+$ double-positive cells, even if those cells comprise less than 1% of the total thymic population. Results for six separate stainings amongst 4-week-old mice and five stainings amongst 7-week-old mice are summarized in Table 3a, b, and a typical plot of CD4 and CD8 staining is shown in Fig. 3.

As can be seen from the plot and the table there are distinct populations of double-positive and CD4 single-positive thymocytes expressing CD45RA. They cannot be accounted for by spill into the $CD45RA^+$ population as they were present in almost identical proportions even when the most stringent gate (e.g. the brightest 5%) was placed on the $CD45RA^+$ cells.

CD45RA expression amongst 16-day foetal thymocytes

Because CD8 is expressed slightly earlier than CD4 as double-negative cells mature to become double positive, we examined cells from the 16-day foetal thymus. At 16 days the foetal thymus comprises mostly double-negative cells, with a few CD8 single-positive cells, few if any double-positive cells and no CD4 single-positive cells (data not shown, Husmann *et al.*, 1988). As can be seen from Table 4, CD45RA could already be detected amongst the CD8 single-positive cells at Day 16.

The surface glycoprotein known as heat-stable antigen (HSA) is present on a small percentage of CD8 single-positive cells, as well as all double-positive cells, and is said to mark functionally incompetent thymocytes in contrast to CD8 single-positive HSA^- cells, which function in the same way as peripheral CD8 single-positive cells (Crispe & Bevan, 1987). All

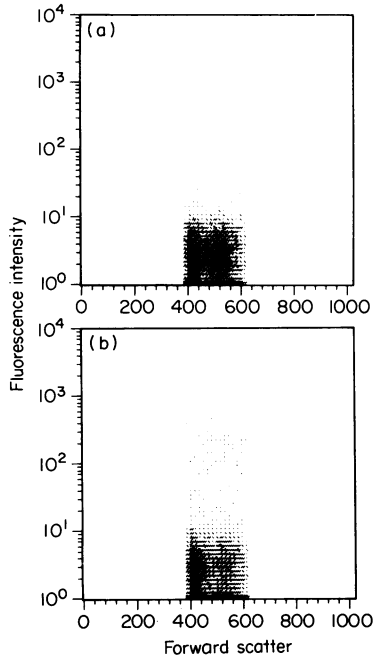

Figure 2. Expression of CD45RA amongst DP blasts (data given in Table 2c, 4-week mouse, experiment 9). The cells were stained with FITC-conjugated anti-CD8, PE-conjugated anti-CD4 and biotinylated anti-CD45RA revealed with a second layer of PE-Texas Red conjugated with streptavidin. Gates were placed at the acquisition level on the DP blast population as defined by CD4, CD8 and by FSC. Cells acquired were analysed for expression of CD45RA. Plot (a) shows the CD45RA-negative control obtained by staining with all three antibodies together with 20 μl of 10 × concentrated unlabelled anti-CD45RA supernatant to inhibit specific anti-CD45RA binding. Plot (b) shows expression of CD45RA by a small but definite subset of blasts.

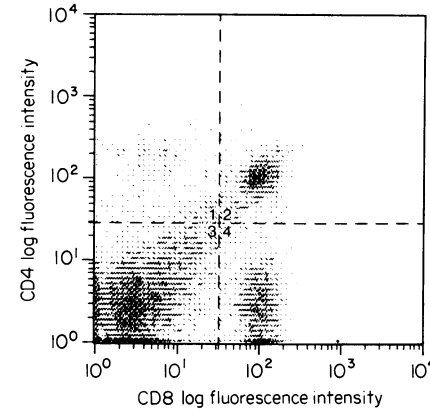

Figure 3. Distribution of CD4 and CD8 expression amongst CD45RA$^+$ thymocytes (data given in Table 3a, experiment 6). Cells were stained with FITC-conjugated anti-CD8, PE-conjugated anti-CD4 and biotinylated anti-CD45RA revealed with a second layer of PE-Texas Red conjugated to streptavidin. Gates were placed at the acquisition level on CD45RA-positive cells, as judged by the inhibited control described in Fig. 1. The cells acquired were then analysed for expression of CD4 and CD8 on the basis of the markers shown. Quadrant (1) CD4$^+$, 4·3%; (2) double positive, 7%; (3) double negative, 63%; and (4) CD8$^+$, 25%.

Table 3. Expression (%) of CD4 and CD8 amongst CD45RA$^+$ thymocytes

Exp.	1	2	3	4	5	6	Mean (SD)
(a) 4 weeks							
DN	68	73	66·5	76	76	63	70·4 (5·4)
DP	16	13	13	12·5	10	7	9·3 (4)
CD4$^+$	5·2	7·2	8·6	4·2	4	4·3	5·6 (1·9)
CD8$^+$	9·3	7·1	10	4·7	9·8	25	11 (7)
(b) 7 weeks							
DN	71·5	74	71	81·5	75		75 (4·3)
DP	13·2	12·5	5·8	9	14		11 (3·4)
CD4$^+$	7·2	6·3	5	2	3		4·7 (2·2)
CD8$^+$	8·2	8	18	7·6	8·4		10 (4·4)

Cells were stained with all three antibodies as described in Table 2.

Gates were then placed at the acquisition level on the CD45RA$^+$ population as determined from controls inhibited with excess unlabelled anti-CD45RA antibody. The percentage of cells in each subset defined by CD4 and CD8 is shown for each experiment, as well as the mean value and the standard deviation (SD) obtained for each group.

(a) Four-week-old mice; (b) 7-week-old mice. Subsets, abbreviations as in Table 2.

Table 4. Expression of CD45RA (%) amongst 16-day foetal thymocyte subsets defined by staining with anti-CD4 and anti-CD8

Exp.	1	2	3	Mean (SD)
Double negatives	5	4·2	3	4·1 (1)
CD8$^+$	2	2	2·5	2·2 (0·3)

Thymocyte suspensions were obtained from pooled thymus lobes of 16-day embryos.

The cells were stained with FITC-conjugated anti-CD8, PE-conjugated anti-CD4 and biotinylated anti-CD45RA revealed with a second layer of PE-Texas Red conjugated with streptavidin. Gates were placed at the acquisition level on double negative or CD8$^+$ single positives, and the expression of CD45RA within each determined. The negative control comprised identical staining together with excess unlabelled anti-CD45RA monoclonal antibody. The data for each of three experiments is shown as well as the mean and standard deviation (SD).

the CD8 single-positive cells in our 16-day thymuses were also positive for HSA (as judged by dual staining with YBM5.10 and anti-Ly-2, data not shown), and thus had an immature phenotype.

DISCUSSION

Using three-colour flow cytometry the presence of CD45RA$^+$ cells has been detected not only amongst single-positive thymocytes but also amongst the double negatives, immature CD8 single-positive and double-positive subsets in mice. Using the same anti-CD45RA monoclonal antibody, RA3-2C2, to purify

double negative CD45RA$^+$ and CD45RA$^-$ populations, Goff, Larssen & Fisher (1990) have demonstrated that the CD45RA$^+$ fraction is greatly enriched for T-cell precursors capable of recolonizing foetal thymuses in organ culture. A similar conclusion has been reached in the rat using an anti-CD45RB monoclonal antibody, MRC-OX22, to enrich or deplete progenitor populations assayed by an intrathymic adoptive transfer assay. In the human, Pilarski *et al.* (1989) demonstrated the presence of a small percentage of CD45RA$^+$CD45RO$^-$ thymocytes; 50% of these cells were single positive and accounted for all single-positive thymocytes with clonogenic potential, as judged by growth *in vitro* in the presence of irradiated feeder cells and concanavalin A. Together, these data suggest that a lineage relationship may exist between the double-negative CD45RA$^+$ thymocytes which are the progenitors and the single-positive CD45RA$^+$ which are functionally competent thymocytes destined to exit to the periphery. In the rat the monoclonal antibody MRC-OX22 has been used, in double staining, to study the expression of the CD45R isoform dependent on the expression of exon B (Law *et al.*, 1990). These studies have shown that the double negative subset of adult rat thymocytes is enriched for the expression of MRC-OX22$^+$ cells and this marker is also present amongst single positives. MRC-OX22$^+$ cells are also seen at Day 16 of foetal life. These data agree with ours, and with those of others (Goff *et al.*, 1990; Pilarski *et al.*, 1989), although the double-positive subset was not closely examined. Law *et al.* (1990) argue that expression of CD45RB occurs early in the double-negative population, is then lost and finally re-expressed prior to exit to the periphery. We feel that their conclusion is premature, particularly as the presence of a minor population of CD45RB cells in the double-positive population would probably not have been detected by the methods used. The crucial question remains whether the double-positive CD45RA$^+$ cells described in our study have clonogenic potential. Technically, this question will be a difficult one because of the small size of the population and the risk of contamination by single-positive cells. Furthermore this raises the vexed question of the precursor potential of double-positive cells in general, most of which undoubtedly die *in vivo*, and cannot be maintained by culture *in vitro*. In spite of the formidable difficulties of contamination in such experiments, a small percentage of double-positive cells seem truly to have clonogenic potential *in vitro* (Howe & Macdonald, 1989). Moreover, these cells have been identified within the blast fraction of the double-positive population (Guidos, Weissman & Adkins, 1989). These cells then might correspond to the small population of double-positive CD45RA$^+$ defined in this study. If these are shown to have clonogenic potential it could imply the existence of a continuous lineage of CD45RA$^+$ cells from double negatives via immature CD8 single-positive and double-positive cells through to single positive and argue against phenotype switching.

If the hypothesis is of a single lineage of CD45RA$^+$ single-positive cells accounting for all mature thymic emigrants, the implication from our data, deduced from the total number of single-positive CD45RA$^+$ cells, is that single-positive cells stay in the thymus for only a few hours. However, recent data from Egerton, Scollay & Shortman (1990) on the kinetics of mature T-cell development in the thymus shows that single-positive cells actually remain in the thymus for several days. It would then be necessary to postulate at least one other lineage of mature thymic emigrants which would be CD45RA$^-$. The discrepancy between the proportion of CD4 single-positive and CD8 single-positive CD45RA$^+$ cells observed in the thymus is also found in the periphery in mice, where 65% of CD8 single-positive spleen cells are positive compared with only 20% of CD4 single-positive cells (Marvel *et al.*, 1989).

These data still leave some questions unanswered. Previous work has shown that the double-negative subset can be further subdivided into several subsets on the basis of expression of markers such as HSA (Wilson *et al.*, 1988; Scollay *et al.*, 1988; Crispe *et al.*, 1987), pgp-1 (Wilson *et al.*, 1988; Hyman *et al.*, 1986), IL-2R (Raulet, 1985), Thy-1 (Kadish & Basch, 1977), CD3 (Scollay *et al.*, 1988; Crispe *et al.*, 1987; Budd *et al.*, 1987b) and Ly-1 (Fowlkes *et al.*, 1985). Further studies would be required to assess the association between expression of these markers and CD45RA and might clarify early thymic maturation pathways. We have not yet determined whether any of the double-negative CD45RA$^+$ cells express the $\gamma\delta$ T-cell receptor, but in the periphery more than 97% of CD45RA$^+$ bear $\alpha\beta$ receptors (our unpublished data) and therefore we think this unlikely. It is also not yet clear whether cells immigrating into the thymus already express CD45RA or whether it is acquired on entry.

We have shown, using 16-day foetal thymuses, that CD8 single-positive CD45RA$^+$ cells are also HSA positive. These cells are believed to represent a developmental stage between double negative and double positive (Paterson & Williams, 1987; Macdonald & Howe, 1988) and this would fit with the lineage proposed here.

In conclusion, we have shown that a fraction of all four subsets of thymocytes defined by CD4 and CD8 expresses CD45RA. Our evidence is compatible with the existence of a continuous lineage of T cells expressing CD45RA, which would develop from double-negative to mature single-positive T cells but which is unlikely to be the sole lineage capable of surviving thymic selection.

ACKNOWLEDGMENTS

The authors would like to thank N. A. Mitchison for helpful discussions, advice and constant support, K. Robertson for preparing most of the antibodies used and D. Wyllie for helpful debate. Grateful thanks to R. Scollay, The Walter & Eliza Hall Institute, Australia for sharing some data. E. B. Lightstone is supported by an MRC Training Fellowship.

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