Clin. exp. Immunol. (1990) **81**, 466–474

Expression of early activation antigen (CD69) during human thymic development

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(Accepted for publication 15 March 1990)

SUMMARY

The novel early activation antigen, EA1, has been shown to be induced by mitogens, antigens and the tumour promoter, phorbol myristate acetate (PMA), on human lymphocytes. This antigen has been designated to be CD69. EA1 has also been shown to be expressed on thymocytes without exogenous activation stimuli. In order to characterize further the expression of EA1 on thymocytes, the ontogeny of its expression was studied. EA1 appeared between 7 and 9·5 weeks of gestation, after colonization of the thymic rudiment with $CD7^+$ T cell precursors, but before the onset of compartmentalization of the thymus into cortical and medullary zones. After cortico-medullary differentiation, the majority of medullary thymocytes expressed EA1 while only a fraction of the cortical thymocytes expressed this antigen. In the fetal and post-natal cortex, EA1 expression appeared to cluster in the subcapsular cortex. $EA1^+$ cells were also scattered throughout the inner cortex. By two-colour fluorocytometric analysis of post-natal thymocytes, it was shown that EA1 was expressed on 30 to 65% of thymocytes. EA1 was expressed on $CD4^+$ $CD8^+$ as well as on the more immature $CD4^-$ $CD8^-$ thymocytes. In contrast to circulating T cells, thymocytes were much less responsive to PMA stimulation for the expression of EA1. Molecular characterization showed that EA1 on thymocytes had the same structure as that of activated peripheral T cells. In addition, thymic EA1 was constitutively phosphorylated. Thus, EA1 expression is acquired early during thymic development after colonization of the thymic rudiment by $CD7^+$ T cell precursors. However, the specific role that EA1 may play in the activation and function of developing thymocytes remains to be determined.

Keywords human thymus T cell activation antigen phosphorylation development CD69

INTRODUCTION

Activation of human lymphocytes by mitogens or antigens results in the expression of a number of surface molecules which are important in their proliferation and differentiation. EA1 is a recently described, early activation antigen which is induced by mitogens, antigens and the tumour promoter phorbol myristate acetate (PMA) on T and B cells (Hara *et al.*, 1986). It is not expressed by monocytes, granulocytes or erythrocytes. Since its initial description, a number of similar or identical molecules have been described. Leu 23 is a heterodimer of 28/32 kD which is induced on lymphocytes by mitogens 1 h after activation. The kinetics of its expression are similar to EA1 (Chen *et al.*, 1988). Interleukin-2 (IL-2) activation of natural killer cells also induces the expression of this antigen (Lanier *et al.*, 1988). We have recently demonstrated that monoclonal antibodies (MoAbs) to EA1 and Leu 23 identify the same molecule (Nakamura *et al.*, 1989). Cosulich *et al.* (1987), have described an early antigen, MLR3, which is a heterodimer of 28/34 kD. Its expression on activated T cells is very similar to that of EA1. It is of interest that a MoAb to MLR3 inhibited IL-1-dependent T cell proliferation, suggesting that MLR3 is involved in the early steps of T cell activation. Unlike EA1 and Leu 23, MLR3 is not expressed on unstimulated thymocytes (Cosulich *et al.*, 1987), but is induced on activated fetal thymocytes (Delia *et al.*, 1988). Recently, Cebrian *et al.* (1988) have identified a new activation

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antigen, designated activation inducer molecule (AIM). The molecular characteristics, the kinetics of expression and activation requirements are similar to those of EA1. Thus, EA1, Leu 23, MLR3 and AIM are very similar. They have recently been classified as CD69 by the 4th International Workshop on Human Leucocyte Differentiation Antigens.

In contrast to peripheral blood T cells, EA1 is found on thymocytes without activation by PMA or mitogens (Hara *et al.*, 1986; Testi, Phillips & Lanier, 1988). To characterize further the expression of EA1 on human thymocytes, immunohistological and biochemical analyses of EA1 on human thymocytes were carried out. Here we report that EA1 appears in the human fetal thymus as early as 9·5 weeks of gestation. During thymic development and in the post-natal thymus, EA1 expression is found concentrated predominantly in medullary zones and scattered in foci throughout thymic cortex. In addition, the EA1 molecule on thymocytes is similar in molecular structure to that of activated T cells and this molecule in constitutively phosphorylated.

MATERIALS AND METHODS

Cell and tissue preparation

Post-natal thymic tissues were obtained from children, ages 7 months to 8 years, undergoing corrective cardiac surgery. One fetal thorax, eight fetal thymus and three fetal liver specimens were obtained as discarded tissues from the Department of Pathology, Duke University Medical Center. These tissues were processed for immunohistological analyses by indirect immunofluorescence as described (Lobach *et al.*, 1985; Denning *et al.*, 1987; Haynes *et al.*, 1988). Single cell suspensions of thymocytes and peripheral blood T cells were prepared according to Jung, Hara & Fu (1984).

In order to select negatively for $CD4^-$ $CD8^-$ cells, thymocytes were incubated for 20 min at 4°C in a mixture of culture supernatants from hybridomas producing MoAbs to CD4 and CD8. Using goat anti-mouse immunoglobulin-coated Petri dishes, the cells were 'panned' twice by the indirect method to deplete $CD4^+$ $CD8^+$ cells as described (Wysocki & Sato, 1978).

Monoclonal antibodies

MoAbs to CD3 (T3I, IgG1) and to EA1, (P8, IgG1) were generated in our laboratory (Hara & Fu, 1985; Hara *et al.*, 1986). MoAbs to CD1 (OKT6, IgG1), CD2 (OKT11, IgG2), CD4 (OKT4, IgG2b), CD8 (OKT8, IgG2a), CD38 (OKT10, IgG1) and HLA-DR (L243, IgG2a) were obtained from American Type Culture Collection (Rockville, MD). MoAbs to CD7 (3A1, IgG1), IL-2R (AT1, IgG1), CD45 (F10-89-4, IgG2a) and transferrin receptor (IgG1) were prepared as described (Haynes, Eisenbarth & Fauci, 1979; Jung *et al.*, 1984; Lobach *et al.*, 1985). AE1, IgG1, (Woodcock-Mitchell *et al.*, 1982) and LFA-1, IgG1 (Sanchez-Madrid *et al.*, 1982) were kind gifts from Dr T.-T. Sun (NYU Medical Center, New York, NY) and Dr T. A. Springer (Harvard Medical School, Boston, MA), respectively. MoAb to EA1 was biotinylated as described (Goding, 1986). For isotype control, NS4.1 (IgM), HDP-1 (IgG1), SS.1 (IgG2a) and NS8.1 (IgG2b), were used.

Immunofluorescence studies

Indirect immunofluorescence assays were carried out on acetone-fixed, 4-μm thick tissue sections (Denning *et al.*, 1987; Haynes *et al.*, 1988a, 1988b). Briefly, thymus tissues were first incubated with an optimal dilution of anti-EA1 ascitic fluid, followed by FITC-conjugated goat anti-mouse immunoglobulin as a secondary antibody. For double-staining experiments, rhodamine-conjugated goat anti-mouse immunoglobulins was used after anti-EA1 MoAb was used as first antibody. The slides were then extensively washed three times and unconjugated mouse immunoglobulin (100 μg/ml) was added to block the available binding sites on the goat anti-mouse immunoglobulin. After three additional washings, FITC-conjugated MoAbs to keratin, AE1, CD2 (OKT11) (Lampson & Levy, 1980), the non-polymorphic determinant of HLA-DR (L243) or LFA-1 were then added. The slides were washed and mounted for fluorescence microscopy.

Flow fluorocytometry

Thymocytes and T cells were incubated with 10 ng/ml of PMA for 0, 2 and 24 h, washed, and stained with a control or anti-EA1 MoAb for 30 min at 4°C. FITC-conjugated goat anti-mouse immunoglobulin prepared in our laboratory was used as a second antibody; 10 000 gated cells were analysed on an Ortho System 50H Cytofluorograf (Ortho Diagnostic System, Westwood, MA) as previously described (Jung & Fu, 1988). For two-colour analysis, the thymocytes were first incubated by MoAb of interest, washed three times and the stained with FITC-conjugated goat anti-mouse immunoglobulin. Available binding sites on the goat anti-mouse immunoglobulin were then blocked with 50 μl of mouse immunoglobulin (100 μg/ml). After three further washes, biotinylated anti-EA1 MoAb was added to the thymocytes, followed by phycoerythrin-conjugated streptavidin (Tago, Burlingame, CA); 20 000 gated cells were analysed on an Ortho 50H Cytofluorograf equipped with a Lexel laser tuned to 488 nm, a 560 nm short-pass dichroic filter mounted at 45° to the orthogonal light beam, a 585 nm band pass filter at the photomultiplier for red fluorescence, a 530 nm band pass filter at the photomultiplier for green fluorescence, and an Ortho fluorescence compensated dual logarithmic amplifier module to compensate for spectral overlap. The red and green fluorescence were collected using three and a half decade logarithmic amplifiers. The collected data were analysed on an Ortho 2151 Data Handler.

Immunoprecipitation and autoradiography

Freshly isolated thymocytes or T cells activated with PMA were labelled with ^{125}I by the lactoperoxidase technique as described (Hara *et al.*, 1986). To compensate for the low expression of EA1 on thymocytes, 50×10^6 thymocytes and 20×10^6 activated T cells per sample were used.

For phosphorylation experiments, cryopreserved thymocytes and freshly isolated T cells were incubated in phosphate-free RPMI 1640 medium with 10% fetal calf serum (FCS) dialysed against the phosphate-free medium at 50×10^6 cells/ml for 1 h. The cells were resuspended in the phosphate-free medium containing 1 mCi/ml of ^{32}P orthophosphate (DuPont, Wilmington, DE) at 50×10^6 cells/ml and labelled for 4 h at 37°C with or without 10 ng/ml PMA. The reactions were stopped with cold phosphate-buffered saline (PBS) containing 10 mM sodium pyrophosphate, 50 mM NaF and 2 mM EDTA. The cells were then washed three times. Labelled cells were solubilized in PBS

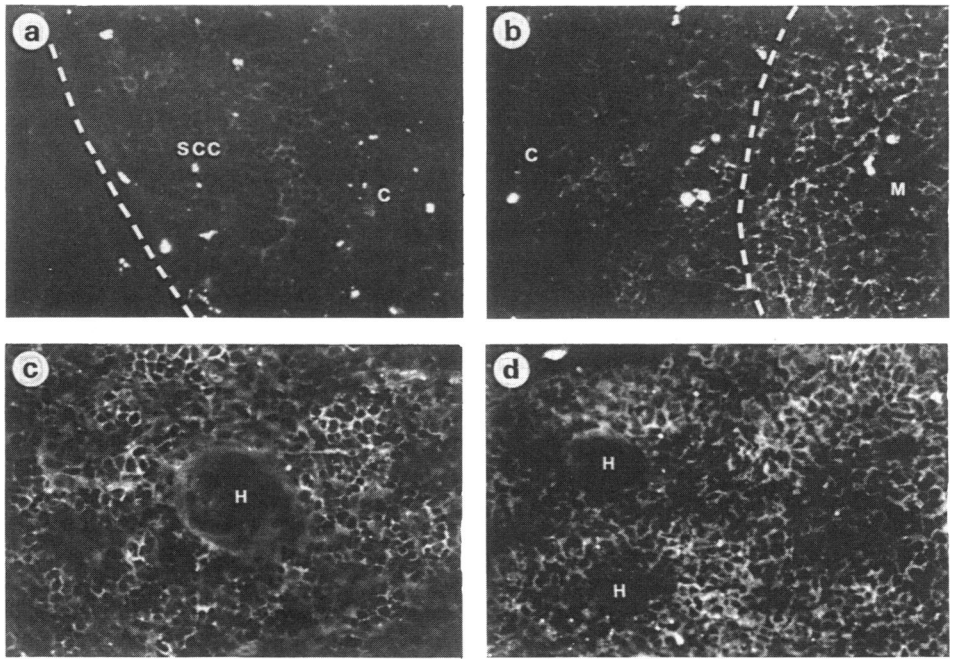

Fig. 1. EA1 expression on post-natal thymus sections. (a) Staining of thymocytes by anti-EA1 MoAb in the cortical regions of postnatal thymus. Scattered cells in the thymus cortex were positive for EA1. These cells tended to localize in the subcapsular cortex (SCC). The dashed line demarcates the capsule of the thymic tissue; (b) cortico-medullary demarcation of thymus. Only scattered faint-staining cells were found in the cortex while clusters of medullary thymocytes expressed EA1; (c and d) medullary section of thymus. Hassall's corpuscles did not express EA1. Clusters of medullary thymocytes were strongly positive. C, cortex; M, medulla; H, Hassall's corpuscles. Magnification ×310.

containing 0·5% Nonidet P-40 (NP-40) and 1 mM phenylmethylsulphonyl fluoride. After centrifugation, supernatants were added to 30 μl of Sepharose 4B, conjugated with goat anti-mouse immunoglobulin (5 mg/ml) that had been incubated with MoAb. The mixture was incubated for 60 min at 4°C with constant mixing, and then washed three times with a buffer containing 10 mM Tris–HCl, pH 7·8 and 0·6 M NaCl. Two additional washings with a buffer containing 10 mM Tris–HCl, pH 8·8, 0·6 M NaCl, 0·1% SDS and 0·05% NP-40 were carried out. The absorbed proteins were released from the Sepharose by boiling for 3 min in a sample buffer containing 0·125 M Tris–HCl, pH 6·8, 2% SDS, 5% 2-ME and 10% glycerol, and were subjected to electrophoresis on 11% polyacrylamide gels in the presence of 0·1% SDS. For non-reducing gels, 2-ME was omitted. Gels were stained, destained and dried. Autoradiography was carried out against X-OMAT AR films at −70°C. For resting T cells and thymocytes without PMA stimulation, 50×10^6 cells were used per sample. For PMA stimulation, 20×10^6 T cells and 50×10^6 thymocytes were used.

Statistical analysis

Student's *t*-test was used for statistical analysis.

RESULTS

Localization of EA1 on thymic tissues

To determine the distribution of EA1$^+$ cells in human thymus, thymic tissues obtained from patients undergoing corrective cardiac surgery were sectioned into 4-μm slices and stained with anti-EA1 MoAb by indirect immunofluorescence. The distribution of EA1 on thymocytes was similar in the five thymuses examined. Representative sections are shown in Fig. 1. Scattered faintly stained cells were seen in the thymic cortex (Fig. 1a). These cells tended to cluster in the subcapsular cortex and scattered throughout the inner cortex. In contrast, most medullary cells appeared strongly positive for EA1. To contrast the distribution of EA1 on cortical and medullary regions of the thymus, Fig. 1b shows the cortico-medullary demarcation which delineates the thymic medulla strongly positive for EA1 from the cortex. Figures 1c and 1d are representative sections of the medulla showing clusters of the EA1$^+$ cells. To confirm that EA1$^+$ cells were indeed thymocytes, double indirect immunofluorescence assays were performed. Using the anti-keratin MoAb AE1, thymic epithelial cells were identified. These epithelial cells did not stain for EA1. Hassall's corpuscles were also negative (Fig. 2). Thymic EA1$^+$ cells were shown to be CD2$^+$ and CD7$^+$ (data not shown). Thus these EA1$^+$ cells were of T cell origin. In the majority of cells, the staining intensities for EA1 were less than that for other antigen such as CD2, LFA-1 and AE1.

EA1 expression in fetal tissues

To determine EA1 expression during ontogeny, fetal thymic tissues from 9·5 weeks to 18 weeks were examined. In addition, the thoracic area of a 7-week-old fetus (no. 46), previously reported to contain CD7$^+$ T cell precursors (Haynes *et al.*, 1988a) was studied (Table 1). At 7 weeks of gestation, CD7$^+$ T cell precursors in the thorax of fetal tissues did not express EA1 (Table 1). Moreover, EA1 was not expressed on CD7$^+$ cells in fetal liver from 10-, 12·75- and 16-week-old fetuses. Thus, CD7$^+$ T cell precursors prior to their entry into the thymic rudiment did not express EA1. At 9·5 weeks (no. 48) scattered

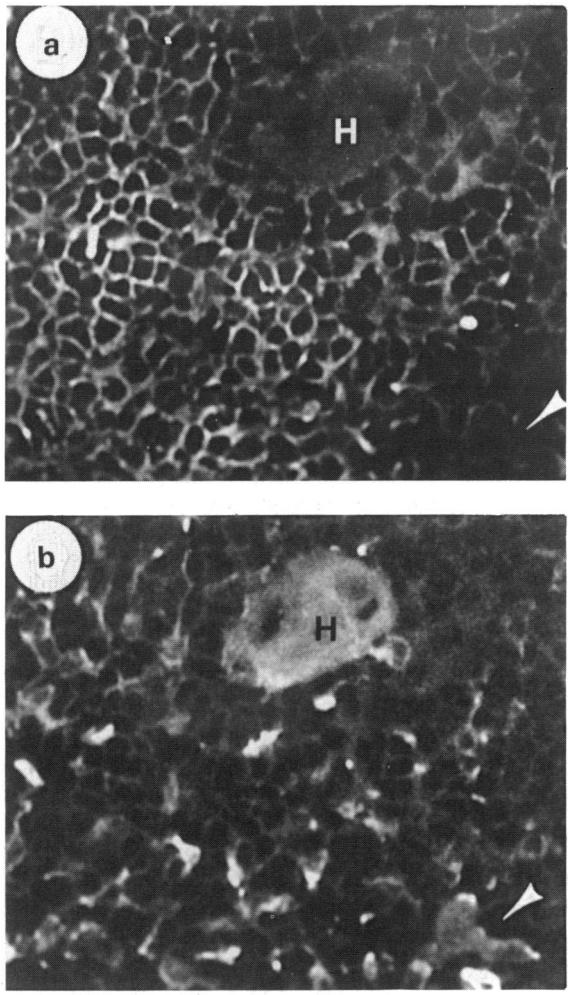

Fig. 2. EA1 expression is not detected on thymic epithelium. A post-natal thymic section was first stained with anti-EA1 MoAb P8 and then with rhodamine-conjugated goat anti-mouse immunoglobulin. After blocking with normal mouse immunoglobulin, the section was then counterstained with FITC-conjugated anti-keratin MoAb AE1. (a) EA1 was detected on thymocytes; (b) keratin was detected on thymic epithelium and on Hassall's corpuscles (H).

EA1$^+$ cells were present, and at 10 weeks EA1$^+$ cells were seen in two thymic tissues studied (Table 1, Fig. 3a). One specimen (no. 22) had a few scattered EA1$^+$ cells, and another (no. 2) had clusters of these cells. Cortico-medullary differentiation begins around 12 weeks of gestation and assumes a post-natal histological appearance by 16 weeks (reviewed by Lobach & Haynes, 1987). By 16–18 weeks, cells strongly positive for EA1 were seen mainly in the medullary area of the fetal thymus (Fig. 3b). However, EA1$^+$ cells were also seen in cortical areas. The patterns of staining were very similar to those of postnatal thymus tissue.

Analysis of EA1 expression on thymocyte subsets

In order to characterize further EA1 expression on post-natal thymus, thymocytes were double stained with MoAb reactive with T cell differentiation antigen (green fluorescence) and anti-EA1 MoAb (red fluorescence). A representative flow fluorocytometric profile is shown in Fig. 4. More than 95% of thymocytes were positive for CD2 and the multiple lineage

Table 1. EA1 expression by thymocytes during fetal development

Specimen no.	Gestational age (weeks)	Source	Markers present*		
			CD45	CD7	EA1
46	7	Thorax	+	+	−
48	9·5	Thymus	+	+	±
2	10	Thymus	+	+	±
22	10	Thymus	+	+	±
40†	12·75	Thymus	+	+	+
5†	15	Thymus	+	+	+
1†	16	Thymus	+	+	+
9†	18	Thymus	+	+	+

* Scores: +, >25% of cells reactive in indirect immunofluorescent assay; −, non-reactive in indirect immunofluorescent assay; ±, <25% of cells reactive in indirect immunofluorescent assay.

† In fetal thymus tissues no. 40, 5, 1 and 9, EA1 reactivity was greatest in central medullary zones.

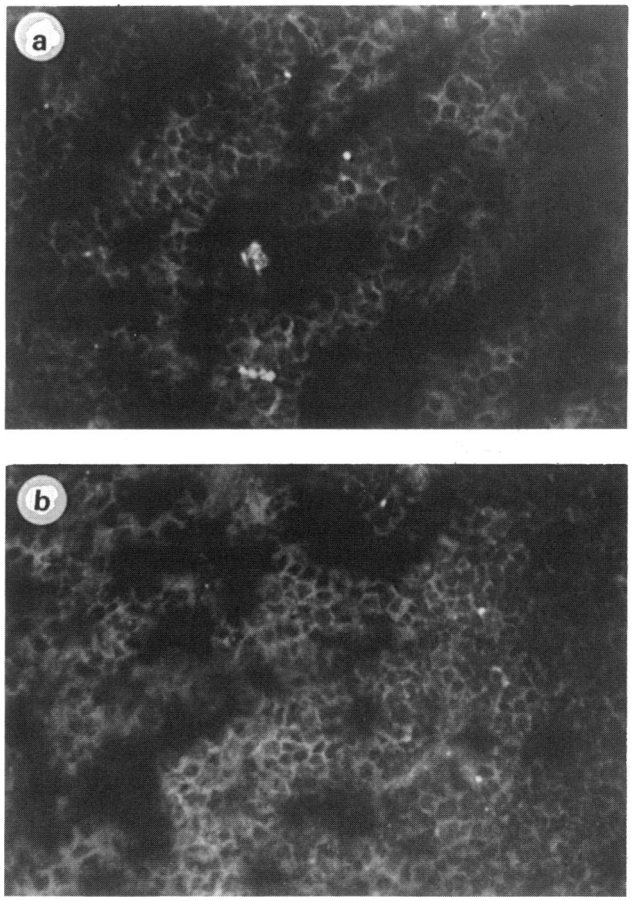

Fig. 3. EA1 expression on fetal thymic tissues. (a) Thymus tissue no. 2, 10 weeks gestation. Cords of thymocytes expressed EA1 antigen; (b) thymic tissue no. 9, 18 weeks gestation. Clusters of EA1$^+$ cells in the medulla area. Magnification × 300.

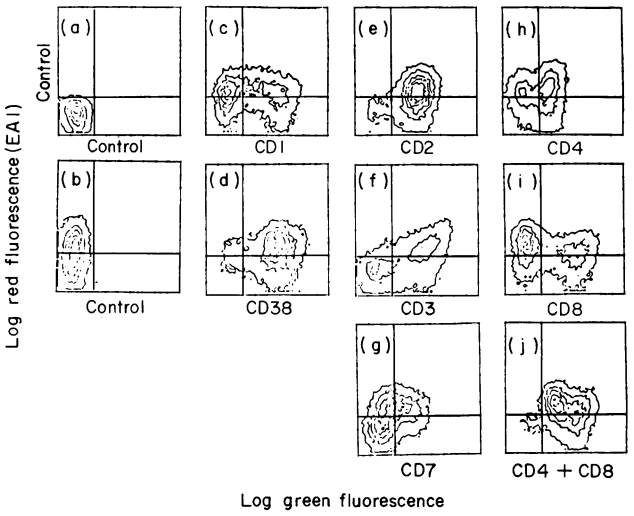

Fig. 4. Two-colour flow fluorocytometric analysis of post-natal thymocytes. Thymocytes were first incubated with control or MoAb of interest and counter-stained with FITC-conjugated goat anti-mouse immunoglobulin. After blocking available sites with mouse immunoglobulin, either control immunoglobulin (a) or biotinylated EA1 was added to the samples, followed by streptavidin-phycoerythrin. (a) Negative control; (b) control immunoglobulin; (c) anti-CD1; (d) anti-CD38; (e) anti-CD2; (f) anti-CD3; (g) anti-CD7; (h) anti-CD4; (i) anti-CD8; (j) anti-CD4 and anti-CD8 were added.

marker CD38 (T10). As reported by Reinherz *et al.*, (1980), 65–70% of thymocytes were positive for CD1. Of the CD1$^+$ thymocytes, 45–50% were EA1$^+$. Although cells strongly positive for CD1 resided predominantly in the cortical region of the thymus (Lobach & Haynes, 1987), the possibility that a subpopulation of medullary thymocytes are weakly positive for CD1 has not been ruled out. Thus, these CD1$^+$ EA1$^+$ thymocytes likely represented the cells seen in the cortex and medulla by immunofluorescence microscopy. In addition, the apparently more numerous CD1$^+$ EA1$^+$ cells revealed by flow cytometry as compared with fluorescent microscopy is likely due to the greater sensitivity of the former method. In order to analyse further the relation between EA1 expression and thymic development, CD4$^+$ and CD8$^+$ thymocytes were studied for their expression of EA1. Of the CD4$^+$ thymocytes, 50–60% were positive for EA1 and 40–45% of the CD8$^+$ cells were positive for EA1. Together, MoAbs to CD4 and CD8 stained over 95% of thymocytes. Thus EA1$^+$ cells were either CD4$^+$ CD8$^-$, CD4$^-$ CD8$^+$ or CD4$^+$ CD8$^+$. In most thymuses examined, the majority of CD3$^+$ or CD7$^+$ thymocytes were positive for EA1. Similar results were obtained from studies of three other thymuses.

Since less than 5% of the thymocytes were CD4$^-$ CD8$^-$ in these analyses, it was not possible to determine whether these double-negative thymocytes were EA1$^+$ without enrichment for this population. Thus, thymocytes were enriched for the CD4$^-$ CD8$^-$ thymocytes by two cycles of negative selection by panning technique (Wysocki & Sato, 1978). After enrichment, a significant number of CD4$^+$ and/or CD8$^+$ thymocytes (30–50%) remained in the preparation. The residual CD4$^+$ or CD8$^+$ thymocytes represent cells with low density of either antigen. This was revealed by flow cytometry. No further attempts were made to deplete these cells. However, these residual CD4$^+$ CD8$^+$ thymocytes could be separated from the CD4$^-$ CD8$^-$ cells by setting appropriate gates in our flow cytometric analysis. A summary of the result of two experiments is shown in Table 2. In the first experiment, 55% and 64·1% of the CD2$^+$ or CD3$^+$ cells were EA1$^+$. Of the cells which expressed low density of CD4 and/or CD8, 62·9% were positive for EA1. Of the thymocytes negative both for CD4 and CD8, 65% were stained positive for EA1. Thus, for the CD4$^-$ CD8$^-$ thymocytes, a similar proportion of the cells subpopulation expressed EA1 as the CD2$^+$, CD3$^+$ or CD4$^+$ CD8$^+$ cells. In contrast, EA1 expression of CD3$^-$ was reduced (39·0% *versus* 64·1%) (Table 2). Although the thymus used in the second experiment had lower percentage of EA1$^+$ cells, similar results were obtained; CD4$^-$ CD8$^-$ thymocytes expressed EA1 proportionally as the other thymocytes, while EA1 expression on CD3$^-$ cells were sharply reduced (Table 2).

Table 2. EA1$^+$ cells in thymocyte populations enriched for CD4$^-$CD8$^-$ cells by negative selection using the panning technique

Gated Populations	EA1$^+$ cells (%)			
	Exp. no. 1		Exp. no. 2	
CD2$^+$	55·0	(90·7)	28·4	(91·5)
CD3$^+$	64·1	(75·4)	28·0	(68·3)
CD3$^-$	39·0	(24·6)	7·6	(31·7)
CD4$^+$ and/or CD8$^+$	62·9	(50·9)	30·1	(35·6)
CD4$^-$CD8$^-$	65·0	(49·1)	27·2	(64·4)

Thymocytes were enriched for CD4$^-$CD8$^-$ cells by panning with MoAb to CD4 and CD8. The resulting preparations were first stained for T cell Ag (green fluorescence) and EA1 expression (red fluorescence), and then analysed by flow cytometry. The doubly stained cells were expressed as a percentage of cells which expressed the T cell differentiation antigen. In parentheses, percentage of thymocytes expressing T cell differentiation antigen in the CD4$^-$CD8$^-$ enriched cell preparation.

EA1 expression on post-natal thymus

It has been shown that normal circulating T cells did not express EA1 but rapidly expressed this antigen on stimulation with PMA (Hara *et al.*, 1986). Shown in Fig. 5 (panels g and h) are cytofluorometric patterns of EA1 expression on peripheral T cells after 2 and 24 h of PMA activation. In the case of thymocytes, 30–65% were EA1$^+$. The staining intensity on these cells was significantly less than that seen on activated T cells (Fig. 5b). This was the case for either freshly isolated thymocytes or cryopreserved thymocytes. To determine whether additional induction of EA1 was possible on these thymocytes, they were activated with PMA (10 ng/ml) and the expression of EA1 was studied. As shown in Fig. 5c and 5d, EA1 expression by thymocytes was enhanced at 2 and 24 h. However, the increased EA1 expression was not as marked as that by PMA-treated peripheral T cells. The progression of EA1 expression was followed for up to 72 h after PMA stimulation with no significant increase as compared with that on 24 h (data not shown).

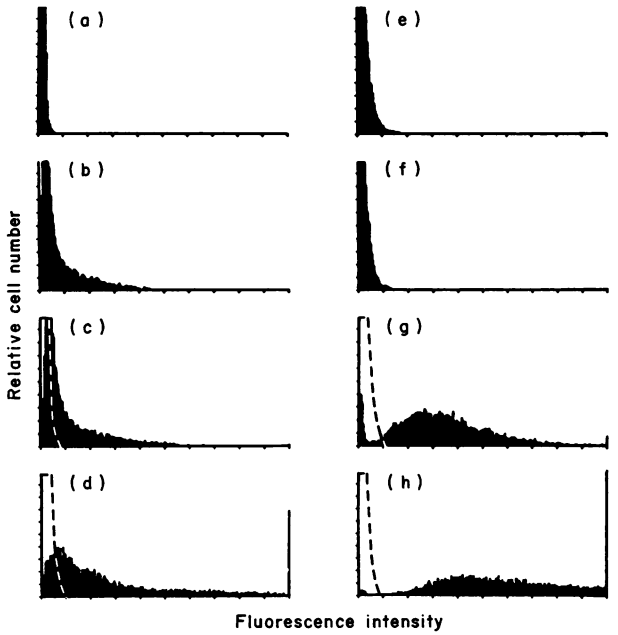

Fig. 5. Flow fluorocytometric analysis of EA1 expression on human postnatal thymocytes (a–d) and peripheral T cells (e–h). Single cell suspensions were incubated in the absence (a, b, e, f) or presence of PMA for 2 h (c and g) or 24 h (d and h). The cells were stained with a control MoAb (a and e; open histograms in c, d, g and h) or anti-EA1 MoAb (solid histograms in b–d, f–h) and analysed on an Ortho 50H Cytofluorograf.

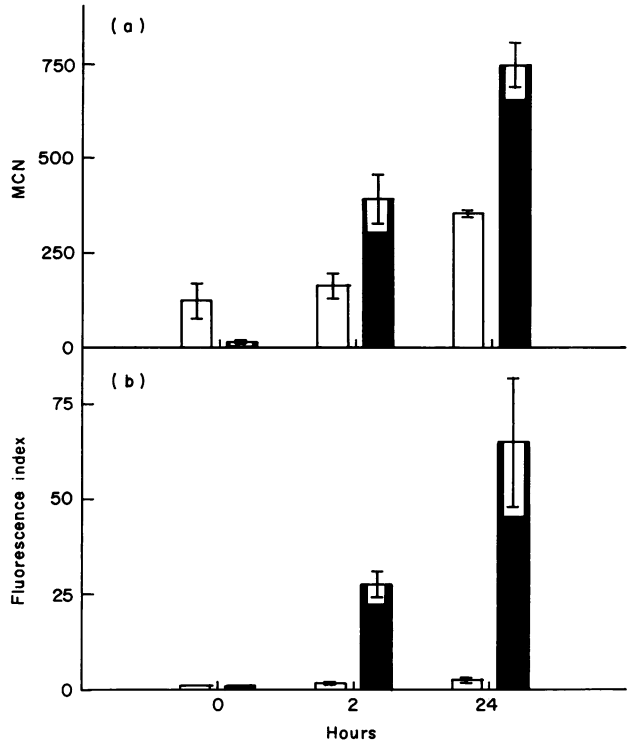

Fig. 6. (a) EA1 expression on thymocytes and T cells. Freshly isolated cells were activated and stained with MoAb to EA1 as described for Fig. 1. The mean channel number of fluorescence (MCN) was obtained using an Ortho system 50 H Data Handler. Mean ± s.e.m. of three specimens; (b) Fluorescence index of thymocytes and T cells at 2 and 24 h after PMA activation.

$$\text{Fluorescence index} = \frac{\text{MCN}_{\text{activated}}}{\text{MCN}_{\text{resting}}}$$

In order to characterize further the difference in EA1 expression in thymocytes and peripheral blood T cells, freshly isolated thymocytes from three specimens were activated for 2 and 24 h with PMA and compared with freshly isolated peripheral T cells. As shown in Fig. 6a, the mean channel number (MCN) of fluorescence of EA1 on thymocytes was significantly higher than that on resting T cells ($P < 0{\cdot}025$). However, 2 h after PMA activation, EA1 expression on T cells was significantly higher than that on the thymocytes ($P < 0{\cdot}05$). The difference was even more pronounced after 24 h ($P < 0{\cdot}01$). To further illustrate the difference in the expression of EA1 on thymocytes and T cells after PMA activation, the fluorescence index (FI) (defined as $\text{MCN}_{\text{ACTIVATED}}/\text{MCN}_{\text{RESTING}}$, with FI of unactivated cells = 1) was calculated. Thymocytes showed little increase in FI ($< 2{\cdot}5$) even at 24 h while FI for peripheral blood T was $27{\cdot}6 \pm 3{\cdot}5$ at 2 h and $65{\cdot}0 \pm 17{\cdot}2$ at 24 h (Fig. 6b). These data indicated that although a thymocyte subset was able to express EA1 in the absence of exogenous stimuli, the ability of EA1 to be induced by PMA on total thymocyte population was significantly reduced.

Molecular characterization of EA1 on thymocytes

To determine whether the EA1 molecule detected on thymocytes was different from that on T cells, thymocytes (50×10^6 cells) or activated T cells (20×10^6 cells) were surface-labelled with ^{125}I. Immunoprecipitation was carried out with anti-EA1 MoAb and the immunoprecipitate was resolved by SDS-PAGE. The EA1 molecule on thymocytes was shown to be a complex of 28/32 kD which was dissociated under reducing conditions (Fig. 7). The electrophoretic mobility of these molecules were identical to those on the activated T cells. In the initial description of EA1, it was thought that the complex was dimeric. With further analysis and better resolving gels, it became apparent that an additional band could be resolved between 32 kD and 28 kD. These bands are products of differential glycosylated polypeptides of a single 24 kD core (Bjorndahl *et al.*, 1988).

EA1 was shown to a phosphoprotein on activated T cells (Hara *et al.*, 1986). To determine whether EA1 was similarly phosphorylated on thymocytes, cryopreserved thymocytes were labelled with ^{32}P and immunoprecipitated with anti-EA1 MoAb. The results are shown in Fig. 8. Lane 2 shows that peripheral T cells did not express EA1. Upon stimulation with PMA, the phosphorylated EA1 was precipitated (Fig. 8, lane 4). In contrast, unstimulated thymocytes were shown to express EA1 and the EA1 molecule was phosphorylated without addition of PMA (Fig. 8, lane 6). Enhanced EA1 phosphorylation on thymocytes by PMA was also demonstrated (Fig. 8, lane 8).

DISCUSSION

In this study, the developmental expression of EA1 (CD69) was studied immunohistochemically. The results indicate that EA1 appears early during T cell ontogeny. Although EA1 is not expressed on 7 week thorax tissue, on fetal liver $CD7^+$ T cell precursor cells at 10, 12·75 and 16 weeks, EA1 is found on some

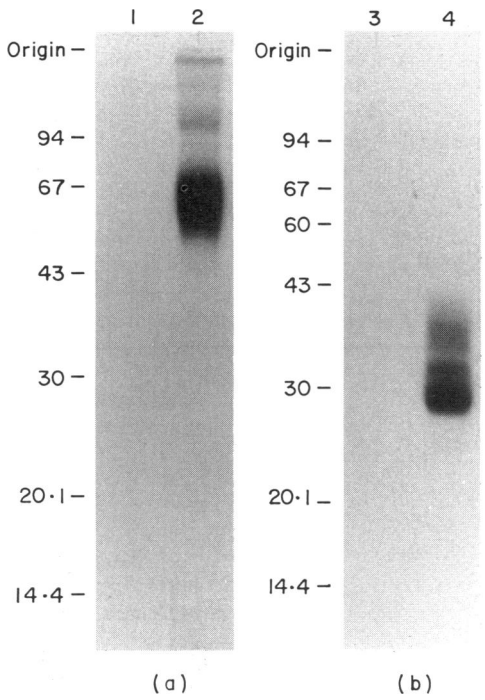

Fig. 7. Immunoprecipitation of ^{125}I-labelled EA1 from thymocytes: 50×10^6 thymocytes were labelled with ^{125}I by lactoperoxidase. The lysate was precleared with Sepharose 4B beads conjugated with goat anti-mouse immunoglobulin antibody. Immunoprecipitation was carried out with the precleared samples. Samples were analysed by SDS–PAGE under non-reducing (a) and reducing (b) conditions. Lanes 1 and 3 were control precipitates with MoAb HPD-1. Lanes 2 and 4 were EA1 precipitated by MoAb P8.

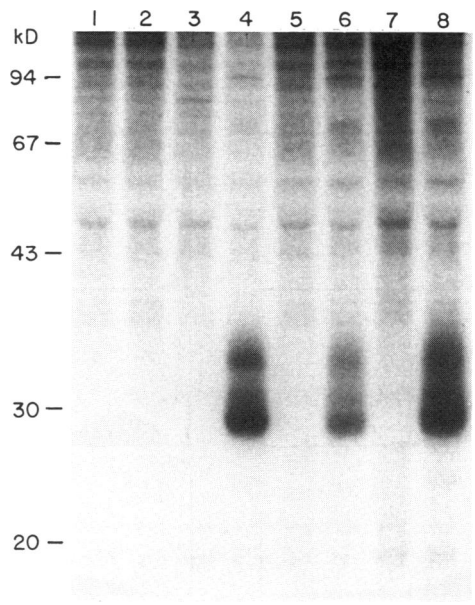

Fig. 8. Immunoprecipitation of ^{32}P-labelled EA1 from thymocytes and T cells. Thymocytes and T cells were incubated for 1 h at 37°C in phosphate-free RPMI 1640 with 10% dialysed FCS and labelled with 1 mCi/ml of ^{32}P-orthophosphate for 4 h at 37° with or without 10 ng/ml of PMA. Then, immunoprecipitation and autoradiography were performed. Resting T cells (5×10^7 cells) did not have ^{32}P-labelled EA1 (lane 2). Phosphorylated EA1 molecules were precipitated from PMA activated T cells (2×10^7 cells, lane 4), resting thymocytes (5×10^7 cells, lane 6) and PMA activated thymocytes (5×10^7 cells, lane 8). Control MoAb precipitation from these cells were shown in lanes 1, 3, 5 and 7, respectively.

thymocytes within the thymic rudiment at 9·5 weeks of gestation. With cortico-medullary differentiation at 14–16 weeks of gestation, EA1 is seen predominantly in the medullary areas, although scattered cells are also seen in the cortical areas. This pattern of EA1 expression is similar to that seen in postnatal thymus. Because EA1 is not expressed on pre-thymic CD7$^+$ T cell precursors in fetal liver or thorax, it appears that EA1 expression on cells of T lineage is acquired intrathymically between 7 and 9·5 weeks. Thus, EA1 expression is among the earliest thymic antigens to appear during ontogeny.

The majority of cortical thymocytes are destined to die within the cortex (reviewed by von Boehmer, 1988). It has been proposed that during the process of differentiation, precursor CD4$^-$ CD8$^-$ T cells in the cortex proliferate and only a small percentage of their progeny survive. These cells developed into the cortical CD4$^+$ CD8$^+$ common thymocytes which further differentiate into CD4$^+$ CD8$^-$ or CD4$^-$ CD8$^+$ 'stage III' medullary thymocytes (Reinherz *et al.*, 1980; Blue *et al.*, 1987). By two-colour analysis on a flow fluorocytometer, it is clear that EA1$^+$ cells are not restricted to any of these thymocyte subsets. Our data indicate that EA1$^+$ cells are found in CD4$^+$ CD8$^+$, CD4$^+$ CD8$^-$ and CD4$^-$ CD8$^+$ cell subsets since combination of both anti-CD4 and anti-CD8 MoAbs identified virtually all the EA1$^+$ thymocytes (Fig. 4). By negative selection, it is also demonstrated here that EA1 is also expressed in a substantial number of subsets of the CD4$^-$ CD8$^-$ thymocytes.

Of special interest in our study is the finding that EA1$^+$ cells are found in the thymocytes expressing relatively high density of CD3, while CD3$^-$ thymocytes have low or no expression of this antigen. Blue *et al.*, (1987) have recently identified a subset of CD4$^+$ CD8$^+$ thymocytes which expressed high levels of CD3. This population was postulated to be the immediate precursors for the 'stage III' medullary thymocytes. Collectively, these data would suggest that EA1 is expressed on cortical thymocytes which have the ability to mature into medullary thymocytes. However, since EA1 is expressed on peripheral blood T cells only upon activation, the possibility that EA1 expression on different thymocyte subsets is an indication of thymocyte activation should also be considered.

Detailed analysis of EA1 has demonstrated that it is a disulphide-linked homodimer consisting of differentially glycosylated core protein subunits (Bjorndahl *et al.*, 1988). Its molecular structure on thymocytes is similar to that expressed on activated T cells. However, unlike the mature T and B cells, a subset of thymocytes expresses EA1 without exogenous stimulation. Additionally, unlike mature T cells, thymocytes are relatively unresponsive to PMA in the expression of EA1 (Figs 5 and 6). PMA activates mature T cells to express EA1 through the protein kinase C pathway (Bjorndahl *et al.*, 1988; Jung *et al.*, 1988), although additional signals, such as calcium influx, also appear to play an important role (Bjorndahl *et al.*, 1988). Thus, it is possible that the inability of a majority of thymocytes to express EA1 in response to PMA may relate to the immaturity

of certain biochemical pathways. Relevant to this discussion is the demonstration of heterogeneous functional competency of T cell receptors in human thymocytes (Weiss *et al.*, 1987). However a subset of thymocytes did respond to PMA and strongly expressed EA1. Whether this represents the relatively mature $CD3^+$, $CD4^+$ or $CD8^+$ medullary thymocytes remains to be determined.

The relative unresponsiveness of thymocytes to PMA regarding EA1 expression is not unique. CD7, a *pan*-T cell antigen normally rapidly downregulated from mature T cell surface by PMA, is resistant to the effect of PMA on thymocytes (Jung & Fu, 1988; Jung *et al.*, 1988). IL-2 receptor expression on the majority of thymocytes is also not induced by PMA, although additional agents such as IL-2, concanavalin A (Con A) or phytohaemagglutinin (PHA) provided the additional signals necessary for IL-2 receptor expression (de Vries *et al.*, 1983; Reem & Yeh, 1985).

Testi *et al.* (1988) have recently reported their study on the expression of Leu 23 antigen on human thymocytes. Because we have demonstrated that Leu 23 is identical to EA1 (Nakamura *et al.*, 1989), their findings are relevant to the present study. Their finding of constitutive expression of thymocytes confirms our original observation that substantial numbers of thymocytes were stained with MoAb to EA1 (Hara *et al.*, 1986). Our finding of phosphorylation of EA1 on resting thymocytes agrees with their results. Although EA1 expression was not markedly induced by PMA, phosphorylation of EA1 was markedly enhanced. This indicates that PMA has considerable effect on thymocytes. Whether the responsiveness is restricted to a subset of thymocytes remains to be clarified. Their finding that approximately 10% of the CD4, CD8 double-positive cells were positive for CD69 agrees with our finding that the majority of the cortical thymocytes are negative. There are minor differences between the findings of Testi *et al.* (1988) and ours: higher percentages of thymocytes were found to be positive in our study; in addition, a substantially higher percentage of $CD4^-$ $CD8^-$ cells was also shown to express EA1. These variations may be due to technical reasons and/or due to inherent differences between thymuses. Despite these differences, the findings of early EA1 expression during ontogeny and its constitutive expression and phosphorylation in post-natal thymocytes, add strong evidence to the hypothesis that this antigen plays a certain important role in T cell ontogeny.

The functional significance of EA1 expression on lymphocytes is not yet known. A recent finding that another MoAb to EA1, G38, as well as MoAb Leu 23 synergize with PMA to activate T cells (Nakamura *et al.*, 1989), suggests a role for EA1 in T cell activation and proliferation. Recently, MoAb MLR3 (Cosulich *et al.*, 1987) was shown to be capable of stimulating thymocytes to proliferate in the presence of a mitogenic dose of PMA (Delia *et al.*, 1988). Although definitive experiments need to be carried out to show that MLR3 and EA1 are identical, the evidence accumulated thus far favour this suggestion. Thus, this observation adds further support that EA1 and related molecules are important in thymocyte activation. Several lymphokines and cytokines have been implicated in the proliferation and differentiation of thymocytes. This includes tumour necrosis factor-alpha, IL-1, IL-2, IL-4 and IL-6 (Palacios, Sideras & von Boehmer, 1987; Spits *et al.*, 1987; Zlotnick *et al.*, 1987; Hodgkin *et al.*, 1988; Ranges *et al.*, 1988). Whether these factors interact with EA1 on thymocytes and T cells needs to be studied further.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health Grants CA34546, AI-25704, CA-29548, CA-28936 and a grant from the Eleanor Naylor Dana Charitable Trust. We are grateful to Craig Wasson, Kay Medina and Bobbi Crummett for their skillful technical assistance in these studies, and to Myra Soden for her assistance in the preparation of the manuscript.

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