

AN INTERMEDIATE CELL IN  
THYMOCYTE DIFFERENTIATION THAT  
EXPRESSES CD8 BUT NOT CD4 ANTIGEN

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In the thymus four lymphoid subsets are identified by CD4 and CD8 antigen expression (1, 2).  $CD4^-CD8^-$  cells are precursors for all other thymocyte populations (3, 4), which include  $CD4^+CD8^-$  and  $CD4^-CD8^+$  medullary subsets that are similar to mature T lymphocytes and contain immunocompetent cells (5–7). Most thymocytes are found in the cortex and are  $CD4^+CD8^+$ . However, these cells have no known function and most of them appear destined for intrathymic death (2, 6, 7).

Rat thymic cells can be further subdivided by expression of the MRC OX-44 antigen ( $M_r$  43,000), which is found on all mature T cells, macrophages, and other leucocytes, but only on 10–15% of thymocytes (8). The  $OX-44^+$  cells include all those with thymopoietic activity or the ability to respond to alloantigens or lectins. Most of the  $OX-44^-$  cells are  $CD4^+CD8^+$  cortical cells, but there are also  $OX-44^-$  subpopulations in the  $CD4^+CD8^-$  and  $CD4^-CD8^+$  sets. In the present study we have established that cells with the  $CD4^-CD8^+OX-44^-$  phenotype constitute an early stage in thymopoiesis, whilst  $CD4^+CD8^-OX-44^-$  cells do not have these properties.

**Materials and Methods**

All antibodies and methods are described in references 8 and 9.

**Results**

*Identification of a Transient Cortical Thymocyte Population.* Cell populations were studied during thymic regeneration in animals given 3 Gy whole-body gamma irradiation. This dose kills cortical but not medullary thymocytes and most dead cells are phagocytosed by macrophages within 48 h of irradiation (10 and Paterson, D., unpublished results). Small numbers of dividing cells were detected in the cortex by bromodeoxyuridine (BUdR) labeling on day 2 after irradiation (not shown), and dividing cortical cells were abundant on days 3 and 4 (Fig. 1, *b* and *c*). For comparison, BUdR-labeled cells in a normal thymus are shown in Fig. 1 *a*. Most cortical cells appearing at day 3 and day 3.5 lacked CD4 antigen expression (Fig. 1 *e*) but were positive for CD8 antigen expression (Fig. 1 *f*). Also, many of the cortical cells were  $OX-44^-$  (Fig. 1 *g*), although this was less clear than for CD4 labeling.  $OX-44^+$  thymocytes include  $CD4^-CD8^-$  cells

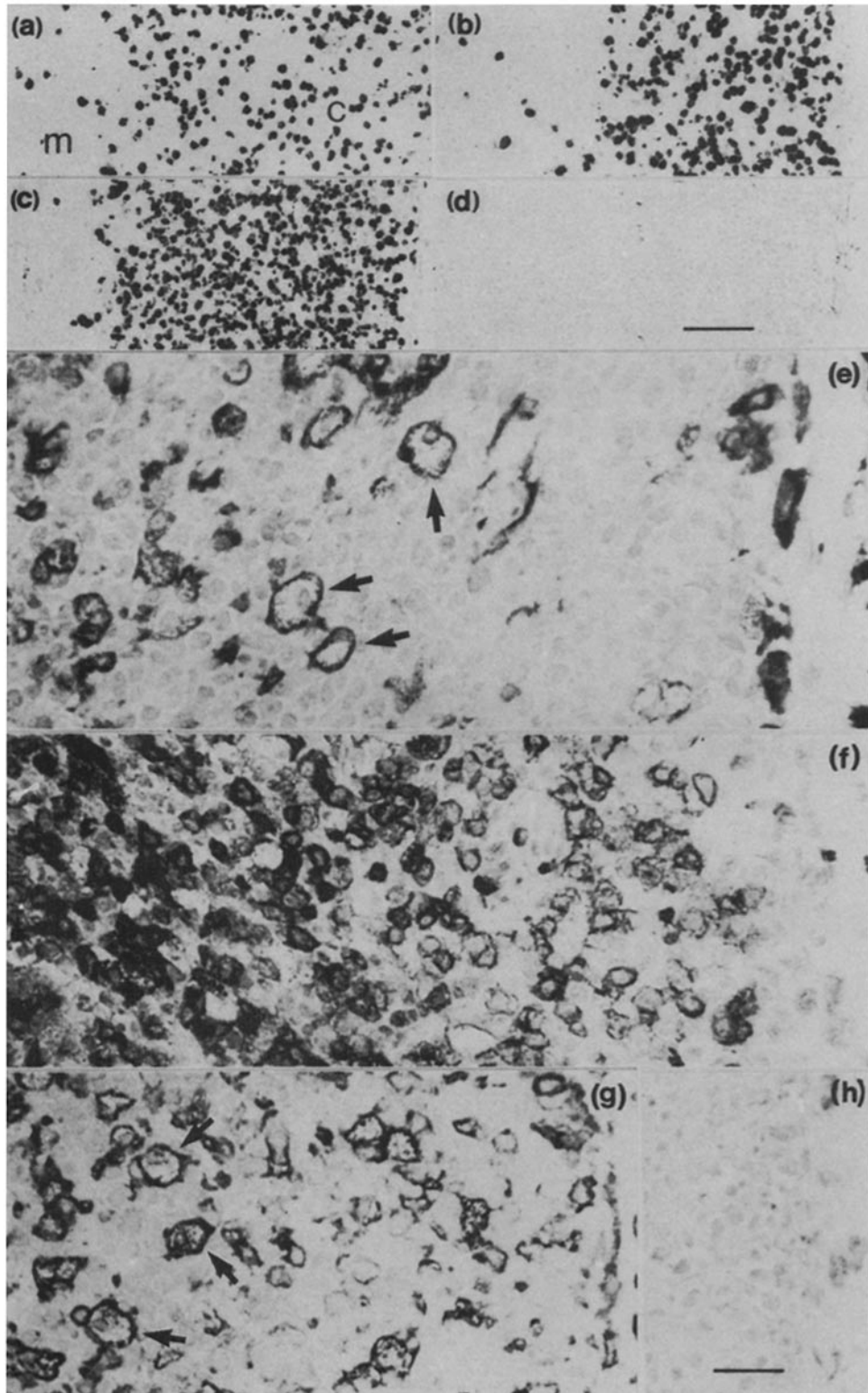


TABLE I  
Proportion of Dividing Cells in Thymocyte Subsets

Thymocyte subset	Percent dividing cells for experiment:							
	1	2	3	4	5	6	7	8
Unfractionated (100%)	7.2	6.5	9.3	6.7	6.4	6.5	5.9	6.1
CD4 <sup>+</sup> CD8 <sup>+</sup> OX-44 <sup>-</sup> (2%)	31.0	—	50.8	32.5	47.7	45.7	—	—
CD4 <sup>+</sup> CD8 <sup>-</sup> OX-44 <sup>-</sup> (2%)	1.1	—	—	1.9	—	—	—	—
CD4 <sup>-</sup> CD8 <sup>-</sup> OX-44 <sup>+</sup> (1-2%)	—	7.0	—	—	—	—	3.7	—
OX-44 <sup>-</sup> (88%)	—	—	—	—	—	—	—	4.6
OX-44 <sup>+</sup> (12%)	—	—	—	—	—	—	—	2.2

Animals were injected intravenously with BUdR at 10 mg/kg body weight 1 h before they were killed. Thymocyte subpopulations were prepared by rosette depletion at 4°C (Exps. 1-7) or by cell sorting (Exp. 8), and were smeared and labeled with an anti-BUdR antibody (Bu-20a) using the immunoperoxidase technique. BUdR<sup>+</sup> cells were estimated by scoring 1,000 cells from each of three slides and taking the mean count. Depleted populations were 93-98% pure, except for Exp. 1 where populations were 84% pure. Numbers in parentheses indicate the percent of total thymocytes that the subsets represent (reference 8). Exps. 5, 7, and 8 used PVG rats, the rest used AO rats.

as well as mature phenotype cells and the CD4<sup>-</sup>,CD8<sup>-</sup> cells are probably located in the cortex. By day 4 the pattern had changed to most cortical cells showing the normal CD4<sup>+</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> cortical thymocyte phenotype, although quantitatively CD4 expression did not attain normal levels until day 5 after irradiation (data not shown). Thus a cell type probably of CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> phenotype had matured to the CD4<sup>+</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> type in cortical regeneration, since it seems unlikely that cell migration could account for the cortical changes between days 3.5 and 4.

*Cell Division within Subsets from Normal Thymus.* CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> cells constitute only ~2% of normal thymus, but if these cells are an intermediate population then a high proportion of cycling cells may be found within this set. This was analyzed by BUdR labeling of thymocytes in vivo (see Table I). The CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> set contained 31-51% BUdR<sup>+</sup> cells. In sharp contrast was the finding that only ~2% of CD4<sup>+</sup>,CD8<sup>-</sup>,OX-44<sup>-</sup> cells were BUdR<sup>+</sup>, while 4-7% of CD4<sup>-</sup>,CD8<sup>-</sup>,OX-44<sup>+</sup> cells were positive, which is similar to the value for unfractionated thymocytes (Table I). This suggests that most CD4<sup>-</sup>,CD8<sup>-</sup>,OX-44<sup>+</sup> cells are in the resting phase.

Although CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> cells show a high percent of BUdR<sup>+</sup> cells, this set accounts for only ~1% of the 7% of labeled cells in the unfractionated population. It seems likely that most BUdR<sup>+</sup> cells are CD4<sup>+</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> since most labeled cells are located in the cortex (Fig. 1a), and when OX-44<sup>-</sup> cells were isolated, the percent of BUdR<sup>+</sup> cells was similar to that for unfractionated cells (Table I and reference 8).

FIGURE 1. mAb labeling of regenerating thymus. AO rats were given 3 Gy irradiation and then killed at timed intervals. Cryostat sections (5 μm) were stained using the immunoperoxidase technique. (a-d) Animals were injected intravenously with 10 mg/kg body weight BUdR 1 h before they were killed. (a) Normal thymus labeled with Bu20a (anti-BUdR mAb): (m) medulla, (c) cortex. (b and c) Day 3 and 4 after irradiation; Bu20a mAb. (d) Day 4; OX-21 (control mAb). (e-h) High magnification views of the cortex, where sections from day 3.5 after irradiation are labeled with (e) CD4, (f) CD8, (g) OX-44, and (h) OX-21 mAbs. Arrows indicate macrophages stained with CD4 or OX-44 mAbs. This assignment is made because rat macrophages are CD4<sup>+</sup>,OX-44<sup>+</sup> and use of macrophage-specific mAbs on other sections identified labeled cells with the morphology of those arrowed above. (a-d) Bar represents 83 μm; (e-h) 28 μm.

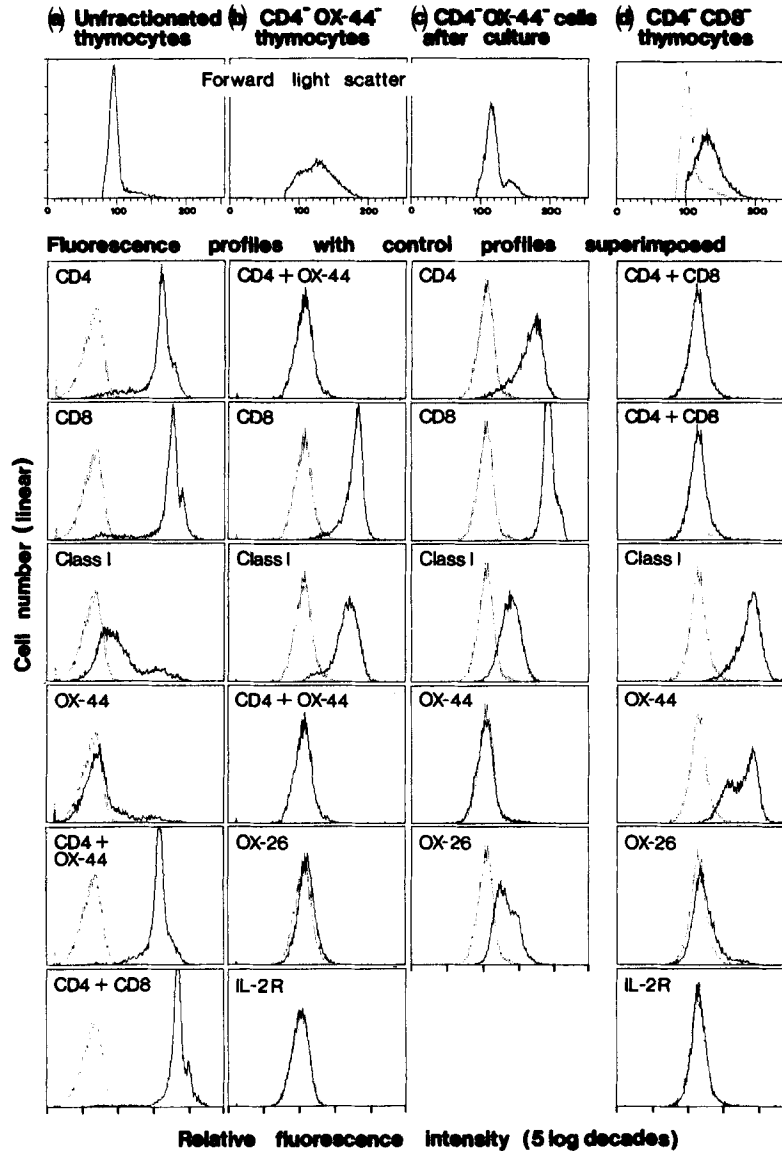


FIGURE 2. Analysis of AO thymocyte subsets on the FACS. (b and d) Populations were prepared by rosette depletion. (a-c) one experiment where the phenotype of  $CD4^+, OX-44^-$  thymocytes before (b) and after 18 h in culture (c) is compared with unfractionated thymocytes (a). Control profiles: OX-21 (a);  $CD4^+, OX-44^-$  (b); OX-21 (c);  $CD4^+ CD8^-$  (d) are superimposed. (a-c) Cells were analyzed on the same scatter and fluorescence gains.

*FACS Analysis of  $CD4^+, OX-44^-$  and  $CD4^+, CD8^-$  Cells.* Forward light scatter analysis shows that  $CD4^+, OX-44^-$  thymocytes are a heterogeneous population of mostly medium and large cells (Fig. 2b). These cells all express CD8 antigen at a level similar to that of unfractionated thymocytes (Fig. 2, a and b), and they also show clear expression of MHC class I antigen. They do not express any

detectable IL-2-R, and little, if any, transferrin receptor (OX-26 labeling) (Fig. 2*b*).

CD4<sup>-</sup>,CD8<sup>-</sup> thymocytes are also a heterogeneous population of mainly medium and large cells (Fig. 2*d*). These cells all clearly express both MHC class I and OX-44 antigens but lack IL-2-R and transferrin receptor expression. Labeling of IL-2-R was with the OX-39 mAb (9) and the negative result was confirmed with a second noncompetitive anti-IL-2-R mAb provided by Dr. M. Dallman.

*In Vitro Differentiation of CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> Thymocytes.* CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> thymocytes prepared by rosette depletion were cultured at  $3-5 \times 10^6$  cells/ml in RPMI/10% FCS for 18 h in the absence of accessory cells. A 7-13% increase in cell number (37% in one case) occurred during the culture period, with recovered cells being 60-88% viable. Viable cells were purified using an Isopaque Ficoll gradient and were then labeled for FACS analysis. Cultured cells were mostly medium sized. Virtually all expressed the CD4 antigen, with most cells as bright as unfractionated thymocytes (Fig. 2, *a* and *c*). This was clearly seen in six experiments. Cultured cells remained CD8<sup>+</sup>,OX-44<sup>-</sup>, showed an increase in transferrin receptor expression (OX-26), and showed a decrease in MHC class I expression (Fig. 2, compare *c* and *b*). IL-2-R expression of these cells has not been studied. Hence, upon in vitro culture, CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> thymocytes express a CD4<sup>+</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> phenotype; this parallels the phenotypic change seen during thymus regeneration after irradiation.

### Discussion

The CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> thymocyte subset has been clearly shown to give rise to cells of CD4<sup>+</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> cortical phenotype in vitro, and the same changes appeared to occur in vivo in thymic regeneration. It seems possible that CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> cells are derived directly from CD4<sup>-</sup>,CD8<sup>-</sup>,OX-44<sup>+</sup> cells. This lineage would be in accord with data on thymic ontogeny in mouse and sheep where CD8 antigen expression preceded that of CD4 antigen (11, 12). Also in the mouse, the CD4<sup>-</sup>,CD8<sup>+</sup> cells can be split into two sets by expression of the J11d antigen (13). All immunocompetent cells are J11d<sup>-</sup> and the CD4<sup>-</sup>,CD8<sup>+</sup>,J11d<sup>+</sup> set may be the mouse equivalent of rat CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> cells.

An outstanding problem is now to determine the origin of the CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>+</sup> and CD4<sup>+</sup>,CD8<sup>-</sup>,OX-44<sup>+</sup> cells that are immunocompetent and found in the medulla. We had previously suggested (8) that loss of the OX-44 antigen may mark the entry of cells into the pathway that results in cell death, but this would not be in accord with an important intermediate role for CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> cells. A key question is whether the medullary cells are rescued from a dead-end pathway by positive selection from CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> or CD4<sup>+</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> cells, and it may be possible to resolve this by determining the progeny of cells after injection into thymuses cultured in vitro (3). Determination of whether TCR chains are expressed in the CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> cells will also be essential.

One interesting additional point resulting from this study is the finding that most CD4<sup>-</sup>,CD8<sup>-</sup> cells lack detectable IL-2-R expression. In the mouse, ~50% of CD4<sup>-</sup>,CD8<sup>-</sup> cells express IL-2-R, but the absence of IL-2-R from the equiva-

lent rat cells adds to the doubt about the functional role of these receptors in early thymic differentiation (14).

### Summary

Thymocytes of CD4<sup>-</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> phenotype have been shown to be an intermediate of thymopoiesis that give rise to cells of CD4<sup>+</sup>,CD8<sup>+</sup>,OX-44<sup>-</sup> normal cortical thymocyte phenotype both in vitro and in vivo during thymic regeneration.

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