# THE MRC OX-44 ANTIGEN MARKS A FUNCTIONALLY RELEVANT SUBSET AMONG RAT THYMOCYTES

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The immunocompetent T lymphocytes that are found in the peripheral lymphoid tissue can be subdivided into three sets on the basis of cell surface marker antigens. The first set to be identified includes the precursors for cytotoxic T cells that are specific for, or restricted by, class I MHC antigens and these are marked by expression of the CD8 antigen (1–3). Complementing this set are the rest of the peripheral T cells that express the CD4 antigen and include T helper cells and precursors for cytotoxic cells whose specificity involves the class II MHC antigens (3–6). The CD4$^+$ cells can be further subdivided into one set that includes cells providing help for B cells and another that contains the cells that initiate the MLR and provide help for CTL (7).

In the thymus 10–15% of the cells have phenotypes apparently similar to peripheral T cells in that they express either CD4 or CD8 antigens (but not both) and contain cells that can function as precursors to cytotoxic T cells (CD4$^-$ CD8$^+$) or T helper cells (CD4$^+$ CD8$^-$) (8–11). These cells are found largely in the medulla, whereas the great majority of the remaining cells express both CD4 and CD8 antigens and are found largely in the cortex (12, 13). Functional activity from the CD4$^+$ CD8$^+$ cells has not been identified in studies in the mouse (9), and these cells have the additional unusual feature of expressing very low levels of class I MHC antigens (14, 15). The CD4$^-$ CD8$^-$ set constitutes only 1–2% of thymocytes but these cells can reconstitute all other populations either in vivo when injected intravenously into irradiated recipients (16), or in vitro when added to fetal thymic lobes in culture (17).

The lineage relationships between the various populations of cells in the thymus are largely unknown and new markers are needed if these are to be resolved. We now describe an antibody called MRC OX-44 that labels all leukocytes in the periphery but only 10–15% in the thymus. Functional studies lead to the working hypothesis that the OX-44$^+$ cells include all thymocytes that are on the pathway to immunocompetent T lymphocytes.

## Materials and Methods

*Animals.* Strains used were HO (RT1$^c$, leukocyte-common antigen [L-CA][1] 1.2), HO.B2 (RT1$^u$), DA (RT1$^a$), and (HO × HO.B2) F$_1$. All were from the Specific Pathogen-free Unit at the Medical Research Council Cellular Immunology Unit, Oxford. HO

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[1] *Abbreviations used in this paper:* L-CA, leukocyte-common antigen; RAM, rabbit anti–mouse F(ab')$_2$; TDL, thoracic duct lymphocyte.

(RT1$^c$, L-CA1.1) rats were maintained under conventional conditions. Chimeric rats were prepared by irradiating HO.B2 rats (9–12 wk) with 950 rad of γ-irradiation (from a $^{137}$Cs source at 87 rad/min) followed by reconstitution with 5–10 × 10$^7$ bone marrow cells i.v. per rat from the F$_1$ hybrid 5–6 h later.

*Antibodies.* A hybridoma secreting the MRC OX-44 mAb (IgG$_1$) was derived from a fusion between BALB/C mouse spleen cells and the mouse myeloma NSO/1 following methods as in Galfre and Milstein (18). The mouse had been immunized with T lymphoblasts from an MLR between purified rat T helper cells and irradiated semiallogeneic rat spleen, as described in Jefferies et al. (19). Rabbit anti–mouse F(ab')$_2$ (RAM) and its fluorescein isothiocyanate (RAM-FITC) and horseradish peroxidase (RAM-peroxidase) conjugates were prepared as previously described (20, 21). Other mAbs used were MRC OX-1, anti-L-CA common determinant (22); MRC OX-8, anti-CD8 (2); MRC OX-18, anti-class I MHC common determinant (15); MRC OX-19, anti-rat CD-5 (Lyt-1) (23); MRC OX-27, anti-class I MHC RT1$^c$ haplotype (19); MRC OX-35 and MRC OX-38, noncompeting anti-CD4 mAbs (19); MRC OX-42, anti-rat C3b receptor (24). ED2, anti-rat tissue macrophages (25), was the kind gift of Dr. C. D. Dijkstra, Free University, Amsterdam, The Netherlands; NDS 58, anti-RT-7.1 (anti-L-CA1.2) (26), was the kind gift of Dr. M. R. Newton, Department of Zoology, University of Oxford; 8G6.1, anti-RT-7.2 (anti-L-CA1.1) alloantigen (27) was the kind gift of Dr. D. L. Greiner, University of Connecticut Health Center, Farmington CT; Bu20a, reactive with bromodeoxyuridine$^2$ was the kind gift of Dr. J.-P. Magaud, Department of Haematology, John Radcliffe Hospital, Oxford. MRC OX-21, anti-human C3b INA, was used as a control mAb.

*Cells.* Thoracic duct lymphocytes (TDL) were collected overnight into ice-cold Dulbecco's A + B medium (DAB) containing 20 U/ml heparin. Thymus, spleen, and cervical lymph nodes were removed aseptically, and either teased apart with forceps or pushed through a square metal mesh of 0.09-mm$^2$ pore size, filtered through lens tissue, and washed twice in DAB/0.2% BSA.

Bone marrow cells were removed from femur and tibia into ice-cold DAB/0.2% BSA by syringing. Cells were filtered, washed twice, and injected in a volume of 1 ml of DAB/0.2% BSA for the preparation of radiation bone marrow chimeras.

For intrathymic injections, thymocytes were prepared in Hepes/RPMI 1640 with 2.5% FCS.

*Immunoprecipitation.* HO TDL were $^{125}$I surface-labeled using the lactoperoxidase/glucose oxidase method (28) and a 1% NP-40 detergent extract was prepared. Material that could bind to OX-44 antibody was precipitated and visualized on 12% SDS-PAGE gels by autoradiography using methods exactly as described by Jefferies et al. (29).

*In Vitro Proliferation Assays.* For the primary semiallogeneic MLR, varying numbers of responder HO thymocytes were cultured with 5 × 10$^5$ irradiated (HO × HO.B2)F$_1$ spleen cells (1,800 rad) for 4 d in RPMI 1640 containing 2.5 × 10$^{-5}$ M 2-mercaptoethanol, antibiotics, and 5% DA serum.

For the Con A stimulation assay, HO thymocytes were incubated for 3 d with 10 μg/ml$^{-1}$ Con A, 5 × 10$^5$ HO–irradiated spleen cells (1,800 rad), and 25 μl of a Con A supernatant (produced by incubating 5 × 10$^6$ HO spleen cells/ml for 24 h with 5 μg/ml$^{-1}$ Con A in the above medium with FCS in place of DA serum).

Culture volumes were 0.2 ml in sterile 96-well U-bottomed microtiter plates at 37°C in a humidified incubator with 5% CO$_2$. 0.5 μCi of [$^3$H]thymidine (Amersham International, Amersham, United Kingdom) was added 18 h before cells were collected onto glass-fiber filters for determination of radioactivity incorporated.

*Staining of Thymus In Situ.* Thymi were removed from HO (4–8 wks) or chimeric rats, dissected, immediately embedded in OCT (R. A. Lamb, London), and frozen in 2-methylbutane cooled by liquid nitrogen. Cryostat sections (5 μm) were cut, dried in a stream of warm air, and fixed in ethanol for 10 min at 4°C. Sections were stained by the immunoperoxidase technique (21), and counterstained with Harris's hematoxylin.

For staining sections with the Bu20a mAb the method of Magaud et al.$^2$ was used.

Briefly, sections were fixed in acetone for 10 min at 4°C, air dried, incubated in 95% formamide for 35 min at 67°C, washed in PBS, and then stained as usual.

*Rosette Depletion.* This was carried out as described by Mason (30). Briefly, thymocytes were labeled with mAb(s), washed, and mixed with SRBC coated with RAM IgG for 20 min at 4°C. Rosettes formed between labeled thymocytes and SRBC-RAM were removed, and the remaining cells were treated with ammonium chloride in Tris buffer to lyse erythrocytes and then washed. The success of depletion was assessed by labeling pre- and postdepletion samples with RAM-FITC, and analyzing on the FACS.

*Thymus Repopulation Assay.* The assay of Goldschneider et al. (27) was used. HO L-CA1.2 rats (5–7 wk old) were given 600 rad and injected intrathymically with HO L-CA1.1 thymocytes 6–8 h later. Donor cells, present in 20 μl of PBS, were injected into four to six sites per thymus at ~3–5 μl per site. Donor reconstitution was assessed by analyzing host thymi on the FACS 14 d later.

## Results

*Expression and Nature of the OX-44 Antigen.* The OX-44 mAb resulted from an immunization of mice with rat T blasts, in which mAbs were first detected by binding to T blasts and then screened with the FACS on other lymphoid cell population to detect any interesting labeling patterns. Fig. 1a shows that all T blasts were labeled by the antibody; this was also the case for lymph node cells although two modes of labeling intensity were seen (Fig. 1b). The more weakly labeled cells were T lymphocytes as shown by the fact that the low intensity peak was shifted to denser labeling when OX-19 mAb, which labels T cells but not most B cells, was added with OX-44 antibody (Fig. 1e). A large fraction of bone marrow cells were labeled (Fig. 1c) and these include the lymphoid and neutrophil populations. The antibody did not label erythrocytes (data not shown) and the negative cells in the bone marrow may be of the erythroid lineage. The labeling of thymocytes was of most interest (Fig. 1d) because in this case only 12% of cells showed detectable labeling. These included the larger cells among the thymocytes as assessed by their scatter profiles in the FACS (Fig. 1f–h). In 22 different labeling experiments of HO rats aged 6–8 wk the mean labeling with OX-44 mAb was 12.2% ± SD 1.9%.

In other studies (data not shown) it was found that OX-44 labeled all nucleated peritoneal cells and dendritic cells obtained from lymph draining the gut. The results of all the labeling studies indicate that OX-44 may label all cells of the lymphoid and myeloid lineages with the exception of the majority of thymocytes.

Preliminary experiments with immunoperoxidase labeling on tissue sections from liver, kidney, and brain show some OX-44 staining. Part of this seemed to be due to a nonspecific nuclear crossreaction, whereas the rest appeared to correspond to the presence of leukocytes in these tissues (data not shown).

Immunoprecipitation studies identified the OX-44 antigen as a broad band of about 43,000 mol wt in the unreduced or reduced state (Fig. 1i). The diffuse nature of the band obtained after SDS-PAGE was not due to technical problems because other antigens run on the same gels gave much sharper bands (not shown). The heterogeneity could be due to diversity in carbohydrate attached to the OX-44 antigen.

*Thymocytes Labeled by the OX-44 mAb.* The relationship of the OX-44⁺ and OX-44⁻ thymocytes to subsets defined by other mAbs was studied by FACS analysis on unfractionated thymocytes or cells obtained after rosette depletion of thymocytes labeled with various mAbs. Fig. 2, *a* and *b*, shows labeling of
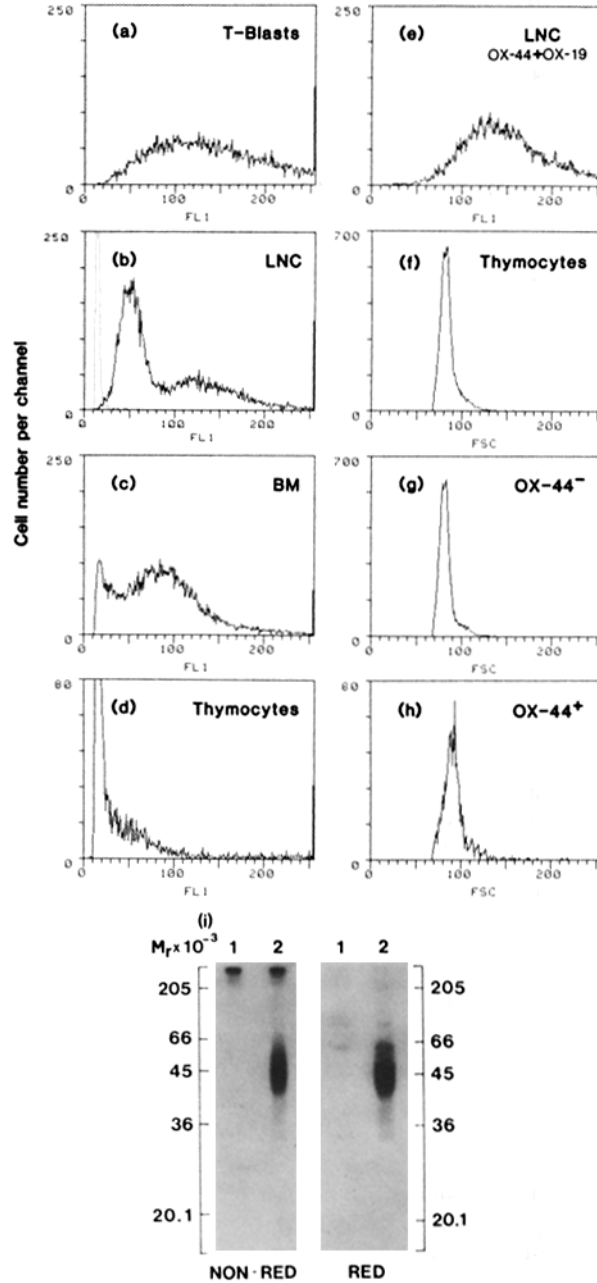
FIGURE 1.   Distribution and nature of the OX-44 antigen. (a–h) Cells were incubated with mAb(s) for 60 min at 4°C, washed, labeled with RAM-FITC, and analyzed on the FACS II (B-D FACS Systems, Becton Dickinson & Co., Sunnyvale, CA). Profiles (a–d) show MRC OX-44 labeling of T lymphoblasts, lymph node cells (LNC) (with negative control), bone marrow (BM), and thymocytes. (e) Labeling of LNC when OX-44 and OX-19 mAbs were used together in the first step. (f and g) Forward light scatter (FSC) profiles of whole, MRC OX-44⁻, and MRC OX-44⁺ thymocytes. FLI, fluorescence. (i) Immunoprecipitation of the MRC OX-44 antigen: material precipitated from $^{125}$I surface-labeled TDL lysate was run reduced (RED) and nonreduced (NON-RED) on 12% SDS-PAGE and visualized by autoradiography. Lane 1, control mAb; lane 2, MRC OX-44.

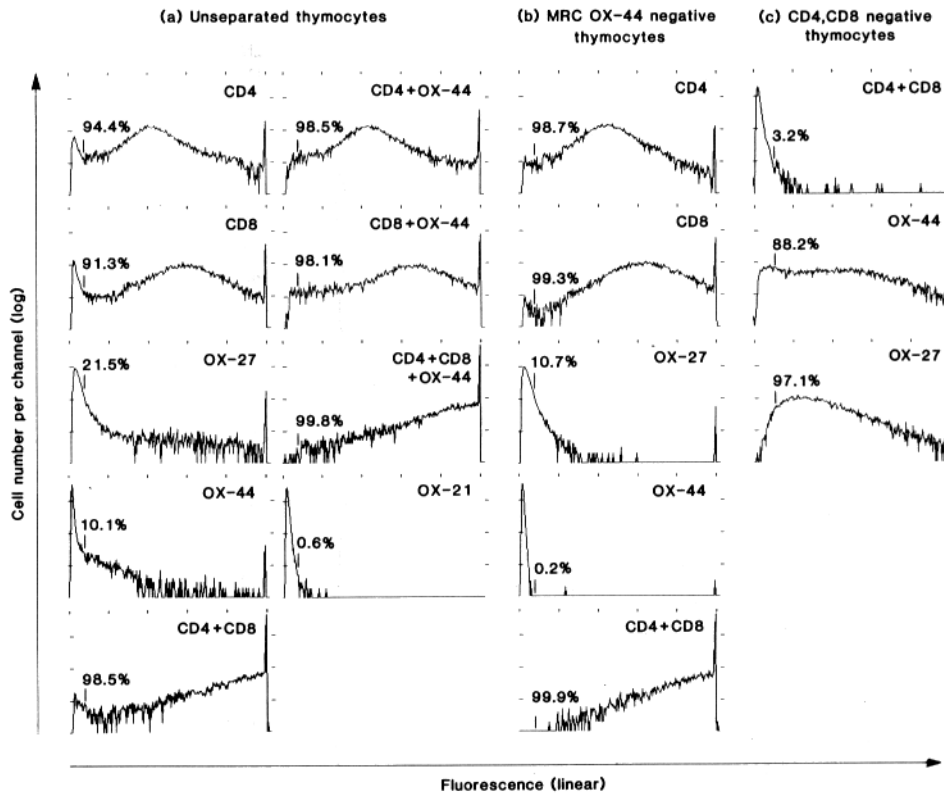(a) Unseparated thymocytes          (b) MRC OX-44 negative          (c) CD4,CD8 negative
                                        thymocytes                     thymocytes

FIGURE 2.   Analysis of thymocyte subpopulations. FACS analysis of (a) unseparated, (b) MRC
OX-44⁻, and (c) CD4⁻ CD8⁻ thymocytes was carried out using single or multiple mAb labeling
in the first step. Thymocyte populations in panels b and c were prepared by rosette depletion.
Figures indicate the percentage of positive cells to the right of the marker. Thymocyte subsets
can be calculated from such analyses, as shown in Table I.

undepleted thymocytes and of OX-44⁻ cells with mAbs against rat CD4, CD8, class I, and MRC OX-44 antigen either alone or in combination with each other whereas Fig. 2c shows labeling of CD4⁻ CD8⁻ cells.

The results for unfractionated thymocytes (Fig. 2a showed that a majority of OX-44⁺ cells were negative for either CD4 or CD8 antigen, or both, inasmuch as when cells were incubated with OX-44 plus antibodies against CD4 or CD8, then the small negative peaks seen with labeling of mAbs against CD4 or CD8 alone were substantially reduced. The CD4⁻, CD8⁻ cells were almost all OX-44⁺ as shown by labeling with combinations of antibodies (Fig. 2, a and b) and (Fig. 2c). In contrast, not all cells singly positive for CD4 or CD8 were OX-44⁺ because small negative peaks remained in labeling of thymocytes with anti-CD4 or CD8 plus OX-44 (Fig. 2a) and in labeling of OX-44⁻ cells with the anti-CD4 or CD8 antibodies (Fig. 2b). The significance of these small peaks is proven by the finding that OX-44⁻ cells were 99.9% labeled by anti-CD4 and CD8 mixed together (Fig. 2b). Most CD4⁺ CD8⁺ cells were OX-44⁻, but calculations of subsets from labeling data as in Fig. 2a always led to the conclusion that a small set of CD4⁺ CD8⁺ OX-44⁺ cells was present. The average percentages from seven experiments for subsets defined by CD4, CD8, and OX-44 mAbs are given in Table I.

TABLE I

*Thymocyte Subsets Calculated from FACS Analysis*

| Subset phenotype | Percent of total population* |
|---|---|
| CD4⁻ CD8⁻ OX-44⁺ | 1.3 ± 0.2 |
| CD4⁺ CD8⁺ OX-44⁺ | 2.2 ± 1.3 |
| CD4⁺ CD8⁺ OX-44⁻ | 85.3 ± 1.3 |
| CD4⁺ CD8⁻ OX-44⁺ | 4.8 ± 0.7 |
| CD4⁺ CD8⁻ OX-44⁻ | 2.0 ± 0.6 |
| CD4⁻ CD8⁺ OX-44⁺ | 2.2 ± 0.7 |
| CD4⁻ CD8⁺ OX-44⁻ | 1.9 ± 0.6 |

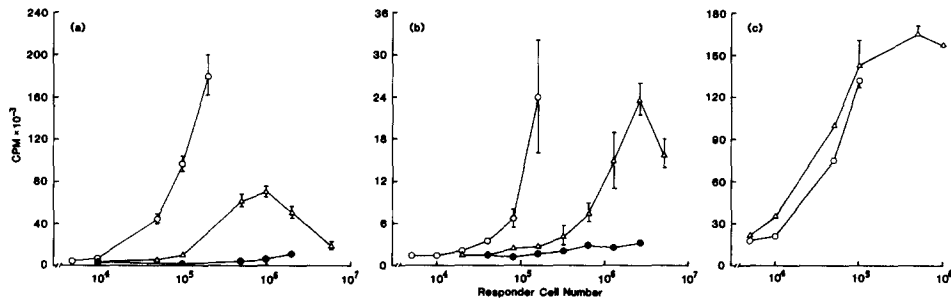* Mean ± SD from seven experiments such as the one shown in Fig. 2.



FIGURE 3.   In vitro proliferative responses of thymocyte populations. The proliferative responses of FACS-purified (O) MRC OX-44⁺ and (●) MRC OX-44⁻ thymocytes are shown in the (a) Con A response and (b) primary MLR. MRC OX-44⁻ thymocytes were 99.8% pure, whereas the MRC OX-44⁺ thymocytes were 94% pure. Cells labeled with MRC OX-44 and RAM-FITC but not run through the FACS were used as the unfractionated thymocyte control (Δ). (c) The Con A response of (O) CD4⁻ CD8⁻ and (Δ) unfractionated thymocytes. CD4⁻ CD8⁻ thymocytes were prepared by rosette depletion followed by cell sorting, and were 99.8% pure. Points are means ± 1 SD using four or six replica wells.

Evidence for all these sets can be directly seen from the labeling profiles in Fig. 2 except for the CD4⁺ CD8⁺ OX-44⁺ set.

mAbs against class I MHC antigens are known to label a subfraction of thymocytes (15) and the OX-44⁺ set was found to overlap with class I⁺ cells. Fig. 2a shows labeling of thymocytes with OX-27 (anti-class I) mAb, and comparison of this with Fig. 2b shows that removal of OX-44⁺ cells resulted in the loss of cells that were heavily labeled with OX-27 mAb. The CD4⁻ CD8⁻ cells were all class I⁺ (Fig. 2c), as were all cells singly positive for CD4 or CD8 (not shown). The class I⁻ fractions are all found in the CD4⁺ CD8⁺ set but a minority of CD4⁺ CD8⁺ cells are weakly positive for class I.

*Proliferation of Thymocyte Subsets In Vitro.*   The Con A stimulation assay (Fig. 3a) and MLR (Fig. 3b) were used to assess the proliferative capacity in vitro of various thymocyte subsets isolated using the FACS or by rosette depletion. In neither assay did OX-44⁻ cells show any proliferation while OX-44⁺ cells were potent responders. The enrichment of activity shown by OX-44⁺ cells in comparison with unseparated thymocytes was consistent with the total activity of unseparated cells being due to the OX-44⁺ cells. It should be noted that OX-44⁻ cells failed to respond despite the fact that, in the Con A stimulation assay, optimal conditions were used including the presence of irradiated syngeneic spleen cells as a source of dendritic cells and T helper cells, as well as a supernatant
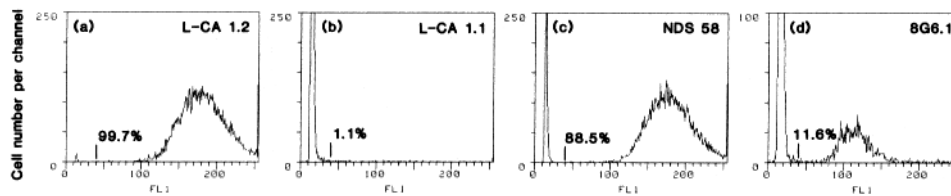
FIGURE 4. Labeling of L-CA allotypes by monoclonal antibodies. The mAb NDS 58 labels
(a) all HO L-CA1.2 thymocytes but (b) no HO L-CA1.1 thymocytes. Thymocytes from an HO
L-CA1.2 rat partially reconstituted with HO L-CA1.1 thymocytes using the assay of Gold-
schneider et al. (27) are labeled with (c) NDS 58 and (d) 8G6.1.

from Con A–activated T cells that contains T cell growth factors. When the
assay was performed using Con A alone, the same results were obtained but the
proliferation was reduced (data not shown).

Because OX-27 (class I) recognizes a subset of thymocytes containing all the
OX-44$^+$ cells, similar experiments to those above were carried out with FACS
purified OX-27$^+$ and OX-27$^-$ cells. The results showed that all cells capable of
proliferation were within the OX-27$^+$ population. The OX-27$^-$ cells gave no
response (data not shown).

The responsiveness of CD4$^-$ CD8$^-$ thymocytes was also tested in the in vitro
assays. In the Con A assay this cell population did show a response (Fig. 3c), but
the level was not enriched in comparison with unseparated cells. The CD4$^-$
CD8$^-$ cells are virtually all OX-44$^+$, but this subfraction of the OX-44$^+$ cells
would appear to contribute little to the enriched response of the total OX-44$^+$
population. The cells most active in proliferation are to be found in the CD4$^+$
CD8$^-$ and/or CD4$^-$ CD8$^+$ fraction of the OX-44$^+$ cells.

*Thymic Regeneration Assay.*    The intrathymic regeneration assay as described
by Goldschneider et al. (27) was used to assay for thymopoietic potency of various
thymocyte subsets. In this assay, rats bred by S. V. Hunt to be congenic for the
RT-7 alloantigen locus, which encodes the L-CA, were used and the donor cells
were distinguished from host by labeling with mAbs against the RT-7 allotypes.
Fig. 4,a and b, shows that the NDS58 mAb labels all thymocytes from the HO
(L-CA1.2) strain whereas the thymocytes from the congenic partner strain HO
(L-CA1.1) are completely negative. The 8G6.1 mAb has the opposite specificity
(not shown). Fig. 4, c and d, shows the labeling of thymocytes from a HO (L-
CA1.2) strain animal injected intrathymically with HO (L-CA1.1) strain thymo-
cytes, and chimerism at the level of 11.3% is clearly demonstrated.

The thymopoietic potency of OX-44$^-$ cells and CD4$^-$ CD8$^-$ cells isolated by
rosette depletion was tested (FACS separation of cells could not be used because
the cell number requiring separation was too great). In experiments 3, 4, and 5
in Table II, the potency of the OX-44$^-$ fraction was ~5–10% of that given by
unfractionated cells whereas in experiments 6 and 7 no thymopoietic activity at
all was shown by the OX-44$^-$ cells. The small amount of activity in experiments
3–5 could be due to residual OX-44$^+$ cells because in experiments 3 and 4
depletions of 74% and 67% efficiency were obtained whereas in experiments 6
and 7 the depletions were 100% effective (100% efficient means postdepletion
labeling was $\leq$ negative control on FACS analysis, insufficient data to estimate
efficiency in experiment 5).

Considerable enrichment of thymopoietic activity was seen in the CD4$^-$ CD8$^-$

TABLE II

*Repopulation of Irradiation-depleted Thymuses by Donor Cells*

| Donor thymo-cyte popula-tion* | Percent donor thymocytes[‡][§] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Exp.: 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Unfractionated cells | | | | | | | |
| 5 × 10^7 | — | — | 27.8, 15.8 | — | — | — | — |
| 2.5 × 10^7 | 20.2, 9.3, 6.3 | — | — | — | 10.1, 0.6 | — | — |
| 2 × 10^7 | — | — | — | 12.3, 12.0 | — | 21.3, 17.7 | 21.2, 15.3 |
| 1 × 10^7 | 18.2, 12.7, 8.4 | 7.6, 5.8 | — | — | — | — | — |
| 5 × 10^6 | 5.0, 4.1 | — | — | — | — | — | 6.4, 4.8 |
| 2.5 × 10^6 | 3.5, 3.1, 0.7 | — | — | — | 2.5, 2.1 | — | — |
| 2 × 10^6 | — | 1.0, 0.3 | 2.5, 2.1 | 5.3, 0 | — | 6.5, 4.9 | — |
| 2 × 10^5 | — | — | — | 0, 0 | — | — | — |
| 1 × 10^5 | — | 0, 0 | 0, 0 | — | — | — | — |
| OX-44^- cells | | | | | | | |
| 5 × 10^7 | — | — | 4.8, 2.2, 1.1 | — | — | — | — |
| 2.5 × 10^7 | — | — | — | — | 5.2, 3.2, 0 | — | — |
| 2 × 10^7 | — | — | — | 2.9, 1.2, 1.1 | — | 0, 0 | 0.1, 0.1, 0 |
| 2.5 × 10^6 | — | — | — | — | 0, 0, 0 | — | — |
| CD4^- CD8^- cells | | | | | | | |
| 2 × 10^6 | — | 17.5, 3.7, 0.4 | 36.8, 23.8 | 10.6, 6.7 | — | — | 30.2, 17.2 |
| 5 × 10^5 | — | — | — | — | — | — | 6.7, 5.2 |
| 2 × 10^5 | — | — | — | 1.1, 0.2 | — | — | — |
| 1 × 10^5 | — | 2.9, 1.1 | 8.4, 1.8, 1.0 | — | — | — | — |

The assay of Goldschneider et al. (27) was used to assess the ability of different thymocyte subsets to repopulate a thymus in vivo. HO congenic strains were used. After 600 rad of irradiation, L-CA1.2 recipients were injected intrathymically with LCA1.1 donor cells, killed 2 wk later, and the percentage of donor thymocytes assessed by FACS analysis. Table summarizes data from all experiments performed.

* Donor populations prepared by rosette depletion, see text for details of purities.
‡ Percent donor cells calculated by subtracting NDS 58 labelling from OX-1 labelling (OX-1 always gave 99.7–99.9% labelling).
§ Each figure represents one animal.

cells (almost all OX-44$^+$, Fig. 2c). This result is in accord with data in the mouse (16) and, in addition, showed that the poor activity of the OX-44$^-$ cells could not be explained by artifacts in the isolation of cells by rosette depletion. The CD4$^-$ CD8$^-$ cells were 10–20 times more potent than the unfractionated thymocytes compared with a theoretical expected enrichment of 70-fold if all the activity were in this subset. The low experimental value may be due to some loss of functional viability in the rosetted cells compared with unfractionated cells, or to the possibility that the thymopoietic activity is due to a subset within the CD4$^-$ CD8$^-$ cells that is recovered with lower efficiency than the bulk CD4$^-$ CD8$^-$ population. A third possibility is that some cells in the OX-44$^+$ set other than the CD4$^-$ CD8$^-$ OX-44$^+$ cells may have thymopoietic activity. It is not likely that the CD4$^-$ CD8$^-$ set contained many cells of lineage other than T lymphoid inasmuch as almost all cells were weakly OX-19$^+$ (rat CD5 or Ly-1) (23) and mAbs recognizing class II MHC, surface Ig, or C3b receptor only gave ~5% labeling (data not shown).

*Localization of the OX-44$^+$ Cells.* This was studied in tissue sections using the immunoperoxidase technique with the results shown in Fig. 5. Unlabeled cells could not be seen in the medulla where positive cells with both lymphoid and dendritic morphology were seen (Fig. 5a). In the cortex most lymphoid cells were OX-44$^-$. This is consistent with the fact that most CD4$^+$ CD8$^+$ cells are OX-44$^-$ and that these cells are known to exist in the cortex, whereas the medulla mostly contains the cells positive for CD4 or CD8 but not both (data for CD8 labeling is shown in Fig. 5c). Small numbers of OX-44$^+$ cells are scattered throughout the cortex: some have irregular morphology, shown by the star;

others are small round cells as shown by arrows in Fig. 5b. All peripheral leukocytes appear to be OX-44$^+$ and thus the sections were stained with various mAbs against macrophages to see if these might account for all the OX-44$^+$ cortical cells. The OX-42 mAb, which labels many rat macrophages and may be the equivalent of mouse Mac-1, labeled few cells in the thymus sections (Fig. 5d) but the ED-2 mAb stained considerable numbers of cortical (but not medullary) macrophages (Fig. 5e) in accord with the data of Dijkstra et al. (25). However, ED-2$^+$ cells did not appear to account for the small rounded cortical cells.

The OX-44$^+$ cells include those that express large amounts of class I MHC antigens, and the localization of class I$^+$ cells was studied in chimeric animals. Class I on leukocytes cannot be visualized in the normal thymus owing to staining of epithelial cells (Fig. 5f), and thus expression of leukocyte class I was studied in HO.B2 (RT1$^u$) animals reconstituted with HO.B2 (RT1$^u$) × HO (RT1$^c$) bone marrow cells. The donor-derived class I could then be detected with the OX-27 mAb which is specific for RT1$^c$ while all class I was detected with the OX-18 mAb which recognizes a common determinant. The results for OX-27 labeling in sections from the chimeric thymus (Fig. 5g) were very similar to those seen with OX-44 labeling in the chimeric (Fig. 5h) or normal thymuses (Fig. 5a). This experiment additionally established that most of the class I in the thymic cortex is derived from epithelial cells in that staining of the chimeric thymuses with the class I-common OX-18 antibody gave the extensive pattern of staining as shown for normal thymus in Fig. 5f (OX-18 data not shown).

Extensive cell division is known to occur in the thymic cortex. This was confirmed in the present series of experiments by injecting animals intravenously with 5-bromo-2' deoxyuridine (10 mg/kg body wt), killing them after 30 min, and detecting cells that had incorporated the metabolite with the mAb Bu20a. A thymus section from such an animal stained with Bu20a is shown in Fig. 5i: there is considerable labeling of cells throughout the cortex, with the highest density in the outer cortical layer, whereas few medullary cells were labeled. Analysis of cells isolated from such a thymus showed that 8.1% of unfractionated thymocytes were Bu20a$^+$, compared with 7.8% for OX-44$^-$ thymocytes. This suggests that most dividing cells in the cortex are CD4$^+$ CD8$^+$, which agrees with the data of Scollay et al. (31) in the mouse. The OX-44$^-$ cells cannot be stimulated to further division with Con A plus growth factors; thus the DNA synthesis in this set seems likely to be occurring in the final divisions of a cell series undergoing terminal differentiation. The situation may be similar to that seen in the avian erythroid series where a set of cell divisions occur along with production of hemoglobin and other erythroid components to produce a nucleated cell that does not divide again (32).

## Discussion

The OX-44 mAb labels a small fraction of thymocytes that mediates functions that are characteristic of the most undifferentiated thymocytes as well as the thymic cells that are most like mature T cells. An anti-rat mAb with similar labeling and molecular properties was described by Matsuura et al. (33) but minimal functional data were reported. An antigen similar to OX-44 has not been described in other species.

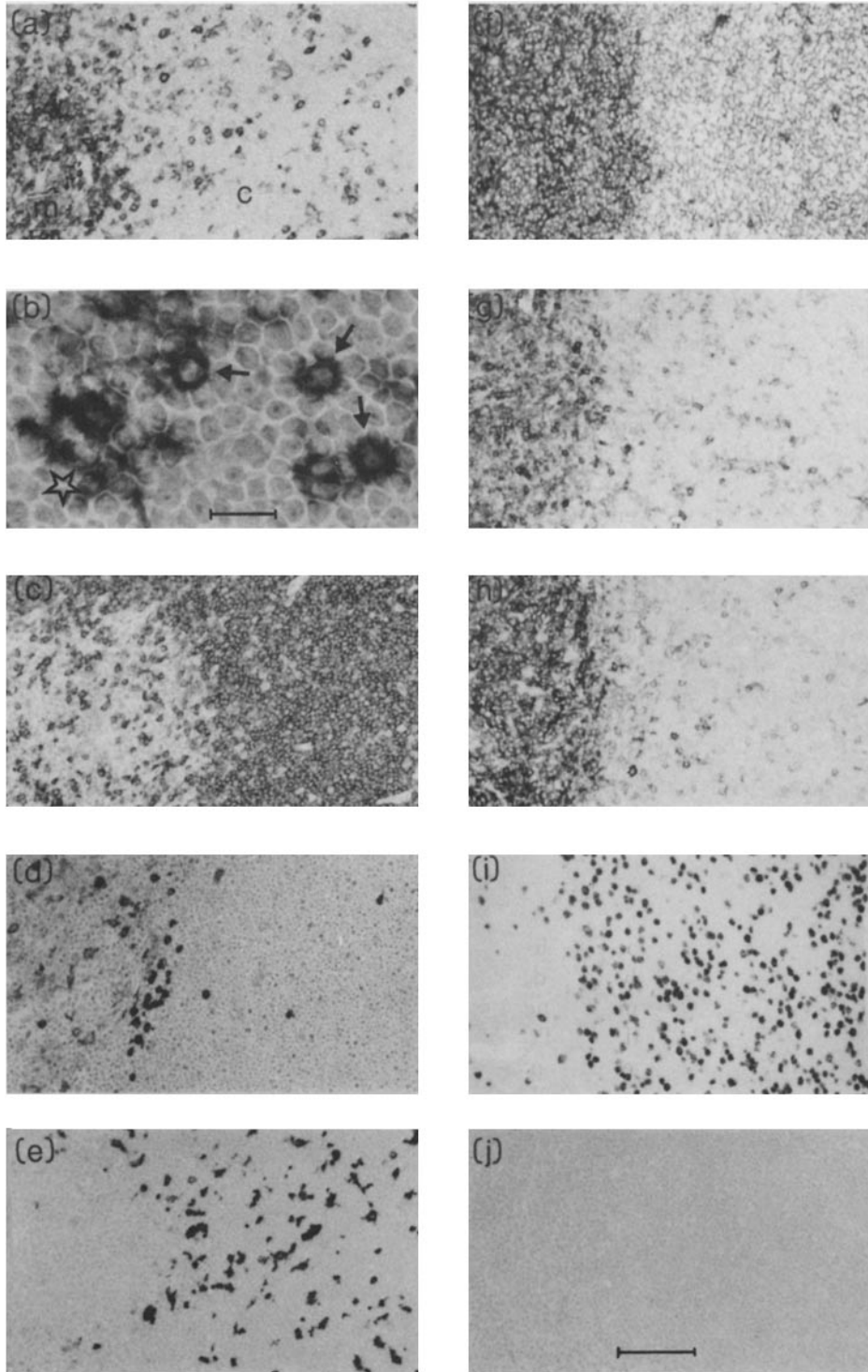From the current studies we derive the working hypothesis that the OX-44$^+$

FIGURE 5.   Monoclonal antibody labeling of thymus. HO thymus cryostat sections (5 μm) were stained with various mAbs using the immunoperoxidase technique. (a) MRC OX-44. m,

cells may include all the cells that are on the differentiation pathway to immunocompetent T cells while the OX-44⁻ compartment represents cells that have failed some test in differentiation and are fated to die in situ. The following factors lead to this view: (a) The OX-44⁻ cells were negative for proliferation in the MLR or by Con A stimulation and had no thymopoietic activity. Furthermore, it seems unlikely that other functions, for example, for T help or cytotoxic activity, would be mediated by OX-44⁻ cells because the cells that mediate these functions would be expected to divide vigorously with Con A stimulation plus accessory cells and growth factors. Rat thymic T helpers are known to have the CD4⁺ CD8⁻ phenotype (8). (b) The OX-44 antigen is a leukocyte-common antigen apart from its absence from most thymocytes. Other antigens are known to be expressed intermittently in a cell lineage (e.g., rat W3/13 antigen is on stem cells and plasma cells but not on pre-B or resting B cells), but in these cases the antigen does not label all peripheral leukocytes as is the case for OX-44. (c) The OX-44 labeling appears to be a more extreme case of a similar pattern shown by class I MHC antigen, which is also heavily expressed on all leukocytes except for the CD4⁺ CD8⁺ thymocytes which are weak or negative. It could be that in thymic differentiation cells that are fated to die first switch off OX-44 expression and then class I.

Regardless of this working hypothesis, the OX-44 antigen allows the definition of new thymic subsets for further study. CD4⁻ CD8⁻ OX-44⁺ cells constitute the much-studied "double negative" cells, whereas the CD4⁺ CD8⁻ OX-44⁺ and CD4⁻ CD8⁺ OX-44⁺ cells correspond closely to mature phenotype T cells. In contrast, the CD4⁺ CD8⁺ OX-44⁺, CD4⁺ CD8⁻ OX-44⁻, CD4⁻ CD8⁺ OX-44⁻ sets have not been previously described and are potentially intermediate cells in the thymic differentiation pathways. In accord with this view is the finding that CD4⁻ CD8⁺ OX-44⁻ cells are a prominent population in thymuses regenerating after irradiation (manuscript in preparation). Further studies on these subsets may help in delineation of pathways for thymic differentiation.

## Summary

A monoclonal antibody called MRC OX-44 is described that labels all myeloid cells and peripheral lymphoid cells but only 12% of thymocytes. The OX-44⁺ thymic cells include most if not all cells found in the medulla but only a small fraction of the cortical cells. Together with CD4 and CD8 antigens, seven subsets of thymic cell were defined and it was notable that most CD4⁻ CD8⁻ cells were OX-44⁺ whereas almost all CD4⁺ CD8⁺ cells were OX-44⁻. In functional tests, the OX-44⁺ cells accounted for all proliferation by thymocytes when stimulated by allogeneic spleen cells or concanavalin A plus growth factors and OX-44⁻ cells were completely negative in these assays. Also, in tests for thymopoiesis

medulla; c, cortex. (b) MRC OX-44, high power of cortex. *Arrows* indicate labeled small mononuclear cells; *star* indicates area of irregular non-thymocyte labeling. Other mAbs used are (c) anti-CD8, (d) OX-42, (e) ED2, and (f) MRC OX-27. Thymus from an MHC class I chimaeric animal is labeled with (g) MRC OX-27 and (h) MRC OX-44. (i) Bu20a labeling of thymus from an animal injected with 5-bromo-2′ deoxyuridine. (j) Control labeling by OX-21 plus RAM-peroxidase. Bar in panel b represents 20 μm; all other sections are at the same magnification with bar in panel j, representing 80 μm.

after intra-thymic injection of cells, all activity was OX-44$^+$. It seems possible that the OX-44$^+$ set may include all functionally relevant cells in the rat thymus.

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