

# A MONOCLONAL ANTIBODY TO A CONSTANT DETERMINANT OF THE RAT T CELL ANTIGEN RECEPTOR THAT INDUCES T CELL ACTIVATION

Differential Reactivity with Subsets of Immature and Mature T Lymphocytes

BY THOMAS HÜNIG, HANS-JOACHIM WALLNY, JILL K. HARTLEY, ALEXANDRA LAWETZKY, AND GEORG TIEFENTHALER

*From the Genzentrum der Ludwig Maximilians Universität, München, D-8033 Martinsried, Federal Republic of Germany*

The TCR has been identified in several species as a heterodimer of two variable chains (reviewed in reference 1) that is associated with a set of invariant polypeptides collectively referred to as CD3. MHC-restricted antigen recognition by both cytotoxic (mostly CD8<sup>+</sup>) and helper (mostly CD4<sup>+</sup>) T cells is mediated by clonotypic heterodimers of the  $\alpha/\beta$  type, while a second type of CD3-associated TCR, termed  $\gamma/\delta$ , has recently been discovered on a small subpopulation of human (2) and mouse (3) T cells.

mAbs to the TCR and CD3 molecules have been instrumental to the discovery and analysis of the TCR complex. Recent work in the mouse system has especially profited from the generation of mAbs to V $\beta$  segments expressed at a frequency detectable in unimmunized T cell populations (4-7), and to the invariant CD3 $\epsilon$  chain (8). No mAb to a constant determinant of the mouse TCR- $\alpha/\beta$  is available, however, that could be used to discriminate TCR- $\alpha/\beta$  and TCR- $\gamma/\delta$  expressing T cells and thymocytes. In the rat system, analysis of T cell maturation and activation has been hampered by a complete lack of TCR- and CD3-specific monoclonal reagents, despite an otherwise excellent collection of mAbs to cell surface molecules.

Here, we describe a new mAb, termed R73, that detects a rat pan T cell surface antigen with the predicted properties of the TCR- $\alpha/\beta$  on mature and immature cells of the T cell lineage and reports its functional effects on resting T lymphocytes.

## Materials and Methods

*Animals.* Young adult Wistar and Lewis rats of both sexes were obtained from the animal quarters of the Max Planck Institute for Biochemistry, Martinsried, FRG, or from the Zentralinstitut für Versuchstierzucht, Medizinische Hochschule Hannover, FRG. Results obtained did not vary significantly between both strains.

*Immunization and Cell Fusion.* Spleen cells from a BALB/c mouse alternately immunized intraperitoneally with rat T blasts and rat erythrocytes (it was also intended to generate an mAb to rat LFA-3) were fused 3 d after an intravenous injection of 10<sup>8</sup> rat erythrocytes with

---

This work was funded by a grant from the Bundesministerium für Forschung und Technologie. Generation of the R73 cell line was funded by Genzentrum e.V.

the nonproducer cell line X63-Ag 8.653 using polyethylene glycol. Supernatants of hybridomas obtained after selection in azaserine/hypoxanthine-containing medium were screened for binding to rat T cell blasts by indirect immunofluorescence and flow cytometry. Positive supernatants were screened at a 1:4 final dilution for the induction of DNA synthesis in  $10^5$  unfractionated rat splenocytes (see below). R73, the only positive culture among ~4,000 hybridomas, was subcloned by limiting dilution.

*mAbs and Fluorochrome Conjugation.* mAbs OX-35, W3/25 (both anti-CD4, reference 9), OX-8 (anti-CD8, reference 10), OX-52 (pan T cell, reference 11), OX-39 (anti-IL-2-R, reference 12) OX-44 (13), and R73 were purified from ascitic fluid using protein A-sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). mAb L180/1 to sheep LFA-3 (14) is of the same isotype ( $\gamma_1$ ) as R73 and was used as a negative control. All OX antibodies were the kind gift of Dr. A. Williams, Oxford, England. They were conjugated with FITC (Sigma Chemical Co., St. Louis, MO) according to standard procedures. Secondary antibodies were FITC-labeled RaMIg Fab<sub>2</sub> and biotinylated RaMIg (both from Dakopatts GmbH, Hamburg, FRG). Avidin-phycoerythrin was obtained from Becton Dickinson GmbH, Heidelberg, FRG.

*Immune Histology.* Detection of the R73 antigen in 5- $\mu$ m cryostat sections of lymphoid organs was kindly performed by Dr. Louise Beyers, Oxford, following published procedures (13).

*Preparation of Cells.* Cell suspensions from thymus, spleen, and pooled lymph nodes were prepared by passing the organs through a metal grid into ice-cold balanced salt solution. Bone marrow was ejected into balanced salt solution from femurs and tibias with a syringe. Resting T cells were isolated from lymph node cell suspensions by passage over nylon wool and by collecting the 50:60% Percoll (Pharmacia Fine Chemicals) interphase after density centrifugation. Thymocyte subsets were isolated by two cycles of "panning" (15) on rabbit anti-mouse Ig (RaMIg)<sup>1</sup>-coated plates to bind thymocytes that had been treated with mouse mAb to various surface markers. Impurities in the subpopulations thus obtained were detected by labeling with RaMIg-biotin and avidin-phycoerythrin, permitting their exclusion by electronic gating when reactivity with FITC-conjugated R73 mAb was studied (see below).

*Immunofluorescence and Flow Cytometry.* All labeling reagents were used at saturating concentrations. For direct immunofluorescence,  $2 \times 10^5$  cells in PBS/0.1% BSA/0.02% NaN<sub>3</sub> were kept for 15 min on ice, and washed. For two-color fluorescence, the cells were reacted with unconjugated mAb to the first marker as above, followed by sequential 15-min exposures to RaMIg-biotin (in the presence of 10% normal rat serum to block anti-rat Ig crossreactivity), avidin-phycoerythrin plus 10  $\mu$ g/ml normal mouse Ig, and FITC-labeled mAb to the second marker.  $2 \times 10^4$  cells per sample were analyzed in a FACScan flow cytometer (Becton Dickinson). Light scatter gates were set to exclude dead cells and erythrocytes. Instrument settings were identical for all experiments shown. Results are displayed as histograms or contour plots generated with the FACScan and Consort 30 software (Becton Dickinson), respectively.

*Radioimmunoprecipitation.* Nylon wool-purified lymph node cells were surface labeled with <sup>125</sup>I (Amersham Buchler GmbH, Braunschweig, FRG) by the lactoperoxidase method as previously described (14). Cells were lysed in lysis buffer (either PBS containing 1% NP-40 and 1 mM PMSF or TRIS-buffered saline, pH 7.5, containing 1 mM EDTA, 0.02% NaN<sub>3</sub>, 1 mM PMSF, and 1% digitonin [Sigma Chemical Co.]). After centrifugation, the lysate was preabsorbed with normal mouse IgG (Sigma Chemical Co.), coupled to cyanbromide-activated sepharose beads (Pharmacia Fine Chemicals). The lysate was then incubated with the respective mAbs coupled to protein A-sepharose for 2 h at 4°C. The beads were washed four times in lysis buffer containing 0.5 M KCl (NP40 lysates only) and two times with lysis buffer, boiled for 5 min in nonreducing or reducing Laemmli sample buffer, respectively (16), and the precipitated immune complexes were analyzed on SDS polyacrylamide gels.

*Cell Culture.* For T cell stimulation assays,  $10^5$  responder cells (unfractionated spleen cells or purified T cells) were cultured in 0.1-ml microcultures for 3 d in RPMI 1640 with 5% FCS in the presence or absence of various dilutions of R73 culture supernatants. In some

<sup>1</sup> Abbreviation used in this paper: RaMIg, rabbit anti-mouse Ig.

groups, the microwell plates (Costar, Cambridge, MA) were precoated overnight with RaMIg ([Dakopatts GmbH]; 40  $\mu\text{g}/\text{ml}$  in carbonate buffer, pH 9.5), while others received  $10^5$  x-irradiated (1,200 rad) spleen cells as accessory cells. Where indicated, 50 U/ml human rIL-2 (Hoechst AG, Frankfurt, FRG) was added. 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine (Amersham, Braunschweig, FRG; 5 Ci/mM sp act) was present in each well for the last 16 h of culture before determining the incorporated radioactivity by standard procedures.

For phenotypic analysis of R73-activated T cells,  $5 \times 10^6$  purified T cells were cultured in 2 ml RPMI 1640 and 5% FCS in tissue culture wells (Cluster 12; Costar) that had first been coated with RaMIg as described above, followed by a 1-h incubation with a 1:200 dilution of R73 or L180/1 ascitic fluid in medium, and three washes.

## Results

**Isolation of mAb R73.** Spleen cells from a BALB/c mouse immunized with rat T cell blasts and rat erythrocytes (see Materials and Methods) were fused with a nonproducer myeloma cell line according to standard procedures. Culture supernatants were first screened for binding to rat leukocytes by indirect immunofluorescence, and positive cultures were tested for the induction of DNA synthesis in unfractionated rat spleen cells. One hybridoma, termed R73, scored positive and a subclone yielding a high-titered tissue culture supernatant was isolated.

**Tissue Distribution of the R73 Antigen.** The tissue distribution of the R73 antigen was investigated by immunofluorescence and flow cytometry and by immune histology. Fig. 1 shows that in young adult animals, the antigen is expressed on  $\sim 46\%$  of splenocytes,  $\sim 63\%$  of lymph node cells, and  $\sim 3\%$  of bone marrow cells. Thymocytes displayed a triphasic distribution of R73 staining. 13% stained with the same intensity as the positive cells found in spleen and lymph nodes, while the majority of thymocytes (68%) formed a distinct subpopulation with lower but clearly measurable antigen expression, and a third population (19%) was R73 $^-$ .

Immune histology of splenic (Fig. 2 A) and lymph node (Fig. 2 B) sections revealed that splenic R73 $^+$  cells reside in the T cell areas of the white pulp and paracortex, respectively. This pattern was indistinguishable from that obtained in serial sections for the pan T cell marker OX-52 (not shown). In the thymus (Fig. 2 C), almost all medullary cells reacted with R73, whereas the cortex contained only scattered R73 $^+$  cells, many of which stained less intensely than medullary thymocytes.

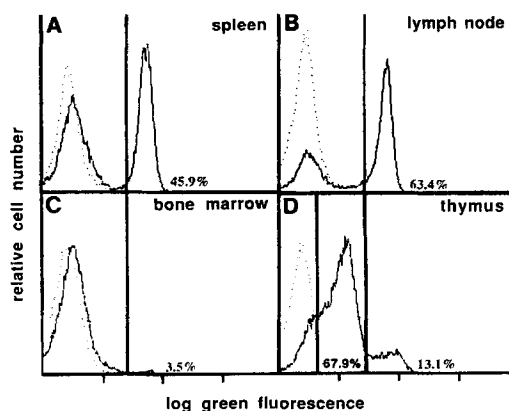


FIGURE 1. Reactivity of mAb R73 with cell suspensions prepared from spleen, lymph nodes, bone marrow, and thymus of young adult rats. Cells were stained with FITC-conjugated mAb R73, and with L180/1-FITC as an isotype-matched negative control.

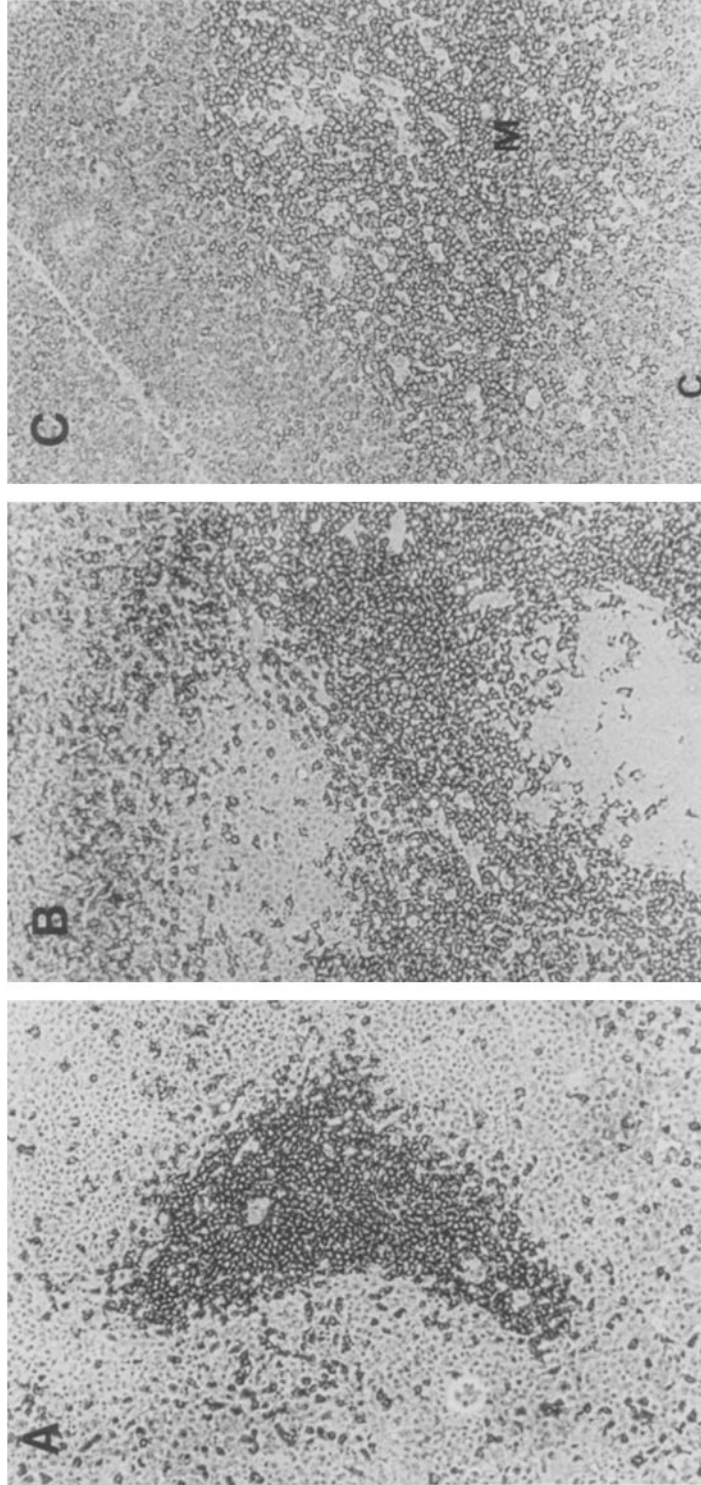


FIGURE 2. Expression of the R73 determinant in situ. Thin sections of rat spleen (A), lymph node (B), and thymus (C) were stained as given in Materials and Methods. M, medulla; C, cortex.

*Distribution of the R73 Antigen on Subsets of Peripheral Lymphocytes.* The above results suggested that R73 detects a pan T cell antigen. It was therefore attempted to define the exact subset(s) of peripheral lymphoid cells reactive with R73 using two-color immunofluorescence and flow cytometry. In the experiment shown in Fig. 3, lymph node lymphocytes were analyzed for coexpression of the R73 antigen with OX-52 (pan T cell), and the CD4 and CD8 markers found on MHC class II and class I restricted peripheral T cells, respectively. Fig. 3 A indicates that all but a very small subpopulation (2-3%) of unseparated OX-52<sup>+</sup> lymph node cells reacted with R73, and that all R73<sup>+</sup> cells also expressed OX-52, indicating that R73 detects a T cell-specific antigen. Accordingly, purified lymph node T cells were analyzed for coexpression of R73 with CD4 and CD8 (Fig. 3, B-D). Virtually all (99.5%) lymph node T cells expressing CD4 (Fig. 3 C) and ~95% of CD8<sup>+</sup> cells (Fig. 3 B) reacted with R73. Simultaneous staining with both anti-CD4 and anti-CD8 in one fluorescence channel vs. R73 in the other revealed that R73 expression is restricted to lymphocytes expressing CD4 or CD8 (Fig. 3 D). It is of interest in this context that rat NK cells are CD8<sup>+</sup> (17), raising the possibility that R73 detects an antigen on all helper and cytotoxic T cells, but not on NK cells.

Very similar results were obtained with rat splenocytes, except that an additional population of larger cells was found that expressed CD4 but was R73<sup>-</sup> (not shown). These cells were negative for the pan T cell marker OX-52 and expressed CD4 at a level below that characteristic of peripheral T cells. Since rat splenic macrophages have previously been shown to express the CD4 antigen (9), we conclude that these were macrophages and that, like in lymph nodes, R73 expression in the spleen is restricted to T lymphocytes.

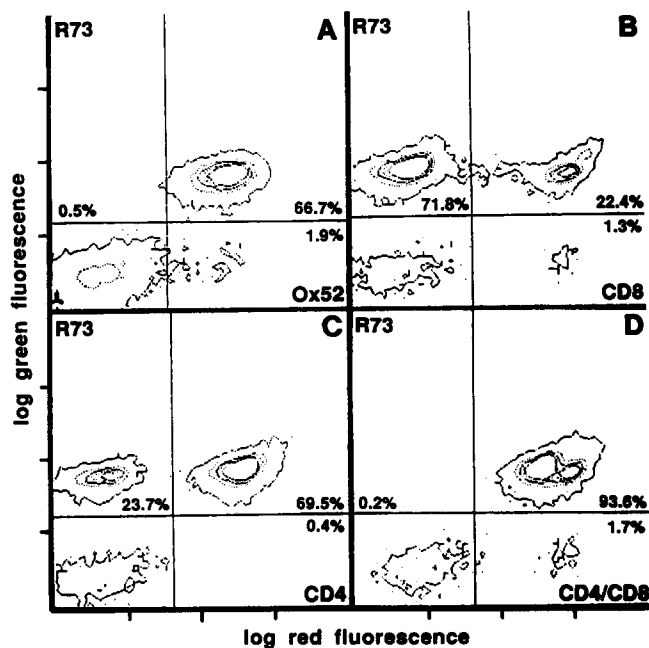


FIGURE 3. Expression of the R73 determinant on subsets of lymph node cells. Unseparated (A) lymph node cells or purified T cells (B-D) were stained with the reagents as indicated in the figure. For details see Materials and Methods. Lowest level shown in contour plots is five cells, graduation is 30 cells/line.

**Biochemical Characterization of the R73 Antigen.** Lymph node cells were enriched for T cells by nylon wool filtration, were surface iodinated, and were lysed with the nonionic detergent NP-40. Radioimmunoprecipitates obtained with R73 and, as a control, normal mouse IgG were analyzed by SDS-PAGE in the absence or presence of a reducing agent (Fig. 4 A). R73 precipitates a broad band characteristic of glycoproteins with a molecular mass of 87 kD without reduction, and two bands of 40 and 46 kD molecular mass upon reduction. Minor bands were also observed that are presently unexplained. This result was consistent with the hypothesis that mAb R73 is specific for the rat TCR- $\alpha/\beta$ . If so, immunoprecipitation from a lysate prepared with a milder detergent such as digitonin, known to preserve TCR/CD3 complexes in other species, should yield one or several bands of smaller molecular mass derived from the invariant CD3 chains noncovalently associated with the TCR. Fig. 4 B shows that under these conditions, an additional band of 26 kD was indeed observed. It is likely that this band represents the CD3  $\delta$  and/or  $\epsilon$  chains that comigrate as the major detectable CD3 components upon precipitation of the mouse TCR/CD3 complex with an anticonotypic antibody (8).

**Requirements for T Cell Activation by mAb R73.** The T cell specificity of mAb R73 suggested that its stimulatory effect on rat splenocytes was directed at T cells. Accordingly, we compared the induction of DNA synthesis by R73 in unseparated spleen cells and in purified T lymphocytes (Fig. 5). Titration of R73 culture supernatant stimulated a dose-dependent response in unfractionated spleen cells (Fig. 5 A). In the experiment shown in Fig. 5 B,  $10^5$  nylon wool-purified lymph node T cells (98% OX-52<sup>+</sup>, 95% R73<sup>+</sup>) were cultured in the presence of serial dilutions of the same R73 culture supernatant. As judged by cell surface staining, the mAb in this super-

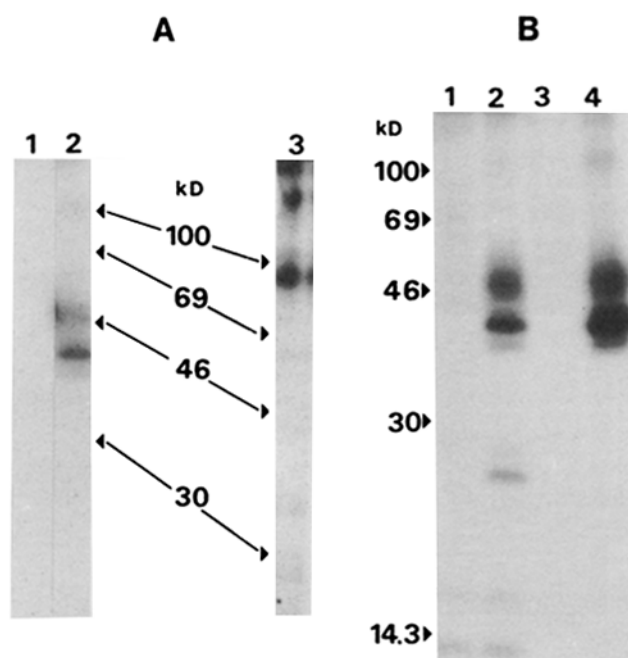


FIGURE 4. Radioimmunoprecipitation of the R73 antigen analyzed by SDS-PAGE. (A) mAb R73 (lanes 2 and 3), or normal mouse IgG (lane 1) were used for precipitation from an NP-40 lysate of surface-iodinated lymph node T cells. (Lanes 1 and 2) Reduced, 12.5% acrylamide; (lane 3) non-reduced, 10% acrylamide. (B) mAb R73 (lanes 2 and 4) or isotype-matched control mAb L180/1 (lanes 1 and 3) were used for precipitation from a digitonin (lanes 1 and 2) or NP40 (lanes 3 and 4) lysate. 15% acrylamide, reduced.

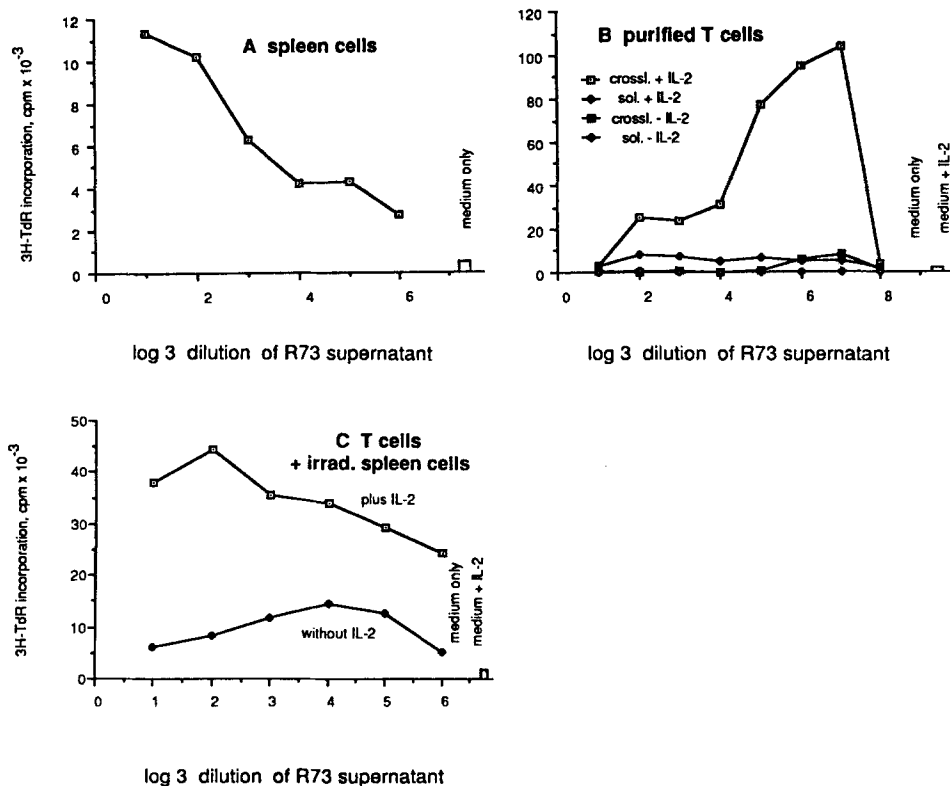


FIGURE 5. T cell activation by mAb R73. Spleen cells (A), or purified lymph node T cells (B and C) were incubated for 3 d with the additions indicated. For details see Materials and Methods. Crossl., wells were coated with RaMIg to crosslink mAb R73. Sol., without crosslinking antibodies.

nant was able to saturate the R73 binding sites available on these T cells with a titer of 1:500 (not shown). Without further additions, no proliferation was observed. Even in the presence of rIL-2, the low level of DNA synthesis induced by IL-2 alone was only slightly enhanced by soluble R73. If, however, the culture wells had been precoated with RaMIg antibodies to crosslink the mAb, a clear-cut response above background was observed that was enhanced by >10-fold by the addition of rIL-2. Under these experimental conditions, a maximum response was observed at a 1,000-fold dilution of the R73 culture supernatant, indicating that soluble, noncrosslinked antibody interfered with T cell activation by antibody bound to the solid phase.

It is shown in Fig. 5 C that T cells from the same preparation did respond to soluble R73 in the presence of irradiated spleen cells as a source of accessory cells. Although exogenous IL-2 was also able to enhance the proliferative response supported by splenic accessory cells, the difference observed in cultures with and without rIL-2 was not as pronounced as in cultures stimulated by antibody crosslinked at the plastic surface (Fig. 5 B).

*Induction of IL-2-R Expression and Modulation of the R73 Antigen.* Fig. 6 shows that purified T cells cultured overnight in the presence of crosslinked mAb R73 responded at a high frequency (>80%) with an increase in cell size (A) and the expression of

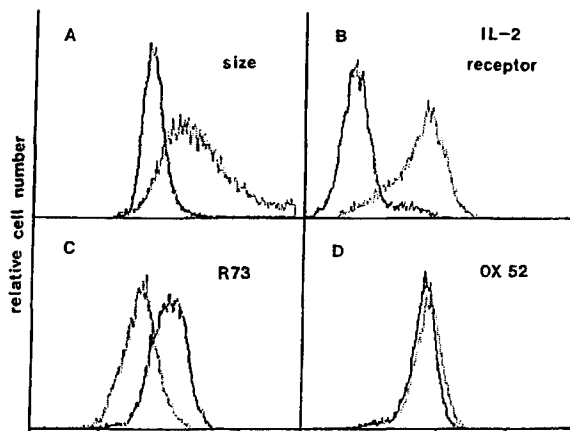


FIGURE 6. Phenotypic changes in T cells after overnight incubation with crosslinked mAb R73. Purified lymph node T cells were cultured for 16 h in the presence of crosslinked mAb R73 (dotted line) or isotype-matched mAb L180/1 as a control (solid line). (A) forward light scatter (linear); (B-D) immunofluorescence (log scale). (B) OX-39-FITC (anti-IL-2-R); (C) R73 plus RaMIg-biotin plus avidin-PE; (D) OX-52-FITC. Negative staining controls (not shown) were within the first log decade.

IL-2-Rs (B). Conversely, expression of the R73 antigen (C), but not of the OX-52 antigen (D) was 3-5-fold reduced on these cells as compared with control cells cultured in the presence of a crosslinked isotype-matched antibody that does not react with rat cells. Crosslinking of the rat CD2 antigen did not affect R73 antigen expression (not shown).

*Expression of the R73 Determinant on Subpopulations of Thymocytes.* The availability of an mAb that is likely to detect all TCR- $\alpha/\beta$  has prompted us to investigate the expression of the R73 antigen on subpopulations of rat thymocytes. In particular, we were interested to investigate a possible correlation between the triphasic profile noted before with regard to R73 staining intensity (Fig. 1) and subpopulations of rat thymocytes previously defined by the markers CD4, CD8, and OX-44. In the thymus, the latter antigen is exclusively expressed on functionally mature CD4 or CD8 single-positive and on CD4<sup>-</sup>CD8<sup>-</sup> double-negative cells, while the major CD4<sup>+</sup>CD8<sup>+</sup> double-positive and the small CD4<sup>-</sup>CD8<sup>+</sup> population from which these double-positive cells mature are OX-44<sup>-</sup> (13).

The two parameter flow cytograms displayed in Fig. 7 make several interesting points with regard to R73 expression on thymocyte subpopulations. Fig. 7A, relating cell size to R73 staining, defines at least three major subpopulations of adult thymocytes: large cells, most of which are R73<sup>-</sup>; intermediate (lymphocyte)-sized cells that express a high level of the antigen; and a large population the size of small cortical thymocytes that contains some negative, but mostly R73<sup>low</sup> cells. Fig. 7, B and C demonstrate the existence of a CD4<sup>-</sup> and of a CD8<sup>-</sup> thymocyte subpopulation that expresses R73 at a level characteristic of peripheral T cells. It will become clear from the following experiments that these are the two functionally mature thymocyte subsets.

Next, thymocyte subsets were prepared by the panning technique and compared with unfractionated thymocytes with regard to R73 staining (Fig. 8). Note that the differences in background staining of the thymocyte subpopulations thus isolated is due to the differences in cell size. Positively selected OX-44<sup>+</sup> cells, shown in Fig. 8B, contain the CD4<sup>-</sup>CD8<sup>-</sup> cells, and in addition, the two functionally mature T cell subpopulations (13). Staining with R73 revealed a negative subpopulation ( $\sim 30\%$ ),



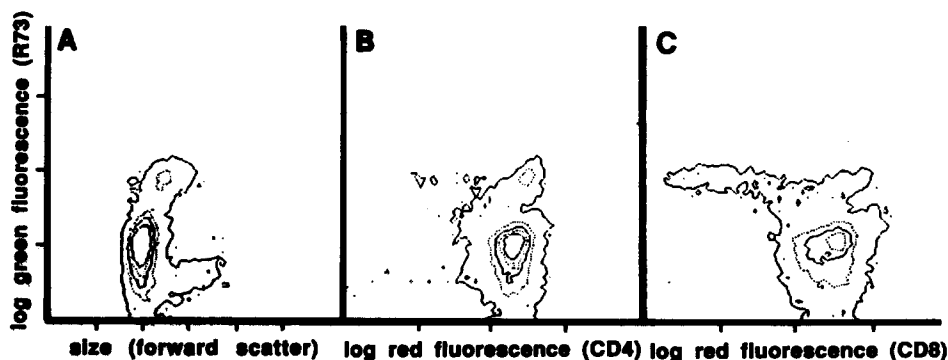


FIGURE 7. Expression of the R73 determinant on subpopulations detected in unseparated thymocytes. Cells were stained and analyzed as given in Materials and Methods. Lowest level in contour plots shown is five cells, graduation is 40 cells/line.

while  $\sim 67\%$  of  $\text{OX-44}^+$  thymocytes expressed the antigen at the same level as peripheral T cells. As shown in Fig. 8 C, the  $\text{R73}^-$  subpopulation could be attributed to the  $\text{CD4}^- \text{8}^-$  cells contained in this preparation. Thus, the vast majority (93.5%) of purified  $\text{CD4}^- \text{8}^-$  thymocytes were found to be negative for the putative  $\text{TCR-}\alpha/\beta$ . In contrast to the majority of  $\text{R73}^- \text{CD4}^- \text{8}^-$  cells, which were all large blasts, the small subpopulation of  $\text{CD4}^- \text{8}^-$  cells that expressed R73 (6.5%) consisted mostly of lymphocyte-sized cells (not shown).  $\text{OX-44}^-$  thymocytes (Fig. 8 D), consisting of  $\sim 90\%$   $\text{CD4}^+ \text{8}^+$  double-positive cells (13, and data not shown), were mostly  $\text{R73}^{\text{low}}$ , but also contained 25%  $\text{R73}^-$  cells as well as a small subpopulation (5.1%) whose antigen density approached that of mature T cells.

Finally, the  $\text{CD4}^- \text{8}^+ \text{OX-44}^-$  thymic blast cells, which are the direct progenitors of double-positive thymocytes, were isolated (18). These cells expressed from undetectable to very low but significant amounts of the putative  $\text{TCR-}\alpha/\beta$  (Fig. 8 E). This

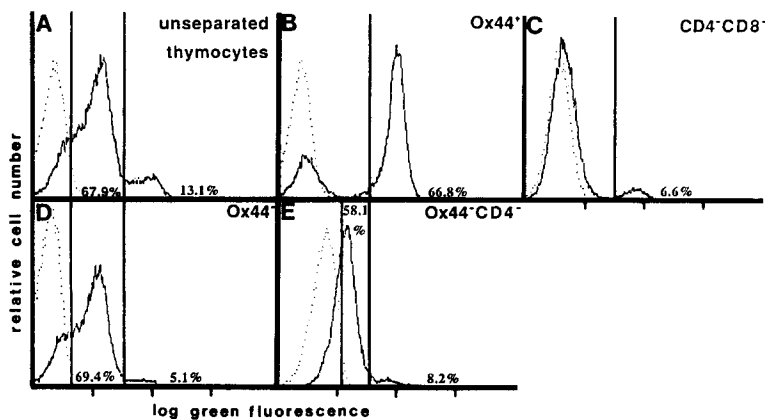


FIGURE 8. Expression of the R73 determinant on isolated thymocyte subsets. Thymocytes were separated by the panning technique into subsets as indicated and stained with FITC-R73 (solid line) or FITC-L180/1 (negative control, dotted line).

low but significant level of R73 staining was observed in all of five independent experiments using various staining protocols.

### Discussion

The conclusion that mAb R73 detects a constant determinant on the rat TCR- $\alpha/\beta$ , although awaiting definite proof from transfection experiments or protein sequence analysis, is based on several independent observations that were compared with the results previously reported for mouse and human TCRs (1). Thus, R73 detects a T cell-specific heterodimeric (40- and 46-kD) cell surface protein that is expressed on the vast majority of (97-98%), but not on all, peripheral rat T cells. This is analogous to the distribution of Ti- $\alpha/\beta$  vs.  $-\gamma/\delta$  expressing T cells in humans (2, 19). The expression of the R73 determinant on all CD4<sup>+</sup> but only ~90-95% of CD8<sup>+</sup> rat T cells is also in agreement with the reported CD8<sup>+</sup> phenotype of rat (17) (and, in part, human, reference 20) NK cells, which are highly enriched in CD8<sup>+</sup>R73<sup>-</sup> spleen cells (A. Lawetzky and T. Hünig, manuscript in preparation). In addition, athymic rats, which have a higher ratio of NK to "classical" T cells, contain up to 75% of R73<sup>-</sup> lymphocytes among their CD8<sup>+</sup> spleen cells (A. Lawetzky and T. Hünig, manuscript in preparation). It remains to be determined, however, what fraction of CD8<sup>+</sup>R73<sup>-</sup> cells are NK cells and/or express Ti- $\gamma/\delta$  receptors as was reported for some human NK cell clones (21).

The T cell specificity of R73 staining observed with isolated leukocytes was in full agreement with its reactivity in thin sections of lymphoid organs (Fig. 2). These experiments also provided the important information that in the thymus, strongly R73<sup>+</sup> cells reside almost exclusively in the medulla. This again correlated well with the CD4 or CD8 single-positive, OX-44<sup>+</sup> phenotype of thymocytes expressing R73 at the level of peripheral T cells (Figs. 7 and 8). Finally, the requirements for T cell activation with mAb R73 fully matched those reported for mAbs to components of the mouse (22) and human (23) Ti/CD3 complex in that antibody crosslinking on an artificial surface or by Fc receptors on accessory cells was required for stimulation. Thus, purified rat T cells responded to crosslinked R73 mAb at a high frequency with blastoid transformation and IL-2-R expression (Fig. 6), but required exogenous IL-2 for proliferation (Fig. 5 B). It may be added that using crosslinked R73, T cell clones can be grown from small numbers of T cells of unknown specificity, an approach that will be helpful for the establishment of clones from scarce sources such as the cerebrospinal fluid or the synovial fluid, where specificity analysis of T cells active in experimental autoimmune disease is amenable only after clonal expansion.

Recent experiments on thymocyte maturation and repertoire selection have raised a number of challenging questions, some of which can now be addressed with the help of the presently described mAb to a constant determinant of the rat TCR- $\alpha/\beta$ . The results presented here show that the vast majority of the CD4<sup>-</sup>8<sup>-</sup> cells known from other systems to contain the most immature thymocytes (24-26) are R73<sup>-</sup>. Only 6% of R73<sup>+</sup> cells were detected in this subpopulation, and they expressed the putative TCR- $\alpha/\beta$  at a level characteristic of peripheral T cells. Moreover, two thirds of this minor subpopulation was of lymphocyte, and not of blast size, leaving only 2% CD4<sup>-</sup>8<sup>-</sup> thymic blasts that express the R73 determinant. The TCR- $\alpha/\beta$  has been identified with the V $\beta$ 8-specific mAb F23.1 on a considerably higher propor-

tion (up to 27%, reference 27) of adult mouse CD4<sup>-</sup>8<sup>-</sup> thymocytes (27–29). A lineage assignment of these cells in thymic differentiation schemes is not possible yet, but they are certainly distinct from the intrathymic stem cell population as judged by their late appearance in ontogeny (29) and their inability to repopulate thymus organ cultures (28).

The rat represents a particularly useful model for the *in vitro* study of thymocyte differentiation due to the availability of a cell surface marker combination that allows the isolation by negative selection of the immature CD8 single-positive blast cells that have been shown to precede CD4<sup>+</sup>8<sup>+</sup> double-positive cells in ontogeny (30–32) and were recently identified as their direct precursors (18). Thus, thymocytes with the phenotype CD4<sup>-</sup>8<sup>+</sup>OX-44<sup>-</sup> acquire the double-positive phenotype upon overnight incubation in suspension culture without overt stimulation (18), a finding that was recently confirmed in the mouse system (33). It is shown here (Fig. 8) that the cells within this committed population of immature rat CD8 single-positive thymocytes display a continuum of R73 antigen density from negative to weakly positive. This is in agreement with the CD3<sup>dull</sup> phenotype of CD8<sup>+</sup> immature mouse thymocytes recently reported by Bluestone et al. (34) but disagrees with the CD3<sup>-</sup> status of these cells described by MacDonald et al. (33). Experiments in progress (T. Hünig, manuscript in preparation) indicate that upon *in vitro* cultivation for 1–2 d, most rat CD8<sup>+</sup> immature thymocytes acquire not only CD4, but also Ti levels characteristic of double-positive thymocytes, i.e., higher than on their precursor cells but distinctly lower than on mature T cells. We therefore speculate that immature CD8 single-positive thymocytes have just successfully rearranged their TCR  $\alpha$  and  $\beta$  loci and are now committed to a pathway where coexpression of CD4, CD8, and intermediate amounts of the TCR makes them available for repertoire selection. This notion is in agreement with several recent reports pointing to the expression of a yet unselected repertoire on CD4<sup>+</sup>8<sup>+</sup> thymocytes (7, 35, 36).

It is hoped that the reagent described in the present communication will be useful for monitoring and manipulation of T cell maturation and activation in the rat system in which the analysis of lymphoid subpopulations is greatly facilitated by the extensive selection of mAbs to cell surface molecules that have been produced by other laboratories.

### Summary

mAb R73 detects a T cell-specific surface molecule consisting of two disulfide-linked subunits of 40 and 46 kD, respectively, on 97% of peripheral rat T cells, as defined by the OX-52 marker. Of the few OX-52<sup>+</sup> R73<sup>-</sup> cells, none are CD4<sup>+</sup> but many express the CD8 antigen known to be present on rat NK cells. mAb R73 is mitogenic for unseparated spleen cells and for purified T cells. In the absence of non-T “accessory cells”, stimulation by R73 requires artificial crosslinking of the mAb and is largely dependent on exogenous IL-2. Overnight incubation of purified T cells with crosslinked R73 mAb induces blastoid transformation, IL-2-R expression, and modulation of the R73 antigen. In the rat thymus, mature medullary cells express the R73 determinant at the same level as peripheral T cells, whereas 94% of CD4<sup>-</sup>CD8<sup>-</sup> thymocytes are R73<sup>-</sup>. The major CD4<sup>+</sup>8<sup>+</sup> thymocyte population contains 25% R73<sup>-</sup> and 70% R73<sup>low</sup> cells. Thymocytes of the CD4<sup>-</sup>CD8<sup>+</sup>OX-44<sup>-</sup> subpopulation that are the direct precursors of CD4<sup>+</sup>CD8<sup>+</sup> cells display a continuum

of R73 antigen density from undetectable to very low levels. We conclude that R73 is most likely directed at a constant determinant of the rat  $\alpha/\beta$  heterodimeric TCR and suggest that CD8<sup>+</sup> immature thymocytes are the first cells in the T cell differentiation pathway to express this molecule at their surface.

We are greatly indebted to A. F. Williams, MRC Cellular Immunology Unit, Oxford, England, for helpful discussions and essential reagents; to Dr. Louise Beyers from the same laboratory for histological staining; and to Dr. E.-L. Winnacker at the Genzentrum for his continued support.

*Received for publication 23 May 1988 and in revised form 27 September 1988.*

### References

1. Marrack, P., and J. Kappler. 1987. The T-cell receptor. *Science (Wash. DC)*. 238:1073.
2. Brenner, M. B., J. McLean, D. P. Dialynas, J. L. Strominger, J. A. Smith, F. L. Owen, J. G. Seidman, S. Ip, F. Rosen, and M. S. Krangel. 1986. Identification of a putative second T-cell receptor. *Nature (Lond.)*. 322:145.
3. Maeda, L., N. Nakanishi, B. L. Rogers, W. G. Haser, K. Shitara, H. Yoshida, Y. Tagagaki, A. A. Augustin, and S. Tonegawa. 1987. Expression of the T-cell receptor  $\gamma$  chain gene products on the surface of peripheral T-cells and T-cell blasts generated by allogeneic mixed lymphocyte reaction. *Proc. Natl. Acad. Sci. USA*. 84:6536.
4. Haskins, K., C. Hannum, J. White, N. Roehm, R. Kubo, J. Kappler, and P. Marrack. 1984. The antigen-specific, major histocompatibility complex-restricted receptor on T cells. VI. An antibody to a receptor allotype. *J. Exp. Med.* 160:452.
5. Staerz, U. D., H.-G. Rammensee, J. D. Benedetto, and M. J. Bevan. 1985. Characterization of a murine monoclonal antibody specific for an allotypic determinant on T-cell antigen receptor. *J. Immunol.* 134:3994.
6. Kappler, J. W., T. Wade, J. White, E. Kushnir, M. Blackman, J. Bill, N. Roehm, and P. Marrack. 1987. The T-cell receptor V $\beta$  segment that imparts reactivity to a class II major histocompatibility complex product. *Cell*. 49:263.
7. MacDonald, H. R., R. Schneider, R. K. Lees, R. C. Howe, H. Acha-Orbea, H. Festenstein, R. M. Zinkernagel, and H. Hengartner. 1988. T-cell V $\beta$  use predicts reactivity and tolerance to MIs<sup>a</sup>-encoded antigens. *Nature (Lond.)*. 332:40.
8. Leo, O., M. Foo, D. H. Sachs, L. E. Samelson, and J. A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA*. 84:1374.
9. Jefferies, W. A., J. R. Green, and A. F. Williams. 1985. Authentic T helper CD4 (W3/25) antigen on rat peritoneal macrophages. *J. Exp. Med.* 162:117.
10. Brideau, R. J., P. B. Carter, W. R. McMaster, D. W. Mason, and A. F. Williams. 1980. Two subsets of T lymphocytes defined with monoclonal antibodies. *Eur. J. Immunol.* 10:609.
11. Robinson, A. P., M. Puklavec, and D. W. Mason. 1986. MRC OX-52: a rat T-cell antigen. *Immunology*. 57:527.
12. Paterson, D. J., W. A. Jefferies, J. R. Green, M. R. Brandon, P. Corthesy, M. Puklavec, and A. F. Williams. 1987. Antigens of activated rat T lymphocytes including a molecule of 50000 M<sub>r</sub> detected only on CD4 positive T blasts. *Mol. Immunol.* 24:1281.
13. Paterson, D. J., J. R. Green, W. A. Jefferies, M. Puklavec, and A. F. Williams. 1987. The MRC OX-44 antigen marks a functionally relevant subset among rat thymocytes. *J. Exp. Med.* 165:1.
14. Hünig, T. 1985. The cell surface recognized by the erythrocyte receptor of T lymphocytes. Identification and partial characterization using a monoclonal antibody. *J. Exp. Med.* 162:890.

15. Wysocki, L. J., and V. L. Sato. 1978. "Panning" for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci. USA.* 75:2884.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T<sub>4</sub>. *Nature (Lond.)*. 227:680.
17. Reynolds, C. W., S. O. Sharrow, J. R. Ortaldo, and R. B. Herbermann. 1981. Natural killer activity in the rat. II. Analysis of surface antigens on LGL by flow cytometry. *J. Immunol.* 127:2204.
18. Paterson, D. J., and A. L. Williams. 1987. An intermediate cell in thymocyte differentiation that expresses CD8 but not CD4 antigen. *J. Exp. Med.* 166:1603.
19. Weiss, A., M. Newton, and D. Crommie. 1986. Expression of T3 in association with a molecule distinct from the T-cell antigen receptor heterodimer. *Proc. Natl. Acad. Sci. USA.* 83:6998.
20. Faure, F., S. Jitsukawa, F. Triebel, and T. Hercend. 1988. CD3/TiγA: a functional γ-receptor complex expressed on human peripheral lymphocytes. *J. Immunol.* 140:1372.
21. Nowill, A., P. Moingeon, A. Ythier, M. Graziani, F. Faure, L. Delmon, M. Rainaut, F. Forestier, C. Bohuon, and T. Hercend. 1986. Natural clones derived from fetal (25 wk) blood. Probing the human T cell receptor with WT31 monoclonal antibody. *J. Exp. Med.* 163:1601.
22. Crispe, I. N., M. J. Bevan, and U. D. Staerz. 1985. Selective activation of Lyt2<sup>+</sup> precursor T-cells by ligation of the antigen receptor. *Nature (Lond.)*. 317:627.
23. Meuer, S. C., J. C. Hodgdon, R. E. Hussey, J. P. Protentis, S. F. Schlossman, and E. L. Reinherz. 1983. Antigen-like effects of monoclonal antibodies directed at receptors on human T cell clones. *J. Exp. Med.* 158:989.
24. Scollay, R., P. Bartlet, and K. Shortman. 1984. T-cell development in the adult murine thymus: changes in the expression of the surface antigens Ly2, L3T4, and B2A2 during development from early precursor cells to emigrants. *Immunol. Rev.* 89:79.
25. Kisielow, P., W. Leiserson, and H. von Boehmer. 1984. Differentiation of thymocytes in fetal organ culture: analysis of phenotypic changes accompanying the appearance of cytolytic and interleukin 2-producing cells. *J. Immunol.* 133:1117.
26. Fowlkes, B. J., L. Edison, B. J. Mathieson, and T. M. Chused. 1985. Early T lymphocytes. Differentiation in vivo of adult intrathymic precursor cells. *J. Exp. Med.* 162:802.
27. Wilson, A., T. Ewing, T. Owens, R. Scollay, and K. Shortman. 1988. T-cell antigen receptor expression by subsets of Ly2<sup>-</sup> L3T4<sup>-</sup> (CD8<sup>-</sup> CD4<sup>-</sup>) thymocytes. *J. Immunol.* 140:1470.
28. Crispe, I. N., M. W. Moore, L. A. Husmann, L. Smith, M. J. Bevan, and R. P. Shimonkevitz. 1987. Differentiation potential of subsets of CD4<sup>-</sup> 8<sup>-</sup> thymocytes. *Nature (Lond.)*. 329:336.
29. Fowlkes, B. J., A. M. Krusbeek, H. Ton-That, M. A. Weston, J. E. Coligan, R. H. Schwartz, and D. M. Pardoll. 1987. A novel population of T-cell receptor α/β bearing thymocytes which predominantly expresses a single Vβ gene family. *Nature (Lond.)*. 329:251.
30. Ceredig, R., D. P. Dialynas, F. W. Fitch, and H. R. MacDonald. 1983. Precursors of T cell growth factor producing cells in the thymus. Ontogeny, frequency, and quantitative recovery in a subpopulation of phenotypically mature thymocytes defined by monoclonal antibody GK-1.5. *J. Exp. Med.* 158:1654.
31. Mackay, C. R., J. F. Maddox, and M. R. Brandon. 1986. Thymocyte subpopulations during early fetal development in sheep. *J. Immunol.* 136:1592.
32. Von Boehmer, H. 1986. Thymus development. *Curr. Top. Microbiol. Immunol.* 126:19.
33. MacDonald, H. R., R. C. Budd, and R. C. Howe. 1988. A CD3<sup>-</sup> subset of CD4<sup>-</sup> 8<sup>+</sup> thymocytes: a rapidly cycling intermediate in the generation of CD4<sup>+</sup> 8<sup>+</sup> cells. *Eur. J. Immunol.* 18:519.
34. Bluestone, J. A., D. Pardoll, S. O. Sharrow, and B. J. Fowlkes. 1987. Characterization

- of murine thymocytes with CD3-associated T-cell receptor structures. *Nature (Lond.)*. 326:82.
35. Kappler, J. W., N. Roehm, and P. Marrack. 1987. T-cell tolerance by clonal elimination in the thymus. *Cell*. 49:273.
  36. Kappler, J. W., U. Staerz, J. White, and P. C. Marrack. 1988. Self-tolerance eliminates T-cells specific for Mls-modified products of the major histocompatibility complex. *Nature (Lond.)*. 332:35.