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Immunology

Molecular characterization of T-cell antigen receptor expression by subsets of CD4$^-$ CD8$^-$ murine thymocytes

(T-cell differentiation/thymus)

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ABSTRACT **Precursors of all T-lineage cells are found in a population of thymocytes that lack the CD4 and CD8 surface glycoproteins. These "double-negative" thymocytes are markedly heterogeneous in their expression of other surface markers and include cells at various stages of development. In this study, CD4$^-$ CD8$^-$ adult murine thymocytes were separated into subsets based on the expression of the "heat stable antigen" (HSA) and of Ly 1 (CD5). The sorted subsets were analyzed directly (without prior expansion in culture) for T-cell antigen receptor (TcR) gene rearrangement and mRNA expression and for TcR and CD3 cell-surface protein expression. Very little surface CD3 or TcR expression was detected on the major HSA$^+$ Ly 1^{low} subset. However, the HSA$^+$ Ly 1^{high}, HSA$^-$ Ly 1^{high}, and HSA$^-$ Ly 1^{low} subsets all contained cells with surface expression of CD3 and TcR. In contrast to previous studies, we found no subset that exclusively expressed either the $\alpha\beta$ or $\gamma\delta$ heterodimer, although the ratio of $\alpha\beta^+$ to $\gamma\delta^+$ varied widely. Two of these three subsets (HSA$^-$ Ly 1^{low} and HSA$^-$ Ly 1^{high}) showed very high usage of Vβ8 gene products in the $\alpha\beta$ heterodimer, but nevertheless included ≈15% non-Vβ8 $\alpha\beta$ forms. All CD4$^-$ CD8$^-$ subsets were found to have extensively rearranged their TcRγ genes and to express γ mRNA. Expression of a high ratio of mature [1.3 kilobases (kb)] to truncated (1.0 kb) β message and presence of α message was largely restricted to subsets with TcR $\alpha\beta$ surface expression.**

An important step toward understanding the process of T-lymphocyte development was the recognition of a 3–4% subpopulation of adult thymocytes that lack the function-associated markers CD4 and CD8, which are present on mature T cells (1, 2). These "double-negative" thymocytes are a major component of embryonic thymus and include many rapidly dividing cells (3). The population also includes precursors capable, on intravenous or intrathymic transfer, of developing into all other thymocyte populations and into mature T cells (4–7).

Recent studies have shown that adult mouse CD4$^-$ CD8$^-$ thymocytes can be divided into discrete subsets by using a variety of other markers (reviewed in ref. 8), including HSA (the "heat stable antigen" recognized by the monoclonal antibodies B2A2, M1/69, and J11d); and Ly 1 (CD5). The developmental relationships of the various subsets have not yet been established.

Despite their apparent "immature" phenotype some CD4$^-$ CD8$^-$ thymocytes express CD3-associated T-cell receptor (TcR) heterodimers—either TcR-$\gamma\delta$ (9) or TcR-$\alpha\beta$ (10–13). It has been suggested, based on analysis of cultured subsets of CD4$^-$ CD8$^-$ cells, that TcR-$\gamma\delta$ is limited to the HSA$^+$ population (14) and that TcR-$\alpha\beta$ is limited to the HSA$^-$ population (6, 12). However, selective growth and differentiation during culture are potential problems with these studies. In this paper, we have used molecular analysis at the DNA, RNA, and protein levels to look at TcR expression in subsets of CD4$^-$ CD8$^-$ cells isolated directly from the thymuses of normal mice, and we have demonstrated both the $\alpha\beta$ and $\gamma\delta$ forms of the TcR in both HSA$^+$ and HSA$^-$ subsets. We also show that despite the exceptionally high usage of Vβ8 gene products amongst the HSA$^-$ cells (10–13), non-Vβ8 $\alpha\beta$ can also be found. To perform these analyses, we have had to develop techniques sensitive enough to allow direct analysis of even minor CD4$^-$ CD8$^-$ subsets. Southern and RNA blot analyses, as well as immunoprecipitation of surface TcR, have been performed with as few as 5 × 10^5 cells.

MATERIALS AND METHODS

Mice. Male 5- to 6-wk-old mice of the CBA/CaH strain, bred and maintained under specific pathogen-free conditions at The Walter and Eliza Hall Institute animal breeding facility, were used throughout.

Preparation of Subpopulations of CD4$^-$ CD8$^-$ Thymocytes. Subsets of CD4$^-$ CD8$^-$ thymocytes were separated by fluorescence-activated cell sorting, as described elsewhere (8), after staining highly purified CD4$^-$ CD8$^-$ thymocytes according to the following protocol: anti-HSA-fluorescein, anti-Ly 1-biotin, phycoerythrin-avidin. The purity of the sorted populations was generally >98%, except for the HSA$^+$ Ly 1^{high} subset, which varied between 90% and 95%.

Lymph Node T Cells. Total lymph node cells were depleted of B cells with magnetic beads coated with affinity-purified sheep anti-mouse immunoglobulin as described (15).

Preparation of RNA and DNA for Hybridization Analysis. DNA and RNA were prepared simultaneously from as few as 5 × 10^5 cells. Cells were washed in mouse tonicity phosphate-buffered saline and then lysed in 150 μl of 0.5% Nonidet P-40 (Sigma)/150 mM NaCl/10 mM Tris·HCl, pH 8.0/2 mM $MgCl_2$/10 mM vanadyl ribonucleoside complexes. The lysate was centrifuged (2000 × *g*; 7 min) to separate the nuclear and cytoplasmic fractions, from which DNA and total cytoplasmic RNA, respectively, were prepared.

Total Cytoplasmic RNA. To the supernatant from the cell lysate (from above) was added an equal volume of 7 M urea/450 mM NaCl/10 mM EDTA/1% $NaDodSO_4$/10 mM Tris·HCl, pH 7.4 (25). After extracting once with phenol/chloroform, RNA was recovered by ethanol precipitation, fractionated on 1% agarose/formaldehyde minisub gels, and transferred to Hybond-N (Amersham).

DNA. The nuclear pellet (from above) was resuspended in 10 μl of nuclei lysis buffer (1% sodium lauroyl sarcosine/6.5 mM EDTA), an equal volume of low gelling temperature

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Abbreviations: TcR, T-cell antigen receptor; HSA, heat stable antigen recognized by the monoclonal antibodies B2A2, M1/69, and J11d.

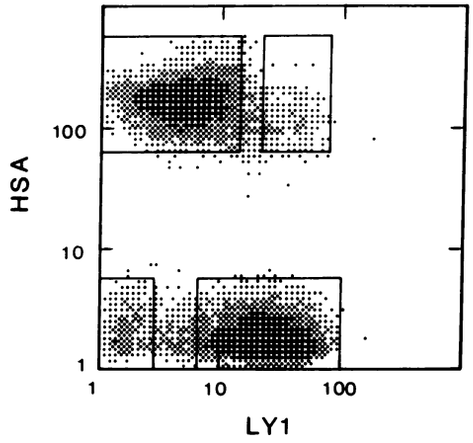

FIG. 1. Isolation by fluorescence-activated cell sorting of four subsets of adult CBA mouse CD4$^-$ CD8$^-$ thymocytes on the basis of HSA (M1/69) and Ly 1 (CD5) expression. Boxes indicate gates used for sorting the four subpopulations.

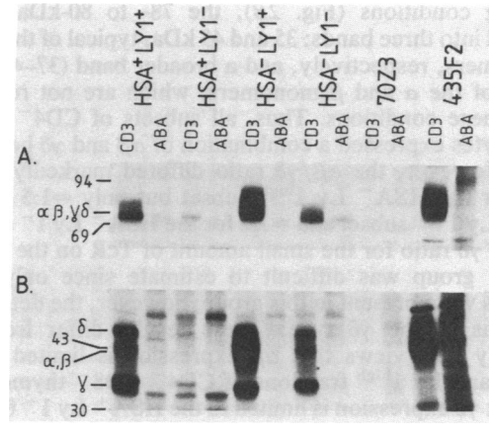

FIG. 2. Analysis of TcR protein chains from the surface of CD4$^-$ CD8$^-$ thymocyte subsets. The subsets defined in Fig. 1 were isolated, surface-labeled with ^{125}I, and immunoprecipitated with either anti-CD3 (CD3, 145-2C11, ref. 22), or, as antibody control, the irrelevant anti-azobenzenearsonate (ABA) as indicated. Samples were separated by PAGE under reducing (*A*) or nonreducing (*B*) conditions. 70Z3 was a TcR-negative pre-B cell line, used as the filler during ^{125}I labeling. 435F2 was a TcR$\alpha\beta$ heterodimer-positive T-cell line. Numbers on left are kDa.

agarose, 2% containing proteinase K (0.5 mg/ml) was added and incubated overnight at 50°C. After 5 min on ice, the solidified agarose was equilibrated against 10 mM Tris·HCl, pH 7.5/1 mM EDTA to remove detergent and proteinase K. After melting (65°C, 5 min), the preparation was digested at 37°C with restriction enzyme. Restricted DNA was separated on an agarose (0.7%) minisub gel and transferred to a Zeta-probe nylon membrane (Bio-Rad) with 0.4 M NaOH.

Hybridization Probes. DNA probes for the TcR α-chain (Pα8; ref. 16), β-chain (86T1; ref. 17), and γ-chain, a 1-kilobase (kb) *Bam*HI/*Ava* I fragment from plasmid 8/10-2γ1.1 (18), were labeled by oligo-labeling with [α-^{32}P]ATP to 1–2 × 10^9 cpm/μg (19).

Immunoprecipitation. Cells (5–10 × 10^5) were surface iodinated by the lactoperoxidase method as described by Goding (20). After the labeling reaction, 5 × 10^6 "filler" cells (70Z3, pre-B-cell line) were added to the washes to reduce the loss of labeled cells. Surface iodinated cells were lysed in a 1% digitonin buffer and then immunoprecipitated by incubating overnight (4°C) with *Staphylococcus aureus* precoated with the appropriate antibody. Immunoprecipitated proteins were resolved by NaDodSO$_4$/PAGE (21) on 10% acrylamide gels in the presence or absence of 50 mM dithiothreitol.

RESULTS

Separation of Subpopulations of CD4$^-$ CD8$^-$ Thymocytes. Adult CBA mouse CD4$^-$ CD8$^-$ thymocytes, prepared by complement-mediated lysis followed by removal of residual CD4 or CD8 positive cells with anti-immunoglobulin-coated magnetic beads, were >99% pure on fluorescence-activated cells sorting analysis. After two-color fluorescent labeling with anti-HSA and anti-Ly 1, they were sorted into four populations, according to the gates shown in Fig. 1. HSA gave a very distinct separation, but Ly 1 gave a more continuous distribution. The two abundant populations, HSA$^+$ Ly 1^{low} and HSA$^-$ Ly 1^{high}, were on average 50% and 29% of CD4$^-$ CD8$^-$ thymocytes. The two minor subpopulations, HSA$^-$ Ly 1^{low} and HSA$^+$ Ly 1^{high}, constituted 12% and 9% of CD4$^-$ CD8$^-$ thymocytes, and for these it required 4–6 hr of sorting to obtain the 5–10 × 10^5 cells used for analyses.

Fluorescence Analysis of Surface CD3 Expression. Two- and three-color fluorescent staining followed by flow-cytometric analysis was used to determine the proportion of cells in each fraction expressing any form of the CD3–TcR complex. Preliminary results of this analysis have been published (10); the full results are summarized in Table 1. The HSA$^+$ Ly 1^{low} subpopulation contained few cells (<2%) expressing CD3, whereas all cells of the HSA$^-$ Ly 1$^+$ subset were CD3$^+$. The minor HSA$^+$ Ly 1^{high} and HSA$^-$ Ly 1^{low} subsets contained a mixture of CD3$^-$ and CD3$^+$ cells.

TcR Surface Protein Analysis. Sorted CD4$^-$ CD8$^-$ thymocyte subsets were surface iodinated and detergent lysates were immunoprecipitated with anti-CD3 antibody. Electrophoresis under nonreducing conditions (Fig. 2*A*) revealed that 78- to 80-kDa material, characteristic of a TcR heterodimer, was detectable in all HSA and Ly 1 subsets, but that the amount varied considerably between subsets. The HSA$^-$ Ly 1^{high} subset expressed the most, the two minor populations (HSA$^+$ Ly 1^{high}, HSA$^-$ Ly 1^{low}) expressed an intermediate amount, and the HSA$^+$ Ly 1^{low} subset expressed trace amounts only. When subjected to electrophoresis under

Table 1. T-cell receptor gene status in subpopulations of CD4$^-$ CD8$^-$ thymocytes

Subpopulation of CD4$^-$ CD8$^-$ thymocytes	% expressing CD3*	% CD3$^+$ cells expressing				DNA rearrangement				mRNA Expression			
						β		γ					
		$\gamma\delta$†	Total $\alpha\beta$	Vβ8 $\alpha\beta$*	Non-Vβ8 $\alpha\beta$‡	GL	R	GL	R	α	β(1.3)	β(1.0)	γ
HSA$^+$ Ly 1$^-$	2 ± 1	2	0	0	0	++	++	+	+++	−	+	++	++
HSA$^+$ Ly 1$^+$	54 ± 27	85	15	<2	15	+	+++	+	+++	±	+	++	++
HSA$^-$ Ly 1$^-$	63 ± 13	66	34	30	4	++	++	+	+++	+	++	+	++
HSA$^-$ Ly 1$^+$	100	23	77	68	9	±	+++	+	+++	++	++	+	++

GL, germ line; R, rearranged.
*Determined directly by surface immunofluorescence.
†Determined by subtracting total $\alpha\beta$ from total CD3.
‡Determined from preclearing experiments, in text.

reducing conditions (Fig. 2*B*), the 78- to 80-kDa bands resolved into three bands: 35 and 45 kDa, typical of the γ and δ monomers, respectively, and a broader band (37–43 kDa) typical of the α and β monomers, which are not resolved under these conditions. Thus, all subsets of CD4$^-$ CD8$^-$ thymocytes expressed a combination of $\alpha\beta$ and $\gamma\delta$ heterodimers. However, the $\alpha\beta/\gamma\delta$ ratio differed markedly, being $\approx$3:1 for the HSA$^-$ Ly 1^{high} subset but only $\approx$1:5 for the HSA$^+$ Ly 1^{high} subset and $\approx$1:2 for the HSA$^-$ Ly 1$^-$ subset. The $\alpha\beta/\gamma\delta$ ratio for the small amount of TcR on the HSA$^+$ Ly 1^{low} group was difficult to estimate since only trace amounts were present in this group; however, the detectable TcR was of the $\gamma\delta$ type. These results differ from the currently held views that $\alpha\beta$ expression is limited to the HSA$^-$ and Ly 1^{high} fractions of CD4$^-$ CD8$^-$ thymocytes, and that $\gamma\delta$ expression is limited to the HSA$^+$ Ly 1$^+$ fraction (7, 11, 12, 14).

It was important to establish to what extent overlap or cross-contamination could explain the mixed $\alpha\beta$ and $\gamma\delta$ forms in the isolated fractions. This was mainly a problem with the minor subsets, and mainly a problem along the Ly 1 axis (since the HSA staining gave a very striking separation, as shown in Fig. 1). When the HSA$^-$ Ly 1^{low} subset was gated very tightly along the Ly 1 axis, the proportion of Vβ8 was not significantly affected. Therefore, it is highly unlikely that the Vβ8$^+$ cells in this subset are derived from overlap from the larger HSA$^-$ Ly 1^{high} Vβ8^{high} subset.

It was also unlikely that the $\gamma\delta$ cells in the HSA$^-$ Ly 1^{high} subset was contributed by other fractions, partly for numerical reasons and partly because no CD3$^-$ cells, or equally likely contaminants, were found in this fraction. The HSA$^+$ Ly 1^{low} fraction, being TcR$^-$, was clearly not contaminated.

The results of the HSA$^+$ Ly 1^{high} fraction of CD4$^-$ CD8$^-$ thymocytes were examined most closely because of the well known difficulty of sorting completely pure "double positives." Several lines of evidence argued against cross-contamination being the source of the mixed $\alpha\beta$ and $\gamma\delta$ TcR in this fraction. Firstly, contamination with the major HSA$^+$ Ly 1^{low} fraction, the most likely because of poor Ly 1 separation, could not explain the presence of either the $\alpha\beta$ or $\gamma\delta$ TcR, since all cells in this fraction are TcR$^-$; however, it may have contributed to the TcR$^-$ component. Secondly, because of the gates chosen and clear staining differences, contamination by simple overlap with HSA$^-$ cells was unlikely. Thirdly, the $\alpha\beta$ TcR in this fraction was quite distinct from that of the HSA$^-$ cells, being low (or negative) for Vβ8 (Table 1). Some experimental checks for contamination were also undertaken. Total HSA$^+$ thymocytes were isolated and subjected to two rounds of fluorescence-activated cell sorting (>98.5% HSA$^+$). Immunoprecipitation with anti-CD3 antibody and PAGE under reducing conditions again revealed 37- to 43-kDa material as well as the 35- and 45-kDa bands. Finally, the possibility of residual contamination of this fraction with mature CD4$^-$ CD8$^+$ or CD4$^+$ CD8$^-$ T cells, which could be Ly 1^{high}, but HSA$^-$ to HSA^{low}, was considered. A thymocyte preparation containing <0.7% residual CD4$^+$ or CD8$^+$ cells was prepared with a still tighter HSA gate, which excluded the lower 30% of HSA$^+$ cells (Fig. 1). Since the CD4$^+$ CD8$^-$ and CD4$^-$ CD8$^+$ cells fall into the lower part of the HSA$^+$ distribution, this should further select against mature contaminants. The sorted HSA$^{very\ high}$ Ly 1^{high} fraction still presented evidence for the same proportion of $\alpha\beta$ TcR$^+$ cells.

Vβ Usage by CD4$^-$ CD8$^-$ Subpopulations. In previous studies (13), we have used fluorescent labeling with the monoclonal antibody F23.1 to determine the proportion of cells in CD4$^-$ CD8$^-$ subsets expressing surface Vβ8; the results are included in Table 1. The striking overusage of products of the Vβ8 gene family was most evident for cells within the HSA$^-$ Ly 1^{high} subset, 70% of which are F23.1$^+$. However, this population also included a significant proportion of $\gamma\delta^+$ cells, so it was not clear whether the TcR of the CD3$^+$ Vβ8$^-$ cells included other non-Vβ8 $\alpha\beta$ receptors. To examine this question directly, CD4$^-$ CD8$^-$ thymocytes were depleted of Vβ8$^+$ cells by lysis with F23.1 antibody and complement. Immunoprecipitation of this Vβ8$^-$ CD4$^-$ CD8$^-$ population (>99.3% F23.1$^-$) with anti-CD3 brought down 37- to 43-kDa material (Fig. 3*A*), representing the non-Vβ8 TcR$\alpha\beta$ heterodimer, as well as the 35- and 45-kDa bands. To obtain an estimate of the relative contribution of Vβ8 and non-Vβ8 gene products to TcR$\alpha\beta$ expression within the HSA$^-$ CD4$^-$ CD8$^-$ subsets, lysates prepared from this fraction were precleared by precipitation with F23.1 and then immunoprecipitated with anti-CD3. A mixture of $\alpha\beta$ and $\gamma\delta$ heterodimers was obtained (Fig. 3*B*, lane B). A comparison of the intensity of the anti-CD3 precipitable 37- to 43-kDa material from the F23.1 precleared (lane B) and control lysate (lane E) indicated that 10–15% of the $\alpha\beta$ heterodimers expressed by the HSA$^-$ CD4$^-$ CD8$^-$ thymocytes do not use Vβ8 gene products. Taken together, these two lines of evidence indicate that there is an exceptionally high, but not exclusive, usage of Vβ8 gene products by the TcR$\alpha\beta^+$ HSA$^-$ CD4$^-$ CD8$^-$ thymocytes.

For the HSA$^+$ CD4$^-$ CD8$^-$ cells, however, very little of the TcR$\alpha\beta$ precipitated with anti-CD3 was also precipitated with F23.1 (results not shown), indicating that no more than a small proportion of the TcR$\alpha\beta^+$ cells utilized Vβ8 gene products. Direct fluorescent labeling with F23.1 also failed to detect positive cells in the HSA$^+$ population (ref. 13; Table 1). In neither case was the sensitivity adequate to distinguish between a low (<30%) Vβ8 usage and a zero usage.

TcRγ Gene Rearrangement and Expression. To examine for TcRγ gene rearrangement, DNA prepared from the HSA and Ly 1 defined CD4$^-$ CD8$^-$ subpopulations, and from CBA

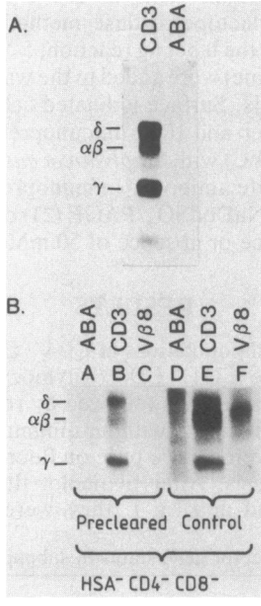

FIG. 3. Vβ region usage by surface TcR of CD4$^-$ CD8$^-$ thymocytes. (*A*) CD4$^-$ CD8$^-$ Vβ8$^-$ thymocytes were isolated by depletion using anti-CD4, anti-CD8, and F23.1. After labeling of surface proteins with ^{125}I, lysates were prepared and immunoprecipitated with anti-CD3 (CD3) or as a control with the irrelevant anti-azobenzenearsonate antibody (ABA). PAGE was performed under reducing conditions. (*B*) Lysates prepared from ^{125}I surface labeled HSA$^-$ CD4$^-$ CD8$^-$ thymocytes were split into two equal fractions. One fraction was precleared with F23.1 (anti-Vβ8; ref. 23) before being immunoprecipitated with either F23.1 or anti-CD3 as analyzed above. The other fraction was immunoprecipitated directly, without preclearing.

mouse kidney as a germ-line control, were digested with *Eco*RI, separated on a 1% agarose gel, and RNA blot filters were hybridized with a γ-chain constant region probe (Cγ) (Fig. 4). In kidney DNA, the Cγ probe hybridized to bands at 10.5 and 15.0 kb. In all thymus-derived samples, including all four CD4$^-$ CD8$^-$ subsets, the intensity of the 10.5-kb band was markedly reduced and additional bands >17 kb that could not be separated were detected. This suggested that TcRγ gene rearrangement was well advanced in most cells in all CD4$^-$ CD8$^-$ subsets and that T-lineage cells were the major component of each subset. RNA blot analysis of the HSA/Ly 1 subsets using the Cγ probe revealed a strong signal for full-length γ message in all CD4$^-$ CD8$^-$ subsets (Fig. 5).

TcRβ Gene Rearrangement. Additional samples of DNA were digested with *Hin*dIII and hybridized with a β-chain constant region probe (Cβ). In interpreting the results in Fig. 4*B*, note that the 9.5-kb band corresponds to Cβ1 and the 3.0-kb band corresponds to Cβ2. Rearrangement to either β-chain constant region reduces the intensity of the 9.5-kb band through deletion or rearrangement of Cβ1, but it does not affect the 3.0-kb fragment, which therefore acts as an internal control (24).

The results shown indicate that the 9.5-kb (Cβ1) band was markedly reduced in the two Ly 1^{high} subsets (HSA$^+$ Ly 1^{high}, HSA$^-$ Ly 1^{high}), indicating extensive rearrangement. On the other hand, the two Ly 1^{low} subsets displayed a prominent 9.5-kb band. Each of these subsets had about half its TcRβ genes in germ-line configuration, but clearly some rearrangement had occurred.

TcRα and -β mRNA Expression. The RNA blot filter, which had previously been hybridized with a Cγ probe (Fig. 5*A*), was stripped and rehybridized successively with Cβ and Cα probes (Fig. 5 *B*, *C*, and *D*). The Cβ probe detected 1.0- and 1.3-kb message in all CD4$^-$ CD8$^-$ subsets, but the ratio of mature (1.3 kb) to truncated (1.0 kb) message varied widely between the subsets. In both the HSA$^+$ populations, the majority of the TcRβ mRNA was 1.0 kb, representing expression from a partially rearranged gene. In the HSA$^-$ Ly 1^{high} and HSA$^-$ Ly 1^{low} subsets, the pattern of TcRβ expression was more typical of lymph node T cells, with more mature (1.3 kb) than truncated (1.0 kb) message. For the HSA$^-$ Ly 1^{high} subset, this was consistent with the finding that β-gene rearrangement was virtually complete and the αβ forms of the TcR were expressed on the surface of most cells. For the HSA$^-$ Ly 1^{low} subset, this mature pattern of TcRβ expression indicated that the rearrangement that had occurred in this population was mostly complete, which was consistent with the finding that some cells in this subset expressed TcRαβ on the surface.

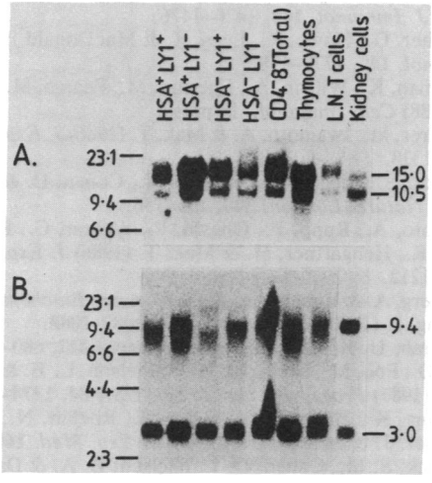

FIG. 4. TcRγ and -β gene rearrangement by CD4$^-$ CD8$^-$ thymocyte subsets. DNA was prepared from the subsets defined in Fig. 1, as well as from other control populations as indicated, using the low gelling temperature agarose-embedding technique. DNA was digested with *Eco*RI and hybridized with a Cγ probe (*A*) or *Hin*dIII and hybridized with a Cβ probe (*B*) in 50% formamide/4× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/7% (wt/vol) NaDodSO$_4$/0.5% skim milk powder/0.5 mg of denatured salmon sperm DNA per ml/1% (wt/vol) polyethylene glycol 6000 containing 1–2 × 10^6 cpm/ml at 42°C for 18 hr. Final wash was in 0.5× SSC/1% NaDodSO$_4$ for 30 min at 65°C. Autoradiography was done with Amersham Hyperfilm with two Cronex Lightning Plus intensifying screens at −70°C for 5 days. Numbers are kb.

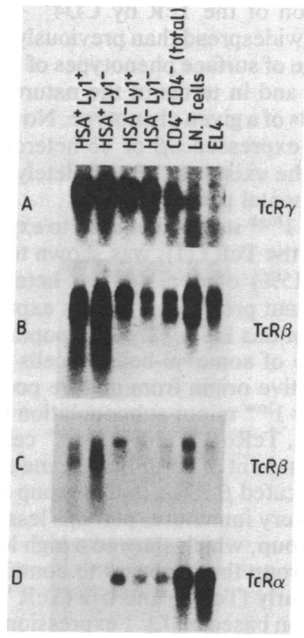

FIG. 5. TcR mRNA expression by CD4$^-$ CD8$^-$ thymocyte subsets. Total cytoplasmic RNA was isolated from the subsets defined in Fig. 1, as well as from the other control population indicated. RNA was separated on a 1% agarose/formaldehyde gel, transferred to Hybond-N (Amersham), and hybridized to ^{32}P-oligolabeled DNA probes as indicated. Filters were prehybridized for 4 hr at 42°C in 50% formamide/3× SSC/1× Denhardt's solution (0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/0.03 mg of denatured salmon sperm DNA per ml. Hybridization was in the same buffer containing 1–2 × 10^6 cpm/ml for 18 hr at 42°C. Membranes were rinsed in 2× SSC/0.1% NaDodSO$_4$ and then washed in 0.5× SSC/0.1% NaDodSO$_4$ for 30 min at 65°C. Autoradiography, as described in legend to Fig. 4, was for 1 day (*C*) or 5 days (*A*, *B*, and *D*).

Hybridization with a Cα probe detected abundant levels of message in the HSA$^-$ Ly 1$^+$ subset and a lower level in the HSA$^-$ Ly 1$^-$ subset, consistent with the level of surface expression of αβ heterodimers by these subsets. No α message was detected in the HSA$^+$ Ly 1$^-$ subset, which expresses no surface TcRαβ. TcRα message was not detected in the HSA$^+$ Ly 1$^+$ subset, despite the fact that αβ heterodimer was detected by immunoprecipitation. This may have been due to the limited amount of material (≈1 μg total RNA) available for analysis. To confirm this, RNA was prepared from a large quantity of total HSA$^+$ CD4$^-$ CD8$^-$ thymocytes (5 × 10^6). When hybridized with a Cα probe, a small amount of mature TcRα message was detected (result not shown). Since the HSA$^+$ Ly 1$^-$ population was surface TcRαβ$^-$ and did not express TcRα message, this TcRα mRNA must have come from the HSA$^+$ Ly 1^{high} subset.

DISCUSSION

The results presented in this study, using direct analysis of freshly isolated thymocyte subpopulations, demonstrate that

surface expression of the TcR by CD4$^-$ CD8$^-$ adult thymocytes is more widespread than previously thought, both in terms of the range of surface phenotypes of the cells expressing surface TcR and in terms of the nature of the receptor expressed by cells of a given phenotype. No subset was found that exclusively expressed $\alpha\beta$ or $\gamma\delta$ heterodimers, perhaps arguing against the existence of completely independent $\alpha\beta$ and $\gamma\delta$ developmental pathways.

The HSA$^-$ Ly 1^{high} subset, believed to express exclusively the Vβ8 form of the TcR (11), was shown to express a small proportion (10–15%) of non-Vβ8 $\alpha\beta$ heterodimers and to include a significant proportion of cells expressing $\gamma\delta$ receptors. The origin of this HSA$^-$ Ly 1^{high} population is obscure, but the presence of some $\gamma\delta$-bearing cells argues against a purely late selective origin from mature postthymic T cells.

The HSA$^-$ Ly 1^{low} minor subpopulation appeared to be a mixture of TcR$^-$, TcR$\alpha\beta^+$, and TcR$\gamma\delta^+$ cells. The presence of a substantial amount of germ-line β gene and the relatively low level of truncated β RNA in this group suggests that the TcR$^-$ cells are very immature, perhaps less mature than the HSA$^+$ Ly 1$^-$ group, which showed a high level of immature message. This group then appears to contain quite separate groups of very early (TcR$^-$) and late (TcR$^+$) cells. The lack of clear separation based on Ly 1 expression complicated the analysis of the TcR expressed on this subpopulation; however, even very tight gating of this population did not significantly affect the proportion of $\alpha\beta^+$ cells (as discussed above), suggesting that the TcR $\alpha\beta^+$ cells in this group were not derived from overlap with the HSA$^-$ Ly 1^{high} subgroup.

The HSA$^+$ Ly 1^{high} minor subgroup, which expressed a high proportion of TcR$\gamma\delta$ as expected from previous studies (14), also included a proportion of cells expressing $\alpha\beta$ receptors. It was of particular interest that the β chains used by this subgroup did not show the exceptionally high usage of Vβ8 characteristic of the HSA$^-$ subsets. Because of the limited material available we could not determine whether the Vβ8 frequency was similar to that in the periphery (20–30%) or if Vβ8 products were underused or excluded.

The HSA$^+$ Ly 1$^-$ cells, the major subset, had partially rearranged β genes, immature β message, no α message, and little surface protein, suggesting an intermediate population in the process of β rearrangement and expression.

The fact that all subsets of CD4$^-$ CD8$^-$ thymocytes had a high level of rearranged TcRγ genes and expressed high levels of γ message indicates that all the isolated subsets were predominantly T cells and suggests that, in the adult, either rearrangement and expression of this locus are very early events, occurring soon after the stem cells seed the thymus, or, alternatively, that rearrangement is occurring prethymically. On the other hand, β-locus rearrangement is clearly an ongoing intrathymic event.

All subsets expressing $\alpha\beta$ heterodimers on the cell surface showed, as expected, rearranged TcRβ genes and expressed α mRNA and mature forms of β mRNA. Any attempt to map the sequence of developmental events leading to TcR$\alpha\beta$ expression must exclude those subsets already "mature" in this respect. Our recent data (5) on the reconstitution of T-lineage cells by intrathymic transfer of CD4$^-$ CD8$^-$ subsets supports the view that the earliest precursors are amongst cells that are CD4$^-$ CD8$^-$ HSA$^-$ Ly 1^{low} and CD3$^-$. The data in this paper support this interpretation and demonstrate the presence in the HSA$^-$ Ly 1^{low} subset of some cells with unrearranged β genes but perhaps rearranged γ genes. However, further analysis of the γ gene in the CD3$^-$ cells amongst the HSA$^-$ Ly 1^{low} population is required to clarify the status of these putative early thymocytes.

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