

Qa-2 EXPRESSION IN THE ADULT MURINE THYMUS

A Unique Marker for a Mature Thymic Subset¹

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The MHC Ag, Qa-2, is expressed on all peripheral T cells, a subset of bone marrow cells, and to a lesser extent on B cells. The Qa-2 Ag is also expressed on 5 to 6% of normal adult murine thymocytes. Through the use of flow cytometry, counterflow centrifugal elutriation and acridine orange staining, we have analyzed the cell surface phenotype, cell size, and cell cycle status of this thymic population. Our studies indicate that Qa-2⁺ thymocytes are large, non-mitotic, G₁ cells which have the cell surface phenotype of CD5⁺, CD3⁺, J11d^{LO} and lack receptors for peanut agglutinin. This population can be further subdivided into three categories; CD4⁺/CD8⁻, CD4⁻/CD8⁺, and CD4⁻/CD8⁻. These data indicate that Qa-2 surface expression can only be detected on thymocytes in the final stages of differentiation. The Qa-2 Ag can be used as a cell surface marker to identify a unique subset of mature thymocytes.

The identification of small thymic subsets and their role in T cell differentiation has been an area of intense investigation. The importance of this area lies in the fact that thymic education accounts for the selection of peripheral T cells which have the ability to recognize and respond to foreign Ag in the context of MHC but are tolerant to the presentation of self-Ag (1, 2). Many details of this selection and differentiation process remain a mystery. According to our present understanding, the differentiation process begins with a precursor thymocyte population (3). These immature cells are able to recolonize an irradiated thymus and are characterized by their lack of expression of the T cell Ag CD4 and CD8, and their low level of CD5 expression (4). According to current models the progeny of this stem cell population undergo a series of selection events which give rise to the different cell types found in the thymus. One of these events is a negative selection step during which thymo-

cytes bearing inappropriate TCR complexes, such as self-reactive cells, are eliminated (5, 6). The thymocytes that have been selected for elimination are most likely contained within the cortical population characterized by the expression of both CD4 and CD8, and their small size (7). However, a minority of thymocytes is spared from the deletion pathway and undergo further differentiation, acquiring a cell surface phenotype characteristic of peripheral T cells, i.e., Thy-1⁺, CD5⁺, and either CD4⁺/CD8⁻ or CD4⁻/CD8⁺ (8, 9). Within this phenotypically mature population are contained all thymocytes capable of displaying T cell functions (8-11). Consequently this mature population is likely to contain all of the thymocytes that will exit the thymus and participate in the peripheral immune response (10, 11). It has become a priority to distinguish the different subsets of thymocytes that make up the mature population to learn the requirements that must be achieved before a cell can exit the thymus.

The cell surface form of Qa-2 is a 40,000 M_r glycoprotein anchored in the cell membrane by means of a phosphatidylinositol bearing glycolipid (12-14). This non-polymorphic class I Ag is expressed on all peripheral T cells and to a lesser extent on peripheral B cells (15, 16). It has been known for some time that a small population of thymocytes also express this determinant (15, 17). It was therefore hypothesized that this Qa-2⁺ thymic subset might describe a population of thymocytes similar to peripheral T cells in phenotype and function. This idea was supported by the recent studies performed by Rabinowitz et al. (18) in which it was demonstrated that the Qa-2⁺ thymocytes are contained within the steroid resistant fraction of cells, that they all express high levels of CD5 and that they can be found in both CD8⁺ as well as CD8⁻ subsets of thymocytes. Furthermore, depletion studies showed that removal of Qa-2⁺ cells eliminated the ability of thymocytes to generate an effective CTL response (18). All of these data indicate that the Qa-2⁺ thymocytes are most likely members of the mature thymic population.

To identify the precise position occupied by Qa-2⁺ cells in the spectrum of T cell programming and differentiation, we performed flow cytometric analyses of the cell surface phenotype, relative cell size, and the cell cycle status of this thymic subset. By correlating the characteristics of Qa-2⁺ thymocytes with those of other mature subsets we have shown that the Qa-2⁺ thymocytes are a subset contained entirely within the mature thymic population, and that the expression of Qa-2 can be used as a criteria by which phenotypically mature thymocytes can be further subdivided.

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MATERIALS AND METHODS

Mice. B6.K3 (H-2^k, Qa-2^a), B6.K4 (H-2^k, Qa-2^b), and C57BL/6 (H-2^b, Qa-2^a) mice were maintained in the Oncology Center animal colony at Johns Hopkins University School of Medicine, Baltimore, MD (19, 20). Mice were used at 6 to 12 wk of age for all experiments.

Antibodies and fluorescent reagents. In most studies Qa-2 Ag expression was detected on strain B6.K3 lymphoid cells using the purified mAb 20.8.4. In this genetic setting, the mAb 20.8.4 recognizes exclusively the Qa-2 Ag (20). The mAb L-243 (IgG2a, anti HLA-DR) served as a negative control (21).

mAb, 20.8.4 (H-2K^b, Qa-2) and L-243 (HLA-DR) were affinity purified and coupled to fluorescein isothiocyanate (U. S. Biochemical Corporation, Cleveland, OH). F(ab) fragments were generated from 20.8.4 by papain cleavage (22) and this preparation was also coupled to FITC. The mAb, Mel-14.D54 (lymph node homing receptor) (23) and J11d.2 (24) were purified using a mouse anti-rat-Sepharose affinity column and conjugated with Sulfo-NHS-biotin (Pierce Biochemical, Rockford, IL) as was the secondary reagent, F(ab')₂ sheep anti-mouse IgG Fc (Pel-Freez Biologicals, Rogers, AK). Biotin conjugates of mAb 53-6.7 (CD8, Lyt-2) and 53.7.3 (CD5, Lyt-1) as well as PE conjugated GK1.5 (CD4, L3T4) were purchased from Becton Dickinson (Mountain View, CA). PE-avidin was purchased from Biomed (Foster City, CA), fluorescein-labeled avidin DCS was purchased from Vector Laboratories (Burlingame, CA). APC-streptavidin was obtained from Becton Dickinson and FITC conjugate F(ab')₂ sheep anti-mouse IgG Fc was purchased from Pel-Freez Biologicals. The hamster mAb, 2C11 (CD3) was used in the form of hybridoma supernatant as described elsewhere (25). The mAb Qa2 (Qa-2) (26) was used to detect Qa-2 expression on C57BL/6 cells in the elutriation studies.

PNA fractionation. The PNA fractionation procedure has been described previously (27). In brief PNA³ (Vector Laboratories) was

³ Abbreviations used in this paper: PE, phycoerythrin; PNA, peanut agglutinin; PNA⁻, thymocytes lacking receptors for PNA; APC, allophycocyanin; AO, acridine orange; MLN, mesenteric lymph node.

diluted to a final concentration of 20 µg/ml in 50 mM Tris HCl, pH 9.5, 150 mM NaCl. Of this solution 4 ml were added to each tissue culture petri plate (Falcon 1029, Falcon Labware, Oxnard, CA) and the PNA was allowed to adhere to the plastic surface 120 min at room temperature. The solution was aspirated from the plates and the treated surface was then washed twice with 20 ml of DPBS and once with 20 ml PBS/1% FCS. A total of 3.5×10^7 thymocytes, at a concentration of 1×10^7 cells/ml in Dulbecco's PBS/5% FCS, were applied to each PNA coated plate. The plates were incubated for 90 min at 4°C. Non-adherent cells in the supernatant were collected with a Pasteur pipette. Any remaining non-adherent cells were collected by gently washing the plates with 10 ml PBS/1% FCS and pooling the resulting cell suspension with the cells collected above. Adherent cells were removed from the plate surface by adding 5 ml PBS/1% FCS/0.2 M galactose and incubating for 5 min at room temperature. The adherent cells were then collected and any cells remaining on the plates were removed by vigorous washing with an additional 5 ml of PBS/1% FCS/0.2 M galactose. In a typical fractionation, 18% of thymocytes were non-adherent to the PNA plates.

Immunofluorescent staining. For single-color analysis, thymocytes were incubated with either Qa2 or MEL-14-biotin. In the case of the MEL-14-biotin samples, antibody binding was detected by adding FITC-avidin. In the case of anti-Qa2 samples, FITC coupled F(ab')₂ sheep anti-mouse IgG Fc was used as a secondary reagent. Analysis was performed on a Coulter Epics flow cytometer (Coulter Electronics, Inc., Hialeah, FL).

For two-color analysis, staining was performed in two stages. The first stage consisted of one of the following biotinylated antibodies: α-CD5, J11d, or MEL-14. The second stage consisted of 20.8.4-FITC and PE-avidin. In the case of two-color analysis with α-CD3, FITC conjugated F(ab) fragment of 20.8.4 was used in the first stage along with the α-CD3 antibody. α-CD3 binding was detected with biotin labeled F(ab')₂ sheep anti-mouse IgG Fc followed by PE-avidin.

For three-color analysis, the first step consisted of α-Qa-2-FITC and α-CD8-biotin followed by incubation with α-CD4-PE and APC-avidin. Analysis was carried out on a Coulter Epics flow cytometer

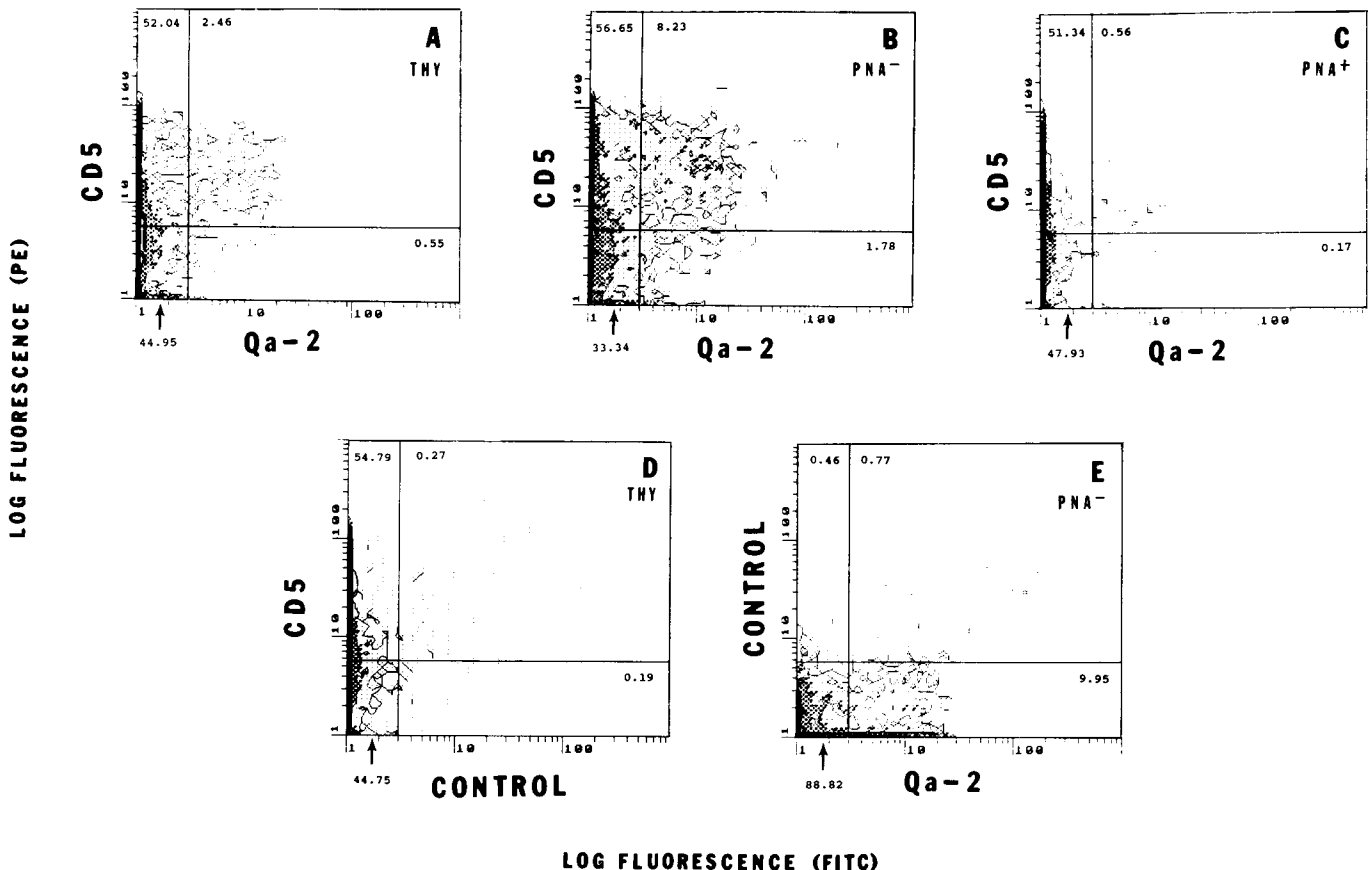


Figure 1. Correlation of Qa-2 and CD5 expression on total and fractionated thymocyte populations. Unseparated thymocytes (A) or thymocytes fractionated into PNA⁺ (C) and PNA⁻ (B) populations were stained with reagents specific for CD5 (biotinylated 53.7.3 plus PE-avidin) and Qa-2 (20.8.4-FITC). The two parameter histograms illustrate the levels of PE and FITC fluorescence as analyzed by flow cytometry. Background levels of fluorescence were determined by staining identical cell samples with secondary reagent alone or with an irrelevant antibody (L243-FITC) (D and E). These levels of background fluorescence were used to set the position of the horizontal and vertical lines which indicate levels of positive fluorescence. Cell percentages are indicated for each quadrant.

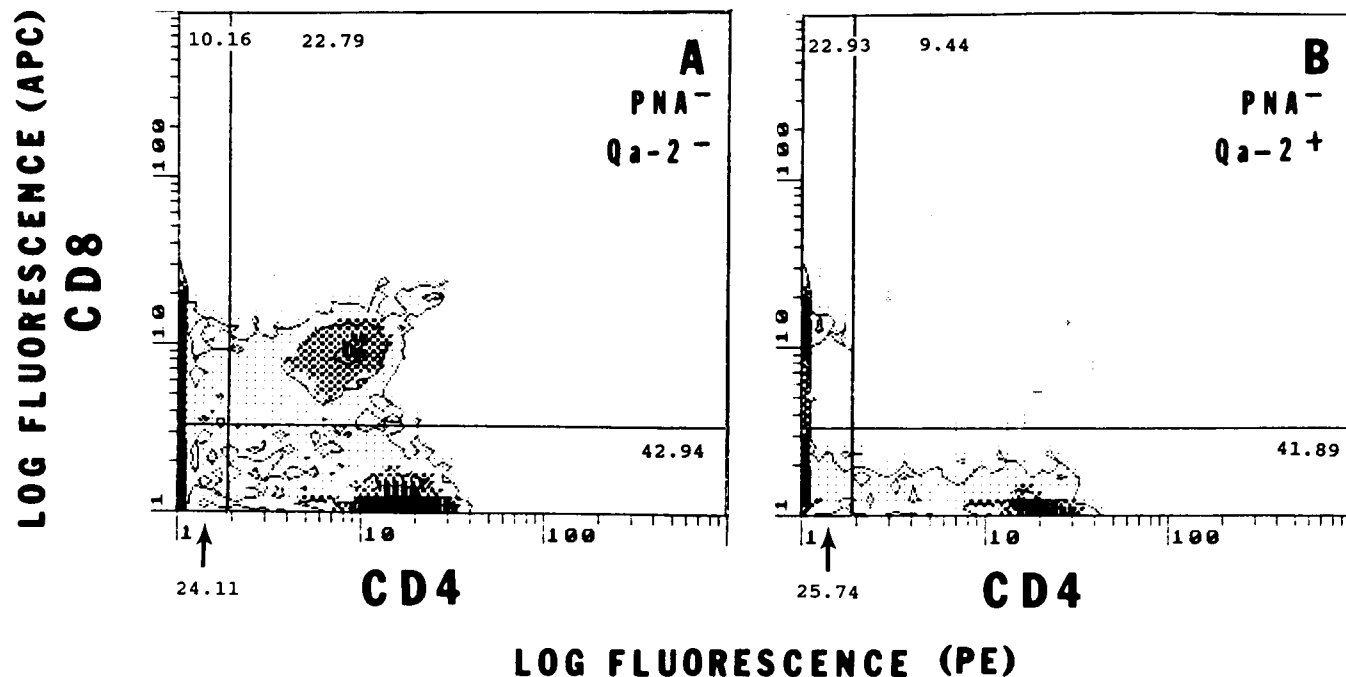


Figure 2. CD4 and CD8 expression on Qa-2⁺ and Qa-2⁻ thymocytes. PNA⁻ thymocytes were incubated with reagents detecting Qa-2 (20.8.4-FITC), CD4 (GK1.5-PE), and CD8 (53-6.7-biotin plus APC-streptavidin). Bit map gating on green (FITC) fluorescence during flow cytometry allowed analysis of CD4 and CD8 expression on Qa-2⁻ (A) and on Qa-2⁺ (B) populations. The two parameter histograms represent levels of PE and APC fluorescence with the horizontal and vertical lines indicating levels of positive fluorescence. The percentage of cells in each quadrant is indicated.

equipped with a dye laser. FITC and PE were excited at 488 nm whereas APC was excited at 600 nm. Emission from these fluorescent molecules were separated by means of a 575 band pass filter which allowed detection of PE, a 525 band pass filter which allowed detection of FITC, and a 630 long pass filter for the detection of APC emission. Qa-2⁺ and Qa-2⁻ populations were separated by gating on log green fluorescence and these gated populations were analyzed simultaneously for PE and APC fluorescence levels.

In all cases, cell samples consisted of 1×10^6 cells at a concentration of 1×10^7 cells/ml. Staining reactions were carried out for 45 min at 0°C in PBS containing 2 mM Na Azide, 2% calf serum. Samples were washed twice between stages of reagent addition and just before FACS analysis in PBS/2% FCS. Saturating amounts of antibodies and secondary reagents were used in each case. Each two parameter histogram represents data collected from 10,000 cells. For each figure, horizontal and vertical lines indicating levels of positive fluorescence were set according to the levels of background fluorescence obtained with an irrelevant antibody (L243-FITC for background green) or secondary reagents alone.

Cell cycle analysis. Cells were stained and analyzed as follows (28): 2×10^5 cells at a concentration of 1×10^6 cells/ml in PBS/10% FCS were placed on ice. Then 0.4 ml of ice cold 0.1% Triton X-100, 0.08 M HCl, 0.15 M NaCl were added to the sample and the mixture was incubated for 15 s on ice. A total of 1.2 ml of ice cold 0.2 M sodium phosphate, 0.1 M citric acid buffer, pH 6, containing 20 μ M AO (Polysciences, Inc., Warrington, PA), 1 mM NaEDTA, 0.15 M NaCl was added. DNA/RNA levels were determined by exciting AO stained cells at 488 nm and measuring emission at 530 nm (RNA levels) and >600 nm (DNA levels).

Counterflow centrifugal elutriation. The separation of murine thymocytes by counterflow centrifugal elutriation has been previously described (29). Briefly, the thymocytes were injected into the inlet stream of a Beckman J-6M centrifuge equipped with a JE-6B elutriator rotor and standard chamber (Beckman Instruments, Palo Alto, CA) and a peristaltic pump (Cole-Palmer Instrument Co., Chicago, IL). Flow rates were determined gravimetrically with a Mettler 3600 balance interfaced to an IBM personal computer. The cells were loaded into the elutriation chamber at a flow rate of 13 ml/min, rotor speed of 3000 rpm, and a temperature of 19°C. Rotor speed was held constant and the cells were eluted by changing flow rates. Flow was increased from 13 to 18 ml/min and first fraction collected by eluting to exhaustion (400 ml collection volume). This procedure was repeated stepwise at 20, 24, and 28 ml/min flow rates. Cells remaining in the chamber, designated the rotor-off fraction, were collected by continuing medium flow after stopping the rotor. The elutriation medium consisted of 0.9% normal saline solution with 100 mg/dl d-glucose, 0.3 mM EDTA, and 50 mg/dl BSA

(Sigma Chemical Co., St. Louis, MO). The pH was adjusted to 7.20 and the medium was sterilized before use.

RESULTS

Qa-2 and CD5 expression on normal and PNA fractionated thymocytes. It had been previously shown that the percentage of Qa-2 expressing thymocytes was increased by cortisone treatment, a procedure that eliminates most of the immature thymic population (18, 30). These Qa-2⁻ cortisone resistant cells were also shown to express high levels of the Ag CD5 (18). However, one concern when using steroid treatment to obtain mature thymocyte populations is the possibility of inducing the expression of cell surface markers that are not normally present. The ability of thymocytes to bind to the lectin PNA has long been used as a marker to isolate cells in various stages of thymocyte differentiation (27, 31, 32).

Thymocytes from adult B6.K3 mice were fractionated by PNA panning and the resulting cell populations were analyzed for Qa-2 and CD5 expression by flow cytometry (Fig. 1). Fig. 1A illustrates the profile of Qa-2 and CD5 expression on normal unfractionated thymocytes. Approximately 3 to 6% of the cells expressed Qa-2 and all of these also expressed the CD5 Ag. In the PNA⁻ thymic population, the proportion of Qa-2⁺ cells increased to 10 to 15% and again all of the cells that expressed Qa-2 also expressed CD5 (Fig. 1B). No Qa-2⁺ cells could be detected in the population expressing receptors for PNA population (Fig. 1C). These results suggested that all of the Qa-2⁺ cells were also CD5⁺ and that this population resided entirely within the PNA⁻ subset. Also illustrated in Figure 1 are the background levels of green (Fig. 1D) and red fluorescence (Fig. 1E) which were obtained with appropriate control reagents. These control experiments are representative of those used to determine the level of positive fluorescence (horizontal and vertical lines) in each of the experiments to follow. It should be noted that

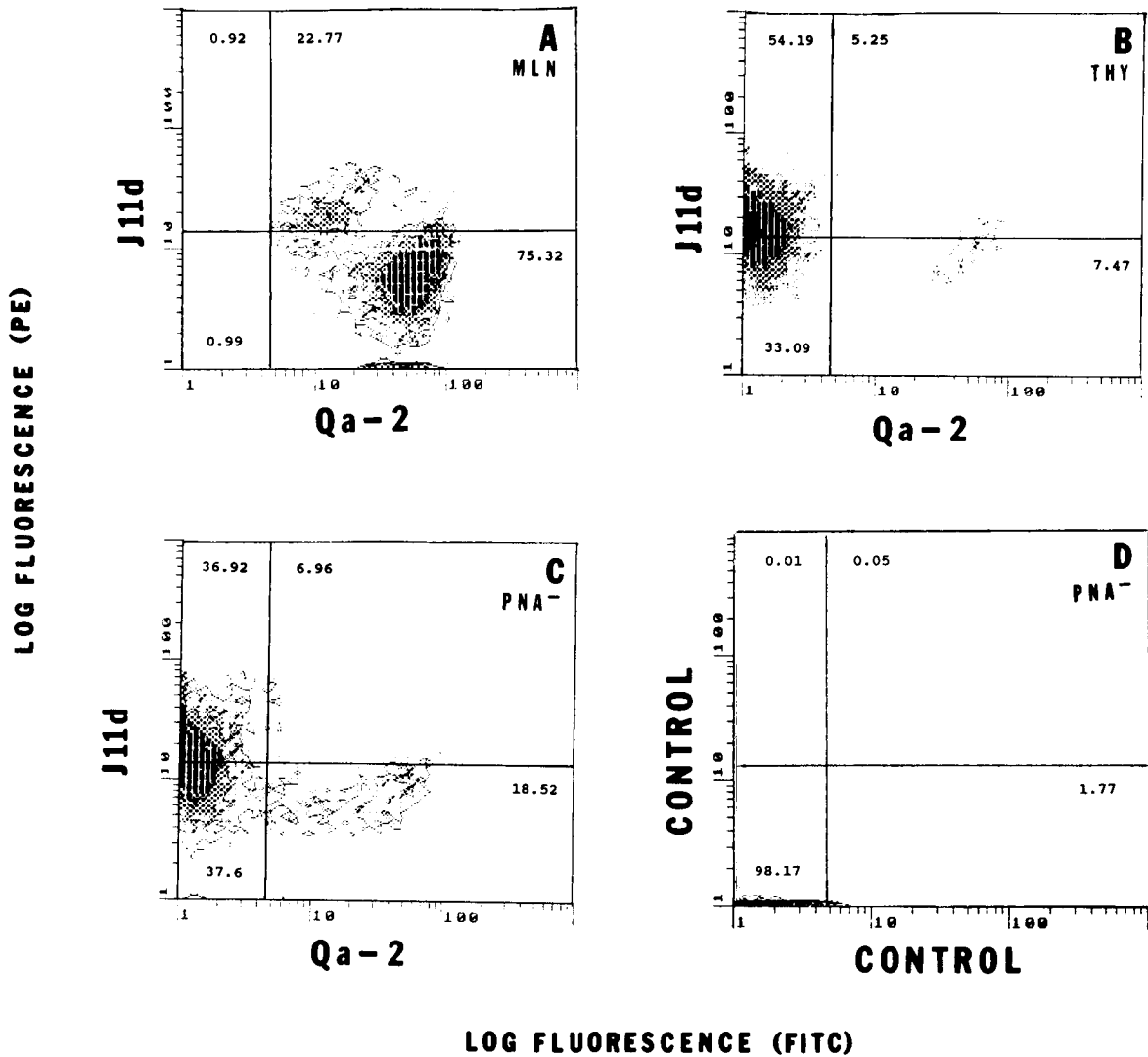


Figure 3. Expression of Qa-2 and the J11d Ag on MLN cells and thymocytes. MLN cells (A), unseparated thymocytes (B), or PNA⁻ thymocytes (C), were incubated with fluorescent reagents recognizing Qa-2 (20.8.4-FITC) and the J11d antigen (J11d.2-biotin plus PE-avidin). The two parameter histograms indicate levels of PE and FITC fluorescence as measured by flow cytometry. In this case the vertical lines indicate the level of positive Qa-2 expression whereas the horizontal line differentiates the J11d^{Lo} and J11d^{Hi} levels of expression. Cell percentages are indicated in each quadrant. The level of background fluorescence with an irrelevant antibody (L243-FITC) and with secondary reagents alone is shown in D.

no Qa-2⁺ cells could be detected when thymocytes from B6.K4 (Qa-2^b) mice were analyzed (data not shown) (20).

CD4 and CD8 expression. Thymocytes can be categorized into stages of differentiation based on their pattern of CD4 and CD8 expression (33). Typically, the vast majority of adult thymocytes is CD4⁺/CD8⁺ (85%) whereas a small subpopulation expresses a mature phenotype of CD4⁺/CD8⁻ (8%), CD4⁻/CD8⁺ (3%), or CD4⁻/CD8⁻ (3%). The levels of CD4 and CD8 expression on Qa-2⁺ and Qa-2⁻ thymocytes were measured by three-color flow cytometry (Fig. 2). When the Qa-2⁻, PNA⁻ cells were analyzed, CD4⁺/CD8⁺ cells were detected, as well as significant levels of single-positive and double-negative cells (Fig. 2A). When the Qa-2⁺, PNA⁻ cells were analyzed, this population was almost exclusively of the single-positive or double-negative phenotype with 42% CD4⁺/CD8⁻, 23% CD4⁻/CD8⁺, and 26% double negative (Fig. 2B). When either unfractionated thymocytes or steroid resistant thymocytes were analyzed, the Qa-2⁺ cells contained similar proportions of CD4⁻/CD8⁺, CD4⁺/CD8⁻, or the CD4⁻/CD8⁻ sets with no significant population of CD4⁺/CD8⁺ cells observed (data not shown).

Based on these studies, Qa-2⁺ thymocytes can be either single-positive or double-negative for CD4/CD8. Furthermore, it should be noted that the Qa-2⁺ cells represent a subpopulation of these cells because a significant number of CD4/CD8 single-positive or double-negative thymocytes are Qa-2⁻.

J11d expression. The mAb, J11d, recognizes several cell surface Ag that are expressed at high levels on immature thymocytes, unprimed B cells, and red blood cells. However, peripheral T cells and thymocytes with phenotypic and functional characteristics equivalent to peripheral T cells, have a reduced level of J11d Ag expression (34). To correlate J11d Ag expression with levels of Qa-2 expression, we performed two-color flow cytometry on MLN cells, normal thymocytes, and PNA⁻ thymocytes (Fig. 3).

The pattern of J11d and anti-Qa-2 staining on MLN cells was as expected. The Qa-2^{Lo} population, made up largely of peripheral B cells (20), had a high level of J11d Ag expression when compared to the Qa-2^{Hi} T cell population (Fig. 3A). We used these high and low levels of J11d staining in the MLN as a guide to our interpretation

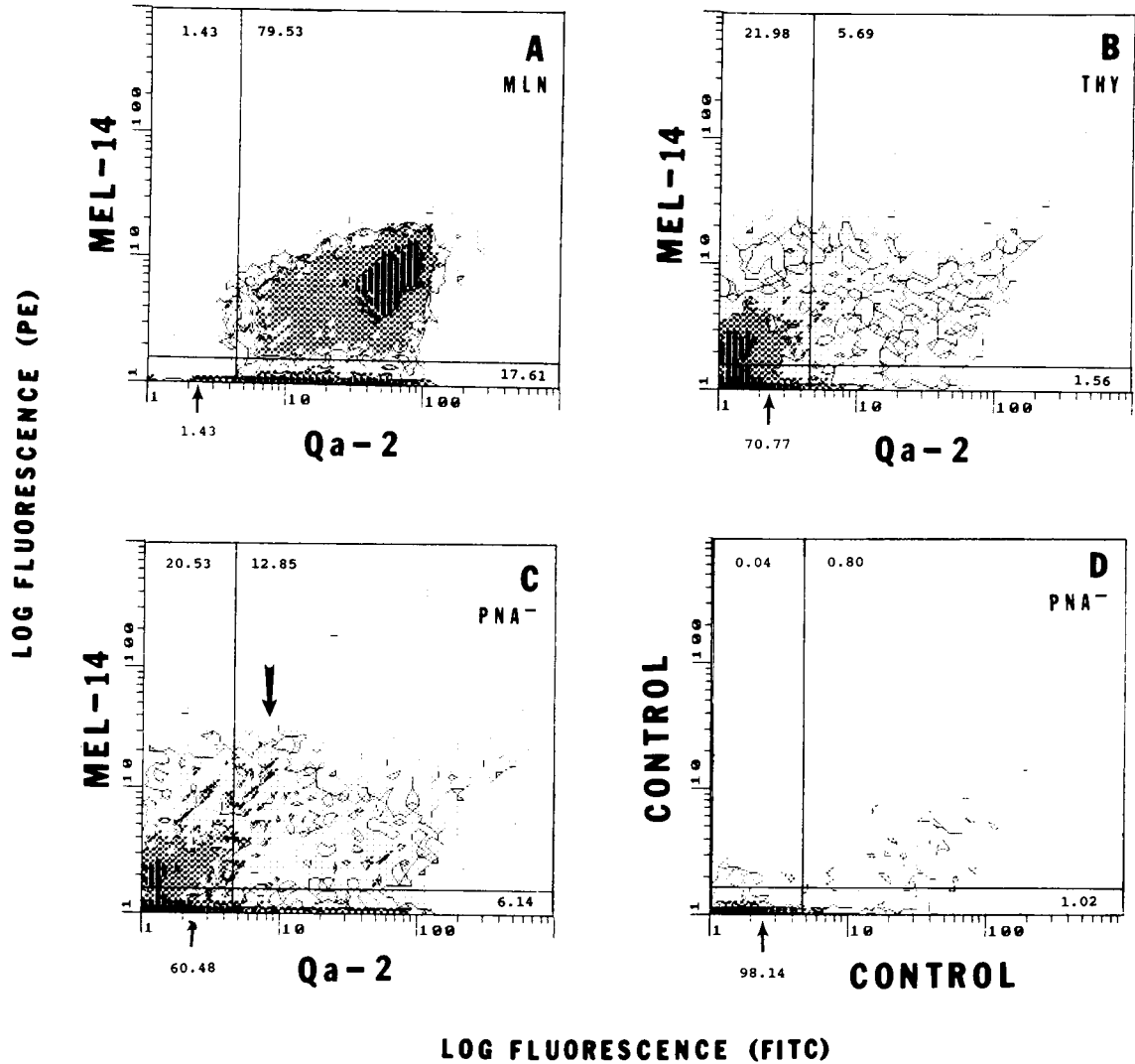


Figure 4. Qa-2 and MEL-14 antigen expression on MLN cells and thymocytes. MLN cells (A), unseparated thymocytes (B) and PNA⁻ thymocytes (C) were stained with fluorescent reagents specific for Qa-2 (20.8.4-FITC) and the lymph node homing receptor (MEL-14.D54-biotin plus PE-avidin). Each panel illustrates the fluorescent levels of PE and FITC as measured by FACS in the form of a two parameter histogram. The horizontal and vertical lines correspond to levels of positive fluorescence. The arrow (C) identifies the MEL-14^{HI} population which expresses reduced levels of the Qa-2 Ag (see text). Percentage of cells found in each quadrant is shown. D illustrates the level of background fluorescence obtained with control reagents.

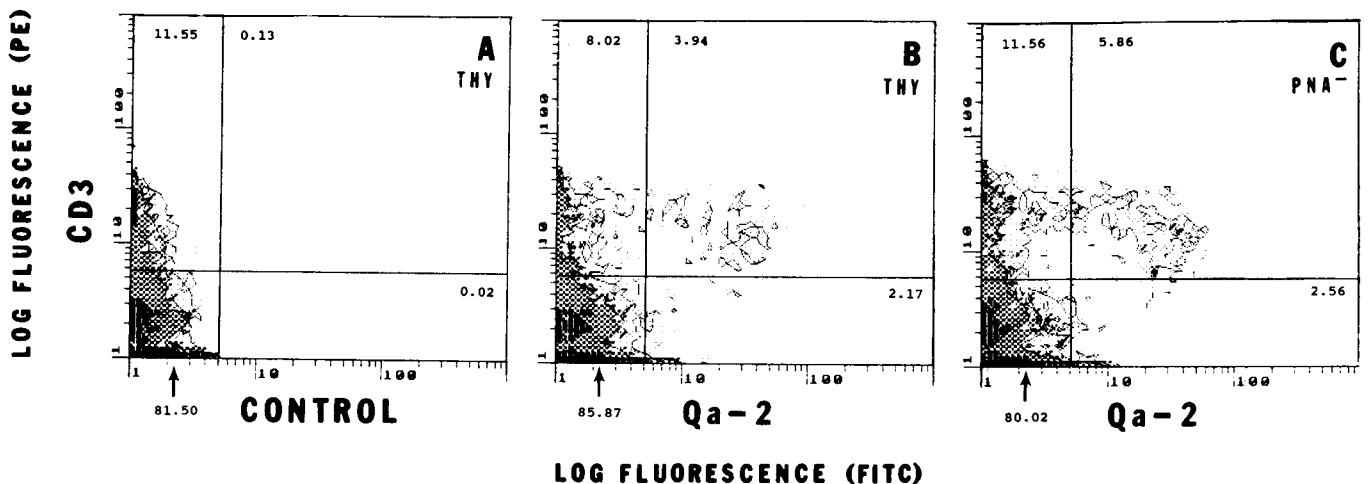


Figure 5. Correlation of Qa-2 and CD3 expression on thymocytes. Unseparated thymocytes (A and B) or PNA⁻ thymocytes (C) were stained with reagents recognizing CD3 (2C11 plus sheep anti-mouse IgG F(ab')₂-biotin plus PE-avidin) and Qa-2 (20.8.4-F(ab)-FITC) (B and C). Alternatively, the CD3 reagents were used along with an irrelevant control reagent (L243-FITC) to illustrate the total population of CD3⁺ cells in the thymus (A). Two parameter histograms represent PE and FITC fluorescence as measured by flow cytometry with positive fluorescent levels indicated by horizontal and vertical lines. The numbers represent the percentage of cells in each quadrant.

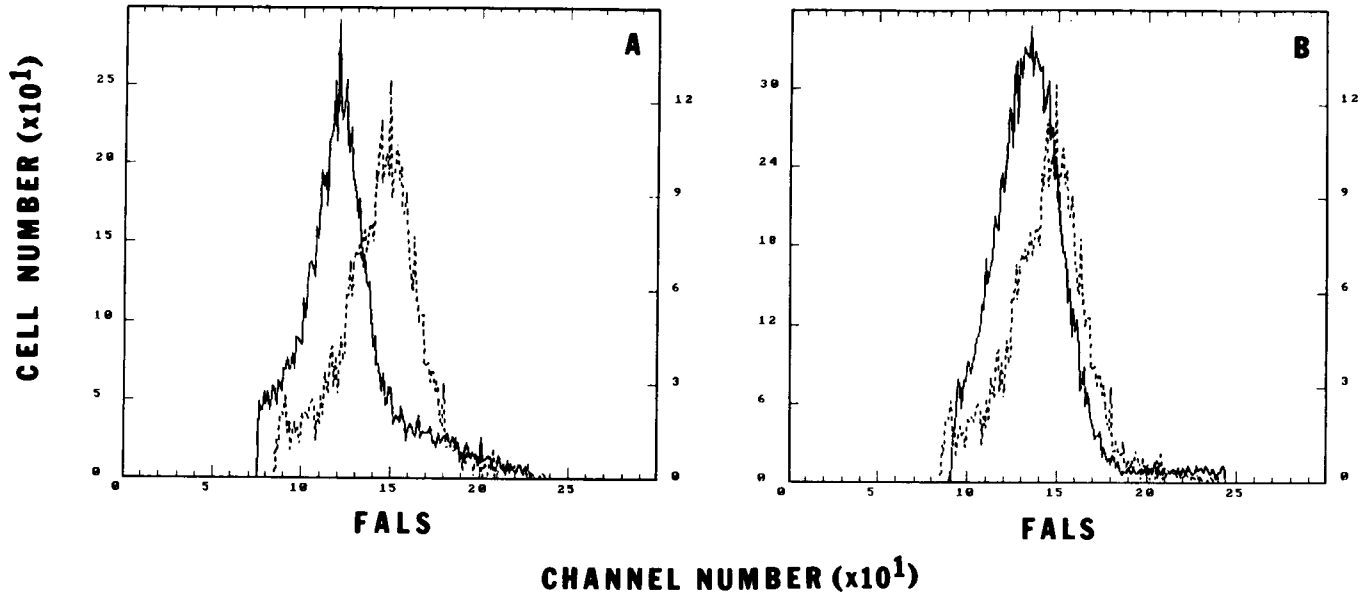


Figure 6. Forward angle light scatter analysis of Qa-2⁺ thymocytes. The FALS profiles, as determined by flow cytometry, were generated from samples of Qa-2⁺ thymocyte (dotted lines, A and B), unseparated thymocytes (solid line, A), and MLN cells (solid line, B). In the case of the Qa-2⁺ thymocyte profile, forward angle light scatter data were collected from only those thymocytes which showed a positive level of Qa-2-specific staining with 20.8.4-FITC. For each histogram the horizontal axis corresponds to the fluorescent channel number, the left-hand vertical axis corresponds to the cell number for the solid line profile and the right-hand vertical axis corresponds to the cell number for the dotted line profile.

of staining patterns found in the thymus. As is illustrated in Figure 3B, the Qa-2⁺ population in normal thymocytes had a level of J11d staining equivalent to peripheral T cells. PNA⁻ thymocytes presented an identical pattern of staining for these two markers (Fig. 3C) with all of the Qa-2 expressing cells being J11d^{LO}. According to these findings, the mature subset defined by Qa-2 expression in the thymus is entirely contained within the J11d^{LO} subset.

MEL-14 Ag expression. The rat mAb, MEL-14, recognizes the lymph node homing receptor that is expressed to some extent by all T cells. However, higher levels of expression are found on peripheral T cells, a small population of subcortical thymic blasts, and a subset of the mature thymocyte population (35, 36). To investigate the possibility that the Qa-2⁺ subset and the MEL-14^{HI} subset are related, two-color flow cytometry was used to correlate the expression of these two Ag.

In Figure 4A, mesenteric lymph node cells were analyzed for co-expression of the MEL-14 and Qa-2 Ag. The majority (90%) of the Qa-2^{HI} peripheral T cells stain positively with the MEL-14 antibody whereas 10% of the Qa-2^{HI} T cells appear MEL-14⁻. The MEL-14⁺ population in the lymph node can be further divided into MEL-14^{LO} and MEL-14^{HI} subsets according to the criteria of Reichert et al. (35) in which case the Qa-2^{HI} lymph node cells are 68% MEL-14^{HI} and 22% MEL-14^{LO}. In the thymus, Qa-2⁺ cells are more heterogeneous in their ability to stain with MEL-14 (Fig. 4B). A clearer demonstration of this fact is seen with the PNA⁻ population of thymocytes (Fig. 4C). Among the Qa-2⁺ thymocytes, 39% of Qa-2⁺ cells express a MEL-14^{HI} phenotype, 29% are MEL-14^{LO}, and 32% are MEL-14⁻. Interestingly, those Qa-2⁺ cells expressing the highest level of MEL-14 Ag express slightly lower levels of Qa-2 Ag (Fig. 4C, arrow).

CD3 Ag expression. The $\alpha\beta$ and $\gamma\delta$ forms of the TCR are both associated with the T3 complex of proteins on the cell surface designated CD3 (37, 38). Figure 5A illustrates the staining pattern observed when the anti-CD3

reagent was used in conjunction with a control antibody. Approximately 12% of thymocytes expressed high levels of CD3. When thymocytes were analyzed for the combined expression of CD3 and Qa-2, virtually all Qa-2⁺ thymocytes also stained with the CD3 antibody (Fig. 5B). Similarly, when PNA⁻ thymocytes were analyzed, all of the Qa-2⁺ cells were CD3⁺ (Fig. 5C). CD3⁺ thymocytes can therefore be divided into two populations based on their expression of Qa-2.

Relative cell size. A direct comparison between the forward angle light scatter profile of Qa-2⁺ thymocytes and the profile generated by the total thymocyte population showed that Qa-2 expressing cells were among the larger cells found in the thymus, with a mean channel number of 144 for Qa-2⁺ cells as compared to a value of 125 for the entire thymocyte population (Fig. 6A). When Qa-2⁺ thymocytes were compared to peripheral lymphocytes, the Qa-2⁺ cells appeared to be larger on the average than lymph node cells (mean channel number value for MLN equal to 136) (Fig. 6B).

Counterflow centrifugal elutriation separates cells on the basis of size and to a lesser extent, density (27, 29). Adult mouse thymocytes were separated by elutriation and the levels of Qa-2 and MEL-14 Ag expression analyzed by flow cytometry (Fig. 7). Positive levels of Qa-2 expression first appeared in the cell fraction eluted at a 24 ml/min flow rate with the bulk of Qa-2⁺ cells eluting in the 28 ml/min fraction. Similarly, the MEL-14^{HI} cells were also eluted in these two fractions. The Qa-2⁺ subset of mature thymocytes is therefore equivalent in size to MEL-14^{HI} thymocytes but is larger in size than an average thymocyte or peripheral lymphocyte.

Cell cycle analysis. Inasmuch as Qa-2⁺ thymocytes were found to be contained in a cell size range that could include large proliferating cells, we used the intercalating dye, AO, to determine the cell cycle position occupied by Qa-2⁺ cells (28, 39). Cells recovered in the elutriated 28 ml/min cell fraction contained levels of DNA equivalent to the diploid state. Additionally, a significant population

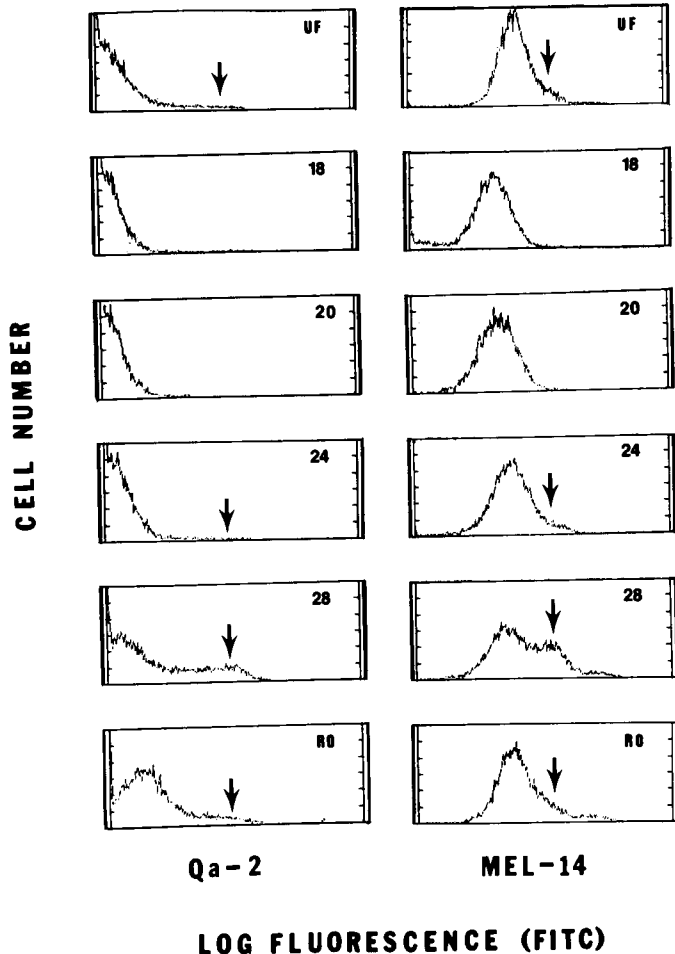


Figure 7. Qa-2 and MEL-14 Ag expression on thymocytes fractionated by counterflow centrifugal elutriation. Samples of unfractionated thymocytes (UF) or of thymocyte fractions generated by counterflow centrifugal elutriation (described in *Materials and Methods*) were stained either with a reagent specific for Qa-2 (Qa2 plus F(ab')₂ sheep anti-mouse IgG Fc-FITC) (left-hand panels) or with reagents specific for the MEL-14 antigen (MEL-14.D54-biotin plus FITC-avidin) (right-hand panels). Each single parameter histogram represents the level of green fluorescence as measured by flow cytometry. The numbers in the upper right hand corner of each panel indicate the buffer flow rate (ml/min) at which the fraction was eluted and panel RO represents the rotor off fraction (see *Materials and Methods*). Arrows indicate the Qa-2⁺ population (left-hand panels) or the MEL-14^{HI} population (right-hand panels).

showed increased levels of DNA indicating the presence of cells undergoing DNA synthesis (Fig. 8A). The RNA staining profile displayed two distinct populations within the 28 ml/min fraction (Fig. 8B). This type of profile is often seen in proliferating populations, with the lower level of RNA being produced by G₀ cells and the higher level of RNA produced by G₁ cells (39). These results agree with our assumption that the elutriated fraction which contained the Qa-2 expressing cells also contained proliferating cells. When Qa-2⁺ thymocytes purified by cell sorting were analyzed, all the cells contained a low level of DNA consistent with the diploid state (Fig. 8A). No significant level of DNA synthesis could be detected. Interestingly, when RNA content was evaluated, all of the Qa-2⁺ cells were found to contain elevated levels of RNA. Such a pattern would indicate that all the Qa-2 expressing thymocytes occupied a position in the cell cycle corresponding to the late G₁ phase.

DISCUSSION

We have used flow cytometric analyses to extensively characterize the phenotype of the Qa-2⁺ cells found in

the adult murine thymus. Cell fractionation and two-color flow cytometry revealed that the Qa-2⁺ lymphoid cells in the thymus are all PNA⁻, J11d^{LO}, CD5^{HI}, and CD3^{HI}. In addition, three-color analysis indicated that the Qa-2⁺ subset is not represented in the CD4⁺/CD8⁺ thymocyte subset but is distributed within the CD4⁺/CD8⁻ (43%), CD4⁻/CD8⁺ (23%), and CD4⁻/CD8⁻ (24%) subsets. Studies from a number of laboratories have demonstrated that those thymocytes that display a mature T cell functional phenotype are characterized as PNA⁻, J11d^{LO}, CD5^{HI}, CD3^{HI} and are found similarly distributed exclusively within the CD4⁺/CD8⁻, CD4⁻/CD8⁺ and CD4⁻/CD8⁻ subsets (8, 9, 33, 40–43). Previous studies have demonstrated that removal of Qa-2⁺ thymocytes eliminated the ability of this population to generate cytotoxic cells in response to alloantigen (18) and that all (>95%) peripheral T cells express high levels of Qa-2 (20). Collectively, this body of information argues that Qa-2 expression by lymphoid cells in the thymus is restricted to a subset of thymocytes that have undergone selection to express high levels of the TCR and have acquired functional programming. Inasmuch as Qa-2 expression is found within the single-positive and double-negative thymocytes, it is likely that Qa-2 is a marker for all functional thymocytes residing in these two subpopulations.

Two-color flow cytometric analysis revealed that the Qa-2⁺ thymocytes are heterogeneous with regard to expression of the MEL-14 Ag. Thus Qa-2⁺ thymocytes can be grouped as either MEL-14⁻ (32%), MEL-14^{LO} (29%), or MEL-14^{HI} (39%) (see Fig. 4). This observation is not unexpected because functional thymocytes capable of proliferation in response to Con A (PTL-p) are distributed among both the MEL-14⁻ and MEL-14^{HI} cells (44). The significance of the MEL-14 heterogeneity among Qa-2⁺ thymocytes is unclear. The possibility that this heterogeneity may reflect cells at various stages of functional development or may mark cells with distinct homing properties awaits further study.

The possibility exists that the Qa-2⁺ cells in the thymus represent a population that fails to exit and is destined for elimination. Observations that argue against this possibility include the phenotype of the Qa-2⁺ thymocytes (18; this report), the finding that all thymocytes capable of generating a cytolytic response to alloantigen are eliminated by treatment with anti-Qa-2 plus C (18) and the observation that all peripheral T cells (>95%) express similar high levels of Qa-2 (20). The argument that Qa-2⁺ thymocytes are a "dead-end" population would imply that thymocytes with demonstrable functional properties are likewise "dead-end," a view not widely held (8, 45–49). Clearly, a demonstration that Qa-2⁺ cells with the phenotypic characteristics of Qa-2⁺ thymocytes exist in the recent thymic emigrant pool (11) will be required to formally exclude this possibility.

We have shown that all Qa-2⁺ thymocytes express high TCR levels (CD3^{HI}) (Fig. 5). Previous studies have shown that mature single positive cells express the α/β form of TCR (50–52). Inasmuch as approximately 65% of Qa-2⁺ thymocytes are single-positive (Fig. 2B), we would expect that the majority of Qa-2⁺ cells expresses the α/β TCR. However, 26% of Qa-2⁺ thymocytes express a double-negative (CD4⁻/CD8⁻) phenotype. Recently, it has been shown that CD3⁺ double-negative thymocytes can express either the τ/δ or α/β TCR heterodimer (52–57). The

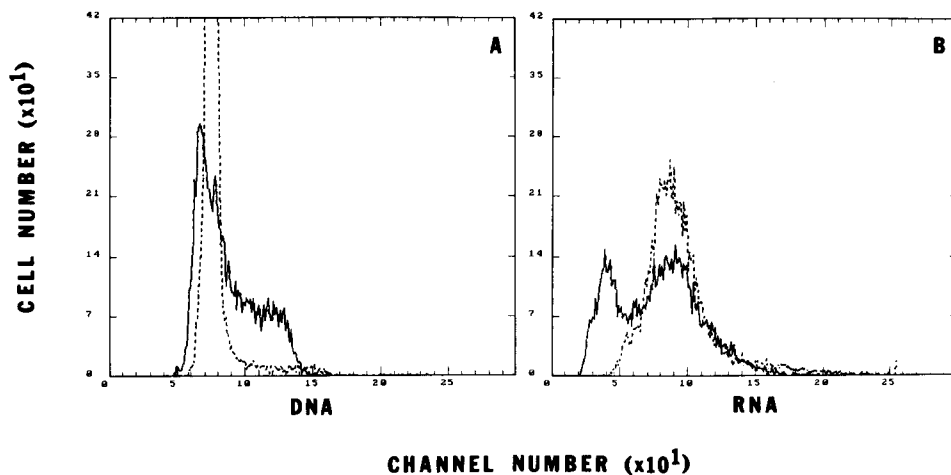


Figure 8. DNA and RNA content of Qa-2⁺ thymocytes. Thymocytes were stained with a Qa-2-specific reagent (20.8.4-FITC) and fluorescent cells were purified by sorting on a FACS. These purified Qa-2⁺ cells (dotted lines) and cells from the 28 ml/min fraction (solid lines) (see Fig. 7) were stained with acridine orange as described in *Materials and Methods*. The quantity of DNA contained within each cell is proportional to the level of green fluorescence (A) and the quantity of RNA is proportional to the level of red fluorescence (B) as measured by flow cytometry.

α/β TCR bearing double-negatives have been found to be exclusively in the CD5⁺/J11d^{LO} subpopulation (51, 55, 56, 58, 59). The τ/δ bearing double-negative thymocytes have been found in both the J11d^{HI} and J11d^{LO} subpopulations (54, 60, 61). Given that Qa-2⁺ thymocytes are all CD3⁺, J11d^{LO}, and CD5⁺, it is possible that Qa-2⁺, CD4⁻/CD8⁻ thymocytes contain both α/β and τ/δ TCR-expressing cells. Positive identification of the TCR types expressed by the Qa-2⁺ thymic subset awaits direct analysis with immunologic reagents specific for each type of receptor.

Forward angle light scatter measurements as well as cell separation by counterflow centrifugal elutriation has shown that the Qa-2⁺ population is of a cell size equivalent to the largest cells in the thymus. Previous studies have shown that thymocytes displaying this size phenotype are part of a proliferating cell population (27). However, cell cycle analysis of the Qa-2⁺ population revealed that these cells contained only diploid amounts of DNA indicating that Qa-2⁺ thymocytes are not a proliferating subpopulation. Interestingly, when the Qa-2⁺ cells were analyzed for RNA content, all cells within this population contained high RNA levels. These observations would indicate that the cells which express Qa-2 in the thymus are not undergoing cell division but rather the entire population is synthesizing large amounts of RNA equivalent to levels in cells at the G₁ stage of the cell cycle.

The Qa-2⁺ cell population represents a subset of the thymocyte subpopulations considered to display a mature phenotype. Of all the cells displaying high levels of the T3/Ti complex (CD3⁺) only 40% are Qa-2⁺. Similarly, the Qa-2⁺ cells represent only 25 and 40% of the CD4⁺/CD8⁻ and CD4⁻/CD8⁺ thymocytes. Thus thymocyte populations that have matured to the point where they express high levels of TCR and are separated into phenotypically distinct subsets can be divided into two categories based on the expression of Qa-2. If it is documented that the Qa-2⁺ set contains all of the functionally mature thymocytes we would speculate that the induction of Qa-2 cell surface expression may mark a specific event in late thymic maturation which confers T cell-like functions on phenotypically mature thymocytes. The high levels of RNA found in Qa-2⁺ thymocytes may be attributable to a need for de novo protein synthesis in cells which have newly acquired functional capacity. Alternatively, cells that display a mature phenotype (e.g., CD3^{HI}) but are Qa-

2⁻ may represent a population that fails to mature or exit the thymus. Additional studies involving biochemical and functional analyses of the Qa-2⁺ thymocyte population must be carried out before these speculations can be proven true.

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