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Use of a monoclonal antibody specifically non-reactive with T cells to delineate lymphocyte subpopulations

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Summary. Rat monoclonal antibody Ml/69.16 reacts with a heat stable antigen of mouse commonly expressed in the majority of cell types in blood, spleen, bone marrow and thymus, including cells of erythroid, myeloid and lymphoid series. However, subpopulations of cells in lymphoid tissues can be identified which are non-reactive with this antibody using the fluorescence-activated cell sorter. All surface Ig positive cells seem to react with Ml/69.16 while more than 96% of Ig negative cells in spleen and lymph nodes are Ml/69.16 negative. Most cells (80%–90%) in the Ml/69.16 negative populations in spleen lymph nodes and bone marrow express Thy-1. Thus, peripheral T cells are specifically non-reactive with this antibody. In contrast, approximately 95% of thymocytes react with Ml/69.16, leaving a minor population which is negative. The negative population (5%) is enriched in cells expressing high amounts of H-2 antigen and those bearing H9/25 antigen which is specific for lymphocyte subsets, indicating that Ml/69.16 negative thymocytes represent a specific subpopulation, possibly 'mature' thymocytes.

Abbreviations: FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; RaMIg, rabbit anti-mouse immunoglobulin antibody; RaRIg, rabbit anti-rat immunoglobulin antibody; DMM, Dulbecco's modified Eagle's medium; RBC, red blood cells; PEC, peritoneal exudate cells.

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INTRODUCTION

Antibodies to cell surface antigens are extensively used for the characterization and fractionation of subpopulations of lymphocytes. For example, anti-Ig and anti-Thy-1 are routine specific reagents for B and T cells respectively (Raff, 1971). While immunoglobulins are expressed exclusively on B cells, the Thy-1 antigen is expressed not only on T cells but also in completely unrelated tissues such as brain (Arndt, Stark, Klein, Müller & Thiele, 1976). Antibodies to widely distributed antigens may nonetheless be of practical importance in defining subpopulations within a given tissue. Indeed, with results using monoclonal antibodies, a large number of surface antigens including one described in this paper are turning out to belong to this category (Milstein & Lennox, 1980; Springer, 1980). In the course of characterizing xenogeneic monoclonal antibodies to mouse cell surface antigens, we described four different antigenic targets (Springer, Galfrè, Secher & Milstein, 1978), namely one specific for and common to all leucocytes (Springer, 1980; Milstein, Galfrè, Secher & Springer, 1979), one specific to macrophages and its precursors (Springer, Galfrè, Secher & Milstein, 1979), a Forssman antigenic determinant present in normoblasts (unpublished observation) and transiently in embryonic cells (Stern, Willison, Lennox, Galfrè, Milstein, Secher, Ziegler & Springer,

1978) and a heat stable antigen present in red cells as well as leucocytes and thymocytes (Springer, 1980). The latter was the target of five different monoclonal antibodies which did not fully overlap in their fine specificity. One of these antibodies, Ml/69.16, has now been used to study in detail the cell distribution of the heat stable antigen. Its most salient characteristic is its absence from peripheral T cells.

Specific absence of common markers from cell subpopulations has been observed in other cases. The most dramatic, perhaps, is the absence of MHC antigens on the majority of thymocytes in humans (Brown, Biberfeld, Christenson & Mason, 1979) and in mouse (Rouse, Ewijk, Jones & Weissman, 1979—see below). The practical importance of such specific non-expression was not easy to appreciate until monoclonal antibodies became available. Therefore we have investigated the use of combinations of monoclonal antibodies each recognizing diverse cell types but non-reactive with specific subpopulations. Subpopulations non-reactive with the Ml/69.16 monoclonal antibody have been isolated using the FACS, then stained with second monoclonal reagents and reanalyzed. We show that such combinations add new dimensions to the analysis of subpopulations and to cell fractionation procedures.

MATERIALS AND METHODS

Mice

Mice used in this study were purchased from OLAC, 1976, Ltd (Bicester, England), and Jackson Laboratories (Bar Harbor, Maine).

Cells and cell lines

Cell lines were as described previously (Springer *et al.*, 1978).

FITC—antibodies and FACS analysis

Methods were as described previously (Springer *et al.*, 1979). In the experiments of Table 1 and Fig. 1, cells treated with culture supernatants (rat monoclonal antibodies) were labelled with fluorescein-labelled (FITC)–F(ab′)$_2$ RaRIg, which had been absorbed with mouse IgG and IgM to prevent cross reactivity with mouse cell surface Ig. For the experiment of Fig. 3 rabbit F(ab′)$_2$ anti-P9 myeloma Fab was fluorescein-labelled and purified by adsorption and elution from a P9 IgG column. Labelling with FITC–anti-P9 followed the previously described procedure with HEPES DMM (10 mM HEPES buffered Dulbecco's modified Eagle's medium) containing 20% foetal calf serum used in the first cycle. For the control, the FITC–anti-P9 Fab was mixed with 5 μl P9 serum before being added to the cells. For double labelling, all samples were first mixed with either FITC–anti-P9 Fab, or P9 plus FITC–anti-P9 as a control, washed and then mixed with Ml clone suppernatants or R4/18.2 (Howard, Butcher, Galfrè, Milstein & Milstein, 1979) as control, washed, and then mixed with FITC–RaRIg and washed.

In some experiments FITC–rabbit anti-mouse Ig (RaMIg) and FITC–RaRIg obtained from Miles Laboratories Limited (Slough, England), which were absorbed with mouse thymocytes and spleen cells respectively, were used for FACS analysis.

Monoclonal antibodies

Production and partial characterisation of monoclonal antibodies Ml/69.16, Ml/22.54, Ml/75.21, Ml/9.47, Ml/89.1 and Ml/9.3 have been described (Springer *et al.*, 1978). Monoclonal antibody H9/25, specific to alloantigen expressed on lymphocyte subsets, has also been described elsewhere (Takei, Waldmann, Lennox & Milstein, 1980b). Culture supernatants of the above hybrids were used as sources of antibodies. Ascitic fluid of monoclonal anti-Thy-1.2 antibody (F7D5) (Lake, Clark, Khorshidi & Sunshine, 1979) was purchased from OLAC, 1976, Ltd. (Bicester, England). Monoclonal anti-H-2K^k antibody (H1000 27R9; Lemke, Hämmerling, Höhman & Rajewski, 1978), also ascitic fluid, was a generous gift from Dr Rajewski (Köln). These antibodies were titrated and used at appropriate dilutions.

RESULTS

Cellular expression of a heat stable antigen

The reactivities of Ml/69.16, Ml/22.54, Ml/75.21, Ml/9.47 and Ml/89.1 were examined by absorption with different tissues and subsequent testing in a standard ^{125}I indirect binding assay (Springer *et al.*, 1978). All gave similar distributions to that of Ml/69.16 (data not shown). Red blood cells (RBC) had the highest antigen concentration. Although kidney, liver and spleen had about 20% and brain and lymph nodes about 3%–6% of the capacity of RBC, this order corresponds with the degree of contamination of these tissues by blood. It is thus not certain whether the antigen is present on cells other than red blood cells in kidney, liver and brain. Screening on a cell panel

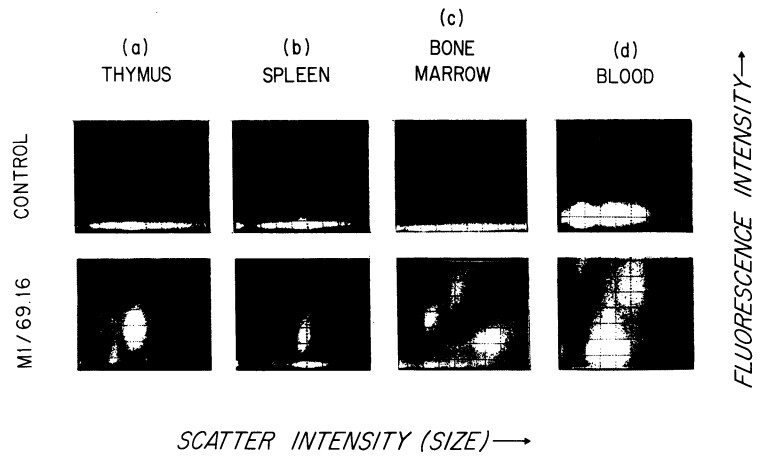

Figure 1. Cells from different tissues labelled with Ml/69.16 supernatants. Cell suspensions were depleted of red and dead cells by Isopaque–Ficoll sedimentation. After incubation with Ml/69 supernatants and washing, cells were labelled with FITC–rabbit F(ab')2 anti-rat IgG. The control was with the non-cross reactive rat anti-histocompatibility antigen monoclonal antibody R4/18.2 (NS1 × rat spleen cells hybrid (Howard *et al.*, 1979)). In each experiment 40,000 cells (or 200,000 blood cells) were counted.

revealed that all these antibodies bound in similar amounts to RBC, but could be subdivided according to their reactivity with leucocytes. Ml/9·47, Ml/22.54, Ml/69.16 and Ml/89.1 bound to splenic lymphocytes, thymocytes, T lymphonas and Abelson lymphomas, but not to the NS1 myeloma line, P815 mastocytoma or $P388D_1$ macrophage line (Springer, 1980). Ml/69.16 bound in larger amounts than the others and therefore most studies below were with Ml/69.16 and the antigen will be referred to as Ml/69.16 antigen. In contrast, Ml/75.21 did not bind to any of the leucocytes or lymphoid cell line tested. These results confirm competitive inhibition studies which demonstrated differences between Ml/75.21, Ml/69.16 and the other three antibodies (Springer *et al.*, 1978).

In conjunction with the FACS, Ml/69.16 showed extraordinary resolution of cell subpopulations (Fig. 1). For example, in bone marrow three Ml/69.16$^+$ populations were distinguished, each of which showed a discrete number of antigen sites per cell and a discrete cell size. Evidence for heterogeneity in the number of antigen sites within a population was also obtained. Ml/69.16 positive and negative subpopulations were clearly separated in the spleen (Fig. 1b) and

Table 1. Distribution of monoclonal antibody-reactive cells in lymphoid tissue*

	Spleen (%)	Lymph Node (%)	Blood lympho-cytes and monocytes (%)	Thymus (%)	Bone marrow (%)	P.E.C., 4 day T.G. (%)	P.E.C., 18 h T.G. (%)
Ml/9.3	99	99	98	100	77	96	97
Ml/69.16†	60	47	61	95	94	55	92
Ml/9.47†	59	n.d.	n.d.	n.d.	n.d.	n.d.	88
Ml/89.1†	57	n.d.	n.d.	n.d.	n.d.	n.d.	88

* Distributions determined with FACS. Results in most cases are averages of several determinations.

† All these antibodies seem to recognize different determinants on a heterogeneous carbohydrate (heat stable) antigen (Springer *et al.*, 1978.)

Ml/9.3 detects a common or leucocyte antigen and was used for comparison.

n.d., Not done.

Table 2. Identification of spleen cell subpopulations by autoradiography

AB	Ag	Erythroblasts	RBC	Lymphocytes	Plasma cells	Neutrophils	Monocytes
Ml/69.16	Heat stable antigen	+	++	>1/2++ <1/2−	−	+	+
Ml/22.25*	Forssman	+	−	−	−	(some+?)	−
Ml/9.3	CLA†	−	−	+	+	+	+

Ml/22/25 and Ml/9.3 were used as complementary controls.
* Occasional large+++unidentifiable cell or cytoplasm from broken cell.
† Common leucocyte antigen.

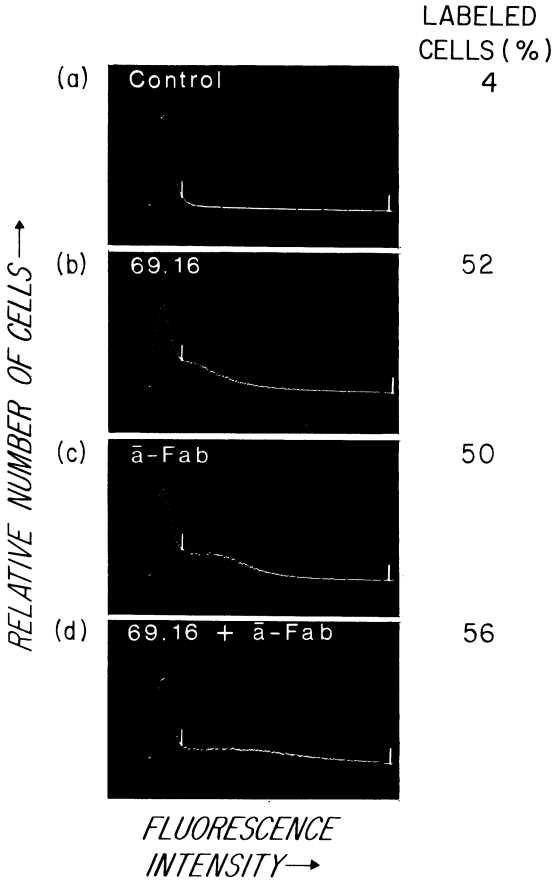

Figure 2. Double labelling of spleen cells with anti-Fab and Ml/69.16. Spleen cells were labelled as indicated in steps 1 and 2 and then with FITC anti-rat IgG in step 3.

	Step 1	Step 2
(a)	P9+FITC anti-P9 Fab	R4/18.2
(b)	P9+FITC anti-P9 FAb	Ml/69.16
(c)	FITC anti-P9 Fab	R4/18.2
(d)	FITC anti-P9 FAb	Ml/69.16

R4/18.2 is an irrelevant rat monoclonal antibody.

were evident though less well separated in blood lymphocytes (Fig. 1d). Only about 50% of the peripheral and spleen lymphocytes are positive for Ml/69.16 (and for Ml/9.47 and Ml/89.1 recognizing the same antigen). Blood monocytes and red cells were labelled by Ml/69.16 as were 92% of 18 h thioglycolate-induced cells (predominantly neutrophils), 94% of bone marrow cells and 95% of thymocytes (Table 1). Autoradiography on spleen cells confirmed the presence of Ml/69.16 antigen on a subpopulation of lymphocytes and on non-segmented and segmented polymorphonuclear leucocytes (Table 2).

On granulocytic and monocytic cells Ml/69.16 and Mac-1 (a macrophage antigen defined by Ml/70 (Springer *et al.*, 1979)) appear to be expressed in a reciprocal manner. Blood monocytes are high in Ml/69.16 and low in Mac-1, as are the neutrophils which predominate in peritoneal exudate after 18 h thioglycolate treatment. The inverse pattern of expression is found on the macrophages which constitute most of the peritoneal exudate cells at 4 days after stimulation (Springer *et al.*, 1979).

The Ml/69.16 negative spleen cells

Spleen cells passed through nylon wool columns to deplete Ig^+ cells showed that Ig^+ and Ml/69.16$^+$, as well as Ml/9.47 and Ml/89.1$^+$ cells, were depleted in parallel (Springer, 1980). This suggested that Ml/69.16 recognized B cells. Therefore BALB/c spleen cells were labelled with both FITC–anti-mouse Fab to label surface Ig, and Ml/69.16 antibody followed by FITC–RaRIg. The number of cells labelled by both reagents together was very close to the number labelled by either reagent alone. Moreover, the intensity of the fluoresence of the two reagents was additive (Fig. 2) indicating that Ml/69.16 and anti-Ig label the same cells.

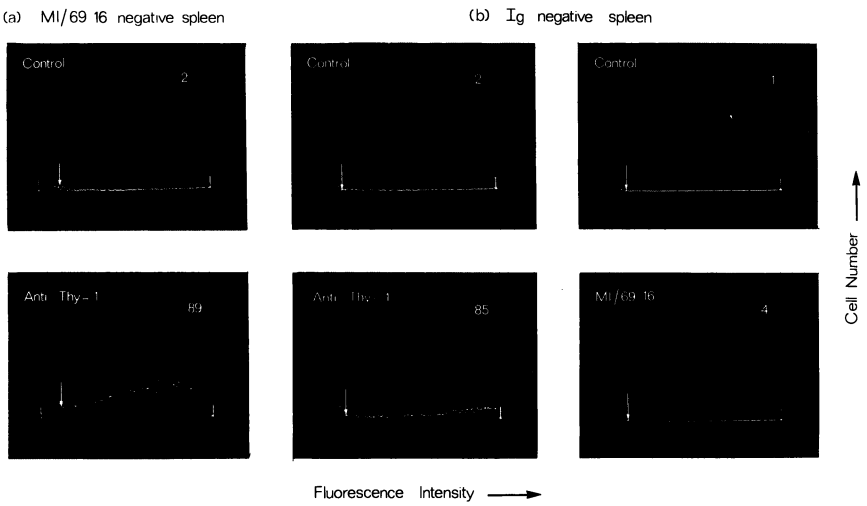

Figure 3. FACS analysis of spleen cell subpopulations. Ml/69.16$^-$ (a) or surface Ig$^-$ (b) spleen cells depleted of red cells from B10BR mice were sorted by the FACS using Ml/69.16 and FITC-RaRIg or FITC-RaMIg alone, respectively. The sorted cells were subsequently analysed for the expression of THy-1 and Ml/69.16. Monoclonal anti-THy-1.2 (F7DS) and FITC-RaMIg or Ml/69.16 and FITC-RaRIg were used. As controls medium was used in place of first antibodies. Numbers in figures show percentage of cells with higher fluorescence intensity than the level indicated by arrows. A total of 20,000 cells was counted in each case.

In another experiment B10.Br spleen cells depleted of RBC were stained with Ml/69.16. The negative cells (approximately 30%) were sorted and analysed for other cell surface markers using the FACS. While no significant percentage of these cells was surface Ig$^+$ nearly 90% expressed Thy-1 antigen (Fig. 3a)). In order to test whether all spleen T cells are Ml/69.16 negative, surface Ig$^-$ (FITC–RaMIg negative) spleen T cells depleted of RBC were sorted and analysed with Ml/69.16. About 85% of surface Ig$^-$ cells expressed Thy-1 antigen as detected by F7D5 (anti-Thy-1.2) while less than 4% of cells in this population were Ml/69.16$^+$ by FACS analysis (Fig. 3b)). Similar results were also obtained with lymph node cells (data not shown). Therefore, probably all peripheral T cells are Ml/69.16$^-$, whereas all B cells are Ml/69.16$^+$. Other spleen cells are also Ml/69.16$^+$ (red cell precursors granulocytes and monocytes) but the 2-3% of nucleated cells which have the phenotypes Ml/69.16$^-$, Thy-1$^-$ and surface Ig$^-$ are yet to be characterized.

Ml/69.16 negative thymocytes

The fact that T lymphocytes are Ml/69.16$^-$ in the spleen and lymph node (and probably in the blood, Table 1) should be contrasted with the fact that 95% of the cells in the thymus are Ml/69.16$^+$. B10.BR thymocytes were divided into Ml/69.16$^+$ and Ml/69.16$^-$ populations. The negative population (5% of thymocytes) was analysed for the expression of other cell surface markers (H-2 and H9/25).

In a preliminary experiment thymocytes were stained with monoclonal anti-H-2K^k (H100-27R9) and analysed by the FACS. A continuous distribution of fluorescent intensity rather than two well-defined subpopulaions was observed. As expected, the majority of the cells were unstained or weakly stained (Fig. 4).

The other marker, H9/25, is an alloantigen recognised by the monoclonal antibody H9/25 (Takei, Galfrè, Alderson, Lennox & Milstein, 1980a) and is expressed by killer T cells, their precursors and plaque forming cells (Takei *et al.*, 1980b). Only 1% of thymocytes were H9/25$^+$ as detected by fluorescence microscopy (Takei *et al.*, 1980a) and hardly detectable by FACS analysis (Fig. 4). Without the fluorescence microscopy results we would have considered the FACS results insignificant.

In contrast to total thymocytes, the Ml/69.16$^-$ subpopulation gave a very different picture on the FACS with both anti-H-2K^k and H9/25. It was found that the H-2K positive cells were enriched from less than 20% to over 50% while H9/25 positive cells were enriched from nearly undetectable (1% by microscopy) to

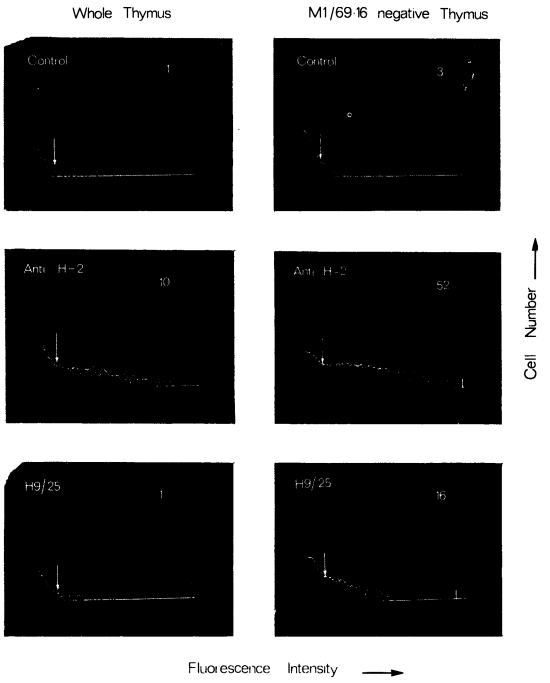

Figure 4. FACS analysis of thymocyte subpopulations. B10.BR thymocytes were fluorescence stained with Ml/69.16 and FITC-RaRIg and the negative population was sorted by the FACS. THe unseparated and the sorted thymocytes were subsequently analysed for reactivity with monoclonal anti-H-2K^k (Lemke *et al.*, 1978), H9/25 (Takei *et al.*, 1980) or tissue culture medium (control) using FITC-RaMIg as described in Fig. 3

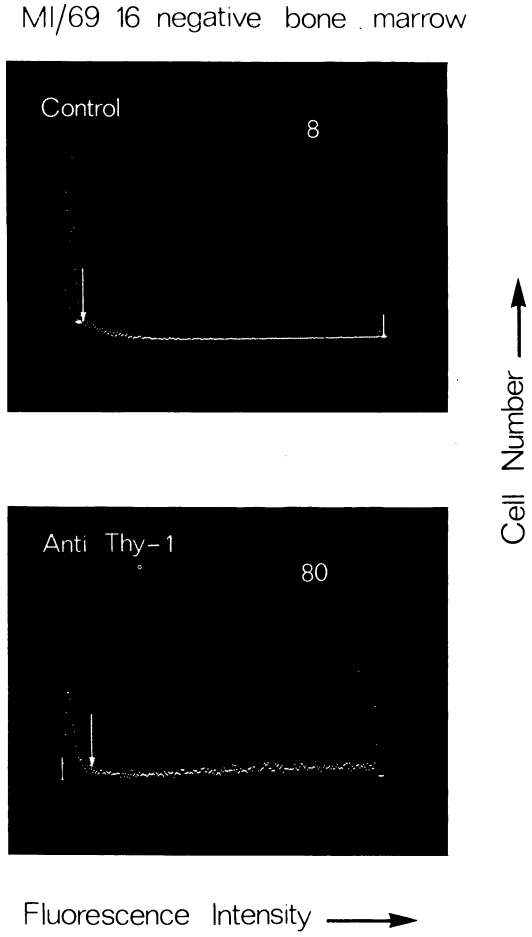

Figure 5. FACS analysis of Ml/69.16$^-$ bone marrow cells. Ml/69.16$^-$ B10.BR bone marrow cells were sorted and subsequently analysed for the expression of Thy-1.2 using F7d5 or tissue culture medium (control) as described in Fig. 3.

15-20% (Fig. 4). The results indicated that Ml/69.16 is not randomly expressed on thymocytes and that the negative cells represent a discrete subpopulation which seems itself to be heterogeneous.

Ml/69.16 negative bone marrow cells

As shown above (Section 3.1) nearly 90% of bone marrow cells are Ml/69.16 positive, including granulomyelocytic cells, erythroid cells and some lymphoid cells. The remaining 10% of cells are non-reactive with Ml/69.16 and in order to characterize them they were sorted by the FACS and tested for the expression of Thy-1 antigen (Fig. 5)). About 80% of the Ml/69.16$^-$ cells were very strongly stained with anti-Thy-1 antibody (F7D5). The nature of the minor population of the bone marrow cells (about 2%) which are negative for both Ml/69.16 and Thy-1 is still unknown.

DISCUSSION

This paper illustrates the practical importance of monoclonal antibodies to widely distributed antigens for the separation and characterization of cellular subpopulations. Antibodies to such antigens have traditionally been considered of little utility for cell separation. However, the myeloma hybrid technique has recently allowed the study of antigenic determinants using single antibody molecular species. This has led to the realization that despite their widespread distribution, many of these antigenic determinants can

serve as highly specific markers of tissue sobpopulations (Milstein & Lennox, 1980; Springer, 1980). We have now shown that the analysis of subpopulations from which the antigenic specificity is missing can be particularly fruitful, since after sorting these can be stained with a second different antibody and reanalysed.

In this paper we have concentrated our attention on antibody Ml/69.16 as the best representative for the recognition of a heat stable antigen. previously characterized as a major mouse antigenic structure recognized by rat monoclonal antibodies (Springer *et al.*, 1978).

Ml/69.16 is, unlike the major transplantation antigen, not widely distributed in other tissues, but it is expressed in the majority of the cell types in blood, spleen, bone marrow and thymus. They include red cells as well as most white cells. On the contrary, the *absence* of Ml/69.16 expression within the lymphoid series is remarkably specific. All peripheral T cells are, within experimental error, Ml/69.16$^-$. Indeed, this negative population is quite pure in that at least 90% of it is Thy-1$^+$ and it does not include measurable numbers of Ig$^+$ cells. This is to be contrasted with the fact that 95% of thymocytes are Ml/69.16$^+$, suggesting that the antigenic specificity is lost concomitantly with maturation and exit from the thymus. Exit from the thymus is also accompanied by increase in H-2 and asialo-GM_1 ganglioside and decrease in Thy-1 and TL antigen expression. It has been proposed that such dramatic changes in antigen expression may play a role in changes in specificity of tissue adhesiveness and homing (Springer, 1980).

The question of the origin and fate of the subpopulation(s) of the 5% Ml/69 negative also remains to be elucidated. Some could be the direct progenitors of the (Ml/69$^-$) peripheral T cells, in which case it is tempting to speculate that the cells enter the thymus as Ml/69$^+$ and exit as Ml/69$^-$. The Ml/69.16$^-$ population in the thymus (5%) is a distinctive subpopulation because it includes most if not all the H9/25$^+$ cells and is very enriched in H-2K^k positive cells. H9/25 is a marker expressed in T killer cells and their precursors as well as in plaque forming cells but not in B memory cells or in T helpers (Takei *et al.* 1980a,b). H9/25$^+$ cells are undetectable in thymus by the FACS, but they are an important fraction (up to 20%) of the Ml/69.16$^-$ population. These Ml/69.16$^-$ thymocytes are also strongly enriched for cells expressing H-2K^k.

That the Ml/69.16$^-$ thymocytes constitute themselves a heterogeneous population is suggested but not proven by the data. Thymocyte subpopulations have been previously indicated by the differential expression of other antigens, mainly TL and Lyt (Cantor & Boyse, 1977). It is possible that the correlation of these markers with the ones we have discribed will reveal further heterogeneity or further clarify the presently defined subpopulations of thymocytes. It is known that there is a reverse correlation between the expression of TL and H-2 in cortical and medullary lymphocytes (Cantor & Boyse, 1977; Schortman, 1977). Similar reverse correlations with putative homologous markers have been observed in human thymocytes (Bradstock, Janossy, Bollum & Milstein, 1980). Therefore, the Ml/69.16$^-$ population which is enriched in H-2 is expected to be depleted of cortical TL positive lymphocytes.

We suggested above that the cells enter the thymus as Ml/69.16$^+$ but whether the precursor in the bone marrow are also Ml/69.16$^+$ is unknown. It is likely that the majority of the Ml/69.16$^-$ cells (8% of the total bone marrow cells) are recirculating T cells since 80% of them are Thy-1$^+$. However there remains 2% of bone marrow cells which are Ml/69.16$^-$ and Thy-1$^-$. It remains to be seen whether these represent a distinct functional subpopulation.

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