

## Monoclonal Antibodies to ThB Detect Close Linkage of *Ly-6* and a Gene Regulating ThB Expression

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**Abstract.** We have generated three hybridomas producing rat monoclonal antibodies to a surface antigen, ThB, that is shared by murine B lymphocytes and approximately 50 percent of murine thymocytes. These antibodies, produced by immunizations with MOPC-104E cells, appear to recognize the same antigen that was previously detected by rabbit and goat antisera to MOPC-104E cells (Yutoku et al. 1974, Yutoku et al. 1976).

Using these antibodies, we have studied a genetic polymorphism that is associated with the level of ThB expression on B lymphocytes but not with the antigen's expression on thymocytes. We present evidence that this trait is controlled by one gene, *Thb*, which we find to be very closely linked to the gene or genes controlling the *Ly-6*, *Ly-8*, *DAG*, and *Ala 1* antigen(s). While the latter four antigens were described as markers on mature T (or activated T and B) lymphocytes, ThB is restricted to immature thymocytes and all B cells. ThB is not expressed on kidney, although some investigators (McKenzie et al. 1977 a, Halloran et al. 1978) report *Ly-6* expression on that tissue. *SJL/J*, *C57BL/10JHz*, *DBA/2J*, and *AKR/J* are among the mouse strains carrying the *Thb<sup>h</sup>* allele, while *BALB/cN*, *CBA/J*, *C3H.SW/SnHz*, and *A/J* carry the *Thb<sup>l</sup>* allele. The ThB antigen has not yet been identified as a glycoprotein after cell-surface iodination, NP-40 solubilization, and immunoprecipitation.

### Introduction

The murine lymphocyte surface antigen, ThB, was originally detected by a cytotoxic rabbit antiserum (RAML) raised to the BALB/c myeloma cell, MOPC-104E (Yutoku et al. 1974). This antiserum contained two types of antibody activity: one cytotoxic for B cells alone and the other cytotoxic for both B cells and thymocytes (Stout et al. 1975). A goat antiserum (G anti-ThB) prepared against the same myeloma cell and absorbed in vivo in BALB/c mice was shown to contain only the

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wells containing approximately  $10^6$  mouse thymocyte feeder cells in 0.1 ml medium. Supernates from these cloned lines (49-h4, 49-g7, 49-f5) were again screened for anti-ThB-like antibody, and cells from the appropriate antibody-containing wells were grown up in 25 or 100 ml culture flasks. The supernates from these culture flasks were harvested for use in subsequent studies. Aliquots of the cells were frozen in 10% dimethyl-sulfoxide, 90% fetal calf serum and stored in liquid nitrogen. Aliquots of the antibody-rich culture medium were either stored at  $-70^\circ\text{C}$  or at  $4^\circ\text{C}$  in the presence of 0.1%  $\text{NaN}_3$ .

*Determination of heavy chain class of the rat monoclonal antibodies.* Antibody-producing hybridoma cultures were fed media containing  $^{35}\text{S}$ -methionine (SJ-204, Amersham Corp., Arlington Heights, Illinois) and their supernates harvested 5 h later. Normal rat serum was added to the culture supernates as carrier protein, and the mixture was reacted with class-specific, anti-rat immunoglobulin antibodies (Miles Laboratory, Elkhart, Indiana) by the Ouchterlony immunodiffusion method. The immunodiffusion slides were washed, dried, and placed under Kodak NS-2T film for the detection of radioisotope-containing precipitin lines.

*Purification of monoclonal antibody from culture supernatants.* Since the heavy chains of all three hybridomas bind protein A from *Staphylococcus aureus*, Cowan Strain I, they were each purified by affinity chromatography on Sepharose-*Staphylococcus aureus* protein A (Pharmacia Fine Chemicals, Piscataway, New Jersey). Sodium acetate (0.2M, pH 4.0) was used to elute bound rat antibody that was then neutralized with 1.0 M Tris-HCl, pH 8.0. Ten to thirty micrograms immunoglobulin per ml supernates were isolated.

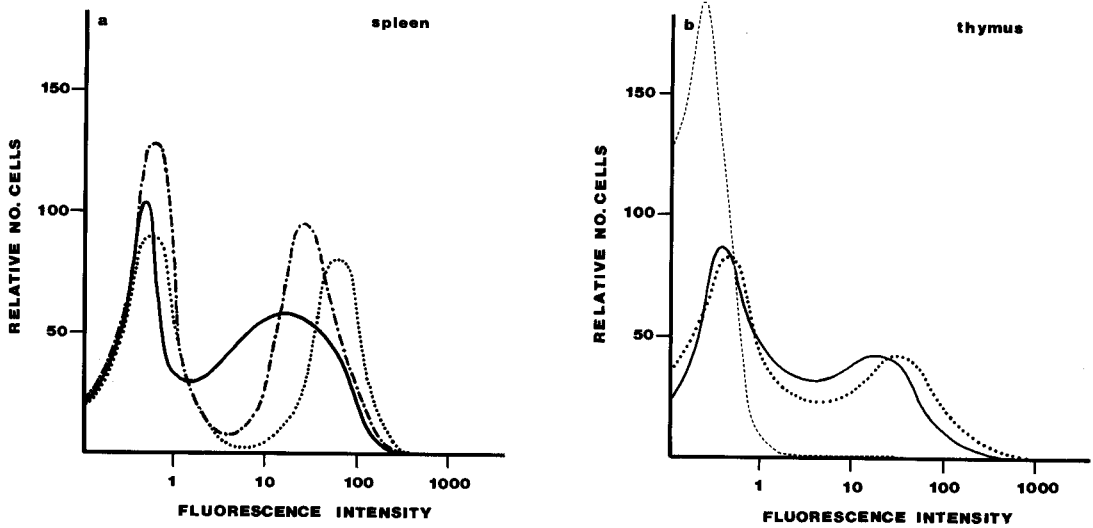
*Immunofluorescence staining and FACS analysis.* Lymphoid cell suspensions were made in RPMI 1640 (Grand Island Biological Co., Grand Island, New York) containing 5% fetal calf serum, and cell viability was determined by acridine orange/ethidium bromide staining (1.0  $\mu\text{g}/\text{ml}$  each, final concentration) (Parks et al. 1979). Peripheral blood lymphocytes were isolated by centrifugation with the density separation medium, Lympholyte M (Accurate Chemical Corp., Hicksville, New York).

For fluorescence staining,  $5 \times 10^5$  cells (90–95% viable) were incubated with 50  $\mu\text{l}$  of antibody-containing supernates. The cells were washed and a fluorescein-conjugated mouse anti-rat immunoglobulin antibody was added as a second-step reagent. (The mouse antibody, kindly provided by J. Ledbetter of this laboratory, was obtained from SJL/J mice repeatedly immunized with rat immunoglobulin.) After 30 min, the cell suspension was again washed and then analyzed on the FACS. All staining procedures were done in the cold and in the presence of 0.1%  $\text{NaN}_3$ .

Stained cells were analyzed for both fluorescence and light scatter (size) on the FACS and, except in two-color fluorescence experiments, logarithmic amplification of the fluorescence signal was employed. Our FACS methods have been extensively described (Herzenberg and Herzenberg 1978).

Biotin-conjugated, purified hybridoma antibody was used in two-color fluorescence experiments and in cross-blocking studies involving the three rat anti-ThB monoclonal antibodies. In these studies, fluorescein-conjugated ( $\text{F}$ ) avidin (Vector Laboratories, Burlingame, California) served as the second-step reagent;  $\text{F}$  avidin binds to the biotin on the antibody molecules. The biotin conjugation procedure has been described (Bayer and Wilchek 1978).

*Cytotoxicity assays.* The microcytotoxicity assay was performed as previously described (Amos et al. 1969, Murphy and Shreffler 1975) with some modifications. Cells were brought to a concentration of  $10^6$  cells/ml in RPMI 1640 containing 2% heat-inactivated fetal calf serum (FCS). Two microliters of this cell suspension were incubated with 2  $\mu\text{l}$  of an appropriately diluted antibody reagent for 15 min at  $37^\circ\text{C}$ . After a single wash, 2  $\mu\text{l}$  of Low Tox rabbit complement (Accurate Chemical Corp., Hicksville, New York), diluted 1/10 in RPMI 1640 without FCS, were added to each well. The cells were again incubated at  $37^\circ\text{C}$  for 30 min, then washed and stained with an ethidium bromide/acridine orange mixture (2  $\mu\text{g}/\text{ml}$  each, final concentration). Live and dead cells were counted under a fluorescence microscope. In all assays, cells exposed to complement alone were more than 90 percent viable. When the peripheral blood lymphocytes of backcross mice were tested for the Ly-6.2 antigen, duplicate aliquots of the isolated lymphocytes were treated with a monoclonal anti-Thy-1.2 antibody (clone 30-H12) (Ledbetter and Herzenberg 1979) plus complement. Since Ly-6.2 is primarily found on T cells by cytotoxicity tests (McKenzie et al. 1977 a, b, Woody 1977, Woody et al. 1977), this served as a control for preferential loss of either the B- or T-cell component of the blood lymphocytes.



**Fig. 1 a and b.** Fluorescence staining profiles with monoclonal anti-ThB. Spleen cells (a) from BALB/cN mice (—), SJL/JHz mice (···), and (BALB/cN × SJL/JHz)F<sub>1</sub> mice (---) were stained with a monoclonal anti-ThB antibody (49-h4) followed by fluoresceinated mouse anti-rat Ig. Stained cells were analyzed on the FACS using logarithmic amplification of the fluorescence signal, and the curves shown were generated. The thymus cells (b) of BALB/cN mice and SJL/JHz mice were stained with the same reagents. The FACS curve for thymus cells stained with fluoresceinated mouse anti-rat Ig alone (---) is also shown in (b).

The same fluorescence staining differences seen in splenic B cells from BALB/c and SJL/J mice are also detected in their peripheral blood B lymphocytes. We therefore analyzed the ThB fluorescence staining patterns of blood lymphocytes from 150 backcross progeny to determine whether or not this trait is regulated by one gene. A representative experiment showing the range of values for mean fluorescence in backcross mice is shown in Table 1. As indicated, the progeny fall into two, nonoverlapping groups on the basis of ThB staining. If only one gene is involved, the ratio of mice with bright ThB fluorescence to mice with dull fluorescence should be 1 : 1. Table 2 summarizes the results of this backcross analysis. The data shown in this table and in Table 1 are in accordance with the one-gene hypothesis. We shall designate the allele responsible for the bright fluorescence phenotype *Thb<sup>h</sup>* and the allele responsible for dull fluorescence *Thb<sup>1</sup>*. The parental phenotypes will be referred to as ThB-h and ThB-1 and the F<sub>1</sub> phenotype as ThB-h/1. We have tested additional inbred mouse strains with the anti-ThB monoclonal antibodies and assign each the *Thb<sup>1</sup>* or *Thb<sup>h</sup>* allele as in Table 3.

In an effort to establish linkage between *Thb* and other genes previously described in the mouse, we examined its strain distribution profile (SDP) in the CXB recombinant inbred (RI) mouse lines developed by Bailey (1971), and the BXH RI lines developed by Taylor (1978). The ThB-h phenotype can be seen to have a SDP identical to that of the Ly-6.2, Ly-8.2, Ala-1.2, and DAG alloantigens (Table 4) and unlike that of other tested markers in these RI strains. In reviewing our inbred mouse strain data on *Thb* (Table 3), we find that all strains expressing the Ly-6.2, Ly-

**Table 4.** Recombinant inbred mouse strain distribution profiles for ThB, Ly-6, Ly-8, DAG, and Ala-1

Antigen	Recombinant inbred lines and phenotypes												
BXH*	2	3	4	5	6	7	8	9	10	11	12	14	19
ThB	B <sup>†</sup>	H	B	B	B	H	H	H	B	B	B	B	H
Ly-6 and Ala-1 <sup>‡</sup>	B	H	B	B	B	H	H	H	B	B	B	B	H
CXB <sup>  </sup>	D	E	G	H	I	J	K						
ThB	B	B	C	C	B	C	B						
DAG, Ala-1	B	B	C	C	B	C	B						
Ly-6, and Ly-8 <sup>§</sup>													

\* C57BL/10 × C3H recombinant inbreds developed by Taylor (1978). Mice purchased from The Jackson Laboratory.

<sup>†</sup> "B" signifies that the RI line has the C57BL/10 phenotype for the alloantigen, "H" signifies the C3H phenotype, and "C" signifies the BALB/c phenotype.

<sup>‡</sup> Data taken from Feeny (1978).

<sup>||</sup> BALB/c × C57BL/10 recombinant inbreds developed by Bailey (1971). Mice purchased from The Jackson Laboratory.

<sup>§</sup> Data taken from Horton and co-workers (1978) and Horton and Sachs (1979).

8.2, Ala-1.2, and DAG antigens, and only these strains, are also of the ThB-h phenotype. This of course suggests close genetic linkage of *Thb* with the gene(s) controlling the antigen(s) described under these designations.

If it were not for the entirely distinct cell and tissue distribution of ThB as compared to this antigen (or antigens) previously described by several different workers, we might have concluded at this point that we had merely rediscovered one or more of the latter antigens. However, knowing that ThB is clearly distinguishable from the other antigen(s) (see below), we attempted to determine how closely linked the *Thb* locus is to the locus or loci controlling Ly-6, Ly-8, DAG, and Ala 1 expression. Of the 150 backcross mice already typed for ThB and listed in Table 2, we have now typed 96 for Ly 6.2 using peripheral blood cells. We found the nonrecombinant phenotypes (Ly-6.2 (+), ThB-h/1) and (Ly-6.2 (-), and ThB-1) in all of these progeny (Table 5).

The fluorescence staining and FACS analysis used for testing the ThB phenotype was done at least twice for each mouse. Similarly, cytotoxicity tests were performed in duplicate and on at least two but usually three occasions. Since Ly-6.2 is primarily a T-cell antigen, a disproportionate loss of T cells during cell preparation might cause a mouse to be incorrectly scored as Ly-6.2 (-). We avoided this error by checking the T-cell recovery in each cell preparation with a cytotoxic anti-Thy-1.2 monoclonal antibody.

It should be noted that initially we thought we had detected some mice of the (Ly-6.2 (-), ThB-h/1) recombinant phenotype among the backcross progeny tested. However, later tests of these putative recombinants and their progeny with the same anti-Ly-6.2 serum, the anti-DAG serum (Sachs et al. 1973), and a monoclonal anti-Ly-6.2 antibody (Tada and Hämmerling, unpublished) showed these mice to be Ly-6.2(+). If the conventional antisera alone had been available, several of the (heterozygous) backcross progeny could not have been unambiguously scored!

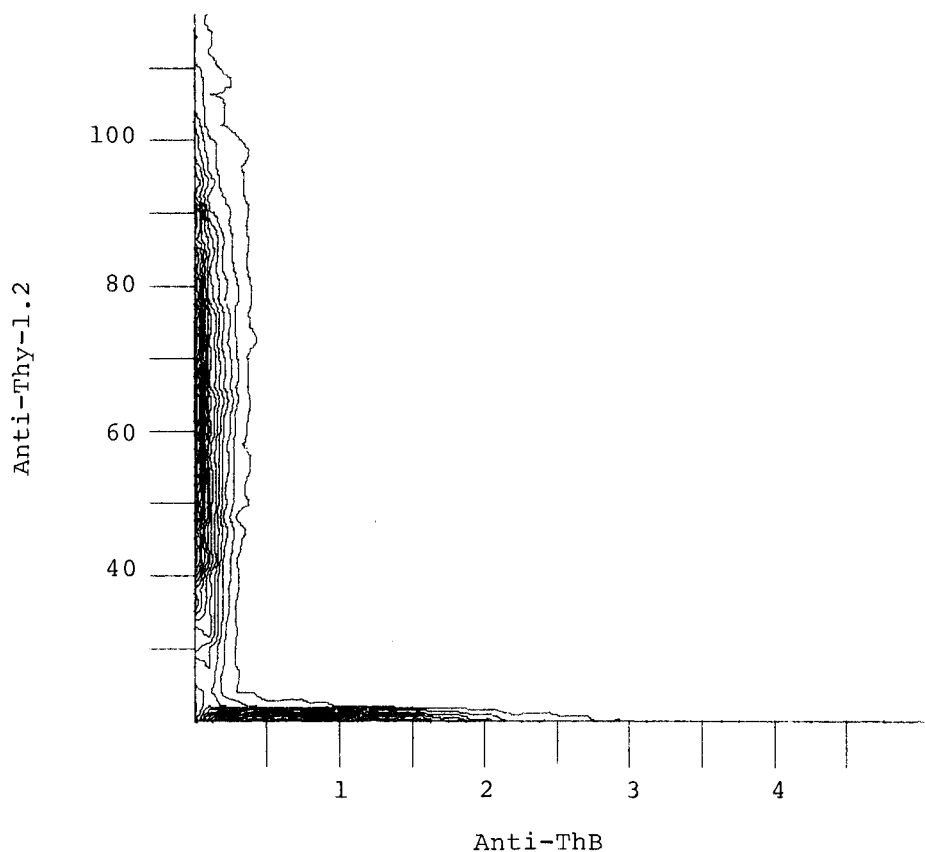
**Table 6.** Expression of ThB and Ly-6.2 on C57BL/10 spleen and thymus

Tissue	Percent cells killed*			
	Anti-ThB <sup>†</sup> alone	Anti-Ly-6.2 <sup>‡</sup> alone	Anti-Ly-6.2 and anti-ThB	Complement alone
Spleen	42 (2)	35 (4)	72 (6)	6 (4)
Thymus	80 (3)	8 (3)	86 (4)	6 (4)

\* Numbers were determined in a microcytotoxicity assay. Samples were prepared in triplicate; standard errors are in parentheses.

<sup>†</sup> Culture supernate from clone 49-h4 used to detect ThB.

<sup>‡</sup> Anti-Ly-6.2 provided by E. Simpson used to detect the Ly-6.2 antigen. (CBA/Ca + *A-Thy-1*<sup>q</sup>)F<sub>1</sub> anti-AKR/Crc (Horton et al. 1978).



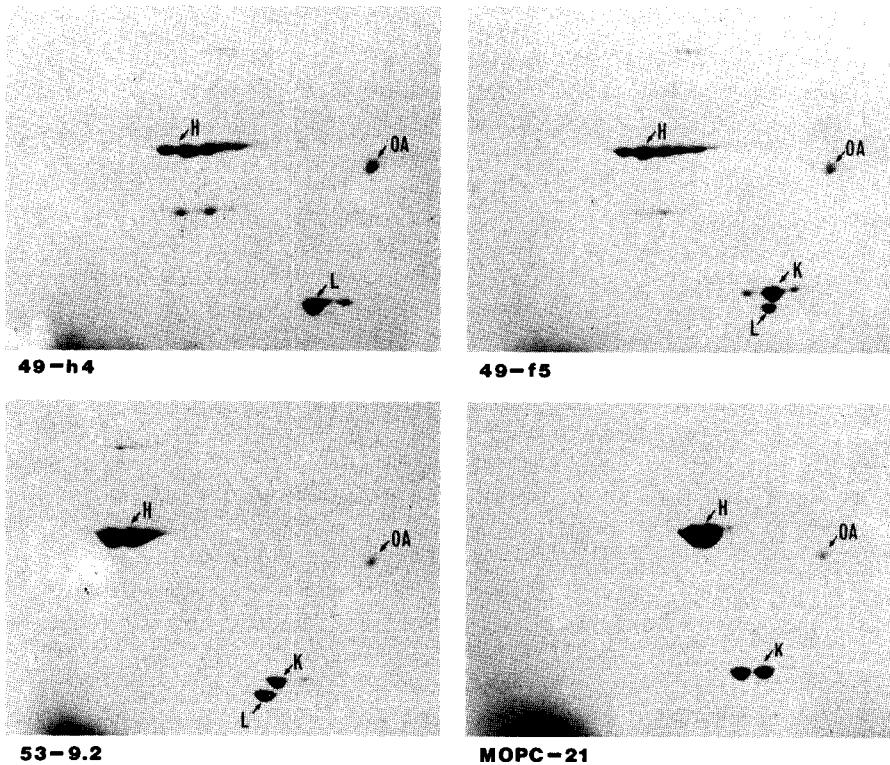
**Fig. 2.** Two-color fluorescence analysis of Con-A-activated T cells stained with anti-Thy-1.2 and anti-ThB. C57BL/10JHz nylon nonadherent T cells were cultured with Con-A as described in *Materials and Methods*. Resulting blast cells were stained with both fluoresceinated rat anti-Thy-1.2 and a combination of biotininated rat anti-ThB and rhodaminated avidin. The two-color fluorescence analysis of the stained cells is represented by a contour map with increasing rhodamine signal (anti-ThB) along the horizontal axis and increasing fluorescein signal (anti-Thy-1.2) along the vertical axis.

In order to establish that the hybridoma antibodies to ThB, like G anti-ThB, were recognizing the B cells and not the T cells in mouse spleen, we subjected the spleen cells of ThB-h mice to two-color fluorescence analysis (Loken et al. 1977). We used ThB-h mouse spleens because of the clearer distinction possible between ThB-positive and ThB-negative cells. However, similar results are obtained in ThB-l mice (data not shown). Spleen cells from SJL/J mice were treated simultaneously with a reagent detecting the Thy-1.2 antigen (<sup>F</sup>rat anti-Thy 1.2 monoclonal antibody, Ledbetter and Herzenberg 1979) and with hybridoma reagents detecting ThB (biotin conjugates of each of the purified monoclonal antibodies were used along with rhodaminated avidin, see *Methods* section). The results of such a two-color fluorescence experiment are shown in Figure 4A. The horizontal axis represents increasing fluorescein signal, and the vertical axis corresponds to an increasing rhodamine signal. A signal from a cell with both fluorochromes will be represented by a dot somewhere in the area between these two axes. A cell with one fluorochrome and not the other will generate a dot lying directly on one of the axes. The data shown indicate that all of the cells react with either 49-h4 or anti-Thy-1.2 but not with both.

A similar double staining experiment with an anti-mouse immunoglobulin antibody and 49-h4 indicated that antigens recognized by these reagents are generally found together on spleen cells (Fig. 4B). The concentration of signals at the origin of the dot plot shown represents cells negative for both markers (primarily T cells). Clearly, most cells are either positive for both immunoglobulin and the 49-h4 target antigen or are negative for both. We can conclude that 49-h4 recognizes an antigen found on B cells and not T cells of spleen. These data, along with the fact that all three of the antibodies we isolated react with thymocytes and spleen cells of ThB-h and ThB-l mice in the same fashion as G anti-ThB, allow us to designate these as "anti-ThB" antibodies.

In order to test whether the three hybridoma antibodies recognize the same or different determinants on the cell, we used a blocking test. All three antibodies were found to completely block the spleen and thymocyte-binding of biotin-conjugated 49-h4 and 49-f5. Neither of these biotin-conjugated anti-ThB monoclonal antibodies is blocked by monoclonal antibodies recognizing other B-cell or thymocyte markers (anti-Lyt-1, anti-Lyt-2, anti-Thy-1.2, anti-T200, anti-Lgp 100, or anti-Igh-5b, data not shown). We conclude that all of the monoclonal anti-ThB antibodies recognize the same or neighboring site(s) on the ThB molecule.

When tested by immunodiffusion against a panel of class-specific, anti-rat immunoglobulin reagents, all three monoclonal antibodies proved to be of the IgG2c subclass. Two-dimensional gel electrophoresis of 49-h4 protein shows it to have lost the NS-1 kappa light chain and to consist of only rat heavy and light chains (Fig. 5B). The heavy and light chains of 49-f5 appear similar to those of 49-h4 by this kind of analysis, but the 49-f5 protein retains the NS-1 kappa light chain (Fig. 5A). While the two clones producing these proteins arose from two separate fusion events, it is possible that the rat parent spleen cell participating in each fusion arose from a single clone in the rat's immune spleen cell population. The protein from clone 49-g7 has not yet been analyzed for light chain composition and heavy-chain isoelectric point. However, we have analyzed a fourth rat anti-ThB antibody that was generated by J. Ledbetter in an independent fusion experiment (Ledbetter and



**Fig. 5.** Two-dimensional electrophoresis of rat anti-ThB monoclonal antibodies. Nonequilibrium pH gradient first dimension gels for each of the proteins shown were run at the same time and under identical conditions (acidic proteins are on the right and basic proteins are on the left). The methodology used was as originally described by O'Farrell and co-workers (1977). The second dimension separations were by SDS polyacrylamide gel electrophoresis on 10 percent slab gels (from top to bottom). Ovalbumin was included as a marker in all samples. Analysis of the MOPC-21 protein is included to establish the position of the MOPC-21 kappa light chain. Arrows point to the immunoglobulin heavy chain (H), rat light chain (L), MOPC-21 kappa light chain (K), and the ovalbumin marker (OA). 53-9.2 is a rat monoclonal antibody generated in a fusion experiment by J. Ledbetter (see text, and Ledbetter and Herzenberg 1979).

also of the IgG2c class (Ledbetter and Herzenberg 1979), and a two-dimensional gel analysis of it is shown in Figure 5C. Like 49-f5, 53-9.2 retains the NS-1 kappa light chain, but its heavy chain is clearly more basic than the other two immunoglobulin heavy chains shown. Since the same rat strain as we used was used in this second fusion, we can conclude that more than one clone of cells in immune Lou rat spleen produces antibodies reacting with ThB. However, all anti-ThB monoclonal antibodies so far examined recognize the same or closely neighboring determinants, and all are of the IgG2c subclass. Rat immunoglobulins of the IgG2c subclass, like mouse IgG3 immunoglobulins (Perlmutter et al. 1978), are often found to recognize carbohydrate determinants (Leslie 1977). It is therefore interesting to note that preliminary information (see *Discussion*) on the ThB antigen indicates that it might be nonprotein in nature.

at *Ly-6*, *Ly-8*, and *Ala-1*. Finally, the BALB-*DAG* congenic mouse strain (Horton and Sachs 1979) carries the DBA/2 allele for the other three markers as well. These genetic data were interpreted as evidence for these antigens being the product of one gene (Horton and Sachs 1979). However, the latter four determinants could represent more than one antigen controlled by closely linked loci. Monoclonal antibodies to the products of this genetic region, like the anti-*Ly-6.2* antibody recently isolated by Tada and Hämmerling (unpublished), should aid in the resolution of this question.

Our data, then, along with that available on the *Ly-6*, *DAG*, *Ala-1*, and *Ly-8* markers, indicate that the genetic region under study probably consists of at least two genes affecting lymphocyte differentiation antigens. Information on the molecular nature of these antigens will help determine their genetic control and possibly contribute to an understanding of their function. In this regard, we have begun studies on the chemical nature of the molecule carrying the ThB determinant. At present, we have no evidence that ThB is a glycoprotein. We have been unable to identify it among  $^{35}\text{S}$ -methionine or  $^{125}\text{I}$ -lactoperoxidase labeled NP-40 cell lysates. In addition, the ThB determinant is insensitive to either trypsin or protease treatment. At present, we are exploring the possibility that the molecule carrying the ThB determinant is a glycolipid. In support of this notion, we find the determinant to be stable to glutaraldehyde fixation, but sensitive to both methanol and acetone fixation. Also, as mentioned above, all of the rat anti-ThB monoclonal antibodies thus far isolated are of an isotype (IgG-2c) associated with anti-carbohydrate antibodies. If the ThB determinant does prove to be carbohydrate or lipid in nature, it will of course affect speculation on the way in which the genetic system we have described must operate. The human ABO blood group system is a well-known precedent for a genetic system involving such nonprotein antigens.

In addition to these and the genetic studies described here, we are continuing the early research that initially distinguished ThB as a potential marker for a lymphocyte precursor. Thymocytes can be clearly discerned from B lymphocytes both antigenically and functionally. However, the ThB determinant is shared by these diverged lymphocyte populations. It is also found on small (lymphocyte size) Ig(-), Thy 1(-) cells in mouse bone marrow as well as on the Ig(+) cells in bone marrow (data not shown). ThB is strictly limited to expression on lymphoid tissues; it is not found on other mouse tissues such as brain, liver, or kidney. For these reasons, we postulate that the ThB determinant is initially expressed early in lymphoid development, survives the branching of the T- and B-cell lineages, and is subsequently lost from the T-cell line during T-cell maturation in the thymus. We have presented evidence that confirms the presence of the ThB determinant on both B lymphocytes and thymocytes. We also find the determinant on a number of plasmacytomas (including the immunogen, MOPC-104E) and on LPS blasts in spleen. We are presently investigating the nature of the ThB(+) cells in mouse bone marrow and are pursuing functional studies of the ThB(+), Ig(+) cells in spleen. Because large quantities of monospecific anti-ThB reagents are now available, these and other studies may move toward a description and, hopefully, an understanding of ThB in lymphoid development.



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