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The Molecular Basis of Immune Cell Function
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MONOCLONAL ANTIBODIES AND THE FLUORESCENCE-ACTIVATED CELL SORTER: COMPLEMENTARY TOOLS IN LYMPHOID CELL BIOLOGY.

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Detection and Analysis of Monoclonal Antibodies Reactive with Cell-surface Antigens.

Our strategy for isolating hybrid clones depends on the use of the fluorescence-activated cell sorter (FACS) both to identify wells containing hybrids that produce antibodies that bind to thymic or splenic subpopulations and to rapidly clone hybrids from positive initial wells[1] before the desired hybrids are overgrown by other cells in the well. The initial fusions were performed according to the procedures of Oi and Herzenberg[2] using 1:1 ratios of NS-1 myeloma cells to spleen cells from rats that had been immunized i.p. with 10^7 mouse thymus or spleen cells and boosted with an equal number of cells four weeks later. Three days after the boost, cells were fused and plated into a 96-well culture dish (Costar #3596, Cooke Engineering, Alexandria, Virginia) at 10^6 cells per well. Growing hybrids were detected in every well within 15 days.

Examples of FACS staining profiles of supernates from three positive wells of the initial culture dish are shown in Fig. 1. The bound monoclonal antibodies in these cases are revealed by the "second step" staining with a fluorescein-conjugated mouse (SJL/J) anti-rat IgG. We used this second step reagent to avoid cross-reaction with mouse immunoglobulin. SJL/J mice were used as donors because their responses to rat Ig were substantially higher than other mouse strains tested. Antibodies that were obtained from the wells shown in Fig. 1 were later found to be detecting the lymphocyte differentiation antigens Lyt-1, Lyt-2, and Thy-1.2.

After the identification of positive initial wells, we used a new modification of the FACS to clone viable hybrids into microtiter plates containing mouse thymocytes as feeder cells[1]. This allowed rapid cloning from each well and avoided overgrowth of the desired hybrid by other cells. The FACS with this cloning modification deposits one cell in each well of a cloning plate, thus avoiding the problems associated with limiting dilution or soft agar cloning of hybrids.

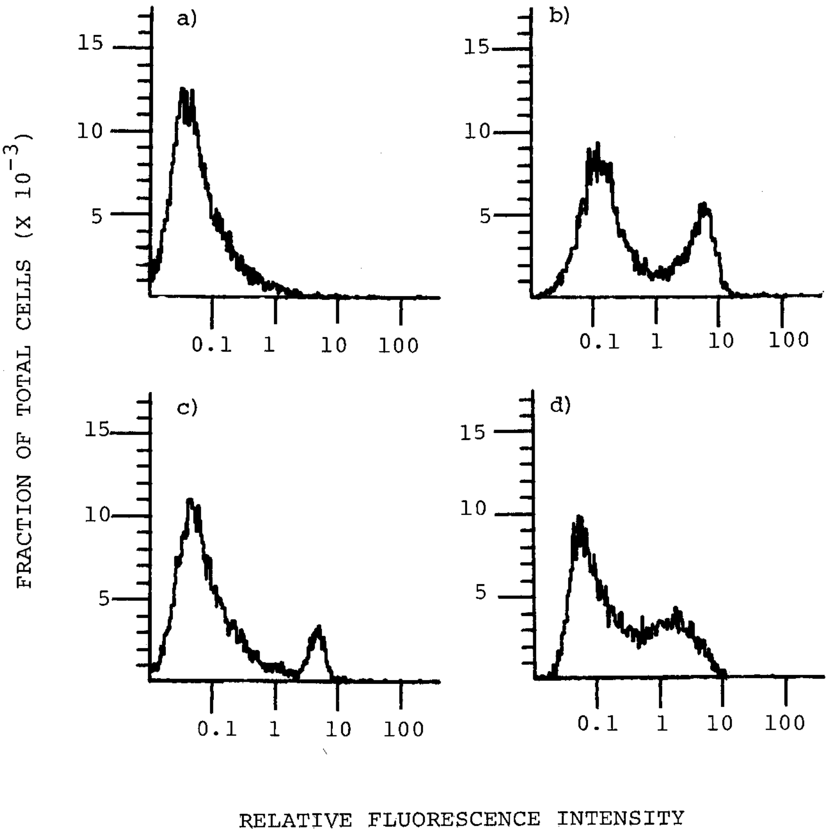


Fig. 1. Immunofluorescent staining of C3H/HeJ spleen cells with supernates from initial hybrid wells. After reaction with hybrid supernates, bound antibody was detected by reaction with FITC-conjugated second step antibody (SJL anti-rat Ig). (A) Background staining with second step only. (B) Monoclonal antibody 30-H12 (anti-Thy-1.2). (C) Monoclonal antibody 53-6.7 (anti-Lyt-2). (D) Monoclonal antibody 53-7.3 (anti-Lyt-1). Stained cells were analyzed using the FACS-II with a logarithmic amplifier.

Nearly all of the antibodies described here were derived from two separate hybridization experiments (H-30 and H-53). FACS analysis showed that 24 of of 96 wells initially tested in H-30 were producing antibody to cell-surface determinants. From these 24 we cloned the 10 which reacted with spleen subpopulations. The antibodies from these 10 clones detect 7 distinct surface antigens. Eighty-five of 96 wells in H-53 were positive in initial FACS analysis and many of these were frozen for later studies. Ten clones have been isolated thus far from H-53 yielding antibodies collectively detecting an additional eight distinct antigenic specificities. All of these rat/mouse hybridoma cell lines have been rather stable in tissue culture and only two have been recloned during the past 4-8 month period when their titers appeared to be dropping.

In our hands, screening for antibody in supernates from hybrid cells with fluorescence staining proved superior to other assays for detection of antibody activity. Positive supernates were also tested by radioimmune binding, cytotoxicity and immunoprecipitation; however, at least one of the antibodies failed to react in each of these assays. The reactivities of the antibodies in these assays are summarized in Table I. Those antibodies characterized further are described in later sections of this paper.

Comparison of FACS data and radioimmune assay (RIA) binding (see Table I) shows that RIA are generally most useful in detecting cell-surface antigens present in relatively large quantities on relatively large numbers of cells. When RIA reactivity was detectable, we used this assay in "cross-blocking" studies to identify clonal products reacting with different antigenic determinants on the same cell-surface molecule (i.e., to test the ability of one antibody to block binding of a different radiolabeled antibody to the target antigen). The cross-blocking assay is useful for detection of monoclonal antibodies against new cell-surface specificities. Positive wells that do not block the binding of any previously defined monoclonal antibody are likely to react with distant antigenic determinants on the same molecule or determinants on different molecules. Thus the cross-blocking assays minimize further studies of "repeat" antibodies and increase the chances of recovery of rare clones producing antibodies to antigens such as Lyt-1 and Lyt-2.

We found the cross-blocking assays extremely valuable for deciding which hybrids to keep when initial FACS screening revealed a large number of wells apparently reacting with the same molecular species. For example, in one of our hybridizations, FACS analysis showed that 34/85 wells had hybrids producing antibodies that gave similar staining profiles to anti-Thy-1 (see Fig. 1).

Another 6/85 wells contained antibody that appeared to stain for T200. (These antigens are both present on thymocytes.) Cross-blocking assays allowed us to screen for hybrids potentially producing antibody to different antigenic determinants on each of these molecules (see Fig. 2).

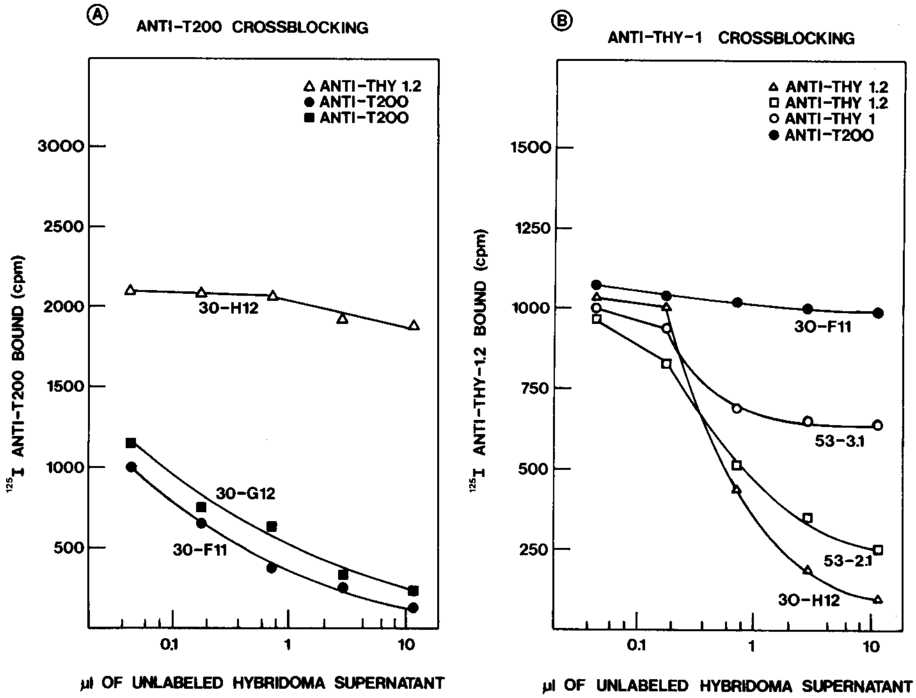


Fig. 2. Crossblocking assays for detection of antibody specificities. (A) Monoclonal antibody supernates were tested for their ability to block the binding of an ^{125}I-labeled anti-T200 monoclonal antibody (30-F11) to immobilized thymocyte membranes. (B) Monoclonal antibody supernates were tested for their ability to block the binding of an ^{125}I-labeled anti-Thy-1.2 monoclonal antibody (30-H12) to immobilized thymoctye membranes.

Many clones produced antibody that completely blocked a known anti-Thy-1.2 or anti-T200 monoclonal. These cells were viably frozen. Hybrid supernates that showed partial blocking of anti-Thy-1.2 or anti-T-200 were selected as candidates for having antibodies that react with different antigenic determinants on the target molecule. This proved to be an effective strategy in that it led to the identification and selection of a clone producing antibody to a framework (non-polymorphic) Thy-1 determinant distinct from the Thy-1.2 determinant (Fig. 2).

Immunoprecipitation and 2-D gel analysis provided a third major assay system for characterizing target determinants. About half of the monoclonal antibodies we obtained reacted with cell-surface glycoproteins that were detectable by immunoprecipitations from NP-40 extracts of cells that were surface-labeled with ^{125}I using the lactoperoxidase technique (see Table I). Immunoprecipitated proteins were characterized by 2-D gel electrophoresis, a system that separates proteins based upon charge-dependent migration properties in the first dimension and size-dependent migration in the second dimension. We used non-equilibrium pH gradient electrophoresis (NEPHGE) in the first dimension to resolve basic as well as acidic proteins and 10% SDS gels in the second dimension[3,4].

Fig. 3 shows the 2-D gel pattern of total thymocyte surface proteins and surface glycoproteins that were specifically precipitated by five of the monoclonal antibodies (Thy-1, Lyt-1, Lyt-2, Lgp100, T30). The monoclonal antibodies listed in Table I gave no detectable immunoprecipitation from NP-40 extracts; however, preliminary evidence suggests that several of these antibodies can precipitate cell-surface glycoproteins from extracts made with other detergents.

<u>Immunochemical Characterization of Lyt-1 and Lyt-2 Glycoproteins.</u>

Three T lymphocyte differentiation antigens, Lyt-1, Lyt-2 and Lyt-3, have been described using conventional cytotoxic alloantisera[5,6]. These antigens are controlled by separate genetic loci. Two alleles are known at each locus, e.g. Lyt-1.1, Lyt-1.2. Genetic mapping studies indicated that the Lyt-1 locus is on chromosome 19[7] and that the Lyt-2 and Lyt-3 loci are closely linked to each other on chromosome 6[8]. The identification of the Lyt-1, Lyt-2 and Lyt-3 alloantigens is currently based upon the presence of cell-surface determinants in Lyt congenic pairs on the C57Bl/6 background[9].

The expression of Lyt antigens distinguishes functional subclasses of T lymphocytes[10,11]. T cells that demonstrate helper activity and T cells that demonstrate delayed-type hypersensitivity responses are killed by anti-Lyt-1 serum but not by anti-Lyt-2 serum[12-15]. In contrast, T cells that demonstrate suppressor activity and T cells with cytotoxic killer activity[10,11,16,17] are killed by anti-Lyt-2 serum but not by anti-Lyt-1 serum. Cells with phenotype

TABLE I

RAT MONOCLONAL ANTIBODIES TO MOUSE CELL-SURFACE ANTIGENS

a) Glycoprotein Differentiation Antigens

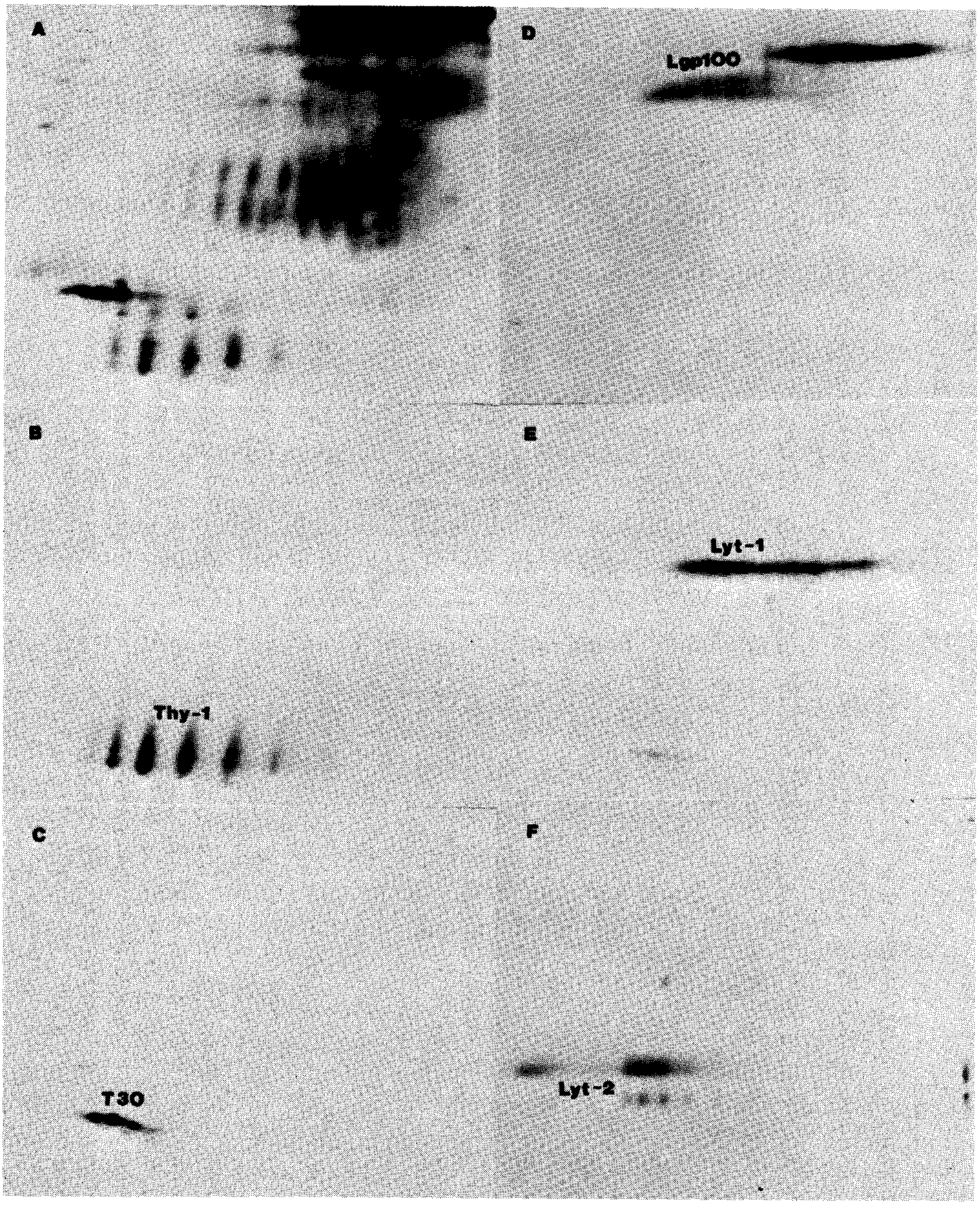
Antibody	Target Antigen	H-Chain Isotype[1]	Cyto-toxicity[2]	RIA[3]	Protein A Binding[4]	FACS Analysis[5] Spleen (%)	T	B	Thymus (%)	Bone Marrow (%)
30-H12	Thy-1.2	IgG_{2b}	+	29	-	37	+	-	>97	<1
53-2.1	"	IgG_{2a}	+	34	+/-	39	+	-	>98	<1
53-3.1	Thy-1	IgM	+	18	-	37	+	-	>98	<1
AD 2.2[6]	"	IgG_{2a}	+	26	-	42	+	-	>98	<1
53-8.1	T30[7]	IgG_{2c}	+	6.9	+	40	+	-	>97	<1
30-C7	Lgp100[7]	IgG_{2a}	-	3.7	-	75	+	+	>93	40
55-6.1[6]	T200	IgG_{2b}	+	3.9	-	90	+	+	>95	55
30-F11	"	"	+	9.2	-	85	+	+	>97	68
30-G12	"	IgG_{2a}	+/-	11	-	89	+	+	>98	65
55-10.2[6]	"	"	-	9.7	-	90	+	+	>95	84
30-F4	"	"	-	9.5	-	92	+	+	>95	72
53-7.3	Lyt-1	IgG_{2a}	+/-	6.2	-	35	+	-	>98	<1
53-6.7	Lyt-2	IgG_{2a}	+/-	9.0	+/-	11	+	-	90	<1
53-5.8	"	IgG_1	-	8.4	-	10	+	-	89	<1

TABLE I (Cont.)

b) Chemically Uncharacterized Differentiation Antigens

Antibody	Target Antigen	H-Chain Isotype[1]	Cyto-toxicity[2]	RIA[3]	Protein A Binding[4]	RBC Aggl.	FACS Analysis[5] Spleen (%)	T	B	Thymus (%)	Bone Marrow (%)
30-H11	--	IgG_{2b}	+	3.7	-	-	43	+	-	>96	49
30-E2	--	IgG_{2b}	+	1.1	-	+	20			<1	<1
30-F1	--	IgG_{2c}	+/-	3.5	+	+	80	+	+	90	66
30-H2	--	IgG_{2a}	-	2.3	-	+	89	+	+	>85	71
53-1.4	--	IgG_1	-	1.2	-	-	75	+	+	>93	55
53-9.2	--	IgG_{2c}	+/-	5.7	+	-	65	-	+	78	20
53-10.1	--	IgG_{2c}	-	1.1	+	+	70	-	+	>90	78
55-9.1[6]	--	IgG_{2b}	+	2.0	n.d.	-	91	+	+	67	95
55-7.2[6]	--	IgG_{2a}	-	4.3	n.d.	-	90	+	+	>98	90
49-h4[6]	ThB	IgG_{2c}	+	3.1	+	-	50	-	+	50	27

1 Determined by double diffusion with class-specific antisera (Miles).
2 Studies of direct cytotoxicity on thymocytes were conducted by Dr. T. Tokuhisa in our laboratory.
3 Radioimmune binding is expressed as specific binding/background binding assayed on thymocyte membranes.
4 Determined by reactivity of the antibodies with ^{125}I-Protein A in radioimmune assays.
5 Determined by fluorescence staining and analysis using the FACS II.
6 These antibodies were produced by Dr. H.S. Micklem, Dr. J. Haaijman and L. Eckhardt in our laboratory.
7 Previously undescribed (see text).


A
B
Thy-1
C
T30
D
Lgp100
E
Lyt-1
F
Lyt-2

Lyt-1^+23^+ appear to be mainly precursors of the above subsets[13] although there are several examples where cytotoxic killer T cells have been removed by cytotoxic treatment with either anti-Lyt-1 or anti-Lyt-2,3[16,18].

The target antigens of the conventional anti-Lyt antisera have been biochemically characterized by immunoprecipitation from detergent extracts of thymocytes that were ^{125}I-labeled by the lactoperoxidase technique. Anti-Lyt-1 antisera precipitated a 67,000 dalton glycoprotein and a glycoprotein of 87,000 daltons that could be labeled with NaB^3H_4 and galactose oxidase but not with the ^{125}I lactoperoxidase method[19]. The Lyt-2 and Lyt-3 antigens were found on cell-surface glycoproteins labeled with the ^{125}I lactoperoxidase method[20]. Immune precipitations with anti-Lyt-3 serum showed a 35,000 dalton protein on SDS gels. Anti-Lyt-2 serum also immunoprecipitated a 35,000 dalton protein. It is not yet clear whether Lyt-2 and Lyt-3 antigens are expressed on the same or different proteins.

The data presented here using monoclonal antibodies confirm and extend the findings with conventional sera. The 2-D gel analysis of immunoprecipitates with antibodies to Lyt-1 and Lyt-2 shows the charge, molecular weight and subunit structure of the lymphocyte surface carrying these determinants (see Fig. 3). Comparative 2-D gel immunoprecipitation studies with C57BL/6 Ly congenic strains show that conventional and monoclonal antibodies precipitate the same molecular species (see Figs. 4 and 5). Our rat antibodies differ from the conventional (allogenic) sera in that ours precipitate both allelic forms of Lyt-1 or Lyt-2 and therefore must recognize framework or species of Lyt-1 or Lyt-2 and therefore must recognize framework or species (non-polymorphic) determinants on the Lyt-1 and Lyt-2 proteins.

The anti-Lyt-1 monoclonal (53-7.3) precipitated a protein of approximately 70,000 daltons that exhibited extensive charge heterogeneity on 2-D gels (see Fig. 4). This molecule ran identically in gels under both reducing and non-reducing conditions; thus it appears to be composed of a single popypeptide chain rather than sulfhydryl linked subunits.

Fig. 3. Two-dimensional gel electrophoresis of cell-surface glycoproteins immunoprecipitated with monoclonal rat antibodies from NP-40 extracts of ^{125}I lactoperoxidase-labeled BALB/c thymocytes. (A) Total ^{125}I-labeled thymocyte proteins; (B) Immunoprecipitate using antibody 30-H12 (anti-Thy-1.2); (C) Immunoprecipitate using antibody 53-8.1 (anti-T30); (D) Immunoprecipitate using antibody 30-C7 (anti-Lgp100a); (E) Immunoprecipitate using antibody 53-7.3 (anti-Lyt-1); (F) Immunoprecipitate using antibody 53-6.7 (anti-Lyt-2). The first dimension separations were by NEPHGE (acidic proteins are on the right and basic portions are on the left). The second dimension separations were by SDS-PAGE on 10% gels (from top to bottom).

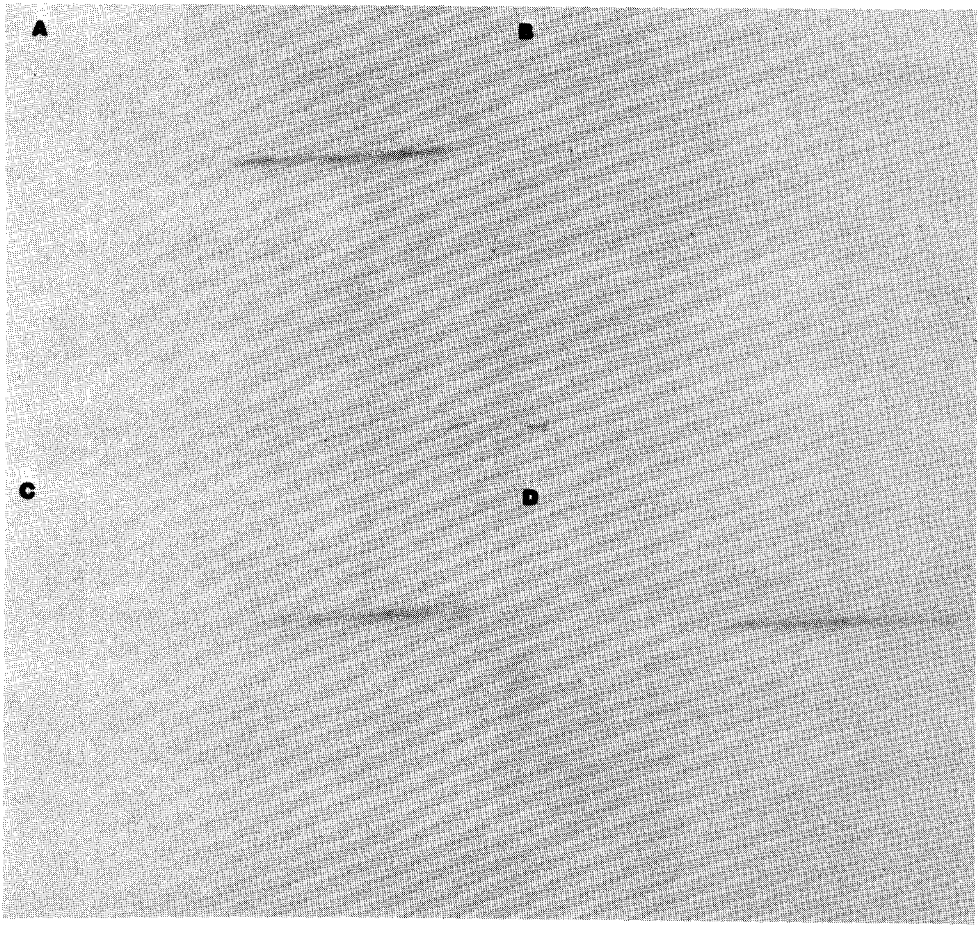


Fig. 4. Comparison of Lyt-1.1 allo antiserum with a monoclonal rat antibody for immunoprecipitation from NP-40 extracts of ^{125}I lactoperoxidase labeled thymocytes of C57BL/6 Ly congenic strains. Immunoprecipitates (A) from C57BL/6.Lyt-1.1 thymocytes using Lyt-1.1 alloantiserum: (B) from C57BL/6.Lyt-1.2 thymocytes using Lyt-1.1 alloantiserum; (C) from C57BL/6.Lyt-1.1 thymocytes using monoclonal rat antibody 53.7.3; (D) from C57BL/6.Lyt-1.2 thymocytes using monoclonal rat antibody 53-7.3. Autoradiograms of the 2-D gels are positioned as described in the legend to Fig. 3. The Lyt-1.1 alloantiserum, a (BALB/c X C57BL/6)F_1 anti-C57BL/6.Lyt-1.1, was generously provided by Dr. M. Tam and Rd. R. C. Nowinski.

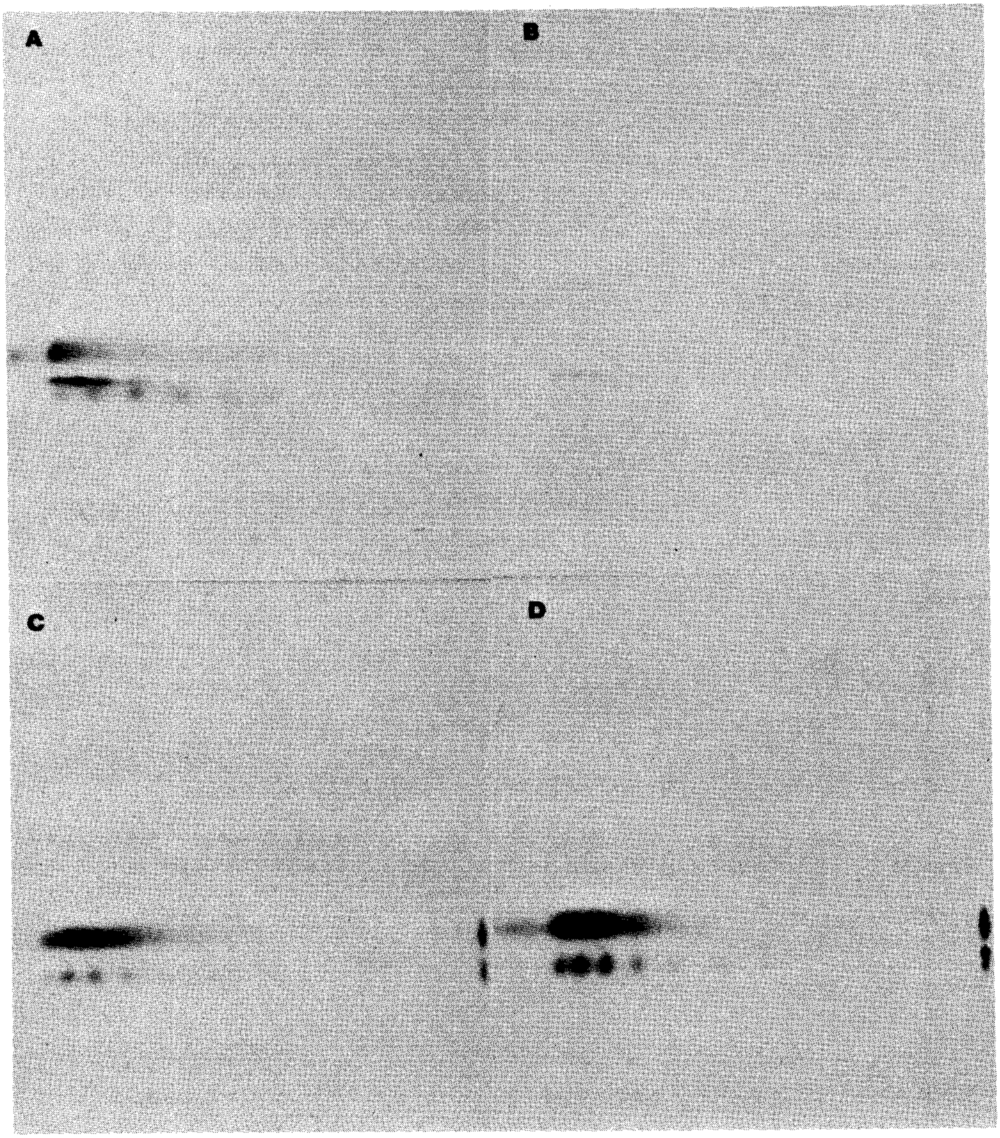


Fig. 5. Comparison of Lyt-2.2 alloantibody with a monoclonal rat antibody for immunoprecipitation from ^{125}I lactoperoxidase-labeled thymocytes of C57BL/6 Ly congenic strains. Immunoprecipitates from: (A) C57BL/6 Lyt-2.2 thymocytes using Lyt-2.2 alloantibody; (B) C57BL/6 Lyt-2.1 thymocytes using Lyt-2.2 alloantibody; (C) C57BL/6 Lyt-2.2 thymocytes using monoclonal rat antibody 53-6.7; (D) C57BL/6 Lyt-2.1 thymocytes using monoclonal rat antibody 53-6.7. Autoradiograms of the 2-D gels are positioned as described in the legend to Fig. 3. The anti-Lyt-2.2 was a monoclonal antibody generously provided by Dr. U. Hämmerling.

In contrast, Lyt-2 appears to consist of two subunits of approximately 30,000 and 35,000 daltons that resolved on 2-D gels under reducing conditions (see Fig. 5). Under non-reducing conditions, the 30,000 and 35,000 dalton proteins are not present; instead a 65,000 dalton glycoprotein appears which runs with the same charge characteristics (extremely basic) as the two small subunits found under reducing conditions (see Fig. 6). Therefore the 30,000 and 35,000 dalton proteins most likely constitute a disulfide-bonded cell-surface molecule. It is conceivable that one of these subunits may be the product of the Lyt-2 gene and the other the product of the Lyt-3 gene.

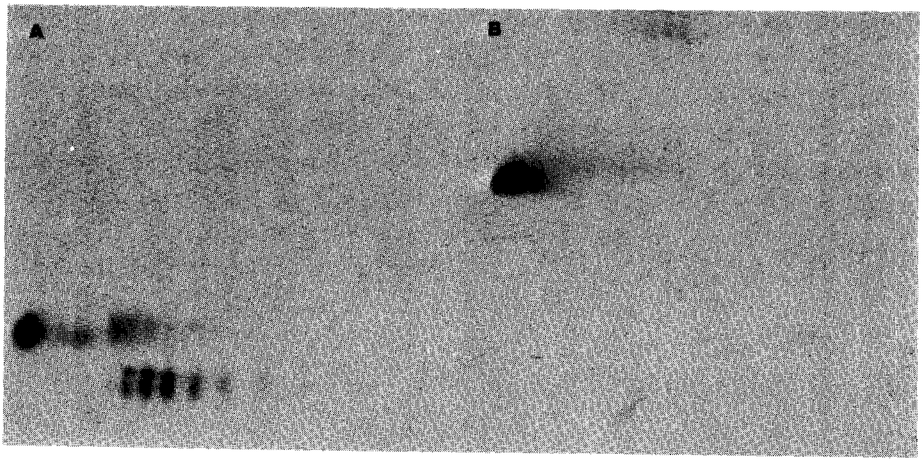


Fig. 6. Reduced vs. non-reduced forms of the Lyt-2 glycoprotein. Thymocytes from BALB/c mice were labeled with ^{125}I using the lactoperoxidase technique and extracted with 0.5% NP-40. Lyt-2 was immunoprecipitated from the cell lysate with monoclonal rat antibody 53-6.7 and divided into two aliquots for (A) electrophoresis under reducing conditions, and (B) electrophoresis under non-reducing conditions. Autoradiograms of the 2-D gels are positioned as described in the legend to Fig. 3.

The above identification of Lyt-1 and Lyt-2 as the target antigens of several of our monoclonal antibodies relied upon the immunoprecipitation of glycoproteins from thymocytes of the C57BL/6 Ly congenic strains. However, in view of the expression of Lyt-1 and Lyt-2 alloantigens on functional subsets of T cells, we feel it is necessary to demonstrate the presence of the immunoprecipitated glycoproteins on functional T cell populations prior to definitively assigning the glycoproteins as Lyt-1 and Lyt-2.

For Lyt-1, FACS analysis (see Table II) indicates that this antigen is present on essentially all thymocytes and peripheral T cells, even though

TABLE II

EXPRESSION OF T CELL DIFFERENTIATION MARKERS AMONG LYMPHOID CELLS[1]

BALB/c Lymphoid Cell Suspension	Monoclonal Antibody Anti-Thy-1.2 (30-H12)	Anti-Lyt-1 (53-7.3)	Anti-Lyt-2 (53-6.7)
Thymus			
all cells	>99	>99	91
cort. res.[2]	>99	>99	50
Spleen			
all cells	34	30	11
nylon passed[3]	87	84	28
Lymph node	78	80	25
Bone Marrow	<1	<1	<1
Periph. Blood Lymph.[4]	61	63	12

[1]Expressed as percentage of cells under the positive peak determined by quantitative fluorescence staining using the FACS-II

[2]Cortisone resistant thymocytes were produced by intraperitoneal injection of 2.5 mg of hydrocortisone acetate per animal 48 hours prior to assay.

[3]Splenic T cells were enriched using nylon columns as described by Julius et al. 1973.

[4]Peripheral blood lymphocytes were purified on Ficoll-paque (Pharmacia).

suppressor T cells are not killed by anti-Lyt-1 and complement. Our 53-7.3 antibody that recognizes the same 70,000 dalton glycoprotein recognized by a conventional anti-Lyt-1.1 serum (Fig. 4) shows a 14-fold variation in the brightness of staining among splenic T cells (Fig. 1), indicating that Lyt-1 is expressed in different quantities on different T cells. If functionally distinct T cells express different amounts of Lyt-1 antigen, then the cells

TABLE III

Ig-1b ALLOTYPE SUPPRESSOR T CELLS EXPRESS Lyt-2 DETERMINANTS DETECTED BY A MONOCLONAL RAT ANTIBODY*

(SJL x BALB/c)F_1 Spleen Cells Transferred (x 10^6)		Anti-DNP Response (RIA)[1]	
DNP-KLH Primed Normal	Unprimed Suppressed[2]	Ig-1a	Ig-1b
10	--	92	76
10	5 (stained, unseparated)[3]	108	24
10	0.5 (Lyt-2 positive)	86	16
10	5 (Lyt-2 negative)	84	72

[1]Units of a standard anti-DNP serum. One unit = 1% of the binding activity of the standard, measured on DNP_{42}-BSA. Response measured 21 days after transfer

[2]Suppressed by exposure to maternal anti-Ig-1b (17)

[3]Stained with monoclonal rat antibody 53-6.7 (anti-Lyt-2). See Figure 1 for staining pattern of spleen cells with this antibody.

*These experiments performed with Dr. T. Tokuhisa and Lee Herzenberg.

with low Lyt-1 expression may be difficult to kill with antibody and complement. This may explain discrepancies among different laboratories in the ability to remove cytotoxic killer T cells with anti-Lyt-1 and complement[16,18]. In fact, recent evidence indicates that cytotoxic T cells can be removed by anti-Lyt-1 and complement[21], indicating that these cells do express low levels of Lyt-1.

For Lyt-2, where a subpopulation of peripheral T cells carries the antigen, FACS staining and separation could be performed. We used our 53-6.7 monoclonal antibody to show that the glycoprotein it detects (Fig. 6) is expressed on allotype suppressor T cells (see Table III). The use of a monoclonal antibody is the key factor in allowing the demonstration of the relationship of a surface molecular structure (the Lyt-2 glycoprotein) to a functional T cell subpopulation (suppressor T cells). In several cases conventionally prepared anti-Ly sera were shown to contain antibodies to other, non-Ly determinants[22,23] and thus Shen and co-workers[9] have advised caution in the use of the standard, non-congenic anti-Ly alloantisera.

Since Lyt-2 is expressed on about only 10% of total spleen cells or 25% of splenic T cells (Table II) it is possible that the Lyt-2 glycoprotein plays a role in the functions of the cells that express it (suppressor and cytotoxic T cells). Recent experiments show that conventional or monoclonal Lyt-2 antisera specifically block the killing ability of cytotoxic T cells[21] (Dr. N. Shinohara, personal communication). However, it is not yet known whether Lyt-2 is involved in the recognition stages or in the effector stages of T cell killing.

ACKNOWLEDGEMENTS

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TABLE III

Ig-1b ALLOTYPE SUPPRESSOR T CELLS EXPRESS Lyt-2 DETERMINANTS DETECTED BY A MONOCLONAL RAT ANTIBODY *

(SJL x BALB/c)F_1 Spleen Cells Transferred (x 10^6)		Anti-DNP Response (RIA)[1]	
DNP-KLH Primed Normal	Unprimed Suppressed[2]	Ig-1a	Ig-1b
10	--	92	76
10	5 (stained, unseparated)[3]	108	24
10	0.5 (Lyt-2 positive)	86	16
10	5 (Lyt-2 negative)	84	72

[1]Units of a standard anti-DNP serum. One unit = 1% of the binding activity of the standard, measured on DNP_{42}-BSA. Response measured 21 days after transfer

[2]Suppressed by exposure to maternal anti-Ig-1b (17)

[3]Stained with monoclonal rat antibody 53-6.7 (anti-Lyt-2). See Figure 1 for staining pattern of spleen cells with this antibody.

*These experiments performed with Dr. T. Tokuhisa and Lee Herzenberg.

with low Lyt-1 expression may be difficult to kill with antibody and complement. This may explain discrepancies among different laboratories in the ability to remove cytotoxic killer T cells with anti-Lyt-1 and complement[16,18]. In fact, recent evidence indicates that cytotoxic T cells can be removed by anti-Lyt-1 and complement[21], indicating that these cells do express low levels of Lyt-1.

For Lyt-2, where a subpopulation of peripheral T cells carries the antigen, FACS staining and separation could be performed. We used our 53-6.7 monoclonal antibody to show that the glycoprotein it detects (Fig. 6) is expressed on allotype suppressor T cells (see Table III). The use of a monoclonal antibody is the key factor in allowing the demonstration of the relationship of a surface molecular structure (the Lyt-2 glycoprotein) to a functional T cell sub-

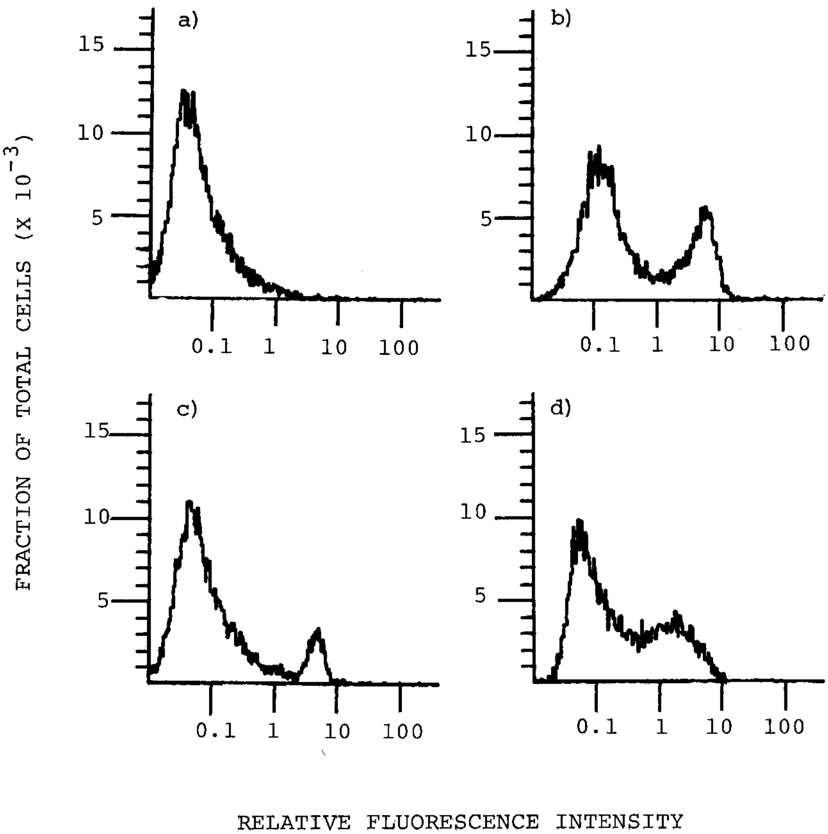


Fig. 1. Immunofluorescent staining of C3H/HeJ spleen cells with supernates from initial hybrid wells. After reaction with hybrid supernates, bound antibody was detected by reaction with FITC-conjugated second step antibody (SJL anti-rat Ig). (A) Background staining with second step only. (B) Monoclonal antibody 30-H12 (anti-Thy-1.2). (C) Monoclonal antibody 53-6.7 (anti-Lyt-2). (D) Monoclonal antibody 53-7.3 (anti-Lyt-1). Stained cells were analyzed using the FACS-II with a logarithmic amplifier.

In contrast, Lyt-2 appears to consist of two subunits of approximately 30,000 and 35,000 daltons that resolved on 2-D gels under reducing conditions (see Fig. 5). Under non-reducing conditions, the 30,000 and 35,000 dalton proteins are not present; instead a 65,000 dalton glycoprotein appears which runs with the same charge characteristics (extremely basic) as the two small subunits found under reducing conditions (see Fig. 6). Therefore the 30,000 and 35,000 dalton proteins most likely constitute a disulfide-bonded cell-surface molecule. It is conceivable that one of these subunits may be the product of the Lyt-2 gene and the other the product of the Lyt-3 gene.

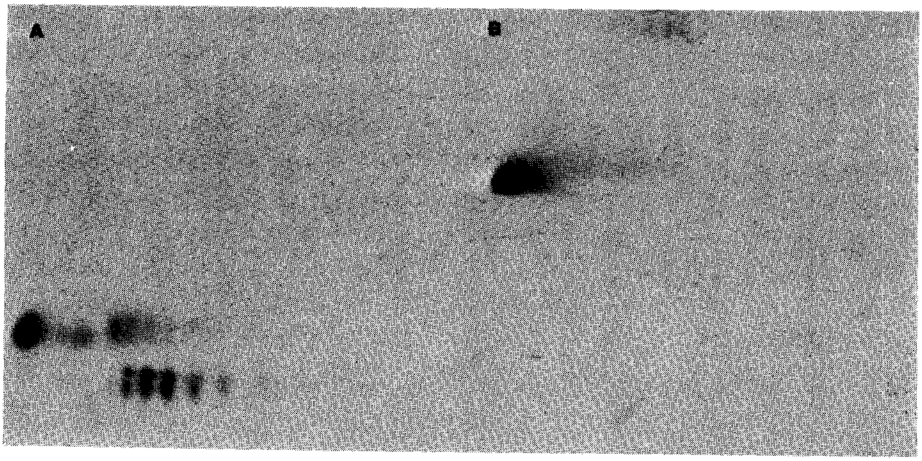


Fig. 6. Reduced vs. non-reduced forms of the Lyt-2 glycoprotein. Thymocytes from BALB/c mice were labeled with ^{125}I using the lactoperoxidase technique and extracted with 0.5% NP-40. Lyt-2 was immunoprecipitated from the cell lysate with monoclonal rat antibody 53-6.7 and divided into two aliquots for (A) electrophoresis under reducing conditions, and (B) electrophoresis under non-reducing conditions. Autoradiograms of the 2-D gels are positioned as described in the legend to Fig. 3.

The above identification of Lyt-1 and Lyt-2 as the target antigens of several of our monoclonal antibodies relied upon the immunoprecipitation of glycoproteins from thymocytes of the C57BL/6 Ly congenic strains. However, in view of the expression of Lyt-1 and Lyt-2 alloantigens on functional subsets of T cells, we feel it is necessary to demonstrate the presence of the immunoprecipitated glycoproteins on functional T cell populations prior to definitively assigning the glycoproteins as Lyt-1 and Lyt-2.

For Lyt-1, FACS analysis (see Table II) indicates that this antigen is present on essentially all thymocytes and peripheral T cells, even though

Another 6/85 wells contained antibody that appeared to stain for T200. (These antigens are both present on thymocytes.) Cross-blocking assays allowed us to screen for hybrids potentially producing antibody to different antigenic determinants on each of these molecules (see Fig. 2).

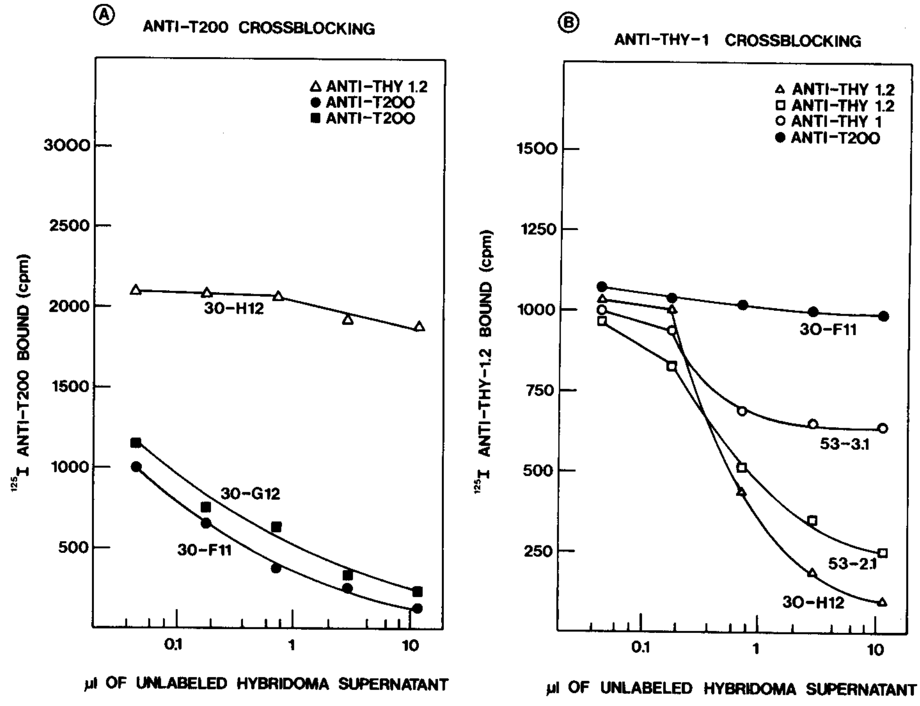


Fig. 2. Crossblocking assays for detection of antibody specificities. (A) Monoclonal antibody supernates were tested for their ability to block the binding of an ^{125}I-labeled anti-T200 monoclonal antibody (30-F11) to immobilized thymocyte membranes. (B) Monoclonal antibody supernates were tested for their ability to block the binding of an ^{125}I-labeled anti-Thy-1.2 monoclonal antibody (30-H12) to immobilized thymoctye membranes.

Many clones produced antibody that completely blocked a known anti-Thy-1.2 or anti-T200 monoclonal. These cells were viably frozen. Hybrid supernates that showed partial blocking of anti-Thy-1.2 or anti-T-200 were selected as candidates for having antibodies that react with different antigenic

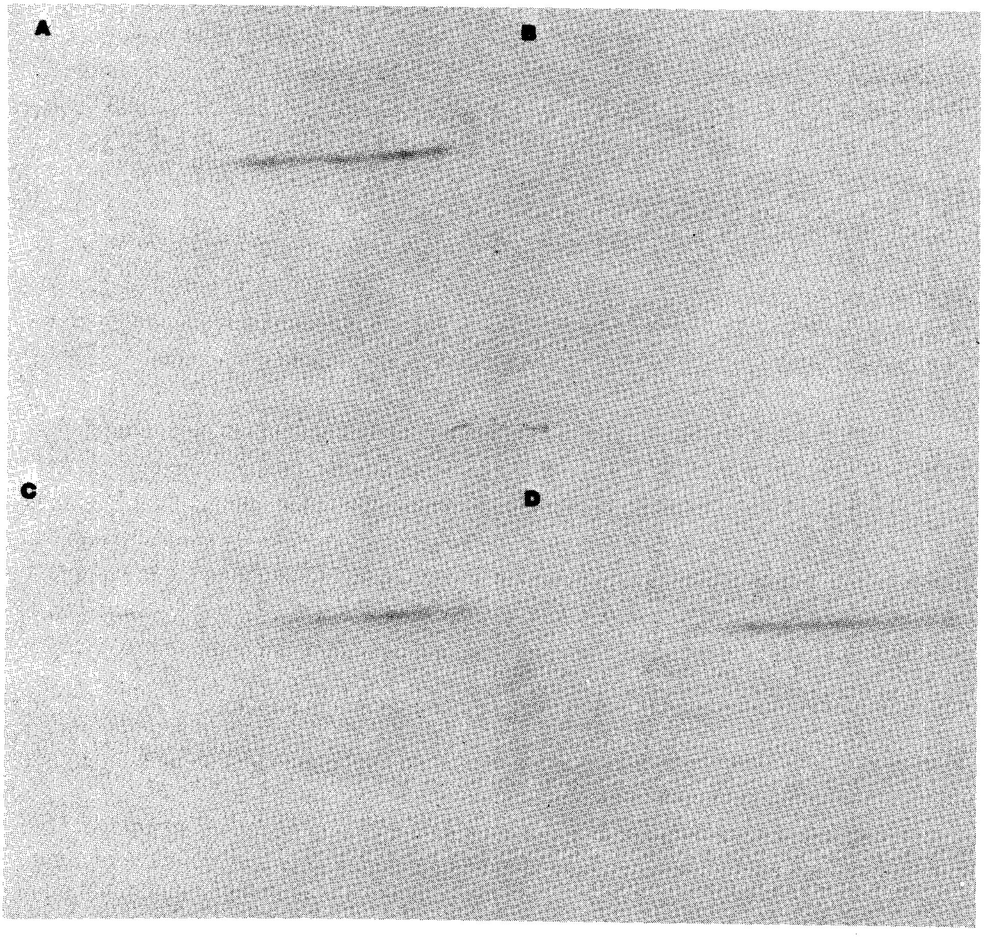


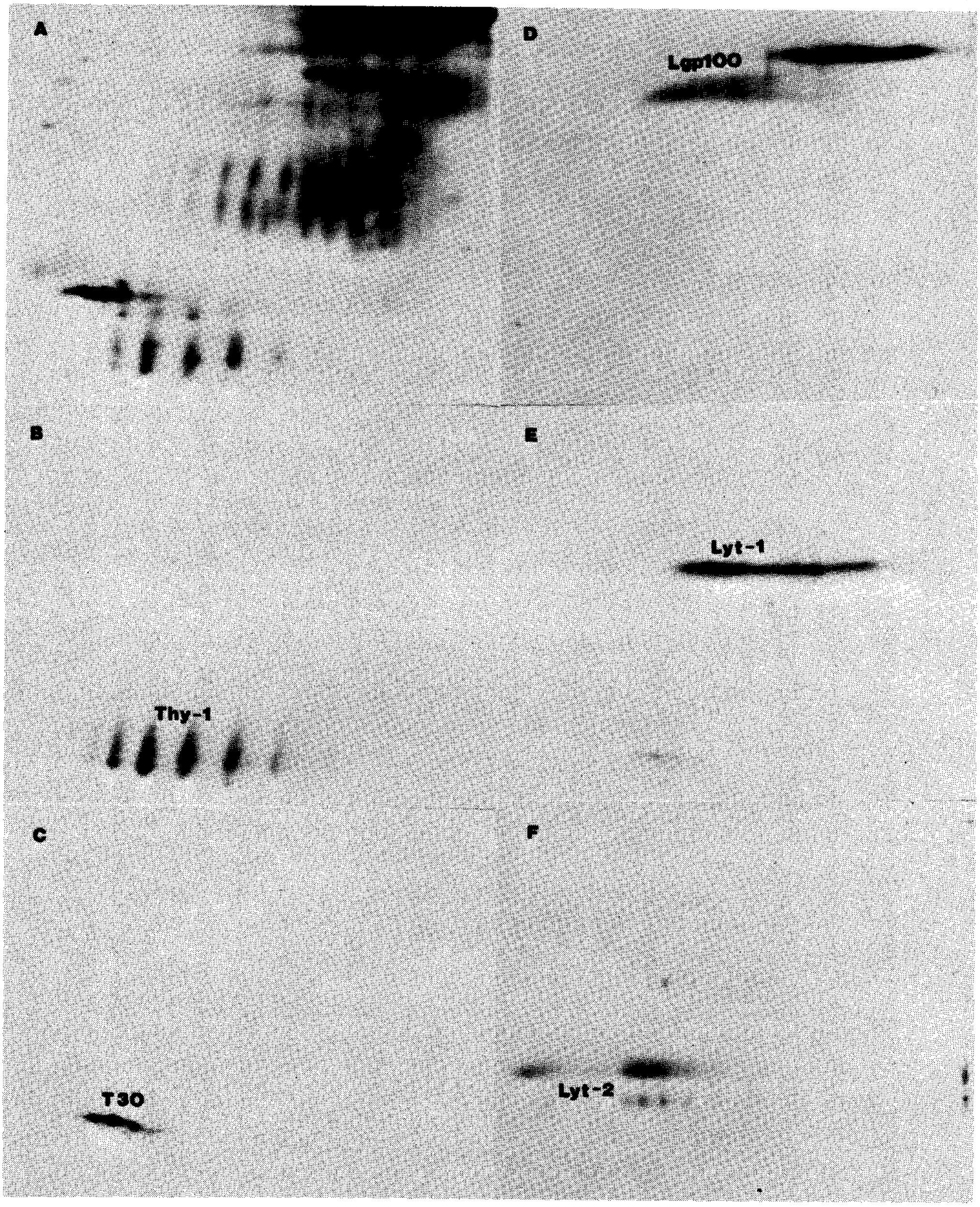
Fig. 4. Comparison of Lyt-1.1 allo antiserum with a monoclonal rat antibody for immunoprecipitation from NP-40 extracts of ^{125}I lactoperoxidase labeled thymocytes of C57BL/6 Ly congenic strains. Immunoprecipitates (A) from C57BL/6.Lyt-1.1 thymocytes using Lyt-1.1 alloantiserum: (B) from C57BL/6.Lyt-1.2 thymocytes using Lyt-1.1 alloantiserum; (C) from C57BL/6.Lyt-1.1 thymocytes using monoclonal rat antibody 53.7.3; (D) from C57BL/6.Lyt-1.2 thymocytes using monoclonal rat antibody 53-7.3. Autoradiograms of the 2-D gels are positioned as described in the legend to Fig. 3. The Lyt-1.1 alloantiserum, a (BALB/c X C57BL/6)F_1 anti-C57BL/6.Lyt-1.1, was generously provided by Dr. M. Tam and Rd. R. C. Nowinski.

TABLE I

RAT MONOCLONAL ANTIBODIES TO MOUSE CELL-SURFACE ANTIGENS

a) Glycoprotein Differentiation Antigens

Antibody	Target Antigen	H-Chain Isotype[1]	Cyto-toxicity[2]	RIA[3]	Protein A Binding[4]	FACS Analysis[5] Spleen (%)	T	B	Thymus (%)	Bone Marrow (%)
30-H12	Thy-1.2	IgG_{2b}	+	29	-	37	+	-	>97	<1
53-2.1	"	IgG_{2a}	+	34	+/-	39	+	-	>98	<1
53-3.1	Thy-1	IgM	+	18	-	37	+	-	>98	<1
AD 2.2[6]	"	IgG_{2a}	+	26	-	42	+	-	>98	<1
53-8.1	T30[7]	IgG_{2c}	+	6.9	+	40	+	-	>97	<1
30-C7	Lgp100[7]	IgG_{2a}	-	3.7	-	75	+	+	>93	40
55-6.1[6]	T200	IgG_{2b}	+	3.9	-	90	+	+	>95	55
30-F11	"	"	+	9.2	-	85	+	+	>97	68
30-G12	"	IgG_{2a}	+/-	11	-	89	+	+	>98	65
55-10.2[6]	"	"	-	9.7	-	90	+	+	>95	84
30-F4	"	"	-	9.5	-	92	+	+	>95	72
53-7.3	Lyt-1	IgG_{2a}	+/-	6.2	-	35	+	-	>98	<1
53-6.7	Lyt-2	IgG_{2a}	+/-	9.0	+/-	11	+	-	90	<1
53-5.8	"	IgG_1	-	8.4	-	10	+	-	89	<1


A
D
Lgp100
B
E
Lyt-1
Thy-1
C
F
T30
Lyt-2