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Xenogeneic Monoclonal Antibodies to Mouse Lymphoid Differentiation Antigens*

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I. INTRODUCTION

Xenogeneic immunizations have the advantage of detecting a wide range of antigenic determinants because many commonly occurring proteins have diverged significantly during the course of evolution and are thus antigenic in other species. The broadness of xenogeneic responses, however, means that the antisera they produce are usually complex and require extensive absorptions to make them specific for a single antigen. This problem has now been overcome by generating hybridomas producing monoclonal antibodies (Köhler & Milstein 1975). These permit dissection of the xenogeneic response so that large amounts of individual antibodies can be obtained, each of which recognizes only one of the determinants recognized by a broadly reactive conventional antiserum.

Williams et al. (1977) used hybridoma monoclonal antibodies obtained after immunizations of mice with rat cells to study rat cell-surface antigens present on subpopulations of rat lymphocytes, i.e., differentiation antigens. Springer et al. (1978a) and Stern et al. (1978) used a similar approach to study mouse lymphocyte antigens. They prepared monoclonal antibodies by immunizing rats with mouse lymphocytes and showed that these monoclonals recognized previously undetected mouse cell surface determinants including a glycoprotein antigen that appears to be specific for macrophages (Springer et al. 1978b). Trowbridge (1978) also used rat anti-mouse immunizations to generate a monoclonal antibody against the non-polymorphic lymphocyte surface antigen T200.

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In this report, we present studies using monoclonal antibodies produced by 24 currently stable hybridomas we generated by fusion of the mouse myeloma line NS1 with spleen cells of LOU/Ws1/M rats immunized with mouse thymus or spleen. The reactivities obtained in this series demonstrate that monoclonal antibodies derived from xenogeneic immunizations can detect either polymorphic or non-polymorphic determinants. Clones in this series produce antibodies detecting the polymorphic determinants Thy-1.2 and Lgp100a, a lymphocyte surface glycoprotein that we recently identified and characterized using one of these monoclonal antibodies (Ledbetter et al. 1979). Several others produce antibodies that detect non-polymorphic determinants on cell-surface molecules previously known to be polymorphic (Thy-1, Lyt-1, Lyt-2). Others identify nonpolymorphic determinants present on lymphocyte subpopulations.

For all the monoclonal antibodies in the series, we present fluorescence-activated cell sorter (FACS) analysis showing the distribution of the target antigens on lymphoid subpopulations. For those antibodies that precipitate detectable amounts of glycoproteins from NP-40 detergent extracts of surface-labeled cells, we show 2-D gel characterization of charge, size, and subunit structure of the target antigens. In addition, we determined the rat IgG subclass of each antibody, and present data on the cytotoxicity and protein A binding properties of each. We also present an interesting relationship which appeared between amount of antibody bound per cell, subclass and direct complement dependent cytotoxicity.

II. DETECTION AND ANALYSIS OF MONOCLONAL ANTIBODIES REACTIVE WITH CELL-SURFACE ANTIGENS

Our strategy for isolating hybrid clones depends on the use of the FACS both to identify initial wells containing hybrids that produce antibodies that bind to thymic or splenic subpopulations and to rapidly clone hybrids from positive initial wells (Parks et al. 1979) before the desired hybrids are overgrown by other cells in the well. The initial fusions were performed according to the procedures of Oi & Herzenberg (1979) using 1:1 ratios of NS1 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 4 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 Figure 1. The bound monoclonal antibodies in these cases are revealed by "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 Figure 1 were later found to be detecting the lymphocyte differentiation antigens Lyt-1, Lyt-2, and Thy-1.2 (see below).

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 (Parks et al. 1979). 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.

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 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 seven distinct surface antigens. Eighty-five of 96 wells in H-53 were positive in initial FACS

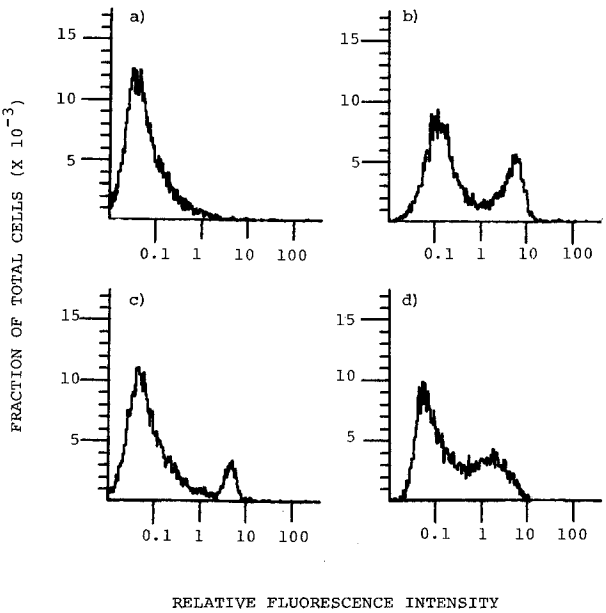

Figure 1. Immunofluorescent staining of C3H/HeJ spleen cells with supernates from initial hybrid wells. After reaction with hybrid supernates, bound antibody was detected by reaction with FITC-conjugated second step antibody (SJL anti-Rat Ig). (*A*) Background staining with second step only. (*B*) Monoclonal 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.

TABLE I

(a) *Rat monoclonal antibodies to mouse cell-surface 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	Thy-1.2	IgG_{2a}	+	34	+/−	39	+	−	>98	<1
53-3.1	Thy-1	IgM	+	18	−	37	+	−	>98	<1
AD 2.2[6]	Thy-1	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	T200	IgG_{2b}	+	9.2	−	85	+	+	>97	68
30-G12	T200	IgG_{2a}	+/−	11	−	89	+	+	>98	65
55-10.2[6]	T200	IgG_{2a}	−	9.7	−	90	+	+	>95	84
30-F4	T200	IgG_{2a}	−	9.5	−	92	+	+	>95	72
53-7.3	Lyt-1	IgG_{2a}	+/−[8]	6.2	−	35	+	−	>98	<1
53-6.7	Lyt-2	IgG_{2a}	+/−[8]	9.0	+/−	11	+	−	90	<1
53-5.8	Lyt-2	IgG_1	−	8.4	−	10	+	−	89	<1

(b)

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

(a) Glycoprotein Differentiation Antigens

(b) Chemically Uncharacterized Differentiation Antigens

[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).

[8] These antibodies are cytotoxic after conjugation with arsanilic acid and use of a rabbit anti-arsanilic acid for sandwich killing.

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

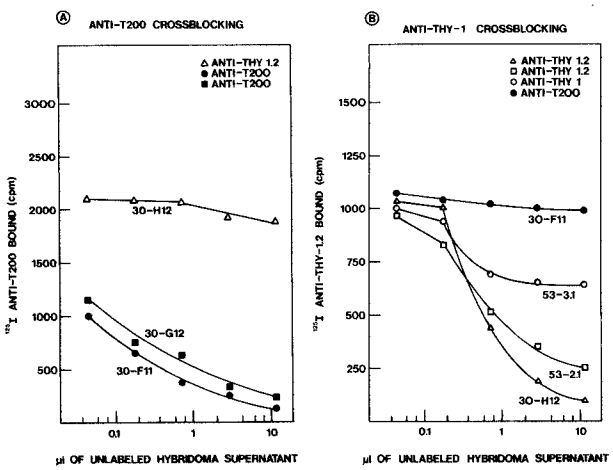

Figure 2. Crossblocking assays for detection of antibody specificities. (*A*) Monoclonal antibody supernates were tested for their ability to block the binding of an ^{125}I-labeled anti-T200 monoclonal antibody (30–F11) to immobilized thymocyte membranes. (*B*) Monoclonal antibody supernates were tested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

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 hybidization, FACS analysis showed that 34/85 wells had hybrids producing antibodies that gave similar staining profiles to anti-Thy-1 (see Figure 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 Figure 2).

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-T200 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 (Figure 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 1). 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 nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension to resolve basic as well as acidic proteins and 10% SDS gels in the second dimension (O'Farrell 1975, O'Farrell et al. 1977).

Figure 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 (see below)). The monoclonal antibodies listed in Table I(b) 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.

In the sections that follow, we present some detailed studies using individual monoclonal antibodies to investigate the expression and biochemical properties

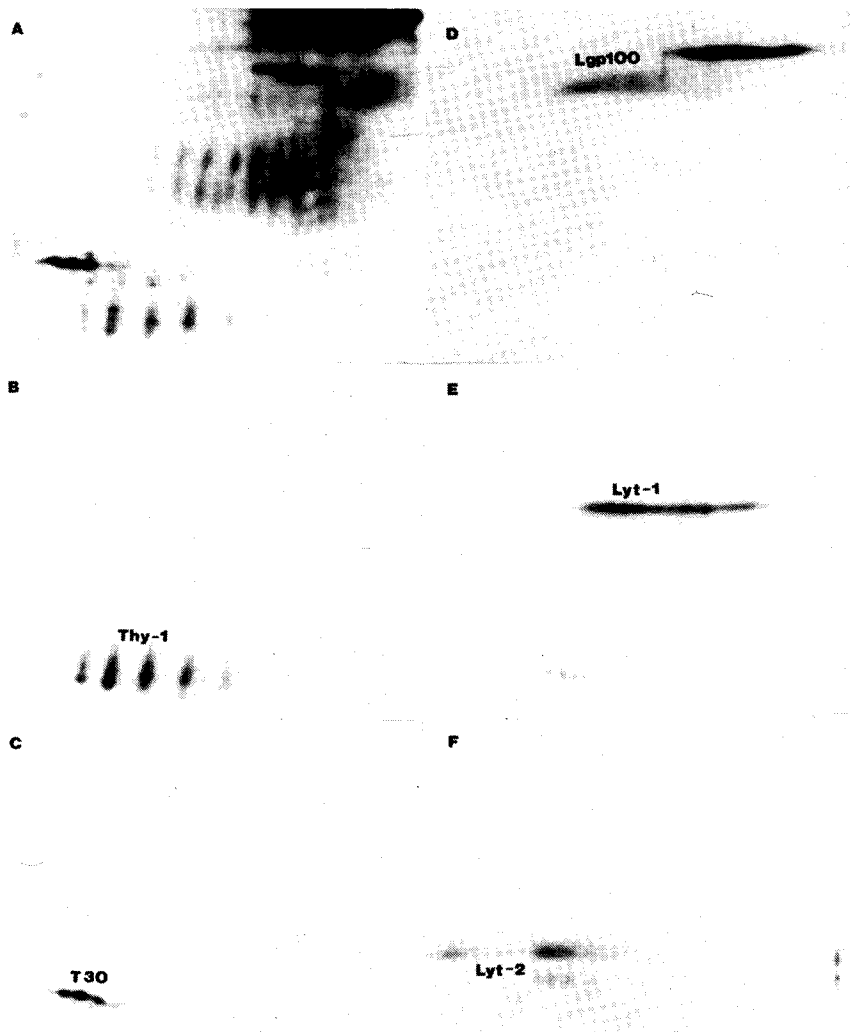

Figure 3. Two-dimensional gel electrophoresis of cell-surface glycoproteins immunoprecipitated with monoclonal rat antibodies from NP-40 extracts of ^{125}I lactoperoxidase-labeled BALB/c thymocytes. (*A*) Total ^{125}I-labeled thymocyte proteins; (*B*) Immunoprecipitate using antibody 30–H12 (anti-Thy-1.2); (*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 non-equilibrium pH-gradient electrophoresis (acidic proteins are on the right and basic proteins are on the left). The second dimension separations were by SDS-PAGE on 10% gels (from top to bottom).

of target antigens. The first sections describe characteristics of Thy-1, Lyt-1, and Lyt-2. Later sections are concerned with previously unknown or uncharacterized differentiation antigens such as ThB, Lgp100, and T30.

The last section departs from this format in presenting a summary of the properties of rat IgG subclasses as determined in the studies of the 24 rat antibodies that constitute our monoclonal series. This section includes an in-depth analysis of differences in cytotoxic efficiency of the various subclasses, possible because all but one of the antibodies studied react with thymocytes.

III. TWO ANTIGENIC SPECIFICITIES OF MOUSE THY-1 RECOGNIZED BY MONOCLONAL ANTIBODIES

The Thy-1 antigen was one of the first mouse lymphocyte differentiation antigens to be discovered (Reif & Allen 1964). Thy-1 is expressed on thymocytes and thymus-dependent cells in the spleen, but is absent on bone marrow cells. It also is expressed in large amounts on brain cells, and in small amounts on epithelial cells and fibroblasts (Barclay et al. 1976, Hilgers et al. 1975, Scheid et al. 1972, Stern 1973). Two Thy-1 alloantigenic determinants, Thy-1.1 and Thy-1.2 are known in mice. These are coded for by alleles of the "Thy-1" locus on chromosome 9 (Itakura et al. 1971, Blankenhorn & Douglas 1972). Thy-1.1 antigenic determinants have also been detected on rat thymocytes with mouse alloantisera, but Thy-1.2 antigenic determinants have not been seen on any rat strains (Douglas 1972, Williams 1977).

Thy-1 has been purified from both rat brain and rat thymus and characterized biochemically (Letarte-Muirhead et al. 1975, Barclay et al. 1975). Two forms of rat thymocyte Thy-1 were identified on the basis of ability to bind to lentil lectin affinity columns. These investigators reported the lentil lectin binding form was a glycoprotein of 25,000 daltons whereas the lentil lectin nonbinding form was a glycoprotein of 27,000 daltons. Rat brain Thy-1 was found to differ from both of these forms in molecular weight and was a glycoprotein of 24,000 daltons.

Amino acid and carbohydrate composition studies of the three forms of rat Thy-1 have been reported (Barclay et al. 1976). These studies indicated that the amino acid compositions are very similar for all three; however, the carbohydrate compositions were markedly different for brain Thy-1 as opposed to either form of thymocyte Thy-1. Smaller carbohydrate differences between thymocyte lentil lectin binding and nonbinding Thy-1 also were apparent. Thus, in the rat, the brain and thymus forms of Thy-1 appear to have the same polypeptide chain associated with different carbohydrate structures.

Mouse Thy-1 antigenic determinants are on a 25,000 dalton glycoprotein similar to the rat molecule. Trowbridge et al. (1975) used antiserum against rat brain that crossreacts with mouse Thy-1 to immunoprecipitate a 25,000 dalton species from mouse thymocytes that were metabolically labeled with radioactive

sugars or by ^{125}I with the lactoperoxidase technique. Several reports have suggested that Thy-1 determinants are expressed on glycolipids (Miller & Esselman 1975, Wang et al. 1978). The monoclonal antibodies we produced, which have staining and cytotoxic properties indistinguishable from coventional anti-Thy-1, precipitate a family of thymocyte glycoproteins with molecular weights of 25,000–30,000 that have biochemical properties very similar to rat

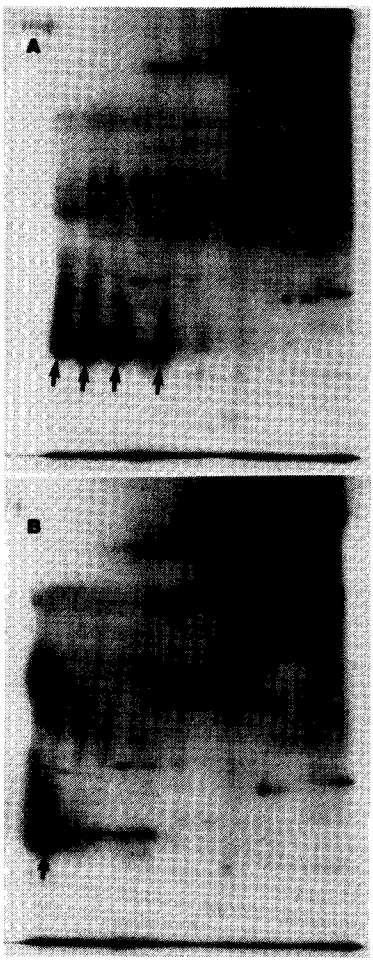

Figure 4. Neuraminidase digestion of thymocyte surface glycoproteins. BALB/c thymocytes were labeled with ^{125}I using the lactoperoxidase technique and then extracted with 0.5% NP-40. (*A*) ^{125}I-labeled thymocyte glycoproteins before neuraminidase digestion; (*B*) ^{125}I-labeled thymocyte glycoproteins after neuraminidase digestion. The arrows in each panel point to the Thy-1 glycoprotein. Autoradiographs of the two-dimensional gels are positioned as described in the legend to Figure 3.

Thy-1. We have not yet examined glycolipids for reactivity with our monoclonal Thy-1 antibodies.

Our monoclonal Thy-1 antibodies identify at least two antigenic determinants on mouse Thy-1. One antibody (30-H12) is specific for a Thy-1.2 antigenic determinant. It stains AKR/Cu (Thy-1.2) thymocytes and immunoprecipitates a glycoprotein from these thymocytes, but it is unreactive with thymocytes from a congenic strain (AKR/J) carrying the Thy-1.1 allele. A second antibody (53–3.1) identifies a framework (non-polymorphic) determinant present on both Thy-1.1 and Thy-1.2 but not on rat Thy-1. This distinction between rat and mouse Thy-1 confirms previous data from absorption studies with conventional xenogeneic antisera showing that mouse and rat Thy-1 each have distinct antigenic determinants (Clagett et al. 1973, Thiele & Stark 1974, Morris & Williams 1975).

The 2-D gel pattern of Thy-1 immunoprecipitated from thymocytes (Figure 3) was obtained with the 30-H12 monoclonal antibody and is identical to patterns obtained with all other anti-Thy-1 antibodies in the series. It shows a family of proteins of 25,000–30,000 daltons with extensive charge and size heterogeneity. Neuraminidase digestions of whole thymocyte extracts (Figure 4) show that the charge heterogeneity of Thy-1 is caused by variable amounts of terminal sialic acid on the glycoprotein. The size heterogeneity of Thy-1 is most likely due to variable amounts of neutral sugars since the series of Thy-1 glycoproteins are divided, based upon size, into a lentil lectin binding fraction (25,000 daltons) and a lentil lectin nonbinding fraction (26,000–30,000 daltons; see Figure 5). These

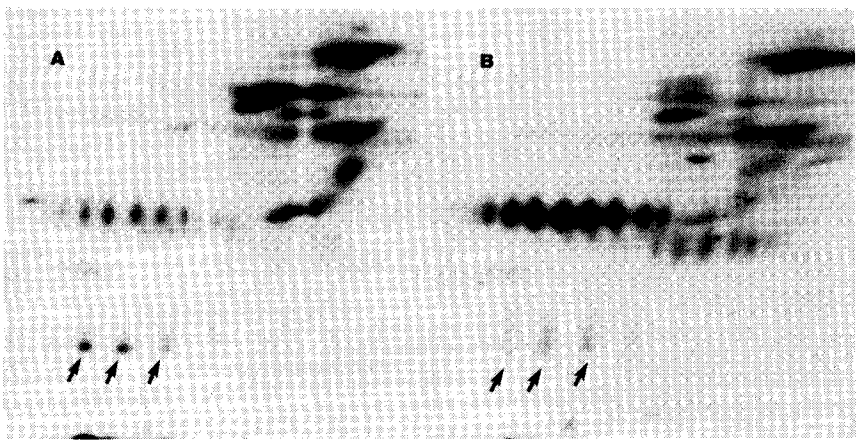

Figure 5. Lentil lectin binding and non-binding surface glycoproteins. C57BL/6 EL4 cells were surface-^{125}I-labeled with the lactoperoxidase technique and extracted with 0.5% NP-40. The cell lysate was then separated into binding and non-binding fractions using lentil-lectin-Sepharose. (*A*) Lentil lectin bound glycoproteins; (*B*) Lentil lectin non-bound glycoproteins. The arrows in each panel point to the Thy-1 glycoprotein. Autoradiographs of the two-dimensional gels are positioned as described in the legend to Figure 3.

data demonstrate that mouse Thy-1, like rat (Williams 1977), most likely consists of a single polypeptide chain with heterogeneity in its glycosylation. Note that the lentil lectin binding forms of mouse thymocyte Thy-1 migrate with an apparently smaller molecular weight on SDS gels compared to the lentil lectin non-binding forms (Figure 5). Therefore the ability of Thy 1 to bind to lentil lectin does not appear to be directly related to its degree of glycosylation.

IV. IMMUNOCHEMICAL CHARACTERIZATION OF LYT-1 AND LYT-2 GLYCOPROTEINS

Three T lymphocyte differentiation antigens, Lyt-1, Lyt-2 and Lyt-3, have been described using conventional cytotoxic alloantisera (Boyse et al. 1968, 1971). 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 have indicated that the Lyt-1 locus is on chromosome 19 (Itakura et al. 1971) and that the Lyt-2 and Lyt-3 loci are closely linked to each other on chromosome 6 (Itakura et al. 1972). 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 (Shen et al. 1976).

The expression of Lyt antigens distinguishes functional subclasses of T lymphocytes (Cantor & Boyse 1975a, 1975b). 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 (Jandinski et al. 1976, Huber et al. 1976, Pickel et al. 1976, Cantor et al. 1976). In contrast, T cells that demonstrate suppressor activity and T cells with cytotoxic killer activity (Cantor & Boyse 1975a, Cantor et al. 1976, Beverly et al. 1976, Herzenberg et al. 1976) are killed by anti-Lyt-2 serum but not by anti-Lyt-1 serum. Cells with phenotype Lyt-1^+23^+ appear to be mainly precursors of the above subsets (Huber et al. 1976) 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 (Beverly et al. 1976, Shiku et al. 1975).

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 (Durda et al 1978). The Lyt-2 and Lyt-3 antigens were found on cell-surface glycoproteins labeled with the ^{125}I lactoperoxidase method (Durda & Gottlieb 1976). 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

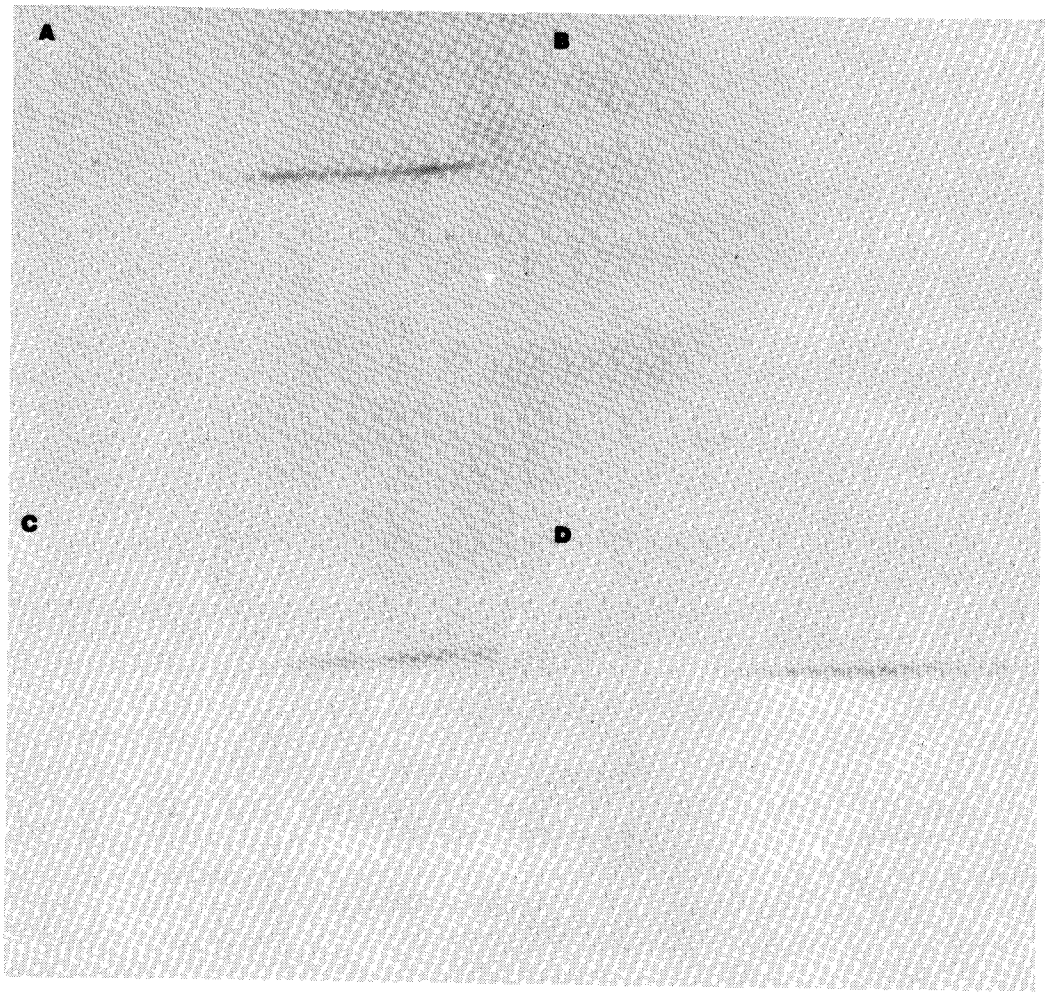

Figure 6. Comparison of Lyt-1.1 alloantiserum with a monoclonal rat antibody for immunoprecipitation from NP-40 extracts of ^{125}I lactoperoxidase labeled thymocytes of C57BL/6 Ly congenic strains. (*A*) Immunoprecipitate from C57BL/6.Lyt-1.1 thymocytes using Lyt-1.1 alloantiserum: (*B*) Immunoprecipitate from C57BL/6.Lyt-1.2 thymocytes using Lyt-1.1 alloantiserum; (*C*) Immunoprecipitate from C57BL/6.Lyt-1.1 thymocytes using monoclonal rat antibody 53-7.3; (*D*) Immunoprecipitate from C57BL/6.Lyt-1.2 thymocytes using monoclonal rat antibody 53-7.3. Autoradiograms of the two-dimensional gels are positioned as described in the Legend to Figure 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 Dr. R. C. Nowinski.

subunit structure of the lymphocyte surface molecule carrying these determinants (see Figure 3). Comparative 2-D gel immunoprecipitation studies with C57BL/6 Ly congenic strains show that conventional and monoclonal anti-

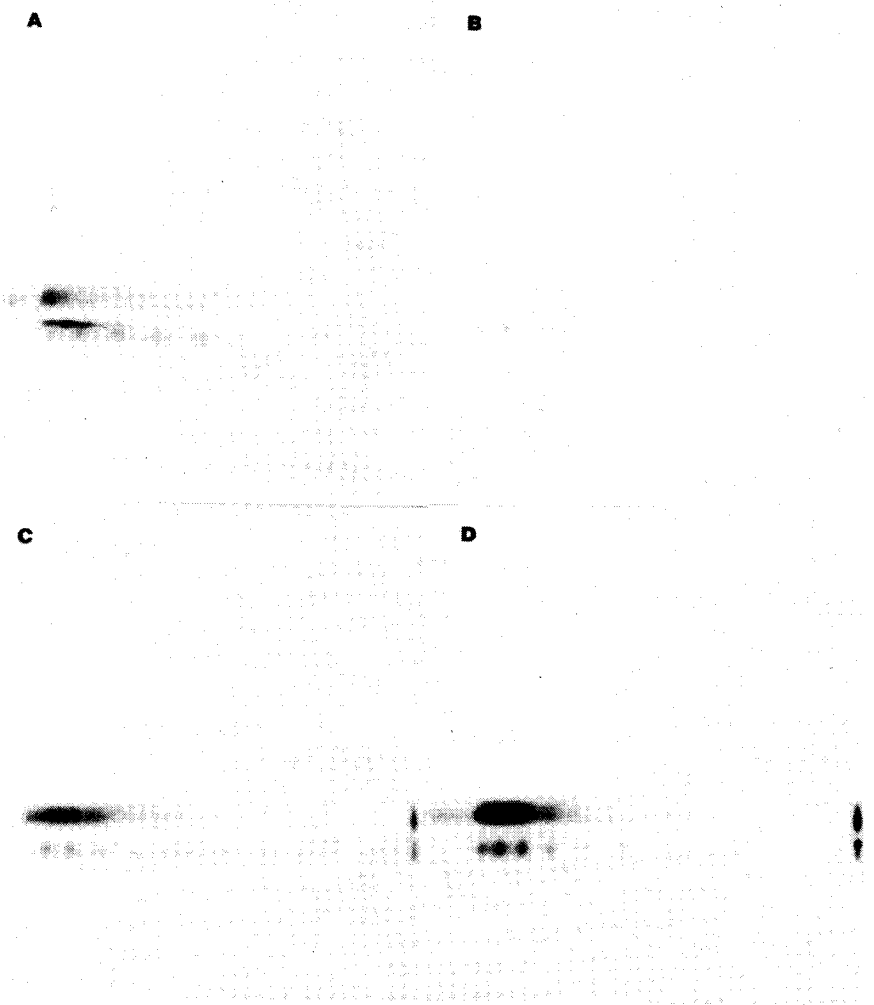

Figure 7. Comparison of Lyt-2.2 alloantibody with a monoclonal rat antibody for immunoprecipitation from ^{125}I lactoperoxidase-labeled thymocytes of C57BL/6 Ly congenic strains. (*A*) Immunoprecipitate from C57BL/6 Lyt-2.2 thymocytes using Lyt-2.2 alloantibody; (*B*) Immunoprecipitate from C57BL/6 Lyt-2.1 thymocytes using Lyt-2.2 alloantibody; (*C*) Immunoprecipitate from C57BL/6 Lyt-2.2 thymocytes using monoclonal rat antibody 53-6.7; (*D*) Immunoprecipitate from C57BL/6 Lyt-2.1 thymocytes using monoclonal rat antibody 53-6.7. Autoradiograms of the two-dimensional gels are positioned as described in the legend to Figure 3. The anti-Lyt-2.2 was a monoclonal antibody generously provided by Dr. U. Hämmerling.

bodies precipitate the same molecular species (see Figures 6 and 7). 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 (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 Figure 6). This molecule ran identically in gels under both reducing and non-reducing conditions; thus it appears to be composed of a single polypeptide chain rather than sulfhydryl linked subunits.

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 Figure 7). 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 Figure 8). 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.

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

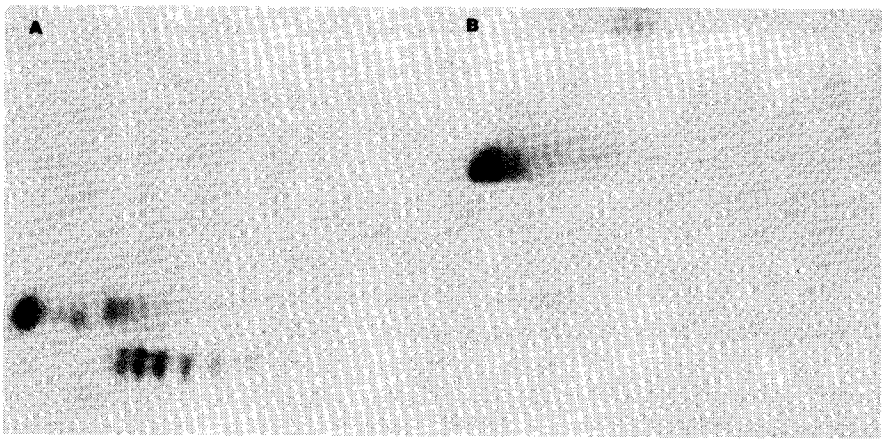

Figure 8. Reducing versus non-reducing forms of the Lyt–2 glycoprotein. Thymocytes from BALB/c mice were labeled with ^{125}I using the lactoperoxidase technique and extracted with 0.5% NP–40. Lyt–2 was immunoprecipitated from the cell lysate with monoclonal rat antibody 53–6.7 and divided into two aliquots for (*A*) electrophoresis under reducing conditions, and (*B*) electrophoresis under non-reducing conditions. Autoradiograms of the two-dimensional gels are positioned as described in the legend to Figure 3.

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 h 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-hypaque (Pharmacia).

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 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 (Figure 6) shows a 14-fold variation in the brightness of staining among splenic T cells (Figure 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 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 (Shiku et al. 1975, Beverley et al. 1976). In fact, recent evidence indicates that cytotoxic T cells can be removed by anti-Lyt-1 and complement (Nakayama et al. 1979), 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 (Figure 8) is expressed on allotype suppressor T cells (see Table III). The use of a monoclonal

TABLE III

Ig-1b allotype suppressor T cells express Lyt-2 determinants detected by a monoclonal rat antibody

(SJL×BALB/c)F_1 Spleen Cells Transferred ($\times 10^6$)		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 (Herzenberg et al. 1975).
[3]Stained with monoclonal rat antibody 53-6.7 (anti-Lyt-2). See Figure 1 for staining pattern of spleen cells with this antibody.

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 (Glimcher et al. 1977, Durda et al. 1979) and thus Shen et al. (1976) have advised caution in the use of the standard, non-congenic anti-Ly alloantisera.

Since Lyt-2 is expressed on only about 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 (Nakayama et al. 1979, 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.

V. IDENTIFICATION OF A NEW LYMPHOID ALLOANTIGEN, LGP100

In the sections above we described monoclonal antibodies that detect non-polymorphic determinants on the Thy-1, Lyt-1 and Lyt-2 glycoproteins. In this section we show that monoclonal antibodies derived from xenogeneic immunization also reveal polymorphic (alloantigenic) determinants which otherwise would rely for detection on the fortuitous choice of an appropriate allogenic strain combination for immunization.

Data in Table IV show the strain distribution of the antigen detected by the

TABLE IV
Expression of Lgp100a[1]

Positive Strains	Negative Strains
A/J	C57BL/6J
AKR/J	C57BL/10J[2]
AKR/Cu	C57L/J
129/J	C57BR/cdJ
BALB/cNHz[2]	C58/J
CBA/N	
C3H/HeJ	
DBA/2J	
SJL/J[2]	

[1]Assayed by fluorescence (FACS) analysis of peripheral blood lymphocytes using monoclonal 30–C7 antibody.
[2]These strains were also tested by immunoprecipitation using monoclonal 30–C7 antibody.

monoclonal 30-C7 antibody. Although most of the common inbred strains stained positively with the monoclonal antibody, a group of five closely related strains were negative (C57BL/6, C57BL/10, C58, C57L and C57BR). Intercross and backcross mice from a positive (BALB/cN) and a negative (C57BL/10J) strain showed that a single (co)dominant gene controls the expression of this determinant. The heterozygous animals express approximately half as much antigen as the homozygous animals by quantitative fluorescence on the FACS (see Figure 9).

Fluorescence staining and FACS analysis show that the 30–C7 antibody reacts with all thymocytes and with both T and B cells in the spleen (Ledbetter et

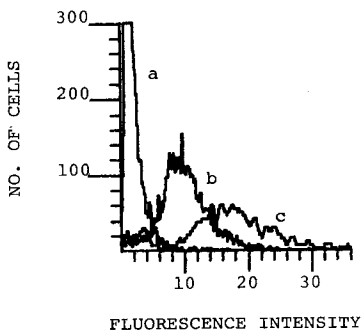

Figure 9. Immunofluorescent staining of peripheral blood lymphocytes (PBL) with monoclonal rat antibody 30–C7 (anti-Lgp100a). After reaction with 30–C7 supernate, bound antibody was detected by reaction with FITC-conjugated second step antibody (SJL anti-rat Ig). (*a*) staining of C57BL/10 PBL; (*b*) staining of (BALB/c X C57BL/10)F_1 PBL; (*c*) staining of BALB/c PBL. Stained cells were analyzed using the FACS-II.

al. 1979). In the bone marrow, 30–C7 reacts with approximately 40% of cells, predominantly those cells in the smaller light scatter peak that contains bone marrow lymphocytes (Loken & Herzenberg 1975). The fluorescence intensity, however, varies markedly with cells from different lymphoid organs. Using saturation levels of the monoclonal antibody, the peripheral blood lymphocytes and lymph node cells stain very brightly, the spleen cells are intermediate in brightness, and the thymus and bone marrow cells stain with a much duller fluorescence (unpublished data).

Immunoprecipitation and 2-D gel electrophoresis using the 30–C7 antibody shows that it reacts with a 100,000 dalton glycoprotein on spleen cells (Ledbetter et al. 1979). Immunoprecipitations were identical from several of the positive strains. Since the antigenic target of 30–C7 is a 100,000 dalton glycoprotein that is expressed on T and B cells in all lymphoid tissues, we named this antigen lymphoid glycoprotein 100 (Lgp100). (This name was agreed to by Dr. Paul Gottlieb, in whose laboratory the same glycoprotein was found as an alloantigen detected by an antibody contaminating an anti-Lyt-2.1 serum) (Durda et al. 1979). Thymocytes have the 100,000 dalton form and a more highly glycosylated (150,000 dalton) form of Lgp100 (see Figure 3).

The gene controlling expression of Lgp100 is located on mouse chromosome 1 near the mixed lymphocyte stimulation (Mls) locus (Festenstein 1976). This linkage was demonstrated by typing 28 informative recombinant inbred (RI) strains from two crosses, C57BL/6J X DBA/2J (BXD) and C57BL/6J X C3H/HeJ (BXH) (Ledbetter et al. 1979). Three recombinants were found among 28 RI strains indicating that Lgp100 is likely to be not more than three centimorgans from Mls. (For methods of linkage analysis using RI strains, see Taylor, 1978).

An allelic form of Lgp100 appears to be expressed in the mouse strains that do not react with 30–C7. An alloantiserum (C3H.SW anti-C57BL/6J thymocytes) immunoprecipitates proteins from ^{125}I lactoperoxidase-labeled C57BL/6 thymocytes with migration properties similar to Lgp100 on 2–D gels. This serum specifically stains intact C57BL/6 thymocytes. Its target antigen, like Lgp100 (Durda et al. 1979, Ledbetter et al. 1979) is highly sensitive to trypsin. We have named the product of the allele detected by the monoclonal 30–C7 antibody as Lgp 100a and the product of the allele detected by the C3H, SW anti-C57BL/6J alloantiserum as Lgp100b.

Antibodies against Lgp100a have also been detected in conventionally prepared alloantisera. In one case anti-Lgp100a antibodies were present in a conventionally prepared anti-Ly-2.1 antiserum (as noted above, Durda et al. 1979). In the other case, we found anti-Lgp100a antibodies were present in an antiserum prepared by immunization of C57BL/6 mice with the AKR leukemia K36 (Ledbetter et al. 1979).

Most of the monoclonal rat anti-mouse antibodies described in this report are

directed against non-polymorphic cell-surface determinants. The two exceptions are 30–H12 (anti-Thy-1.2) and 30–C7 (anti-Lgp100a). Interestingly, in both cases, the LOU rat (used as immunized donor) carries a homologous antigen that shows strong cross-reactivity with the product of the other known mouse allele, i.e., Thy-1.1 and Lgp100b. The presence of Thy–1.1 antigenic

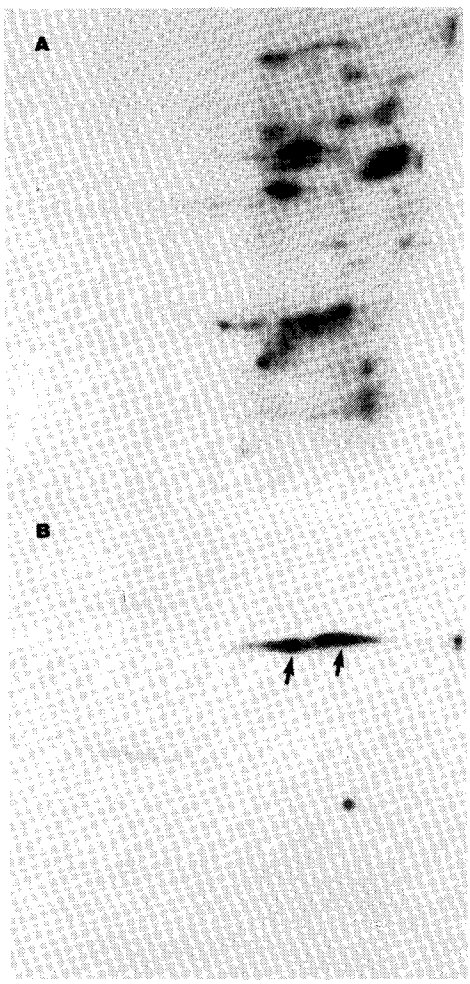

Figure 10. Immunoprecipitation of a rat thymocyte surface protein using a mouse alloantiserum (anti-Lgp100b). LOU/Wsl/M rat thymocytes were surface-labeled with ^{125}I using the lactoperoxidase technique and extracted with 0.5% NP–40. The gel in (*A*) shows the total ^{125}I-labeled proteins, and the gel in (*B*) shows the immunoprecipitate using a C3H.SW anti-C57BL/6 thymocyte (anti-Lgp100b) alloantiserum. The two-dimensional gels are positioned as described in the legend to Figure 3. The arrows in Panel (*B*) show the proteins that were specifically precipitated by the alloantiserum.

TABLE V

Expression of differentiation antigens recognized by monoclonal rat antibodies[1]

BALB/c Lymphoid Cell Suspension	Monoclonal Antibody 49-h4[2] (anti-ThB)	30-H11	30-F1
Thymus			
all cells	50	>95	>90
cort. res.[3]	< 3	>95	>90
Spleen			
all cells	50	40	75
nylon passed[4]	5	92	5
Lymph node	n.d.	75	35
Bone marrow	27	50	66
Perip. Blood Lymph.[5]	n.d.	60	80
Comments:	stains small bone marrow lymphocytes	stains large bone marrow cells	agglutinates erythrocytes

[1]Expressed as percentage of cells under the positive peak determined by quantitative fluorescence staining using the FACS-II.
[2]Studies of ThB were conducted by L. Eckhardt in our laboratory.
[3]Cortisone resistant thymocytes were produced by intraperitoneal injection of 2.5 mg of hydrocortisone acetate per animal 48 h prior to assay.
[4]Splenic T cells were enriched using nylon columns as described by Julius et al. 1973.
[5]Peripheral blood lymphocytes were purified on Ficoll-hypaque (Pharmacia).

determinants on rat thymus has been described (Douglas 1972). The anti-Lgp100b serum strongly stains all rat thymocytes and specifically immunoprecipitates a prominent rat cell-surface protein from surface ^{125}I-labeled rat thymocytes that resolves on 2-D gels with a molecular weight of approximately 90,000 (Figure 10).

VI. IDENTIFICATION OF MORE DIFFERENTIATION ANTIGENS

The 53–8.1 antibody reacts with all thymocytes, causing a tight agglutination of these cells. In the spleen, 53–8.1 reacts with T cells but not with B cells. No cells in the bone marrow express the determinant recognized by this antibody. Thus, the 53–8.1 antibody recognizes a T cell differentiation antigen. Immunoprecipitations from NP-40 extracts of surface ^{125}I-labeled thymocytes show that 53–8.1 recognizes a 30,000 dalton protein that is distinct from Thy-1 (Figure 3). We have named this new T cell differentiation antigen T30.

Many of our monoclonal antibodies that react with lymphocyte surface antigens do not appear to immunoprecipitate glycoproteins from NP-40 extracts of ^{125}I lactoperoxidase-labeled cells (Table I(b)). The target antigens of these

TABLE VI

*Expression of normal lymphocyte differentiation antigens on lymphoid tumors**

Tumor Type	30-H12 (anti-Thy-1.2)	30-G12 (anti-T200)	30-H11	30-E2	30-F1
B lymphomas	0/4	4/4	0/4	3/4	4/4
Plasmacytomas	0/12	5/12	2/3	1/3	0/4
Thymic lymphomas	8/8	8/8	2/2	0/4	5/5
Peripheral T lymphomas	7/7	6/6	5/5	n.d.	1/7
Myeloid tumors	1/8	3/3	3/3	0/3	0/3

*Expressed as number of positives/total number tested.

antibodies may be glycoproteins that either (1) do not contain exposed tyrosines and thus are not labeled by the lactoperoxidase technique; (2) are not solubilized by 0.5% NP-40; or (3) are extracted into but not immunoprecipitable from 0.5% NP-40. Alternatively, these antibodies may recognize carbohydrate determinants on membrane glycolipids that are not detectable on SDS gels.

Although we have not yet characterized the antigenic targets of these antibodies, FACS analysis indicates that nearly all are "differentiation antigens" present on subpopulations of lymphocytes and/or other lymphoid cells. Some representative data on expression of such antigens detected by different monoclonal antibodies are presented in Table V. Two of these antibodies detect determinants on both thymocytes and B cells; however, these two antibodies must be detecting different determinants because only one of them reacts with erythrocytes. The tissue distribution pattern of determinants detected by some of these antibodies are striking compared with the conventional view of developmental relationships among lymphoid cells.

The presence of the same antigenic determinant(s) on thymocytes and B cells, while absent on mature T cells, has been described before. This determinant(s) has been called ThB (Yutoku et al. 1974, 1976, Stout et al. 1975). A gene controlling the level of this antigen or antigens on B cells has been found and its chromosomal location identified (Eckhardt, in preparation).

VII. EXPRESSION OF LYMPHOCYTE SURFACE ANTIGENS ON MOUSE LYMPHOCYTE TUMORS

A wide variety of lymphoid tumors was examined in immunofluorescence studies for expression of determinants detected by the monoclonal antibodies described above. These studies were done in collaboration with Dr. Noel Warner (University of New Mexico) and will be described in detail elsewhere. A summary of the results is shown in Table VI.

With one exception, the reactivities of the antibodies with cells of normal

lymphoid tissues correlated with their reactivities with the lymphoid tumors. For example, an anti-Thy-1.2 monoclonal reacted with all thymic and peripheral T cell lymphomas (originating in Thy–1.2 animals). Thymic lymphomas stained brighter than peripheral T cell lymphomas just as thymocytes stained brighter for Thy-1.2 than peripheral T cells (Cantor et al. 1975). B cell lymphomas and plasmacytomas were not stained by this antibody. One of eight myeloid tumors was Thy-1.2 positive (WEHI–3).

Similarly, the 30–H11 antibody, which detects an antigen present on T but not B cells, reacts with all T cell lymphomas and no B cell lymphomas. Other monoclonal antibodies (30–C7 and 30–F1) react with a pattern of lymphoid tumors that correlate with their reactivities with cells of normal lymphoid tissues. This supports the concept that lymphoid tumors are representative of specific stages of normal differentiation and tend to express "appropriate" surface antigens.

In one case, tumor reactivity provides the only indication thus far of the normal cells on which a determinant detected by a monoclonal antibody may be expressed. The 30–E2 antibody reacts with 20% of spleen cells but does not react with thymocytes or bone marrow cells (Table I). We were unable to determine whether it detects a T or a B cell antigen, however, because it stains its target population very dully. By screening lymphoid tumors, we observed that 30–E2 reacted with three of four B cell tumors and one of three plasmocytomas, but did not react with any T cell lymphomas or myeloid tumors (Table IV). This suggests that 30–E2 may react predominantly with a part of the B cell population during a restricted stage of B cell maturation.

The reaction of anti-Thy-1.2 (30–H12) with the WEHI–3 tumor constitutes the major exception to the correlation between tumor and normal cell expression of determinants. The WEHI–3 cell line is a myelomonocytic leukemia that phagocytoses latex beads, produces colony stimulating factor in culture and has a granulocytic type 90° light scatter (Dr. Noel Warner, personal communication). It is the only one of eight myeloid tumors tested that carries Thy–1.2 determinants. The molecular species on WEHI–3 carrying these determinants shows some differences in 2–D gel migration properties compared to the Thy–1.2 molecular species present on thymocytes (not shown).

VIII. DIRECT CYTOTOXICITY AND PROTEIN A BINDING PROPERTIES OF RAT IMMUNOGLOBULIN SUBCLASSES

Previous studies of rat immunoglobulins relied upon the use of myeloma proteins produced by tumors that occurred spontaneously in the LOU/Ws1/C rat strain (Bazin et al. 1972, 1973). These myeloma proteins have been used to characterize the rat immunoglobulin classes and subclasses (Bazin et al. 1974) and to study their biological properties (Medgyesi et al. 1978). However, the

interactions of the rat immunoglobulins with the complement system could only be studied indirectly with anticomplementary and complement fixing activity of aggregated myeloma proteins (Medgyesi et al. 1978). The studies of IgG_{2b} immunoglobulins were further hampered since myelomas secreting immunoglobulins of this subclass are rare (Bazin et al. 1974).

In the sections above we described 24 monoclonal rat antibodies that react with lymphocyte surface antigens. These antibodies were assayed for their ability to kill target cells in the presence of rabbit complement and for their levels of binding to thymocyte membranes. The relationship between antibody subclass, relative antigen density and direct complement dependent cytotoxicity is shown in Figure 11.

All of the IgG_{2b} subclass antibodies could kill thymocytes regardless of antigen density. In contrast, the IgG_{2a} subclass antibodies could kill thymocytes only if their target antigen was present in high densities. For example, two IgG_{2a} anti-Thy-1 antibodies were cytotoxic, whereas other IgG_{2a} antibodies that recognized antigens present in smaller amounts were non-cytotoxic or marginally cytotoxic. Similarly, the cytotoxicity of the IgG_{2c} subclass antibodies appeared to be related to the density of their target antigen (Figure 11).

Therefore the IgG_{2b} antibodies exhibit very efficient complement-dependent cytotoxicity, whereas the IgG_{2c} and IgG_{2a} antibodies exhibit varying levels of complement-dependent cytotoxicity related to the density of their target

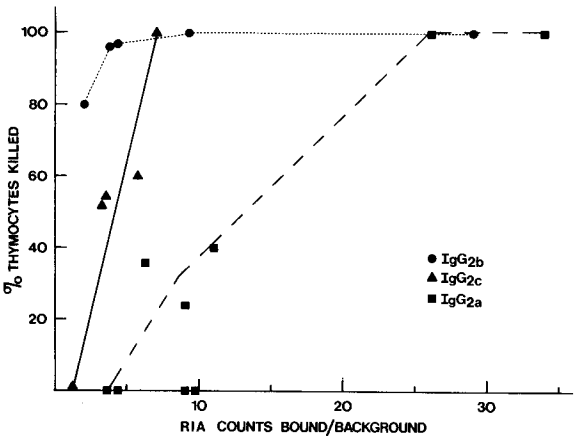

Figure 11. Relationship of antigen density and rat IgG subclass to direct complement dependent cytotoxicity. Each monoclonal rat antibody was measured for its ability to kill target thymocytes with rabbit complement. Cytotoxicity measurements, reported as percentages of thymocytes killed were obtained using saturation levels of antibody in assays with $<10\%$ background. Levels of antibody binding were determined with a radioimmunoassay that used purified thymocyte membranes bound to the plastic wells of a microtiter plate as targets. Binding was measured using saturation levels of each antibody and an ^{125}I-labeled SJL anti-rat Ig as second step reagent.

antigens. Two of our monoclonal antibodies are of the IgG_1 subclass, and neither of these was able to kill thymocytes in the presence of complement (not shown).

The monoclonal rat antibodies were also assayed for their reactivity with ^{125}I protein A in solid-phase radioimmune assays against thymocyte membranes. Binding to *Staphylococcus aureus,* protein A provides a sensitive method for detection of bound antibodies, for immunoprecipitation of membrane glycoproteins (Kessler 1976), and for purification of monoclonal antibodies from culture supernates (Oi et al. 1978).

All of the IgG_{2c} subclass monoclonal antibodies efficiently bound ^{125}I protein A at pH 7.0, whereas none of the IgG_{2a}, IgG_{2b} or IgG_1 antibodies bound ^{125}I protein A under these conditions (Table I). However, at pH 8.6 several of the IgG_{2a} antibodies bound to protein A and were purified from culture supernates on protein A-Sepharose columns.

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