INTERSPECIES SPLEEN-MYELOMA HYBRID PRODUCING MONOCLONAL ANTIBODIES AGAINST MOUSE LYMPHOCYTE SURFACE GLYCOPROTEIN, T200*

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Fusion of myeloma cells and normal antibody-forming cells (1, 2) can be used to obtain cell hybrids producing specific monoclonal antibodies against individual cell surface differentiation antigens (3-5). We have previously identified a large surface glycoprotein on mouse thymus-derived cells referred to as T200 that is a major differentiation antigen of mouse lymphoid tissue (6-8). We report here the selection of a rat spleen cell-mouse myeloma hybrid producing monoclonal antibodies to this molecule.

Materials and Methods

Cell Lines. The cell lines used in these studies were S194/5.XX0.BU.1, a bromodeoxyuridine-resistant mutant of a mouse myeloma that originally secreted IgA but now is a nonproducer (kindly provided by Dr. R. Hyman, Salk Institute), T1M1 (Thy-1⁻c), a Thy-1⁻ mouse thymus-derived (T) lymphoma, BW5147.G.1.4 Ouaᴿ.1 and S49.1.T.2.3, Thy-1⁺ T lymphomas referred to as BW5147 (Thy-1⁺) and S49 (Thy-1⁺), respectively, RAW253.1.TB.1, an Abelson virus-induced cell line, S194/2.C.M.D.3.3.3 (kindly provided by Dr. W. Raschke, Salk Institute), and MPC.11.TG.7.Ouaᴿ.1, two mouse myelomas. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Details of the origin and some of the properties of these cell lines can be found in references 9-11.

Cell Fusion. S194/5.XX0.BU.1 myeloma cells (10⁷) and spleen cells (10⁸) from Lewis rats immunized by 4-6 i.p. injections of 1-4 × 10⁷ T1M17 (Thy-1⁻c) or BW5147 (Thy-1⁺) lymphoma cells were fused with polyethylene glycol 1500 (BDH lot 6022810, 35 or 40% vol/vol) 3-5 days after the rats had been given their last immunization (3). Cell hybrids were selected in HAT medium and their production of antibodies against the lymphoma cells used for immunization was tested by an antibody-binding assay essentially as described by Williams et al. (4). Hybrid cells were cloned by the method of limiting dilution in Falcon microtiter plates (BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.) (Catalogue no. 3040).

Antibody Binding Assays. Rabbit IgG antibodies against rat IgG were purified by affinity chromatography and gel filtration and then labeled with ¹²⁵I (12). Trace binding assays (rabbit anti-rat IgG antibodies, ~25 μCi/μg, 1.68 × 10⁶ cpm/assay) were used to detect cell hybrids producing antibodies against mouse lymphoma surface antigens and saturation binding assays (rabbit anti-rat IgG antibodies at 100 μg/ml, 0.4 μCi/μg) were used to estimate the number of antibody binding sites per cell (13). The number of anti-rat IgG antibodies bound per rat IgG molecule on the cell surface under these conditions was determined by a radioimmune assay as described previously (13).

Biochemical Methods. Lactoperoxidase-catalyzed cell surface iodination, immunoprecipitation techniques, and sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)[1]

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[1] *Abbreviations used in this paper:* B, thymus-independent; M_r, molecular weight; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; T, thymus-dependent.

were performed as described previously (6–8). To analyze the secreted products of cell hybrids, cells (5×10^5/ml) were incubated for 24 h with [³H]leucine (50 μCi/ml) in Dulbecco's modified Eagle's medium and the labeled Ig in the supernates was isolated by immunoprecipitation and examined by SDS-PAGE. The concentration of antibody in culture supernates was estimated by a radioimmune assay (12) suitable for measuring rat IgG over a range of 10–400 ng. Protein was measured by the method of Lowry et al. (14) by using bovine serum albumin as the standard.

Results

Selection of Cell Hybrids. Using a cell fusion procedure and subsequent selection conditions essentially as previously described by Köhler and Milstein, Galfré et al., and Williams et al. (1–4), we obtained cell hybrids between the mouse myeloma, S194/5.XX0.BU.1, and rat spleen cells immunized against mouse lymphoma cells. At an optimal concentration of polyethylene glycol (40% vol/vol in these experiments), most or all of the 48 wells of the Linbro tissue culture plates into which the polyethylene glycol-treated cell suspensions were divided contained cell hybrids.

We tested 376 supernates from cultures of cell hybrids obtained from 20 separate fusion experiments for antibodies specific for surface antigens of mouse lymphoma cells by the double antibody binding assay described by Morris and Williams (13). Highly significant antibody binding (>3-fold background) was detected in supernates from 71 cultures (19%). We then tested 34 of these supernates for antibodies capable of precipitating labeled molecules from detergent extracts of lymphoma cells labeled by lactoperoxidase-catalyzed iodination. Only one positive culture supernate was found which was from a cell hybrid (designated I3/2) derived from a fusion between S194/5 myeloma cells and rat spleen cells immunized against T1M1 (Thy-1⁻c) cells. SDS-PAGE analysis of the labeled species precipitated by I3/2 supernate showed that a single iodinated species of apparent molecular weight (M_r) 190,000 was present (Fig. 1). From the apparent M_r and the amount of radioactivity associated with the labeled molecule we tentatively identified this molecule as the major lymphoid cell surface glycoprotein, T200, which we had previously studied (6–8). The remaining experiments reported here were designed to test this idea and to characterize the I3/2 spleen-myeloma hybrid and the immunoglobulin it produces.

Specificity of I3/2 Antibody. The specificity of I3/2 antibody and the nature of the surface molecules with which it reacts were investigated in several ways. First, the binding of I3/2 antibodies to normal mouse lymphoid cells and a variety of mouse lymphoid cell lines was measured at saturating amounts of both I3/2 antibodies and the second antibody reagent (¹²⁵I-labeled, affinity-purified rabbit IgG anti-rat IgG) (Table I). The results of these studies show that the antigen recognized by I3/2 antibodies is present on mouse thymocytes, spleen, and bone marrow cells and all the lymphoid cell lines tested except for MPC.11.TG.7.Ouaᴿ.1 myeloma cells.

Because of problems in measuring I3/2 antibodies in a conventional radioimmune assay for rat IgG (see later), the ratio of rabbit anti-rat IgG antibodies to I3/2 antibodies bound to the cells under these conditions could only be determined approximately. However the value obtained of 4:1 is similar to previous estimates for the ratio of first and second antibodies in similar saturation
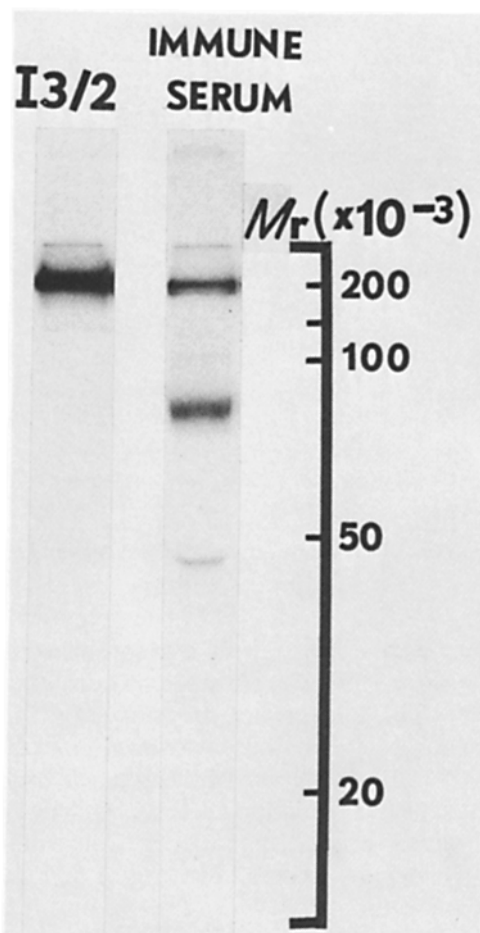
FIG. 1. Analysis of the specificity of the monoclonal antibody secreted by I3/2 hybrid. The figure shows an autoradiograph (1 day exposure) of a 10% SDS-polyacrylamide gel of the labeled antigen precipitated by I3/2 antibodies from a detergent extract of T1M1 (Thy-1$^+$) cells previously iodinated by the lactoperoxidase technique. Also shown for comparison is a similar autoradiograph of the iodinated species precipitated by serum from a rat immunized with T1M1 (Thy-1$^-$) cells in exactly the same way as the donor of the spleen cells used for the fusion from which the I3/2 myeloma hybrid was selected.

binding assays (13). If this ratio is accurate, then the number of antigenic sites for I3/2 antibodies on the surface of each lymphoid cell can be calculated to be either 50% or 25% of the values given in Table I, depending upon whether one or both binding sites of the I3/2 antibodies are occupied. From this calculation it can be estimated that thymocytes and spleen cells have 50,000–100,000 molecules per cell of I3/2 antigen, BW5147 (Thy-1$^+$) cells, 200,000–400,000 molecules per cell (a similar antigen density to spleen cells and thymocytes since their surface area is about fivefold less than that of BW5147 (Thy-1$^+$) cells), while bone marrow cells and the other lymphoid cell lines express lesser amounts of the antigen.

The detailed results of a saturation binding assay in which the binding of I3/

TABLE I

Saturating Binding of I3/2 Supernate to Mouse Lymphoid Tissues and Lymphoid Cell Lines

Mouse lymphoid tissues		Molecules bound per cell ($\times 10^{-5}$)*		
		Exp. 1	Exp. 2	Exp. 3
Thymus		1.57	2.05	2.1
Spleen		2.85	2.35	1.7
Bone marrow		1.42	1.19	
Mouse lymphoid cell lines				
Tumor type	Cell line			
Lymphoma	BW5147 (Thy-1$^+$)	11.4	8.89	10.7
	T1M1 (Thy-1$^-$ c)	4.34	3.67	
Abelson virus induced	RAW253.1.TB.1	2.72	2.27	
Myelomas	MPC.11.TG.7.Oua^R.1	Not detectable (<0.2)		
	S194/5.XXO.BU.1	1.84	2.52	
	S194/2.C.M.D.3.3.3	0.89	0.99	

* Molecules of ^{125}I-labeled rabbit anti-rat IgG antibody bound per cell determined as described in Materials and Methods.

2 antibodies to thymocytes and spleen cells was measured is shown in Fig. 2. It can be seen that clear saturation was obtained at quite low amounts of culture supernate ([3] Fig. 6). The background binding of ^{125}I-labeled anti-rat IgG antibodies to spleen cells preincubated with tissue culture medium is higher than that to thymocytes. One factor contributing to the high binding of anti-rat IgG antibodies to mouse spleen cells in the absence of I3/2 antibody is that the anti-rat IgG antibodies cross-react with mouse Ig. Consequently the background binding to spleen cells includes specific binding of anti-rat IgG antibodies to immunoglobulin-bearing cells.

The distribution of I3/2 antigen on the nonlymphoid tissues, thymus, and spleen was also investigated by quantitative absorption (Fig. 3). Consistent with the saturation binding assays, spleen cells expressed slightly more antigen than thymocytes. Little or no absorption of I3/2 antibodies by erythrocytes, brain or liver was detected. However, because of the relatively large numbers of spleen cells and thymocytes required to absorb I3/2 antibodies, the lower limit of detection of I3/2 antigen was 10–20% of the amount found on spleen and thymus.

The nature of the molecules on spleen cells and thymocytes with which I3/2 antibodies react was determined by immunoprecipitation of the labeled antigens from detergent extracts of thymocytes, normal spleen cells, and spleen cells from an adult-thymectomized, irradiated and bone-marrow reconstituted mouse (B spleen) labeled by lactoperoxidase-catalyzed iodination (Fig. 4). It can be seen that I3/2 antibodies react with iodinated molecules from thymocytes and spleen cells in the mol wt range of about 190,000–220,000 as judged by comparison of their electrophoretic mobilities with that of rabbit skeletal muscle myosin (M_r 200,000). Three labeled components from normal spleen were resolved on the gels. The intermediate component from normal spleen was not found on B spleen cells suggesting this is derived from peripheral T lymphocytes. The
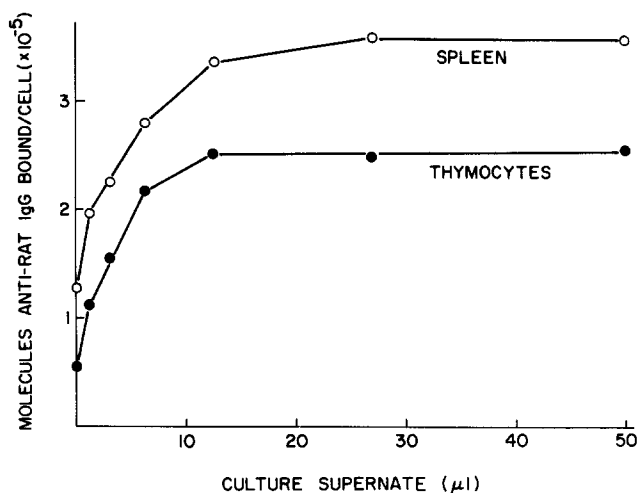
FIG. 2. Saturating binding of I3/2 antibodies to BALB/c mouse thymocytes and spleen cells. Cells (2.5 × 10⁶) were incubated for 1 h at 0°C in a total vol of 0.1 ml with various amounts of spent culture supernate from I3/2 cells. After washing, the target cells were then incubated for 1 h with affinity-purified rabbit anti-rat IgG antibodies (100 μg/ml; 0.4 μCi/μg), washed, and the bound radioactivity measured in a Nuclear-Chicago gamma counter (Searle Diagnostics Inc., Subsidiary of G. D. Searle & Co., Des Plains, Ill.).
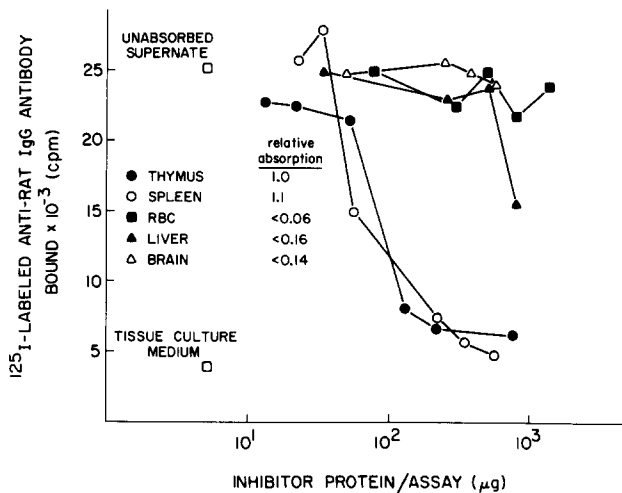

FIG. 3. Relative absorption of I3/2 antibody by mouse tissues. Equal volumes of I3/2 antibody at a dilution which was limiting in the trace antibody binding assay were incubated for 1 h at 0°C with various amounts of cells or tissue homogenates prepared from 4- to 6-wk-old BALB/c mice. The mixtures were centrifuged and the supernates assayed in duplicate for residual binding in BW5147 (Thy-1⁺) cells. The figure shows the inhibition curves obtained and the relative absorption of the mouse various tissues calculated from the μg protein required for 50% absorption of I3/2 antibodies.

slowest migrating component from normal spleen is probably derived from B lymphocytes since it is also found on B spleen cells and is likely to be the same molecule previously identified on B-cell lymphoblasts induced by lipopolysaccharide (6). The most rapidly migrating species from normal spleen is only
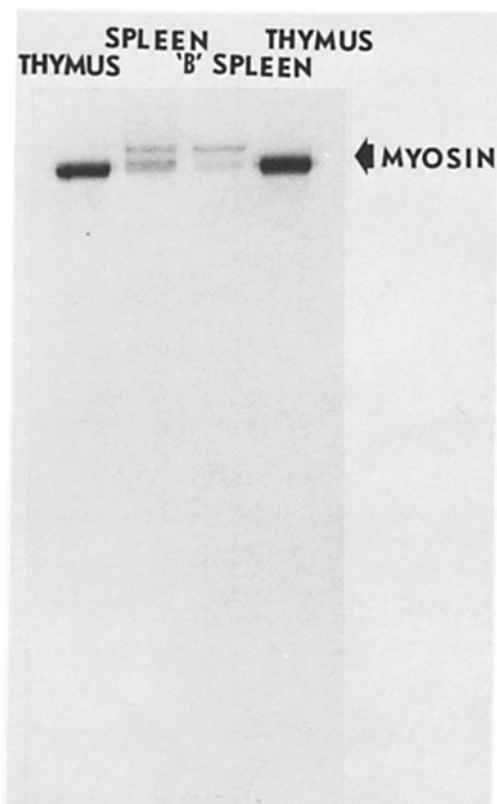
FIG. 4.   Analysis of the surface molecules of thymocytes and spleen cells recognized by I3/2 antibodies. The figure shows an autoradiograph (2 day exposure) of a 5–15% gradient SDS-polyacrylamide gel of the iodinated surface molecules of thymocytes and spleen cells from normal BALB/c mice and of spleen cells from an adult-thymectomized, irradiated and bone marrow-restored BALB/c mouse (kindly provided by Dr. G. Dennert, Salk Institute) precipitated from detergent extracts of the cells by I3/2 antibody.

reduced slightly on B spleen cells and therefore is derived from either B lymphocytes or a radiation-resistant lymphoid cell. One striking fact illustrated by Fig. 4 is that although spleen cells express more I3/2 antigen than thymocytes, the efficiency of labelling by lactoperoxidase-catalyzed cell surface iodination is disproportionately low relative to thymocytes. This observation should be taken into account in the interpretation of previous studies in which the existence of a high molecular weight T-lymphocyte-specific antigen was inferred largely on the basis of differences in the labeling patterns of iodinated spleen and thymocytes (15).

Finally, since I3/2 antibodies bound to the surface of spleen cells it was of interest to determine whether this interaction induced cell proliferation. The results of the mitogenesis assay performed as described in reference 16 showed that even at antibody concentrations sufficient to saturate all the I3/2 spleen cell antigens, lymphocyte mitogenesis was not induced.

Properties of I3/2 Hybrid Cells.   The I3/2 hybrid was selected from a fusion experiment in which a suboptimal concentration of polyethylene glycol (35%)
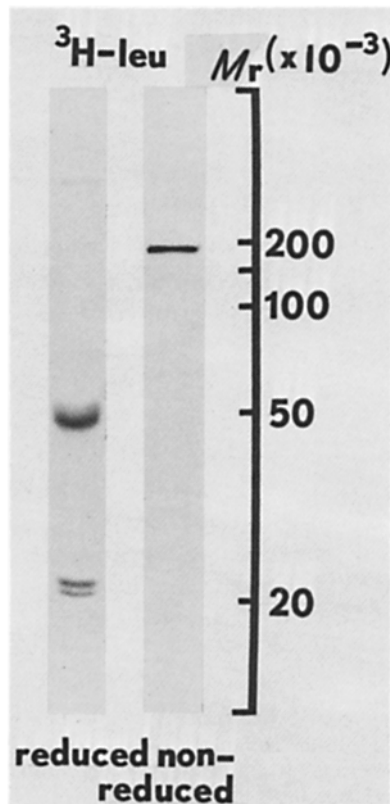
FIG. 5. Analysis by SDS-PAGE of the secreted antibody of I3/2 myeloma hybrid cells. [³H]leucine labeled I3/2 antibody was precipitated by rabbit anti-rat IgG serum and analyzed on a 10% polyacrylamide gel either after reduction with 2-mercaptoethanol or unreduced in the presence of iodoacetamide. The labeled species were visualized by fluorography (3 day exposure).

was used. Since only growth of hybrid cells was obtained in 5 of 48 wells it was very likely I3/2 cells arose from a single fusion event. Subclones were isolated at limiting dilution from I3/2 cells which had been frozen in liquid N_2 and all were active.

The amount and properties of the monoclonal antibody produced by I3/2 hybrid cells was studied. As shown in Fig. 5, the cells produce an Ig with an M_r before and after reduction that suggests the secreted antibody is an IgG. The mouse myeloma, S194/5.XX0.BU.1, is a nonsecreting variant of cells originally producing IgA (9). It is clear from Fig. 5 that I3/2 cells do not produce mouse IgA. Also, no free immunoglobulin light chains are seen in the nonreduced sample of the secreted immunoglobulin. This observation and the results of preliminary immunoprecipitation experiments with anti-Ig sera from which mouse-rat cross-reactive antibodies were removed by absorption both suggest that the two species of light chains resolved on SDS-PAGE are both derived from rat I3/2 antibodies. Quantitative analysis of the amount of rat IgG in spent culture supernate of I3/2 cells gave results that suggested at first that the antibody concentration was 0.2–0.4 μg/ml. However, as shown in Fig. 6, we
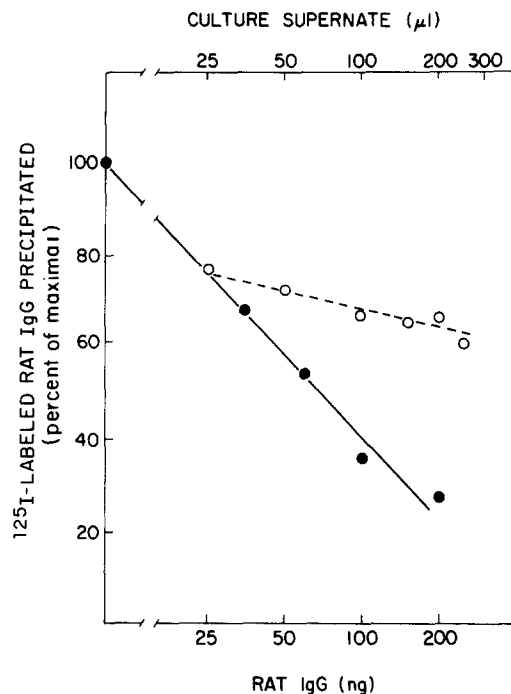
FIG. 6.   Anomalous inhibition of I3/2 culture supernates in a radioimmune assay for rat IgG. Inhibition of binding of ^{125}I-labeled rat IgG (Miles Laboratories) by various amounts of rat IgG and I3/2 culture supernate at a limiting dilution of rabbit anti-rat IgG serum was determined in duplicate. Antigen-antibody complexes were precipitated by goat anti-rabbit IgG serum in the presence of carrier normal rabbit serum. ●, rat IgG; O I3/2 supernate.

found that the inhibition curve of I3/2 supernates had a less steep slope than that of normal rat IgG indicating that the anti-rat IgG antiserum used in the assay had a lower affinity for I3/2 antibodies than the bulk of normal rat IgG. From a comparison of the slopes of the two inhibition curves it can be calculated that measurements of I3/2 antibody from the normal IgG standard curve underestimate the true amount of I3/2 antibody by about fivefold. Thus the concentration of specific antibody in spent I3/2 culture medium is probably about 1-2 μg/ml. Another interesting observation was that I3/2 antibodies were not cytotoxic in the presence of guinea pig complement and had only a modest cytotoxic titer (50% kill at 1:256 supernatant dilution) if rabbit serum was used as the complement source (R. Hyman, Salk Institute, unpublished results). Both the weak cytotoxic titer of the I3/2 antibodies and the anomalous results of the radioimmune assay suggested I3/2 antibodies may be a minor IgG subclass that fixes complement poorly. The ability of antisera (Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.) specific for rat IgG_{2a}, IgG_{2b} and IgG_{2c} subclasses (17) to precipitate leucine-labeled I3/2 antibodies was tested. The results showed that rabbit anti-rat IgG_{2b} reacted with I3/2 antibodies as efficiently as a polyvalent anti-rat IgG serum. In contrast, goat antisera specific for rat IgG_{2a} and IgG_{2c} did not precipitate more radioactivity than the control normal goat serum (Table II). It is concluded therefore that the monoclonal I3/2

TABLE II
Identification of the IgG Subclass of I3/2 Antibodies

Antiserum	I3/2 Antibody precipitated
	cpm
Normal rabbit serum	464
Polyvalent rabbit anti-rat IgG	21,382
Rabbit anti-rat IgG$_{2b}$	20,363
Normal goat serum	5,028
Goat anti-rat IgG$_{2a}$	3,628
Goat anti-rat IgG$_{2c}$	4,063

Secreted [^{3}H]leucine-labeled I3/2 protein was isolated from culture supernates by gel filtration on Biogel P-10. Samples of supernate (28,000 cpm; 0.025 ml) were incubated with 0.1 ml of the antisera shown at dilutions of 1:10, 1:50, 1:250, and 1:1250 in duplicate tubes containing 0.005 ml of the appropriate normal serum as carrier. After 60 min at 4°C antigen-antibody complexes were precipitated by the addition of either goat anti-rabbit IgG or donkey anti-goat IgG. The washed precipitates were dissolved in 0.1 ml of 10% SDS, 0.9 ml of H$_2$O added, and samples counted in a triton-toluene scintillation mixture. The results show the maximal radioactivity precipitated by each antiserum.

antibody is an IgG$_{2b}$ immunoglobulin. To my knowledge the ability of rat IgG$_{2b}$ immunoglobulin to fix complement is unknown (18).

Finally, another question raised by the selection of a mouse myeloma-rat spleen hybrid producing monoclonal antibody against a mouse lymphoid antigen is whether the hybrid expresses this antigen.

As shown in Table I, the mouse myeloma, S194/5.XX0.BU.1, parent of the I3/2 hybrid, expresses a moderate amount of the I3/2 antigen on its surface. Since direct binding could not be used to measure the amount of I3/2 antigen on the surface of the hybrid cells, we attempted to detect the synthesis of the antigen by metabolically labeling the cells with ^{3}H-mannose and immunoprecipitation of the antigen with I3/2 antibodies. Synthesis of I3/2 antigen was not detected in these experiments and it is possible therefore that the selection of this cell hybrid was dependent upon the loss of the antigen either by chromosome segregation or some other mechanism.

Discussion

The studies of the specificity of the monoclonal antibody produced by the rat spleen-mouse myeloma hybrid I3/2 establish that the antibody reacts with the high molecular weight glycoprotein of mouse thymocytes and T cells which we have previously referred to as T200 (6–8). The I3/2 antibody has enabled us for the first time to determine the amount of T200 on these cells by an indirect antibody binding assay. The estimate of 50,000–100,000 T200 molecules per thymocyte indicates that on a weight basis the thymocyte plasma membrane contains similar amounts of T200 and Thy-1 glycoproteins. Together these two glycoproteins probably account for a substantial fraction of the total thymocyte plasma membrane protein. The present studies have also confirmed our previous conclusions (6, 7) that B lymphocytes express high molecular weight

glycoproteins antigenically related to T200 that have a different electrophoretic mobility on SDS-PAGE.

The availability of large amounts of monoclonal antibody specific for T200 glycoprotein is significant for several reasons: purification of T200 and the related glycoproteins of mouse B lymphocytes and detailed investigation of their chemical and biological properties should now be possible; also studies, similar to those already in progress on Thy-1 glycoprotein (11), on the biosynthesis and turnover of T200 glycoprotein in cultured lymphoma cells can be attempted; and finally, the availability of I3/2 antibody and other lymphoid tissue-specific monoclonal antibodies may enable the development of highly specific immunosuppressive reagents in the mouse. It is clear that similar antibodies against human lymphocyte antigens have potential clinical use.

Summary

A cell hybrid has been selected from fusion of a mouse myeloma and rat spleen cells immunized against mouse lymphoma cells that produces monoclonal antibody against the mouse lymphocyte surface glycoprotein, T200. Antibody binding assays employing the monoclonal antibody show that there are about 50,000–100,000 molecules of T200 glycoprotein on mouse thymocytes and that similar antigens are present on spleen and bone marrow but not detected on nonlymphoid tissues. Examination of the labeled molecules precipitated from detergent extracts of spleen cells and thymocytes iodinated by the lactoperoxidase technique by SDS-PAGE confirm that there are structural differences between the antigens found on B and T lymphocytes. The B-cell glycoprotein consists of at least one component of apparent mol wt 220,000 on SDS-PAGE, while the T-cell glycoprotein has an apparent mol wt of about 190,000.

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