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69

A RABBIT ANTISERUM TO A THYMUS EXTRACT SPECIFIC FOR MOUSE THYMUS-DERIVED CELLS[1]

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A heteroantiserum has been prepared in rabbits against an EDTA eluate of mouse thymus. The globulin fraction of the unabsorbed serum precipitates macromolecules of several molecular weights eluted from various lymphomas. It is highly cytotoxic to both Thy-1 (θ)-positive lymphomas and normal thymus cells, but is also cytotoxic to some bone marrow cells. After absorption with insolubilized serum proteins and bone marrow cells, the serum is highly specific for mouse thymus-derived cells.

Indirect immunofluorescence of thymus cells assessed by rapid-flow fluorescent measurements gives a single sharp distribution; the activity of thymus-derived cells is specifically removed from primed spleen cells by the absorbed serum plus complement when tested in an in vivo transfer system. The antiserum does not discriminate between Thy-1 (θ) alleles.

Alloantisera recognizing antigenic specificities which show a restricted tissue specificity have been used in the study of certain aspects of cell differentiation and membrane architecture (1). Antisera raised against specific surface antigens can remove a particular class of cells in a mixed population (2). Preparation of heteroantisera with restricted specificity toward a given tissue has been difficult due to the presence in these sera of antibody specificities with a wide tissue distribution (3–9). In addition, heteroantisera are not able to detect allelic differences between antigens (5). One reason for the presence of broadly reacting specificities in heteroantisera is that whole cells, rather than purified cell surface antigens, are usually used as immunogens (10).

To prepare a rabbit antiserum specific for mouse thymocyte antigens, we have attempted to make use of the fact that certain membrane components are preferentially extracted by EDTA at low ionic strength (11, 12). We found the EDTA eluate from thymus cells to be an active immunogen. The gel electrophorogram of this eluate indicates that several membrane components of thymus cells are extracted by this procedure. Some of these membrane components may be specific to thymus-derived cells. The specificities of the heteroantiserum made against the thymus eluate have been compared to those recognized by alloantisera by studying patterns of cytotoxicity and fluorescence on both normal lymphoid cells and on several tissue culture lines. *In vivo* reconstruction experiments have been used to show that treatment of spleen cells with the heteroantiserum and complement selectively removes thymus-derived cells.

MATERIALS AND METHODS

Antisera

Anti-Thy-1.1 and anti-Thy-1.2 antisera were prepared according to the procedure of Reif and Allen (13). The antigens Thy-1^a and Thy-1^b are the alleles of the Thy-1 locus which determine specificites Thy-1.1 and Thy-1.2, respectively (14). These alleles were previously called θ-AKR and θ-C3H.

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Cell lines

See References 15, 16 for the history and methods of culture of many of the lines used. The cell line S49.1TB.2 referred to as S49(Thy+) is a BALB/c lymphoma carrying the Thy-1.2 antigen. S49.1TB.2/θ 5x.3.2, referred to as S49(Thy−), is a derivative of S49.1TB.2 which lacks Thy-1.2 antigen (16). BW5147 is an AKR lymphoma carrying the Thy-1.1 antigen. T1M1, clone 4, referred to in this paper as T1M1(Thy+) is a C57BL/6 line expressing the Thy-1.2 antigen whereas clone 7, referred to as T1M1(Thy−) does not express detectable Thy-1 antigen by either direct cytotoxicity or absorption (16). P3.6.2.8.1 is a BALB/c myeloma which does not express the Thy-1 antigen (17). None of these cell lines expresses surface immunoglobulin except for P3.6.2.8.1 which is lysed by antimouse immunoglobulin and complement (18).

Buffers

The following buffers were used:

Buffer I. Phosphate-buffered isotonic solution (PBS):[4] NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 1.15 g, NH_2PO_4 0.2 g, $MgCl_2 \cdot 6H_2O$ 0.1 g, $CaCl_2$ 0.1 g, water to make 1 liter.

Buffer II. Buffer I with 2% bovine serum albumin, Armour Pharmaceutical Co., Kankakee, Ill., Fraction V.

Buffer III. Buffer I with 10^{-5} M KI and no calcium salt.

Buffer IV. Buffer III with 2% bovine serum albumin (BSA).

Buffer V. Buffer III with 0.2% BSA.

Buffer VI. Buffer I with 1 mM EDTA and no calcium and magnesium salts.

Preparation of EDTA eluates

An eluate of thymus cells was used as a source of antigen to prepare the heteroantiserum. Thymus tissue was collected in buffer II from BALB/c female mice of approximately 3 to 4 weeks of age. A cell suspension from thymus was made by teasing the capsule and removing the debris by passing through a wire mesh. Cells were centrifuged three times in buffer V and the final pellet was resuspended in twice the volume of the cell pellet. An eluate from these cells was prepared by treating the cells with an equal volume of a 10 mM neutralized solution of EDTA in distilled water. Both the cell suspension and the eluting solution were prewarmed to 37°C. The addition of EDTA solution was made quickly and the tube was shaken gently. The hypotonic shock in conjunction with EDTA releases the antigenic material into the supernatant. After incubation for 2 min at 37°C the cells were centrifuged at 1200 × G for 5 min and the supernatant was kept on ice. Extracts were freshly made before each use. The total amount of protein in the supernatant prepared from 10 thymuses (approximately 2×10^9 cells) is usually about 200 μg.

Preparation of heteroantiserum

Approximately 100 μg of the EDTA extracts in 1 ml mixed with 3 ml complete Freund's adjuvant were injected intradermally into two rabbits once every week for 3 weeks and every 2 weeks for 4 weeks. Serum was collected 1 week after the last injection, was fractionated by 35% saturated ammonium sulfate, and was heated to 56°C for 30 min before use.

Absorption of rabbit antithymus eluate

To remove antimouse immunoglobulin activity in the heteroantiserum (see *Results*), rabbit antithymus eluate (*unabsorbed*) was absorbed with Sepharose 4B immunoabsorbent coupled with C57BL serum (19). This serum was further absorbed with C57BL bone marrow cells (about 1×10^8 packed cells at 4°C for 30 min for 10 mg protein) to make thymus-specific heteroantiserum (*absorbed*). Antibody activity against BSA, also present in this antiserum, was removed from certain preparations by passage of the serum through BSA-Sepharose immunoabsorbent columns.

Iodination of intact cells and preparation of radioactive eluate

Cells used for iodination were either freshly derived thymus cells or suspension cultures of lymphoma lines.

The thymus cells were washed twice at 4°C and finally resuspended in buffer V. The lymphoma cells were washed at least six times in buffer III at room temperature to remove serum components from the growth medium which have a tendency to remain attached to cells.

[4] Abbreviations used in this paper: PBS, phosphate-buffered saline; BSA, bovine serum albumin; SDS buffer, 8 M urea 1% sodium dodecyl sulfate.

The iodination reaction was performed at room temperature in a reaction mixture containing 1.5×10^9 thymus cells (or 2×10^8 lymphoma cells) in 1.5 ml of buffer V (in buffer III for lymphoma lines) containing 10^{-5} M KI, 3.3×10^{-7} M lactoperoxidase (Calbiochem, Los Angeles, Grade B), 1 mCi of carrier-free ^{125}I (or ^{131}I) (New England Nuclear Corp., Boston) to which 10 μM hydrogen peroxide were added to start the reaction. Incubation was carried out for 15 min with additions of hydrogen peroxide at 0, 5, and 10 min.

Reaction with thymus cells was stopped by adding an equal volume of cold buffer II before centrifugation. The labeled cells were washed three times with cold buffer to remove residual components of the reaction mixture. The pellet from the last wash was resuspended in two volumes of prewarmed buffer VI. After 2 min at 37 °C, the cells were centrifuged and the supernatant was collected. This step was repeated once and the two supernatant preparations were pooled.

Analysis of immune precipitates of the cell eluate

A double precipitation test was used in all these experiments. Eluates were treated separately with each of the following six sera: alloantisera against Thy-1.1 and Thy-1.2, congenic antiserum against Thy-1.2, normal mouse serum, unabsorbed rabbit antiserum against BALB/c thymus eluates, and normal rabbit serum. Rabbit anti-normal mouse immunoglobulin and goat antirabbit immunoglobulin for double precipitation were absorbed with the lymphoma lines T1M1 (Thy+) and S49 (Thy+). The reaction mixture for double precipitation was made in the following manner: To 0.5 ml of the radioactive eluate, 5 μl of one of the six sera listed above were added. At the end of incubation at 37 °C for 10 min, 50 μl of the appropriate double precipitating serum were added. The mixture was incubated for another 1 hr at 37 °C and then stored at 4 °C for 8 hr.

The mouse alloantisera and the rabbit antisera were adjusted to approximately the same cytotoxic titer. The rabbit antiserum was not absorbed with cell lines. For comparison, therefore, the normal rabbit serum was used undiluted and unabsorbed. Under these conditions, the normal rabbit serum gave a relatively high background in gel electrophorograms (see Fig. 3*B*) and seemed to contain many specificities also present in immune serum which can be removed by absorbing the normal rabbit serum with the lymphoma lines.

The precipitate was washed at least six times at 7000 $\times$ G for 1 min with buffer VI containing 2% of the serum used in the growth medium of the lymphoma lines or with 2% BSA in the case of thymus eluate.

The final pellet was dissolved in pH 8.5 urea: SDS buffer (8 M urea 1% sodium dodecyl sulfate 0.5 M Tris pH 8.5) reduced with 0.1 M dithioerythritol (Calbiochem) for 1 min in a boiling water bath ~100 °C and finally alkylated with excess iodoacetamide (0.25 M). This was dialyzed overnight against a buffer containing 0.1% SDS, 0.5 M urea, 0.01 M phosphate buffer and 0.001 M dithioerythritol, pH 7.2.

The SDS-urea gel electrophoresis was performed according to the method of Maizel (20). The electrophoresis time was approximately 3 hr. The gels were frozen and cut into 1-mm slices which were counted directly in a Packard gamma counter.

In all cases except where it is specified otherwise, the cells were labeled with ^{125}I. Mouse myeloma protein S63-(IgA) radioactively labeled with ^{131}I was added to the washed immune precipitate as a marker and the mixture was reduced and alkylated before gel electrophoresis. The specific activities of cell eluates from different cell lines are not normalized. As a routine procedure the precipitations were performed on equal aliquots of a freshly made cell eluate, and the results presented in a figure are derived from the same eluate preparation. Approximately 90% of the input counts are recovered within 4 cm from the meniscus of the gel.

Serologic tests

Direct cytotoxic tests, qualitative absorption, and quantitative absorption tests on cell lines were done as previously described (17), except that sera and cells were suspended in a modified Eagle's medium without bicarbonate and containing 2% fetal bovine serum certified free of γ-globulin (Grand Island Biological, Co., Grand Island, N. Y.).

The cytotoxic assay on normal lymphoid cells was carried out by adding 5×10^6 cells in

200 μl of diluent to 250 μl of diluent or diluted antibody. Fifty microliters diluent or neat guinea pig serum were added to give a final concentration of 1:10, the tubes were incubated for 45 min at 37°C, and viability was tested by trypan blue staining.

Immunofluorescence staining and analysis by electronic fluorescent cell sorter

Fluorescent antibody staining was carried out as described in (21). Briefly 0.25 ml of diluted rabbit antithymus eluate was added to 5×10^6 packed cells and the mixture was incubated at room temperature for 15 min. After washing, 0.25 ml of fluorescent goat antirabbit γ-globulin was added, and incubation was carried out at room temperature for 15 min. Washing after each step was done by the fetal calf serum gradient method. Cells were loaded into an electronic fluorescent cell sorter and analyzed with respect to the fluorescence distribution of these stained cells. Details of the technique are in (21, 22).

Reconstitution experiment

Spleens were removed from C3H.SW/Hz mice primed with 4×10^8 sheep red blood cells intravenously and 10 μg Piromen endotoxin (Pseudomonas polysaccharide (Piromen) Flint Laboratories, Morton Grove, Ill.) intraperitoneally 5 weeks previously. Aliquots of spleen cells were treated with *absorbed* rabbit heteroantiserum and complement as for cytotoxic testing or fluorescence. The treated spleen cells were injected intravenously with 4×10^8 sheep red cells into 4- to 6-month-old CWB mice given 800 R whole-body irradiation 1 day previously. (CWB is a strain congenic with C3H.SW differing in immunoglobulin allotype. C3H.SW is Ig^a whereas CWB is Ig^b, cf. Ref. 23.) Certain groups were also given 5×10^7 thymocytes from 4- to 6-week-old C3H.SW mice. Recipient spleens were assayed for direct and indirect plaque-forming cells 7 days after cell transfer (23). Indirect plaque-forming cells were developed with a polyvalent rabbit antimouse immunoglobulin (23).

RESULTS

Comparison of rabbit heteroantiserum and alloantisera against Thy-1.1 and Thy-1.2. Analysis on gels. The EDTA eluate of BALB/c thymus cells which was used to immunize the rabbits is composed of several components as seen in Figure 1. Some of the slow-moving components with molecular weights higher than the heavy chain marker are probably residual lactoperoxidase (m.w. 78,000) and BSA (m.w. 65,000). The components with intermediate mobilities between the marker immunoglobulin subunits are probably specific to thymus. The major component migrating about the middle of the gel (shaded portion of Fig. 1) consists of about 20% of the input radioactivity and the molecular weight of this component is calculated to be approximately 35,000 (±10%).

Rabbits immunized with such a heterogeneous material will give rise to antibodies recognizing several specificities. In order to determine some of the specificities of the rabbit antiserum we compared, by gel electrophoresis, the precipitation patterns of anti-Thy-1 alloantisera and the rabbit heteroantiserum with eluates from several lymphoma lines.

For example, the lymphoma BW5147 expresses the Thy-1.1 specificity. When equal aliquots of an eluate from BW5147 are precipitated with anti-Thy-1.1, anti-Thy-1.2 and the rabbit heteroantiserum and the immune precipitates are run on gel electrophoresis, the

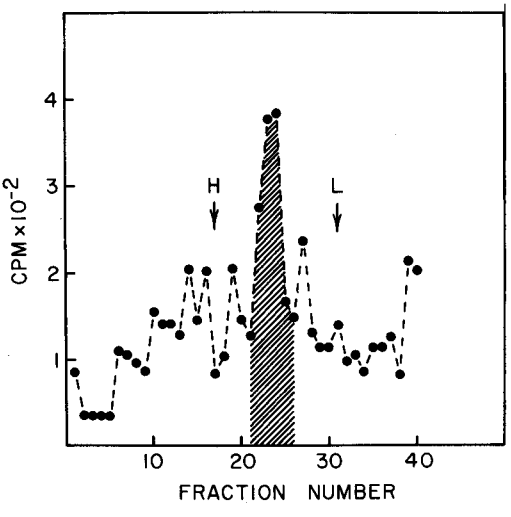

Figure 1. The radioactive eluate from BALB/c thymus cells was prepared as explained in *Methods*. The eluate was lyophilized, reduced with dithioerythritol and alkylated with iodoacetamide before acrylamide gel (7.5%) electrophoresis in SDS and urea. Migration is toward the anode. Electrophoresis time is approximately 3 hr.

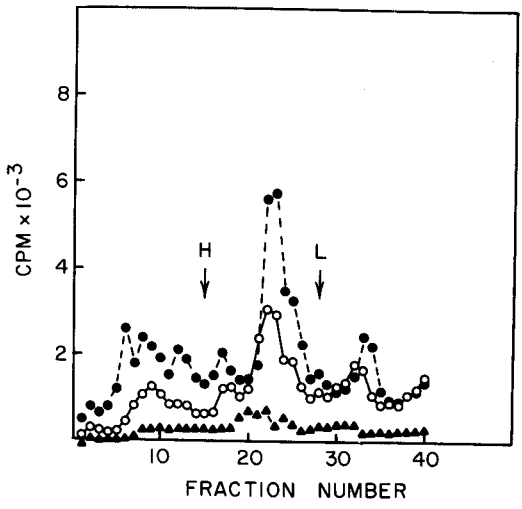

Figure 2. SDS-urea gel electrophoresis of immune precipitates of BW5147, Thy-1.1+, eluate. The same eluate in equal volumes was used for three precipitations. Anti-Thy-1.1 (○ ○ ○), anti-Thy-1.2 (△ △ △), and rabbit antisera (● ● ●). Length of electrophoresis about 3 hr. Separate gels are matched according to the common internal markers H and L.

patterns shown in Figure 2 are obtained. Little precipitation is seen with anti-Thy-1.2. Anti-Thy-1.1 gives a pattern similar to that of total thymus eluate (Fig. 1) with a major component migrating in about the middle of the gel. The rabbit antiserum precipitates components similar to those brought down by the anti-Thy-1.1 serum but in much higher amounts.

An experiment with an eluate from the lymphoma T1M1 (Thy+) which carries the Thy-1.2 specificity is shown in Figure 3. The alloantisera are shown in Figure 3*A*. The control serum anti-Thy-1.1 in this case precipitates a considerable amount of material (in comparison to normal mouse serum). This material may represent antigens other than Thy-1 seen by this serum (24, 25). The specific anti-Thy-1.2 serum also gives a heterogeneous precipitate but shows a peak well above the control at about the middle of the gel as well as several smaller peaks in the heavier region of the gel (compare with Fig. 2). A congenic anti-Thy-1.2 serum (kindly supplied by Dr. L. J. Old) gave a similar pattern. The rabbit

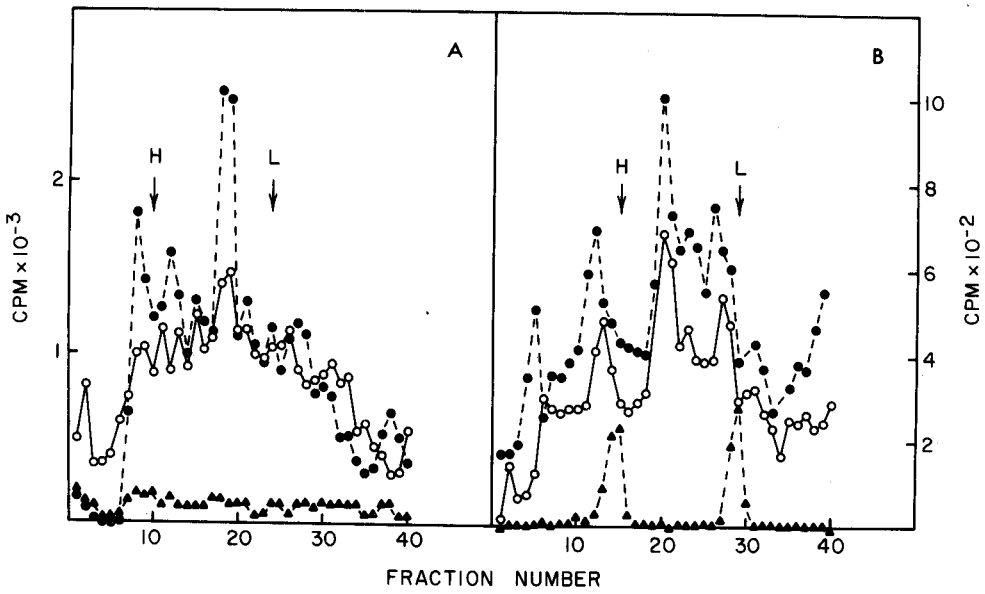

Figure 3. A, procedure as described under Figure 2. Allotype sera anti-Thy-1.2 (● ● ●), anti-Thy-1.1 (○ ○ ○), normal mouse serum (△ △ △). Eluate from T1M1 (Thy+), Thy-1.2+. *B*, procedure similar to the one described under Figure 2. Rabbit antiserum (● ● ●), normal rabbit serum (○ ○ ○), and H and L chains (△ △ △) showing complete reduction and separation. Eluate from T1M1 (Thy+) lymphoma, Thy-1.2+. The amount of precipitation by the normal rabbit serum is high in this particular experiment, probably because undiluted rabbit serum used in this case contained some antimouse thymus activity.

TABLE I

Precipitation of extracts from Thy-1.2-positive and -negative lymphomas

	^{125}I Counts/Min ($\times 10^{-3}$)	
	T1M1 (Thy$^+$) (Thy-1.2-positive)	T1M1 (Thy$^-$) (Thy-1.2-negative)
A. Precipitation by Anti-Thy-1.2 (Alloantiserum)		
Total acid precipitable		
In eluate	60	70
By anti-Thy-1.2 alloantiserum	12	4.9
With anti-Thy-1.1 alloantiserum (control)	7.5	4.2
Specific-nonspecific precipitable		
As % of total acid precipitable	$\frac{(12-7.5)}{60} = 7\%$	$\frac{(4.9-4.2)}{70} = 1\%$
B. Precipitation by Rabbit Anti-Thymus Eluate (Heteroantiserum)		
Total acid precipitable		
In eluate	60	70
By rabbit antithymus serum	2.5	0.62
By normal rabbit serum (nonspecific control)	0.2	0.67
Specific-nonspecific precipitable		
As % of total acid precipitable	$\frac{(2.5-0.2)}{60} = 4\%$	$\frac{(0.62-0.67)}{70} = 0$

heteroantiserum is shown in Figure 3*B*. Normal rabbit serum gives several peaks, perhaps due to antimouse thymus antibody found in normal rabbit sera (see *Materials and Methods—Immune precipitation*). The heteroantiserum gives a large peak in the middle of the gel at the same position as that seen with anti-Thy-1 as well as several other peaks.

The results in Figures 1, 2, and 3 indicate that some allelic specificities recognized by the two alloantisera are detected in eluates from thymus cells bearing two corresponding alleles of Thy-1 antigen. It seems likely, and is compatible with the results given below, that the Thy-1 antigen is in the eluate and appears in the gel electrophorogram of the specific precipitates. Direct evidence on this point could be obtained if a purified Thy-1 antigen were available. The heteroantiserum includes several specificities present in both Thy-1 alloantisera, as well as specificities against other determinants and is not able to discriminate between the two alleles of Thy-1.

Additional support for the idea that the rabbit antiserum recognizes a specificity in common with that recognized by anti-Thy-1.2

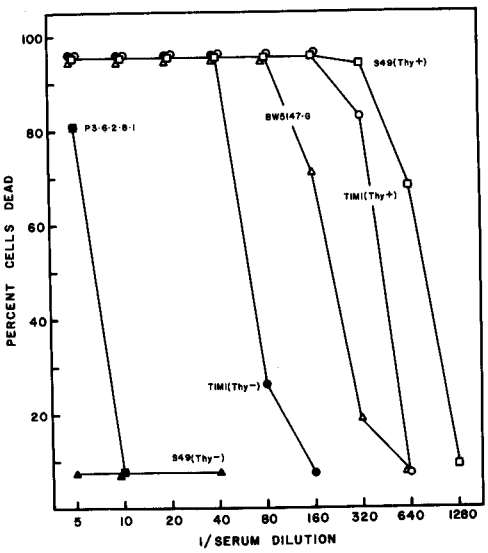

Figure 4. Cytotoxicity of rabbit heteroantiserum for various cell lines. The test was done as described in Reference 17. Guinea pig complement was used at a dilution of 1:3. S49 (Thy+) and T1M1 (Thy+) are Thy-1.2-positive lymphomas. S49 (Thy−) and T1M1 (Thy−) are Thy-1.2-negative variant lines. BW5147.G is a Thy-1.1-positive lymphoma. P3.6.2.8.1 is a myeloma which does not express Thy-1 antigen.

TABLE II

Analysis of rabbit heteroantiserum by qualitative absorption[a]

Indicator Cell T1M1 (Thy+) Serum Absorbed with:	% Dead Indicator Cells for Serum Dilution:				Absorption
	1:40	1:80	1:160	1:320	
Nothing	>95	43	28	<10	–
CE/J thymus (Thy-1.2)	<10	<10			+
CE/J brain (Thy-1.2)	<10	<10			+
AKR/J thymus (Thy-1.1)	<10	<10			+
AKR/J brain (Thy-1.1)	<10	<10			+
T1M1 (Thy+) (Thy-1.2)	<10	<10			+
T1M1 (Thy−) (Thy-1.2-neg. variant)	>95	66	27	<10	–
S49 (Thy+) (Thy-1.2)	<10	<10			+
S49 (Thy−) (Thy-1.2-neg. variant)	>95	78	29	<10	–

Indicator Cell T1M1 (Thy−) Serum Absorbed with:	% Dead Indicator Cells for Serum Dilution:			Absorption
	1:15	1:30	1:160	
Nothing	>95	27	<10	–
CE/J thymus (Thy-1.2)	<10	<10		+
CE/J brain (Thy-1.2)	>95	15	<10	–
AKR/J thymus (Thy-1.1)	<10	<10		+
AKR/J brain (Thy-1.1)	>95	22	<10	–
T1M1 (Thy+) (Thy-1.2)	>95	15	<10	–
T1M1 (Thy−) (Thy-1.2-neg. variant)	<10	<10		+
S49 (Thy+) (Thy-1.2)	>95	29	<10	–
S49 (Thy−) (Thy-1.2-neg. variant)	>95	10	<10	–

[a] Absorption test carried out as in Reference 17. The ratio of cells to serum was approximately 1:4. Complement and serum controls showed <10% dead.

comes from the observation that an eluate from a Thy-1.2-negative clone, T1M1 (Thy−), derived from the Thy-1.2-positive lymphoma T1M1 is precipitated in much reduced amounts by the alloantiserum or the heteroantiserum (Table I). The loss of Thy-1 antigen from T1M1 (Thy−) as evidenced by cytotoxic tests (16) corresponds with the loss of precipitability of the eluate prepared from these cells.

Analysis of the rabbit heteroantiserum by cytotoxicity on cell lines. The precipitation data suggested that the heteroantiserum is recognizing an antigen similar to Thy-1 as well as other specificities. This supposition was confirmed by direct cytotoxic tests with unabsorbed antiserum on a number of lymphoma and myeloma lines. Figure 4 shows that the Thy-1-positive lines T1M1 (Thy+), and BW5147.G are all killed by the rabbit heteroantiserum. The heteroantiserum does not discriminate between alleles since both Thy-1.2, T1M1 (Thy+) and S49 (Thy+), and Thy-1.1, BW5147.G, carrying lines are killed. The Thy-1-negative variant S49 (Thy−) is not killed by the heteroantiserum.

The myeloma cell P3.6.2.8.1 is only killed by the unabsorbed antiserum at low dilution. Other myelomas tested showed no or weak killing. Tests of normal rabbit serum showed no killing with any line.

The cell line T1M1 (Thy−) expresses no detectable Thy-1.2 antigen, but it is killed quite well by the heteroantiserum. This result indicates that the heteroantiserum is recognizing an additional thymic specificity which is not Thy-1-like. A characteristic of the mouse Thy-1 antigen is that it is absorbed by brain as well as thymus (13). Using T1M1 (Thy+) as an indicator cell in qualitative absorption tests, we could show (Table II) that the heteroantiserum is recognizing one specificity present on thymus, brain, and Thy-1-carrying lymphoid lines. This specificity is not expressed in appreciable amounts on the Thy-1.2-negative variant lines, T1M1 (Thy−) and S49 (Thy−). With T1M1 (Thy−) as in-

dicator cells, another specificity could be detected which is present in thymus but not in brain. This specificity is not detected in appreciable amounts on the Thy-1.2-positive lines, T1M1 (Thy+) and S49 (Thy+), in this test, but more extensive absorption of the heteroantiserum with T1M1 (Thy+) does result in some loss of titer when the absorbed serum is tested on T1M1 (Thy−).

Specificity of the heteroantiserum for normal mouse lymphoid cells. The results in Table III (original globulin fraction) show that all or nearly all thymus cells are recognized by the heteroantiserum. A small proportion (10 to 15%) of bone marrow cells are also recognized. Since bone marrow-derived cells are recognized by anti-immunoglobulin antibodies (2), it is possible that some of the labeling of bone marrow cells may be due to anti-immunoglobulin antibody in the heteroantiserum. Ouchterlony and radioimmunoprecipitation tests (26) showed a contamination of anti-γG_{2a} antibody. This activity was removed by absorption with a Sepharose 4B immunoabsorbent coupled with C57BL serum. This serum was further absorbed with C57BL bone marrow cells (see *Methods*) to give an *absorbed* globulin fraction.

Cytotoxic and immunofluorescence tests using this absorbed serum (Table III, absorbed globulin fraction) show that activity against thymus cells remains, while activity against bone marrow cells is reduced. Only about 5% of bone marrow cells are now stained by the heteroantiserum and fluorescent goat antirab-

TABLE III[a]

Cytotoxic and immunofluorescence tests of rabbit heteroantiserum before and after absorption with Sepharose-conjugated mouse serum and bone marrow

	Original Globulin Fraction		Absorbed Globulin Fraction[b]	
	% killed trypan blue	% labeled by indirect immunofluorescence	% killed trypan blue	% labeled by indirect immunofluorescence
Thymus	96	100	96	97
Bone marrow	14	10	6	5
Spleen			9	30

[a] Mice used for these experiments are 6 to 8 weeks old C57BL/10 Hz. 1:200 dilution of original (0.16 mg/ml) and 1:40 dilution of absorbed (0.8 mg/ml) antibodies were used for trypan blue tests. 1:200 dilution of original and 1:160 (0.2 mg/ml dilution of absorbed antibody were used for immunofluorescent technique.

[b] Serum was absorbed with a Sepharose 4B coupled with C57BL serum and further with C57BL bone marrow cells (see *Methods*).

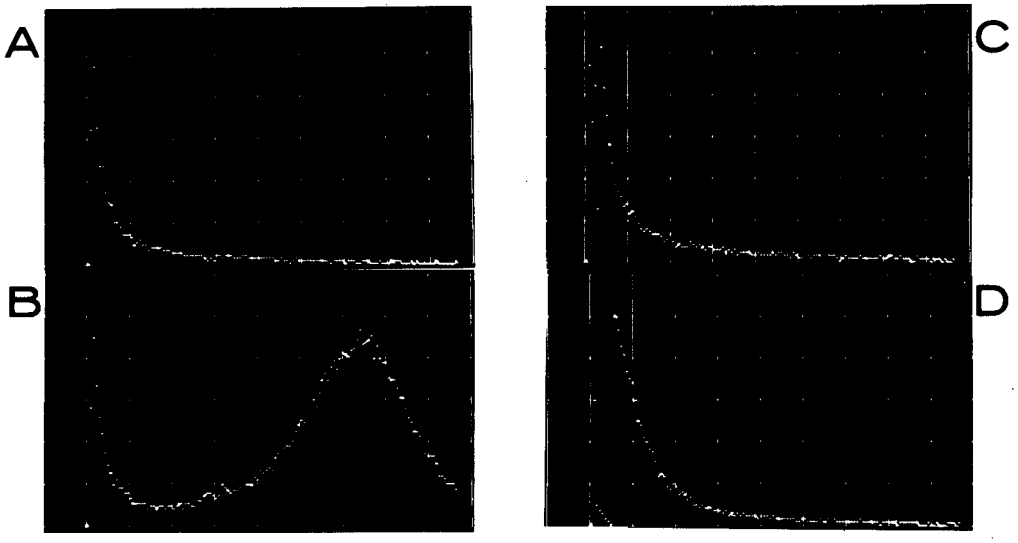

Figure 5. Signal analysis of fluorescently stained thymus (*A* and *B*) and bone marrow (*C* and *D*) cells. Four-week-old C57BL mice were used. In *A* and *C* the heteroantiserum has been omitted. Ordinate, no. of cells; abscissa, fluorescent signal intensity.

TABLE IV

Abrogation of the adoptive secondary response to sheep red cells by absorbed rabbit antiserum and complement[a]

Group	Spleen Cells Injected (SRBC Primed)	*In Vitro* Treatment	Thymus Cell Supplement	No. of Irradiated Recipients	pfc/spl $\times 10^{-2}$	
					Direct	Indirect
1	2×10^6	Normal rabbit serum and C		3	2.9	33.6
2	2×10^6	R anti-T and C		3	<1	<1
3	3×10^6	Stained with R anti-T and F-G anti-Rγ		3	8.8	38.7
4	2×10^6	R anti-T and C	5×10^7	3	2.5	36.0
5			5×10^7	3	<1	0

[a] See *Methods*.

bit γ-globulins. About 30% of spleen cells are labeled with the absorbed serum in immunofluorescence, although a somewhat lower proportion (about 10%) are killed when complement is added. Thymus cells, stained with the sandwich technique, show strong patchy or ring patterns. Bone marrow cells show faintly dotted patterns.

The distribution of fluorescent intensity obtained with a fluorescent cell analyzer and sorter (21, 22) shows no discernible peak with bone marrow cells (Fig. 5*D*). Thymus cells show a high and symmetric peak (Fig. 5*B*) indicating that the amount of surface antigens reacting with the heteroantiserum is nearly equal from cell to cell. This peak disappears if the heteroantiserum is absorbed with thymus cells. Omitting the heteroantiserum (Figs. 5*A* and *C*) gives only background staining.

Abrogation of the secondary immune response by the heteroantiserum and its restoration by thymus cells. When sheep red blood cell-primed spleen cells are treated with heteroantiserum and complement and transferred to irradiated hosts, the plaque-forming cell response of the transferred cells is abolished (Table IV, groups 1 and 2). The abrogation is complement dependent, since treatment with antiserum (without complement) does not affect the plaque-forming cell response (group 3). The response is restored if the treated spleen cells are supplemented with 5×10^7 thymus cells (group 4).

DISCUSSION

The use of EDTA extraction has provided a means for making a heteroantiserum which seems to be quite specific for thymic antigens, containing only small amounts of contamination against γG_{2a}-globulins and possibly some antibody to bone marrow cells. Both antibodies are readily removed by absorption. Electrophorograms of immune precipitates from eluates of cells whose surface has been radioactively labeled with iodine by the lactoperoxidase method show similar patterns whether the rabbit heteroantiserum made against EDTA eluates or alloantisera made against cells bearing the thymus cell-specific Thy-1 antigen are used (Figs. 2 and 3). The electrophorograms, however, are quite heterogeneous indicating that all of the antisera are recognizing several entities. A positive identification of each of these components and their relation to cytotoxic specificities can only be determined when purified antigens become available. However, the gel patterns suggest that the rabbit heteroantiserum and anti-Thy-1 alloantisera probably recognize similar components, although the rabbit serum does not distinguish between alleles as do the mouse alloantisera.

Direct evidence that the heteroantiserum is recognizing a thymus specificity similar to the Thy-1 alloantigen comes from the results of cytotoxic tests on various established murine lymphoid lines (Fig. 4). Thy-1-positive lymphomas are highly sensitive to the antiserum, while Thy-1-negative myelomas show little cytotoxicity. Also, the Thy-1-negative variant S49 (Thy−) is not killed by the antiserum although the parental S49 (Thy+) line, which expresses the Thy-1 antigen, is killed. By absorption (Table III), using the Thy-1-positive lymphomas as indicator cells, thymus, and

brain removes activity from the heteroantiserum while two Thy-1-negative lymphoid cell lines do not. All of these results support the idea that the heteroantiserum is chiefly directed against a molecule bearing a Thy-1-like specificity.

The specificity recognized by the heteroantiserum seems to differ from MSLA (5) and the Ly antigens (24), since the latter are not absorbed by brain. It is also different from a heteroantiserum raised against brain (Ref. 9 and unpublished results of E. Golub and R. Hyman).

Evidence that the heteroantiserum is also recognizing a specificity other than Thy-1 specificity but is killed by the heteroantiserum (Fig. 4). The results of qualitative absorption tests with T1M1 (Thy−) as the indicator cell (Table III) indicate that the specificity present on T1M1 (Thy−) is distinct from the Thy-1 antigen since it is not present on brain—a tissue which is known to express Thy-1 antigen (13). It is present on thymus but must be present in rather small amount on the two Thy-1-bearing lymphomas tested since these cells do not absorb detectable activity. We have not been able to find evidence of specific precipitation of this specificity when eluates of iodine-labeled T1M1 (Thy−) cells are precipitated with the heteroantiserum (Table I). Either the antigen is present in too small an amount in the eluate to be detected or it is not labeled efficiently by the lactoperoxidase method.

The results of cytotoxicity and fluorescence tests on normal mouse lymphoid organs (Table IV) give further support to the idea that the major specificity recognized by the heteroantiserum is limited to thymocytes and thymus-derived lymphocytes in a manner similar to the Thy-1 alloantigen. Thymus cells react strongly and uniformly with the rabbit antiserum, while about 20 to 30% of the cells in spleen react with the rabbit heteroantiserum—similar to results found by others for Thy-1 antigen and distinguishing the major specificity seen from those such as TL which are found only in the thymus (1). Treatment of sheep red cell-primed spleen cells with the absorbed rabbit antiserum and complement abolishes the ability of these cells to give plaque-forming cells in a cell transfer system (Table IV). Plaque-forming cells can be generated if thymocytes are added to the treated spleen. Thus the absorbed serum depletes the spleen of thymus-derived, cooperator cells but does not deplete other cells required for the adoptive response. These results are also similar to the results of others with Thy-1 alloantisera (2, 23).

The results taken together indicate that the rabbit antiserum we have made against an EDTA eluate of thymus reacts predominantly with antigens specific to thymus-derived lymphocytes. The major specificity seen by cytotoxicity and immunofluorescence behaves operationally in a similar manner to the Thy-1 alloantigen although it does not show allelic specificities. The serum (with complement) can be used to deplete spleen populations of thymus-derived cells and can also be used (with the rapid-flow cell sorter) to enrich for them. Since the method used to prepare this heteroantiserum is simple and provides large amounts of high titer antiserum specifically active against thymus-derived cells, we believe that it may be useful to immunologists studying cell interactions in the immune response and to others who want antisera which recognize particular cell types.

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