THE Ly-3 ANTIGENS ON MOUSE THYMOCYTES: IMMUNE PRECIPITATION AND MOLECULAR WEIGHT CHARACTERIZATION*

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Boyse and co-workers have identified several systems of murine isoantigens called Ly antigens (for lymphocyte antigens) (e.g. Ly-1, Ly-2, Ly-3, and Ly-5) which appear to be restricted to thymic lymphocytes and peripheral T lymphocytes (1–3). Each system, defined by cytotoxic alloantisera, appears to be determined by a genetic locus which contains two alternative alleles (e.g., $Ly\text{-}2^a$ and $Ly\text{-}2^b$) governing expression of two alternative cell surface antigenic specificities (e.g. Ly-2.1 and Ly-2.2, respectively). The *Ly-1* locus has been shown to reside in linkage group XII (4), and the *Ly-2* and *Ly-3* loci are extremely closely linked to each other in linkage group XI of the mouse (5).

Using the antibody-blocking test (6), Boyse and co-workers have obtained evidence that the Ly-2 and Ly-3 antigenic determinants are topologically in close proximity on the cell surface. The close genetic and topological linkage of Ly-2 and Ly-3 have prompted Itakura et al. (5) to suggest that Ly-2 and Ly-3 may actually comprise a single complex locus, *Ly-2,3*, and that their respective antigenic determinants may reside upon a single molecule.

Although the functions of the molecules bearing the Ly antigenic specificities (hereafter referred to as the Ly antigens) are unknown, recent evidence using the C57BL/6 inbred strain (phenotype *Ly-1.2*, *Ly-2.2*, and *Ly-3.2*) suggests that different functional subclasses of peripheral T lymphocytes may bear different sets of Ly antigens (7, 8). Peripheral T cells bearing only the Ly-1.2 specificity appear to perform a helper function in the humoral and perhaps the cellular immune response. Killer cells or their precursors and suppressor T cells appear to bear both the Ly-2.2 and Ly-3.2 specificities but not Ly-1.2. The majority of peripheral T-cells appear to bear all three specificities (Ly-1.2, Ly-2.2, and Ly-3.2) and may be precursors of the functional subclasses bearing a restricted selection of Ly antigens.

A genetic marker in the V region of mouse immunoglobulin L chains (called the I_B-peptide marker) (9) has been demonstrated to be very closely linked to the

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Ly-2 and *Ly-3* genetic loci in linkage group XI (10, 11). In inbred and congenic strains, complete correlation was observed between expression of this V-region marker on approximately 5% of normal serum IgG L chains and expression of the Ly-3.1 surface antigenic specificity on thymic lymphocytes. The close genetic linkage of a V_L-region polymorphism with a thymic lymphocyte surface antigen which may be determined by a complex genetic locus raised the question of whether the molecules bearing the Ly-3.1 antigenic specificity might contain L chains or L-chain V regions.

Techniques involving radiolabeling of cell surface components, dissociation of the plasma membrane with detergents, precipitation with specific alloantisera, and analysis by sodium dodecyl sulfate (SDS)[1]-polyacrylamide gel electrophoresis (PAGE) have been used to determine the approximate molecular weights of polypeptide chains comprising a large number of cell surface antigens (12–18). We have used such methods to characterize the polypeptides of the Ly-2 and Ly-3 antigens present on thymic lymphocytes.

Materials and Methods

Mice. C57BL/6J mice (Ly-2.2,Ly-3.2 phenotype) were obtained from The Jackson Laboratory, Bar Harbor, Maine. Breeding stock of the congenic C57BL/6-*Ly-2*^{a},*Ly-3*^{a} (Ly-2.1,Ly-3.1 phenotype) and C57BL/6-*Ly-2*^{a} (Ly-2.1,Ly-3.2 phenotype) strains were the generous gift of Dr. E. A. Boyse of the Memorial Sloan-Kettering Cancer Institute, New York.

Antisera and IgG. Alloantiserum specific for Ly-3.1 was produced by successive intraperitoneal injections of (CBA/H-T6J × SJL/J)F_1 female mice with C58/J thymocytes as described by Boyse and co-workers (2). Antiserum specific for Ly-2.1 (C57BL/6-*H-2*^{k} anti-CE/J thymocytes) was the gift of Dr. E. A. Boyse and Dr. F-W. Shen, Memorial Sloan-Kettering Cancer Institute. Normal (CBA/H-T6J × SJL/J)F_1 and C57BL/6J sera were obtained from unimmunized female mice. Normal serum IgG from C57BL/6J and (CBA/H-T6J × SJL/J)F_1 mice was isolated by starch zone electrophoresis and two successive gel filtrations on Sephadex G-200.

Rabbit anti-mouse IgG (RAMIG) was produced by immunization of a New Zealand albino rabbit with C57BL/6J IgG. Goat anti-rabbit IgG (GARIG) was obtained from Gateway Immunosera Co., Cahokia, Ill.

Lactoperoxidase. Lactoperoxidase with an A_{412}/A_{280} ratio of 0.9 was purified from raw skim milk essentially as described by Rombauts et al. (19). Aliquots were stored at −70°C at a concentration of 4 mg/ml until use.

Other Materials. $Na^{125}I$ and $Na^{131}I$, both carrier free with an activity of 2 mCi/ml, were obtained from New England Nuclear, Boston, Mass. NaB^3H_4 with a specific activity of 10.5 Ci/mmol was a product of Amersham/Searle Corp., Arlington Heights, Ill. Nonidet P-40 was purchased from Particle Data, Inc., Elmhurst, Ill. Trypsin, grade B, was obtained from Calbiochem, La Jolla, Calif. Soybean trypsin inhibitor, a product of Sigma Chemical Co., St. Louis, Mo., was kindly provided by Dr. Richard O. Hynes (MIT Center for Cancer Research). Phosphate-buffered saline (PBS) consisted of NaCl, 8.0 g/liter; KCl, 0.2 g/liter; anhydrous Na_2HPO_4, 1.15 g/liter; KH_2PO_4, 0.2 g/liter; $CaCl_2$ dihydrate, 0.1325 g/liter; and $MgCl_2$, 0.1 g/liter. Bovine serum albumin (BSA), alcohol dehydrogenase (yeast), myoglobin (equine skeletal muscle), cytochrome C (horse heart), aprotinin (trasylol) and galactose oxidase (*Polyporus circinatus*, Type 1) were obtained from Sigma Chemical Co. Lysozyme (egg white) was purchased from Worthington Biochemical Corp., Freehold, N. J.

Preparation of Thymocytes. All procedures employing intact thymocytes were performed in an ice bath or at temperatures below 7°C except for iodination and trypsinization.

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[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; GARIG, goat anti-rabbit IgG; NMS, normal mouse serum; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RAMIG, rabbit anti-mouse IgG; SDS, sodium dodecyl sulfate.

Thymocytes from 1- to 3-mo-old mice were teased into cold PBS (2 ml PBS/thymus). Cell suspensions were filtered through 50-guage stainless steel mesh to remove large aggregates and were washed three times by centrifugation at 140 *g* for 8 min and suspended in 1 ml of PBS. Cell numbers were determined in a hemocytometer. Cell viability was consistently approximately 95% as determined by trypan blue exclusion.

Procedure for Iodination of Thymocyte Cell Surfaces. Thymocytes were iodinated by a procedure similar to that of Haustein et al. (20). Aliquots containing 10^7 thymocytes were centrifuged in capped conical polystyrene tubes at 140 *g* for 8 min. One such aliquot of cells was iodinated for each specific or control precipitation to be performed. Supernates were removed and 20 μl of lactoperoxidase (A_{280} = 2.80) at a concentration of 2.0 mg/ml in PBS was added. 30 μl of neutralized ^{125}I (300 μCi) in PBS was next added to each tube. The iodination reaction, initiated by the addition of 10 μl of 0.03% H_2O_2 in PBS (diluted from a 30% stock solution just before use) was carried out at 30 ± 1°C. Unless otherwise stated the reactions were terminated after 5 min by the addition of 2 ml of cold PBS containing 5 mM KI (PBS-KI).

After centrifugation at 140 *g* for 8 min the cells were washed three times in 2 ml of PBS-KI by centrifugation and resuspension. After the second and third centrifugation steps the cell suspensions were transferred to new polystyrene tubes. After the final wash the cell pellets were suspended in 60 μl of PBS for detergent extraction.

Solubilization of Iodinated Cell Surface Components. Cell surface components were solubilized using the nonionic detergent Nonidet P-40 (NP-40) essentially as described by Vitetta et al. (21). To iodinated cell suspensions (60 μl) was added a quantity of 2% (wt/vol) NP-40 in PBS sufficient to bring the NP-40 concentration to 0.5%. These suspensions were allowed to stand at room temperature for 15 min. In order to insure that samples to be treated with normal serum and antiserum contained comparable amounts of ^{125}I label, the NP-40 extracts from the several parallel iodinations were pooled into a 1 ml polyethylene tube. Nuclei and cellular debris were removed by centrifugation in an Eppendorf centrifuge 3200 at 3,200 *g* for 15 min. The supernate containing the solubilized iodinated cell surface components was divided into the appropriate number of aliquots which were then treated in various ways as described below.

Trypsinization of the Iodinated Cells. 10^7 thymocytes were iodinated as described above. The incorporation of ^{125}I was terminated by the addition of 10 μl of 1 M KI and 1 ml of trypsin (10 μg/ml) in PBS. Trypsinization was performed at room temperature for 10 min and was terminated by the addition of 1 ml of soybean trypsin inhibitor (20 μg/ml) in PBS (22). Cells were then washed as described above.

Absorption of Antisera. Aliquots of anti-Ly-3.1 serum (25 μl) were diluted to 50 μl with PBS. Each aliquot was then mixed with a wet cell pellet containing 6 × 10^8 thymocytes from the appropriate strain. After incubation at 4°C for 1 h with occasional shaking, suspensions were centrifuged at 3,200 *g* for 5 min and the supernates were recovered.

Absorption with C57BL/6-*Ly-2*^{a},*Ly-3*^{a} thymocytes resulted in removal of all cytotoxic activity for Ly-3.1-positive thymocytes. Absorbed antisera were stored frozen at −70°C until use.

SDS-PAGE. SDS-PAGE was performed on 5, 10, and 15% (wt/vol) polyacrylamide gels (0.6 × 11 cm) using a modification of the method of Weber and Osborn (23). Immune precipitates were suspended in 55 μl of a solution containing 0.05 M Tris/Cl, pH 8.4, 8 M urea, and 2% SDS. Mouse IgG labeled with ^{131}I using chloramine-T (24) was usually added as an internal molecular weight marker. After the addition of 5 μl of 2-mercaptoethanol and 5 μl of an 0.05% solution of bromophenol blue, the samples were incubated at 55°C for 1 h to promote solubilization and reduction of the components present in the immune precipitate. Immediately before electrophoresis, the samples were placed in a boiling water bath for 2 min. Electrophoresis was generally performed at 3 mA/tube for 15 h at room temperature. After this period of time the bromophenol blue-tracking dye had migrated to a position approximately 8 cm from the origin. Gels were then removed from the tubes and frozen at −70°C in 50% glycerol (vol/vol). Gel slices (2 mm in length) were placed in tubes and counted using a Packard Autogamma Scintillation Counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

"Clearing" of NP-40 Supernates. Before precipitation with anti-Ly-3.1 serum, NP-40 extracts were preprecipitated (or "cleared") with normal mouse serum (NMS) and RAMIG to remove ^{125}I-labeled mouse IgG and any other labeled material which might be nonspecifically included in an immunological precipitate. To 80 μl of NP-40 extract containing 6–9 × 10^6 cpm was added 25 μl of (CBA/H-T6J × SJL/J)F_1 normal serum. A small quantity of ^{131}I-labeled (CBA/H-T6J × SJL/J)F_1

IgG was also added to determine the efficiency of immune precipitation. After incubation at 37°C for 30 min, 50 μl of RAMIG was added, and incubation was continued at 37°C for an additional 30 min. This quantity of RAMIG had been shown to be optimal for precipitating all of the mouse IgG present in 25 μl of NMS. Samples were then stored at 4°C for 1 h before removal of the precipitates by centrifugation. Supernates were cleared a second time before specific precipitation with anti-Ly-3.1 serum was attempted. An NP-40 concentration of 0.5% was maintained throughout by the addition of appropriate quantities of 2% (wt/vol) NP-40.

The precipitates obtained from clearings and specific precipitation (see below) were washed three times by centrifugation and resuspension in 1 ml of cold PBS. Between each wash the suspension was transferred to a clean glass tube.

Precipitation of Ly-3.1 by Indirect Immune Precipitation. To cleared NP-40 extracts, 25 or 50 μl of anti-Ly-3.1 serum and 5 μl of ^{131}I-labeled IgG were added, followed by incubation at 37°C for 30 min. RAMIG (50 μl) was added to precipitate all mouse IgG present in the solution. Samples were incubated at 37°C for 30 min and then at 4°C for 12 h. The NP-40 concentration was maintained at 0.5% as described. Immune precipitates were washed three times by centrifugation and resuspended in 1 ml of cold PBS as described above.

Labeling of Surface Galactosyl Residues with NaB^3H_4. The procedure employed was similar to that of Gahmborg and Hakamori (25). In summary, 10^7 thymocytes in 0.5 ml of PBS were treated with 0.1 ml of galactose oxidase (10 U) in PBS at 22°C for 2 h. Cells were washed once by centrifugation with 2 ml of PBS, and resuspended in 0.5 ml of PBS. 5 μl of a solution containing NaB^3H_4 (1 mCi) in 0.1 M NaOH was added, and samples were incubated at room temperature for 30 min. Cells were then washed twice with 2 ml of PBS and subjected to NP-40 lysis as described previously. Clearing before specific immune precipitation was as described above, except that samples were incubated at 4°C for 12 h after the first clearing and for 6 h after the second clearing. Specific immune precipitation was as described previously.

Gel Filtration of Mixtures of NP-40 Extracts and NMS or Anti-Ly-3.1 Serum on Sephadex G-200. To NP-40 extracts of ^{125}I-labeled cells were added 50 μl of anti-Ly-3.1 serum or NMS and a small quantity of ^{131}I-labeled mouse IgG as an internal marker. Appropriate amounts of 2% (wt/vol) NP-40 in PBS were added to maintain a concentration of 0.5%. Solutions were incubated at 37°C for 30 min to promote the formation of immune complexes. Each solution was then subjected to gel filtration at 4°C on a column (0.8 × 24 cm) of Sephadex G-200 in PBS containing BSA (2 mg/ml) and NP-40 (0.5%, wt/vol). To each eluted fraction (0.2 ml) containing ^{125}I-labeled macromolecular material was added a small quantity of ^{131}I-labeled (CBA/H-T6J × SJL/J)F_1 IgG to assess the efficiency of the subsequent precipitation. RAMIG (2 μl) was then added and the solutions were incubated at 37°C for 30 min. GARIG (30–50 μl) was then added in quantity sufficient to precipitate all of the IgG in the added RAMIG. Samples were incubated at 37°C for 30 min and then overnight at 4°C. Precipitates were recovered by centrifugation, washed three times with 2 ml of cold PBS, and subjected to SDS-PAGE as described above.

Comparisons of Experiments Employing Different Thymocytes and/or Sera. Experiments which compare the activities of various antisera and/or NMS were performed in parallel on identical NP-40 extracts, and all procedures and reagents employed were otherwise identical. When ^{125}I-labeled NP-40 extracts of thymocytes from different congenic or unrelated inbred strains were compared, iodinations and other procedures were performed in parallel under conditions as identical as possible.

Results

Indirect Precipitation of Ly-3.1 from NP-40 Extracts of C57BL/6-Ly-2^a,Ly-3^a Thymocytes. C57BL/6-*Ly-2^a,Ly-3^a* thymocytes (Ly-3.1 positive) were labeled with ^{125}I and NP-40 extracts were prepared as described (see Materials and Methods). Extracts were cleared twice, anti-Ly-3.1 serum or NMS was added to supernates, and immune complexes and uncomplexed mouse IgG were precipitated with RAMIG. Precipitates were dissolved in SDS buffer, reduced with 2-mercaptoethanol, and subjected to PAGE in 10% gels as described. As shown in Fig. 1 (panels *a* and *b*), specific anti-Ly-3.1 serum results in precipitation of an

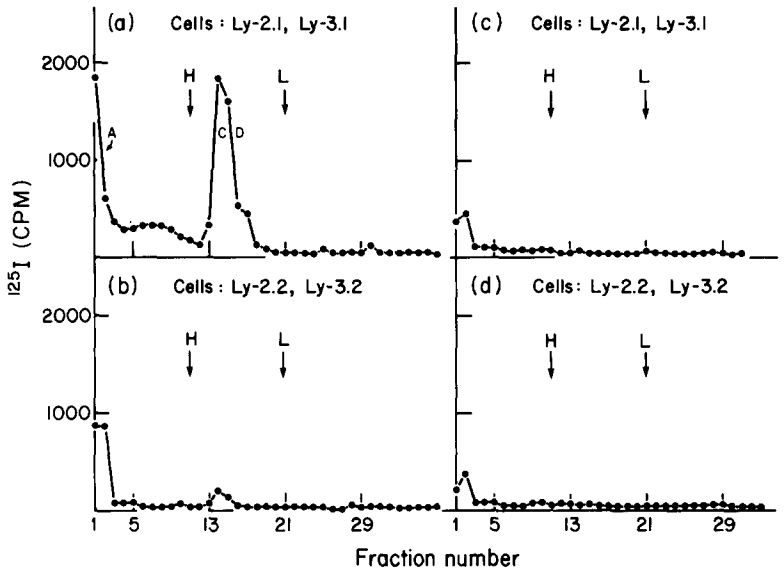

FIG. 1. The effect of Ly-3 phenotype on the immune precipitation of Ly-3.1 from NP-40 extracts of iodinated thymocytes. Gel profiles are of reduced immune precipitates electrophoresed on 10% polyacrylamide-SDS gels. Cells: refers to the phenotype of the thymocytes employed. Ly-2.1,Ly-3.1-positive thymocytes were obtained from C57BL/6-*Ly-2*^{a},*Ly-3*^{a} mice and Ly-2.2,Ly-3.2-positive cells from C57BL/6J mice. Immune precipitation was by an indirect method with the first serum (25 μl of anti-Ly-3.1 serum [*a* and *b*] or NMS [*c* and *d*]). The precipitating antiserum is 50 μl of RAMIG. NMS refers to normal serum from (CBA/H-T6J × SJL/J)F_1 mice. The positions of heavy and light chains from ^{131}I-lableled mouse IgG are indicated by H and L, respectively. Peaks A, C, and D are described in text.

^{125}I-labeled component of apparent molecular weight between that of immunoglobulin H and L chains which is absent from precipitates formed with NMS.

When the identical protocol was followed using ^{125}I-labeled Ly-3.1-negative C57BL/6J (Fig. 1, panels *c* and *d*) or C57BL/6-*Ly-2*^{a} thymocytes which differ from C57BL/6-*Ly-2*^{a},*Ly-3*^{a} at both Ly-2 and Ly-3, and at Ly-3 only, respectively, no significant difference was observed between gels of NMS- and antiserum-treated extracts. In contrast, anti-Ly-3.2 serum precipitated from NP-40 extracts of C57BL/6J thymocytes a component of apparent molecular weight identical to the Ly-3.1-related molecule shown in Fig. 1*a*. Moreover, results obtained using specific anti-Ly-3.2 serum and NP-40 extracts of C57BL/6-*Ly-2*^{a},*Ly-3*^{a} thymocytes were identical to those obtained with NMS.

Indirect Precipitation of Ly-3.1 Using Absorbed Antisera. As a further specificity control, anti-Ly-3.1 serum was absorbed with a number of C57BL/6-*Ly-2*^{a},*Ly-3*^{a} thymocytes sufficient to remove all cytotoxic activity for Ly-3.1-positive thymocytes (see Materials and Methods). Similar aliquots were absorbed with equivalent numbers of C57BL/6J thymocytes (Ly-3.1 negative). Aliquots of normal (CBA/H-T6J × SJL/J)F_1 serum were also absorbed with thymocytes of each strain. Cleared ^{125}I-labeled extracts of C57BL/6-*Ly-2*^{a},*Ly-3*^{a} cells were then precipitated with each absorbed antiserum and NMS, and precipitates were subjected to SDS-PAGE on 5% gels (Fig. 2).

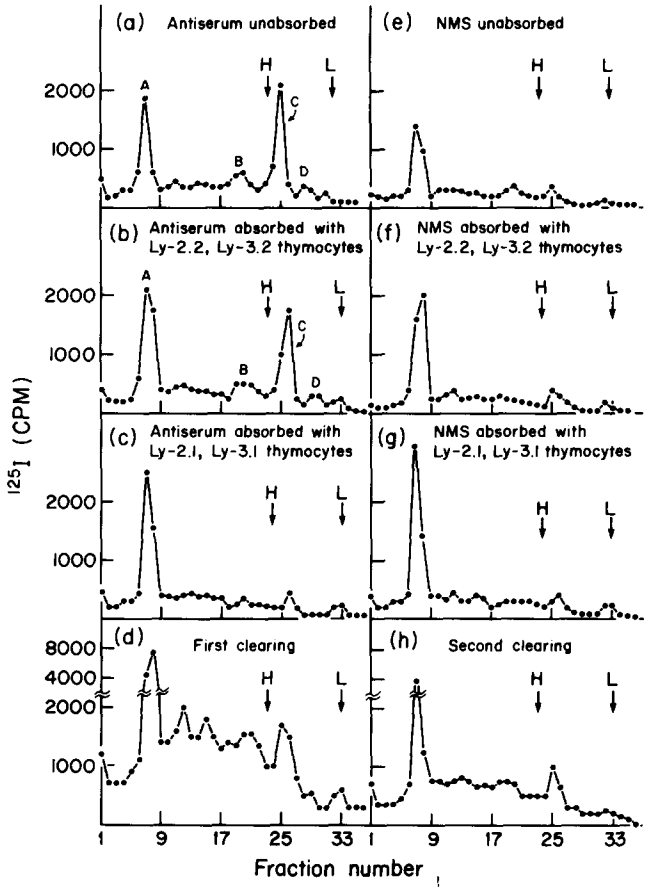

FIG. 2. The effects of absorption (see Materials and Methods) of anti-Ly-3.1 serum with Ly-3.1-positive (C57BL/6-*Ly-2^a,Ly-3^a*) or Ly-3.1-negative (C57BL/6J) thymocytes upon the immune precipitation of Ly-3.1 from NP-40 extracts of C57BL/6-*Ly-2^a,Ly-3^a* thymocytes. Designations NMS, H, and L are as in Fig. 1. Reduced samples were electrophoresed on 5% polyacrylamide-SDS gels. Included for comparison in the two bottom panels are the gel profiles of immune precipitates resulting from the first and second clearings of the NP-40 extracts with NMS and RAMIG (25 μl and 50 μl, respectively). Peaks A–D are described in text.

Antiserum absorbed with Ly-3.2-positive C57BL/6J thymocytes resulted in polyacrylamide gel patterns virtually identical to those obtained with unabsorbed antiserum (Figs. 2*b* and 2*a*, respectively). In contrast, absorption with C57BL/6-*Ly-2^a,Ly-3^a* thymocytes drastically reduced the quantity of the major Ly-3.1 component detectable by these procedures and resulted in a gel pattern indistinguishable from that obtained with NMS (Figs. 2*c* and 2*e*, respectively). Gels of precipitates made with absorbed NMS were indistinguishable from those obtained with unabsorbed normal serum (Figs. 2*f*, 2*g*, and 2*e*).

SDS-PAGE of Precipitates Obtained by Clearing NP-40 Extracts. Immunological precipitates obtained from clearings of ^{125}I-labeled NP-40 extracts of C57BL/6-*Ly-2^a,Ly-3^a* thymocytes with NMS and RAMIG were subjected to SDS-PAGE as described (Figs. 2*d* and 2*h*). It is apparent that a

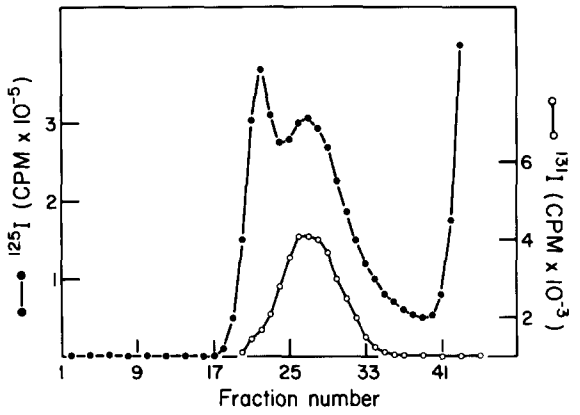

FIG. 3. Sephadex G-200 gel filtration of an NP-40 extract of ^{125}I-labeled C57BL/6-*Ly-2*^{a},*Ly-3*^{a} thymocytes. The extract (100 μl) was mixed with 50 μl of anti-Ly-3.1 serum and 5 μl ^{131}I-labeled mouse IgG and the mixture was incubated for 30 min at 37°C before gel filtration at 4°C. The column was developed with PBS containing NP-40 (0.5%) and BSA (2 mg/ml). Fractions of 0.2 ml were collected.

considerable amount of radioactivity is precipitated and is distributed throughout the gel including at the position of the Ly-3.1 component.

Gel Filtration of NP-40 Extracts Containing NMS or Anti-Ly-3.1 Serum. In order to obviate the need for clearing, we added aliquots of NMS or anti-Ly-3.1 serum to NP-40 extracts of ^{125}I-labeled C57BL/6-*Ly-2*^{a},*Ly-3*^{a} thymocytes, and after incubation at 37°C for 30 min, these extracts were subjected to gel filtration on columns of Sephadex G-200 as described in the Materials and Methods (Fig. 3). Immune complexes formed between antibody and macromolecular material should have molecular weights greater than that of uncomplexed IgG and should be eluted from the column earlier than the bulk of the IgG. Such immune complexes could then be precipitated from individual column fractions using optimal amounts of RAMIG and GARIG antisera.

Fractions from the gel filtrations of C57BL/6-*Ly-2*^{a},*Ly-3*^{a} samples treated with NMS or antiserum were subjected to immune precipitation with RAMIG and GARIG (Table I). It is apparent that a significantly higher percentage of total ^{125}I-labeled material in each fraction is precipitated in the case of anti-Ly-3.1 serum-treated NP-40 extracts than from extracts treated simultaneously and in identical fashion with NMS. Comparable fractions from gel filtrations of extracts which received neither NMS nor antiserum yielded results identical to those obtained with NMS. When extracts of Ly-3.1-negative C57BL/6J thymocytes were substituted and everything else was kept the same, no difference was observed between NMS- and anti-Ly-3.1 serum-treated samples. Thus the differences between NMS- and antiserum-treated samples in Table I appear to be Ly-3.1 specific.

A small amount of ^{131}I-labeled IgG was added as an internal marker to each column fraction before precipitation. The proportion of the total ^{131}I-labeled material in each fraction which appeared in the precipitate is taken as a measure of the efficiency of precipitation of mouse IgG in that fraction. The elution position of uncomplexed mouse IgG in that fraction. The elution position

TABLE I

*Immune Precipitation of Gel Filtration Fractions of Anti-Ly-3.1 Serum- and NMS-Treated NP-40 Extracts of ^{125}I-Labeled Ly-3.1-Positive Thymocytes**

Serum added to NP-40 extract	Column fraction	Total ^{125}I in fraction (cpm × 10^{-5})	Percent of total ^{125}I precipitated	Percent of ^{131}I-labeled IgG precipitated
(CBA/H-T6J × SJL/J) F_1 normal serum (50 μl)	a	0.04	5.80	76.0
	b	0.19	5.44	76.6
	c	0.59	5.82	75.5
	d	1.41	5.95	71.6
	e	1.71	4.84	75.5
	f	1.53	3.09	61.0
	g	1.14	1.92	55.6
	h	1.15	0.82	44.4
Anti-Ly-3.1 serum (50 μl)	a	0.03	13.40	78.3
	b	0.28	10.42	74.2
	c	1.13	9.11	68.1
	d	1.94	8.22	68.5
	e	1.94	5.97	75.6
	f	1.44	3.53	58.0
	g	1.18	1.61	44.3
	h	1.13	0.75	34.9

* NP-40 extracts of ^{125}I-labeled C57BL/6-*Ly-2^a,Ly-3^a* thymocytes (Ly-3.1 positive) were treated with either NMS or anti-Ly-3.1 serum at 37°C for 30 min. Treated extracts were then subjected to gel filtration at 4°C on Sephadex G-200 (see Fig. 3) and fractions of 0.2 ml were collected. Fractions a–h from each gel fraction (which are comparable to Fig. 3, fractions 17–24) were subjected to immune precipitation as described in the Materials and Methods. A small amount of ^{131}I-labeled mouse IgG was added to each fraction before precipitation as an internal standard to test the efficiency of the precipitation (see Materials and Methods and text).

of uncomplexed mouse IgG in gel filtrations of NMS- and antiserum-treated extracts is indicated by the inhibition of precipitation of ^{131}I-labeled IgG seen in fractions 6 through 8 from both gel filtrations (Table I). This position is identical to that determined by prior standardization of the columns with ^{131}I-labeled IgG. Thus when NMS or antiserum is added to extracts before gel filtration, the percentage of ^{125}I-labeled material precipitated is maximal in fractions which precede the elution position of uncomplexed IgG.

Analysis of Immune Precipitates from Column Fractions by SDS-PAGE. Immune precipitates from gel filtration fractions (see Table I) were subjected to SDS-PAGE under reducing conditions as described in the Materials and Methods. SDS-PAGE profiles of comparable gel filtration fractions from antiserum-treated and NMS-treated extracts are presented in Figs. 4*a-d* and 4*e-h*, respectively. It is apparent that the anti-Ly-3.1 serum-treated samples contain significant quantities of a radioactive component of apparent molecular weight between that of IgG H and L chains, while NMS-treated samples contain only trace amounts of radioactivity in this portion of the gel. Specificity controls were performed which were similar to those described for the indirect precipitation from cleared NP-40 extracts (see Figs. 1 and 2). These controls included

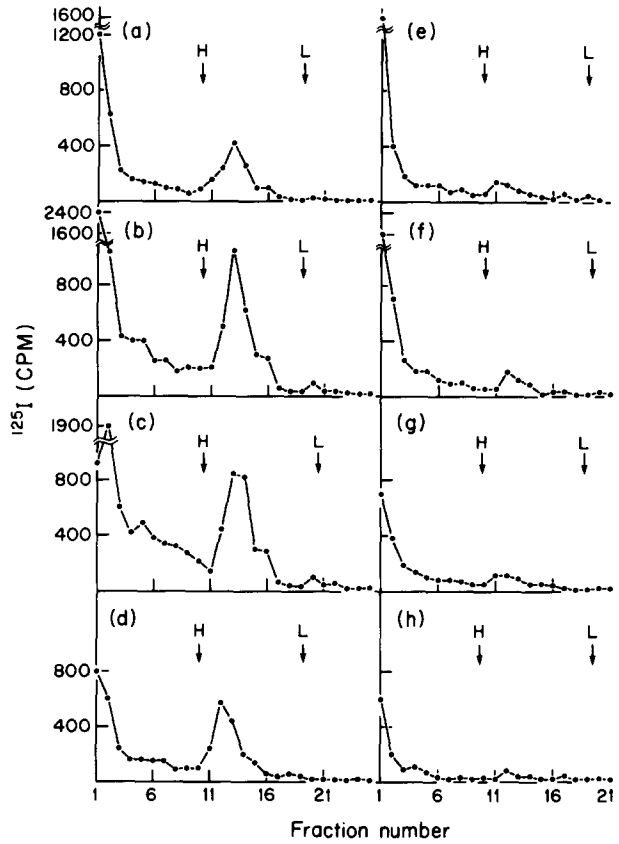

FIG. 4. SDS-PAGE (10% gels) of reduced immune precipitates from successive fractions obtained by gel filtration on Sephadex G-200 of NP-40 extracts of ^{125}I-labeled C57BL/6-*Ly-2^a,Ly-3^a* thymocytes. NP-40 extracts were incubated with 50 μl of anti-Ly-3.1 serum (left column, panels *a*, *b*, *c*, and *d*) or NMS (right column, panels *e*, *f*, *g*, and *h*) before gel filtration. The protocol employed for immune precipitation of each fraction is described in the Materials and Methods. The gel filtration profile of the antiserum-treated extract is presented in Fig. 3; the profile of the NMS-treated sample is similar. The correspondence of fractions is as follows: panels *a* and *e* are equivalent to Fig. 3, fraction 19; *b* and *f* to fraction 20; *c* and *g* to fraction 21; and *d* and *h* to fraction 22. Designations H, L, and NMS are as in Fig. 1 legend.

iodination of Ly-3.1-negative thymocytes from strains congenic with C57BL/6-*Ly-2^a,Ly-3^a*, and absorption of anti-Ly-3.1 serum with Ly-3.1-positive and Ly-3.1-negative thymocytes from C57BL/6J congenic strains. In all cases the results were consistent with the Ly-3.1 antigen being composed, at least in part, of a component with an apparent mol wt in the range of 30,000–40,000 daltons.

Effect of Trypsinization of Cells Upon Specific Precipitation with Anti-Ly-3.1 Serum. ^{125}I-labeled C57BL/6-*Ly-2^a,Ly-3^a* thymocytes were subjected to mild proteolysis with trypsin. Anti-Ly-3.1 serum was then added to NP-40 extracts of trypsinized and control cells and mixtures were subjected to gel filtration on Sephadex G-200 as described above. Comparison of the gel filtration profiles indicated that trypsinization led to a 60% loss of ^{125}I-labeled macromolecular material as compared with the untreated control. Immune precipitates from

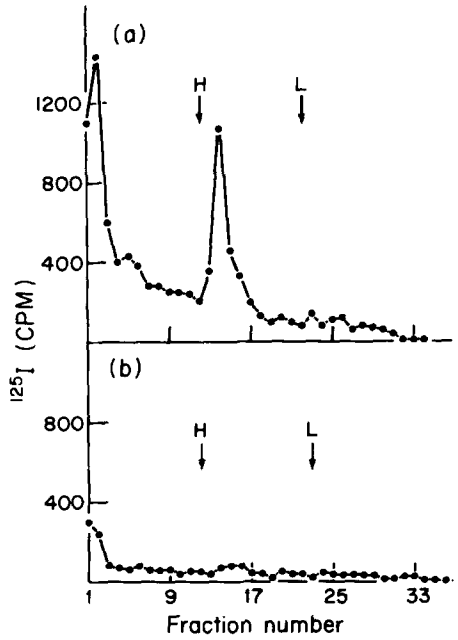

FIG. 5. The effect of trypsinization upon the immune precipitation of Ly-3.1. NP-40 extracts of trypsinized and untreated ^{125}I-labeled C57BL/6-*Ly-2^a,Ly-3^a* thymocytes were mixed with 50 μl of anti-Ly-3.1 serum and then subjected to gel filtration on Sephadex G-200. Fractions were submitted to immune precipitation as described, and precipitates containing maximal amounts of ^{125}I were reduced and subjected to SDS-PAGE under reducing conditions on 10% gels. Panel *a*, control; and panel *b*, trypsinized. Designations H and L are in Fig. 1 legend.

column fractions were prepared and subjected to SDS-PAGE as described, and gels of comparable fractions of trypsin-treated and untreated samples are presented in Fig. 5. It is apparent that digestion with trypsin resulted in degradation or loss from the cell surface of molecules reactive with anti-Ly-3.1 serum.

Molecular Weight of Ly-3.1. ^{125}I-labeled material specifically precipitated with anti-Ly-3.1 serum was extracted from polyacrylamide gels and re-electrophoresed under reducing conditions in 5, 10, and 15% polyacrylamide gels in the presence of appropriate protein markers of known molecular weight. The logarithm of molecular weight was plotted as a function of mobility in the gel (23, 26) (Fig. 6). From its mobility in 5% polyacrylamide gels, the major Ly-3.1-specific component detectable by these procedures has an apparent mol wt of approximately 39,800 daltons. As determined in 10 and 15% gels, this component has an apparent mol wt of approximately 35,000 daltons.

Effect of Pronase on the Major Ly-3.1-Specific Component. The major Ly-3.1-specific component was extracted from gels and subjected to digestion with pronase in order to determine whether it was protein in nature. Whereas untreated material was 100% precipitable with 10% TCA, less than 10% of the pronase-treated material was precipitable. Thus by the criterion of susceptibility to digestion by a proteolytic enzyme, at least a portion of the molecule bearing the Ly-3.1 antigenic specificity must be a polypeptide.

Other ^{125}I-Labeled Components. Precipitates from cleared NP-40 extracts

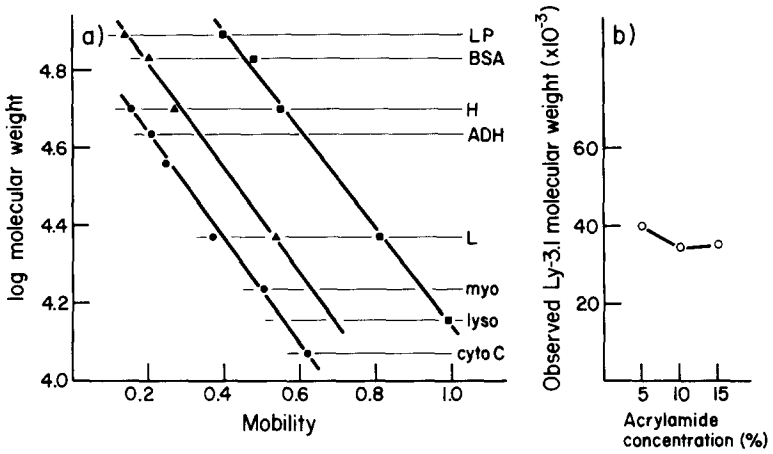

FIG. 6. Determination of the apparent molecular weight of Ly-3.1. Ly-3.1 extracted from SDS-polyacrylamide gels was re-electrophoresed on 5, 10, and 15% gels in the presence of various proteins of known molecular weight. LP, lactoperoxidase (bovine); H, mouse IgG heavy chain; ADH, alcohol dehydrogenase (yeast); L, mouse IgG light chain; myo, myoglobin (equine skeletal muscle); lyso, lysozyme (egg white); and cyto C, cytochrome C (horse heart). Mobility is determined relative to a bromophenol blue dye marker. Panel *a*: (■—■), 5% gels; (▲—▲), 10% gels; and (●—●), 15% gels. Panel *b*: (○—○), observed molecular weight of Ly-3.1 (peak C, Figs. 1*a* and 2*a*).

and from fractions obtained by the gel filtration procedure contain a major ^{125}I-labeled component which does not enter 10% gels (e.g., Fig. 1*a*, peak A) and which appears on 5% polyacrylamide gels under reducing conditions to have a mol wt of approximately 180,000 daltons (Fig. 2, peak A). Comparable amounts of this material are precipitated from thymocyte extracts of all strains employed in this study in the presence of NMS or specific antiserum. It appears to become associated with any immunological precipitate which forms in its presence, and its identity remains unknown.

It is apparent from Figs. 2*a* and 2*b* that besides the major detectable component of Ly-3.1 (peak C), two other ^{125}I-labeled components (peaks B and D) are precipitated from C57BL/6-*Ly-2*^{a},*Ly-3*^{a} NP-40 extracts by anti-Ly-3.1 serum and are detectable by SDS-PAGE under reducing conditions. These vary in quantity in different experiments, and, as determined on 5% gels, components B and D have apparent mol wt of approximately 55,000 and 30,000 daltons, respectively. Component D is present on 10% gels as a shoulder on the low molecular weight side of the major Ly-3.1-specific component (e.g., Fig. 1*a*). The relevance of these minor peaks to Ly-3.1 is not known, since ^{125}I-labeled material is sometimes present at positions B and/or D of gels containing material precipitated with NMS (e.g., Fig. 2*e*).

Precipitation of cleared Ly-3.1-positive extracts with NMS usually results in a small amount of a ^{125}I-labeled component at the approximate position of the major Ly-3.1 component (Fig. 2*e*). A similar component is often observed when extracts of C57BL/6J cells (Ly-3.1 negative) are precipitated with anti-Ly-3.1 serum. When displayed on 10% polyacrylamide gels, the maximum of this NMS-precipitated peak is usually displaced at least one gel slice towards the origin from the maximum of the major detectable Ly-3.1 component.

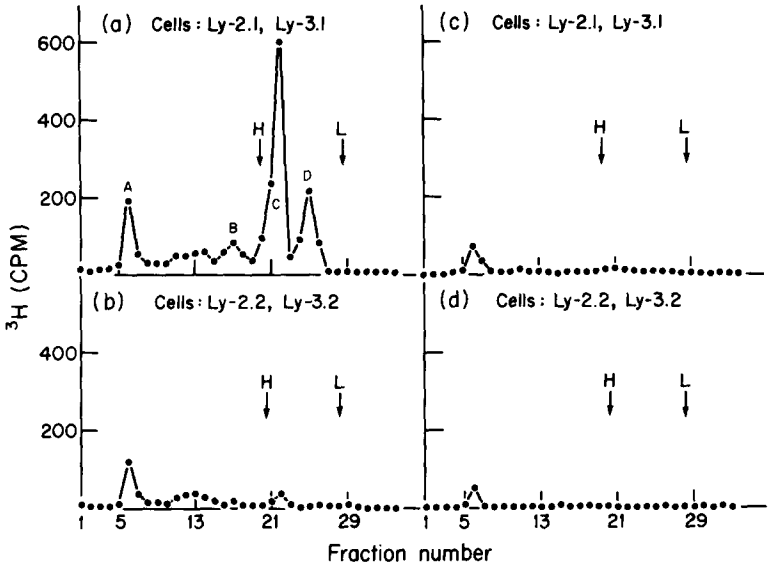

FIG. 7. The effect of Ly-3 phenotype on the immune precipitation of Ly-3.1 from NP-40 extracts of thymocytes labeled with ^{3}H using galactose oxidase and NaB^3H_4 (see Materials and Methods). Immune precipitation was as described above, and SDS-PAGE was performed under reducing conditions in 5% gels. Left column treated with anti-Ly-3.1 serum; right column treated with NMS. Cells: refers to the phenotype of the thymocytes employed as described in Fig. 1 legend. Designations H, L, and NMS are as above. Peaks A–D are as described in text.

Precipitation of Ly-3.1 Labeled using Galactose Oxidase and NaB^3H_4. Cell surface-associated galactose residues of C57BL/6-*Ly-2^a,Ly-3^a* thymocytes were labeled using galactose oxidase and NaB^3H_4 as described in the Materials and Methods. NP-40 extracts were cleared, precipitated with either NMS or anti-Ly-3.1 serum, and subjected to SDS-PAGE on 5% gels under reducing conditions (Fig. 7). In agreement with the ^{125}I studies, a major Ly-3.1-specific component was observed with an apparent mol wt of 35,000 daltons. Thus it appears that this major Ly-3.1 component is a glycoprotein containing galactose residues. ^{3}H-labeled components were also observed at positions B and D in the gel as well as in the region corresponding to a mol wt of 180,000 daltons (peak A), suggesting that these components are also glycoproteins. Galactose oxidase plus NaB^3H_4 labeling yielded higher D/B and D/C peak height ratios than enzymatic iodination (compare Figs. 7*a* and 2*a*), suggesting that component D is more readily labeled by the former method.

Specific Immune Precipitation of Ly-2.1. NP-40 extracts of ^{125}I-labeled Ly-2.1-positive and Ly-2.1-negative thymocytes were treated with NMS or anti-Ly-2.1 serum and precipitated as described for Ly-3.1. SDS-PAGE profiles of the immune precipitates are presented in Fig. 8. It is apparent that precipitation of ^{125}I-labeled surface components of C57BL/6-*Ly-2^a* thymocytes with anti-Ly-2.1 serum results in a gel pattern indistinguishable from that obtained after precipitation of labeled C57BL/6-*Ly-2^a,Ly-3^a* extracts with anti-Ly-3.1 serum (Figs. 8*a* and 2*a*, respectively). Treatment of labeled NP-40 extracts of C57BL/6J thymocytes (Ly-2.1 negative) with anti-Ly-2.1 serum yields a gel pattern indistin-

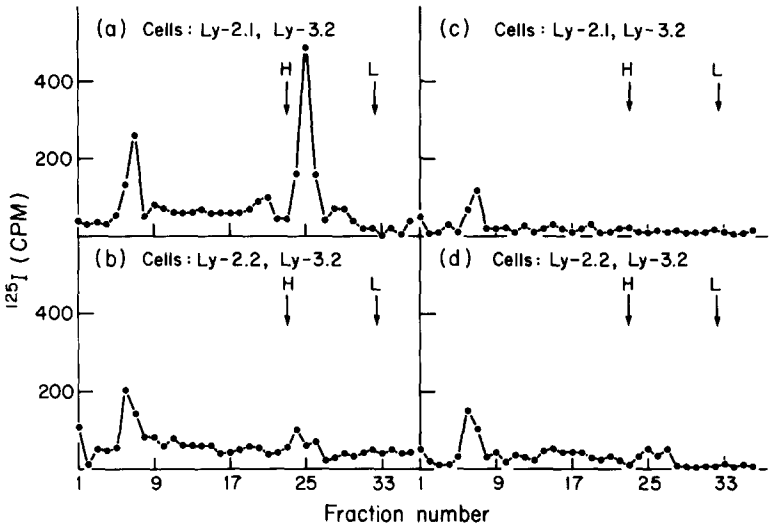

FIG. 8. The effect of Ly-2 phenotype on immune precipitation of Ly-2.1 from NP-40 extracts of iodinated thymocytes. Immune precipitation was as described above, and SDS-PAGE was performed under reducing conditions in 5% gels. Left column treated with anti-Ly-2.1 serum; and right column treated with NMS. Cells, refers to phenotype of thymocytes employed: Ly-2.1,Ly-3.2-positive thymocytes were obtained from C57BL/6-*Ly-2*^{a} mice, and Ly-2.2,Ly-3.2-positive cells from C57BL/6J mice. Designations NMS, H, and L are as in Fig. 1 legend.

guishable from that obtained with NMS (Figs. 8*b* and 8*d*). Thus the thymocyte surface component bearing the Ly-2.1 antigenic specificity appears to show the same labeling pattern and molecular weight species as the molecule bearing Ly-3.1.

Discussion

The use of alloantisera as indicators of cell surface polymorphisms in inbred mice has led to the identification and genetic mapping of a large number of murine cell surface antigens (27–29). Antigens which are represented on widely varying cell types are generally assumed to play some general role in the interaction or maintenance of cellular function, while those surface antigens represented on more restricted cell populations (i.e., differentiation antigens) are thought to be involved in the peculiar origins and/or properties of those cell types (29).

The relative ease in obtaining lymphocytes and in characterizing their surface components by cytotoxic and absorption assays using alloantisera has led to the identification of a large number of alloantigens on T and B lymphocytes. While the presence of such antigens on lymphocytes of known location or function is useful as an identifying characteristic of such cells, determination of the role, if any, of a surface molecule in mediating that function requires its chemical characterization.

The chemical characterization of mouse lymphocyte and other cell surface antigens has revealed that a number of such molecules fall into two basic groups

composed of polypeptides of characteristic molecular weights. The H-2, *T* locus, and thymus leukemia antigens appear to consist of large glycoprotein subunits with mol wt of approximately 45,000 daltons and a small subunit with a mol wt of 12,000 daltons which resembles human β_2-microglobulin (12, 15–18). A major glycoprotein subunit of mol wt 35,000 marks the second molecular weight class of surface antigens which includes the *I*-region-associated antigens (or Ia antigens) coded for by genes located within the *H-2* complex (13, 14, 30). Allogeneic effect factor, which is released by T lymphocytes activated by alloantigens, appears by gel filtration to be in this molecular weight range (31) and it has been demonstrated to bear Ia antigenic determinants (32). In addition, an antigen-specific suppressive T-cell factor which contains antigenic specificities determined by the *K* end of the *H-2* complex (*H-2K*, *I-A*, and/or *I-B*) has a mol wt by gel filtration of between 35,000 and 60,000 daltons (33).

The present studies indicate that the thymocyte cell surface constituents which bear the Ly-3.1 and Ly-2.1 alloantigenic determinants each contain a glycoprotein of apparent mol wt 35,000 which contains a galactose-containing carbohydrate moiety. This places them in the same molecular weight range as the *I*-region-associated antigens (13, 14, 30–33), although no other similarity is implied by this comparison. The apparent absence of a polypeptide of mol wt 22,500 suggests that typical immunoglobulin L chains containing side chains susceptible to iodination under the conditions employed are not a constituent of the Ly-3.1 antigen. If the Ly-3.1 thymocyte surface antigen had contained L chains, one might have concluded that a single genetic locus codes for the structures of the I_B-peptide marker in mouse V_L regions and the Ly-3.1 thymocyte surface antigen. This would have explained the close genetic linkage of these traits (10, 11). Both traits might still be governed by the same structural genetic locus if it is demonstrated by peptide mapping and by amino acid sequence analysis that the Ly-3.1 polypeptide contains a region which is homologous to immunoglobulin L-chain V regions. If no such homologies are found, there are three other possibilities: (*a*) Both traits may be controlled by the same genetic locus which does not directly code for their structure; (*b*) a single locus may code for the structure of one of the two traits, and that trait may govern the other; and (*c*) the two traits may be governed by closely linked but distinct genetic loci.

It is likely that the *Ly-3*^{a} and *Ly-3*^{b} alleles code for the polypeptide portions of the glycoproteins of mol wt 35,000 which bear, respectively, the Ly-3.1 and Ly-3.2 antigenic specificities. However, we have not excluded hypothesis (*a*), in that alleles at the Ly-3 locus might govern expression of glycosylating enzymes which have different substrate specificities and which generate the polymorphisms observed in a fashion analogous to the human blood group substances (34).

If Ly-3.1-positive T cells were needed to stimulate the synthesis of I_B-positive L chains, then absence of this determinant might result in a I_B-negative phenotype. It is unlikely that such stimulation is by conventional T-cell helper activity, since Cantor and Boyse have demonstrated using C57BL/6 mice that helper T cells are Ly-3.2 negative (7), and it is likely that helper T cells of inbred strains bearing the *Ly-3*^{a} allele are Ly-3.1 negative. Peptide maps of serum Ig L

chains from (AKR × BALB/c-nu/nu)F_3 nude mice of the *Ly-2*^{a},*Ly-3*^{a} genotype prepared by Boyse and Shen (see reference 11) indicated a quantity of I_B-positive L chains indistinguishable from that of the AKR/J parent. Thus the absence of a thymus and a normal complement of peripheral T cells did not influence the frequency of I_B-positive L chains in serum IgG, thus eliminating one form of hypothesis (*b*). Moreover, it had previously been demonstrated that I_B-positive serum IgG is apparently not autoantibody directed towards the Ly-3.1 antigenic specificity (10). Thus it appears that the *Ly-3* locus probably does not cause the appearance of the I_B phenotype, at least not by the mechanism discussed.

Thus unless structural homology is demonstrated between the polypeptide bearing the Ly-3.1 antigenic specificity and immunoglobulin V_L regions, it is likely that the Ly-3 thymocyte surface antigen and the I_B-peptide marker in mouse V_L regions are determined by closely linked but distinct genetic loci [hypothesis (*c*)].

Treatment of cleared NP-40 extracts of C57BL/6-*Ly-2*^{a},*Ly-3*^{a} thymocytes with anti-Ly-3.1 serum or anti-Ly-2.1 serum followed by RAMIG results in precipitation of components of identical molecular weight as revealed by SDS-PAGE (Figs. 2 and 8). This is true both for ^{125}I labeling of polypeptides using lactoperoxidase (Figs. 2 and 8) and for ^3H labeling of carbohydrate moieties using galactose oxidase and NaB^3H_4. These antisera precipitated glycoproteins of mol wt 35,000 only from NP-40 extracts of thymocytes of C57BL/6 congenic strains with Ly phenotypes for which the antisera were specific. Therefore both the Ly-2.1 and Ly-3.1 antigenic specificities are present on molecules containing polypeptides of this molecular weight. This similarity in molecular weight and the close genetic linkage of the loci governing the Ly-2 and Ly-3 surface antigens suggest the possibility that these polypeptides may be homologous in primary structure due to evolution from a common precursor gene, as has been suggested for immunoglobulin C-regions (35–37) and for the *H-2D* and *H-2K* surface antigens (28).

Studies using the antibody-blocking test have suggested that the Ly-2 and Ly-3 antigenic specificities are topologically adjacent on the cell surface, and therefore may be present on the product of a single gene (2, 5). Preliminary results obtained in our laboratory suggest that extensive precipitation of ^{125}I-labeled C57BL/6-*Ly-2*^{a},*Ly-3*^{a} thymocytes with anti-Ly-3.1 serum and RAMIG reduces to zero the amount of 35,000 mol wt component precipitated by subsequent treatment with anti-Ly-2.1 serum and RAMIG. If these results are borne out by further experiments of the type described, then we can conclude that the polypeptides bearing the Ly-2.1 and Ly-3.1 antigenic specificities are present on the same quarternary complex in the NP-40 extract and probably on the thymocyte surface as well. Studies to determine the molecular weight of the Ly-2 and Ly-3 antigens before reduction are in progress. Determination of whether the Ly-2.1 and Ly-3.1 antigens are on the same or different polypeptides will very likely require peptide mapping studies and amino acid sequence analysis.

Summary

Specific anti-Ly sera were employed to precipitate Ly antigens from Nonidet P-40 extracts of mouse thymocytes labeled with ^{125}I using lactoperoxidase and

with NaB^3H_4 using galactose oxidase. Thymocytes from mice of the congenic strains C57BL/6J (Ly-2.2, Ly-3.2 positive), C57BL/6-*Ly-2*^{a},*Ly-3*^{a} (Ly-2.1,Ly-3.1 positive) and C57BL/6-*Ly-2*^{a} (Ly-2.1,Ly-3.2-positive) were used as sources of labeled antigens and as immune adsorbants to permit evaluation of the specificity of each anti-Ly serum employed. Results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions are consistent with the Ly-3.1 antigen containing a glycoprotein subunit with an apparent mol wt of 35,000 daltons. Specific precipitates obtained using anti-Ly-2.1 serum yielded SDS-PAGE profiles identical to that obtained with anti-Ly-3.1 serum, suggesting that the Ly-2 and Ly-3 antigens have the same molecular weight distribution. The relationships of these results to the observed close genetic and topological linkage of Ly-2 and Ly-3 and to the genetic linkage of these loci with the I_B-peptide marker, a mouse V_κ-region polymorphism, are discussed.

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