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Immunology

Genetic polymorphism of IgD-like cell surface immunoglobulin in the mouse

(IgD/lymphocyte antigen receptors/alloantisera/immunoglobulin allotypes/staphylococcal protein A)

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ABSTRACT **Lymphocyte surface antigens from spleen cells of several mouse strains were studied by cell surface radioiodination, extraction with detergent, incubation with various antisera, and separation of complexes using protein A-containing staphylococci as a solid phase adsorbent. Complexes were then dissociated and analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.**

Using this technique and an alloantiserum prepared in C57BL mice against CBA spleen cells, four distinct specific peaks of radioactivity were found with CBA spleen cells. These corresponded to H-2 and Ia antigens, immunoglobulin light chain, and a heavy chain previously proposed to be the murine homolog of the human δ chain. With the same serum, B10.BR spleen cells revealed only H-2 and Ia antigens, whereas C57BL.Ig^e (allotype congenic) spleen cells showed only the light chain and "δ" chain peaks. Depletion of immunoglobulin from the surface-iodinated preparations resulted in removal of the light chain and "δ" chain peaks.

The tissue distribution and membrane expression of this "δ" chain antigen was then studied by indirect immunofluorescence with various C57BL derived alloantisera and lymphoid cells from C57.Ig^e allotype congenic mice. Significant numbers of positive cells were found in spleen, lymph nodes, and Peyer's patches, whereas few if any positive were found in bone marrow or thymus. No reaction was found between this molecule and alloantisera to any of the previously described immunoglobulin allotypes. It is proposed that these alloantisera to spleen cells recognise one allelic form of the murine "δ" chain coded for by a gene locus closely linked to the known structural genes for mouse immunoglobulin heavy chains. The designation *Ig-5* is proposed for this new immunoglobulin heavy chain locus.

Implicit in the clonal selection theory of acquired immunity is the notion that the lymphocyte surface receptors for antigen are identical copies of the antibody product which a given cell is programmed to secrete upon activation. This notion, of receptor equals product, has recently been modified in regard to the Ig constant region (1). Many lines of evidence now suggest that lymphocyte receptors in several species consist of an IgM-like molecule and an IgD-like molecule. Several groups have recently reported the presence of a murine B cell surface Ig heavy chain which migrates slightly faster than μ chain in sodium dodecyl sulfate (NaDodSO$_4$) polyacrylamide gel electrophoresis (2–5), but is not generally precipitable by antisera against any of the known Ig classes. On the basis of this lack of reactivity with known class specific antisera, its localisation on the lymphoid cell surface, and its reported susceptibility to proteolysis, this molecule has been postulated to be the murine homolog of human IgD, although its mobility in NaDodSO$_4$-PAGE is not identical to human δ chain (Warr and Marchalonis, submitted). However, definitive characterisation of this chain has not yet been made, principally due to the lack of specific allo- or heteroantisera, and to the failure to identify the molecule from normal serum or from any myeloma serum.

Based on a report by Kessler (6), a method was developed in this laboratory for the identification of cell surface iodinated alloantigens using protein A-containing staphylococci as a solid phase adsorbent for IgG. While testing a variety of different anti-H-2 antisera, we unexpectedly encountered alloantigens bearing a striking resemblance to the IgD-like molecule. Since these antigens were found to be Ig in nature, and distinguishable in appropriate strains of allotype congenic mice, it was evident that this molecule was an allelic form of an immunoglobulin. Previous studies (7, 8) have documented the existence of a genetic polymorphism of murine Ig heavy chain genes coding for γ2a (*Ig-1* locus), α (*Ig-2*), γ2b (*Ig-3*), and γ1 (*Ig-4*). As it was found in both surface iodination studies and in indirect immunofluorescence that this new Ig was not determined by any of these loci, we propose the designation of the *Ig-5* locus coding for the murine "δ" chain gene.

MATERIALS AND METHODS

Animals. CBA/H, C57BL/6 (C57), and B10.BR mice were from Hall Institute stocks and used at 6–12 weeks of age. C57BL.Ig^e is an allotype congenic partner strain to C57BL/6 produced by appropriate backcrossing and selection for *Ig-1^e* allotype (derived from NZB mice). At the fourteenth backcross generation, mice were inbred and homozygotes for *Ig-1^e* selected as the nucleus of the inbred homozygous congenic line (C57BL/6.Ig^e N14F5) (abbreviated C57.Ig^e). Backcrossing has also continued to the nineteenth generation (C57BL/6.Ig^e N19), these mice being heterozygous b/e at the *Ig-1* locus. Testing of C57.Ig^e mice for serum allotypes was performed as previously described (7), and these mice were shown to be homozygous for NZB type at the *Ig-1*, *Ig-3*, and *Ig-4* loci.

Antisera. Alloantisera were prepared by immunisation of C57BL/6 mice with spleen cells from CBA, BALB/c, or B10.D2 mouse strains. Immunisation usually consisted of 6 weekly intraperitoneal injections of approximately 20 × 10^6 spleen cells without adjuvant. Mice were pool bled 2 weeks later. C57 anti-CBA spleen-1 serum was made as follows. C57 mice were injected intraperitoneally with approximately 50 × 10^6 CBA spleen, lymph node, and thymus cells emulsified in complete Freund's adjuvant, boosted three times in saline and bled 7 days after the last injection. All anti-spleen cell sera were tested by radioimmunoassay (7) for precipitating activity against myeloma proteins of each of the known serum-derived Ig classes (IgM, IgA, IgG$_1$, IgG$_{2a}$, IgG$_{2b}$, and IgG$_3$). All sera were totally negative. Anti-H-2K^k serum was a generous gift from Dr. I. F. C. McKenzie. It was produced by multiple intraperitoneal injections of

Abbreviations: Ig, immunoglobulin; NP-40, Nonidet P-40; NaDodSO$_4$-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; EBSS, Eisen's balanced salt solution. MAID, membrane associated immunoglobulin detaining protein.

(A.TL × B10.D2)F_1 mice with lymphoid cells from A.AL mice, until a high cytotoxic titre was obtained. Anti-Ia^k serum was produced by multiple weekly intraperitoneal injections of A.TH mice with 5×10^6 spleen cells from A.TL mice.

Antisera against standard Ig allotypes were prepared as previously described (7). Fluorescent goat anti-mouse μ, $\gamma 1$, and $\gamma 2$ Ig sera were obtained from Meloy Laboratories (U.S.A.). Rabbit anti-mouse IgM serum (R640) was produced as described elsewhere (9), and had activity against both μ and light chains. Rabbit anti-mouse μ chain serum (R647) was produced by repeatedly passing a rabbit anti-mouse IgM serum (predominantly anti-μ) over a column of mouse IgG coupled to Sepharose 4B until no further antibody bound.

Staphylococci. A culture of *Staphylococcus aureus* positive for protein A (6 mg of rabbit IgG bound per milliliter of packed bacteria at 50% saturation) was obtained from Dr. C. Cheers of the Department of Microbiology, University of Melbourne, inoculated into a flask of 1 litre of heart infusion broth (Difco Laboratories, Detroit, Mich.), and grown overnight at 37°. Harvesting, fixation, and heat killing were essentially as described by Kessler (6).

Radioiodination. Spleen cell surface radioiodination with ^{125}I and detergent extraction with NP-40 (BDH, Melbourne) was as described by Goding *et al.* (10) using red cell- and dead cell-depleted preparations (5). Typically 20–40% of counts became associated with the cells. Extracts were not dialysed.

Isolation of Cell Surface Molecules. Detergent lysates of labeled cells were centrifuged at 12,000 × *g* for 20 min; 200 μl aliquots of the supernatants were transferred to miniature conical capped centrifuge tubes (Camelec Medical Products catalog number MCP 5505C), mixed with 5 μl of antiserum, and held at room temperature for 1 hr. Fifty microliters of a 10% suspension of staphylococci were then added, and the contents mixed. After a further 10 min, the tubes were centrifuged for 3 min in a Beckman Microfuge. Bacteria were washed three times in phosphate-buffered saline (PBS) at which stage the counts were stable. Bound material was eluted by suspending the bacteria in $NaDodSO_4$-PAGE sample buffer (9), and heating to 100° for 5 min. Analysis of radiolabeled molecules by $NaDodSO_4$-PAGE was as previously described (9). The mobility of standards in the gels was determined by running human IgG in the unreduced gels, or a mixture of human IgM and IgG for the μ, γ, and L chain standards in the reduced gels. The mobilities of $H\text{-}2K^k$ and Ia^k molecules were determined from parallel gels run on radioiodinated material reacted with specific antisera. The mobility of the "δ" chain was similarly determined with rabbit anti-mouse IgM serum containing anti-light chain antibody.

Immunoglobulin Depletion of Radioiodination Extracts. The centrifuged lysate of radioiodinated CBA splenocytes was run slowly (flow rate <5 ml/hr) over a column containing 1.0 ml of Sepharose 4B conjugated with 5 mg of the IgG fraction of rabbit anti-mouse IgM serum (R640), and equilibrated with 0.1% NP-40 in PBS. The adsorption was repeated once.

Immunofluorescent Procedures. Lymphoid cell suspensions were prepared from various tissues and washed twice in Eisen's balanced salt solution (EBSS). Approximately 10^6 cells to be tested were suspended in 100 μl of EBSS and reacted with 10 μl of the relevant mouse serum. After 30 min at 4°, cells were washed twice, supernatants removed and 100 μl of fluorescent goat anti-mouse $\gamma 1$ and $\gamma 2$ Ig (7.5% vol/vol of each reagent) added. After a further 30 min at 4° and two washes, the cells were suspended in three drops of 1% paraformaldehyde in PBS and examined for fluorescence using a Zeiss microscope with HP-100 mercury lamp and appropriate filters. In one series of experiments cells were first incubated at 37° for 2 hr with various rabbit antisera (1:10 dilution) prior to incubation with mouse antisera.

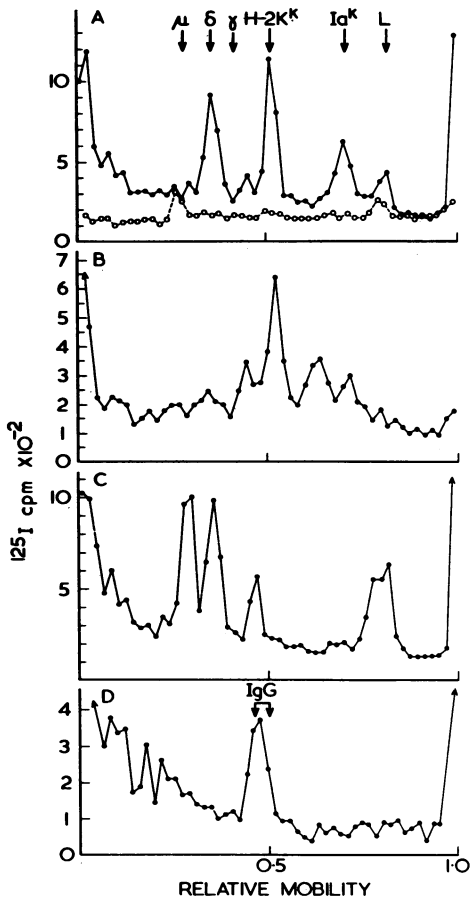

FIG. 1. $NaDodSO_4$-PAGE analysis of ^{125}I-labeled CBA lymphocyte membrane proteins recognised by alloantiserum. (1A and 1D) CBA splenocyte membrane proteins bound by C57BL anti-CBA spleen-1 serum (● - - - ●), or by staphylococci alone (○ - - - ○). (1B) CBA thymocyte membrane proteins bound by C57BL anti-CBA serum. (1C) CBA splenocyte membrane proteins bound by rabbit anti-mouse IgM serum. Gels 1A–1C were of 10% acrylamide, 0.25% bisacrylamide concentration, and samples were reduced with 2-mercaptoethanol (9). Gel D was of 5% acrylamide, 0.125% bisacrylamide concentration, and samples were not reduced.

RESULTS

Cell Surface Iodination of CBA Spleen Cells. Surface iodinated proteins were extracted from CBA spleen cells and assayed with various antisera. In the absence of specific antisera, only traces of iodinated cell surface material bound to the staphylococci (Fig. 1A). On the other hand, when various antisera to cell surface proteins were included, specific peaks of radioactivity corresponding to the antigens in question were seen (Fig. 1). Thus, the method should, in principle, allow identification of any cell surface antigen for which an antiserum with appropriate antibody class can be made.

During the characterisation of cell surface molecules recognised by a variety of alloantisera, it was noted that an an-

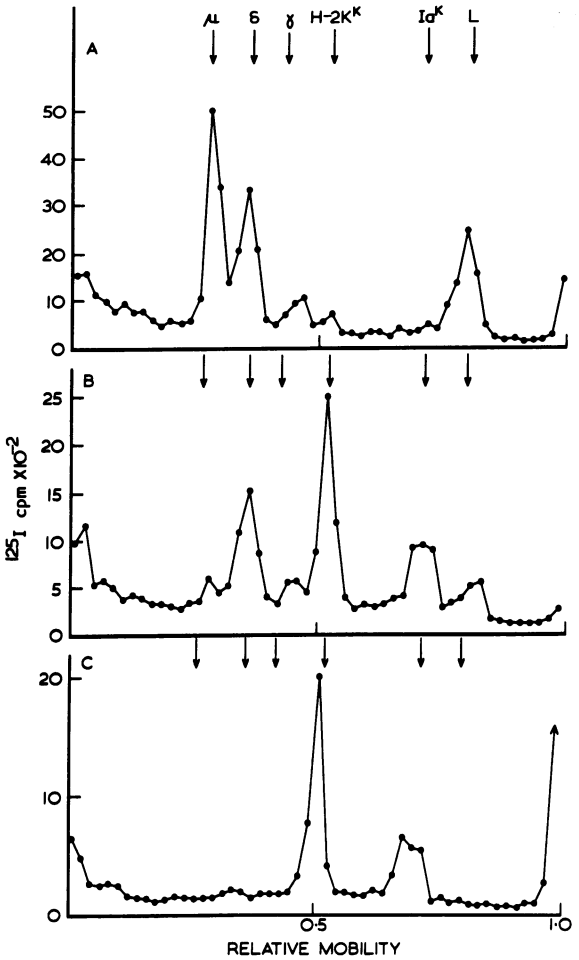

FIG. 2. NaDodSO$_4$-PAGE analysis of ^{125}I-labeled CBA splenocyte membrane proteins recognised by alloantiserum. (2A) CBA splenocyte membrane proteins bound by rabbit anti-mouse IgM serum. (2B) CBA splenocyte membrane proteins bound by C57BL anti-CBA serum. (2C) Immunoglobulin depleted CBA splenocyte membrane proteins bound by C57BL anti-CBA serum. All gels were of 10% acrylamide, 0.25% bisacrylamide concentration. All samples were reduced with mercaptoethanol (9).

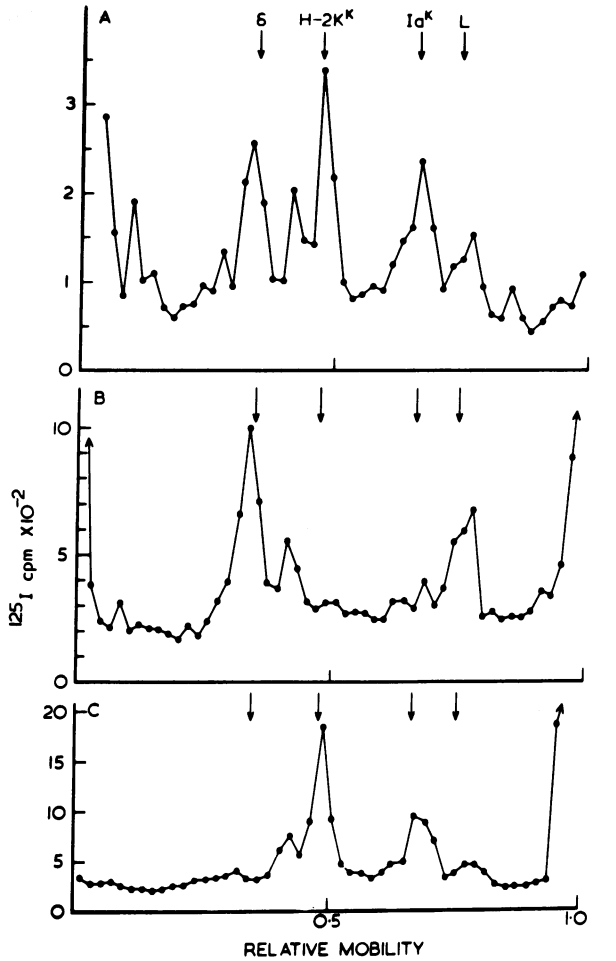

FIG. 3. NaDodSO$_4$-PAGE analysis of ^{125}I-labeled splenocyte membrane proteins from congenic mice recognised by C57 anti-CBA serum; (3A) CBA cells; (3B) C57.Ig^e cells; (3C) B10.BR cells. All gel conditions as for Fig. 2.

tiserum produced in C57 mice against CBA spleen cells reacted with five iodinated molecular species recognisable on gels run after sample reduction. As illustrated in Fig. 1A, two of the peaks of radioactivity showed mobilities consistent with their being H-2 and Ia, while the other two peaks resembled the "δ" and light chains of B cell surface Ig (Fig. 1C). The fifth peak observed in all gels may be associated with the Fc receptor or membrane-associated immunoglobulin detaining protein (MAID) (11) activity, and has been discussed elsewhere (9). This observation suggested that the antiserum may have recognised alloantigenic polymorphism in the IgD-like molecule on the B cell surface. This possible interpretation was supported by the observation that the antiserum recognised only one major component on CBA thymocytes, which resembled H-2 in its mobility (Fig. 1B). Thymocytes possess only very small amounts of Ia antigens (10), and immunoglobulin is not readily detectable in detergent lysates of surface radioiodinated T cells (9). Further, when the material isolated from CBA splenocytes was analysed without prior reduction on 5% gels, one major peak of radioactivity with mobility similar to intact IgG was observed (Fig. 1D). That the C57 anti-CBA serum was recognising determinants on the IgD-like molecule was also strongly suggested by an Ig depletion experiment (Fig. 2). Passage of iodinated CBA splenocyte extract over a column of anti-mouse IgM immunoadsorbent (containing anti-light chain antibodies) before reaction with C57 anti-CBA serum removed both the "δ" and light chain-like peaks from the gels, but left the peaks corresponding to H-2 and Ia antigens unaffected apart from slight dilution (Fig. 2).

Cell Surface Iodination of Congenic Strain Spleen Cells. In order to further analyse the possible genetic background required for the production of these molecules recognised by the C57 anti-CBA serum, experiments were performed using radioiodinated congenic strain-derived spleen cells (Fig. 3). In the case of B10.BR (Fig. 3C), only gene products controlled by the *H-2* complex should be detected by this serum. Peaks corresponding exactly in mobility to H-2K^k and Ia^k were seen, but the peaks corresponding to "δ" and light chains were no longer present.

To test whether the apparent Ig peaks observed with CBA spleen cells were linked to the known Ig heavy chain structural genes, spleen cells from C57.Ig^e mice (Fig. 3B) were investigated for surface antigens recognised by C57 anti-CBA alloantiserum. The NZB and CBA γ chain allotypes crossreact extensively, and it was hoped that a similar cross-reaction might pertain with the putative "δ" chain allotypes. Spleen cells from C57.Ig^e mice were surface radioiodinated, extracted, and the antigens detected by C57 anti-CBA

Table 1. Presence of anti-Ig-5^a antibody in various alloantisera

Test serum	Percent fluorescent cells	
	C57.Ig^e	C57
C57 anti-CBA spleen-1	26.8 ± 1.3 (5)	3.4 ± 0.8 (4)*
C57 anti-CBA spleen-2	32.6 ± 3.3 (4)	2.4 ± 0.3 (2)
C57 anti-BALB/c spleen	16.3 ± 6.2 (4)	3.4 ± 0.6 (3)
C57 anti-B10.D2 spleen	6.0	5.9
C57 normal serum	3.6 ± 1.0 (3)	3.5 ± 1.1 (3)
C57 anti-allotype sera†	4.8 ± 0.3 (4)	2.3 ± 0.4 (4)

* Number of individual determinations in parentheses.
† Mean values shown for four different allotype sera (see *Materials and Methods*) including anti-Ig-1^a, Ig-1^e, Ig-3^a, and Ig-4^a.

serum analysed. In this combination, only gene products closely linked to Ig heavy chain structural genes should be detected. The results are shown in Fig. 3. Peaks of radioactivity corresponding to "δ" and light chain were seen, but there were no peaks corresponding to H-2 or Ia antigens. A peak of radioactivity which migrated a little behind the H-2K^k marker, seen with both preparations, may represent the Fc receptor or MAID protein.

Detection of Membrane Bound Ig-5^a by Various Alloantisera. Four mouse alloantisera were tested by immunofluorescence for the presence of antibodies reactive against lymphoid surface antigens associated with allotype-linked genes. All sera were prepared in C57BL/6 mice and were tested on C57.Ig^e speen cells. The presence of such antibodies was detected by the second reagent, fluorescent goat anti-mouse $\gamma 1/\gamma 2$ serum. This serum reacted with an average of 3% spleen cells in C57 mice or C57.Ig^e mice when normal mouse serum was used as first reagent. As shown in Table 1, three different noncongenic anti-spleen cell sera showed the clear presence of antibodies reactive to surface antigens on C57.Ig^e cells but not on C57 cells. In all cases, only distinctly bright patched or capped fluorescent cells were scored, and for all sera, at least two counts on over 200 total cells were performed on coded samples. The congenic H-2 serum and four different antiallotype sera, with reactivities to Ig-1^a, Ig-1^e, Ig-3^a, or Ig-4^a all failed to show this reaction. Furthermore, as the three noncongenic anti-spleen cell sera failed to show by radioimmunoassay any precipitating activity to any myeloma protein of all known Ig classes, it is unlikely that the allotype-linked gene product detected in the C57.Ig^e mice is due to any of the presently known serum immunoglobulins.

Table 2. Tissue distribution of Ig-5^a bearing cells

Lymphoid cell suspension*	Percent fluorescent cells†	
	C57.Ig^e	C57
Spleen	27.4 ± 5.4	3.2 ± 1.1
Mesenteric lymph node	21.5 ± 3.3	3.3 ± 1.2
Peyer's patch	49.2 ± 10.2	7.8 ± 2.7
Bone marrow	4.6 ± 0.2	1.8 ± 0.5
Thymus	<1	<1

* Lymphoid cell suspensions reacted with particular C57 antiserum, followed by fluorescent goat anti-mouse γ_1/γ_2 serum.
† Mean values for three separate determinations with sera C57 anti-CBA spleen-1 and -2, C57 anti-BALB/c.

Tissue Distribution. Lymphoid cell suspensions from various sources were prepared from C57 or C57.Ig^e mice and tested with all three noncongenic anti-spleen cell sera. With spleen, lymph node, and Peyer's patch cells, a significant percentage of positive cells was found in C57.Ig^e mice (Table 2). Of particular significance was the failure of these three sera to detect any significant number of fluorescent cells in either bone marrow or thymus of C57.Ig^e mice.

Allelic Exclusion. Although these alloantisera contain reactivity to only one allele of the proposed *Ig-5* heavy chain locus, some indication that this locus is subject to allelic exclusion was found in a comparison of the percentage of fluorescent spleen cells from allotype homozygous (C57.Ig^e N14F3) or heterozygous (C57.Ig^e N19) mice of similar age: 32.6 ± 3.3% of cells were positive in homozygous spleen cell suspensions, and 13.1 ± 2.9% in heterozygotes. The intensity of fluorescence appeared similar on the positive cells from homozygotes or heterozygotes. Control (C57) cells were 3.5 ± 1.1% positive. These results suggest that allelic exclusion of the *Ig-5* locus occurs.

Independence of Membrane IgM and Ig-5^a Positive Immunoglobulin. As the average fraction of Ig-5^a positive cells in spleen is approximately 30% and that of IgM cells is 40% (Table 3), it is most likely that a considerable proportion of B lymphocytes in spleen express both of these Ig molecules on their cell surface. To determine whether these molecules are independent, blocking studies were performed with rabbit anti-Ig sera prior to testing for the presence of μ chains or Ig-5^a positive Ig. Two rabbit antisera were used that have been characterised by surface iodination studies (Fig. 4). Serum R647 bound only μ chains and serum R640 contained antibodies to μ and light chains. Both antisera considerably inhibited the subsequent binding of fluorescent anti-μ sera

Table 3. Inhibition of anti-Ig-5^a binding by heterologous anti-immunoglobulins

Pretreatment (37°, 2 hr)	Test reagent sera	Percent fluorescent cells	
		C57.Ig^e	C57
Normal rabbit serum	Fl-anti μ	38.1*	36.1*
Rabbit anti-μ (R647)	Fl-anti μ	6.4† (17%)	8.4† (23%)‡
Rabbit anti-K, μ (R640)	Fl-anti μ	3.3† (9%)	5.7† (16%)
Normal rabbit serum	Anti-Ig-5^a, § Fl-anti γ_1/γ_2	26.2	2.4
Rabbit anti-μ (R647)	Anti-Ig-5^a, Fl-anti γ_1/γ_2	32.1 (>100%)	1.5
Rabbit anti-K, μ (R640)	Anti-Ig-5^a, Fl-anti γ_1/γ_2	2.8 (11%)	1.5

Fl = fluorescein conjugated.
* Nineteen and 14%, respectively, of positive cells with fluorescent caps.
† Sixty to 90% of remaining positive cells had fluorescent caps.
‡ Percent of relevant control value shown in parentheses.
§ C57 anti CBA spleen-1 serum.

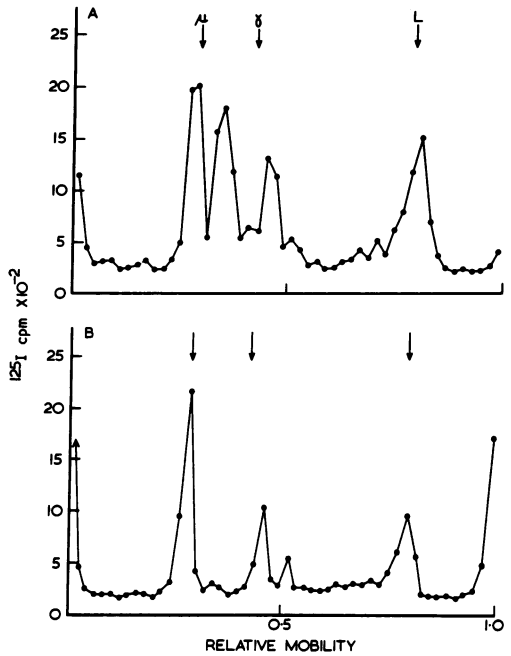

FIG. 4. NaDodSO$_4$-PAGE analysis of ^{125}I-labeled CBA splenocyte membrane proteins recognised by rabbit anti-mouse immunoglobulin sera. (4A) Material bound by rabbit anti-mouse IgM (R640) which contained antibodies to μ and light chains. (4B) Material bound by rabbit anti-mouse μ chain (R647). Gel conditions as for Fig. 2.

with spleen cells of C57 or C57.Ig^e origin (Table 3). However, only the anti-light chain containing serum inhibited the binding of anti-Ig-5^a antibodies. Hence, it is proposed that the Ig-5^a bearing molecules are independent on the cell membrane from IgM molecules.

DISCUSSION

Evidence has been presented that murine alloantisera to spleen cells can recognise, besides H-2 and Ia determinants, Ig molecules on the surface of lymphoid cells. This cell surface Ig is of a class which has not yet been unequivocally identified. Our studies indicate that it bears a unique alloantigenic determinant, determined by an allele which we tentatively name *Ig-5^a*, this locus being linked to other immunoglobulin heavy chain genes.

As this present study has not yet involved a complete characterisation of the isolated chain, several possibilities of the *Ig-5^a* gene product might be considered. (*i*) A non-immunoglobulin molecule: negated by the Ig depletion experiments and blocking of fluorescence with anti-light chain sera. (*ii*) K or λ light chains: negated by failure of alloantiserum to precipitate in radioimmunoassay any K or λ myeloma protein, and positive cell frequency in immunofluorescence less than with anti-K and greater than expected for anti-λ. In all species tested, light chain and heavy chain genes are unlinked. Definitive elimination of the possible presence of a new *unique* light chain associated only with the IgD-like molecule will require chain isolation studies. (*iii*) γ or α chains: negated by failure of alloantisera to precipitate myeloma proteins, lack of reactivity with anti-α, or -γ (3), and negative results with known allotype antisera. (*iv*) μ chain: alloantisera do not precipitate serum IgM and frequency of cells is less than that of μ positive cells. Anti-μ specific serum does not inhibit anti Ig-5^a binding. (*v*) δ chain: our data clearly indicate that the Ig-5^a antigen is expressed on the Ig chain previously described to be the murine homolog of human δ chain. Our results show that this chain is coded for by a new Ig heavy chain gene, but do not in themselves add anything further to the debate as to whether this should be termed the murine IgD molecule.

The description of the *Ig-5* locus may permit the production of monospecific antisera for the IgD-like molecule on murine splenocytes. Attempts to generate such antisera using cross immunisations of allotype congenic mice with lymphoid cells, and to identify the complementary *Ig-5* allele, are in progress. Studies on the possible biological effects of antisera to the Ig-5^a antigens are being presently assessed.

These studies raise several other issues. The heavy chain of immunoglobulin isolated from murine T cells is distinct from μ chain in its electrophoretic mobility (5) and possibly differs from μ chain antigenically (12). The question of whether or not this molecule bears Ig-5 determinants is being examined. The present data indicate that caution must be exercised in the interpretation of many experiments involving mouse alloantisera. For example, noncongenic antisera to H-2, Thy-1, and Ly-4 may contain antibodies to products of the *Ig-5* locus.

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