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Immunoglobulin Isoantigens (Allotypes) in the Mouse

V. Characterization of IgM Allotypes

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Abstract. Murine antisera raised against allogeneic lymphoid cells often contain antibodies to IgM allotypes. Rarely, allotypic antibodies to IgM have been found after immunization with *B. pertussis* anti-*B. pertussis* conjugates. Using both types of antibodies, we have defined a new constant-region locus for both secreted and membrane-bound μ chains. This locus, *Ig-6*, is closely linked to the previously described H-chain constant-region loci (*Ig-1* through *Ig-5*) and is subject to allelic exclusion. We have identified three alleles and four antigenic specificities of *Ig-6*.

Introduction

Allotypic variation in the immunoglobulin heavy chains of the mouse reflects genetic polymorphism of a series of linked genetic loci that code for these polypeptides. Genetic polymorphism in six of these heavy-chain classes has been reported: Ig-1 (IgG_{2a}), Ig-2 (IgA), Ig-3 (IgG_{2b}), Ig-4 (IgG_1), Ig-5 (IgD), and Ig-6 (IgM) (Herzenberg and Herzenberg 1978, Goding *et al.* 1976, Warner *et al.* 1977). Following the nomenclature of Herzenberg and Herzenberg (1978), the allelic forms for these genes are assigned according to the *Ig-1* allele of the type strains in which they are found and are designated by small letters (i.e., *Ig-1*^{a}, *Ig-1*^{b}, etc.). These genes are closely linked to each other, since no recombinations have been observed in extensive backcross studies (Herzenberg *et al.* 1965, Herzenberg and Herzenberg 1978, Potter and Lieberman 1967).

In this paper we extend our genetic and serologic analysis of the IgM allotypes. These allotypes are expressed on both the monomeric (8S) form of IgM on the surface of lymphocytes, and in the pentameric (19S) form found in serum. Three alleles have been identified for the *Ig-6* locus (termed *Ig-6*^{a}, *Ig-6*^{b}, and *Ig-6*^{e}) and involve at least four antigenically distinct specificities.

The anti-IgM activity in allotype sera raised by two different methods was detected by immunofluorescence, SDS polyacrylamide gel electrophoresis (PAGE), and direct radioimmune precipitation of myeloma proteins. The first type of antiserum was prepared against cell-surface IgM by immunizing mice with spleen

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cells from animals differing in both *H-2* and allotype. These complex antisera were tested on cells from animals that were allotype congenic with the serum producers so that the antibodies recognizing the *H-2* complex, as well as other gene products, would not react with the target cells. The second type of anti-Ig-6 serum was made against secreted IgM. A small minority of 'conventional' antiallotype sera raised against *B. pertussis* anti-*B. pertussis* complexes (Herzenberg and Warner 1968) were found to recognize IgM allotypes. This paper describes the specificity of these two types of sera towards IgM, and the cross-reactivities between the *a*, *b* and *e* alleles. Preliminary reports of this work have been published previously (Warner *et al.* 1977, Herzenberg *et al.* 1976).

Materials and Methods

Mouse Strains. The strains of mice used in this study are shown in Table 1. The BALB/cN, BAB/14, CSW, CWB, SJL, and SJA animals were raised at Stanford University while the C57BL/6, C57BL/6.*Ig*^{e}, ASW, NZB, and CBA animals were raised at the Walter and Eliza Hall Institute, Melbourne, Australia. The allotype congenic strains were produced by repeated backcrossing to the parental strain with selection for the allotype being introduced. The Ig^b allotype in BAB originated from C57BL/Ka and was backcrossed 14 generations with BALB/cN prior to inbreeding the animals. C57BL/6.*Ig*^{e} received the Ig^e allotype from NZB and underwent 14 backcross generations with C57BL/6 prior to inbreeding. The SJA animals obtained the Ig^a allotype from BALB/cN and underwent nine backcross generations before inbreeding.

Antisera Production. In the production of most antiallotype sera, animals were injected with 10^7 spleen cells in saline from donor animals differing in both *H-2* and allotype. After four weekly injections, the animals were bled weekly and boosted with 10^7 spleen cells monthly. The strain combinations used to produce antisera include SJL anti-BALB/c, SJA anti-BAB/14, and SJA anti-CWB. One set of antiallotype sera were made by immunizing C57BL/6 mice with spleen cells from CBA/H mice using complete Freund's adjuvant for the first injection as previously described (Goding *et al.* 1976). Conventional allotype sera were prepared by immunization with antibody-coated *B. pertussis* organisms (Herzenberg and Warner 1968).

The goat antimouse IgM (G-anti-MIgM) was prepared by injecting purified MOPC-104E into a goat. This serum was absorbed on Sepharose coupled myeloma proteins S-8 (IgA) and GPC-8 (IgG_{2a}) to remove activities directed against these proteins. By radioimmune precipitation this serum reacted only with IgM and not with IgA or IgG_1, IgG_{2a}, or IgG_{2b}. This serum precipitated only IgM from cell lysates as detected by PAGE. The G-anti-MIgG was a mixture of three sera prepared against the myeloma proteins RPC-5, MPC-11, and MOPC-21. These sera were absorbed to remove activity to other heavy chain classes and shown by radioimmune assay to react only with the class of

Table 1. Mouse Strains Used

Strain	Allotype	H-2	Allotype-Congenic Partner
BALB/cN	Ig^a	*d*	BAB/14
BAB/14 (BALB/cN.*Ig*^{b})	Ig^b	*d*	BALB/c
C57BL6	Ig^b	*b*	C57BL/6.*Ig*^{e}
C57BL/6.*Ig*^{e}	Ig^e	*b*	C57BL/6
SJL	Ig^b	*s*	SJA
SJA (SJL.*Ig*^{a})	Ig^a	*s*	SJL
CBA	Ig^a	*k*	
CSW (C3H.SW)	Ig^a	*b*	CWB
CWB (C3H.SW.*Ig*^{b})	Ig^b	*b*	CSW
ASW	Ig^e	*s*	
NZB	Ig^e	*d*	

immunoglobulin to which they were raised. The rabbit antimouse Ig (R-anti-MIg) was prepared by injecting rabbits with a mixture of purified myeloma proteins MPC-31, GPC-8, MPC-11, MOPC-21, and MOPC-104E. This antiserum detects all classes of mouse immunoglobulins. The rabbit anti-κ (R-anti-κ) was prepared against normal mouse IgM purified by starch block electrophoresis and Sephadex G-200 filtration. It contained antibodies directed against both μ and κ chains. The rabbit antimouse IgM (R-anti-MIgM) was prepared in a similar manner and was extensively absorbed with polyclonal mouse IgG coupled to Sepharose. The sheep antimouse IgM was prepared in the same way as the R-anti-MIgM. goat antirabbit Ig (G-anti-RIg) was prepared by injecting the 45% saturated $(NH_4)_2SO_4$ precipitate of normal rabbit serum into a goat. The conjugation of these sera with fluorochromes followed the protocol of Cebra and Goldstein (1965). (In this paper 'F*' denotes an antiserum that is labeled with fluorescein while 'R*' denotes a serum labeled with rhodamine.) Normal mouse sera from DBA/2, AKR, A/J, RIII, and SEA/Gn were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Staining and Analysis of Fluorescence. Typical immunofluorescence staining followed the protocol of pelleting 2×10^6 splenic lymphocytes and resuspending them in 40 μl of phosphate buffered saline (PBS) containing 10% fetal calf serum (FCS) and 0.1% NaN_3. Two μl of alloanti-serum was added and the suspension incubated either at room temperature or on ice for 20 minutes. The staining suspension was layered on top of FCS and pelleted. The cells were resuspended in 40 μl of PBS containing F*G-anti-MIgG and incubated again for 20 minutes. The cells were then washed and the amount of fluorescence per cell obtained by quantitative analysis using a fluorescence-activated cell sorter (FACS) (Becton Dickinson FACS Division, Mountain View, California) (Loken and Herzenberg 1975). Only live cells as determined by their forward-angle light scattering characteristics (Loken and Herzenberg 1975) were analyzed for their fluorescence. Alternately, cell suspensions were stained as previously described (Warner *et al.* 1975) and analyzed for percentage of fluorescent cells by fluorescence microscopy.

Cell-surface immunoglobulins were removed from B lymphocytes by incubating the cells under conditions where the added antibodies would cap and strip. Cells were resuspended in an appropriate concentration of antiserum and incubated for 30 minutes at 37° C for an additional hour. The extent of immunoglobulin removal was determined by restaining the cells with the original antiserum and analyzing them on the FACS. Comparing the amount of stain on the cells before the stripping process to the amount of strain after stripping, it was determined that more than 90% of the total immunoglobulin was removed using a R-anti-MIg, while IgM was removed using G-anti-MIgM.

Gel Analysis. The outer membranes of spleen cells were radioiodinated with ^{125}I by the lactoperoxidase technique (Goding *et al.* 1975), and solubilized with the nonionic detergent Nonidet P-40 (Shell Chemicals) (Goding *et al.* 1976). After overnight dialysis against PBS, extracts were divided into 200 μl aliquots and centrifuged at 10,000 *g* for 20 minutes. For precipitation analysis, 1–2 μl of the appropriate antiserum were added to the supernates, and after 1 hour of incubation at room temperature, immune complexes were isolated by binding to *Staphylococcus aureus* bacteria (Kessler 1975). The staphylococci were washed three times, and the complexes eluted by boiling in SDS-sample buffer for 5 minutes. The eluate was analyzed by SDS-PAGE as previously described (Goding *et al.* 1975).

Radioimmune Precipitation. Radioimmune precipitation was carried out as previously described (Herzenberg and Warner 1968). Purified myeloma proteins were labeled with ^{125}I according to the protocol of Greenwood and coworkers (1963). To 50 μl of ^{125}I-labeled antigen (approximately 10 μCi/μg; 0.1 μg/ml in 0.05 M tris, pH 7.4, containing 3% bovine serum albumin) was added 50 μl of antiserum diluted in 0.05 M tris pH 7.4, containing 10% normal rabbit serum. The mixture was held at 37° C for 2 hours and at 4° C overnight, and then centrifuged at 15,000 *g* for 10 minutes. Fifty μl of the supernate were removed and counted. The percentage precipitation was calculated by reference to tubes containing antigen and diluent, but no antibody. In some experiments, a specific anti–γ-chain antibody [polymerized on albumin carrier with ethyl chloroformate (van der Loo *et al.* 1977)] was used to enhance the precipitation of the complexes. To assess the inhibition of precipitation, the antibody concentration that gave just less than maximal precipitation was chosen. One to five μl of inhibitor was added to 50 μl of ^{125}I-labeled antigen. After mixing, 50 μl diluted antiserum was added and precipitation carried out as above.

Proteins. Myeloma proteins used in this study include: MOPC-104E (IgM, λ of BALB/c origin); C.BPC112 (IgM, κ of C.B/20 (BALB/c.*Ig*^{b}) origin) kindly supplied by Dr. Michael Potter, NCI; HPC-

76 (IgM, κ of BALB/c origin); HPC-208 [IgM arising in (BALB/c × NZB)F_1]; HPC-82 [IgG_{2a} of (NZB × NZC)F_1 origin]; and HPC-149 (IgG_{2a} of BALB/c origin). These proteins were purified by combinations of zone electrophoresis, ion-exchange chromatography, euglobulin precipitation, and gel filtration. The purity of each protein was checked by SDS-PAGE and was estimated to be greater than 95%.

Immunoglobulins were coupled to Sepharose activated by CNBr using the technique of van der Loo and coworkers (1975). The antiallotype sera were absorbed by adding the antisera to these solid-phase proteins in a 5 : 1 v/v ratio. This suspension was incubated at room temperature for 30 minutes and the serum was collected from the mixture. Additional absorptions did not affect the staining of cells by these sera.

Lymphomas. Cells of several established tissue culture lines of B lymphomas were kindly provided by Dr. Alan Harris of the Walter and Eliza Hall Institute, Melbourne, Australia. The cell lines (with strain of origin and type) are as follows: WEHI-231 [(BALB/c × NZB)F_1, B lymphoma]; WEHI-259 [(BALB/c × NZB)F_1, B lymphoma]; WEHI-279 (NZC, B lymphoma); ABE-8 (BALB/c, pre-B lymphoma); WEHI-267 (BALB/c, Ig-secreting B lymphoma); and PU-5-IR (BALB/c, macrophage tumor).

Results

Antisera Recognize Allotype-Linked B-Cell–Surface Markers

Using indirect immunofluorescence, a serum raised in SJA mice against spleen cells from BAB [SJA anti-(BAB spleen)] reacted with approximately half of SJL spleen cells. The cells were stained with this serum followed by F*G-anti-MIgG as described in the 'Materials and Methods' section. The viable cells from this preparation, as detected by forward-angle light scattering (Loken and Herzenberg 1975), were analyzed for fluorescence using a FACS and the resulting histogram is presented in Figure 1. The fluorescence, expressed in terms of arbitrary fluorescein units, is linear so that a cell having 40 units of fluorescein has twice the number of fluorochromes as a cell expressing 20 units of fluorescein (Loken and Herzenberg 1975).

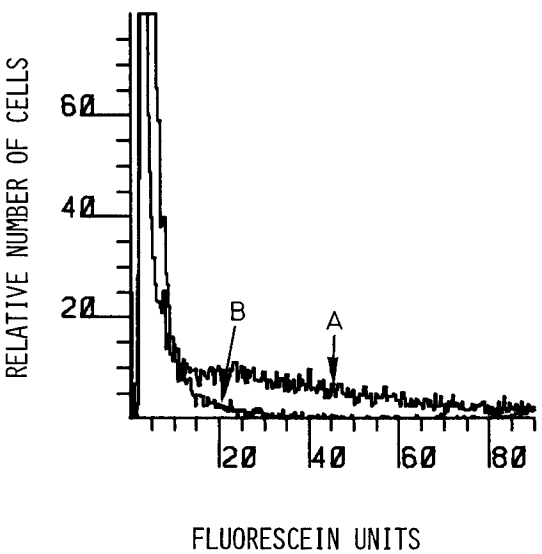

Fig. 1. Spleen cells from SJL (*A*) or SJA (*B*) animals were reacted with SJA anti-(BAB spleen) followed by F*G-anti-MIgG. Analysis of the fluorescence of live lymphocytes on the FACS indicates that this serum reacts only with SJL cells

The staining profile of the SJL cells is compared to the staining of SJA cells that are allotype congenic with SJL, using this SJA anti-(BAB spleen) serum (see Fig. 1). It is evident that this SJA anti-(BAB spleen) serum reacts only with SJL spleen cells and not with SJA cells, indicating that the determinant(s) is allotype linked. Approximately half of the SJL cells have fluorescence greater than 15 units, while the few SJA cells having this fluorescence are equivalent to the number of cells stained with the second step alone, i.e., the IgG-bearing cells in spleen. The proportion of cells expressing this allotype-linked cell-surface determinant is the same as the proportion of cells stained with a R-anti-MIg that detects all surface immunoglobulins. Approximately 50% of SJL spleen cells, 30% of lymph node cells, and 0% of thymocytes are stained using this SJA anti-(BAB spleen) serum.

The conclusion that this surface marker is indeed on B cells was made from double-staining experiments. SJL spleen cells were first stained with anti-μ (F*G-anti-MIgM) followed by SJA anti-(BAB spleen) and R*G-anti-MIgG. Examination of the double-labeled cells using fluorescence microscopy showed that those cells exhibiting green fluorescence also exhibited red fluorescence.

The immunoglobulin nature of the cell-surface determinant was determined by removing the surface Ig by capping and stripping. Spleen cells stripped of their immunoglobulin with R-anti-MIg could no longer be stained with additional R-anti-MIg followed by F*G-anti-RIg. These stripped cells could not be stained with the SJA anti-(BAB spleen) serum. Under these conditions neither Ia nor *H-2* was removed from cell surfaces. Similar data have been obtained with an SJL anti-(BALB spleen) that detects the a allotypic determinants when tested on SJA cells.

Further evidence for the immunoglobulin nature of these determinants was obtained by comparing the staining of SJA, SJL, and the allotype heterozygote (SJA × SJL)F_1. It was found that about half the B cells in the allotype-heterozygote animals were labeled with the antiallotype reagents. The histograms obtained when SJL, SJA, and (SJA × SJL)F_1 were stained with SJA anti-(BAB spleen) are shown in Figure 2. The reduced number of B cells labeled in the allotype-heterozygous animals with the antiallotype serum suggests that the determinant detected by this serum

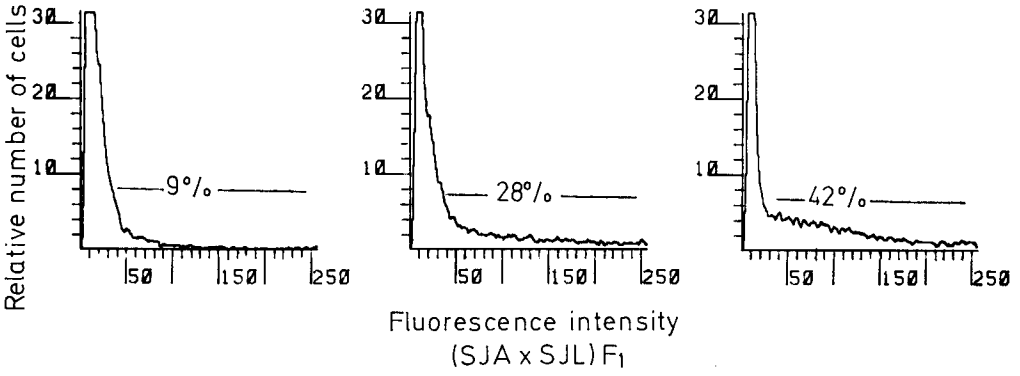

Fig. 2. Spleen cells from SJA, (SJA × SJL)F_1, and SJL mice were reacted with SJA anti-(BAB spleen) followed by F*G-anti-MIgG. The fluoresence profiles of live cells on the FACS demonstrates that the serum reacts with cells of the F_1 hybrid and SJL cells. The percentage of reacting cells is determined as the percentage of total cells with intensity greater than 30 fluorescence units. These values are shown for each population

undergoes allelic exclusion. The proportion of cells stained in the F_1 by this serum varied from animal to animal, ranging from 15% to greater than 30% in the spleen, even though the total number of B cells remained at approximately 50%. This may reflect individual variations of the relative proportion of each allotype expressed.

Alloantisera Detect Cell-Surface IgM

Although these alloantisera were expected to have anti-IgD allotype reactivity as reported by Goding and coworkers (1976), analysis showed that many of them also recognized allotypic determinants on IgM. Two such sera which react strongly with IgM, but not with IgD were selected for PAGE analysis. An SJA anti-(BAB spleen) serum and an SJA anti-(CWB spleen) serum were incubated with ^{125}I-labeled SJL spleen membrane lysates and the ensuing complexes precipitated with *S. aureus* bacteria. Complexes were solubilized, and the results of PAGE analysis are shown in Figure 3. It is evident that a peak of counts that migrates in these gels with the same mobility as IgM is specifically precipitated by this serum. In contrast, these sera when used with surface-iodinated SJA cells do not detect this component, and give a pattern identical to that obtained with normal SJA serum.

This reactivity was also detected using immunofluorescence. Using a G-anti-MIgM on SJL cells, the cell-surface IgM was removed by reacting the cells at 37° C for 60 minutes. The cells could no longer be stained with F*G-anti-MIgM indicating that all surface IgM was removed. The quantitative fluorescence analysis of SJL cells stained with an SJL anti-BAB serum before and after the stripping process is shown in Figure 4. Removing the IgM from the cell surface greatly reduces their staining by the alloantiserum. In a similar experiment, SJL cells were reacted with saturating amounts of G-anti-MIgM in the presence of azide to prevent capping. These cells also could not be stained with SJA anti-(BAB spleen) serum. Again, similar results

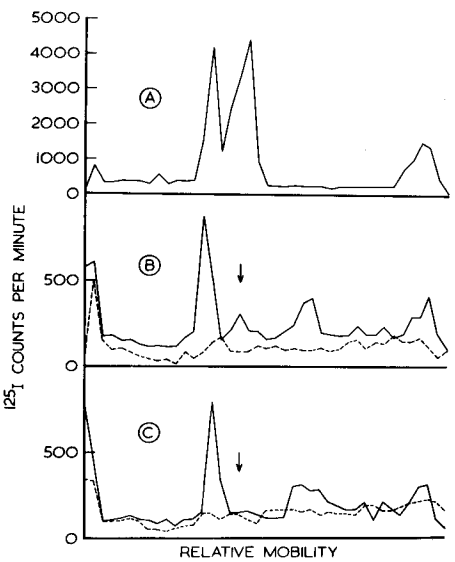

Fig. 3. SDS-PAGE analysis of ^{125}I-labeled C57BL/6 spleen-cell–surface antigens recognized by anti-immunoglobulin antisera: (*A*) Rabbit-antimouse κ (containing antibodies to both μ and κ chains); (*B*) Alloantiserum SJA-anti-(BAB spleen); (C) Alloantiserum SJA-anti-(CWB spleen). The *solid lines* are the experimental values, while the *broken lines* indicate the controls with no antiserum added. All gels contained 10 acrylamide with 0.25% bisacrylamide and all samples were reduced with 2-mercaptoethanol. The *arrow* marks the mobility of mouse δ chains of λ, γ and, L chains

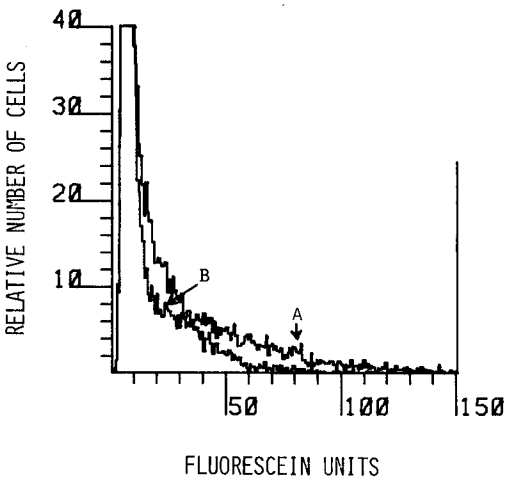

Fig. 4. FACS analysis of spleen cells from SJL stained with SJA-anti-(BAB spleen) followed by F*G-anti-MIgG: (*A*) Cells stained before IgM removal; (*B*) Cells stained after IgM removal. Cell-surface IgM was removed by reacting the cells with G-anti-MIgM under capping and stripping conditions

were obtained with the reciprocal SJL anti-(BALB spleen) serum when tested on SJA cells.

Antispleen Antisera Detect Secreted IgM

The alloantisera prepared against spleen-cell–surface IgM react not only with cell-surface IgM, but also with secreted IgM. Absorption of the SJA anti-(BAB spleen) serum with insolubilized IgM myeloma of the b allotype, C.BPC112, or with normal serum from b-allotype animals, removed the binding activity detectable by immunofluorescence (Fig. 5). None of the activity was removed when the serum was absorbed with BALB normal mouse serum. The activity of the reciprocal serum, SJL anti-(BALB spleen), was similarly removed by absorption with normal BALB serum, but not with normal C57BL/6 serum.

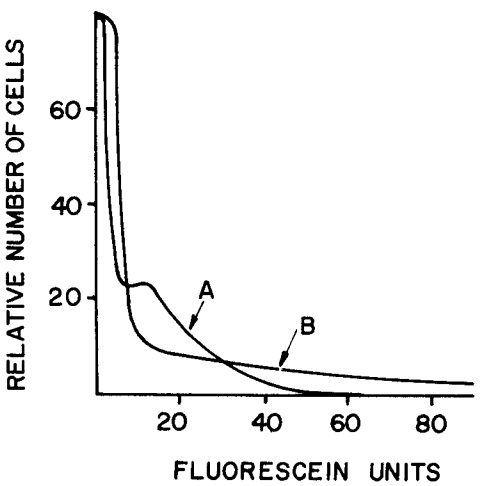

Fig. 5. FACS analysis of spleen cells from SJL mice reacted with SJA-anti-(BAB spleen) serum after (*A*) and before (*B*) absorption with Sepharose-coupled C.BPC112

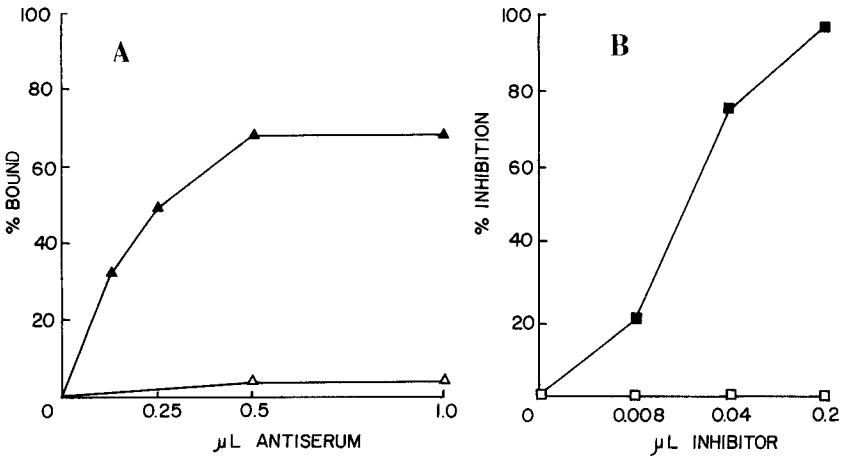

Fig 6. (*A*) Binding of ^{125}I-labeled IgM myelomas by SJA anti-BAB antiserum. Labeled antigen (1 ng) was incubated overnight with antiserum, and IgG precipitated by polymerized goat antimouse γ chain: ▲ = C.BPC112; △ = MOPC-104E. (*B*) Competition of serum proteins for the binding of Ig-6b (C.BPC112) to SJA anti-BAB serum: □ = Normal BALB serum (Ig^a); ■ = Normal C57BL serum (Ig^b)

The reactivity of various antispleen alloantisera with secreted IgM was also demonstrated by specific precipitation of radiolabeled IgM myelomas. SJA anti-(BAB spleen) serum could precipitate ^{125}I-labeled C.BPC112 myeloma protein (an IgM myeloma of the *b* haplotype), but not MOPC-104E (an IgM myeloma of the *a* haplotype) (Fig. 6). However, precipitation of C.BPC112 by this antiserum required a sandwich (G-anti-MIgG) step. Precipitation was haplotype specific, since it was inhibited by normal serum of the *b* haplotype (C57BL/10), but not by normal serum of the *a* haplotype (BALB/c) (Fig. 6). In the reciprocal direction, SJL anti-(BALB/c spleen) precipitated ^{125}I-labeled MOPC-104E, but not ^{125}I-labeled C.BPC112 (legend to Fig. 6). Similarly, C57BL/6 anti-(CBA spleen) serum *directly* precipitated ^{125}I-labeled HPC-76 (an IgM myeloma of the *a* haplotype) (Fig. 8), but not ^{125}I-labeled C.BPC112 (data not shown).

Anti-Ig-6 Sera Produced by Immunization with Complexes

In addition to raising alloantisera directed towards IgM by immunization of animals with whole spleen cells, anti-Ig-6a reactivity was found in some sera raised using traditional methods of antiallotype serum production, i.e., by injecting serum from CBA animals, which had been previously immunized with B. pertussis, into C57BL/6 animals in the form of *B. pertussis* anti-*B. pertussis* complexes (Herzenberg and Herzenberg 1978). These alloantisera, produced against secreted IgM, could also react with both cell surface IgM and secreted IgM, as demonstrated by PAGE, immunofluorescence, and radioimmune precipitation.

The PAGE analysis of ^{125}I-labeled CBA spleen cell lysates precipitated with C57BL/6 anti-(CBA serum) is shown in Figure 7. It is evident that this serum specifically precipitates IgM as shown by the presence of two chains that have the

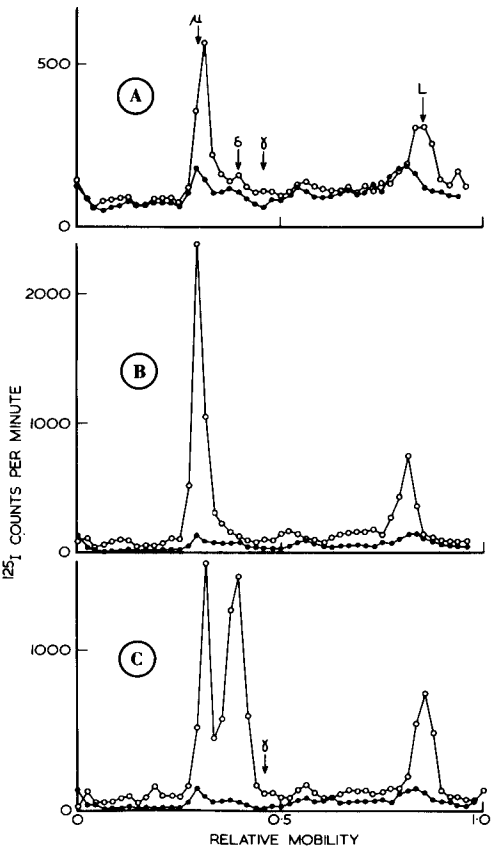

Fig. 7. SDS-PAGE analysis of ^{125}I-labeled CBA spleen-cell–surface antigens recognized by anti-immunoglobulin antisera. (*A*) C57BL/6 anti-(CBA serum) raised against *B. pertussis* anti-*B. pertussis* complexes (Serum G_1A2). (*B*) Rabbit-antimouse IgM antiserum containing anti–μ-chain antibodies only. (*C*) Rabbit-antimouse-κ (containing antibodies to both μ and κ chains): ○○○ = experimental; ●●● = control (no antiserum). All gels contained 10% acrylamide and 0.25% bisacrylamide and all samples were reduced with 2-mercaptoethanol. *Arrows* indicate mobilities of λ, δ, γ, and L chains

mobility of μ and light chains. This precipitation can be compared to the center gel pattern in Figure 7, where a heterologous R-anti-MIgM is used in the precipitation. When a heterologous R-anti-κ reagent is used, a third peak is observed, having the mobility of δ chain. Similar data have been obtained with the iodinated cell-surface proteins of a monoclonal B-cell lymphoma, WEHI-231, which has been shown to synthesize and bear membrane immunoglobulin of only IgM, and not IgD type (Gutman *et al.*, manuscript in preparation).

The reactivity of another C57BL/6 anti-(CBA serum) towards cell-surface IgM was tested by indirect immunofluorescence using F*G-anti-MIgG as a second step. This serum reacted with spleen cells from animals expressing the a allotype, but not with cells expressing the b allotype (Table 2). Staining of a-allotype spleen cells was blocked by preincubation of the cells with heterologous R-anti-MIgM. Thymus cells from BALB/c animals did not stain with these reagents.

Several lymphoma cell lines derived from Ig^a-type mice were also tested by immunofluorescence using these sera (Table 3). Only those tumor cells that express surface IgM (WEHI-231, WEHI-259, and WEHI-279) were stained with the C57BL/6 anti-(CBA serum). Lymphoma cells that exhibit κ, but not μ chains [WEHI-267 (IgA) and ABE-8(κ)] did not stain with this reagent. A macrophage cell line, PU-5-IR, originating in BALB/c, also could not be stained.

Table 2. Strain Distribution of Ig-6[a] Positive Cells

Strain	Ig Allotype	Fluorescent Cells[a] (%)
BALB/c	a	24.6
CBA/H	a	25.9
NZC	a	32.1
C57BL	b	<4
SJL	b	<4
NZB	e	<4
C57BL.*Ig*[e]	e	10.0[b]

[a] Immunofluorescence with C57 anti-CBA (GA-20) and fluorescent goat antimouse γ_1/γ_2
[b] Very weak reaction

Table 3. Reaction of Murine Lymphoid Cell Lines with Anti-Ig-6a Sera

Tumor Line	Cell Type	Type of Membrane Ig	Reactivity with α-Ig-6a[a]
WEHI-231	B lymphoma	IgM	>90
WEHI-259	B lymphoma	IgM	>90
WEHI-279	B lymphoma	IgM	>90
ABE-8	Pre B lymphoma	κ[b]	<1
WEHI-267	B lymphoma	IgA	<1
PU-5-IR	Macrophage	nil	<1

[a] Percentage of cells showing strong immunofluorescence after treatment with C57 anti-(CBA serum), (GA-20) antiserum, and F*R-anti-MIgG. Control cells treated with normal mouse serum and F*R-anti-MIgG showed no reactivity
[b] Cell-surface immunoglobulin present in extremely small amounts and κ-chain reactivity only detected

Like the sera raised against cell-surface IgM, the sera prepared by immunization with antigen-antibody complexes react with allotypic determinants on secreted IgM. Figure 8 shows the ability of two sera prepared by immunization with complexes and one serum prepared by immunization with cell-surface IgM to precipitate radiolabeled myeloma proteins. Two different C57BL/6 anti-(CBA sera), GA-20 and GA-26, precipitate ^{125}I-labeled HPC-149, an Ig-1a myeloma, whereas only GA-20 and the C57BL/6 anti-(CBA spleen) serum, (MA-3), precipitate HPC-76. These results show that both GA-20 and GA-26 have anti-Ig-1a activity, but only GA-20 also has anti-Ig-6a activity. The serum MA-3 has only the anti-Ig-6a but not anti-Ig-1a reactivity.

The difference between GA-20 and GA-26 shows that although the two sera were prepared in the same manner, they have different reactivities. Representative results from screening of selected sera are presented in Table 4, and indicate that most sera prepared against complexed immunoglobulins do not contain anti-Ig-6a antibody, although all of the sera are able to precipitate an IgG_{2a} myeloma. To date, none of the CBA anti-(C57BL/6 serum) (reciprocal antisera which have anti-Ig-1b activity) have been found to have anti-Ig-6b antibody.

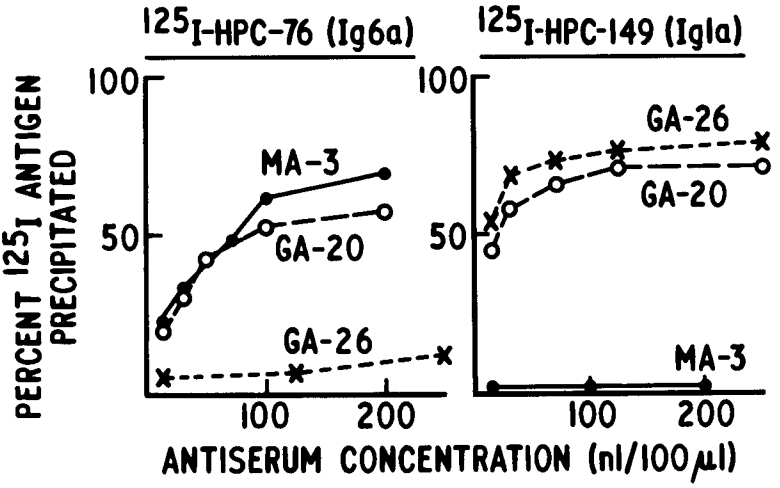

Fig. 8. Radioimmune precipitation of ^{125}Iodinated IgM (HPC-76) and IgG (HPC-149) by alloantisera. Sera GA-20 and GA-26 were raised in C57BL/6 animals against *B. pertussis* anti-*B. pertussis* complexes made from CBA serum. MA-3 is a C57BL/6 anti-(CBA spleen) serum

Table 4. Correlation of Radioimmune Assay and Fluorescence Analysis of Anti-allotype Sera

Serum Pool	Precipitation in Radioimmune Assay[a]		Fluorescent Reaction[b]	
	HPC-76 (Ig-6[a])	(HPC-82 (Ig-1[a])	WEHI-231	BALB/c Spleen
GA-7	++	+	++	++
G1A-2	++	++	++	++
GA-20	++	++	++	++
GA-21	++	++	++	++
GA-27	±	++	±	nt[c]
GA-28	±	++	±	±
GA-4	–	++	–	–
GA-6	–	++	–	–
G1A-5	±	++	–	nt
GA-25	–	++	–	nt
GA-26	–	++	–	–
GA-15	–	++	–	–
GA-16	–	++	–	–
GA-17	–	++	–	–

[a] ++ = >50% at 1:100; ± = 10–49% at 1:100
[b] ++ = >20% positive cells; ± = 6–20%; (–) = values <6%
[c] nt = not tested

Definition of IgM Allotype Determinants

The cross-reactions between IgM allotypes of different mouse strains were tested using the alloantisera produced by both types of immunizations. The results obtained to date have defined four specificities as follows:

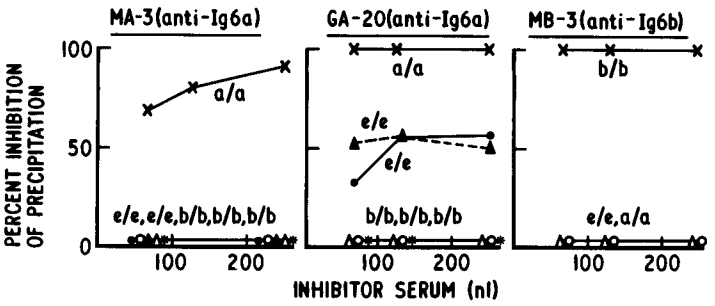

Fig. 9. Radioimmune analysis of the cross-reactivities between allotypic determinants. ^{125}I-labeled HPC-76 was precipitated by either MA-3 [C57BL/6 anti-(CBA spleen)] or GA-20 [C57BL/6 anti-(CBA serum)]. ^{125}I-labeled C.BPC112 was precipitated by MB-3 [ASW anti-C57BL/6 spleen)]. The addition of normal serum from BALB/cN (a/a); BALB/c.*Ig*^{b}, NZB.*Ig*^{b}, and C57BL/6 (b/b); or NZB and C57.*Ig*^{e} (e/e) was used to inhibit the precipitation of the myeloma proteins

Ig-6.1 The cross-reactions between the a, b, and e allotypes were investigated by the inhibition of precipitation method (Herzenberg and Herzenberg 1978) using HPC-76–iodinated IgM (BALB-c derived) with C57 anti-(CBA spleen) antiserum MA-3, and C57 anti-(CBA serum) antiserum GA-20 (Fig. 9). The results shown in Figure 9a involve antiserum MA-3 and inhibition with whole serum from strains BALB/c (a/a), NZB and C57.*Ig*^{e} (e/e), and C57, BALB/c.*Ig*^{b}, and NZB.*Ig*^{b} (b/b). Only Ig-6a–derived serum shows inhibition of precipitation defining specificity Ig-6.1 as follows: Ig-6.1—a: positive, b: negative; and e: negative.

Ig-6.2 The results with the same iodinated HPC-76 antigen and inhibitor sera, but with antiserum GA-20, are shown in Figure 9b. Again, BALB/c serum completely inhibits, but in this instance both e-allotype–derived sera partially inhibit, indicating the presence of at least two specificities, one common to *a* and *e* allelic products, and the other present only on a-type IgM. On a *minimal definition* of specificities, the latter is assumed to be 6.1, and the former is now defined as 6.2 (Ig-6.2—a: positive; b: negative; and e: positive).

Ig-6.3 In studies on different strain combinations used in attempts to raise anti-IgM antisera, several antisera were produced in e-allotype recipients against b-allotype–derived donor spleen cells. These particularly included ASW anti-(C57BL/6 spleen) (serum MB-3), and A.TL anti-(C57BL/6 spleen). The results shown in Figure 9c demonstrate that serum from b-allotype mice were fully capable of inhibiting this reaction using C.BPC-112 ^{125}I-labeled myeloma, whereas sera from both a- and e-allotype mice could not do so. This reaction thus defines a different specificity termed 6.3 (Ig-6.3—a: negative; b: positive; and e: negative).

Ig-6.4 Several antisera were raised by immunization of a-allotype mice with spleen cells from b-allotype donors, and were also tested by inhibition of precipitation reactions with ^{125}I-labeled C.BPC-112. The results shown in Table 5 demonstrate

Table 5. Cross-Reactions Between Allotypes Detected by Anti-Ig-6[b] Inhibition of Binding of ^{125}I C.BPC112 to SJA Anti-BAB[a]

Experiment Number	Strain	Ig Allotype	Inhibition[b] (%)
1	BALB/c	a	0
2	CBA	a	0
2	CSW	a	0
1	C57BL/6	b	100
1	SJL	b	90
1	DBA/2	c	0
1	AKR	d	100
2	AKR	d	100
1	A/J	e	100
2	A/J	e	65
2	NZB	e	100
2	CE/J	f	0
2	RIII	g	0
1	SEA/Gn	h	0

[a] One μl of normal mouse serum was added to inhibit the precipitation. Excess of G-anti-MIgM bound 80% of the counts of the labeled C.BPC112, whereas 70% bound to saturating amounts of SJA anti-BAB antiserum. In Experiment No. 1 60% of the counts were specifically bound to MB-1, SJA anti-(BAB spleen) in the absence of inhibitor while in Experiment No. 2 40% of the counts were specifically bound to MB-1

[b] Figures given are $\pm 5\%$

Table 6. IgM Allotypic Antigenic Specificities

Strain Type	Allele	*Ig-6* Locus
BALB/c	*a*	1 2 – –
C57BL/6	*b*	– – 3 4
NZB	*e*	– 2 – 4

that sera from mouse strains of allotype b, d, or e could completely inhibit precipitation, whereas all other sera tested did not show any inhibition under the conditions used. Since this specificity is present on both b and e types, it cannot be 6.3, and hence represents a fourth specificity termed 6.4 (Ig-6.4—a, c, f, g, and h: negative; b, d, and e: positive).

Based on these results, four allotypic specificities of IgM molecules have thus been identified, and permit the distinction of three different alleles of the *Ig-6* locus. This is shown in Table 6, where the same type strains and allelic designation are used as for the *Ig-1* locus (Herzenberg and Herzenberg 1978).

Expression of Ig-6 Determinants on Myeloma Proteins

The partial inhibition of precipitation of iodinated HPC-76 (Ig-6a) by serum from Ig^e mice (Fig. 9b) with antiserum GA-20, is due to the detection of both specificities 6.1

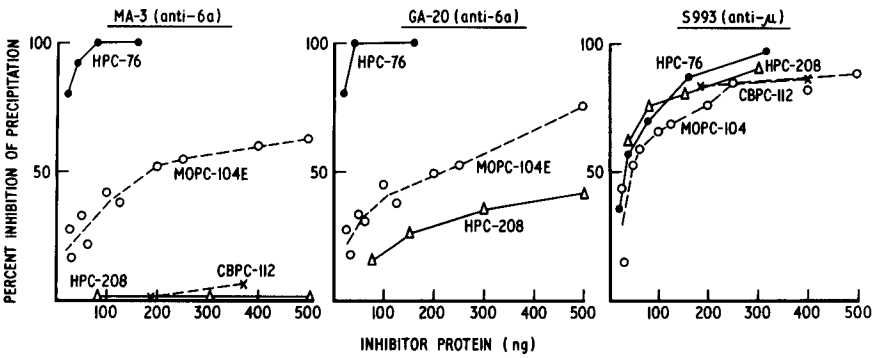

Fig. 10. Analysis of Ig-6 specificities on different myeloma proteins by radioimmunoprecipitation. ^{125}I-labeled HPC-76 was precipitated with MA-3 [C57BL/6 anti-(CBA spleen)], GA-20 [C57BL/6 anti-(CBA serum)], or sheep-antimouse IgM (S993). Graded amounts of purified IgM myeloma proteins were added to inhibit these precipitations. The proteins and their strain of origin (and allotype) are: HPC-76 and MOPC-104E, BALB/c (Ig-6a); HPC-208 (BALB/c × NZB)F_1 Ig-6^e; and C.BPC112, BALB/c.*Ig*^{b} (Ig-6b)

and 6.2 on HPC-76, whereas the e allelic product lacks 6.1. It was therefore of interest to determine whether an IgM myeloma HPC-208 that arose in a (BALB/c × NZB)F_1 mouse was of Ig-6a or Ig-6e type. The HPC-208 protein gave only partial inhibition of precipitation of HPC-76 by serum GA-20, and no inhibition of precipitation of HPC-76 with antiserum MA-3 (Fig. 10). In this respect, HPC-208 therefore behaved in exactly the same fashion as serum from NZB mice (Fig. 9). It is thus likely that HPC-208 inherited expression of the NZB (*Ig*^{e}) parental allele rather than the BALB/c (*Ig*^{a}) allele, and is consistent with allelic exclusion in IgM secreting plasma cell tumors, as in murine IgG-secreting plasmacytomas (Herzenberg and Warner 1968).

It is also of note that HPC-76 and MOPC-104E are different in their expression of Ig-6a determinants, as shown in Fig. 10a. The precipitation of ^{125}I-HPC-76 by MA-3 is only partially inhibited by MOPC-104E. Similarly, MOPC-104E caused only weak and partial inhibition of precipitation of HPC-76 by serum GA-20 (Fig. 10b). It is clear, however, from the results shown in Fig. 10c, that HPC-76, HPC-208, C.BPC112, and MOPC-104E are all capable of completely inhibiting the precipitation of HPC-76 by heterologous (sheep) anti-μ.

The reason for the difference in behavior between MOPC-104E and HPC-76 in radioimmunoassay is not clear. Unlike the μ determinants detected by heterologous antisera, at least some of the Ig-6 allotypic determinants are relatively labile, and are easily destroyed by heat, acid, and thiocyanate (Warner, manuscript in preparation). It is possible that the MOPC-104E protein used was in some way defective in allotype expression for this reason, although all batches (from several different sources) showed the same effect. Whatever the reason, the use of MOPC-104E for testing anti-Ig-6a activity of various sera may lead to variable results.

Discussion

The existence of allotypic markers on both secreted and cell-surface IgM in mice has been demonstrated in this paper by several different procedures using alloantisera

raised against both cell-surface and secreted IgM. Both types of alloantisera react with cell-surface IgM, as shown by PAGE analysis and quantitative immunofluorescence, and also react with secreted IgM, as shown by serum absorption and by the direct precipitation of radiolabeled myeloma proteins.

The most effective method of producing antiserum against IgM allotypes was by spleen-cell immunization between strain combinations differing in H-2 type as well as in allotype. These sera may potentially have many reactivities to cell-surface components, so that tests for cell-surface IgM were performed in a manner that detected only anti-Ig activity, by utilizing cells from allotype-congenic strains. Under these conditions, the reactivity of these alloantisera with splenic B cells from the congenic partner of the antiserum produced indicates that the determinants identified by these sera are controlled by genes in the heavy-chain–locus region. These determinants are expressed on B cells, as shown by double-staining with a heterologous anti-IgM, and *are* immunoglobulin in nature, as shown by the removal of allotype staining by stripping the cells of their surface immunoglobulin and by other immunochemical procedures.

Since there are two major classes of Ig on cell surfaces (Vitetta and Uhr 1975), the reactivity toward IgM must be distinguished from reactivity to IgD. Although the PAGE of spleen-cell lysates precipitated with alloantisera showed only a μ-like rather than a δ-like heavy chain, this criterion of electrophoretic mobility by itself is insufficient proof that the serum identifies IgM allotypes (Sitia *et al.* 1977, Parkhouse and Cooper 1977). The evidence that these sera do recognize cell-surface IgM comes from the removal of the immunofluorescence staining by absorption with IgM myeloma proteins, removal of reactivity by stripping the cells with heterologous anti-IgM, blocking the staining with heterologous anti-IgM, and by reaction with cell-surface immunoglobulin of the B-lymphoma line WEHI-231 that possesses only IgM and not IgD. Reactivity towards secreted IgM was directly shown by direct radioimmune precipitation of IgM myeloma proteins, and by the removal with solid-phase serum globulins (Herzenberg *et al.* 1976) of antibodies responsible for cell-surface immunofluorescent staining. This latter approach also showed that the determinants recognized on the secreted molecules are the same as those detected on the cell surface.

It has been shown that sera prepared by spleen-cell immunization can also react with cell-surface IgD (Goding *et al.* 1976). In testing sera produced in this manner, both anti-IgD and anti-IgM reactivity could be detected. Varying amounts of the two reactivities could be found in sera from individual animals. These two reactivities were distinguished by the ability of the sera to be absorbed by solid-phase myeloma proteins. Some sera lost almost all of their reactivity against cell-surface determinants after absorbing them with IgM myeloma proteins of the appropriate allotype, and therefore were designated as having predominantly anti-IgM reactivity. Other sera lost almost none of their reactivity against cell-surface determinants and were predominantly anti-IgD in nature. Most sera, however, had both anti-IgM and anti-IgD reactivity and were made specific for IgD by absorption with IgM myeloma proteins.

Considerable variation was found in the ability of different mouse strains to produce anti-Ig-6 antibodies. The highest titer anti-Ig-6a serum was found when C57BL/6 animals were injected with CBA spleen cells or when SJL animals were injected with BALB/c spleen cells. Very little, if any, anti-Ig-6a activity was detected when B10.D2 mice were immunized with CBA cells or when B10.BR mice were immunized with either C3H or C3H.SW spleen cells. Anti-Ig-6b serum could be raised in CBA against C57BL/6 cells or in SJA against either BAB or CWB cells.

Neither the immunization of 129/J mice with B10.A cells or of C3H.SW mice with B10.BR cells produced detectable anti-Ig-6b activity. However, a different type of anti-Ig-6b serum was produced in e-allotype animals by injecting C57BL/6 cells into either A.TL or A.SW recipients. This reactivity was not detected in serum from C57BL.Ig^e animals immunized with SJL or B10.Br cells. Immunization between congenic mice, SJL and SJA, C57BL/6 and C57BL.Ig^e, as well as BALB and BAB, produced no serum with anti-Ig-6 activity. The production of anti-Ig-6 serum may require that the immunizing donor and the recipient have several genetic differences in addition to the immunoglobulin allotypes. The difference between strain combinations that will result in anti-IgM production and those that will not may be associated with *Ir* genetic control of antiallotype production as in the case of anti-IgA and anti-IgG antibody production (Lieberman *et al.* 1972) or may involve some cell-surface components acting as carrier molecules for the allotypic determinants.

Many of the problems encountered with the serum prepared by spleen-cell immunization with respect to multiple cell-surface reactivities were avoided using antiserum prepared by immunization with pertussis anti-pertussis complexes. Although these sera reacted with several classes of secreted immunoglobulins, they do not have reactivities against cell-surface markers such as H-2, and thus can be tested on cells from any mouse strain. The reactivity of these sera prepared against secreted IgM were similar to those prepared against cell-surface IgM, as demonstrated by all methods used.

Unfortunately, however, the production of anti-Ig-6 serum using antigen-antibody complexes was inconsistent and few of the immunized animals produced anti-Ig-6 in addition to anti-Ig-1 and anti-Ig-4. The reason why some animals responded to the immunization regime while most did not has not been determined. Only anti-Ig-6a serum was produced in this manner, since of more than 100 sera screened for anti-Ig-6b antibody, none were found to be positive.

A difference in the specificities of the sera raised against spleen cells and complexes was noted in studying the cross-reactivities between the various allotypes. Significant cross-reaction between the *Ig-6a* and *Ig-6b* alleles was demonstrated in an antiserum prepared against secreted IgM, while this cross-reaction was not observed using a corresponding antiserum against spleen cells (Fig. 9). The difference between these sera indicates that the serum raised against secreted IgM detects at least two antigenic determinants (6.1 and 6.2), whereas the serum raised against spleen cells detects only 6.1. From the available data we conclude that both specificities are expressed on secreted IgM, and that 6.1 is expressed on cell-surface IgM; however, it has not been determined whether 6.2 is also expressed on cell-surface IgM. Similarly, the antiserum detecting specificity 6.2 has been used only to demonstrate its presence on secreted IgM, and cell-surface expression is as yet undetermined. Specificity 6.4 detected by several antisera raised in a-allotype animals against spleen cells of b-allotype was shown to detect both secreted and cell-surface IgM, and was readily detected on both cell-surface and secreted IgM.

Various studies on the immunoglobulin heavy-chain genes have shown that these genes are extremely closely linked on the chromosome. No recombinants between the *Ig-1* and the other *Ig* loci have been discovered in about 3000 informative mice (Herzenberg and Herzenberg 1978, Potter and Lieberman 1967). The linkage of *Ig-1* and *Ig-6* is similarly quite strong. The testing of the inhibition of HPC-76 with sera from BALB/cN, BAB/14, BALB/c.Ig^b, and C57.Ig^e indicates that the *Ig-6b* and *Ig-1b* alleles and *Ig-6e* and *Ig-1e* alleles were inherited together. Since 14–20 backcross generations were involved in the derivation of these strains, close linkage is indicated for *Ig-6* and *Ig-1*.

The use of CWB cells to raise an anti-Ig-6b antiserum in SJA animals indicates that both the *Ig-1* and *Ig-6* alleles were inherited together in these strains. The derivation of these lines involved 23 backcross generations, so that no recombinants have been detected in the approximately 40 independent backcross generations that were involved in the production of these various congenic animals.

In view of recent data indicative of recombinations between the V_H and IgG_{2a} C_H genetic regions (Riblet *et al.* 1975), it will be of interest to determine whether these recombinations are indeed between V_H and C_H, rather than within C_H-region genes.

The present report further extends the range of defined genetic polymorphisms of murine immunoglobulin heavy-chain genes. It is perhaps ironic that a period of approximately 10 years has elapsed between the definition of the first four heavy-chain loci and the last two, both of which determine cell-surface immunoglobulins, which in turn are receiving considerable attention in relation to their roles as antigen receptors in the total scheme of B-cell differentiation. The availability of anti-Ig-5 and Ig-6 antisera should thus provide valuable tools for the further investigation of the cellular and genetic control of immunoglobulin synthesis (Goding *et al.* 1977, Goding 1978), and the methods used to derive these reagents will hopefully stimulate further investigations of a similar nature on human IgM and IgD.

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