

EVIDENCE FOR IDENTITY OR CLOSE ASSOCIATION OF THE Fc  
RECEPTOR OF B LYMPHOCYTES AND ALLOANTIGENS  
DETERMINED BY THE *I<sub>r</sub>* REGION OF  
THE *H-2* COMPLEX

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(Received for publication 10 June 1974)

Studies in several species, including mouse and man, have shown that B lymphocytes bear on their surface membranes a receptor (termed the Fc receptor) which specifically binds heat-aggregated or antigen-complexed immunoglobulin (complexed Ig<sup>1</sup>) (1-6). The receptor is distinct from lymphocyte surface Ig (2-4) and the C3 receptor (6), and appears to be a trypsin-resistant protein or glycoprotein (4). Binding to this receptor is apparently not dependent on complement (2, 3, 5, 6), pH, temperature, or divalent cations (3). The receptor is specific for a site on the Fc portion of the complexed immunoglobulin molecule (2, 4, 5), and the integrity of this site is apparently dependent on intact disulfide bond(s) (4). The receptor appears to play a role in antibody-dependent lymphocyte-mediated cytotoxicity (4).

Recent studies in this laboratory and elsewhere have uncovered a system of murine lymphocyte surface alloantigens which are determined by genes in the *I<sub>r</sub>* region of the *H-2* complex (7-11, footnote 2). These have been termed Ia (*I<sub>r</sub>*-region associated) antigens (12). Multiple antigenic determinants appear to be involved and at least some of these are shared by strains of mice which differ in *H-2* haplotype. Ia antigens appear by a variety of criteria to be expressed primarily on B lymphocytes (7, 11). They can be detected serologically in at least three ways: (a) by sera raised via immunizations between congenic-resistant inbred strains of mice which differ only in the *I<sub>r</sub>* region of the *H-2* complex (8-11); (b) by testing of anti-*H-2* antisera on strains which differ in *H-2* haplotype but which share Ia antigens (7); and (c) by absorbing anti-*H-2* antisera with T cells of the donor *H-2* haplotype, leaving activity for B-cell alloantigens.<sup>2</sup> Ia antigens appear to play a role as stimulators in mixed

<sup>1</sup> Abbreviations used in this paper: Agg, heat-aggregated human Ig; BSA-PBS, 2% bovine serum albumin in phosphate-buffered saline, pH 7.2 or 8.0, 0.02% Na Azide; EAC, sheep red blood cell-19S rabbit antishsheep red blood cell antibody-mouse complement complexes; FI, fluorescein isothiocyanate; Ig, immunoglobulin; PBS, phosphate-buffered saline, pH 7.2 or 8.0, 0.05 M PO<sub>4</sub> and 0.15 M NaCl; RAMIG, rabbit antimouse immunoglobulin; Rho, tetramethylrhodamine isothiocyanate.

<sup>2</sup> Sachs, D. H., and J. L. Cone. 1974. *I<sub>r</sub>*-associated mouse B-cell alloantigens: demonstration of multiple Ia specificities in *H-2* alloantisera following absorptions with T cells tumors. Manuscript submitted for publication.

lymphocyte cultures (13, 14). Isolation of Ia antigens using membrane solubilization techniques has indicated that they are proteins and/or glycoproteins of mol wt  $\sim 30,000$  (15, 16).

The purpose of the present experiments was to evaluate possible relationships between the Fc receptor and Ia antigens. Data from these studies indicate that the receptor for complexed Ig and alloantigens controlled by the *Ir* region of the *H-2* complex are identical or closely associated on the B-lymphocyte surface membrane.

#### Materials and Methods

*Animals.*—Adult male mice of all strains were used. Strains A.SW/Sn, A/J, AKR/J, C57BL/10Sn (B10), B10.A/SgSn, B10.D2/nSn, B10.BR/SgSn, C57BL/6 (B6), B10.A(2R), and (B6A)F<sub>1</sub> were purchased from Jackson Laboratories, Bar Harbor, Maine. Strain B10.A(4R) was obtained from our own breeding colony established from stock kindly provided by Dr. J. Stimpffling, The McLaughlin Institute, Great Falls, Mont. Mice of strain A.TL were the kind gift of Dr. D. C. Shreffler, Univ. of Michigan. The genetic compositions of the *H-2* complexes of these strains are listed in Table I.

*Preparation of Cells.*—A single cell suspension from spleen or thymus was prepared and red blood cells and their precursors, as well as granulocytic cells, were removed by density flotation as described by Boyum (17). The lymphocyte preparation was washed three times with 2% bovine serum albumin in phosphate-buffered saline, 0.02% Na Azide (BSA-PBS) pH 7.2 and then resuspended at  $20 \times 10^6$ /ml in BSA-PBS pH 7.2 or 8.0 as required (see below). Preparations contained >95% small lymphocytes by the criteria of morphology and phagocytosis of 1  $\mu$  polystyrene beads, and were >96% viable (trypan blue exclusion). Cells of lym-

TABLE I  
Origin of the *H-2* Haplotypes of the Mouse Strains Used\*

Strain	Haplotype map of <i>H-2</i> complex†			
	K	<i>Ir</i>	Ss	D
B6	b	b	b	b
B10	b	b	b	b
A/J	k	k	d	d
B10.A	k	k	d	d
B10.A(2R)	k	k	d	b
B10.A(4R)	k	k b	b	b
(B6A)F <sub>1</sub>	k/b	k/b	d/b	d/b
B10.D2	d	d	d	d
B10.BR	k	k	k	k
AKR	k	k	k	k
A.SW	s	s	s	s
A.TL	s	k	k	d

\* Adapted from the review by Shreffler and David.<sup>3</sup>

† Vertical lines indicate presumed crossover positions of recombinant haplotypes.

<sup>3</sup> Shreffler, D. C., and C. S. David. 1974. The *H-2* major histocompatibility complex and the I immune response region: genetic variation, function, and organization. Manuscript submitted for publication.

phomas EL4 and RDM 4, maintained in ascites form in B6 and AKR mice respectively, were obtained by paracentesis and further purified as described above.

*Antisera Used for the Detection of H-2 and Ia Antigens.*—Immunizations were carried out as previously described (7, footnote 2). The antisera used in this study were: B10.A anti-B10, B10 anti-B10.A, B10.A anti-B10.D2, B10.BR anti-B10.D2, B10.D2 anti-B10.BR, A/J anti-A.SW, and BN rat anti-B10.D2. Each of these antisera detected antigens controlled by the whole *H-2* complex (including Ia antigens) when tested on the donor strain (7, footnote 2). In order to specifically detect Ia antigens the antisera were tested on strains which differed in *H-2* haplotype but shared Ia specificities (7), or the antisera were absorbed with T cells of the donor *H-2* haplotype (either thymocytes or lymphoma cells of T-cell origin), leaving only activity against B-cell alloantigens.<sup>2</sup> All antisera were decomplemented by heating at 56°C for 30 min and were ultracentrifuged to remove material >10S as previously described (18) immediately before use. An Fab preparation from B10 BR anti-B10.D2 serum was prepared by trypsin digestion of the 7S fraction according to published methods (19). This preparation was judged free of residual intact IgG by chromatographic size on Sephadex G-200 and by complete loss of cytotoxic activity. The preparation contained minimal Fc by Ouchterlony double diffusion using rabbit antimouse Fab and rabbit antimouse Fc (kindly provided by Dr. R. Asofsky, NIH) as developing reagents.

*Other Antisera and Antigen-Antibody Complexes.*—Purified staphylococcal nuclease (nuclease) and mouse antibody to nuclease purified by affinity chromatography were prepared using methods previously described (20, 21). Goat antimouse Ig was prepared by immunizing a single goat with 1 mg of immunoelectrophoretically pure mouse Ig in complete Freund's adjuvant subcutaneously three times (2 wk apart). The goat was bled 1 wk after the last injection. Based on precipitin curves, antigen-antibody complexes were formed in antigen excess for each test by mixing 30  $\mu$ l of mouse antinuclease (1.8 mg/ml in normal saline) and 4  $\mu$ l of nuclease (1.0 mg/ml in normal saline), and incubating for 30 min at 23°C.

*Fluorescence.*—

*Surface Ig:* Fluorescein isothiocyanate (Fl) and tetramethylrhodamine isothiocyanate (Rho)-conjugated rabbit antimouse Ig (heavy and light chains) (RAMIG) were purchased from Cappel Laboratories, Downingtown, Pa. (lots no. 7282 and 7184). These antisera were unabsorbed and were tested for specificity by immunoelectrophoresis, Ouchterlony double diffusion, lack of reactivity with thymocytes, and inhibition of activity by purified mouse Ig. These antisera were routinely ultracentrifuged to remove material >10S as described (18) immediately before use. Equal amounts of lymphocytes ( $20 \times 10^6$ /ml in BSA-PBS pH 7.2) and RAMIG (Fl or Rho) (0.5 mg/ml in PBS 7.2) were mixed and incubated 30 min at 4°C. The cells were then washed three times with BSA-PBS pH 7.2, and wet mounts were prepared in the same medium. Slides were read under alternate ultraviolet and phase microscopy as previously described (4). A minimum of 200 small lymphocytes per preparation were evaluated. The criterion of fluorescence positivity was uniform punctate staining over the entire cell surface.

*Aggregated Ig binding:* Previous studies in mice have shown that Ig complexes prepared with immunoglobulin from other mammalian species or from mice bind equally well to mouse B lymphocytes (1, 22). Therefore, the assay utilizing Fl-conjugated heat-aggregated human Ig complexes (Fl-Agg) developed for humans and described in detail (4) was applied to mouse lymphocytes without modification. Briefly, equal amounts of Fl-Agg, or, as controls, deaggregated Fl-conjugated 7S human Ig (both at 1–2 mg/ml in PBS pH 8.0) and lymphocytes ( $20 \times 10^6$ /ml in BSA-PBS pH 8.0) were mixed and incubated for 30 min at 23°C. The cells were then washed three times and wet preparations were made, all in the same medium. Microscopy was performed as described for surface Ig. The criterion of positivity was three or more fluorescent complexes bound per cell.

*Inhibition studies:* For inhibition by antigen-antibody complexes, lymphocytes ( $25 \mu$ l at  $20 \times 10^6$ /ml in BSA-PBS pH 8.0) were combined with 34  $\mu$ l of mouse antinuclease-nuclease

complexes which had been formed in antigen excess (see above). In control tubes lymphocytes were mixed with equivalent amounts of either medium, mouse antinuclease alone, or nuclease alone. Preparations were mixed and incubated for 30 min at 23°C. The cells were then washed three times with BSA-PBS pH 8.0 and assayed for the binding of Fl-Agg as described above.

For inhibition by antisera, lymphocytes (25  $\mu$ l at  $20 \times 10^6$ /ml in BSA-PBS pH 7.2) were combined with 100  $\mu$ l of deaggregated antisera at various dilutions, or, as controls, normal mouse serum (from the same strain as the antiserum in each test), immune antiserum nonreactive with the test lymphocytes, or medium. Preparations were mixed and incubated for 30 min at 4°C. The cells were then washed three times with BSA-PBS pH 7.2 or 8.0 and assayed for surface Ig or aggregated Ig binding as described above. It should be noted that under these conditions F1RAMIG detected both surface Ig and bound antibody. All antisera were used at dilutions which were on the plateau of cytotoxic killing for the strain of cells being tested (7, footnote 2).

*C3 Rosettes.*—These were performed according to published methods (23) with reagents kindly provided by Dr. E. Jaffe, NIH.

#### RESULTS

*Binding of Ig Complexes to Mouse Lymphocytes.*—The binding of Fl-conjugated heat-aggregated human Ig (Fl-Agg) to mouse spleen lymphocytes of various strains is listed in Table II. In each animal tested the number of cells binding Ig complexes was approximately the same as the number which stained for surface Ig. The control experiments using deaggregated Fl-conjugated 7S human Ig were completely negative, indicating that the binding of the Fl-Agg was not due to either natural human antibody against mouse or to nonspecific binding of human Ig by some mouse lymphocytes. As is evident in Table II, different mice, even of the same strain, varied in the number of lymphocytes which bore surface Ig and bound Ig complexes, and both markers varied synchronously. This probably reflects normal variation in the number of splenic lymphocytes bearing these markers. Cells which bound aggregates were heterogeneous in the amount bound, which is analogous to the case in humans (3).

In order to determine whether the aggregated human Ig was binding to the same sites as bind mouse antibody-antigen complexes, B10 spleen lymphocytes were incubated with mouse antinuclease-nuclease complexes, washed thoroughly, and assayed for the binding of Fl-Agg. The lymphocytes which had been incubated with mouse antibody-antigen complexes were almost completely inhibited in their ability to bind Fl-Agg (4.5% positive vs. 48.5% positive for lymphocytes incubated in medium alone). In contrast, cells incubated with antigen or antibody alone were unaffected (49.0% and 46.5% positive, respectively). Thus it appeared that heat-aggregated human Ig complexes bind to the same sites which bind mouse antibody-antigen complexes. Previous studies have shown that the binding of Ig complexes requires the Fc portion of the Ig molecule (2, 4, 5, 22) and the site is termed the Fc receptor.

The above results suggested that the Ig complexes were binding to B cells. To further evaluate this system, B10 thymocytes were tested for their ability to bind these complexes, and B10 spleen lymphocytes were double labeled with

TABLE II  
*Binding of Ig Complexes to Mouse Spleen Lymphocytes*

Strain	Animal no.	Surface Ig	Aggregated Ig binding*
			% Positive
B6	1	42.5	38.5
	2	40.7	41.3
B10	1	42.0	42.5
	2	47.5	46.0
B10.A	1	48.0	46.0
	2	38.0	39.0
B10.A(2R)	1	46.0	44.5
	2	51.0	48.5
B10.A(4R)	1	54.0	56.0
B10.D2	1	42.5	44.5
	2	40.0	38.5
B10.BR	1	47.5	51.5
	2	44.5	42.0
(B6A)F <sub>1</sub>	1	57.0	58.0
A/J	1	48.0	49.5
A.TL	1	53.0	52.5
	2	47.0	46.0
A.SW	1	49.0	46.0
	2	40.5	42.5

\* Control experiments with deaggregated FI-conjugated 7S human Ig always showed <0.5% positive cells.

RhoRAMIG and FI-Agg. Less than 2% of thymocytes bound aggregates (compared with 53.0% in the spleen of the same animal), and the surface Ig-bearing and aggregate-binding lymphocyte populations in mouse spleen were essentially overlapping (49.0% showed both markers, 4.0% only surface Ig, and 2.0% only aggregated Ig binding). Further, various modifications in the assay were made in an attempt to demonstrate binding to T cells. These included preincubation and washing of lymphocytes at 37°C before testing, and incubation of cells and Ig complexes at different temperatures (4°, 23°, and 37°C), different pH's (7.2 and 8.0), and in different media (2% BSA-PBS and Delbeco's PBS supplemented with 5% deaggregated fetal calf serum). Binding of Ig complexes to thymocytes or thymus-derived (non-Ig bearing) spleen lymphocytes could not be demonstrated under any of these conditions. Thus, the heat-aggregated human Ig complexes appeared to be binding only to B lymphocytes.

*Inhibition of Binding of Complexed Ig to B Lymphocytes by Anti-H-2 Antisera.*—Antisera raised between strains of completely different *H-2* haplotype contain antibodies against antigenic specificities determined by any of the regions of the *H-2* complex (including Ia antigens) when tested on the strain against which the antiserum was raised.<sup>2</sup> Therefore, as a first test of a possible

relationship between Ia alloantigens and the Fc receptor, mouse spleen lymphocytes of various strains were incubated with anti-*H-2* antisera, washed, and assayed for their ability to bind aggregated Ig complexes. As shown in Table III, lymphocytes which were pretreated with these antisera were markedly inhibited in their ability to bind Ig complexes. Also shown are control experiments which indicated: (a) that normal mouse sera from the same strains as those which produced the antisera caused no such inhibition, (b) that no inhibition was caused by immune mouse serum tested on lymphocytes from the same strain which produced the antiserum, and (c) that antibody was indeed bound to the target lymphocytes as indicated by fluorescence of T cells with

TABLE III  
*Inhibition by Anti-H-2 Antisera of the Binding of Ig Complexes to Mouse B Lymphocytes*

Strain	Serum	Surface Ig	Aggregated Ig binding
		% Positive	
B6	Normal B10.A	43.0	42.0
	B10.A anti-B10	91.0	11.5
B10	Normal B10.A	52.5	52.0
	B10 anti-B10.A	53.0	51.5
B10.A	B10.A anti-B10	96.0	6.5
	Normal B10	49.5	47.0
B10.D2	B10 anti-B10.A	>99.5	7.5
	Normal B10.A	48.5	48.0
B10.BR	B10.A anti-B10.D2	91.5	9.5
	Normal B10.BR	55.0	54.0
	B10.BR anti-B10.D2	>99.5	6.0
A.SW	Normal B10.D2	53.0	51.0
	B10.D2 anti-B10.BR	99.5	5.5
A.SW	Normal A/J	48.0	48.5
	A/J anti-A.SW	>99.5	8.0

FIRAMIG and increased intensity of fluorescence on B cells resulting from the detection of both surface Ig and bound antibody. Further, since all of the anti-*H-2* antisera were raised between congenic-resistant strains which only differed at *H-2*, antibodies against non-*H-2* antigens could not have been responsible for the inhibition. It should be noted that while inhibition was never complete, the residual positive cells bound very few aggregates. Since little is known about the relative affinities of binding of the antibodies and of Ig complexes, it seems possible that some aggregates were able to displace antibody and bind to the lymphocyte. Nevertheless, the possibility that some complexes were binding to sites not coated by the antisera could not be ruled out.

A series of experiments was performed to evaluate the specificity of inhibition of Ig complex binding by anti-*H-2* antisera. One possible artifact appeared to be that *H-2* antigen-antibody complexes might be shed from the cell surface

and then bind to the Fc receptor via the Fc of the complexed antibody, resulting in inhibition of aggregate binding. To investigate this possibility a Fab preparation from B10.BR anti-B10.D2 antiserum was prepared and tested for ability to inhibit aggregated Ig binding (Table IV). As seen in Exp. A, the Fab preparation inhibited the binding of aggregates to B cells, but not as effectively as the whole antiserum from which it was made. A possible interpretation of this result was that the Fab, being univalent, bound less avidly to the lymphocytes. This possibility was supported by the observation that the Fab-coated cells stained with F1RAMIG were less fluorescent, quantitatively and qualitatively, than the cells coated with the whole antiserum, despite the fact that the concentrations had been adjusted to be equivalent in

TABLE IV  
*Inhibition by Anti-H-2 Fab of Ig Complex Binding to Mouse B Lymphocytes*

Exp.	Strain	Serum	% Positive	
			Surface Ig	Aggregated Ig Binding
A	B10.D2	Normal B10.BR	56.5	55.0
		B10.BR anti-B10.D2	>99.5	6.0
		Fab (B10.BR anti-B10.D2)	91.0	18.5
B*	B10.D2	Normal B10.BR	54.5	53.5
		B10.BR anti-B10.D2	99.5	5.5
		Fab (B10.BR anti-B10.D2)	—	9.5
	B10.BR	Normal B10.BR	55.5	53.5
		Fab (B10.BR anti-B10.D2)	—	52.0

\* In experiment B, cells incubated with B10.BR anti-B10.D2 Fab were not washed before the addition of F1-Agg. Volumes of incubations with F1-Agg in other portions of experiment B were adjusted so as to be equivalent. Cells incubated with B10.BR anti-B10.D2 Fab and not washed could not be incubated with F1RAMIG due to cross-reactions. Reasons for these modifications are detailed in the text.

the number of combining sites. Therefore, a second experiment was performed in which cells incubated with anti-H-2 Fab were not washed before incubation with the aggregates (Exp. B). Under these conditions the Fab preparation produced inhibition nearly equal to that of the whole antiserum. The control (Fab plus cells with which the antibodies did not react) indicated that this increased inhibition was specific and not due either to a reaction between Fab fragments and F1-Agg or to residual Fc in the Fab preparation. Thus, it appeared that shedding of complexes was not the explanation for the inhibition of Ig complex binding to B cells produced by anti-H-2 antisera.

In order to determine whether other antisera bound to B lymphocytes could inhibit Ig complex binding, lymphocytes were incubated with unconjugated goat antimouse Ig, washed, and tested for binding of Ig complexes (Table V). No inhibition of Ig complex binding was produced by the antimouse Ig antiserum. Controls indicated that the antiserum had bound to the B cells (inhibi-

TABLE V  
*Failure of Antimouse Ig to Inhibit Binding of Ig Complexes to Mouse B Lymphocytes\**

Antiserum	Surface Ig	Ig complex binding
	% Positive	
Normal mouse serum	43.0	42.0
Anti- <i>H-2</i>	97.0	8.0
Unconjugated goat antimouse Ig	7.5‡	40.5

\* Strain B6 was used. The normal serum was B10.A and the anti-*H-2* antiserum B10.A anti-B10. There was no cross-reaction between the antimouse Ig and the FI-Agg as assessed by Ouchterlony double diffusion.

‡ Positive cells were extremely faint (i.e. ±).

tion of F1RAMIG fluorescence) and that the binding of aggregates to the same cells could be inhibited by the appropriate anti-*H-2* antiserum. Similar results were obtained with antisera specific for antigens determined by the K and D regions of the *H-2* complex (These results will be detailed below). These data provided further evidence for the specificity of the inhibition of Ig complex binding by some antibodies in anti-*H-2* sera.

As another criterion of specificity it seemed important to determine whether the anti-*H-2* antisera would inhibit detection of other receptors on B lymphocytes (i.e. nonspecific steric inhibition). This possibility was evaluated with two separate experiments. In the first experiment, lymphocytes were incubated with anti-*H-2* antisera, washed, and evaluated for EAC binding. In the second experiment, B10.D2 lymphocytes were incubated with BN rat anti-B10.D2 antiserum, washed, and evaluated for surface Ig. A rat antiserum was required in order to allow detection of lymphocyte surface Ig despite the presence of bound antibody. Such rat antisera have previously been shown to detect *H-2* and Ia specificities (7). Also, the reagents in this experiment (BN rat anti-B10.D2 and F1RAMIG) were exhaustively absorbed with insoluble mouse Ig and rat Ig respectively, in order to prevent cross-reactions. The results (Tables VI and VII) showed that no inhibition of EAC binding or surface Ig staining was produced by antisera which caused marked inhibition of Ig complex binding. Thus, no nonspecific steric inhibition could be detected in either system. The results presented in this section indicated that some antibodies in anti-*H-2* antisera inhibited the binding of aggregated Ig complexes to the Fc receptor of mouse B lymphocytes, and this inhibition appeared to be specific by several criteria.

*Inhibition of Binding of Complexed Ig to B Lymphocytes by Antisera Specific for Ia Alloantigens.*—In order to determine whether the inhibition of Ig complex binding produced by anti-*H-2* antisera was due to antibodies against Ia alloantigens contained in those sera, lymphocytes were incubated with antisera specific for Ia antigens, washed, and assayed for Ig complex binding (Table VIII). Specificity for Ia antigens was produced by testing anti-*H-2* antisera



TABLE VI  
*Failure of Anti-H-2 Antiserum to Inhibit the Binding of EAC to Mouse B Lymphocytes\**

Antiserum	Surface Ig	Aggregated Ig binding	EAC binding
		<i>% Positive</i>	
Medium	51.0	49.0	38.5
Normal mouse serum	50.5	47.5	40.0
Anti-H-2	89.0	13.5	39.0

\* Mouse strain B10 was used. The normal serum was B10.A and the anti-H-2 antiserum was B10.A anti-B10.

TABLE VII  
*Failure of Anti-H-2 Antiserum to Inhibit Detection of Surface Ig on Mouse B Lymphocytes\**

Antiserum	Surface Ig	Aggregated Ig binding	
		<i>% Positive</i>	
Medium	46.0	47.0	
Normal BN rat serum	46.5	47.5	
BN rat anti-B10.D2	48.0	5.5	

\* Mouse strain B10.D2 was used. Reasons for using rat antiserum and other details are presented in the text.

TABLE VIII  
*Inhibition by Anti-Ia Antisera of Binding of Ig Complexes to Mouse B Lymphocytes*

Exp.	Strain	Antiserum	Surface Ig	Aggregated Ig binding
			<i>% Positive</i>	
1	B6	Normal B10.A	43.0	42.0
		B10.A anti-B10.D2	44.5	13.0
2	B10	Normal B10.A	53.0	53.5
		B10.A anti-B10.D2	53.5	9.5
3	B10.D2	Normal B10.A	48.5	48.0
		B10.A anti-B10	48.0	12.5
4	B6	Normal B10.A	44.0	42.5
		B10.A anti-B10 absorbed (EL4)	45.5	13.5
5	B10	Normal B10.A	56.0	54.5
		B10.A anti-B10 absorbed (EL4)	55.5	9.0
6	B10	Normal B10.A	46.5	47.0
		B10.A anti-B10 absorbed (B10 thymocytes)	46.0	6.5
7	B10.BR	Normal B10.D2	48.5	46.5
		B10.D2 anti-B10.BR absorbed (RDM4)	48.0	9.5
8	A.SW	Normal B10.A	40.5	42.5
		B10.A anti-B10 absorbed (EL4)	39.5	9.5

against strains which differed at *H-2* but shared Ia specificities (Exp. 1-3) (7), by absorbing anti-*H-2* antisera with T cells from the donor strains leaving specificity for B-cell alloantigens (Exp. 4-7),<sup>2</sup> or by a combination of the two methods (Exp. 8). Anti-Ia antisera produced marked inhibition of Ig complex binding in every experiment. Controls indicated that: (a) the antisera were specific for B-cell alloantigens in that no appreciable increase in the percentage of cells staining with the F1RAMIG was observed following pretreatment with anti-Ia antibodies (compare to results obtained when detecting antigens of the whole *H-2* complex in Table III), and (b) normal mouse serum from the same strains as produced the antisera caused no such inhibition. Further, it should be noted that these anti-Ia specificities are components of the same anti-*H-2* antisera for which a number of specificity controls were described (see above). Two preparations of the same antiserum, one absorbed with a T-cell lymphoma and the other with normal thymocytes, produced equivalent inhibition (Exp. 5 and 6). The inhibition of Ig complex binding produced by antisera against the whole *H-2* complex (Table III) and antisera specific for Ia antigens (Table VIII) appeared to be equivalent. This was further supported by experiments in which an antiserum against the whole *H-2* complex (B10.A anti-B10) and two antisera specific for Ia antigens, one by absorption (B10.A anti-B10 absorbed with EL4), and one by cross-reactivity (B10.A anti-B10.D2), when serially diluted and tested on the same lymphocytes (from a single B10 spleen), showed identical titers. Thus, antibodies contained within antisera against the whole *H-2* complex but specific for Ia alloantigens produced inhibition of binding of Ig complexes to the Fc receptor of B lymphocytes. However, these experiments did not rule out the possibility that antibodies against antigens determined by the K or D regions of the *H-2* complex could also produce such inhibition.

*Failure of Antisera Against the K and D Regions of the H-2 Complex to Inhibit Binding of Ig Complexes to Mouse Lymphocytes.*—Antiserum-strain combinations were selected such that specificity for antigens determined by the K or D regions was obtained (refer to Table I). To examine D region specificities a B10.A anti-B10 antiserum was tested on B10.A(2R) and B10.A(4R) cells, and to examine K region specificities an A/J anti-A.SW antiserum was tested on A.TL cells. The results, presented in Table IX, indicate that no inhibition was produced by antibodies directed only against the K or D region specificities. Controls indicated that the K or D region specific antibodies had bound to the test cells as shown by F1RAMIG staining of T cells and increased intensity of fluorescence on B cells resulting from the detection of both surface Ig and bound antibody. Further, cytotoxic testing of these combinations revealed killing of nearly all lymphocytes (both B and T).<sup>4</sup> Other controls indicated that both the antisera and lymphocytes used in these experiments could inhibit

<sup>4</sup> Sachs, D. H. Unpublished observation.

TABLE IX  
*Failure of Antisera Against Antigens Determined by the K or D Regions of the H-2 Complex to Inhibit Ig Complex Binding to Mouse B Lymphocytes*

Exp.	Strain	Antiserum	Specificity of this combination	Surface Ig	Aggregated Ig binding
<i>% Positive</i>					
A	A.TL	Normal A/J	—	55.0	53.5
		A/J Anti-A.SW	K region of <i>H-2</i> complex	>99.5	54.0
		B10 anti-B10.A	<i>H-2</i> complex except K region	>99.5	6.5
	A.SW	Normal A/J	—	48.0	48.5
		A/J anti-A.SW	Whole <i>H-2</i> complex	>99.5	8.0
B	B10.A(2R)	Normal B10.A	—	49.0	50.5
		B10.A anti-B10*	D region of <i>H-2</i> complex	81.0	49.0
		B10 anti-B10.A	<i>H-2</i> complex except D region	>99.5	12.5
	B10.A(4R)	Normal B10.A	—	50.5	53.0
		B10.A anti-B10	All regions of <i>H-2</i> complex except K and <i>Ir1</i>	96.0	48.5
		B10 anti-B10.A	K and <i>Ir1</i> regions of <i>H-2</i> complex	>99.5	10.5
	B10	Normal B10.A	—	46.5	47.0
		B10.A anti-B10	Whole <i>H-2</i> complex	94.5	5.0

\* Because of the poor response of B10.A mice to the D region specificities of B10 (24) this serum, even when undiluted, was not on the plateau of cytotoxic activity. This probably explains the incomplete staining observed with FIRAMIG in this case. However, other anti-*H-2* and anti-Ia antisera produced marked inhibition of Ig complex binding at similar points on their cytotoxic titration curves, indicating that the failure to obtain blocking in this instance was significant.

Ig complex binding or be inhibited in their ability to bind aggregates, respectively, when used in the appropriate combinations.

The experiment with lymphocytes from B10.A(4R) mice was particularly interesting since, when using antiserum B10.A anti-B10, one might expect to detect antibodies against antigens determined by the *Ir-IgG* region (25). Since there is evidence that this region does determine some Ia antigens (9), this result suggests a possible deletion of Ia antigen(s) in the *Ir-IgG* region of the B10.A(4R), as has previously been proposed (13).

Further evidence for a lack of relationship between *H-2* K and D region specificities and the Fc receptor was obtained by testing mouse lymphoma cells of T-cell origin (EL4 and RDM 4). These tumor cells expressed *H-2* antigens but not Ia antigens as determined by direct cytotoxicity and absorption of cytotoxic activity,<sup>2</sup> and did not bind Ig complexes. The results of this section provided strong evidence for the lack of a relationship between antigens determined by the K or D regions of the *H-2* complex and the Fc receptor,

and thus indicated the necessity of anti-Ia activity to produce the inhibition of binding of Ig complexes to B lymphocytes.

*Codominant Expression of Ia Alloantigen.*—The apparent existence of numerous Ia antigens (7–11, footnote 2) raised the question of whether B-cell alloantigens determined by different genes were expressed on all B lymphocytes or on different B lymphocytes (e.g. allelic exclusion) within the same strain. Therefore, (B6A)F<sub>1</sub> lymphocytes were incubated with reciprocal antisera, each of which recognized the Ia antigens of one parent but not the other (B10.A anti-B10 and B10 anti-B10.A), and then assayed for aggregate binding (Table X). It was expected that if allelic exclusion occurred in this system only about half of the B lymphocytes would be inhibited in their ability to bind Ig complexes, whereas if all B lymphocytes bore the Ia antigens of each parent, then no inhibition would be seen (since approximately half of the Ia antigens of each B cell would not be recognized by antibody). Surprisingly, each of the antisera produced marked inhibition of Ig complex binding to the F<sub>1</sub> lymphocytes. That this was not due to shedding of antigen-antibody complexes was shown by the control experiment using a mixture of B10 and B10.A lymphocytes, where, as expected, only partial inhibition was caused by each antiserum. Thus, it was apparent that most, if not all, of the B lymphocytes expressed at least certain of the Ia antigens separately inherited from each parent. However, the results did not rule out allelic exclusion of individual Ia specificities since each of the reciprocal antisera recognize multiple Ia antigens.<sup>2</sup> The reason why marked inhibition (instead of no inhibition) was obtained was less clear. The most trivial explanation would be that if some Fc receptors are inhibited, then the rest cannot be detected because of the relative insensitivity of the direct fluorescent technique. A second possibility would be that distinct parental genes determined different portions of the same molecule. There is some chemical evidence to contradict this explanation (15). Finally, Ia antigens may be clustered on the cell surface such that antibodies reactive with some serve to block all. Experiments are in progress to differentiate between these possibilities.

TABLE X  
*Codominant Expression of Parental Ia Alloantigens on B Lymphocytes from (B6A)F<sub>1</sub> Mice*

Strain	Antiserum	Surface Ig	% Positive	
			Aggregated Ig binding	
(B6A)F <sub>1</sub>	Normal B10.A	58.0	57.5	
	B10.A anti-B10	92.5	9.5	
	B10 anti-B10.A	99.5	7.5	
Mixture (½ B10 + ½ B10.A)	Normal B10.A	59.5	57.0	
	B10.A anti-B10	78.5	41.5	
	B10 anti-B10.A	80.0	39.0	

## DISCUSSION

Immunoglobulin complexes, composed of F1-Agg have been shown in the present studies to bind to mouse B lymphocytes of a variety of strains, but not to either thymocytes or thymus-derived (non-Ig bearing) spleen lymphocytes under a variety of conditions. Results with this assay confirm most previous studies of the binding of Ig complexes to mouse lymphocytes (1, 2, 5, 6), and offer the advantages of being both simple and rapid. Further, the binding of Agg complexes was shown not to be due to either natural human antibodies against mouse antigens nor nonspecific binding of human Ig by mouse lymphocytes. Such complexes were also shown to bind to the same sites as mouse antibody-antigen complexes. There is much evidence that Ig complexes (either heat-aggregated or antigen-antibody) require an intact Fc in order to bind to lymphocytes (2, 4, 5, 22), and the binding site has been termed the Fc receptor.

These results of aggregate binding to mouse lymphocytes differ from those of Anderson and Grey (22), who found that aggregated Ig bound not only to B lymphocytes but also to some thymocytes and thymus-derived spleen lymphocytes. This discrepancy might be accounted for by differences in sensitivity of the assays used, or by differences in the source of Ig used for heat aggregation. The radioautographic assay employed by those workers may have been more sensitive than the present fluorescent system, thereby allowing detection of receptors on T cells. The second difference, potentially more important, was that they used mouse myeloma proteins. In a single experiment (Table II in Ref. 22) they found that aggregates of human Ig bound as well as mouse Ig to mouse spleen lymphocytes (40% vs. 38% respectively), while in contrast human Ig aggregates showed little binding to mouse thymocytes (similar to the present study). Thus, the receptor for Ig complexes on T cells may differ in specificity from the Fc receptor of B cells. The inhibition studies reported here apply only to the Fc receptor of B lymphocytes.

The present studies have shown that anti-*H-2* antisera are able to markedly inhibit the binding of Ig complexes to B lymphocytes. A series of experiments indicated that this inhibition was not due to the artifact of shedding of *H-2* antibody-antigen complexes, nor to nonspecific steric inhibition. Further experiments showed that the antibodies within anti-*H-2* antisera which were responsible for this inhibition were specific for alloantigens associated with the *Ir* region. Antisera specific for these Ia antigens produced inhibition whereas antisera specific for antigens determined by the K or D regions of the *H-2* complex did not. Finally, evidence was obtained that Ia antigens are codominantly expressed on every B lymphocyte. These data indicate that the Fc receptor and a series of alloantigens controlled by the *Ir* region of the *H-2* complex are identical or closely associated on the B-lymphocyte surface membrane.

Proof that the Fc receptor and Ia antigens are identical (as opposed to a close physical association) will require studies at the molecular level. Of interest in this regard is

the report by Vitetta, et al. (16), using lymphocyte solubilization techniques, of the isolation of Ia antigens via immunoprecipitation. These authors noted in their discussion that: "... immune complexes formed with control sera (including NMS) appeared to bind a portion of Ia molecules.", and that: "analyses by acrylamide gel electrophoresis of the reduced immunoprecipitates obtained with RAMIG disclosed an additional peak of radioactivity that migrated like Ia." These observations, although not interpreted by the authors, may indicate chemical identity between the Fc receptor and Ia antigens.

Little is presently known of the functional significance of either the Fc receptor or the Ia antigens in the immune response. Further, despite their similar genetic localization, the relationship between genes determining Ia antigens and genes controlling immune responsiveness to many immunogens (Ir genes) (26) is unclear. It is also unknown whether there are different Fc receptors with different specificities on B lymphocytes, although there is some evidence consistent with this possibility (4). If such different Fc receptor specificities exist, they may correlate with the different serologically defined specificities of Ia antigens. In any case, the identity or close association between a receptor for antibody and a series of alloantigens determined by the *Ir* region of the *H-2* complex is intriguing and raises the possibility that Fc receptor/Ia antigens play a role in control of the immune response.

Most immunogens for which genetic control of the immune response has been demonstrated are thymus-dependent, with cooperation between T and B cells being required for efficient antibody response (26). Also, it has recently been shown for several thymus-dependent antigens that the "help" provided by T cells can be replaced by factors derived from antigen-stimulated activated T cells (27-29). Such factors are antigen specific, but there is conflicting evidence as to their biochemical nature (27, 29). Finally, evidence has been obtained that in certain cases of Ir gene control, the defect in low responders appears to be expressed at the level of the B cell (29-32). It has been proposed that such unresponsiveness at the B-cell level is to the haptenic determinant rather than the carrier portion of the immunogen (33). Thus, it is possible that cooperation between T and B cells requires a receptor site on the B lymphocyte which recognizes either T cells or an antigen-specific T-cell factor. We wish to propose that this receptor is Fc receptor/Ia antigens. The proposed interaction between a T-cell factor and Fc receptor/Ia antigens might provide the second signal hypothesized by the Bretcher-Cohn model for B-cell activation (34).

The B-lymphocyte Fc receptor has clearly demonstrated capacity to bind complexed antibody. However, the antigen-specific T-cell helper factor need not be immunoglobulin. It seems possible that this T-cell factor might either contain antibody or, alternatively, a product of the Ir genes which may or may not have structural similarity to the Fc portion of complexed Ig. In either case the specificity of the Fc receptor/Ia antigens for complexed Ig might serve as a means for regulation of the immune response by feed-back inhibition due to antigen-antibody complexes (35).

In addition to B lymphocytes, there is much evidence that other cell types can also bind immunoglobulin, including some T lymphocytes (22), activated T lymphocytes (22, 36, 37), monocytes and macrophages (38, 39), and basophils (40). The present observations raise the question of whether the Ig-binding site(s) of these other cell types may also be associated with Ia antigens. If this were the case, it is possible to envision a relationship between Ir genes and several diverse aspects of the immune response including the ability of T cells to bind antigen via cytophilic antibody (41), the ability of antibody to augment the helper effect of activated T cells (42), the ability of antibody to stimulate phagocytosis in macrophages (43), and the antigen-triggered IgE-dependent release of mediators from basophils (44).

#### SUMMARY

Immunoglobulin complexes, composed of heat-aggregated human Ig, were shown to bind to mouse B lymphocytes of a variety of strains, but not to either thymocytes or thymus-derived (T) lymphocytes under a variety of conditions. It was shown that this binding was not due to either natural human antibodies against mouse nor to nonspecific binding of human Ig by mouse lymphocytes. Such complexes were shown to bind to the same sites which bind mouse antibody-antigen complexes. This site is known as the Fc receptor.

The binding of Ig complexes to mouse B lymphocytes was markedly inhibited by pretreatment of the lymphocytes with anti-*H-2* antisera. A series of experiments indicated the specificity of this result, including the fact that this inhibition was shown not to be due to the artifact of shedding of *H-2* antibody-antigen complexes, nor to nonspecific steric inhibition.

The antibodies within anti-*H-2* antisera which were responsible for this inhibition were specific for alloantigens associated with the *Ir* region of the *H-2* complex (Ia antigens). Antiserum specific for these Ia antigens produced inhibition, whereas antisera specific for antigens determined by the K or D regions of the *H-2* complex did not. Evidence was obtained using F<sub>1</sub> hybrid cells that at least some Ia antigens of both parental types are expressed on every B lymphocyte (i.e. codominant expression).

These data indicate that the Fc receptor and a series of alloantigens controlled by the *Ir* region of the *H-2* complex are identical or closely associated on the B-lymphocyte surface membrane. This observation may have implications for the mechanism of control of the immune response.

Grateful acknowledgement is made to M. Kubicek and J. L. Cone for invaluable assistance, and to Dr. W. D. Terry for helpful discussions.

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