VARIATIONS IN THE RESPONSES OF C57BL/10J AND A/J MICE TO SHEEP RED BLOOD CELLS

I. Serological Characterization and Genetic Analysis*

By DONALD M. SILVER, IAN F. C. McKENZIE, and HENRY J. WINN

(*From the Division of Immunology, Harvard Medical School; the Transplant Unit, General Surgical Services, and the Department of Surgery, Harvard Medical School at the Massachusetts General Hospital, Boston, Massachusetts 02114*)

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Antibodies can be divided into a number of classes on the basis of distinctive structural characteristics of the constant portions of their heavy chains. These structural characteristics have no apparent influence on specificity but are responsible for variations in biological properties of the molecules which provide for great versatility in immune responses. Antibodies of similar or identical specificity may appear in any of the subclasses of immunoglobulins, and there is, accordingly, considerable variety among the products of responses to single antigenic determinants. Unfortunately, there is little specific information available on the biological significance of this remarkable versatility, and the only known roles of some immunoglobulin classes involve their participation in pathologic processes. There is, moreover, relatively little known about the factors which determine the relative proportions of molecules of different immunoglobulin classes that appear in any particular response, and neither structural nor functional relationships between different classes have been elucidated. Of special interest in connection with the latter is the relationship between IgM and IgG antibodies of similar specificities. These are the classes most commonly found in immune responses, and their sequential appearance in response to many antigens has been taken to imply a relationship of fundamental significance (1). It has even been suggested that the same cells in which IgM is synthesized early in responsiveness may, subsequently, switch to the exclusive synthesis of IgG molecules of identical specificity (2, 3). The evidence in favor of this interpretation is not regarded as conclusive, however, and this important issue must be regarded as open to further investigation.

A new approach to the problem is suggested by the results of studies on variations among inbred strains of mice in response to injections of sheep red blood cells (SRBC)[1] (4). Some strains were found to respond initially by forming only 19S antibodies which on hyperimmunization were replaced almost en-

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[1] *Abbreviations used in this paper*: 2-ME, 2-mercaptoethanol; SRBC, sheep red blood cells; VBS, Veronal-buffered saline.

tirely by 7S molecules. In other strains, notably C57BL/10, the responses, even after multiple injections of SRBC, consisted largely in the elaboration of 19S antibody. We have undertaken further investigation of these strain differences with a view of obtaining data bearing on the factors which channel immune responses into synthesis of different classes of antibodies and of broadening our understanding of the relationships between these different classes.

Materials and Methods

Animals.—Male and female mice of strains B10.A, C57BL/10J, and A/J were purchased from the Jackson Laboratory, Bar Harbor, Maine. F_1 hybrid mice obtained from matings between C57BL/10J females and A/J males were raised in our laboratories as were mice from backcross matings between female F_1 hybrids and male A/J mice.

Antisera.—Mice were injected intraperitoneally with 0.1 ml of a 2% suspension of sheep red blood cells (SRBC) in 0.9% saline. The SRBC, which were obtained from the same animal for all experiments, were collected in sterile Alsever's solution and stored at 4°C for 1 wk before use. For injection the cells were washed three times with saline and resuspended at a concentration of 4×10^8 cells/ml (2%). The mice were injected at weekly intervals and bleedings were made at various points throughout the course of immunization. In each case a small amount of blood was obtained from a tail vein 6 days after the most recent injection; the blood was collected in 4 volumes of 0.9% saline, the erythrocytes were removed by centrifugation, and the diluted plasma was stored at −20°C.

Serology.—Assays for hemagglutinins and hemolysins were carried out with Microtiter plates (Cooke Engineering Co., Alexandria, Va.). For hemagglutination, immune plasma which had been diluted 1:5 in saline was added in volumes of 0.025 ml to an equal volume of 0.9% saline or 0.1 M 2-mercaptoethanol (2-ME) in 0.9% saline. The mixtures were incubated at 37°C for 30 min after which time twofold serial dilutions were made in saline using the microtiter apparatus, and 0.025 ml of a 2% suspension of SRBC was added to each well. The plates were then allowed to stand at room temperature for 2 hr after which they were examined for evidence of cell agglutination. The titer was taken as the reciprocal of the highest dilution of antiserum in which the SRBC showed a settling pattern distinctive from that observed in wells that did not contain antiserum.

For determinations of hemolysins all reagents were prepared in Veronal-buffered saline (VBS), pH 7.4, as described by Mayer (5). Immune plasma was mixed with VBS or with 0.1 M 2-ME as for hemagglutination, and serial twofold dilutions of the plasma were made in volumes of 0.025 ml. To each dilution was added 0.025 ml of guinea pig complement which had been diluted 1:10 and 0.025 ml of a 2% suspension of SRBC. The plates were incubated at 37°C for 30 min and centrifuged at 1000 rpm for 1 min after which the mixtures were read for hemolysis. The titer was taken as the reciprocal of the highest dilution of serum in which there was clear evidence of hemolysis. This reading was made by direct inspection since it was found that this procedure led to the same titers as those found when released hemoglobin was determined spectrophotometrically at 4100 A. Preliminary tests had shown that 2-ME as used in this test did not interfere with the hemolytic properties of guinea pig complement.

The reproducibility of the tests was measured by repeating the assays for a single antiserum in the presence or absence of 2-ME. Of nine assays, six gave identical titers both before and after treatment with 2-ME. In three cases a difference of one dilution was recorded in the end points.

Chromatography.—1 ml of serum was layered onto a 2.5 × 100 cm column of Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and eluted at room temperature with 0.15 M saline in 0.015 M tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.2. The absorbance of the effluent material was determined at 2800 A and the fractions of individual

peaks were pooled. The volume of each pool was reduced by ultrafiltration to that of the starting serum (1 ml), and the concentrated materials were tested by immunodiffusion with commercially available goat anti-mouse immunoglobulin reagents specific for heavy-chain determinants (Meloy Laboratories, Springfield, Va.).

TABLE I

*Serological Characterization of the Response of C57BL/10J and A/J Mice to SRBC**

No. of injections‡	Titer§			
	C57BL/10J		A/J	
	Hemolysin	Hemagglutinin	Hemolysin	Hemagglutinin
1	80 (0)	20 (0)	160 (0)	40 (0)
	160 (0)	80 (0)	40 (0)	10 (0)
	160 (0)	80 (0)	160 (0)	80 (0)
	40 (0)	10 (0)	80 (0)	40 (0)
	160 (0)	40 (0)	160 (0)	40 (0)
5	20,480 (0)	640 (20)	10,240 (10,240)	20,480 (10,240)
	5120 (80)	640 (80)	640 (320)	1280 (640)
	2560 (0)	160 (20)	1280 (1280)	1280 (640)
	10,240 (160)	1280 (80)	2560 (1280)	1280 (320)
	10,240 (0)	160 (10)	2560 (2560)	10,240 (10,240)
9	5120 (40)	640 (80)	640 (320)	10,240 (5120)
	10,240 (320)	1280 (40)	320 (320)	2560 (1280)
	2560 (40)	1280 (160)	320 (320)	5120 (5120)
	5120 (80)	640 (80)	640 (640)	20,480 (10,240)
	5120 (40)	1280 (40)	640 (320)	40,960 (20,480)
19	10,240 (40)	5120 (320)	80 (80)	20,480 (20,480)
	5120 (80)	1280 (40)	320 (160)	20,480 (20,480)
	10,240 (80)	2560 (80)	320 (320)	20,480 (10,240)
	10,240 (160)	5120 (160)	160 (160)	10,240 (10,240)
	5120 (80)	2560 (40)	320 (320)	20,480 (20,480)

* Mice were injected intraperitoneally at weekly intervals with 0.1 ml of SRBC (4×10^7 cells) and were bled 6 days after the first, fifth, ninth, and nineteenth injections.

‡ The same mice were used for each bleeding, but the order in which individual titers are presented is not the same for each injection.

§ Figures in parentheses represent titers after treatment of the antisera with 2-ME.

RESULTS

Serological Analysis of the Response of A/J and C57BL/10J Mice to Single and Multiple Injections of SRBC.—Female mice of strains A/J and C57BL/10J were injected intraperitoneally at weekly intervals with 0.1 ml of a 2% suspension of SRBC, and serum was prepared from individual bleedings made 6 days after the first, fifth, ninth, and nineteenth injections. These sera were tested for hemagglutinins and hemolysins before and after treatment with 2-

ME and representative results of these tests are summarized in Table I. Data are presented in detail from tests carried out on five mice of each strain; the same patterns of response have been observed in over 100 mice.

In mice of both strains the response to a single injection of SRBC consisted of low levels of antibodies which were completely inactivated by 2-ME, and sera obtained from all animals had higher titers in hemolysin tests than in hemagglutination tests. After multiple injections of SRBC there were marked differences in the serological responses of mice of the two strains, and these differences persisted even after 19 injections. Antibodies produced by C57BL/10J mice throughout the entire course of immunization were highly sensitive to treatment with 2-ME and were in every instance more active as hemolysins than as hemagglutinins. The level of hemolytic activity reached a peak after five injections and remained relatively constant thereafter while hemagglutinin titers continued to rise in response to repeated injections. The activity of sera obtained from A/J mice that had received multiple injections of SRBC showed increasing resistance to 2-ME. After five injections serum titers were, with one exception, unaffected or reduced by a single doubling dilution after treatment with this reagent; after 19 injections there was no detectable effect of 2-ME on serological activity. Hemagglutinin titers increased throughout the course of immunization, whereas hemolytic activity increased after the first few injections but declined after subsequent injections. Thus hyperimmunization is accompanied in both strains by a marked increase in the ratio of hemagglutinin to hemolysin activity though the activities are mediated by different classes of immunoglobulins in the two strains.

The distinctive patterns of immune responses that have been described here were also observed in mice that were injected by the intravenous or subcutaneous routes and in mice that were splenectomized before the start of immunization. Patterns of responsiveness were, moreover, not influenced by the sex of the immunized mice nor by variations in the dose of SRBC administered over the range 4×10^6 to 4×10^8 cells per injection.

The Relationship of 2-ME Sensitivity to the Molecular Size and Class of Antibodies Produced in Response to SRBC.—The principal distinction between antibodies produced in A/J mice and those formed in C57BL/10J mice in response to injections of SRBC is the high sensitivity to 2-ME of antibodies produced in the latter strain even after hyperimmunization. It was important, therefore, to relate this property to some more clearly defined parameter of immune responsiveness. While sensitivity to 2-ME is most often associated with IgM molecules, it cannot be taken as an absolute criterion for classification. We have, accordingly, attempted to characterize antibodies produced in mice of both inbred strains on the basis of their behavior on columns of Sephadex G-200. Sera obtained from bleedings made 6 days after the ninth injection of SRBC were applied to columns and eluted with Tris buffer. Three protein peaks appeared in the eluates and the fractions from individual peaks were

pooled and brought to the volume of the starting material. The first peak, which emerged in the void volume of the column, and the second peak, which appeared immediately after the void volume, containing detectable antibodies to SRBC and were shown by immunodiffusion tests to contain immunoglobulins. The final protein peak which was eluted from the column contained no detectable antibody or immunoglobulin.

Hemolysin and hemagglutinin titers of the materials contained in the first two peaks are presented in Table II. The activity present in the first peaks obtained from sera produced in either A/J or C57BL/10J mice was greatly reduced after treatment with 2-ME, whereas the activity of the second peaks

TABLE II

*The Relationship Between Sensitivity to 2-ME and the Molecular Size of Mouse Antibodies to SRBC**

Strain	Protein peak‡	Reaction with§		Titer‖	
		Anti-μ	Anti-γ	Hemolysin	Hemagglutinin
C57BL/10J	1st	+	−	40,960 (20)	640 (40)
"	2nd	−	+	512 (256)	128 (64)
A/J	1st	+	−	160 (40)	640 (80)
"	2nd	−	+	20,480 (20,480)	40,960 (40,960)

* Sera were pooled from mice which had been injected intraperitoneally nine times at weekly intervals with 0.1 ml of SRBC (4×10^7 cells) and bled 6 days after the last injection.

‡ Protein peaks were obtained by fractionating whole serum on columns of Sephadex G-200. The 1st peak was contained in the void volume; the 2nd peak appeared immediately after elution of the void volume.

§ Tests were made with goat antisera specific for heavy-chain determinants of mouse immunoglobulins.

‖ Figures in parentheses represent titers after treatment of the antisera with 2-ME.

was undiminished or reduced by only one doubling dilution after similar treatment.

When the serum fractions were tested in immunodiffusion tests with antisera specific for heavy-chain determinants of various classes of immunoglobulins, the material present in the first peaks precipitated only with an anti-μ reagent, and material in the second peaks precipitated only with anti-γ reagents. There is thus strong evidence that in these studies treatment with 2-ME can be used to distinguish IgM antibodies from those of other subclasses.

Genetic Analysis of the Differences in Responsiveness between A/J and C57BL/10J Mice.—Differences in the responses of A/J and C57BL/10J mice to SRBC were clearly discernible at a relatively early point in the course of immunization, and the genetic analysis of the variations in responsiveness was based therefore on the study of serum samples obtained after five injections. Fur-

thermore, since the distinction between the responses of the two strains can be more readily drawn from the results of hemolysin rather than hemagglutination tests, only the former technique was used in this study. Immune responses were studied in male and female mice derived from a cross between C57BL/10J females and A/J male mice and from matings in which female F_1 hybrids were backcrossed to A/J males. Responses of a small group of B10.A female mice were also studied because of their genetic relationships with the two strains of mice used for the bulk of this work. B10.A is a subline of C57BL/10 in which the *H-2A* complex has been substituted for *H-2B* after an initial cross between C57BL/10J and A/J (*H-2A*). Thus mice of the B10.A

TABLE III

*Serological Characterization of the Response to SRBC in B10.A, (C57BL/10J × A/J)F_1 Hybrids, and (F_1 × A/J) Backcross Mice**

Hemolysin titers (range)	% Reduction in activity by 2-ME	Proportion of mice
	%	
(C57BL/10J × A/J)F_1		
1280–5120	87.5–93	3/17
1280–5120	>96	14/17
(F_1 × A/J) Backcross		
640	50	1/32
640	75	1/32
320–640	87.5	3/32
160–20,480	>93	27/32
B10.A		
160–5120	87.5–93	6/8
640–1280	>96	2/8

* Mice were injected intraperitoneally five times at weekly intervals with 0.1 ml of SRBC (4×10^7 cells) and were bled 6 days after the last injection.

line differ from C57BL/10J mice at the *H-2* locus, but the two lines are otherwise very similar, if not identical. In view of the influence of genes within or near the *H-2* complex on immune responses to a variety of antigens, we sought to determine their importance in the present study.

The results of these immunizations are presented in Table III. The hemolytic titers of the sera of all of 17 (C57BL/10J × A/J)F_1 hybrids were significantly reduced after treatment with 2-ME. In 14 of the sera the titers were lowered by five or more doubling dilutions; in 2 sera there was a reduction of four dilutions; and the titer of the remaining serum fell off by three dilutions. The responses of the hybrid mice were thus indistinguishable from those observed in the C57BL/10J parental strain suggesting that the trait observed in the latter animals was dominant to that seen in A/J mice.

The sera from backcross mice showed a wider range of sensitivity to 2-ME although 30 of the 32 sera listed could be placed in the range observed for sera obtained from C57BL/10J mice. Of the remaining two sera, one could quite clearly be classified with A/J sera and the other was of questionable classification. Thus the ratio of backcross mice which behaved like A/J mice can be taken as 1/16 or 1/32. The size of this ratio is a measure of the minimal number of independently segregating loci at which there are allelic genes exerting an influence on the trait under study; the proportion of mice showing A/J-like responses should be equal to $(1/2)^n$ where n is the number of loci involved. Hence the values 1/16 or 1/32 are consistent with three to six loci in the present situation ($P = 0.005$). This number can, of course, be determined more precisely by testing a larger number of backcross mice, but it seems very unlikely that the estimate will be reduced.

Although all of the sera from B10.A mice were distinctively more sensitive to 2-ME than were sera from A/J mice, they contained, on the whole, a slightly greater proportion of 2-ME-resistant activity than did the sera from C57BL/10J mice. Clearly, genes of the *H-2* complex do not by themselves play a decisive role in the response to SRBC, but their participation in some form is not excluded by our results.

DISCUSSION

While the data presented in this paper show striking and consistent differences in the responses of A/J and C57BL/10J mice to the injection of SRBC, the basis for such differences is difficult to establish, principally for two reasons. First, SRBC are likely to contain a number of substances that are potentially antigenic for mice and it is virtually impossible to determine the extent to which individual animals respond to each of these various antigens. Secondly, the serological tests used for the detection and measurement of antibodies to SRBC depend not only on the concentrations of antibodies but also on their avidities (including valence and affinity) and on distinctive structural characteristics of the constant portions of their heavy chains. The location and distribution of antigens on SRBC may also exert some influence on the outcome of these tests.

Caution is, therefore, indicated in the interpretation of the data presented here but several clear-cut differences in the responses of the two strains do emerge. C57BL/10J mice respond by forming a preponderance of high molecular weight antibodies that appear in increasing concentrations after multiple injections of SRBC. Thus, while there is no large-scale switch to the production of 7S antibodies, there is maturation of the response in the sense that larger quantities of antibodies are formed in response to secondary than to primary stimulation. Furthermore, the large increase in the ratio of hemagglutinin/hemolysin activity which accompanied hyperimmunization suggests a change in the character of the IgM antibodies, perhaps an increase in average affinity

of their combining sites. A/J mice respond initially with the production of low levels of 19S antibody with no detectable 7S activity. Multiple injections of SRBC lead to the formation of antibodies which are largely IgG and which also show an increasing ratio of hemagglutinin/hemolysin activity. Thus, both strains make good primary and secondary responses to SRBC, and there is evidence for some sort of maturation of responses in both strains. The major difference in their responses lies in the class of antibody which appears after multiple injections of antigen.

This difference might be traceable to differences in the antigens to which mice of the two strains respond, in which case genetically determined variation may be related to the action of *Ir* genes of the type previously described in mice and guinea pigs (6). It is possible, for example, that C57BL/10J mice are strongly stimulated by antigens which cause an obligatory 19S response, whereas A/J mice make little or no response to such antigens. A reciprocal relationship would obtain for a second group of antigens for which much of the 7S antibody of A/J mice is specific. This scheme differs from the descriptions of the action of other *Ir* genes insofar as the responsiveness of the A/J mice is not expressed in F_1 hybrids (6).

Alternatively, the mice of both strains could be responding to the same antigens but there could be a defect in the ability of C57BL/10J mice to switch from the formation of 19S to 7S antibodies. It is not easy to see how such a defect could operate through the action of dominant genes at several loci, but there is very little known about the factors which determine the paths of immune responsiveness or the relative quantities of various classes of immunoglobulins which are formed in various circumstances of immunization. In this connection it is important to stress here that C57BL/10J mice do not suffer a generalized defect in their ability to make 7S responses, although they are thought to have a propensity for prolonged synthesis of IgM molecules.

Whether the explanation lies in responses to different antigens or to a failure of a switching mechanism, further study of this experimental system through the use of plaque assays and in the context of the cell cooperation model (7, 8) for production of antibodies to SRBC should provide useful information bearing on the problems related to the biological relationships of IgM and IgG molecules.

SUMMARY

In response to repeated injections of sheep red blood cells, C57BL/10J mice produce predominantly 19S antibody in increasingly higher amounts, while A/J mice initially produce 19S antibody and then switch to produce increasing 7S antibody titers. In an F_1 generation all mice responded like the C57BL/10J mice. Backcross data implied genetic control involving at least three loci.

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