HUMORAL ASPECTS OF THE IMMUNE RESPONSE TO HOMOGRAFTS

II. Relationship Between the Hemagglutinating and Cytotoxic Activities of Certain Isoimmune Sera*

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Isoimmune sera raised in one line of inbred mice against tissues of another exhibit *in vitro* the capacity to agglutinate erythrocytes of the donor strain (1–3) and to sensitize nucleated donor cells for lysis by complement (4, 5). Such antisera have been shown to produce paradoxical effects *in vivo*, in some cases producing passive antigraft immunity (4, 6, 7) and in other cases producing the phenomenon of "enhancement" of graft survival (4, 8, 9). It has been suggested (10) that these effects might be explained by the existence in such sera of two classes of isoantibodies, one possessing hemagglutinating activity and reacting with test homografts in such a manner as to enhance their survival, the other possessing cytotoxic activity and mediating graft destruction. Medawar (11, 12) has indeed suggested the existence of two distinct classes of isoantigens, cytoplasmic "H antigens" responsible for serum hemagglutinin production, and nuclear "T antigens" responsible for the production of transplantation immunity. On the other hand, hemagglutination and cytotoxicity may merely be different expressions of the activity of a single species of isoantibody, capable of damaging a homograft under some circumstances and of protecting it under others.

It seemed possible to discriminate between these alternatives by comparing the hemagglutinating and cytotoxic activities of isoimmune sera which had been raised in various ways, absorbed in various fashions, or subjected to physicochemical fractionation. The finding in some sera or serum fractions of discrepancies between hemagglutinating and cytotoxic activity would suggest that two antibody species were present, while the finding of consistently parallel levels of these two activities would argue for their dependence on a single antibody species. The present report is concerned with a study of sera

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raised against living or dead homologous cells, with or without the use of Freund's adjuvant, and harvested at various times during the course of immunization. The cytotoxic and hemagglutinating activities of these sera were compared, and some of the sera were subjected to absorbtion with donor erythrocytes or with living or lyophilized nucleated cells of the donor strain. The results and some of the implications of these experiments are described in this communication.

Materials and Methods

Immunization of mice of the BALB/c line with tissues derived from C57Bl/6 mice was carried out as described earlier (10). Hemagglutinin titrations were carried out by the "dextran" technique of Gorer and Mikulska (13) and cytotoxic antibody titrations were performed by a modification (10) of the method described by Gorer and O'Gorman (5). Absorbtions were performed by incubating 0.2 ml. aliquots of serum (diluted 1 to 5 with physiologic saline) for an hour at 4°C. with equal volumes of the absorbents.

RESULTS

Antibody Response of BALB/c Mice to Injections of Living or Lyophilized C57Bl/6 Mouse Spleen Cells.—It has already been reported that the incorporation of Freund's adjuvant enhances the cytotoxic isoantibody response to immunization with homologous tissue and that the antibody response to lyophilized tissue is generally weaker than that to living homologous tissue (10). In the present study, the hemagglutinating antibody response was found to be similarly influenced. The primary response to the injection of living spleen cells was characterized by the appearance of both cytotoxic and hemagglutinating antibodies in relatively low titer. Subsequent booster injections resulted in a prompt rise in titer of both activities, such stimulation being especially effective after the antibody titers had declined during an interval of a month or more following primary immunization. Similarly, antisera harvested after primary stimulation with lyophilized tissue showed little or no cytotoxic or hemagglutinating activity, while booster injections resulted in a brisk secondary response. Further booster injections given at a time when antibody titers were already high had, as might be expected, relatively little effect. Especially noteworthy was the speed with which antibodies appeared during the booster response, nearly maximum titers being achieved within 6 days after the injection. In general, the kinetics of the primary and secondary responses were quite typical of those seen in other, more classical, immunization procedures. In Fig. 1 are shown the cytotoxic and hemagglutinating antibody titers of sera obtained at various times during an experiment in which BALB/c mice were immunized with lyophilized C57Bl/6 splenic tissue. The effect of adjuvant is seen in the earlier appearance of antibodies and in the higher titers of both cytotoxic and hemagglutinating antibodies found after booster injections.

Comparison of Cytotoxic and Hemagglutinating Activities of Isoimmune Sera.— In the experiment illustrated in Fig. 1 there was a general parallelism between the hemagglutinating and cytotoxic activities of the sera drawn at various intervals. In this and other experiments the hemagglutinating antibody titer was usually found to be several fold higher than the cytotoxic antibody titer, due

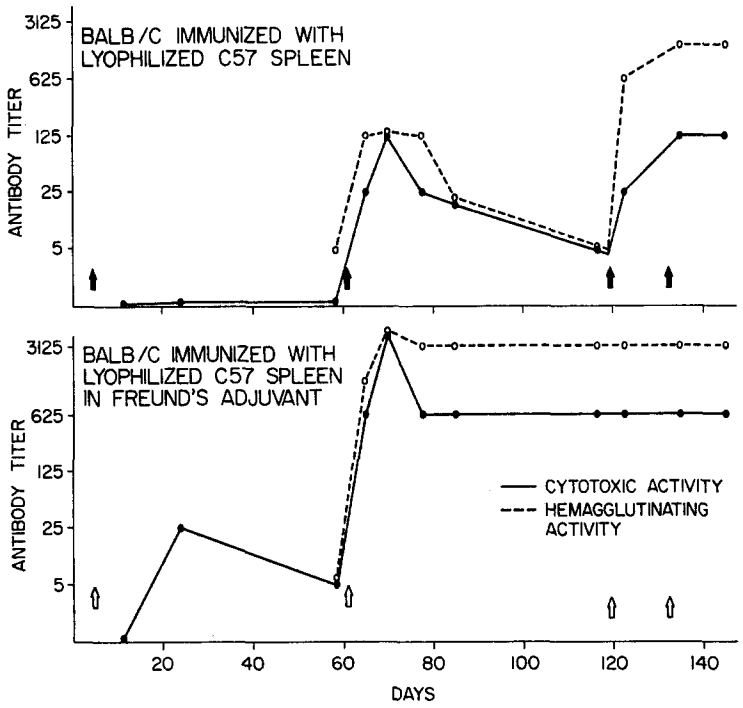

FIG. 1. Cytotoxic and hemagglutinating antibody titers of pooled sera obtained at intervals during immunization of BALB/c mice with lyophilized C57Bl/6 spleen. Initial injections of 5 mg. were given on day 0, and the mice were boosted as indicated by the arrows. All injections were made into the foot-pads. In this experiment, hemagglutinating antibody determinations were performed only on those sera drawn after the 50th day of immunization.

to intrinsic differences in the sensitivities of the antibody assay systems used. In Table I it may be seen that the sensitivity of the cytotoxic antibody test is dependent on the concentration of target cells in the test suspension. The inoculum used as routine in these studies contained approximately 2×10^4 cells/c.mm. and was chosen to provide sufficient cells that the proportion of lysed cells could conveniently be estimated by the examination of a few high power fields. The choice of a lower cell concentration for these titrations would have resulted in cytotoxic antibody titers quite comparable in magnitude to the hemagglutinin titers obtained.

The general correspondence between these titers is of some interest since, if the cytotoxic and hemagglutinating activities of isoimmune sera were due to the presence of two distinct antibody species, it is unlikely that these would always occur in the same relative proportions. That is, it might be expected that a

TABLE I

Sensitivity of the Cytotoxic Assay with Respect to the Concentration of Target Cells

Serum dilution	No. of cells per c.mm. in test system		
	2.5×10^5	5×10^4	1×10^4
1/20	++++	++++	++++
1/40	++	++++	++++
1/80	±	++++	++++
1/160	0	+	++++
1/320	0	0	++++
1/640	0	0	++
1/1280	0	0	0

Each tube contained 0.2 ml. of the suspension of E.L.4 ascites tumor cells in balanced salt solution and 0.2 ml. serum dilution. After incubation at 37°C. for 15 minutes, 0.2 ml. undiluted guinea pig complement was added to each tube and incubation was continued for ½ hour. Then 1.0 ml. 0.1 per cent eosin Y in balanced salt solution was added, a drop of the suspension was examined microscopically, and the percentage of cells showing nuclear staining and cytoplasmic lysis was estimated.

+, 0 to 25 per cent cells lysed; ++, 26 to 50 per cent cells lysed; +++, 51 to 75 per cent cells lysed; ++++, 76 to 100 per cent cells lysed.

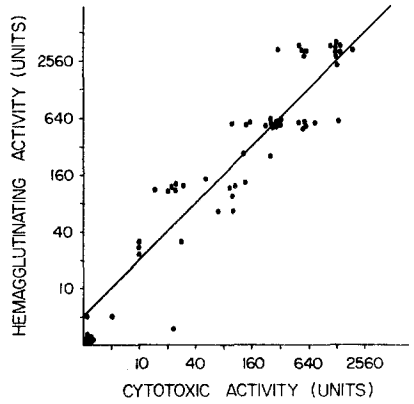

FIG. 2. Comparison between cytotoxic and hemagglutinating activities of 60 pools of serum, drawn at various intervals after immunization of BALB/c mice with C57Bl/6 spleen. Freund's adjuvant was used in the immunization of some of the groups of mice, and sera directed against either lyophilized or living tissue are represented. No serum pool tested showed a high cytotoxic activity but low hemagglutinating activity, and *vice versa*.

given immunizing material or procedure would result in the production of antisera of high cytotoxic but low hemagglutinating antibody content or *vice versa*. Such a discrepancy has not been found, although numerous sera of high or low titers, produced against lyophilized or living tissue, with or without Freund's adjuvant, harvested early or late in the course of immunization, have been tested. Fig. 2 shows that within the limits of the experimental error inherent in the assay techniques there was always a reasonably good correspondence between the two titers.

Attempts at Differential Absorbtion of Cytotoxic and Hemagglutinating Activities of Isoimmune Sera.—It was anticipated that absorbtion with living nucleated cells, lyophilized tissue preparations and erythrocytes would provide information useful from two points of view. First, it seemed possible that differential absorbtion of cytotoxic or hemagglutinating activity might be accomplished with one or another of these agents, as suggested by Brent (14); the selective removal of hemagglutinating activity by absorbtion with erythrocytes, for example, would argue for the dependence of this activity on an antibody species different from that involved in the cytotoxic phenomenon. On the other hand, any appreciable absorbtion of cytotoxic activity by either lyophilized tissue or erythrocytes would in itself be of considerable interest, as such preparations are thought not to contain significant amounts of transplantation antigens (11, 12, 14).

TABLE II

Absorption of BALB/c Anti-C57Bl/6 Sera

Serum	Cytotoxic titer			Hemagglutinin titer		
	Before absorption	After absorption with C57		Before absorption	After absorption with C57	
		Lyophilized liver	Erythrocytes		Lyophilized liver	Erythrocytes
19C0125	16	0	32	128	0	256
19C0131	16	0	16	256	0	128
19C0208	16	0	32	128	0	32
19C0215	16	0	16	64	0	64
19C0324	16	0	16	128	0	128

Cytotoxic titers are expressed as the reciprocal of the serum dilution giving approximately 50 per cent lysis of the test E.L.4 cells. Hemagglutinin titers are expressed as the reciprocal of the highest serum dilution giving unequivocal macroscopic agglutination of the test erythrocytes.

Five isoimmune sera, obtained from BALB/c mice at various times after immunization with lyophilized C57Bl/6 splenic tissue and Freund's adjuvant, were absorbed with packed C57Bl/6 erythrocytes or lyophilized C57Bl/6

liver homogenate. Serial twofold dilutions of the absorbed and unabsorbed sera were made, and the hemagglutinating and cytotoxic activities were determined. The results of this experiment, shown in Table II, indicate that absorbtion with lyophilized liver resulted in complete removal of both cytotoxic

TABLE III

Absorption of Anti-C57Bl/6 Sera with Fresh Isologous and Homologous Liver Homogenates

Serum	Cytotoxic titer			Hemagglutinin titer		
	Before absorption	After absorption with		Before absorption	After absorption with	
		BALB/c liver	C57Bl/6 liver		BALB/c liver	C57Bl/6 liver
CBA anti-C57Bl/6	80	30	5	1280	1280	160
BALB/c anti-C57Bl/6	80	60	10	1280	1280	320
BALB/c anti-C57Bl/6	50	40	5	640	320	5

TABLE IV

Absorption of Isoimmune Sera by Lyophilized Isologous and Homologous Liver Homogenates

Serum	Antibody titer	Before absorption	After absorption with		
			BALB/c liver	C57Bl/6 liver	C57Bl/6 erythrocytes
BALB/c anti-C57	Cytotoxic*	192	96	6	192
	Hemagglutinating	768	768	96	1536
BALB/c anti-C57	Cytotoxic*	320	n.d.‡	80	n.d.
	Hemagglutinating	5120	n.d.	640	n.d.
C57 anti-BALB/c	Cytotoxic§	80	n.d.	80	n.d.
	Hemagglutinating	5120	n.d.	5120	n.d.

* Titer determined against E.L.4 cells.

‡ n.d., not done.

§ Titer determined against BALB/c lymph node cells.

and hemagglutinating activity, while no consistent change in titer was observed following absorbtion with erythrocytes.

In another experiment, 50 per cent suspensions of fresh liver tissue from C57Bl/6 and from BALB/c mice were subjected to homogenization in a Waring blender. The sediments obtained on centrifugation were washed twice and suspended in equal volumes of physiologic saline solution, and were then used to absorb aliquots of a CBA anti-C57Bl/6 serum and two BALB/c anti-

C57Bl/6 sera. Determination of hemagglutinating and cytotoxic activities of these sera showed (Table III) that absorbtion with isologous liver resulted in only slight decreases of titer, which could be attributed to dilution occurring during absorbtion, but absorbtion with C57Bl/6 liver homogenate resulted in appreciable and proportionate reduction of both cytotoxic and hemagglutinating titers.

The results of absorbtion with isologous and homologous lyophilized liver and with homologous erythrocytes are illustrated in Table IV; again, a marked reduction in both antibody activities was observed following the absorbtion with homologous liver while absorbtion with isologous liver or homologous erythrocytes had no appreciable effect. The absorbtion produced by the lyo-

TABLE V

Absorption of Cytotoxic and Hemagglutinating Antibodies from BALB/c Anti-C57Bl/6 Serum by Homologous Erythrocytes

Serum	Hemagglutination at serum dilution of					Cytotoxicity at serum dilution of		
	1/20	1/40	1/80	1/160	1/320	1/20	1/40	1/80
Unabsorbed	++++	++++	++++	++	0	++++	++	0
Absorbed twice with C57Bl/6 erythrocytes	++++	+	0	0	0	0	0	0
Absorbed twice with BALB/c erythrocytes	++++	++++	++++	+	0	++++	++	0

philized C57Bl/6 liver preparation was specific, in that the same preparation did not absorb cytotoxic or hemagglutinating activity from C57Bl/6 anti-BALB/c serum. The relative ineffectiveness of erythrocytes in these absorbtion experiments may be related to their relatively low content of isoantigens (4) and to the fact that the absorbtions were carried out under conditions different from those known to be essential for the demonstration of hemagglutination. When similar absorbtions were carried out in the presence of 2 per cent dextran and normal human serum (13), absorbtion by erythrocytes of both hemagglutinating and cytotoxic activity was unequivocal (Table V).

Electrophoretic Fractionation of Isoimmune Sera.—Starch block electrophoresis of three pools of isoimmune BALB/c anti-C57Bl/6 sera was carried out, and the antibody activities of the resulting fractions were determined. Fig. 3 shows that both the hemagglutinating and cytotoxic activities were found in the gamma globulin region and the activity curves do not indicate that any significant separation of these activities had been accomplished. These results

are consistent with, but of course not necessarily indicative of, the existence of a single antibody responsible for both effects.

DISCUSSION

No evidence was obtained in these experiments for the existence in isoimmune murine sera of separate antibodies responsible for the hemagglutination

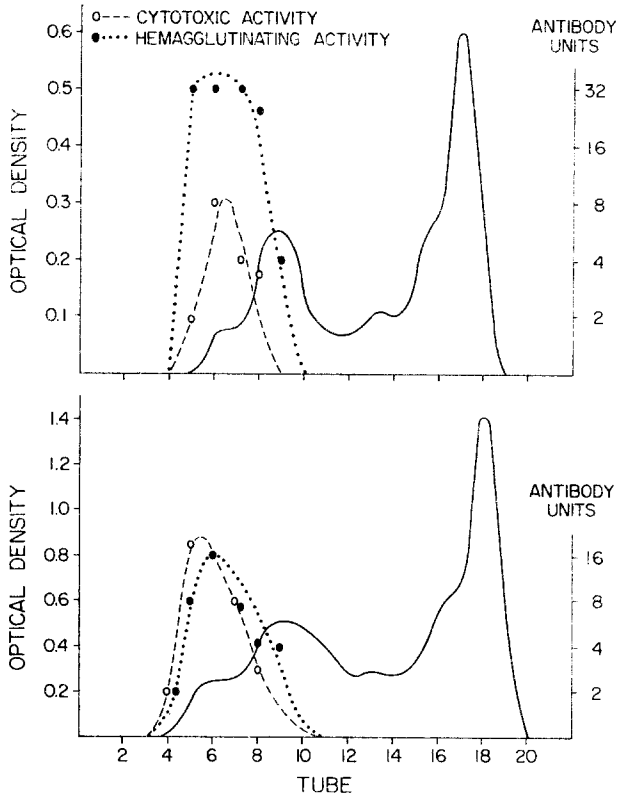

FIG. 3. Cytotoxic and hemagglutinating activity of eluted fractions obtained by starch block electrophoresis. The solid lines represent the protein concentrations in the fractions of these two pools of BALB/c anti-C57Bl/6 sera. The peak of the gamma globulin fraction of a control human serum was found in tube 6. Both of the isoantibody activities were maximal in this region, and no significant separation was demonstrable.

and cytotoxicity shown by these sera. Rather, the data are consistent with the interpretation that the same antibody or antibodies are responsible for both effects. It is possible that the use of other variables might have yielded different results, and further experiments with other strain combinations and antigenic materials are under way.

The demonstration that lyophilized tissue preparations are capable of inducing the formation of antibodies with both cytotoxic and hemagglutinating

activity, and are furthermore capable of absorbing both activities from isoimmune sera, was a somewhat unexpected finding. Much evidence has been cited (4, 8, 9, 11, 12, 14) in support of the view that immunization with dead or lyophilized tissue leads to the production of hemagglutinating antibodies mediating the phenomenon of graft enhancement, while the occurrence of cytotoxic antibodies capable of killing homologous cells *in vivo* or *in vitro* has been reported only after immunization with living cells. Gorer (4), however, has called attention to the reports by Snell *et al.* (15, 16) on the induction of graft immunity by immunization with lyophilized tissue, and Kaliss (17) has described an intriguing dose-response relationship in which immunization with small doses of lyophilized tissue resulted in antigraft immunity while larger doses produced enhancement. While the present experiments indicate that the antibody response to lyophilized tissue is qualitatively similar to that produced by immunization with living tissue, it is quite possible that quantitative differences may be of great importance in determining the reaction of the immunized host to test grafts. It has already been established in passive serum transfer experiments that small doses of antiserum may cause graft enhancement while larger doses produce immunity (18). Differences in the capacity of living and lyophilized tissue to induce delayed hypersensitivity of the tuberculin type may also be implicated in these *in vivo* phenomena.

It should be pointed out that the *in vitro* absorbtion of isoantibodies provides the basis for an assay of isoantigens in tissues. In particular, the cytotoxic assay lends itself readily to quantitative work, and in preliminary experiments has provided a more rapid and sensitive method for the assay of isoantigens than is possible with the bio-assays now in use. The validity of this approach, of course, is predicated on the assumption that the isoantigens detectable by this method are indeed those involved in the various histocompatibility phenomena. Evidence bearing on this point will be presented in a subsequent communication.

SUMMARY

Isoimmune sera, prepared in BALB/c mice by immunization with living or lyophilized tissue from C57Bl/6 mice, were found to possess both hemagglutinating and cytotoxic activity for donor cells. Comparative titrations indicated a good correspondence between these two activities. Absorbtion with living or dead donor tissue caused removal or proportionate reduction of both activities. The results are consistent with the existence in isoimmune sera of a single class of isoantibodies, capable of agglutinating donor cells or of sensitizing them for complement lysis. Living and lyophilized tissue differ quantitatively, but not qualitatively, in their capacity to induce formation of these antibodies.

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