THE ROLE OF NONCLASSICAL, Fc RECEPTOR-ASSOCIATED, Ag-B ANTIGENS (Ia) IN RAT ALLOGRAFT ENHANCEMENT*

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The ready availability of inbred strains, ability to withstand microvascular surgery, and the ease of production of immunological enhancement have made the rat a popular and valuable model for organ transplantation. A number of studies in the mouse employing skin allografts have demonstrated the central role of gene products of the major histocompatibility *H-2* complex in the rejection process. Fragmentary understanding of the rat major histocompatibility gene complex (MHC)[1] has limited the general applicability of data obtained by studying rat organ transplantation. Because important differences exist between the immunobiology of skin and organ allografts (1), it is important to develop a full understanding of the rat MHC. Recent studies have indicated that rat gene products identical to or closely linked to the serologically defined Ag-B system function as immune response genes (2, 3) and stimulate the mixed lymphocyte culture response (4). These studies suggest, therefore, that the rat MHC bears close similarity to the more extensively mapped MHC regions of primates and the mouse.

The phenomenon of passive enhancement of tumor and tissue allografts (1, 5) has been shown to be associated with only minimal alteration in cellular immune responsiveness in the host (6). In contrast, immunopathological (7, 8) and humoral studies (6–9) have emphasized the depression of host humoral responsiveness and suggest an impairment of B–T-cell cooperation (6). Still, the mechanism by which passive immunization produces this deviation in the immune response remains speculative. An important recent advance has been the finding that, while antibodies of different classes (10) and physicochemical characteristics (11) can induce enhanced graft survival, antibodies specific for the products of the *K* and *D* regions of the mouse MHC are unnecessary (12). Similarly, in the rat,

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[1] *Abbreviations used in this paper:* AEF, allogeneic effect factor; BUN, blood urea nitrogen; EA, rabbit antisheep (IgG)-coated SRBC; EAC, rabbit antisheep (IgM) plus complement (C3)-coated SRBC; HIS, hyperimmune allogeneic sera; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; PAS, platelet-absorbed sera; RAS, red blood cell-absorbed sera; SAS, splenocyte-absorbed sera; SRBC, sheep red blood cells.

heart allografts appear equally well enhanced by an alloantiserum whether it has been absorbed with Ag-B antigen-bearing donor red blood cells (RBC) or not (13). Such studies, while not eliminating the possible importance of spontaneously reversed rejection episodes (14), strongly suggest that antigens other than the products of the *K* and *D* regions of the mouse MHC and of analogous "serologically-defined" Ag-B antigens in the rat are of prime importance in passive enhancement.

The present study shows that a rat alloantiserum, which has been extensively absorbed with donor strain RBC or platelets, is as effective as the unabsorbed antiserum in inducing renal graft enhancement. Furthermore, absorbed antisera inhibit the mixed lymphocyte culture and specifically block donor lymphocyte Fc receptors. We suggest that the specificities of antibodies of predominant importance in passive enhancement in rat allotransplantation are directed against antigens analogous to those coded for by the *Ir* region of the mouse MHC (15), since the *I* region antigens (Ia) and Fc receptors seem to be closely related (16). An hypothesis unifying our present knowledge of the rat MHC and the immunologic alterations observed during the induction of enhancement is presented.

Materials and Methods

Animals. Inbred rats, weighing ~200 g, Buffalo (BF) Ag-B^6, Brown-Norway (BN) Ag-B^3, Lewis (Lew) Ag-B^1, Wistar Furth (WF) Ag-B^4, and ACI Ag-B^2 were purchased from Microbiological Associates, Bethesda, Md., as were the following F_1 hybrids: (Lew/BN)F_1, (Lew/BF)F_1 and (Lew/ACI)F_1. August (AUG) rats, Ag-B^5, were kindly provided by S. M. Poiley, National Cancer Institute, Bethesda, Md. F_2 hybrids from (Lew/BN)F_1 × (Lew/BN)F_1 matings and either homozygous at the *Ag-B* locus [(Lew/Lew), (BN/BN)] or heterozygous (Lew/BN) were kindly provided by Dr. R. M. Williams, Harvard Medical School, Boston, Mass. AKR male mice, deficient in C5 complement, were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Kidney Transplantation. Rat renal allografts were performed after bilateral nephrectomy of the recipients, as previously described (6, 17). Animals in experimental groups received intravenous injections of a particular hyperimmune serum (HIS), Lewis anti-BN, immediately after transplantation, whereas control rats received no therapy. Blood urea nitrogen (BUN) levels were measured weekly in each animal. Rats dying within the 1st wk after surgery were autopsied and removed from the study if an obvious technical failure had occurred.

Hyperimmune Allogeneic Sera (HIS). Lew rats were immunized with a single BN skin graft followed by biweekly injections for 2 mo of pooled BN lymphocytes (10^7–10^8) harvested from spleen, bone marrow, and lymph node. Animals were bled by aortic puncture 10 days after the last injection, and the sera were pooled and heat inactivated for 30 min at 56°C. Two pools (no. 23 and 26) with the same lymphocytotoxicity and hemagglutinating titers were used.

RBC-Absorbed Sera (RAS). Heparinized BN blood was washed two times with saline and the buffy coat removed. The remaining cells were filtered through a packed cotton column, and after centrifugation the top fourth of packed RBC were discarded. The remaining RBC were layered onto a Ficoll-Hypaque density gradient (sp gr 1.094) and centrifuged at 300 *g* for 15 min. The sedimented RBC were washed twice in normal saline and checked for purity by acridine orange staining. HIS was then absorbed with these BN RBC by mixing 1 vol of HIS with 2 vol of RBC for 4 h at 37°C, followed by a 12 h incubation at 4°C with constant stirring. This absorption procedure was repeated three times.

Platelet-Absorbed Sera (PAS). Citrated BN blood was diluted with normal saline (1:4) and centrifuged at 200 *g* for 10 min. The supernate was aspirated and centrifuged twice at 200 *g* before pelleting the platelets at 1,000 *g* for 20 min. The absence of leukocytes was confirmed by acridine orange staining. Repeated absorptions of HIS were performed as above, utilizing the platelets from 40–50 ml of BN blood for absorption of 1 ml of HIS. The absorptions were performed at 37°C for 4 h, followed by a shorter (4 h) period at 4°C.

Thymus- and Splenocyte-Absorbed Sera (TAS-SAS). HIS was absorbed three times with either 1.2×10^9 per ml of Ficoll-Hypaque purified splenocytes or thymocytes. Incubations were at 37°C for 4 h, followed by a 4-h period at 4°C with constant agitation. All the absorbed sera were then diluted (1:3) with RPMI 1640 medium and sterilized by micropore filtration (HAWP 02500; 0.45 Millipore Filter Corp., Bedford, Mass.). Samples of sera were stored at −20°C.

Acridine Orange Staining. Five drops of saline saturated acridine orange dye (National Analine) were added to 10 ml of 95% ethylalcohol. The diluted dye was poured over clean slides and dried (18). One small drop of a cell preparation was plated on the stained slide and examined under a cover slip with blue illumination (Zeiss microscope, #B050 mercury lamp, K490 excitor, and 510 suppression filters; Carl Zeiss, Inc. New York).

Preparation of $F(Ab')_2$. Pure IgG was prepared from 10 ml of HIS and PAS previously dialyzed for 2 h against 0.05 M Tris buffer (pH 7.6) and placed upon a 50-ml column of DEAE 52 cellulose (Whatman Lab., England) in the same buffer. The eluted IgG was immunochemically pure, as assessed by immunoelectrophoresis with rabbit antirat serum (Cappel Lab., Downington, Pa.). The IgG was then digested with pepsin (2,560 U/mg, Sigma Chemical Co., St. Louis, Mo.) at an enzyme/substrate ratio of 1:40 in acetate (0.075 M) saline (0.005 M) buffer (pH 4.5) for 18 h at 37°C. An equal amount of pepsin was added before an additional 4-h incubation; the reaction was stopped by raising the pH to 8 with solid Tris. Immunoelectrophoretic analysis revealed a triple pattern characteristic of a mixture of $F(Ab')_2$ and nondigested IgG. After dialysis against phosphate-buffered saline (pH 7.6) at 4°C, the preparation was applied to a 100 cm × 1.5 cm G-200 Sephadex column. Three peaks were obtained: undigested IgG (19.5%), $F(Ab')_2$ (49%), and a small diffuse peak of a lower molecular weight which contained the Fc fragments (30.7%). The first and second peaks gave single but not identical bands upon immunoelectrophoretic analysis against rabbit anti-IgG and were, respectively, the undigested IgG fraction and $F(Ab')_2$ fraction. Rabbit antirat IgG $F(Ab')_2$ was prepared in the same manner, but an enzyme/substrate ratio of 1:100 and a single 18-h digestion was employed. The $F(Ab')_2$ preparations were adjusted to a concentration of 5 mg/ml.

Antibody Elution from Platelets. The pooled pellet of platelets used for absorption was incubated in a citrate buffer (pH 3.2) at 37°C for 2 h with constant agitation. The mixture was centrifuged at high speed, and the supernate was collected and immediately buffered to pH 7.2. A second elution was performed on the same platelet pellet.

Assessment of the Sera. Each antiserum was tested for complement-dependent cytotoxicity titer against ^{51}Cr-labeled splenocytes, as previously described (19), and for hemagglutination titer using BN RBC in a microcapillary method (20). $F(Ab')_2$ fragments were devoid of cytotoxicity, but were shown to inhibit the complement-dependent cytotoxicity assay: ^{51}Cr-labeled spleen BN splenocytes were preincubated for 40 min at room temperature with HIS $F(Ab')_2$, washed, and the cytotoxicity assay was performed using these preincubated target cells. HIS $F(Ab')_2$ fragments retained the hemagglutination titer of undigested HIS.

Mixed Lymphocyte Culture (MLC). MLC was performed with 2-mercaptoethanol (2-ME) (Sigma Chemical Co.) supplemented media, as previously reported (19). Briefly, cells were harvested under sterile conditions from spleen after exsanguination of the animals. 0.75×10^6 responding cells were mixed with an equal number of mitomycin C-treated stimulating cells, in microtiter plates (Cooke Laboratory Products, Alexandria, Va.) (flat bottom 18–29A). The test sera were added to the MLC in various dilutions and adjusted to a 10% final serum concentration with normal Lew rat sera.

EA Rosette Assay. Sheep red blood cells (SRBC) (Baltimore Biological Laboratories, Baltimore, Md.) less than 1-wk old, were washed three times, adjusted to 1%, and mixed with an equal volume of various dilutions of the IgG-enriched fraction (Sephadex G-200) of rabbit anti-SRBC immune serum (Baltimore Biological). A 1/3,000 dilution of IgG was adopted as the optimal sensitizing dose. After 1 h at 37°C followed by 1 h at 4°C the cells were washed twice, and the indicator cells were stored for up to 4 wk without loss of activity. Rosettes were formed by mixing 0.5×10^6 lymphocytes in 50 μl of RPMI 1640 with 50 μl of 1% sensitized SRBC and 25 μl of SRBC absorbed fetal calf serum (FCS) (Grand Island Biologicals, Grand Island, N. Y.) and centrifuging at 200 *g*. After 45 min incubation at 37°C the cells were gently resuspended and a drop placed on an acridine orange-stained microscope slide for examination. The number of rosettes were determined using dark field illumination after the number of fluorescent cells in the same field were

enumerated. This method permits definition of the nuclear morphology of the cells; only mononuclear cells were counted. A rosette was defined as three or more RBC bound to a mononuclear cell.

In rosette inhibition experiments, the lympyhocytes were preincubated with various dilutions of the test sera for 45 min at room temperature and then washed once before using for rosette formation.

EAC Rosettes. The first peak of the G-200 elution of rabbit anti-SRBC serum, enriched in IgM, was added at a 1:2,000 dilution to 2% SRBC, incubated for 30 min at 37°C and 30 min at 4°C, washed twice, and incubated for 30 min at 37°C with a 1:5 dilution of C5-deficient AKR mouse serum. The rabbit antisheep (IgM) plus complement-coated SRBC (EAC) were washed twice, resuspended to 1% in a mixture of equal parts of RPMI 1640 and isotonic EDTA (0.04 M), and stored for up to 4 wk at 4°C. EAC rosettes were formed by mixing 0.5×10^6 lymphocytes in 0.8 ml of RPMI/EDTA solution with 0.2 ml of 1% EAC, incubating for 15 min at 37°C, and centrifuging at 200 *g* for 5 min with immediate resuspension. Experiments performed with IgM-sensitized RBC (but not incubated with AKR mouse serum) did not result in the formation of rosettes. Rosette inhibition experiments were performed as described for rabbit antisheep (IgG)-coated SRBC (EA) rosettes.

Results

In Vivo Study

DOSE DEPENDENCE OF HIS ENHANCING EFFECT. The enhancing effect of HIS upon rat renal allografts has been described in detail by our laboratory (6, 7). 1/2 ml of HIS was able to prolong indefinitely the survival of (Lew/BN)F_1 kidneys transplanted into a Lew host (Fig. 1). The Lew recipients exhibited slightly increased BUN levels at the end of the 2nd wk after transplantation with a subsequent decline to approximately normal values. All the animals receiving 0.5 ml of HIS became long-term survivors. Rats receiving 0.3 ml showed a marked increase in BUN (155±70 mg/100 ml) and two of six recipients died 2 wk postgrafting. The remaining four rats recovered from rejection episodes and became long-term survivors with renal function similar to that of animals receiving 0.5 ml of HIS. Three of six rats receiving 0.1 ml of HIS died, and all exhibited a marked increase in the BUN (220±110 mg/100 ml). Interestingly, the three animals surviving the 3rd wk recovered excellent renal function and became long-term survivors. The control transplanted group consisted of nine rats that did not receive any serum. All of these animals exhibited a major and early rise in the BUN level, and eight died within the first 20 days after surgery, while one recovered and became a long-term survivor. It is evident that HIS protected (Lew/BN)F_1 transplants in Lew recipients, and that this enhancing effect is dose dependent as assessed by the mortality rate and severity of rejection crises.

STRAIN SPECIFICITY OF THE ENHANCING HIS ACTIVITY. Previously published experiments on the specificity of enhancement using different enhancing models are in conflict (14, 21). We tested the ability of the anti-BN HIS to protect semiallogeneic kidneys transplanted into Lew recipients (Table I). When (BF/Lew)F_1 and (ACI/Lew)F_1 kidneys were transplanted into Lew hosts all the recipients died within 2 wk except for one animal which spontaneously became a long-term survivor. The group injected with 0.5 ml of Lewis anti-BN HIS, which was able to enhance 100% of (Lew/BN)F_1 donor kidneys, as shown above, was ineffective with (BF/Lew)F_1 or (ACI/Lew)F_1 kidneys as shown in Table I. One treated (BF/Lew)F_1 graft became a long-term survivor as in the control group.

These results show that, in the three inbred strains tested, the ability of the

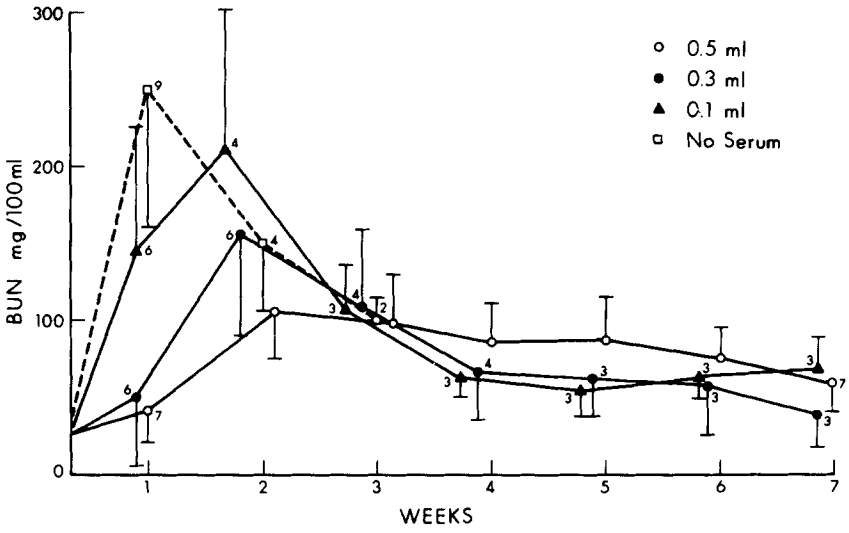

FIG. 1. Dose response effect of HIS in rat renal allografts. Lew rat recipients of (Lew/BN)F_1 grafts receiving 0.5 ml (○), 0.3 ml (●), 0.1 ml (▲), or no HIS (□). The number adjacent to the mean ± SD of BUN represents the number of surviving rats in each group at each time period.

HIS to enhance semiallogeneic grafts is restricted to grafts expressing the BN MHC haplotype.

ENHANCING EFFECT OF THE ABSORBED SERA. Three successive absorptions of HIS with BN RBCs resulted in maximal removal of cytotoxic antibodies; a fourth absorption did not remove additional cytotoxicity. The 18% specific lysis produced at the 1:6 dilution of the RAS plateaued over the next two $\log_2$ dilutions and then declined sharply (Fig. 2). RAS was almost completely devoid of anti-BN hemagglutinating activity, while unabsorbed HIS had an end point titer of 1/1,728. Calculations from the hemagglutination titer showed that less than 0.7% of the hemagglutinating antibody activity remained after the absorption. Five Lew rats receiving (Lew/BN)F_1 kidneys and injected with 0.5 ml of RAS all became long-term survivors. Moreover, the mean posttransplant BUN levels were equal to those in the group enhanced with nonabsorbed HIS (Fig. 3).

PAS exhibited lower cytotoxicity against ^{51}Cr-labeled BN splenocytes than did RAS, but with the same plateau over the early serial dilutions (Fig 2). No hemagglutinating activity was detected after platelet absorption. The PAS also produced a good enhancing effect as shown by the survival rate and renal function of this group of grafted rats (Fig. 3).

TAS and SAS completely depleted of both cytotoxic and hemagglutinating activity were unable to protect (Lew/BN)F_1 transplanted kidneys; all five rats in each group died within 2 wk (Fig. 3).

We conclude that RAS and PAS are able to produce enhancement of renal allografts at doses comparable to those of the unabsorbed HIS. Anti-Ag-B antibodies, as defined by classical serological techniques, remaining after absorption could not be quantitatively sufficient to explain this effect as shown by the HIS dose response studies (Fig. 1). Therefore, in vitro studies of HIS and its

TABLE I
Specificity Control of LEW Anti-BN Antiserum

Combination donor → host	BUN per week					Survival days
	1	2	3	4	5	
	mg/100 ml					
(LEW/BN)F_1 → LEW						
Control*	245					7
	—					7
	220					8
	330					7
	280	120				16
	440					10
Enhanced‡	55	90	175	140	140	>35§
	70	70	65	40	45	>35§
	25	190	100	65	65	>35§
	25	70	90	70	90	>90
	60	80	60	105	70	>35§
	40	75	90	80	60	>90
(LEW/BF)F_1 → LEW						
Control*	290					23
	300					10
	280	55	55	60	50	>90
	340					9
	420					8
Treated‡	370					11
	410					8
	275					12
	280	65	190	120	110	44
	—					7
(LEW/ACI)F_1 → LEW						
Control*	285					9
	270					8
	350					8
	280					12
	250					11
Treated‡	—					7
	330					9
	—					7
	380					7
	380					8

* Transplanted animals without injection of serum.
‡ Animals receiving 0.5 ml of LEW Anti-BN HIS at the time of transplantation.
§ Animals used for other experiments 5 wk after successful transplantation.

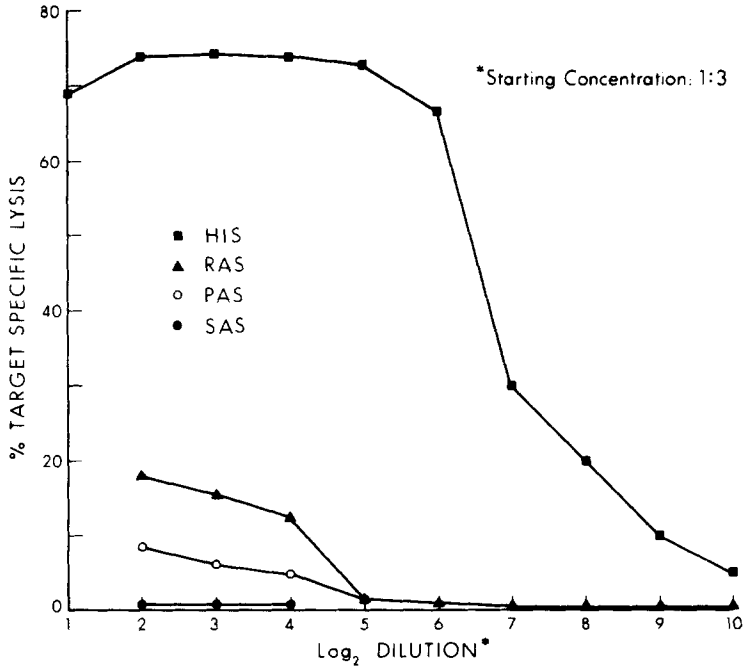

FIG. 2. Lymphocytotoxicity titer of various absorbed sera. The results are expressed as percent target specific lysis according to the formula: (Ex − SR)/(Ft − SR) × 100, where Ft is the mean counts per minute (cpm) obtained from supernates after freezing and thawing aliquots of ^{51}Cr spleen target cells. Spontaneous release (SR) is the mean cpm obtained from the supernate of target cells incubated either with test anti-sera or complement alone. Ex is the mean cpm obtained after incubation of target cells with the test sera and complement. HIS is Lew anti-BN. Absorption with BN spleen (SAS) removes all activity, while some degree of cytotoxicity against spleen is retained after exhaustive absorption with RBC (RAS) or platelets (PAS).

absorbed derivatives were performed in an attempt to define the antigen(s) recognized by PAS and RAS.

In Vitro Studies

EFFECT OF THE SERA ON MLC. When HIS was added to Lew plus BN_m MLC, the [^{3}H]thymidine uptake was strongly inhibited. A normal proliferative response occurred only with dilutions of HIS equal to or greater than 1:1,200 (Fig. 4). The HIS also blocked the reciprocal BN plus Lew_m MLC. The specificity of the HIS inhibitory effect upon MLCs was studied by using Lew responder cells with stimulator cells harvested from four other inbred strains (BF, WF, ACI, and AUG) differing at the major histocompatibility locus (*Ag-B*). High concentrations of HIS inhibited each of these MLC combinations (Fig. 4 a). However, higher dilutions of HIS (1:640) inhibited (approximately 60%) only the specific Lew plus BN_m combination (Fig. 4 *a*), and none of the other four combinations were blocked at these dilutions. HIS tested at high concentrations (1:10) in the reciprocal MLC combinations, i.e. Lew_m as stimulator cells, diminished only slightly the [^{3}H]thymidine incorporation of the WF, ACI, and AUG responders,

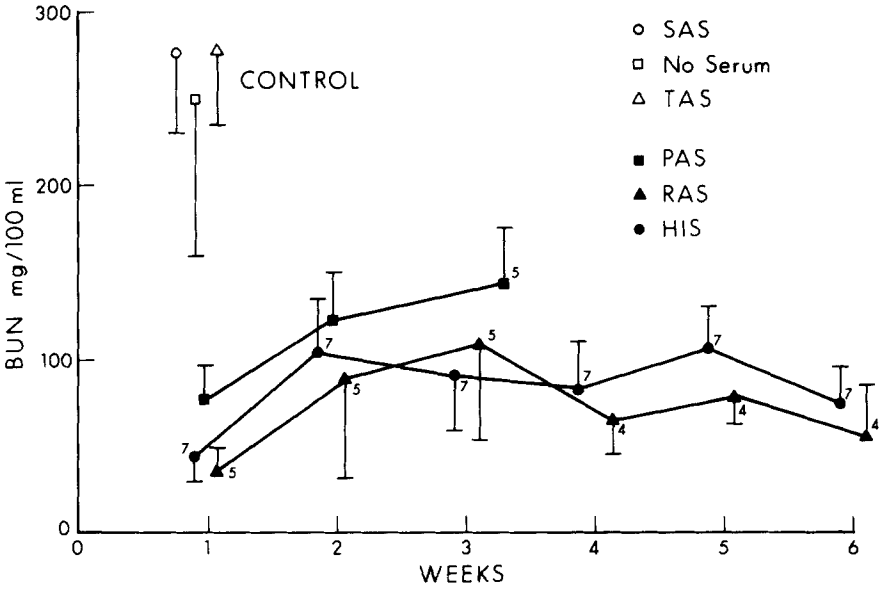

FIG. 3. Enhancing ability of absorbed HIS. The symbols represent the mean ± SD of the BUN level of Lew rats transplanted with a (Lew/BN)F_1 kidney. Spleen- and thymus-absorbed sera were ineffective, while platelet or RBC absorbed sera were comparable to the Lew anti-BN HIS. 1/2-ml doses were given in each case.

but, as previously noted, abolished the response of BN responders (Fig. 4 c). MLCs performed in the presence of PAS also inhibited all MLC combinations with Lew responder cells; however, higher concentrations of PAS than HIS were required for inhibition (Fig. 4 b). The specific inhibitory effect of PAS at high dilutions on BN_m × Lew combinations was less pronounced than with the HIS. TAS and SAS were unable to block the MLC even at a 10% concentration (Fig. 4 b). Sephadex G-200 fractions of HIS, RAS, and PAS (with fractions of Lew NRS as controls) were tested in MLCs and demonstrated that blocking activity was confined to the IgM and IgG-enriched fractions, suggesting that the active component is an antibody. Thus, HIS blocked MLCs between Lew responder cells and BF_m, WF_m, ACI_m, and AUG_m cells, indicating shared specificity between these strains. However, at high dilutions (up to 1:640) HIS was able to discriminate the specific immunizing BN strain. In the reciprocal MLC (Lew_m), high concentrations of HIS specificity blocked the BN plus Lew_m combination. Absorption with BN thymocytes or spleen cells, but not platelets, removed the inhibitory effect on the MLC.

EFFECT OF THE SERA ON FC AND COMPLEMENT RECEPTORS. When Lew, WF, BF, ACI, AUG, or BN spleen lymphocytes were preincubated for 45 min in 10% HIS or normal Lew sera before the EA rosetting procedure, HIS abolished the capacity of BN cells, but not those of other strains, to rosette with EA (Table II). RAS and PAS were also strongly inhibitory to BN EA rosettes. Rabbit antirat IgG inhibited EA rosette formation, but this effect was not strain specific. Blocking of FITC-IgG aggregate binding (16) was also produced by HIS and PAS. SAS was devoid of any blocking activity. Antibodies eluted from platelets

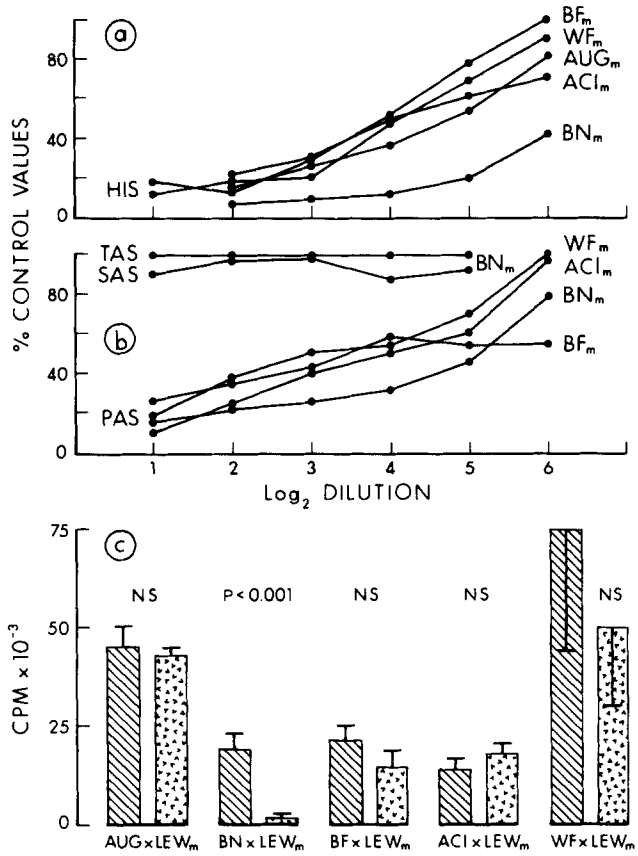

FIG. 4. Effect of HIS, PAS, TAS, and SAS on the MLC. (a) Lew lymphocytes are the responding cells of a one-way MLC performed with various rat strains in the presence of various concentrations of HIS. (Log_2 dilution of 1 is 10% HIS). Each point represents the mean values obtained when Lew cells were mixed with mitomycin-treated cells (BN_m, WF_m, AUG_m, ACI_m) in the presence of HIS. (b) Same as (a), but MLCs were performed in the presence of absorbed sera as indicated. (c) Here Lew_m lymphocytes are used as stimulating cells and mixed with the other allogeneic cells as responders in the presence of 10% HIS, shown by the right hand speckled bar, or in 10% normal rat serum on the left. Only the BN × Lew_m combination is inhibited.

(used for anti-Ag-B absorption), having a hemagglutinating titer of 1:1,920, were unable to block rosette formation, evidence that the membrane antigens blocked by the absorbed sera are not classical Ag-B.

To rule out the possibility that antigen-antibody complexes were causing the specific blocking phenomenon, additional control experiments were performed. Supernates collected after the incubation of HIS with BN lymphocytes failed to inhibit EA rosettes formed by Lew and BN cells. Furthermore, when WF, BF, ACI, or Lew cells were mixed with an equal number of BN cells during the preincubation step with HIS, inhibition of the rosette score averaged 58% (Table III), indicating that the inhibitory effect of HIS upon EA rosettes is restricted to cells expressing the BN genotype. Furthermore, $F(Ab')_2$ obtained from PAS was

TABLE II
Effect of Various Sera on EA and EAC Rosettes

	EA rosettes						EAC rosettes		
	BN	LEW	WF	ACI	AUG	BF	BN	LEW	BF
				%				%	
NRS	19	22	13	13	14	11.5	27	20	18
HIS	0	23	12	12	10	11	26	21	16
RAS	4	19	14			11			
PAS	2	24	11			12			
$F(ab')_2$*	3	17				19			
RαIgG‡	0	0	1	0.5					
Platelet eluate	19								

* $F(ab')_2$ fraction prepared from PAS (Control LEW 17%, BF 20%).
‡ Rabbit anti-rat IgG (same results were found using $F(ab')_2$ from RαIgG).

TABLE III
Effect of HIS on BN Cells Mixed with Nonrelated Cells

	NRS	HIS	NRS	HIS	NRS	HIS	NRS	HIS
WF	16	14						
BUF			13	13				
ACI					18	21		
LEW							11	12
BN	18	0	25	2	18	0	12	1
BN + WF	20	6						
BN + BUF			15	5				
BN + ACI					17	7		
BN + LEW							13	8

NRS, Preincubation with normal LEW serum.
HIS, Preincubation with LEW anti-BN HIS serum.

able to inhibit EA rosettes with BN cells, but not with Lew and BF lymphocytes (Table II). These experiments indicate that the specific rosette inhibitory effect of the sera is not due to formation of free antigen-antibody complexes but is due to the binding of an antibody either to the Fc receptor itself or to a structure spatially closely related on the cell surface. The fact that $F(Ab')_2$ fragments are inhibitory indicate that inhibition is caused directly by antibody binding and not as a secondary result of binding with subsequent Fc site interaction with Fc receptors.

Further study of the genetic segregation of the HIS "anti-Fc" activity was performed in F_1 and F_2 generations of Lew and BN parental strains. The degree of inhibition of EA rosette formation by the (Lew/BN) F_1 cells (compared to BN cells) was assessed after preincubation with various dilutions of HIS. High concentrations (1:10) of the HIS inhibited BN rosettes by 85% compared to 44% for the (Lew/BN)F_1 cells (Fig. 5). At higher dilutions the discriminatory effect persisted: a 1:1,000 dilution did not affect (Lew/BN)F_1 EA rosetting capacity, but

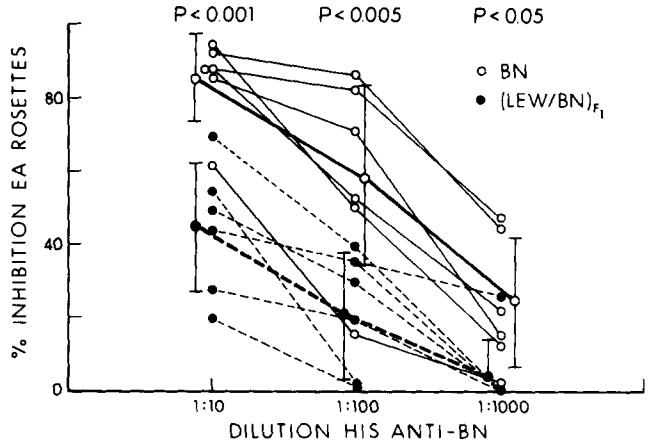

FIG. 5. Inhibition of EA rosette formation of (Lew/BN)F_1 and BN lymphocytes after preincubation with HIS. The degree of inhibition is greatest with BN (O) cells compared to (Lew/BN)F_1 (●) cells at all dilutions of HIS. Individual experiments are shown along with the means ± SD for each group.

TABLE IV
Inhibition of EA Rosettes in F_2 LEW × BN Litter Mate after Preincubation with LEW Anti-BN HIS

HIS	LEW/LEW*	LEW/BN*	BN/BN*
1:10	15‡	75	85
1:100	4	25	28
1:1,000	0	0	39

* Rats typed by cytotoxicity assay and MLC.
‡ Result expressed as percent of inhibition; control values are obtained after incubation with normal LEW serum.

reduced BN rosette formation by 24%. Typing of (Lew/Lew)F_2, (Lew/BN)F_2 and (BN/BN)F_2 rats showed an absence of EA blocking activity in the homozygote (Lew/Lew), while a strong inhibition was found in the BN/BN homozygote. The heterozygote (Lew/BN) showed a lesser degree of blocking. Data from one of two experiments performed with F_2 animals are shown in Table IV.

In contrast to its effects on EA rosette formation, HIS was completely ineffective in blocking EAC rosette formation by cells of the three strains tested, using the same preincubation procedure used for EA rosette inhibition (Table III).

Discussion

This study demonstrates that HIS Lew anti-BN antibodies specifically enhance renal grafts expressing the BN genotype. The administration of 0.5 ml of hyperimmune Lewis anti-BN alloantiserum (HIS) at the time of kidney transplantation greatly reduced the initial rejection crisis and insured the survival of all (Lew/BN)F_1 kidneys in Lew hosts; however, 0.1 ml enhanced only half the animals, and all such survivors exhibited a strong initial rejection episode (Fig.

1). (BF/Lew)F_1 or (ACI/Lew)F_1 kidneys transplanted in Lew hosts were not at all enhanced by Lewis anti-BN sera. The immunological specificity discerned for the MHC is in agreement with studies performed in the mouse tumor model (21).

When Lewis anti-BN HIS was absorbed with BN platelets or RBC, the enhancing properties persisted, even though little or no anti-Ag-B activity, as assessed by lymphocytotoxicity and hemagglutination, was present. These data clearly indicate that no correlation exists between the presence of classical anti-Ag-B antibodies and the passive enhancement properties of HIS, as previously thought by some authors (22), and is in agreement with other observations showing no correlation between anti-Ag-B titers and enhancing potency (23). The antibodies remaining in the PAS and RAS are directed to antigens present on lympocytes, but not on platelets or RBC. Such sera are similar with regard to low cytotoxicity levels to anti-Ia sera described in the mouse (24). These *I*-region specificities are not represented on the RBC and platelet membranes and are preferentially expressed on B cells. Unpublished experiments performed in this laboratory demonstrate that rat platelets are unable to stimulate a proliferative response when mixed with allogeneic lymphocytes, over a wide range of platelet/lymphocyte ratios, and over a prolonged time-course, suggesting also a lack of MLR determinants on rat platelet membranes. However, in the absence of congenic strains in the rat one can only suggest a strict analogy with the mouse. Although traces of anti-Ag-B may remain after absorption, this is an unlikely explanation for the enhancing activity, since a dose of HIS of one-fifth of the normal dose injected no longer enhanced grafts, while the RAS containing less than 1% of the total amount of the hemagglutinating activity originally present was fully effective. These in vivo observations are in agreement with the experiments performed in the rat heart allograft model (13) in which donor RBC-absorbed August (Ag-B^1) anti-Wag (Ag-B^2) serum was as effective as unabsorbed serum in promoting long-term cardiac allograft survival. Our data show not only that the transplant recipients become long-term survivors, but also that the early rejection episode is abrogated, additional evidence that enhancing activity is fully retained in the RAS (Fig. 1 and 3).

Two other possible explanations for the enhancing effect of absorbed HIS must be excluded before the conclusions drawn regarding anti-*I* region specificities can be considered tenable. Firstly, is the possible effect of antigen-antibody complexes formed during the absorption procedure with platelets and RBC. Thymus and spleen absorbed sera should also contain such complexes, but they do not enhance allografts. Furthermore, PAS and RAS do not block the formation of EA rosettes by lymphocytes that do not express the BN haplotype, a phenomenon which would be highly likely if immune complexes were present. Davies and Alkins (13) analyzed the IgG peak of an erythrocyte-absorbed serum (DEAE purified ^{125}I-IgG) by filtration through a Sephadex G-200 column and found no difference between the location of the IgG peak immunoprecipitated by an antirat IgG serum and that detected by isotopic markers, strong evidence against complex formation. Secondly, the PAS and RAS could contain antireceptor antibodies, Lewis anti(Lew anti-BN), which have been detected in some HIS that produce enhancement (25). Such anti-idiotypic antibodies have been re-

ported in sera obtained after hyperimmunization procedures (26); however, the HIS we have used lack such a factor (6).

When tested in vitro, the PAS as well as HIS blocked proliferation in MLC. Combinations of BN plus Lew_m and Lew plus BN_m are both blocked by the HIS, findings in agreement with results in humans (27), showing that alloantisera block either the responding or stimulating cells as long as the serum has specificity to alloantigens on one of the cell populations. When Lew cells were responding, strict specificity to BN alloantigens was discerned only at low concentrations of HIS or PAS (Fig. 4 a). High concentrations of HIS inhibited the MLC's with "unrelated" *Ag-B* haplotypes. However, with Lew as stimulator cells (Lew_m) HIS blocked only the MLC with BN-responding cells (Fig. 4 c). Therefore, the phenomenon of "nonspecific" MLC blocking at high serum concentrations involved only the stimulator cells, indicating that the cross-reactivity is directed to a target cell antigen different from the MLR recognition structure present on responding cells. For example, Lew antibodies on ACI cells which block Lew recognition of ACI do not prevent the ACI response to Lew. MLR-S antigens have recently been shown to be linked to the MHC (*Ag-B* complex) in the rat (4). They are therefore analogous to *I*-region specificities (Ia) which in the mouse are often defined as public antigens (15) and which appear to be targets for the anti-*I*-region antisera which block the MLC (28). In present studies the lack of strict strain specificity in vitro of the Lew anti-BN HIS is in contrast with the specific BN genotype specificity of in vivo enhancement. The biological relevance, therefore, of the in vitro MLC blocking at high serum concentrations is unclear. The problem may be quantitative in that very much larger doses of Lew anti-BN HIS might be able to enhance donor grafts of a different genotype; alternatively, it is likely that the cross-reacting antibodies are not directed to the critical *I*-region coded recognition-response membrane region.

Various antisera, including heterologous anti-IgG and anti-B cell sera and anti-Ly4.2, have been reported to block mouse lymphocyte Fc receptors (29). Particularly relevant, however, was the demonstration by Dickler and Sachs (16) that an anti-Ia serum raised in congenic mouse lines specifically blocked lymphocyte Fc receptor binding of IgG aggregates, while antibodies directed against the antigenic products of the *K* and *D* ends of the MHC were ineffective. This suggests that anti-Ia serum contains an antibody specific for the Fc receptor (and hence the Fc receptor is itself an Ia antigen) or alternatively that the Fc receptor and Ia are closely associated on the lymphocyte surface. In the present study, this phenomenon was investigated in the rat using EA rosette formation as an index of Fc receptor function. The results are strikingly similar to those in the mouse. PAS and RAS have properties very similar to mouse anti-Ia sera as they are poorly cytotoxic and show immunologic specificity in their capacity to block the Fc receptor. Nonspecific blockade of Fc receptors did not occur, as shown by controls using the $F(Ab')_2$ fragments of PAS (Table II). Moreover, the absence of Fc receptor blocking by the eluate from platelets used to absorb HIS shows that Ag-B antigens are analogous in this regard to the gene products of the *K* and *D* series in the mouse. However, lack of knowledge of the exact location of the *I* region in the rat (2, 3) and lack of congenic strains makes closer comparison unreasonable. The antigen recognized by the anti- "Fc-recep-

tor" antibody in these experiments appears to be genetically linked to the major Ag-B specificities detected by lymphocytotoxicity, as shown by strain specificity and its segregation with Ag-B in F_2 generations (Table IV). The possibility of serotyping for major locus linked *I*-region specificities by EA rosette inhibition is suggested by these results.

The ability of homozygous BN/BN cells to form EA rosettes was more completely inhibited by HIS than was that of heterozygous F_1 (Lew/BN) cells. This finding, of practical importance in interpreting results obtained with cells of various haplotype combinations, could be due to decreased efficiency of antibody binding when antigen density is reduced or to a degree of heterogeneity of gene expression among individual F_1 cells. These findings differ from those obtained in the mouse, using the technique of inhibition of aggregated IgG binding by anti-Ia antisera (16), in which maximal inhibition occurred with both parental and F_1 cells.

Our results, taken as a whole, indicate that alloantibodies inhibiting Fc receptor binding are directed against MHC-linked determinants different from the classical "serologically-defined" Ag-B antigens. Since antibodies to the latter are not required for enhancement, our studies parallel those in which antibodies raised by immunizing mice differing only by the *I* and *K* regions of the MHC, and absorbed with RBC, have been reported to enhance mouse skin grafts (12). In addition, it has been reported (30) that skin grafts performed in humans where there was HLA antigen identity, but mixed lymphocyte response nonidentity, survived longer (22 vs. 11.7 days) in preimmunized hosts than in nonimmunized recipients, suggesting that non-HLA antibodies can play a role in human graft enhancement.

In the light of these findings it may be possible to explain the previously mentioned report showing lack of strain specificity of enhancing alloantisera (14). DA anti-Lew serum enhanced AS_2 kidneys transplanted into DA hosts; however, the antiserum was not cytotoxic for AS_2 lymphocytes. AS_2 and Lew rats differ at the *Ag-B* locus, but share some antigenic specificity as shown by cross-absorption studies. It is possible that the shared specificities are, in light of the present data, *I*-region antigens.

Current knowledge about the mechanism of enhancement is far from complete (1); however, several observations, including those presented in this paper, suggest a tenable hypothesis. Firstly, Ia antigens are present on passenger leukocytes (31, 32) and possibly on endothelium of the graft. Secondly, the most important functional alteration of the enhanced host appears to be abrogation of its capacity to produce IgG-mediated lesions in the graft (6, 7), rather than the comparatively minor alterations of cell-mediated immunity (6, 33). This alteration of the humoral immune response of the host closely mimics the impaired IgG response seen when T–B collaboration is lacking during the induction phase of immunity (34). Thirdly, both the studies presented here and those previously mentioned in the mouse (12) suggest that antibodies directed against Ia antigens are enhancing. Fourthly, allogeneic effect factor (AEF), prepared from the supernate of mouse lymphocyte cultures, is able to boost the in vitro IgG response of allogeneic cells to antigen. This allogeneic effect is abrogated by passing the AEF-containing supernate through an anti-Ia immunoabsorbent column, suggesting identity between AEF and Ia (35).

It is therefore hypothesized that enhancing antiserum acts by virtue of its content of specific anti-"Fc receptor" (Ia) antibodies, which bind to Ia antigens present in, or coming from, the allograft and impair the allogeneic effect which normally results from graft-host contact and so decreases the IgG response which is of prime importance in the rejection reaction. This hypothesis is consistent with the available data concerning the mechanism of passive enhancement (1) and could explain how an antiserum directed against target antigens can affect the host immune response, thus unifying the old central and peripheral blockade theories regarding the mechanism of action of enhancing sera, neither of which alone have provided adequate explanation for the phenomenon.

Summary

The ability of a hyperimmune Lew anti-BN serum (HIS) to induce enhancement of (Lew/BN)F_1 kidneys transplanted into Lew recipients was compared to that of the same antiserum that had been depleted of hemagglutinating anti-Ag-B antibodies by absorption with Brown-Norway (BN) RBC-absorbed sera (RAS) or platelet-absorbed sera (PAS). The RAS and PAS were as effective as the unabsorbed HIS in abrogating early rejection as assessed by renal function and promotion of long-term survival. The absorbed sera retained the capacity to block the mixed lymphocyte culture (MLC) between Lew and BN lymphocytes and to a lesser degree the MLC between Lew and BUF, WF, AUG, and ACI lymphocytes; however, strain specificity was clearly evident at high antiserum dilutions. Similarly, these absorbed sera retained the capacity to block the Fc receptor of BN lymphocytes, and this effect was completely strain specific. In contrast, hemagglutinating and cytotoxic antibodies eluted from platelets used for antiserum absorption did not react with Fc receptors as assessed by rabbit antisheep (IgG)-coated SRBC (EA) rosette formation. $F(Ab')_2$ fragments of PAS also blocked EA rosettes. On the other hand, complement rosettes (EAC) were not inhibited by the HIS. The antibodies were therefore directed against the Fc receptor itself or a structure spatially or functionally closely related to it. Both the Fc receptors and the enhancing capacity of the antisera were strictly specific for the BN genotype. It is suggested that the anti-"Fc receptor" antibody could play an important role in the induction of enhancement by impairing host T–B collaboration as a result of its binding to graft allogeneic "Fc receptors" which appear to be analogous to the major histocompatibility complex (MHC)-coded Ia antigens of the mouse.

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