IMMUNE RESPONSE GENES CONTROLLING
RESPONSIVENESS TO MAJOR TRANSPLANTATION ANTIGENS

Specific Major Histocompatibility
Complex-linked Defect
for Antibody Responses to Class I Alloantigens*

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The experiments described in this paper are concerned with the mechanism by which major histocompatibility antigens delivered on allogeneic cells induce an antibody response in vivo. The matter is of some interest in view of the characteristic anomalies associated with alloreactivity in general. Does the response of an appropriately immunized animal reflect the outcome of an alloreaction, or of a conventional immune response in which alloreactivity plays no significant part? The issue hinges on the nature and specificity of help provided to the B cell precursors of alloantibody-forming cells. Do helper cells exist for alloantibody production in vivo, and if they do, are they specific for the immunizing alloantigen alone, for the immunizing alloantigen restricted by the *I* region products of the donor, or for the immunizing alloantigen restricted by the *I* region products of the responder? Only in the last case would one describe the cellular mechanism of the alloantibody response as conventional.

Our experiments show that the last case provides the best description of the in vivo antibody response of rats to the *RT1A*-region specific (class I, mouse H-2K/D-like) alloantigens, and we therefore conclude that for practical purposes, the alloantibody response is indeed largely, if not entirely, conventional in mechanism. This analysis is based on the discovery and properties of potent major histocompatibility complex (MHC)[1]-linked immune response (*Ir*) genes governing the response of the alloantigen immunized recipient. There is a precedent for such *Ir* genes in the early studies of Stimpfling and Durham (1) and Dorf and Stimpfling (2) on the response of B10 congenic mice to the H-2D^b private specificity H-2.2. Our own studies confirm the existence of such *Ir* genes and extend the findings to demonstrate that the level of responsiveness of the recipient is largely independent of the form in which the immunizing alloantigen is delivered. We further demonstrate that the responses to

two epitopes on the same alloantigenic molecule are controlled concordantly, and that low-responder B cells are normally unable to participate in the antibody response. To account for our findings, we locate the *Ir* gene effect to the helper mode of the immune response, and suppose that major alloantigens delivered on viable cells must normally be reprocessed by host antigen-presentation systems before the induction of helper cells.

Materials and Methods

Animals.   The rat strains used and their MHC haplotypes are listed in Table I. All the rats used were bred in our department (Iap sublines). The PVG-congenic series is described in a communication to the *Rat News Letter* (4). The properties of the *A* and *B* regions of the rat MHC have been described elsewhere (5–11). Briefly, the *A* region specifies class I alloantigenic molecules and the *B* region specifies mouse H-2Ia-like molecules and functions.

Immunizations.   For the production of alloantisera, animals were immunized by subcutaneous (s.c.) and intraperitoneal (i.p.) routes. 1 ml of a suspension of pooled spleen and lymph node cells in either Dulbecco's phosphate-buffered saline (PBS) with no added serum or Dulbecco's A and B salts solution containing 2% fetal calf serum (DAB/FCS) was distributed among five s.c. sites (2 X 0.1 ml to shoulders and flanks, and 0.1 ml to the base of the tail) and the peritoneal cavity (0.5 ml). For the demonstration of secondary allo-plaque-forming cells (allo-PFC) against RT1A^a (Table IV), 1 ml of a similar cell suspension was injected into the lateral tail vein. The doses of cells used for immunization were normally in the range of 12–50 X 10^6 pooled spleen and lymph node cells ($\frac{1}{12}$–$\frac{1}{3}$ donor equivalents) per recipient. There was no noticeable effect of dose over this range. Further details of immunization protocols are given in

TABLE I
Rat Strains Used*

Strain	MHC haplotype	
	A region	B region
AGUS	*l*	*l*
AO	*u*	*u*
AUG	*c*	*c*
DA	*a*	*a*
PVG (previously also called HO)	*c*	*c*
MHC congenic strains on the PVG background		
PVG-*RT1*^{a} (DA) N6F > 2	*a*	*a*
PVG-*RT1*^{l} (AGUS) N9F > 2	*l*	*l*
HO.B2 (AO)‡ N7F > 2	*u*	*u*
PVG-*RT1*^{u} (AO) N7F?N6F > 2	*u*	*u*
MHC recombinants		
PVG.R1 (congenic) N8F6F > 2	*a*	*c* haplotype name *r1*
R8§	*a*	*u* haplotype name *r8*

* All the rat strains described here except the R8 line are available from OLAC Ltd., Shaw's Farm, Blackthorn, Bicester, Oxon, Eng.

‡ The HO.B2 congenic strain was derived by Ford and Simmonds (3). It was further backcrossed on to the PVG background in our laboratory and the resulting line was renamed PVG-*RT1*^{u} (AO). Throughout the text, HO.B2 and PVG-*RT1*^{u} rats will not be distinguished and will all be referred to by the latter name.

§ The recombinant haplotype *r8* was recently discovered in a (DA X AO)F$_2$ population. The rat bearing it was crossed to PVG. A line homozygous for *r8* was extracted from the progeny of this cross by full sibling mating with appropriate selection. Thus, the rat stock R8 is undoubtedly heterozygous at many loci outside the MHC.

figure and table legends. A single exception to the range of immunizing doses described above was in the secondary intravenous (i.v.) immunization of the PVG + PVG-RTI^u → (PVG × PVG-RTI^u)F$_1$ chimeras (Table V), where a dose of only 10^6 spleen and lymph node cells was used. Rats were bled either by cardiac puncture or from the tail 7–11 d after immunization. All antisera were inactivated by heating at 56°C for 30 min and were stored at −20°C.

Preparation of Heat-killed Homogenized Cells. Suspensions of pooled spleen and lymph node cells in PBS were incubated for 30 min at 56°C. At this stage, the viability of the cells was assessed by Trypan Blue dye exclusion: 100% of cells examined took up the dye over a 10-min period of observation. Subsequently, the cell population was homogenized for 2 min on ice using a Polytron Homogenizer (Kinematica GMBH, Luzerne, Switzerland) at setting 4.

Complement-dependent Lymphocytotoxicity Tests. These were conducted essentially as described previously (8) except that V-bottomed microtiter plates (M25A; Sterilin Ltd., Teddington, Middlesex, Eng.) were used. In some cases, the assay was adapted to accommodate serial threefold dilution series of antiserum. Freshly prepared lymph node cells, depleted of dead cells by centrifugation over Ficoll-Isopaque solution (12) were labeled with Na$_2$^{51}CrO$_4$ and used as target cells (2.5 × 10^4 per well of microtiter plate). Titers were defined as the concentration at which the antiserum released one-half of the ^{51}Cr counts released at the cytotoxic plateau. Spontaneous release of label in the absence of serum was always <12% of the total input whereas plateau releases were always >60% of total input.

Monoclonal Antibodies Against Defined Determinants of $RTIA^a$. The four monoclonal antibodies R2/10P, R2/15S, R3/13, and R3/47 have been described in detail elsewhere (13–16). R2/10P and R3/13 define the P epitope, and R2/15S and R3/47 the S epitope of RT1A^a molecules. All molecules carrying the P epitope also carry the S epitope, and antibodies binding to the two determinants are noncompetitive. Some of the monoclonal antibody supernatants used contained mouse immunoglobulin (Ig) chains derived from the parent mouse myeloma X63. To indicate the chain composition of the supernatants used here, the letters H and L denote the rat-derived heavy and light immunoglobulin chains, G and K the X63-derived mouse heavy and light Ig chains. Monoclonal antibodies internally labeled with [^{3}H]lysine have been described elsewhere (17).

Hemolytic Plaque Assays for Allo-PFC. The principle of this assay has been described in a recent publication (18). Donors were immunized intravenously with alloantigen-bearing viable lymphocytes. Spleen cells were taken 7 d after immunization (see above), washed in DAB/FCS, and diluted in an appropriate volume of DAB/FCS. Cunningham plaque chambers were filled with a mixture containing 100 μl spleen cell suspension, 2 drops of RPMI 1640 medium, 1 drop of monoclonal antibody directed against the P (R2/10P, R3/13) or S (R3/47) epitopes of the RT1A^a molecule, 1 drop of DA erythrocytes (RBC), and 2 drops of guinea pig serum absorbed with rat RBC (two parts guinea pig serum to one part packed rat RBC) used at a concentration (from ½ to ⅐ depending on batch) at which nonspecific lysis of the RBC layer was undetectable. The drops were delivered from a 23-gauge hypodermic needle from which the bevel had been removed. Chambers were incubated for ∼1 h at 37°C and plaques were read by eye without magnification.

Hemolytic Plaque Assay for Anti-Sheep RBC (SRBC) Antibody-forming Cells. The assay was performed as described for allo-PFC except that the monoclonal antibody was replaced by RPMI 1640 and the guinea-pig serum was absorbed at a ratio of four parts serum to one part packed SRBC.

Competitive Inhibition by Antiserum of the Binding of Internally Radiolabeled Monoclonal Alloantibodies to RBC Targets. 2.5 × 10^6 strain DA RBC, were incubated at 4°C with serial dilutions of antiserum or normal serum in a total volume of 0.05 ml in V-bottomed microtiter trays. Trays were shaken periodically. After 1 h, 0.025 ml of an appropriate dilution of culture supernatant containing monoclonal anti-RT1A^a antibody internally labeled with [^{3}H]-L-lysine ([^{3}H]Lys) was added to each well. The plates were incubated at 4°C for a further 60 min. The RBC pellets were washed three times with 0.2 ml DAB/FCS, collected with a semiautomatic harvester (Otto Hiller, Madison, Wisc.) using a saline wash on to glass-fiber filters (GF/A; Whatman Biochemicals Ltd., Maidstone, Kent, England), and counted in a β-scintillation spectrometer (Packard Tri-carb 3255, Packard Instrument Co., Inc., Downers Grove, Ill.). The

[^{3}H]Lys-labeled antibody-containing supernatants used were R2/15S.HL and R3/13.HLGK prediluted in DAB/FCS to 1/500 and 1/30, respectively, relative to original supernatant.

Radiation Chimeras. (PVG × PVG-*RT1^u*)F$_1$ ♀ recipients (10–12 wk old) were given a total mid-body dose of 900-rad 16-MeV x rays from an electron linear accelerator at New Adden-brookes Hospital, Cambridge, England (MEC SL75/20; Phillips Group; Amsterdam, The Netherlands). Within 4 h of irradiation, recipients were given an i.v. injection of fetal liver cells pooled from embryos at 14–18 d gestation in 1 ml DAB/FCS. Viable cells were not counted, but the injected dose of cells was equivalent to 1–2 embryos per recipient. In the experiments described, F$_1$ recipients received either PVG, PVG-*RT1^u*, or an equal mixture of the two fetal liver suspensions. The three classes of chimeras are referred to in the text as $c \rightarrow (c \times u)F_1$ $u \rightarrow$ $(c \times u)$F$_1$ and $c + u \rightarrow (c \times u)F_1$, respectively, where c and u refer to the *RT1* haplotypes.

All chimeras were immunized with 1 ml 1% SRBC i.v. at ~6 wk after reconstitution to demonstrate restoration of T cell function (19). The chimeric status of most animals was demonstrated by appropriate cytotoxic alloantisera on peripheral blood lymphocytes before use (Fig. 5) or on spleen lymphocytes at the time of assay (Table V). In no case could cells of recipient origin be detected (<10%). In the $c + u \rightarrow (c \times u)$ chimeras, the level of reconstitution with the two parental populations was approximately equal.

Assays of Origin of Plaque-forming Cells (PFC) in Radiation Chimeras Using Cytotoxic Alloantisera. The origin of allo-PFC and anti-SRBC PFC in $c + u \rightarrow (c \times u)$ chimeras was demonstrated by elimination of PFC with specific cytotoxic alloantisera and complement essentially as described elsewhere (20, 21). As a control for nonspecific loss of PFC, suspensions were also incubated with normal type AO serum instead of antiserum. To eliminate PFC of *RT1^c* origin, the antiserum S.245 (AO × PVG.*RT1^u*)F$_1$ anti-PVG was used; to eliminate PFC of *RT1^u* origin, the antiserum S.207 (AGUS × PVG)F$_1$ anti-PVG-*RT1^u* was used. In one group, a mixture of the two antisera was used. In all cases, the final concentration of each antiserum in the incubation mixture was 1/36. At this concentration, the anti-*RT1^c* antiserum had previously been shown to eliminate 98% of allo-PFC of $(c \times u)$ origin whereas the anti-*RT1^u* antiserum eliminated 93%, compared with normal AO serum controls. After incubation with antiserum and complement, suspensions were washed and resuspended to their original volumes. Aliquots were then assayed for PFC against DA RBC and SRBC. Data are expressed in PFC/spleen derived from each suspension after serum treatment.

Results

General Features of the Immune Response to RT1A^a and RT1A^c. Our attention was drawn to the possibility that antibody responses to MHC alloantigens in the rat were significantly influenced by dominant genetic factors in the responder by the large difference in the primary and secondary anti-RT1^a lymphocytotoxic antibody titer between PVG(*RT1^c*) rats and (AO × PVG)F$_1$ hybrids (*RT1^u/RT1^c*) immunized against DA(*RT1^a*) lymphoid cells (Fig. 1A and B). Since RT1B antigens (class II) are present on only a subpopulation of peripheral lymphocytes (5, 9), it was likely that the greater part of the difference in titer was in the response to RT1A^a antigens (class I).

The relatively feeble response of the low-responder strain PVG to RT1A^a antigens was still more reduced if recipients were immunized with PVG.R1 (*RT1A^{a}B^c*) (see Table I) instead of DA(*RT1A^{a}B^a*) lymphoid cells (Fig. 1C and D). We attribute the difference in response to RT1A^a antigens with these two different immunogens primarily to elimination of allogeneic effects because of the *RT1B*-region incompatibility associated with the DA cells. Incompatibility at non-MHC loci may also contribute to the greater response against RT1A^a delivered on DA cells (e.g., "inter-molecular help" [22]). Certainly, the response of PVG against PVG.R1 is always small, whereas that of PVG to DA or to PVG-*RT1^a* can vary greatly depending on the means and frequency of immunization. Thus, antisera obtained from animals

Target Lymphocytes PVG-$RT1^a$ ($A^a B^a$)

Target Lymphocytes PVG.R1 ($A^a B^c$)
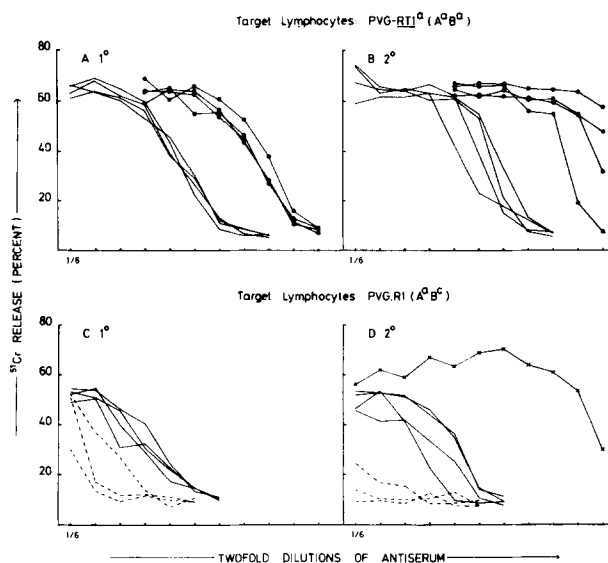
TWOFOLD DILUTIONS OF ANTISERUM

Fig. 1.   The antibody response to $RT1^a$ antigens. Influence of responder genotype, immunogen genotype and immunization protocol. (A and B) Four PVG rats (—) and four (AO × PVG)F₁ rats (●) were immunized against DA on days 0 and 32. They were bled on day 7 (A, primary response) and day 39 (B, secondary response), and the sera assayed against PVG-$RT1^a$ lymph node cells. (C and D) The sera from the four PVG rats immunized against DA (A and B) were reassayed against PVG.R1 lymph node targets (—), and compared with sera from three PVG rats immunized against (PVG.R1 × PVG)F₁ on days 0 and 39 and bled on days 11 and 48 (---). (×) Titration of an antiserum obtained from a single PVG immunized against DA on days 0 and 247 and bled on day 254. Immunizations and complement-dependent lymphocytotoxic assays on ⁵¹Cr-labeled targets were conducted as described in Materials and Methods. The final concentration of antiserum in the first well of each titration was 1:6.

primed with a skin graft can be very strong in these combinations, but not in the combination PVG anti-PVG.R1. One high-titered PVG anti-DA serum is illustrated in Fig. 1 D. This was obtained from an animal left unimmunized for 254 d between its priming and boosting injections of lymphocytes. Such anti-$RT1A^a$ responses from PVG rats immunized against DA or PVG-$RT1^a$ rule out the possibility that PVG rats lack B cells specific for determinants of the $RT1A^a$ molecules.

Because the difference between high and low responders to $RT1A^a$ was greatest after immunization with PVG.R1 cells, these cells were used as the immunogen in the genetic analysis of responsiveness that follows. Appropriate controls for differential allogeneic effects in low- and high-responder recipients of PVG.R1 cells are included in a later section of this paper.

Differential responsiveness to the $RT1A^c$ antigens of PVG was seen by immunizing low-responding PVG.R1 ($RT1A^a B^c$) or high-responding (PVG.R1 × PVG-$RT1^u$)F₁ recipients with PVG lymphoid cells. This comparison was more impressive than between DA (low) and (AO × DA)F₁ (high) responding to the same immunogen, presumably again on account of the elimination of $RT1B$-region allogeneic effects and background incompatibilities in the low-responder combination.

Genetics of Responses to $RT1A^a$ and $RT1A^c$.   19 (AO × PVG)F₁ × PVG backcross progeny were immunized against $RT1A^a$. The responses (Fig. 2 A) fell into two nonoverlapping groups in both primary and secondary responses: typing for $RT1^u$,
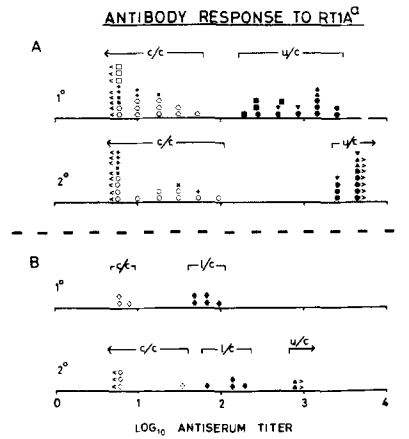
FIG. 2. The genetics of responsiveness to RT1A^a. Accumulated data of primary and secondary lymphocytotoxic titers of rats of various genotypes immunized against (PVG × PVG.R1)F$_1$ or PVG.R1. All immunizations were congenic, the only disparity between responder and immunogen being the *RT1A^a* region. Each symbol represents the response of one animal.

Responder	Symbol	Genotype	Immunogen
Panel A			
PVG	×	*c/c*	*rl/c*
(AUG × PVG)F$_1$	+	*c/c*	*rl/c*
(AO × PVG)F$_1$	▲	*u/c*	*rl/c*
(AO × PVG)F$_1$ × PVG backcross progeny	○	*c/c*	*rl/c*
(AO × PVG)F$_1$ × PVG backcross progeny	●	*u/c*	*rl/c*
(PVG × PVG-*RT1^u*) × PVG backcross progeny (N12)	□	*c/c*	*rl/c*
(PVG × PVG-*RT1^u*) × PVG backcross progeny (N12)	■	*u/c*	*rl/c*
PVG-*RT1^u/RT1^c* (N7)	▼	*u/c*	*rl/c*
Panel B			
Primary response			
(PVG-*RT1^l* × PVG)F$_1$ × PVG backcross progeny	◇	*c/c*	*rl/c*
(PVG-*RT1^l* × PVG)F$_1$ × PVG backcross progeny	◆	*l/c*	*rl/c*
Secondary response:			
(PVG-*RT1^l* × PVG)F$_1$ × PVG backcross progeny	◇	*c/c*	*rl/rl*
(PVG-*RT1^l* × PVG)F$_1$ × PVG backcross progeny (a different litter from primary response)	◆	*l/c*	*rl/rl*
(AO × PVG)F$_1$	▲	*u/c*	*rl/rl*

Secondary immunizations were performed between 29 and 39 d after the primary. Bleeds were taken between 7 and 11 d after immunization. All antisera were titrated against lymph node cells from DA donors. The symbols > and < indicate that the titer of the antiserum was greater than, or less than, that plotted. See Materials and Methods for definition of titer in these assays.

the segregating MHC of AO origin, revealed that all members of the high-responding group carried this haplotype, whereas all low responders were *RT1^c/RT1^c* homozygotes. That the *u* haplotype was determining high-responsiveness was confirmed by the finding that (PVG × PVG-*RT1^u*)F$_1$ rats made high-titered anti-A^a responses, and that in a (PVG × PVG-*RT1^u*)F$_1$ × PVG backcross again only the *u/c* animals responded well. There was no clear evidence of *Ir* genes in the genetic background either in the (AO × PVG)F$_1$ × PVG backcross, or in the response of four (AUG × PVG)F$_1$, all of which were low responders (AUG carries *RT1^c* on a different genetic background from PVG).

We also studied the effect of including the *RT1^l* (AGUS) haplotype in the responder
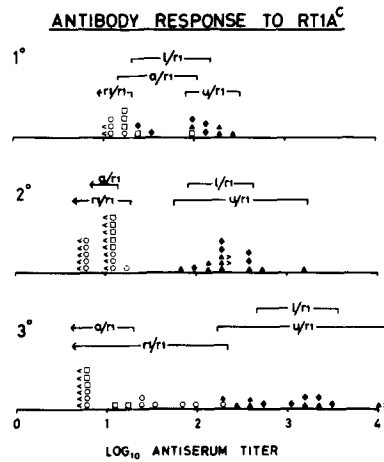
Fig. 3. The genetics of responsiveness to RT1A^c. Accumulated data of primary, secondary, and tertiary lymphocytotoxic titers of sera from various rats immunized against PVG. All immunizations were congenic, the only genetic disparity between responder and immunogen being the RT1A^c region. Each symbol represents the response of one animal.

Responder	Symbol	Genotype	Immunogen
PVG.R1	O	rl/rl	c/c
(PVG-RT1^a × PVG.R1)F$_1$	□	a/rl	c/c
(PVG-RT1^l × PVG.R1)F$_1$	◆	l/rl	c/c
(PVG-RT1^u × PVG.R1)F$_1$	▲	u/rl	c/c

Secondary immunizations were performed 21 or 25 d after the primary, and tertiary immunizations 45, or 46 d after the primary. All bleeds were taken 7 d after the appropriate immunization. All antisera were titrated against lymph node cells from PVG donors. The symbols > and < indicate that the titer of the antiserum was greater than, or less than, that plotted. See Materials and Methods for definition of titer in these assays.

genome. Progeny of an [l(AGUS)/c] × c backcross were immunized against A^a and the l(AGUS)/c heterozygous progeny were found to respond better than the c/c rats. When comparing the responses of $l(AGUS)/c$ rats with those of u/c rats, however, it was clear that the former made significantly weaker responses than the latter (Fig. 2 B).

Fig. 3 shows results of the analysis of responsiveness to RT1A^c. PVG.R1 responded poorly to PVG. Inclusion of the RT1^u or RT1^l(AGUS) haplotype in the responder produced high responsiveness. The (PVG-RT1^a × PVG.R1)F$_1$ however, was a low responder to PVG. Since all the animals involved in these experiments were congenic on the PVG background, we again conclude that the MHC of the responder governs the level of the response.

Is High Responsiveness to RT1A^a or RT1A^c a Consequence of a Strong Graft-vs.-Host Reaction (GVHR)? The data in Figs. 2 and 3 are consistent with MHC-linked *Ir* gene-control of the responses to the RT1A^a antigen, different haplotypes determining high, intermediate, or low responsiveness. It is apparent however that differential allogeneic effects could have influenced the results in some of the immunizations described. For example, when PVG.R1 (A^aB^c) or (PVG.R1 × PVG)F$_1$ (A^aB^c/A^cB^c) lymphocytes were used as immunogen in PVG (A^cB^c) or (PVG × PVG-RT1^u)F$_1$ (A^uB^u/A^cB^c) recipients, the injected cells could have made a GVHR against the A^u and B^u alloantigens of the high responder, which are absent from the low responder. (The

RTIB region encodes the strong GVHR-stimulating determinants of the rat [11].) It was possible that this GVHR caused a positive allogeneic effect in the high responder, leading to a high antibody response to the immunizing alloantigen $RT1A^a$. A similar situation could also arise in the immunization of (PVG.R1 × PVG-$RT1^u$)F$_1$ with PVG lymphoid cells.

To control for this possibility, high- and low-responder animals were immunized in combinations in which allogeneic effects were equalized (Table II). The responses of the low-responder PVG against PVG.R1 lymphoid cells were compared with the responses of the high-responder (PVG × PVG.$RT1^u$)F$_1$ against (PVG.R1 × PVG.$RT1^u$)F$_1$ lymphoid cells. Appropriate immunizations were also compared in the response against RT1A^c alloantigens. In both these cases, the residual allogeneic effects are limited to the response of the immunizing cells to the *A* region alloantigens of one haplotype of the recipient, that is, to RT1A^c in the case of immunization against RT1A^a, and to RT1A^a in the case of immunization against RT1A^c. Table II shows that the distinction between high and low responders was maintained in both immunizations in the absence of a major alloreaction against *B* region products. This experiment suggests, therefore, that the immune response difference is correctly described in terms of *Ir* genes in the responder. It is of interest to note, however, that the response of (PVG × PVG.$RT1^u$)F$_1$ to PVG.R1 appeared slightly but consistently stronger than the response to (PVG.R1 × PVG.$RT1^u$)F$_1$ leaving open the possibility that an allogeneic effect may contribute in part to the magnitude of the high response. In the same context, however, the immunization (PVG-$RT1^a$ × PVG.R1)F$_1$ anti-PVG is a typical low-response combination (Fig. 3), despite the possibility of a full haplotype GVHR (anti-A^{a}B^a) by the immunizing inoculum against the responder. Finally, it can be seen in Table II (lines iv and viii) that even the deliberate inclusion of an irrelevant full haplotype host-vs.-graft reaction in the responses PVG anti-(PVG.R1 × PVG-$RT1^u$)F$_1$ and PVG.R1 anti-(PVG × PVG-$RT1^u$)F$_1$ did not produce consistent high responsiveness, although the primary response in the latter combination was much increased. Further evidence that allogeneic effects are not responsible for our observations is provided in the next section.

From the results of the experiments described so far, we conclude that the differential responsiveness of rat strains to RT1A^a and RT1A^c alloantigens is due to MHC-linked *Ir*-genes. We shall refer to these *Ir* genes as *Ir-RT1A^a* and *Ir-RT1A^c*, respectively.

Does Ir Gene Complementation Contribute to High Responsiveness in Ir-RT1A^a? The previous experiments established that differential responsiveness to RT1A^a and RT1A^c alloantigens was controlled by MHC-linked *Ir* genes in the responder. They did not, however, enable us to distinguish between dominant high responsiveness and *Ir*-gene complementation because all high-responder animals tested were heterozygotes at the MHC. To resolve this question without introducing the complication of further allogeneic effects, we first assayed the primary responsiveness of $c \rightarrow (c \times u)F_1$ and $u \rightarrow (c \times u)F_1$ radiation chimeras to the RT1A^a alloantigens of (PVG.R1 × PVG-$RT1^u$)F$_1$ lymphoid cells. This immunization presents a single haplotype A^a incompatibility to both groups of chimeras, and minimizes graft vs. host (GVH) differences to a single A^c incompatibility in the $c \rightarrow (c \times u)F_1$ group (assuming any relevant GVH to be limited to the lymphomyeloid target from the fetal liver donor. The A^c antigens will, of course, be present in the nonlymphoid compartment of both groups

TABLE II
Experiment to Test Role of In Vivo Allogeneic Effects in Observed Immune Response Differences

Responders	Immunogen	Antiserum titer			HVG*	GVH*
		1°	2°	3°		
i. PVG	PVG.R1	<6, 17, 24	<6, <6, <6	24, 48, ‡	2 × A^a	2 × A^c
ii. (PVG × PVG-*RT1^u*)F$_1$	PVG.R1	17, 192, 1,086	2,172, 3,072, 4,344	3,072, 3,072, 3,072	2 × A^a	1 × (A^{u}B^u × A^c)
iii. (PVG × PVG-*RT1^u*)F$_1$	(PVG.R1 × PVG-1*RT1^u*)F$_1$	48, 96, 136	768, 1,086, 1,536	543, 1,536, 1,536	1 × A^a	1 × A^c
iv. PVG	(PVG.R1 × PVG-*RT1^u*)F$_1$	12, 34, 68	12, 17, 34	ND§	1 × (A^{u}B^u × A^a)	2 × A^c
v. PVG.R1	PVG	<6, <6, 68	<6, <6, <6	<6, 8, 68	2 × A^c	2 × A^a
vi. (PVG.R1 × PVG-*RT1^u*)F$_1$	PVG	96, 96, >192	34, 271, 384	384, 543, ‡	2 × A^c	1 × (A^{u}B^u × A^a)
vii. (PVG.R1 × PVG-*RT1^u*)F$_1$	(PVG × PVG-*RT1^u*)F$_1$	34, 34, 192	271, 271, 384	271, 384, 768	1 × A^c	1 × A^a
viii. PVG.R1	(PVG × PVG-*RT1^u*)F$_1$	96, >192, >192	<6, <6, 24	ND	1 × (A^{u}B^u × A^c)	2 × A^a

Three animals in each group were immunized s.c. and i.p. on days 0, 21, and 52 with 1/6 of a donor pool of spleen and lymph node cells (see Materials and Methods) and were bled on days 7(1°), 28(2°), and 59(3°).
* These columns indicate the specificity of host-vs.-graft (HVG) and graft-vs.-host (GVH) reactions for each of the listed immunizations. The gene dose of each incompatible haplotype for each HVG or GVH reaction is indicated by the multiplier.
‡ One rat died before the third bleed.
§ Not done.

of chimeras). Four $u \rightarrow (c \times u)F_1$ chimeras responded very well, whereas seven $c \rightarrow$ $(c \times u)$F$_1$ chimeras were virtually unresponsive (Fig. 4 A).

In a second experiment, we attempted to avoid allogeneic effects altogether by immunizing the chimeras with heat-killed and homogenized PVG-RTI^a lymphoid cells. Two $u \rightarrow (c \times u)F_1$ chimeras produced large secondary responses to RT1A^a whereas two $c \rightarrow (c \times u)F_1$ chimeras remained unresponsive (Fig. 4 B).

Recently, a new MHC recombinant haplotype *r8* (A^aB^u) was discovered (23) with which a conventional test of the response of the *u* haplotype to A^a could be carried out in the absence of a *B*-region disparity. PVG-RTI^u, (PVG × PVG-RTI^u)F$_1$, and PVG rats were immunized with lymphoid cells from R8 (a noninbred line homozygous for the *r8* haplotype, see Table I) and the secondary responses to RT1A^a analyzed. In Table III, it can be seen that the former two types of rat made good responses, whereas the PVG responded poorly despite, in their case, an anti-B^u disparity in the immu-
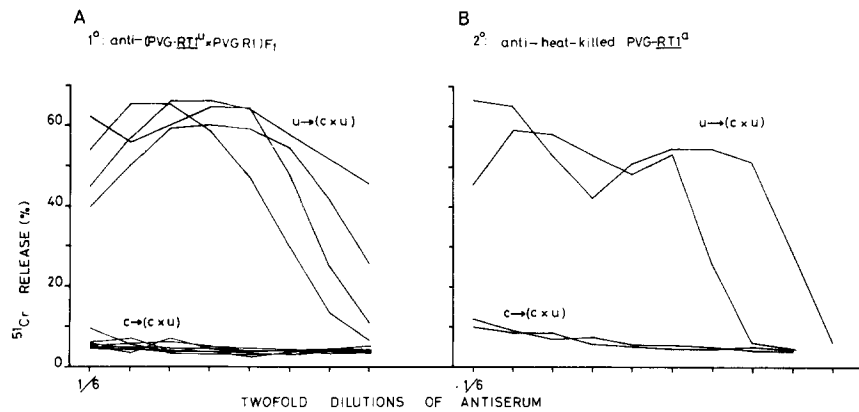

Fig. 4.   Antibody responses of $c \rightarrow (c \times u)$ and $u \rightarrow (c \times u)$ chimeras against RT1A^a. (A) A primary response: ~8 mo after their reconstitution with parental fetal liver cells, seven $c \rightarrow (c \times u)$ and four $u \rightarrow (c \times u)$ chimeras were immunized with live (PVG.R1 × PVG-RTI^u)F$_1$ spleen and lymph node cells. Bleeds were taken 11 d later and sera assayed on PVG.R1 targets. (B) A secondary response: ~7 mo after their reconstitution with parental fetal liver cells, two $c \rightarrow (c \times u)$ and two $u \rightarrow (c \times u)$ chimeras were immunized with heat-killed and homogenized PVG-RTI^a lymphoid cells. 33 d later, they were immunized in the same way, and after 7 d they were bled for serum. The sera were assayed on PVG.R1 targets. Complement-dependent lymphocytotoxicity tests were performed as described in Materials and Methods. The final concentration of antiserum in the first well of each titration was 1/6.

TABLE III

Use of the R8 Strain to Demonstrate that RTI^u per se Is a High-responder Haplotype

Responding strain	Immunogen	Secondary serum lymphocytotoxic titer
PVG	R8 (A^aB^u)	34, 96, 136
PVG-RTI^u	R8 (A^aB^u)	1,536, 1,536, 1,536
(PVG × PVG-RTI^u)F$_1$	R8 (A^aB^u)	543, 1,086, 1,536

Three animals in each group were immunized s.c. and i.p. on days 0 and 27 with 1/6 of a donor pool of R8 spleen and lymph node cells. They were bled for serum on day 34 and assayed against PVG.R1 lymphocytes.

nization. This experiment, therefore, confirms that the u haplotype, in its own right, confers high responsiveness to RT1A^a.

Ir-RT1A^a Controls the Response to Two Distinct Epitopes on the Same Molecules. Our earlier work has shown the presence of two topographically separated epitopes, P and S, on the RT1A^a molecules (14, 15). The P and S epitopes are defined by monoclonal antibodies, which we have used in two complementary assays to demonstrate that *Ir-RT1A^a* controls the level of response to both epitopes concordantly. In the first experiment, we assayed the ability of secondary immune sera from high- and low-responder donors to compete with internally labeled monoclonal antibodies for binding to the P and S sites of RT1A^a molecules on DA erythrocytes (Fig. 5). It is evident that antisera from high-responder donors had a much higher competitive inhibition titer for both monoclonal antibodies than antisera from low responders.

Our second experiment excluded the possibility that the concordant competitive inhibition titers were a result of the bulk of the antiserum being directed against an undefined site lying between P and S and capable of inhibiting the binding of both P- and S-directed monoclonal antibodies. To do this, we exploited the phenomenon
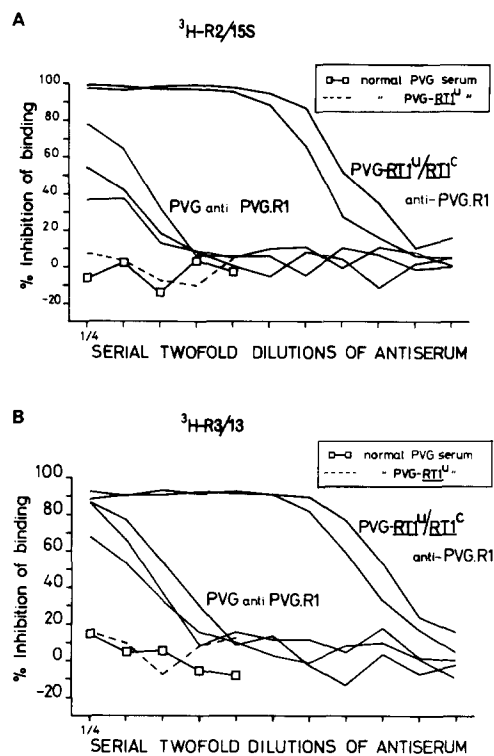

Fig. 5. Inhibition by antisera from high- and low-responder rats of the binding of internally labeled monoclonal antibodies to two different epitopes of RT1A^a molecules. The antibodies used were [^{3}H]Lys-R2/15S, which binds to the S site of RT1A^a and [^{3}H]Lys-R3/13, which binds to the P site (15). The antisera were from secondary bleeds from the three PVG rats and from the two PVG-*RT1^u/RT1^c* (N7) heterozygotes plotted on Figure 2 A. The assay was conducted as described in Materials and Methods. The final antiserum concentration in the first well of each titration was 1/4. 0% inhibition was defined from the counts in wells where medium was substituted for serum. The failure of inhibition of binding to reach 100% in B is attributed to a nonspecific component of binding present in the [^{3}H]Lys-R3/13 preparation used.

of "synergistic lysis" (15) in the development of anti-RT1A^a allo-PFC (18). Anti-RT1A^a allo-PFC from a hyperimmune spleen do not form plaques on DA erythrocyte targets because the lytic efficiency of single monoclonal IgG alloantibodies is low. However, if an excess of a monoclonal anti-RT1A^a antibody is added exogenously to the Cunningham plaque chamber, large numbers of plaques are seen. The rationale for this synergistic effect is that when two monoclonal IgG antibodies are bound noncompetitively to two different epitopes of a single molecule, activation of complement is extremely efficient. In the case of development of allo-PFC, one of the two antibodies is added exogenously, and the other is contributed by the PFC. Table IV shows the secondary anti-RT1A^a PFC responses of seven (PVG × PVG-*RT1^u*)F$_1$ × PVG backcross progeny typed for their possession of the *RT1^u* haplotype. It is clear that *Ir-RT1A^a* controls the size of the PFC responses that are synergistic with both the anti-P and anti-S antibodies. Because the phenomenon of synergistic lysis depends on noncompetition between the two antibody components, it is clear from this and the competitive inhibition assay that *Ir-RT1A^a* must control the immune response to two distinct determinants on the same molecule.

Origin of Anti-RT1A^a PFC in c + u → (c × u)F$_1$ Radiation Chimeras. Our next experiment showed that *Ir-RT1A^a* probably acts at the level of T lymphocyte-B lymphocyte collaboration. Using cytotoxic alloantisera and complement, we analyzed the origin of anti-RT1A^a allo-PFC in fully reconstituted double parental fetal liver radiation chimeras, where one parental donor was of low-responder (*RT1^c*) and the other of high-responder (*RT1^u*) genotype. To demonstrate chimerism in the population of PFC precursors, we also analyzed PFC secreting antibody to an antigen, SRBC, to which the response is not under *Ir* gene control. Table V shows that >90% of anti-RT1A^a allo-PFC were of high-responder (*RT1^u*) origin, whereas anti-SRBC PFC were

TABLE IV

Secondary Spleen Allo-PFC Responses of (PVG × PVG-RT1^u) × PVG Backcross Progeny to RT1A^a

Animal	*RT1^u*	Numbers of PFC per spleen in presence of*		
		No additional antibody	R2/10P	R3/47
Controls				
(AO × PVG)F$_1$ unimmunized	+	36 (0.2)	144 (0.8)	72 (0.4)
(AO × PVG)F$_1$ No. 1	+	950 (5)	26,600 (140)	3,800 (20)
(AO × PVG)F$_1$ No. 2	+	309 (1.6)	31,800 (165)	5,790 (30)
(PVG-*RT1^u* × PVG) × PVG backcross progeny				
Serial No. 2	−	403 (1.8)	1,120 (5)	179 (0.8)
No. 11	−	96 (0.6)	96 (0.6)	32 (0.2)
No. 12	−	63 (0.4)	94 (0.6)	63 (0.4)
No. 3	+	1,080 (5)	25,800 (120)	4,300 (20)
No. 4	+	1,160 (5)	36,000 (155)	4,640 (20)
No. 13	+	203 (1.4)	7,980 (55)	1,450 (10)

Animals were immunized by the following schedule: day 0, 1/5 donor per recipient (s.c. and i.p. injections); day 8, primary bleed; day 33, 1/5 donor per recipient (i.v.); day 40, rats killed for spleen PFC analysis. Immunizing spleen and lymph node cells came from N9 or N10 PVG-*RT1^{r1}*/*RT1^c* heterozygotes.
* Details of the plaquing techique are given in Materials and Methods. The figures in parentheses are the numbers of PFC per 10^6 spleen cells.

TABLE V

Responder Origin of Anti-RT1A^{a} PFC in Tetraparental Radiation Chimeras [c + u → (c × u)F$_1$]

Serum treatment	Percent spleen cells dead	Number of plaques per spleen vs.	
		DA RBC (percent reduction of control)	SRBC (percent reduction of control)
Rat 1			
NRS + complement (C')	ND*	20,500 (control)	69,500 (control)
anti-RT1^{u} + C'	39.6	1,750 (91.4)	29,000 (58.3)
anti-RT1^{c} + C'	39.4	19,500 (4.8)	44,000 (36.7)
anti-RT1^{u} + anti-RT1^{c} + C'	76.8	1,875 (90.8)	4,700 (93.2)
Rat 2			
NRS + C'	ND	775 (control)	61,500 (control)
anti-RT1^{u} + C'	37.3	<25 (96.8)	25,500 (58.5)
anti-RT1^{c} + C'	40.6	625 (19.4)	41,500 (32.5)
anti-RT1^{u} + anti-RT1^{c} + C'	73.6	<25 (96.8)	5,350 (91.3)

This table shows that essentially all the PFC making antibody against RT1A^{a}-S epitopes in c + u → (c × u)F$_1$ chimeras are of u (high-responder) origin. The PFC simultaneously developed against SRBC are of both c and u origin, demonstrating the chimeric state of the population of antibody-forming cell precursors.

The chimeras were immunized with (PVG.R1 × PVG-RT1^{u})F$_1$ spleen and lymph node cells (22 × 10^{6} cells s.c. and i.p.) 42 d after reconstitution, and boosted 18 d later with 10^{6} (PVG.R1 × PVG-RT1^{u})F$_1$ lymphoid cells and 1 ml 1% SRBC given i.v. in a single injection. Spleens were removed 5 d later for assay of PFC against DA RBC and SRBC as described in Materials and Methods. PFC against DA RBC were developed in the presence of the monoclonal alloantibody R2/10P.HL (anti-RT1A^{a}-P epitope).

Before incubation with antisera, PFC were developed against both target erythrocytes. Chimera 1 gave 83,000 PFC against DA and 301,000 against SRBC; chimera 2 gave 2,500 PFC against DA and 231,000 against SRBC. After incubation with normal serum and complement as described in Materials and Methods, between 23.1 and 31% of PFC were recovered against the two targets. Nonspecific loss of PFC during incubation with antiserum and complement has been reported elsewhere (20). Both chimeras were fully and approximately equally reconstituted with the two parental populations, assayed by Trypan Blue dye exclusion on spleen cell suspensions after the plaque assay and confirmed by the origin of anti-SRBC PFC.

* Not determined.

approximately one-third of *RT1^{c}* origin and two-thirds of *RT1^{u}* origin. Although the anti-SRBC response was similar in magnitude in both chimeras assayed, the anti-RT1A^{a} responses were very different, for unknown reasons. Interestingly, however, even the very small anti-RT1A^{a} response of the poorer responder was essentially exclusively mediated by *RT1^{u}* cells. The limitations of the experimental technique would not allow us to identify a very small (<10%) *RT1^{c}*-derived component in the anti-RT1A^{a} response of either chimera.

Discussion

This paper deals with the mechanisms that control antibody responses to the class I (mouse H-2K/D-like) major alloantigens of the rat. We have found that these responses are under potent, MHC-linked *Ir*-gene control. In general, immunization with viable allogeneic cells raises technical problems of interpretation because of the possible intervention of allogeneic effects, mediated by immunogen against responder or vice versa, in modulating response magnitude. A major part of this study is

therefore devoted to showing that although allogeneic effects may indeed influence response magnitude, particularly in tending to raise the response of low responders, they are unable to override the *Ir*-gene effects. Since the immune response measured was against the class I alloantigens, it was possible in appropriate strain combinations to minimize allogeneic effects in high- and low-responder combinations using viable immunogens (Table II). Finally, it was possible to show that heat-killed and homogenized allogeneic cells would immunize high responders and fail to immunize low responders.

It is known from primary amino acid sequence (24) and from sequential precipitation studies that the A region of $RT1^a$ specifies at least two class I molecules. We are not at present in a position to say whether *Ir-RT1A^a* controls the antibody response to epitopes on both molecules. Certainly, our results are compatible with this interpretation. The alternative possibility is that the differential responsiveness controlled by *Ir-RT1A^a* applied to only one of the RT1A^a molecules, whereas both $RT1^c$ and $RT1^u$ are low responders to the second molecule. The possibility that response to both RT1A^a molecules is controlled concordantly because of a high degree of structural similarity between the two class I products of the $RT1^a$ haplotype is presently under investigation.

The properties of the *Ir* genes uncovered in this study are conventional in several respects. The haplotype $RT1^c$ determines the low-response phenotype in the response to RT1A^a, whereas $RT1^u$ determines dominant high responsiveness. Similarly, the haplotype $RT1^{rl}$ determines low responsiveness to RT1A^c, whereas again $RT1^u$ determines dominant high responsiveness. There is apparently no *trans*-complementation involved in the response to RT1A^a among the alleles that we have studied. Second, *Ir-RT1A^a* controls concordantly the antibody response to two independent epitopes on the RT1A^a molecule, a result strictly analogous to the concordant control of antibody responses to poly(L-Tyr, L-Glu)-D,L-Ala--L-Lys (T,G-A--L) determinants and to dinitrophenyl (DNP) on the immunogen DNP-T,G-A--L (25). Third, in tetraparental radiation chimeras, the alloantibody against RT1A^a was made exclusively by PFC of high-responder origin, again in strict conformity with the findings of Press and McDevitt (26) in the response of mice to T,G-A--L. In view of the demonstrated T cell dependence of alloantibody responses in mice and rats (27, 28) and the absence of a B cell defect in our low-responder strain (Fig. 1) we feel entitled to conclude that the *Ir* gene controlling the response to the RT1A^a molecules determines the specificity with which T cell help is delivered to the B cell.

Because of the nature of the antigenic molecules involved in these experiments, this result is of some interest. It is now impossible not to consider that MHC-linked immune responses genes of a conventional type reflect an aspect of MHC restriction in the helper-cell compartment. The difference between low and high responsiveness in conventional systems is undoubtedly a function of the different consequences of presentation of the immunizing antigen in association with the low- and high-responder MHC allelic products. Because the *Ir* gene effects in the response to RT1A^a antigens are essentially independent of the form of delivery of the antigen, we conclude that these transmembrane molecules, even when delivered on viable cells, must be processed by the recipient and ultimately presented to the immune system on host antigen-presenting cells. In other words, the native membrane context of the immunizing alloantigens becomes irrelevant to the outcome of the immunization: the

immunizing form is determined exclusively by the host. This conclusion is similar to that proposed by Matzinger and Bevan (29) and by Korngold and Sprent (30) for MHC-restricted cytotoxic responses to minor transplantation antigens, and more recently by Weinberger et al. (31) in a study of the conditions required for immunization of in vitro cultures with the mouse class I alloantigen H-2K^k associated with lipid vesicles. In these last experiments, although the readout was anti-H-2K^k cytotoxic responses, the authors concluded that H-2K^k molecules were presented to helper cells in the system by an Ia-bearing presenting cell.

Our experiments by no means exclude the possibility that class I alloantigens can immunize for help in their native configuration on viable donor cells. For example, in the immunization of PVG-$RT1^u$ against PVG.R8, the viable immunogen carries the RT1A^a molecule as well as the $RT1B^u$ product, which we assume to be the relevant determinant for high responsiveness by analogy with other Ir genes in the rat (7). We cannot tell in this case whether the response seen is a result of antigen handling by the host or of direct presentation by the donor cells. It is unfortunate that this issue may not be easy to resolve because it would be interesting to know whether the native configuration of a transmembrane molecule capable of forming its own restricting interactions could be the same as the processed configuration in which the molecule is itself restricted by another membrane component.

It is worth remarking that the failure of PFC of low-responder origin to participate in the antibody response to RT1A^a is another clear example where MHC restriction imposed on a helper cell population at the antigen-presentation stage is also visited on the B cells at the effector stage of the response. The conservation of helper cell restriction specificity from induction to effect has now been observed in three antibody responses under Ir gene control (26, 32; and this study) and in experimentally biased responses to erythrocyte antigens in vivo (33) and in vitro (34). Why restriction at the level of induction of the B cell apparently fails in other experimental protocols (35–37) is still unexplained and will presumably remain so until the molecular basis of T-B interaction in linked-recognition systems is further clarified.

The properties of the antibody response to class I transplantation antigens described in this paper suggest that the serologically defined epitopes P and S on RT1A^a behave like structurally and topographically distinct haptens on a molecule with undefined carrier determinants. Similar considerations apply to the RT1A^c product, although in this case, distinct haptenic groups have not yet been defined. In view of the obvious antigenic complexity of class I alloantigens, it is interesting that Ir gene control of the response to these molecules should be so clear-cut and easy to demonstrate. The implication is that at least one regulatory component in the response has a range of specificity considerably narrower than the total antigenic complexity of the molecule. It is possible that there is only one effective carrier epitope on the class I molecules, and that failure to recognize this epitope is the cause of low responsiveness. Alternatively, the antigens may present a multiplicity of carrier epitopes to both high-, and low-responder strains, and Ir gene control may be through the presentation of a suppressor epitope in low-responder strains. The clearest precedent for a simple-suppressor model of Ir gene control over complex molecules comes from the properties of the N-C determinant of lysozyme, which induces a suppressive response in low-responder mice that can override help through other carrier determinants (38). In this

case, the MHC linkage of the *Ir* gene apparently results from MHC-restricted recognition of the suppression epitope in the low-responder strain (39, 40).

The experiments described in this paper identify a conventional mechanism of T cell-mediated help for antibody responses to a major transplantation antigen. We shall show[2] that apparently similar *Ir* gene control exists for the rejection of skin allografts bearing the same major antigens and for the development of specific cytotoxic T cell function in vivo. These experiments suggest that the *Ir* gene defect extends its influence to several modalities of the immune response, but in all cases, we are inclined to localize the primary defect to antigenic recognition required for the delivery of specific help to effector-cell precursors, rather than to the effector modalities themselves.

Summary

We have identified two major histocompatibility complex (MHC)-linked *Ir* genes that control the antibody responses made by rats against class I major alloantigens. We have named these genes *Ir-RT1A^a* and *Ir-RT1A^c*. These *Ir* genes determine responsiveness of the immunized animal in a typical codominant fashion. There is no evidence so far for *trans*-complementation between low-responder haplotypes. Detailed studies of *Ir-RT1A^a* indicate that it controls the antibody response to at least two distinct alloantigenic determinants on RT1A^a molecules. These class I molecules thus behave like hapten-carrier conjugates when the response against the carrier is under *Ir* gene control. Analysis of the origin of alloantibody-forming cells in tetraparental radiation chimeras indicates that *Ir-RT1A^a* must control the provision of effective help to B cells. In many respects, therefore, the properties of *Ir-RT1A^a* are broadly similar to those described for *Ir* genes controlling antibody responses to conventional antigens. The existence of apparently conventional *Ir* genes controlling the antibody response to major alloantigens strongly suggests that the processing of these transmembrane molecules by host antigen-presenting cells is a prerequisite for immune induction, and that it is the MHC of the responder rather than that of the allograft to which T helper cells are restricted in alloimmune responses in vivo.

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