THE GUINEA PIG *I* REGION

II. Functional Analysis

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In the accompanying report we have presented a structural analysis of seven Ia antigens coded for by genes in the major histocompatibility complex (MHC)[1] of the guinea pig (1). The seven Ia antigens were organized into three distinct groups each with a characteristic structure. We have previously demonstrated that an intimate association existed between *Ir* gene product function and the Ia antigens of the guinea pig MHC in that alloantisera prepared by cross-immunization of inbred strain 2 and strain 13 guinea pigs specifically blocked the activation of T lymphocytes from immune guinea pigs by antigens, the response to which is controlled by *Ir* genes (2). In the present report we will extend these studies and demonstrate that an association exists between individual Ia specificities and specific *Ir* genes. In addition, we will present evidence that antisera raised against the Ia antigens of a nonresponder animal are capable of specifically inhibiting *Ir* gene product regulated activation of cells of a responder animal that bears a cross-reactive Ia specificity which is associated with Ia molecules normally linked to the involved *Ir* gene product.

Materials and Methods

Animals. Inbred strain 2, strain 13, and outbred guinea pigs were obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md.

Antigens. A copolymer of L-glutamic acid (60%) and L-lysine (40%) (GL) with an average mol wt of 40,000 was purchased from the New England Nuclear Corp., Pilot Chemicals Div., Boston, Mass. Ovalbumin (OVA) and a copolymer of L-glutamic acid (50%) and L-tyrosine (50%) (GT), mol wt 14,500 were purchased from Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill. Purified protein derivative of tuberculin (PPD) was purchased from Connaught Medical Research Laboratory, Willowdale, Ontario, Canada. 2,4-dinitrophenyl (DNP)-GL and DNP-guinea pig albumin (GPA) were prepared as previously described (3).

Immunization of Guinea Pigs. Solutions of each antigen in saline were emulsified with an equal volume of complete Freund's adjuvant [(CFA) containing 0.4 mg/ml *Mycobacterium tuberculosis* H37Ra; Difco Laboratories, Detroit, Mich.]. Each animal received 0.1 ml of emulsion in each

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[1] *Abbreviations used in this paper:* GA, linear copolymer of L-glutamic acid, L-alanine; GL, copolymer of L-glutamic acid (60%) and L-lysine (40%); GPA, guinea pig albumin; GT, copolymer of L-glutamic acid (50%) and L-tyrosine (50%); MHC, major histocompatibility complex; NGPS, normal guinea pig serum; OVA, ovalbumin; PPD, purified protein derivative of tuberculin.

foot pad. Strain 2 animals received a total of 100 μg of DNP-GL and 100 μg of OVA; strain 13 animals and outbred guinea pigs received a total of 500 μg of GT and 1 μg of DNP-GPA.

Determination of Responder or Nonresponder Status. Responsiveness to GT was determined both by assessment of delayed skin hypersensitivity and measurement of serum antibody (4). Responder status after immunization with 1 μg of DNP-GPA was determined solely on the basis of the concentration of anti-DNP antibodies in the sera as assayed by the Farr technique with ^{3}H-ε-DNP-lysine (5).

Preparation of Anti-Ia Sera. The methods of preparation and the specific donor/recipient combinations used are described in the accompanying report (1). All antisera were sterilized by Millipore filtration and heat inactivated at 56°C for 45 min before use.

Preparation of Macrophages and Peritoneal Exudate Lymphocytes. Immunized guinea pigs were injected intraperitoneally with 25 ml of sterile mineral oil (Marcol 52; Humble Oil & Refining Co., Houston, Texas) and the resulting peritoneal exudate was harvested 3-4 days later. This cell population which consisted of 75% macrophages, 10% neutrophils, and 15% lymphocytes was used as a source of macrophages for antigen pulsing. Peritoneal exudate lymphocytes were obtained by passing the peritoneal exudate cells over a rayon wool adherence column (6).

Technique of Brief Antigen Exposure. The unfractionated peritoneal exudate cells (10×10^6/ml) were incubated for 60 min at 37°C in Hanks' balanced salt solution in the presence of 25 μg/ml of mitomycin C (Sigma Chemical Co., St. Louis, Mo.) and the appropriate concentration of antigen. The final concentrations of antigens used were: DNP-GL, 100 μg/ml; DNP-GPA, 1 μg/ml; OVA, 100 μg/ml; and PPD, 100 μg/ml. The antigen-pulsed macrophages were washed four times to remove unbound antigen.

In Vitro Assay of DNA Synthesis. Antigen-pulsed macrophages (1×10^6/ml) were mixed with an equal volume of column-purified lymphocytes (2×10^6/ml) in medium RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) containing L-glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% normal guinea pig serum (NGPS) or 5% anti-Ia serum. 0.2 ml of this cell suspension was then cultured in round bottom microtiter plates (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Arlington, Va.) for 3 days at 37°C in 5% CO_2 in air; 18 h before harvesting 1.0 μCi of tritiated thymidine (sp act 6.7 Ci/mmol, New England Nuclear Corp.) was added to each well. The cells were then harvested with the aid of a semiautomated microharvesting device and the amount of radioactivity incorporated into DNA measured (7). The results of triplicate cultures are expressed as total counts per minute per culture.

Preparation and Analysis of Radiolabeled Ia Antigens. Lymph node cells from outbred guinea pigs were internally labeled with ^{3}H-leucine in short-term culture and the radiolabeled antigens analyzed as described in the accompanying report (1).

Results

Inhibition of Strain 2 T-Lymphocyte Proliferation. In studies reported several years ago from this laboratory we demonstrated that when immune lymphocytes from $(2 \times 13)F_1$ animals were cultured in vitro, anti-2 serum inhibited the response to DNP-GL (an antigen the response to which is controlled by an *Ir* gene linked to the strain 2 MHC), while anti-13 serum inhibited the response to GT (an antigen the response to which is controlled by an *Ir* gene linked to the strain 13 MHC) (2). Because 13 anti-2 serum had since been shown to contain antibodies to two distinct Ia antigens (Ia.2 and Ia.4), we examined the relative capacity of functionally monospecific anti-Ia.2 and anti-Ia.4 sera to inhibit the proliferative response of immune strain 2 T lymphocytes. A 13 anti-2 serum which contained activity to both Ia.2 and Ia.4 produced a marked inhibition of the proliferative response to DNP-GL as well as to OVA and PPD (Table I). A monospecific anti-Ia.2 serum produced a marked inhibition of the response to DNP-GL and a modest inhibition of the response to PPD. On the other hand, a monospecific anti-Ia.4 serum had relatively little effect on the response to DNP-GL, but still produced a 40–50% inhibition of the PPD response. In general, anti-

TABLE I

Inhibition of the In Vitro Proliferative Response of Strain 2 T Lymphocytes by Anti-Ia Sera

	Antigen	Serum			
		NGPS	Anti-Ia.2,4	Anti-Ia.2	Anti-Ia.4
Exp. 1	0	4,633*	2,689	5,838	3,997
	DNP-GL	97,031	3,591	12,515	86,473
	OVA	86,827	15,762	69,858	75,862
	PPD	192,022	27,429	123,777	89,733
Exp. 2	0	7,501	3,980	5,581	9,721
	DNP-GL	77,857	5,266	21,651	62,710
	OVA	105,753	12,994	62,738	85,306
	PPD	100,751	18,501	46,682	58,302

* Results are expressed as cpm per tube; each value is the mean of three to four determinations.

Ia.2,4 serum produced a much greater inhibition of the responses to PPD and OVA than did the monospecific anti-Ia.2 and anti-Ia.4 sera.

Two Ia antigens (Ia.5 and Ia.6) have been characterized by cross-reaction and are demonstrable on both strain 2 and strain 13 cells. In strain 2 animals Ia.5 is a determinant on the same molecule as Ia.4 (1). We have not as yet been able to localize Ia.6 to a specific Ia molecule of the strain 2 guinea pig. We next evaluated the ability of antisera to these cross-reactive Ia specificities to inhibit the proliferative response of strain 2 cells (Fig. 1). Antisera to Ia.5 raised either against strain 2 cells (anti-Ia.4,5) or against strain 13 cells (anti-Ia.3,5) had a negligible effect on the response of strain 2 cells to DNP-GL, but produced a 30–40% inhibition of the response to PPD. An antiserum which contained activity to both Ia.5 and Ia.6 also produced little inhibition of the DNP-GL response. The results of these experiments confirm the studies with the monospecific Ia.2 and Ia.4 sera which demonstrate that the gene controlling responsiveness to DNP-GL is closely associated with Ia.2 and not with Ia.4. Antisera raised against determinant Ia.5 shared by the two strains which is present on the same molecule as Ia.4 fail to inhibit the DNP-GL response. Because we have not as yet identified a specific *Ir* gene linked to Ia.4, one could argue that the failure of antisera to this antigen to inhibit the DNP-GL response merely reflects a relatively low titered serum. It should be noted that all the sera used were relatively equivalent in cytotoxic titer for strain 2 cells and in general there is a strong correlation between the cytotoxic activity and the ability of an antiserum to inhibit T-cell proliferation. Furthermore, while the antisera to Ia.5 and Ia.6 only produced modest inhibition of the proliferative responses of strain 2 cells to OVA and PPD, these same sera were capable of producing marked inhibition of the proliferative responses of strain 13 cells (see below).

Inhibition of Strain 13 Lymphocyte Proliferation. 2 anti-13 serum (anti-Ia.1,3,7) produced a marked inhibition of the proliferative response of strain 13 cells to GT, DNP-GPA, and PPD (Table II). A monospecific anti-Ia.1 serum specifically inhibited the response of strain 13 cells to GT, but had relatively little effect on the response of these cells to DNP-GPA. Conversely, an anti-Ia.3 serum

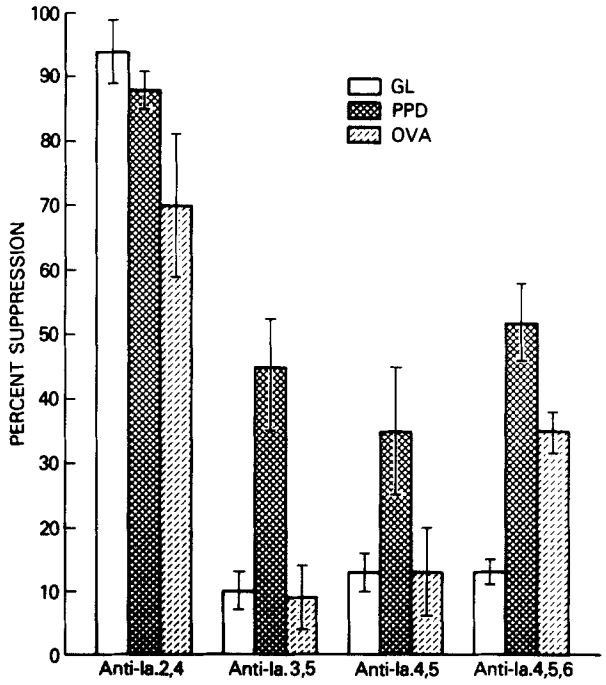

FIG. 1. Inhibition of strain 2 T-lymphocyte proliferation by anti-Ia sera. Strain 2 T cells were cultured with antigen-pulsed macrophages in the presence of 5% NGPS or anti-Ia serum. Results are expressed as: Percent suppression = 100 × [1 − (Δcpm in anti-Ia serum)/(Δcpm in NGPS)].

TABLE II

Inhibition of the In Vitro Proliferative Response of Strain 13 T Lymphocytes by Anti-Ia Sera

	Antigen	Serum			
		NGPS	Anti-Ia.1,3,7	Anti-Ia.1	Anti-Ia.3
Exp. 1	0	4,740*	6,710	6,215	2,384
	GT	58,729	24,004	16,887	52,374
	DNP-GPA	78,577	10,477	68,728	19,000
	PPD	242,133	65,233	65,729	86,988
Exp. 2	0	4,630	3,409	2,971	3,422
	GT	50,161	5,821	11,444	36,692
	DNP-GPA	60,982	6,688	42,052	10,931
	PPD	81,953	18,087	35,175	48,626

* Results are expressed as cpm per tube; each value is the mean of three to four determinations.

specifically inhibited the response of strain 13 cells to DNP-GPA but had relatively little effect on the GT response. These studies suggest a specific association exists between Ia.1 and *Ir-GT* and Ia.3 and *Ir-DNP-GPA*.

We next evaluated the capacity of antisera to the cross-reactive Ia antigens (Ia.5 and Ia.6) to inhibit the proliferative response of strain 13 cells (Fig. 2). Antisera to Ia.5 raised either against strain 2 cells (anti-Ia.4,5) or against strain

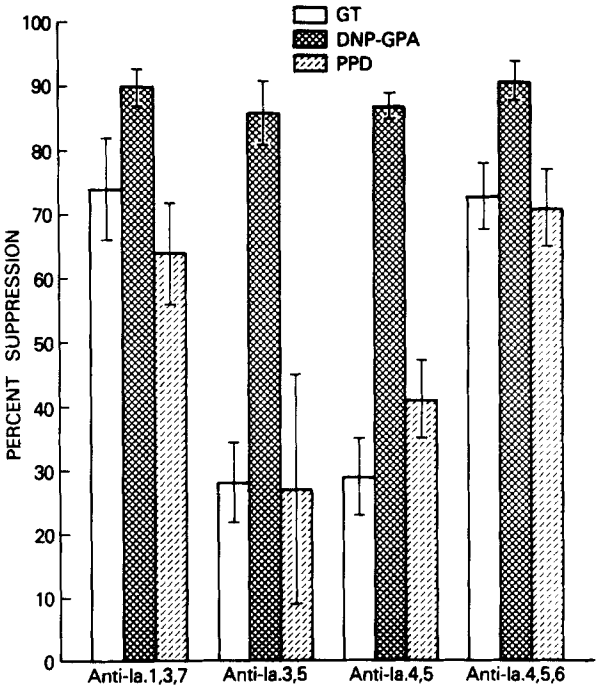

FIG. 2. Inhibition of strain 13 T-lymphocyte proliferation by anti-Ia serum. Strain 13 T cells were cultured with antigen-pulsed macrophages in the presence of 5% NGPS or anti-Ia serum. See legend of Fig. 1 for calculations.

13 cells (anti-Ia.3,5) produced a marked inhibition of the proliferative response to DNP-GPA and produced only slight inhibition of the response to GT. In contrast, an antiserum which contained activity to both Ia.5 and Ia.6 produced marked inhibition of the proliferative responses to both DNP-GPA and GT. As Ia.3 and Ia.5 are borne on the same molecule, as are Ia.1 and Ia.6, these studies suggest the specific association of Ia.3,5 and *Ir-DNP-GPA* and Ia.1,6 and *Ir-GT*. In addition these studies demonstrate that anti-Ia sera raised against the nonresponder strain 2 haplotype are capable of producing marked specific inhibition of *Ir* gene product function in cells derived from the responder strain 13 haplotype.

Ir-Ia Associations in the Outbred Guinea Pig Populations. Another approach to the demonstration of specific associations between a given *Ir* gene and a specific Ia antigen is to examine populations of outbred guinea pigs for an association between individual Ia specificities and a given *Ir* gene. 182 outbred guinea pigs were identified whose cells were lysed by anti-Ia.1,3,7. Of these 182 animals, 150 were responders to both GT and DNP-GPA ($GT^+DNP\text{-}GPA^+$), 19 were $GP^-DNP\text{-}GPA^+$, and 13 were $GT^+DNP\text{-}GPA^-$; no $GT^-DNP\text{-}GPA^-$ animal was identified in this group. When the cells from these animals were typed with a specific anti-Ia.3 serum, all the $GT^+DNP\text{-}GPA^+$ and all the $GT^-DNP\text{-}GPA^+$ animals bore Ia.3, but only one of the $GT^+DNP\text{-}GPA^-$ animals was found to be Ia.3 positive. It thus appears that the DNP-GPA *Ir* gene is closely associated with Ia.3 in the outbred as well as the inbred population.

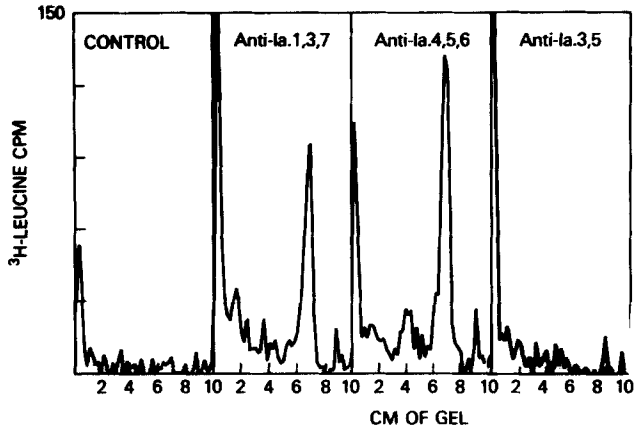

FIG. 3. Sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis patterns of Ia antigens derived from ^{3}H-leucine-labeled lymph node cells from an outbred GT$^+$DNP-GPA$^-$ guinea pig. Under nonreducing conditions, both anti-Ia.1,3,7 and anti-Ia.4,5,6 sera detect only a single peak of 26,000–27,000 daltons (Ia.1,6). No peak was seen when the extract was reacted with anti-Ia.3,5 serum.

We have been able to perform only limited serologic studies in the outbred guinea pig population with the antiserum to Ia.1 as this serum was found to contain antibodies to additional Ia specificities not present in the inbred strains. An alternative approach to demonstrate an association between Ia.1 and *Ir-GT* in the outbred population is the biochemical analysis of the radiolabeled Ia antigens from animals that are GT$^+$DNP-GPA$^-$ and GT$^-$DNP-GPA$^+$. Two animals of each type were studied in detail. An antigen preparation from ^{3}H-leucine-labeled lymph node cells from a representative GT$^+$DNP-GPA$^-$ outbred animal was analyzed with anti-Ia.1,3,7 anti-Ia.4,5,6, and anti-Ia.3,5 (Fig. 3). Reaction of the labeled cell extract with anti-Ia.1,3,7 serum and electrophoresis under nonreducing conditions detected only a single peak of 26,000–27,000 daltons; a peak with similar characteristics was detected with an anti-Ia.4,5,6 serum and no peak was seen when the extract was reacted with anti-Ia.3,5 serum. Thus, on the basis of structural characteristics, only Ia.1 and Ia.6 could be detected in the labeled extract from this GT$^+$DNP-GPA$^-$ outbred animal. Ia antigens with the molecular characteristics of Ia.7 (single component of 58,000 daltons) or Ia.3,5 (two components of 33,000 and 25,000 daltons) could not be detected in this outbred animal using serum prepared against strain 13 cells. Further evidence for the association of Ia.1 and *Ir-GT* in the outbred population was derived from an experiment where an antigen preparation from ^{3}H-leucine-labeled lymph node cells from a representative GT$^-$DNP-GPA$^+$ animal was analyzed with anti-Ia.1,7 and anti-Ia.3,5 sera (Fig. 4). The anti-Ia.1,7 serum used was contaminated with antibodies to one of the antigens (B.3) of the *B* region, the guinea pig homologue of the mouse *H-2K* or *H-2D* regions (8). As this particular outbred animal bore the B.3 antigen, a peak with the structural characteristics of a classic histocompatibility antigen (40,000 daltons) was detected in the labeled extract. In addition, on the basis of structural characteristics, Ia.7 (single component 58,000 daltons) and Ia.3,5 (two components of 33,000

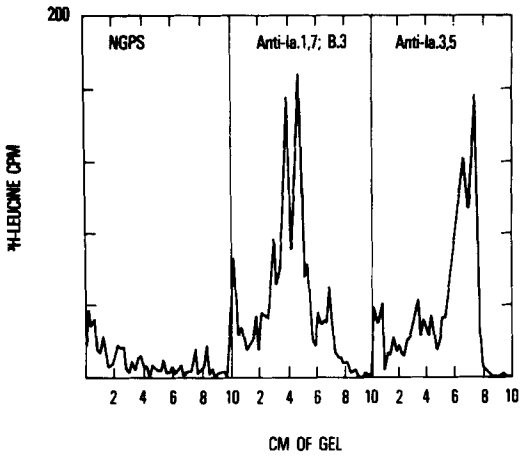

FIG. 4. Sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis patterns of Ia antigens derived from ^{3}H-leucine-labeled lymph node cells from an outbred GT$^-$DNP-GPA$^+$ guinea pig. Under nonreducing conditions anti-Ia.1,7, B.3 serum detects a peak of 58,000 daltons (Ia.7) and a peak of 40,000 daltons (B.3). Anti-Ia.3,5 serum detects two peaks of 33,000 and 25,000 daltons.

and 25,000 daltons) could be detected. A peak with the molecular characteristics of Ia.1,6 (single component of 26,000 daltons) was absent and this Ia antigen is not shared by this outbred animal and inbred strain 13 animals.

Discussion

In this report we have examined the question of whether an association exists between specific Ia antigen genes and *Ir* genes which are encoded within the same haplotype. Anti-Ia sera directed against multiple specificities of either the strain 2 or strain 13 *I* region produced a marked inhibition of the in vitro antigen-induced proliferative response both under the control of specific *Ir* genes and not under unigenic control. Functionally monospecific anti-Ia sera were selective in their inhibitory profile. Thus, an antiserum directed against the Ia.1 antigen of the strain 13 *I* region produced a marked inhibition of the proliferative response to GT and had relatively little effect on the response to DNP-GPA; an antiserum directed against the Ia.3 antigen of the strain 13 *I* region had the opposite effect and produced a marked inhibition of the DNP-GPA response, but had little effect on the response to GT. We could thus establish an association between Ia.1 and *Ir-GT*, and Ia.3 and *Ir-DNP-GPA*. Similar studies in strain 2 animals lead to an association between Ia.2 and *Ir-DNP-GL*.

The results of these studies in inbred animals were confirmed by examining the association of *Ir* genes and Ia antigens in the outbred guinea pig population. Serologic studies demonstrated that a close association existed between Ia.3 and *Ir-DNP-GPA*. Biochemical studies of the Ia antigens of GT$^+$DNP-GPA$^-$ and GT$^-$DNP-GPA$^+$ animals confirmed that an association existed between Ia.1 and *Ir-GT* in the outbred population. Thus, GT$^+$DNP-GPA$^-$ animals bore an Ia antigen also borne by inbred strain 13 animals with the structural characteristics of Ia.1, while GT$^-$DNP-GPA$^+$ animals did not bear an antigen detectable by

Ir genes	PLL, GA	?	?	STRAIN 2
Ia genes	2	4,5, (6)	(6)	GUINEA PIG
Ir genes	DNP-GPA	?	GT	STRAIN 13
Ia genes	3,5	7	1,6	GUINEA PIG

FIG. 5. Organization of the *I* region of strain 2 and strain 13 guinea pigs.

anti-Ia.1 antiserum. We have previously demonstrated a functional association between Ia.1 and *Ir-GT* and Ia.3 and *Ir-DNP-GPA* in that only macrophages from an outbred animal with the appropriate Ia antigen and *Ir* gene could present the antigen to a strain 13 T cell capable of responding to both antigens (9).

The results of the structural studies in the accompanying report (1) and the functional experiments presented here have allowed us to organize the *I* region of strain 2 and strain 13 guinea pigs into three subregions (Fig. 5). The assignment of an *Ir* gene to a given region is based on an association of that *Ir* gene with an Ia antigen in outbred populations, on the ability of anti-Ia sera to block T-cell responses controlled by that *Ir* gene, and on the association between *Ir* genes and Ia antigens in macrophage-T-cell interaction. Although we have not presented the results of experiments with the linear copolymer L-glutamic acid, L-alanine (GA), experiments of the type described in this report have demonstrated a close association between *Ir-GA* and the Ia.2 antigen of the strain 2 *I* region. The organization presented in Fig. 5 does not imply order on the chromosome or allelic assignment, but is arranged by structural homology. Thus, in strain 2 animals Ia.2 was found on a molecule in which the 33,000 and 25,000 dalton chains are probably noncovalently associated, while in strain 13 animals Ia.3,5 was found on a molecule with the same structural characteristics. Ia.4,5 of strain 2 and Ia.7 of strain 13 animals were found on molecules in which the 33,000 and 25,000 dalton chains are linked by disulfide bonds. Ia.1,6 of strain 13 animals was found on a single chain molecule of 26,000 daltons; the subregion assignment of Ia.6 in strain 2 animals has not as yet been made and the existence of this third subregion in strain 2 animals is inferred from the data obtained from strain 13 animals.

Although the mechanism of inhibition of T-cell proliferation by anti-Ia sera and the cellular site of action of these sera are as yet poorly defined, the studies with the antisera to the cross-reactive antigens Ia.5 and Ia.6 support the concept that it is antibodies to Ia antigens rather than antibodies to unique determinants of *Ir* gene products that are responsible for the inhibition of T-cell proliferation. Thus, antibodies of Ia.5 and Ia.6 which can be raised against the lymphoid cells of nonresponder strain 2 animals can specifically inhibit the response of responder strain 13 cells to DNP-GPA and GT, respectively. However, we still cannot exclude the possibility that the antisera to the cross-reactive Ia specificities might still contain activity to unique determinants of *Ir* gene products. It has been demonstrated by a number of laboratories that the response to several antigens in the mouse is under dual *Ir* gene control and that two complementary *Ir* genes must be present in order to mount a specific response (10). It is possible that strain 2 and strain 13 guinea pigs might share one *Ir* gene which controls responsiveness to GT or DNP-GPA. In such circum-

stances, anti-Ia.5 and anti-Ia.6 sera might contain antibodies to unique determinants of *Ir* gene products which do not exhibit polymorphism in the two strains.

If one can draw an analogy to the murine K and/or D antigens, one might define Ia.5 and Ia.6 as "public" specificities of Ia antigens. Thus, although antisera to these antigens are capable of producing as marked an inhibition of antigen-induced T-cell proliferation as antisera to the "private" Ia specificities, Ia.5 and Ia.6 do not appear to be the relevant determinants in certain *I*-region-mediated functions. For example, the interaction of antigen-pulsed macrophages and immune guinea pig T lymphocytes requires that macrophage and T cell be homologous for either the entire *I* region or at least one subregion. In spite of the fact that strain 2 and strain 13 animals share Ia.5 and Ia.6, no significant T-cell activation is observed when macrophages and immune lymphocytes from the two strains are co-cultured (11). This situation is analogous to the situation in the mouse where the public specificities of *H-2D* or *H-2K* molecules are not targets for cell-mediated lympholysis (12).

The existence of three *I* regions in the guinea pig MHC each coding for a protein with distinctive structural characteristics has raised the possibility that each region might perform a different function in the regulation of the immune response. At the present time, our data suggest that each *I* region performs an identical function. The regions coding for the Ia.1 and the Ia.3,5 antigens of the strain 13 *I* region have been studied in greatest detail. Antisera to either region are capable of completely inhibiting antigen-induced T-cell proliferation the response to which is under the control of *Ir* genes which map in that region. Furthermore, deficiencies of either the Ia.1 or the Ia.3 subregion do not permit effective macrophage-T-cell interaction when macrophages are pulsed with an antigen controlled by an *Ir* gene which maps to that subregion. The guinea pig MHC is unique in that at least two subregions of the *I* region appear to perform identical functions in the interaction of immunocompetent cells. In the mouse all the genes which control macrophage-T-cell or T-cell-B-cell interaction have been mapped to the *I-A* subregion of the murine MHC (13, 14), although *Ir* genes have been identified in both *I-B* and *I-C* (12, 15).

The approach we have used in this series of papers is to combine serologic, structural, and functional studies in order to further our understanding of the role of the MHC in the regulation of the immune response. This approach has allowed rapid progress to be made in the genetic dissection of the guinea pig MHC in spite of the availability of only two inbred strains and no informative recombinant animals.

Summary

We have examined whether an association exists between specific Ia antigen genes and *Ir* genes which are encoded within the same haplotype. Functionally monospecific sera to the Ia antigens of the guinea pig MHC were selective in their ability to inhibit antigen-specific T-cell proliferation and we were thus able to demonstrate an association between individual Ia specificities and specific *Ir* genes. The results of these studies in inbred animals were confirmed by examining the association of *Ir* genes and Ia antigens in the outbred guinea pig population. Of great interest was the observation that antisera made against cross-reactive Ia antigens of strains lacking specific *Ir* genes would still inhibit

immune responses of strains possessing the *Ir* gene, if the *Ir* gene was associated with that Ia antigen in the responder strain.

Received for publication 14 February 1977.

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