GENETIC CONTROL OF COMBINING SITES OF INSULIN ANTIBODIES PRODUCED BY GUINEA PIGS*, ‡

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Several reports (1–3) where large molecular weight natural-occurring antigens and markedly heterogeneous antigenic material were used have demonstrated a statistically significant relationship of genetic factors to the immune response. More recently Levine, Ojeda, and Benacerraf (4, 5) have been able to demonstrate a genetic control of the immune response of guinea pigs immunized with hapten–poly-L-lysine conjugates.

Previous evidence (6) from this laboratory demonstrated that rabbits and guinea pigs produce insulin antibodies toward several determinants on the insulin molecule. The portions of the insulin molecule toward which antibodies were produced appeared to be an individual characteristic which varied from animal to animal. It was therefore postulated that genetic factors controlled insulin antibody production. Supporting evidence (7) for genetic control of insulin antibody production demonstrated that inbred strain 2 guinea pigs produced antibodies to portions of beef insulin to which strain 13 guinea pigs could *not* produce antibodies.

Additional supporting evidence that genetic factors control the configuration of the combining sites of insulin antibodies produced by guinea pigs is presented in this communication. Data consistent with the possibility that control of the antibody-combining site configuration may involve more than one gene and not multiple alleles at a given gene locus is also presented.

Methods and Materials

Antigens.—

Imsulin: Two preparations of insulin were used in these studies. Lilly insulin (Lot 917106)[1] was a preparation of zinc crystals of beef insulin with a potency of 27.0 units/mg. Novo insulin (Lot 03355)[2] was a commercial preparation of 10 times recrystallized beef insulin with a biological potency of 22.0 units/mg.

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[2] We wish to thank Mr. H. Juncher, Novo Industri A/S, Copenhagen, Denmark, for the generous supply of 10 × crystalline beef insulin.

Alum-precipitated insulin (API) was prepared by suspending 50 mg insulin in 10 ml buffered saline (0.01 M phosphate buffer pH 7.4) and then adding 0.5 ml of 1 per cent alum, $AlNH_4(SO_4)_2 \cdot 12H_2O$.

Insulin in Freund's adjuvant was prepared by adding 1.5 ml (7.5 mg) API to 10 ml Freund's complete adjuvant (Hyland Laboratories, Los Angeles).

Antisera.—

Guinea pig antisera to novo insulin: Antisera to novo insulin were developed in adult male and female guinea pigs by injecting 0.2 ml insulin in Freund's adjuvant into each paw pad. They were desensitized 3 to 4 weeks later by the successive subcutaneous injections of 1, 10, and 100 μg API at 45 minute intervals and a second 100 μg API 1 hour later. The animals were constantly watched and 0.5 ml of 1:100,000 epinephrine was injected intraperitoneally when symptoms of anaphylaxis appeared. Following each series of inoculations, 4 ml of 50 per cent glucose was given intraperitoneally to minimize hypoglycemic reactions.

One week following the desensitization procedure 7 to 10 ml samples of blood were taken by cardiac puncture. Fourteen and 21 days postcardiac puncture the animals were again desensitized. Between 28 and 31 days postcardiac puncture, the animals were again bled. In this manner, 3 to 5 ml of antiserum were obtained from each animal at monthly intervals.

Blood samples were allowed to stand at room temperature, 25°C, 2 hours and then overnight at 4°C. The following morning the serum was separated by centrifugation (3000 *g*, 10 minutes at 3°C), divided into aliquots, tested for antibody activity, and stored at −80°C until used. Complement activity of all antisera was inactivated by incubation at 56°C for 30 minutes prior to being used.

Animals.—The inbred strain 2 (str 2), strain 13 (str 13), and F_1 hybrids (str 2 × str 13) were received from several sources.[3] The F_2 hybrids ($F_1 \times F_1$) were raised in our laboratory. The inbred animals, in all cases, originally came from the National Institutes of Health, Bethesda, Maryland; however, because of breeding difficulties, many of these animals were not strictly offspring from brother-sister matings. Consequently, there may have been some errors due to breeding of which we were not aware.

The partially inbred rabbits (AO1 and AO2) were obtained from Jackson Memorial Laboratories, Bar Harbor, Maine. They are the strain III albino rabbits which, at the time, had a coefficient of inbreeding of approximately 0.70. These animals were immunized as previously described (6).

Solutions.—

Veronal-buffered saline (VBS): The diluent used for complement and immune hemolysis titrations was veronal-buffered saline, pH 7.4 (8), containing 5×10^{-4} M $MgCl_2$ and 1.5×10^{-3} M $CaCl_2$.

Insulin: The insulin solution was made up at a concentration of 1 mg/ml. Insulin was dissolved in 0.01 M NaOH, equal to one-half the desired final volume, and diluted to final volume with 0.22 M phosphate buffer, pH 7.4. This stock insulin solution was stored at 4°C and discarded if not used within 3 days.

Complement: Adsorbed complement (C'_a) was used for the hemolytic titration of antibodies. Pools of fresh guinea pig sera were adsorbed at room temperature (25° C) for 10 minutes, while gently agitated, with 10 mg/ml of the sheep erythrocyte stroma prepared according to

[3] The authors are grateful to Dr. Charles W. McPherson, D.V.M., Chief, Animal Production Section of National Institutes of Health, Bethesda, Maryland, Dr. Roy Walford, Department of Pathology, University of California, at Los Angeles School of Medicine, Los Angeles, and Dr. Charles Cochrane, Division of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California, for supplying the majority of the animals used in these experiments.

the method of Eylar *et al.* (9). It was divided into 2 ml aliquots and stored at −80°C. C'_a preparations maintained their complement activity for at least 6 months when prepared in this manner.

Titration of complement (10): Titers of C'_a were determined by 50 per cent hemolytic end points. A unit of complement is defined as the smallest amount of C'_a which will cause lysis of 50 per cent of the sensitized cells (1 ml of a 1.25 per cent suspension) in a final volume of 1.5 ml after 30 minutes at 37°C.

Erythrocyte preparation: Sheep red cells were stored as sheep blood collected in an equal volume of Alsever's solution (11). This suspension was stored at 4°C and used for a period of 14 days.

Bis-diazo-benzidine (BDB): BDB was prepared by a modification of the method described by Kabat and Mayer (12). To prepare approximately 200 ml stock BDB, 0.92 gm benzidine and approximately 6.8 ml sodium nitrite were used. The reaction was carried out in the presence of ice crystals. The final product was a pale yellow solution. This material was empirically standardized by adding 7 ml of 0.11 M phosphate buffer pH 7.4 to 0.5 ml BDB (1:15 BDB). A deep reddish brown color developed immediately and the solution became turbid within 90 seconds (±5 seconds). In the event the solution did not immediately turn deep reddish brown, the onset of turbidity was also prolonged. A few crystals of benzidine were then added and the solution retested. When the turbidity developed rapidly (less than 80 seconds), small amounts of sodium nitrite were added to the solution. When the desired preparation was obtained, the stock BDB was divided into 0.5 ml aliquots and stored at −80°C. Under these conditions BDB was active for at least 9 months.

Preparation of insulin-sensitized erythrocytes (insulin cells): Insulin cells were prepared by conjugating insulin to the surface of washed sheep erythrocytes with bis-diazo-benzidine, as previously described (13).

Titration of insulin antibody activity: Insulin antibodies were measured by a modification of the immune hemolysis technique previously described (6). The hemolysis of insulin cells by antibodies in the presence of excess C'_a has been shown to be proportional to antibody concentration with this method. Antisera, serially diluted with VBS to a final volume of 0.5 ml, were cooled in an ice bath and 20 50 per cent hemolytic units of C'_a in 0.3 ml were then added. Finally the cells (0.2 ml of a 2.5 per cent suspension) were added and evenly suspended by gentle shaking. All samples in a given experiment were incubated in a 37°C water bath for 30 minutes, the non-hemolyzed cells separated by centrifugation at 3°C at approximately 2000 *g* for 5 minutes, and the supernatant solutions decanted into 2 ml of water. The degree of hemolysis was measured by the extinction of each sample at 580 mμ with a Beckman model DB spectrophotometer.

By measuring the area of the hemolytic titration curve, it is possible to obtain precise measurements of relative antibody concentrations (6). The hemolytic titration curve areas obtained from 5 aliquots of the same antiserum simultaneously tested were estimated by planimetry. The standard deviation calculated from the mean area of the 5 aliquots was found to be ±1.5 per cent.

Preparation of insulin-cellulose (IC) aggregate with BDB: Insulin (60 mg) was dissolved in 10 ml 0.01 N NaOH, to which 10 ml 0.22 M phosphate buffer pH 7.4 was then added. This solution was cooled in an ice bath and while constantly being stirred 0.5 ml of stock BDB was added. Theoretically this should have resulted in equimolar concentrations of insulin and BDB assuming insulin to have a molecular weight of 6000. After 20 minutes incubation, 280 mg of PABC (*p*-aminobenzyl ether of cellulose) obtained from the California Corp. for Biochemical Research, Los Angeles (cellex PAB) were added to the insulin-BDB solution. This mixture was incubated at 4°C for approximately 18 hours, with constant stirring. The PABC-BDB-insulin (IC) was then washed 6 times with distilled water. The washed IC was suspended in 3.4 ml of VBS containing 150 mg per cent bovine serum albumin (VBS-alb) and stored at 4°C.

To prepare the antibody-saturated insulin aggregate (AB_s-IC), 0.3 ml of the stock IC was diluted with 15 ml of VBS-alb, centrifuged at approximately 2000 *g* for 10 minutes at 4°C, and the supernatant discarded. Three aliquots (2 ml) of insulin antiserum were then successively incubated with IC for 15 minutes at room temperature while being shaken. Following the last incubation with the third aliquot of saturating antiserum and subsequent centrifugation, the AB_s-IC was evenly suspended in 8 ml of VBS-alb, divided into eight 1 ml aliquots, centrifuged, and the supernatants discarded. Diluted antisera (AB_t) were added to the 8 AB_s-IC aliquots to test for the presence of antibodies capable of binding to exposed antigenic determinants on the AB_s-IC.

RESULTS

Experimental Procedure.—These experiments were designed to test whether genetic factors control the configuration of the combining sites of insulin antibodies from inbred str 2, str 13 guinea pigs, the F_1 (str 2 × str 13), and F_2 ($F_1 \times F_1$) hybrids.

Differences in configuration of the insulin antibody-combining sites were discerned by measuring the percentage of antibodies in a test antiserum (AB_t) which bound to the exposed insulin determinants on an insoluble insulin complex (IC) which had been saturated with antibodies from a different antiserum (AB_s).

The details of this system have been previously described (6).

In a typical experiment an aliquot of stock insoluble insulin aggregate (IC) is saturated with antibodies from a single antiserum (AB_s) to form an insulin aggregate saturated with antibodies (AB_s-IC). Aliquots of AB_s-IC (usually 8) are each incubated with various test antisera (AB_t). If a significant percentage of antibodies in the AB_t are bound to the AB_s-IC it is assumed that the test antiserum contained antibodies which conformed to antigenic determinants to which the antibodies in AB_s could not conform. Such antibodies were considered to have combining-site configurations different from any of the antibodies in AB_s.

The validity of these experiments requires that the IC be saturated with antibodies from the saturating antiserum (AB_s) prior to the addition of the test antiserum AB_t. In all experiments, saturation of IC with AB_s was accomplished by repeatedly incubating IC with 3 fresh aliquots of AB_s. In the vast majority of experiments the IC appeared to be saturated with insulin antibodies following the second incubation. The supernatant AB_s aliquot following the third incubation very rarely showed a decrease in insulin antibodies. Saturation was not accomplished on two occasions when very low titered AB_s was employed; these results were considered not valid.

The per cent binding of AB_t to AB_s-IC was calculated as previously described (6) from the difference in area under the curve obtained by the hemolytic titration of *non*-incubated AB_t with the area under the curve obtained by the hemolytic titration of AB_t which had been incubated with AB_s-IC.

A decrease of 10 per cent or more of the immune hemolysis titer of AB_t which was incubated with AB_s-IC was attributed to antibodies in AB_t directed

to portions of the insulin molecule to which the antibodies in the saturating antiserum (AB_s) could not bind. An alternative possibility was the dissociation from AB_s-IC of antibodies which had reduced ability or were less capable of causing hemolysis of insulin cells. Conceivably, antibodies in AB_t could react with those portions of the insulin molecule from which such antibodies had

TABLE I

Similarity in Combining Sites of Insulin Antibodies from Guinea Pigs of the Same Strain

Str 2			Str 13		
AB_s	AB_t	AB_t bound	AB_s	AB_t	AB_t bound
		per cent			*per cent*
Pool*	C-12	0.5	Pool‡	D-22	0.5
	CB-2	0.5		DB-2	0.5
				DB-3	0.5
C-1	C-2	0.5			
	C-3	0.5	D-2	D-1	0.5
				D-3	0.5
C-9	Pool*	0.5			
			D-7	D-6	0.5
C-17	C-18	0.5			
	C-19	0.5	D-15	Pool‡	0.5
	C-25	0.5			
	C-26	0.5	D-32	D-15	0.5
				D-24	0.5
CB2	C-12	0.5			
			D-B2	Pool‡	0.5

* This pool was a combination of approximately equal aliquots of antisera from 7 str 2 guinea pigs.

‡ This pool was a combination of approximately equal aliquots of antisera from 5 str 13 guinea pigs.

dissociated resulting in a net decrease in the immune hemolysis titer of incubated AB_t. Evidence has been presented (6) demonstrating that decreased immune hemolysis titers of incubated AB_t are *not* due to dissociation of insulin antibodies from AB_s-IC during the period of incubation with the test antisera.

Under these circumstances the following criteria were considered necessary to support the postulate that genetic factors control the configuration of the insulin antibody-combining sites in guinea pigs:

1. No differences in configuration of antibody-combining site should be demonstrable in guinea pigs of the same strain; *i.e.*, str 2 AB_t should not bind to str 2 AB_s-IC or str 13 AB_t should not bind to str 13 AB_s-IC.

2. Differences in configuration of antibody-combining site should be demonstrable when antisera from str 2 guinea pigs are compared to antisera from str 13 guinea pigs.

3. Segregation of such differences in configuration of antibody-combining sites should be demonstrable when insulin antibodies from hybrid guinea pigs (F_1 and F_2) were compared to insulin antibodies from str 2 and str 13 parents.

TABLE II

Overlap of Combining Sites of Insulin Antibodies from Str 2 and Str 13 Guinea Pigs

Str 2	Str 13 AB_t (9)*		Str 13	Str 2 AB_t (9)*	
AB_s- IC	No. Exp.	No. Bound‡	AB_s-IC	No. Exp.	No. Bound‡
6	14	0	6	13	0

* Designates the number of different antisera employed.

‡ Designates the number of test antisera in which 10 per cent or more of the insulin antibodies bound to exposed antigenic sites on AB_s-IC.

TABLE III

Configurational Differences of Combining Sites of Insulin Antibodies Produced by Str 2 (C) and Str 13 (D) Guinea Pigs

Guinea pig AB_t	Per cent of AB_t bound to AB_s-IC saturated with:	
	AO1*	AO2*
C/D‡	95/0	99/0
C17/D15	70/0	57/0
C18/D24	41/4	35/0
C19/D32	30/5	36/0
C25	26	23
C26	54	59

* AO1 and AO2 are 2 different individual insulin antisera from partially inbred rabbits.

‡ Str 2 (C) and Str 13 (D) antiserum pools previously described, see Table I.

Similarity in Combining Sites of Insulin Antibodies Produced by Guinea Pigs of the Same Strains.—Table I summarizes the results observed in a group of experiments in which str 2 antisera were tested against insulin aggregates saturated with str 2 antibodies and str 13 antisera were tested against insulin aggregates saturated with str 13 antisera.

Ten experiments were performed testing the binding of insulin antibodies from 8 individual and 1 pooled str 2 antisera (AB_t) to insulin aggregates which had been saturated with antibodies from 4 individual and 1 pooled str 2 antisera (AB_s-IC). None of the 9 str 2 test antisera (AB_t) contained demonstrable antibodies with combining sites different than the antibodies in the 5 str 2 (Table I).

Likewise, in 10 experiments none of 9 test antisera (AB_t) from str 13 guinea pigs contained significant amounts of insulin antibodies capable of binding to AB_s-IC saturated with insulin antibodies from 6 str 13 (Table I).

Because of the shortage of inbred animals it was necessary and also considered desirable to use a pooled str 2 and a pooled str 13 antiserum in these experiments. The str 2 pool was made up of approximately equal aliquots of antisera

TABLE IV

Combining Site Overlap of Insulin Antibodies Produced by F_1 Offspring Compared with Antibodies Produced by Str 2 and Str 13 Guinea Pigs

F_1 AB_t *vs.* Str 2 AB_s-IC			F_1 AB_t *vs.* Str 13 AB_s-IC		
Str 2 AB_s	F_1 AB_t	AB_t-bound	Str 13 AB_s	F_1 AB_t	AB_t-bound
		per cent			*per cent*
Pool*	40	6.0	Pool‡	40	0.5
	41	0.5		41	0.5
	43	3.5		43	0.5
	44	0.5		44	0.5
	45	0.5		45	0.5
	46	0.5		46	38.2←
	47	0.5		47	0.5
	48	0.5		48	0.5
	53	0.5		53	0.5
CB2	41	0.5	DB2	41	0.5
	44	0.5		44	0.5
	45	0.5		45	0.5
	46	0.5		46	59.0←
				48	0.5

← Indicates significant binding.

* This pool was a combination of approximately equal aliquots of antisera from 7 str 2 guinea pigs.

‡ This pool was a combination of approximately equal aliquots of antisera from 5 str 13 guinea pigs.

from 7 guinea pigs and the str 13 pool was made up of approximately equal aliquots of antisera from 5 guinea pigs.

These experiments indicate that inbred guinea pigs of the same strain produce antibodies toward similar determinants on the insulin molecule. Consequently, it is probable that the configurations of the combining sites of insulin antibodies produced by guinea pigs are similar in animals of the same strain.

Configurational Differences of Combining Sites of Insulin Antibodies from Str 2 and Str 13 Guinea Pigs.—Differences in combining sites of insulin antibodies from str 2 guinea pigs tested against insulin aggregates saturated with antibodies from str 13 guinea pigs could *not* be demonstrated. Nor were differences in

combining sites of antibodies from str 13 guinea pigs noted when tested against AB_s-IC saturated with antibodies from str 2 guinea pigs (Table II).

In 14 experiments where 9 str 13 antisera were tested against insulin aggregates saturated with 6 different str 2 antisera, none of the str 13 antisera contained significant amounts of antibodies capable of binding to insulin aggregates saturated with antibodies from str 2 antisera. Likewise, in 13 experiments, with 9 str 2 AB_t, *no* demonstrable antibodies were noted which were capable of binding to AB_s-IC saturated with 6 str 13 AB_s. Therefore, in 27 experiments, differences between str 2 and str 13 could not be demonstrated directly (Table II).

Configurational differences of insulin antibody combining sites between str 2 and str 13 guinea pigs were noted only when tested against 2 insulin aggregates, each saturated with antibodies from one of 2 partially inbred rabbits (A01 and A02) Table III. In 12 experiments, each of 6 different str 2 antisera contained antibodies which bound (25 to 95 per cent) to portions of the insulin molecule to which antibodies from neither of the 2 rabbits could bind. Whereas, in 8 experiments, none of the str 13 antisera contained significant (10 per cent or more) amounts of antibodies which could bind to either of the 2 insulin aggregates saturated with antibodies from the partially inbred rabbits. Therefore, str 2 guinea pigs produce antibodies to portions of the insulin molecule to which str 13 guinea pigs cannot produce antibodies.

The results suggest that str 2 guinea pigs produce antibodies to antigenic sites on insulin which are in close proximity to the binding sites of the antibodies produced by str 13 guinea pigs. The inability to demonstrate binding of antibodies from str 2 guinea pigs to insulin aggregates saturated with str 13 antibodies and *vice versa* is probably best explained by overlap of the respective antigenic determinants and interference due to steric hindrance. The determinants on insulin of antibodies from the partially inbred rabbits are probably in close proximity to the determinants of the antibodies from str 13 guinea pigs but more distant from the determinants of antibodies produced by str 2 guinea pigs. Consequently, antibodies in AB_t from str 13 animals did *not* bind and antibodies in AB_t from str 2 animals did bind to AB_s-IC saturated with antibodies from partially inbred rabbits. It therefore is likely that the configurations of the combining sites of a significant percentage of insulin antibodies in str 2 antisera are different than the configurations of the antibodies in str 13 antisera.

Combining Site Overlap of Insulin Antibodies Produced by F_1 Offspring Compared to Antibodies Produced by Str 2 and Str 13 Guinea Pigs.—Twenty-seven experiments employing 9 individual F_1 antisera were done against 2 AB_s-IC saturated with 2 str 2 AB_s and also against 2 AB_s-IC saturated with 2 str 13 AB_s (Table IV). One of the str 2 AB_s was a pool made up of antisera from 7 animals, and one of the str 13 AB_s was a pool made up of antisera from 5

animals. One F_1 antiserum (46) contained significant amounts of antibodies with combining sites different than the antibodies in both str 13 AB_s. This same antiserum (46) did *not* contain insulin antibodies with combining sites different than the two str 2 AB_s. One other F_1 animal (40) contained (6 per cent) a few (not significant) antibodies which combined with insulin sites different from

TABLE V

Configurational Differences of Combining Sites of Insulin Antibodies Produced by F_1 Animals Compared to Antibodies Produced by Str 2 and Str 13 Guinea Pigs

F_1 AB_t *vs.* Str 2 AB_s-IC			F_1 AB_t *vs.* Str 13 AB_s-IC		
Str 2 AB_s	F_1 AB_t	AB_t-bound	Str 13 AB_s	F_1 AB_t	AB_t-bound
		per cent			*per cent*
Pool*	62	63.0←	Pool*	62	42.2←
	63	0.5		63	3.5
	64	49.0←		65	0.5
	65	0.5			
	66	0.5			
	67	0.5	D15	61	1.6
				62	60.8←
				63	2.2
C14	61	39.5←		64	99.0←
	62	80.1←			
	63	0.5			
	64	71.1←	D25	61	2.6
				62	75.1←
				63	2.0
				64	88.7←
			DB2	64	39.0←

← Indicates significant binding.

* See footnotes Table I.

those saturated with str 2 antiserum pool. Therefore, in 24 of 27 experiments, antibodies in the F_1 antisera conformed to determinants on the insulin molecule in close proximity to the determinants with which the antibodies in str 2 or str 13 antisera conformed. It is not known whether the significantly different combining sites of the antibodies in the one (46) F_1 antiserum is a true characteristic of F_1 antisera or whether an unrecognized error in breeding may have taken place. It appears, therefore, that the great majority (possibly all) of insulin antisera from F_1 (str 2 $\times$ str 13) hybrids do not contain discernable antibodies capable of binding to exposed determinants on insulin aggregates saturated with antibodies produced by str 2 or str 13 animals. Consequently, the configuration of the insulin antibody-combining sites of F_1 animals is not sufficiently different

from the configuration of antibody combining sites from str 2 and str 13 animal to be demonstrable in these experiments.

Configurational Differences of Combining Sites of Insulin Antibodies Produced by F_2 Animals Compared to Antibodies Produced by Str 2 and Str 13 Guinea Pigs.—Segregation of the differences in antibody-combining site configuration was noted when F_2 ($F_1 \times F_1$) antisera were tested against AB_s-IC saturated with str 2 and str 13 antisera (Table V). Such differences were noted in 11 of 22 experiments in which 7 F_2 AB_t were tested against 2 str 2 AB_s-IC and also against 4 str 13 AB_s-IC (Table V). The pooled str 2 AB_s and str 13 AB_s used in

TABLE VI

Overlap of Insulin Antibody-Combining Sites of F_1 and F_2 Hybrid Guinea Pigs Compared to Antibody-Combining Sites of Related Animals

No. F_1 AB_s-IC	Str. 2 AB_t (4)*		Str 13 AB_t (5)*		F_2 AB_t (6)*	
	No. Exp.	No. Bound‡	No. Exp.	No. Bound‡	No. Exp.	No. Bound‡
4	7	0	7	0	11	0
No. F_2 AB_s-IC	No. Str 2 AB_t (4)*		Str 13 AB_t (4)*		F_1 AB_t (9)*	
	No. Exp.	No. Bound‡	No. Exp.	No. Bound‡	No. Exp.	No. Bound‡
5	10	0	10	0	18	0

* Designates the number of different test antisera employed.

‡ Designates the number of test antisera in which 10 per cent or more of the insulin antibodies bound to exposed antigenic sites on AB_s-IC.

these experiments were aliquots of the same pooled antisera previously described.

Two (62 and 64) of the F_2 AB_t contained a significant percentage of insulin antibodies with combining site configurations different from the antibodies in str 2 and also str 13 antisera. One (61) of the F_2 AB_t contained insulin antibodies with combining site configurations different from the str 2 antibodies but similar to the str 13 antibodies. The remaining 4 (63, 65–67) F_2 antisera contained insulin antibodies to determinants which overlapped the determinants to which the insulin antibodies in both str 2 and str 13 AB_s were directed.

It appears therefore, that F_2 offspring are capable of producing antibodies directed to portions of the insulin molecule to which neither of the inbred grandparents can make antibodies. These findings, therefore, are consistent with the possibility that the configuration of the antibody-combining site in guinea pigs is controlled by more than one gene and not by different alleles at a given gene locus.

Overlapping of Insulin Antibody-Combining Sites of F_1 and F_2 Animals Com-

pared to Antibody-Combining Sites of Related Animals.—The combining sites of insulin antibodies engendered in F_1 (str 2 × str 13) guinea pigs were found to differ from the combining sites of antibodies from inbred parents when the F_1 antisera were used to saturate insulin aggregate (Table VI). Str 2 and str 13 AB_s overlapped most F_1 AB_t (Table IV), and only half of the F_2 AB_t (Table V). In these experiments, 4 different F_1 AB_s-IC's were prepared and tested against 4 str 2, 5 str 13, and 6 F_2 test antisera (AB_t) in a total of 25 experiments, Table VI. F_1 animals apparently produced antibodies which overlap or sterically hinder the binding of insulin antibodies from both inbred parents and also their hybrid offspring (F_2). In this regard, the antibodies produced by F_1 animals are different from the antibodies produced by str 2 and str 13 parents. Most

TABLE VII

Insulin Antibody-Combining Sites of F_1 and F_2 Hybrid Guinea Pigs Compared to Antibody-Combining Sites from Other F_1 and F_2 Animals Respectively

No. F_1 AB_s-IC	F_1 AB_t (9)*		No. F_2 AB_s-IC	F_2 AB_t (6)*	
	No. Exp.	No. Bound*		No. Exp.	No. Bound*
4	19	1‡	5	12	2§

* See footnotes, Table VI.

‡ Antibodies (13 per cent) from F_1 AB_t (46) bound to AB_s-IC saturated with F_1 AB_s (43).

§ Antibodies (26 per cent) from F_2 AB_t (64) and also antibodies (21 per cent) from F_2 AB_t (67) both bound to AB_s-IC saturated with F_2 AB_s (65).

(possibly all) F_1 animals produce antibodies which are not capable of binding to AB_s-IC saturated with str 2 or str 13 AB_s (Table IV). Whereas, about half of F_1 animals produce antibodies capable of binding to AB_s-IC saturated with str 2 or str 13 AB_s (Table V). None of 6 F_2 AB_t contained antibodies which bound to any of 4 F_1 AB_s-IC in 11 experiments (Table VI). It appears, therefore, that the F_1 hybrid antibodies have combining sites which conform to more determinants on the insulin molecule than both of the inbred parents. However, the combining sites of antibodies produced by F_1 animals are not sufficiently different to be capable of binding to either str 2 or str 13 AB_s-IC.

A total of 38 experiments were performed with 5 F_2 AB_s-IC and AB_t from 4 str 2, 4 str 13, and 9 F_1 guinea pigs (Table VI). The F_2 antisera were capable of overlapping and preventing antibodies of all the AB_t tested from reacting with antigenic sites on the insulin molecule. Since the F_2 animals produce antibodies which react with both str 2 and str 13 AB_s-IC (Table V), it would appear that F_2 offspring can make antibodies which conform to a greater number of antigenic sites on the insulin molecule than antibodies produced by F_1 animals.

It appears, therefore, that the variations in configurations of the insulin antibody-combining sites of F_1 animals are intermediate in comparison with F_2 animals.

These observations are considered to be additional evidence that genetic factors control the configuration of the insulin antibody-combining sites.

Insulin Antibody-Combining Sites of F_1 and F_2 Hybrid Guinea Pigs Compared to Antibody-Combining Sites from Other F_1 and F_2 Animals Respectively.—Nineteen experiments were performed in which 9 F_1 AB_t were tested against 4 F_1 AB_s-IC. One F_1 AB_t (46) antiserum contained significant amounts of antibodies with combining site configurations different than the F_1 AB_s (43) used to saturate the IC (Table VII). It is interesting that this is the same *and only* F_1 animal (46) whose antiserum contained significant amounts of antibodies with combining site configurations different than inbred str 13 animals (Table IV). It is possible that the combining site differences in the antibodies from this F_1 (46) guinea pig are a true characteristic of F_1 antisera. It is probably more tenable in view of the marked disparity with respect to all the other 8 F_1 animals to tentatively explain the unique characteristics of F_1 (46) antibodies as due to an unrecognized error in breeding. In which case it would be likely that the configurations of the combining sites of insulin antibodies amongst F_1 animals are similar to each other. It is anticipated that with additional F_1 animals this tentative explanation will be substantiated.

Twelve experiments were performed where F_2 antisera were compared with each other (Table VII). Two different AB_t (F_2 64 and 67) were found to have antibodies with combining site configurations different from one F_2AB_s (65). Such differences were not demonstrable in the remaining 10 experiments. In this regard, it is interesting that such differences were found in 4 of 21 experiments with mongrel guinea pigs (6).

Much larger groups of F_1 and F_2 animals must be tested to establish similarity of insulin antibody-combining sites amongst F_1 animals and differences amongst F_2 animals. With the exception of one of the 31 experiments performed, the results observed here are consistent with the other results which have been presented.

DISCUSSION

No configurational differences of antibody-combining sites were noted in guinea pigs of the same strain (Table I). Configurational differences in insulin antibody-combining sites were noted between str 2 and str 13 guinea pigs (Table III). Segregation of differences in antibody combining site configurations was noted in F_1 and F_2 guinea pigs (Tables V and VI). It is therefore, quite probable that genetic factors control the configuration of insulin antibody-combining sites in guinea pigs.

Furthermore, insulin antibody-combining sites from F_2 animals can have configurations that are different from the configurations of insulin antibodies noted in either of the 2 inbred parental strains (Table V). It is therefore also

possible that the configurations of the combining sites of insulin antibodies produced in guinea pigs is controlled by more than one gene and not by multiple alleles at a given gene locus.

It is possible that these configuration differences of the F_2 antibodies may be due to segregation of residual heterozygosity of the parental inbred str 2 and str 13. No significant quantitative or qualitative differences were noted within either of these strains (Table I). Consistent quantitative differences, however, were noted in the amounts of antibodies amongst the various str 2 AB_t that bound to both AB_s-IC saturated with partially inbred rabbit antisera. Whether such quantitative differences are an expression of residual heterozygosity may possibly be discerned by comparisons within and between different familial lines of the inbred str 2 and str 13 animals.

Considerable evidence recently reviewed by Edelman and Gally (14) suggests that the configuration of the antibody-combining site of gG is formed by sequences of amino acids in contiguous portions of both the light (λ) and heavy (γ) chains. In which case, it would be likely that at least 2 genes are involved in dictating the configuration of the antibody-combining site of gG.

The results presented here are in one way or another probably an expression of all the immunoglobulins in the antisera which are employed. Sufficient animals have not been used to estimate the number of genes involved in determining the configuration of the combining sites of the guinea pig insulin antibodies. It is therefore unwarranted to relate these results to the well documented (15) structure of human and rabbit gG.

These experiments are concerned only with the binding of antibodies to insulin. It would be presumptive therefore to speculate at which stage or stages of immunization the gene or genes involved influence the insulin antibody-combining site differences which have been demonstrated in these experiments.

SUMMARY

1. Genetic factors control the configuration of combining sites of guinea pig insulin antibodies.

2. It is possible that the configuration of the combining sites of guinea pig insulin antibodies is controlled by more than one gene and not by multiple alleles at a given gene locus.

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