INSULIN ANTIBODY VARIATIONS IN RABBITS AND GUINEA PIGS AND MULTIPLE ANTIGENIC DETERMINANTS ON INSULIN*, ‡

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Previous studies (1) demonstrated *in vivo* neutralization of insulin by guinea pig antisera but no neutralization by rabbit antisera. These observations suggested that rabbit antibodies were directed to antigenic determinants on insulin which were different from the determinants to which guinea pig antibodies were directed. This possibility was tested by measuring the ability of antibodies in one antiserum to bind to an insoluble insulin complex saturated with antibodies from a different antiserum. Rabbit antisera tested in this manner contained antibodies which bound to insulin sites to which guinea pig antibodies could not bind. More rarely, certain guinea pigs produced antibodies which bound to insulin sites to which rabbit antibodies could not bind. Similar antibody differences were noted within each of these two species, *i.e.*, rabbits produced antibodies which bound to insulin sites to which other rabbit antibodies could not bind, and occasional guinea pigs produced antibodies which bound to insulin sites which other guinea pig antibodies could not bind.

Studies with I^{131}-labeled antisera show that the antiserum differences noted are caused by insulin antibodies directed to different determinants and not because of differences in relative dissociation of antibodies from the same determinant on the insulin molecule.

These results demonstrate that more than one antibody molecule can simultaneously react with an insulin molecule. Implicit within these observations is the probability that insulin has a multiplicity of antigenic determinants. The determinants to which insulin antibodies are directed appear to be a characteristic of each animal immunized. It is postulated therefore that genetic factors direct production of antibodies toward specific antigenic determinants when insulin is the antigen.

Materials and Methods

Antigens

Insulin.—Three preparations of insulin were used in these studies. Lilly insulin (Lot 719106)[1] was a preparation of zinc crystals of beef insulin with a potency of 27.0 units/mg. Some hyperglycemic factor was present in this preparation. Novo insulin (Lot HPA61)[2] was a preparation of zinc crystals of pork and beef insulin (80 per cent pork and 20 per cent beef) with a potency of 22.9 units/mg. Novo insulin (Lot 03355) was a commercial preparation of ten times recrystallized beef insulin with a biological potency of 22.0 units/mg.

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).

Antisera

Rabbit Antisera to Lilly and Novo Insulin.—Antisera to Lilly and Novo insulin were developed in adult, male and female, New Zealand white rabbits by injecting 0.2 ml insulin in Freund's adjuvant into each toe pad. 3 to 4 weeks later 0.5 ml of intravenous API was given as a booster and followed by another 0.5 ml within 48 to 72 hours. A sample of blood (3 to 4 ml) for testing was taken within 7 to 19 days following the second booster.

When high levels of antibody were observed, 50 ml of blood was taken from the marginal ear vein. When antibody levels were low, the booster injections were repeated and another sample of blood was obtained and tested. Following all antigen inoculations, the rabbits received 30 ml of 50 per cent glucose subcutaneously to minimize hypoglycemic reactions. Infections were minimized by adding 125 mg terramycin to 100 ml of the glucose solution.

Blood samples were allowed to stand at room temperature (25°C) for two hours and then stored overnight at 4°C. The following morning the serum was separated by centrifugation at 3,000 g for 10 minutes. Insulin antibody level of the serum was then determined, divided into aliquots, and stored at −80°C until used.

Rabbit antisera were inactivated at 56°C for 30 minutes and adsorbed for 10 minutes at room temperature with 10 mg of formalin-treated lyophilized sheep erythrocytes per ml.

Guinea Pig Antisera to Lilly and Novo Insulin.—Antisera to Lilly and Novo insulin were developed in adult male and female mongrel 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 μg, 10 μg, 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.

1 week following the desensitization procedure, an animal was selected at random and a 7 to 10 ml test sample of blood was taken by cardiac puncture. If an adequate antibody titer was observed, all the guinea pigs which had been immunized were anesthetized with intraperitoneal nembutal (0.5 ml of 60 mg/ml), and exsanguinated from the carotid artery within

1 to 2 days after the test sample was obtained. Guinea pig antisera were treated in the same manner as the rabbit antisera except that they were not adsorbed with sheep erythrocytes.

Solutions

Veronal-Buffered Saline (VBS).—The diluent used for titrating complement and insulin antisera was VBS pH 7.4 (2) containing 5×10^{-4} M $MgCl_2$ and 1.5×10^{-3} M $CaCl_2$ per liter.

Insulin.—Insulin was dissolved in 0.01 N NaOH equal to one-half the desired volume. To this was added an equal volume of 0.22 M phosphate buffer pH 7.4. Insulin solutions were stored at 4°C and discarded 3 days after preparation.

Complement.—Adsorbed complement (C_a') was used for the hemolytic titration of insulin antibodies. Pools of fresh guinea pig sera were adsorbed with 10 mg/ml of the S_2 fraction of sheep erythrocyte stroma (3) at room temperature (25°C) for 10 minutes while gently agitated and then centrifuged at approximately 2,000 g at 3°C for 15 minutes.

An aliquot was tested for complement activity and the remainder divided into 2 ml aliquots quick frozen in a dry ice–acetone bath 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 (C_a') (4–7).—All titrations of C_a' were carried out at 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 sensitized cells (1 ml of 1.25 per cent suspension) in a final volume of 1.5 ml after 30 minutes at 37°C.

Sheep Erythrocytes.—Sheep erythrocytes were stored as sheep blood collected in an equal volume of Alsever's solution (8). This suspension was stored at 4°C and used for a period of 14 days.

Preparation of Insulin-Sensitized Erythrocytes (Insulin-Cells).—Insulin-cells were prepared by conjugating insulin to the surface of washed sheep erythrocytes with *bis*-diazobenzidine (BDB) as previously described (9).

Titration of Insulin Antibody Activity.—Insulin antibodies were measured by a modification of the immune hemolysis technique previously described. 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 (9). Antisera, serially diluted with VBS to a final volume of 0.5 ml, were cooled in an ice bath and twenty 50 per cent hemolytic units of C_a' in 0.3 ml was 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 then incubated in a 37°C water bath for 30 minutes and the non-hemolyzed cells were then separated by centrifugation at 3°C at approximately 2000 g for 5 minutes. The supernatant solutions were decanted and 2 ml of water was added to each sample. The degree of hemolysis was measured by the extinction of each sample at 580 mμ with a Beckman model DB spectrophotometer.

This method is valid and reliable when antisera are standardized and large pools of standardized C_a' are prepared. It is also necessary to test the reagents (C_a', BDB, insulin, or cells) when any is changed. Specific inhibition of immune hemolysis was performed in each experiment by adding a known amount of insulin (50 μg) in 0.05 ml to samples of diluted antisera prior to the addition of C_a'. Specificity of the system is controlled in this manner since the inhibition of hemolysis with insulin in this system has been demonstrated to possess a high degree of specificity (10).

Insulin Cellulose Stroma (ICS) Aggregates.—An insoluble insulin aggregate prepared by conjugating insulin to a mixture of cellulose and sheep erythrocyte stroma with BDB has been previously described (3). Three types of ICS aggregates were prepared; one with Lilly insulin and the other two with preparations of Novo insulin. These ICS aggregates were washed twice with distilled water, lyophilized, and stored at 3°C until they were used. The ability of insulin antibodies to bind to ICS was observed for at least 3 months after they had been prepared in this manner.

Experimental Approach.—These experiments were designed to test whether antibodies in different antisera were directed to different antigenic determinants on insulin. A system was devised which enabled the estimation of the degree to which antibodies in one antiserum react with an insoluble insulin complex (ICS) which had been saturated with antibodies from a different antiserum.
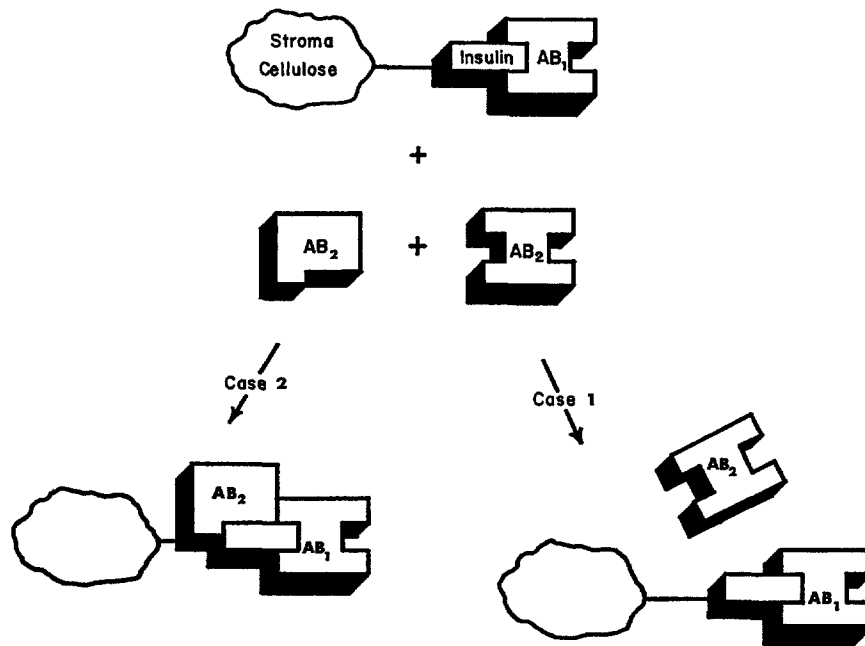

Fig. 1. Binding of various insulin antibodies to insoluble insulin aggregates saturated with antibody.

Theoretically if only one portion of the insulin molecule acts as the immunological determinant, then no reaction between insulin antibodies (AB_2) and an insulin-antibody complex prepared with a different antiserum (AB_1) would be expected, Case 1, Fig. 1.

If, however, antibodies are directed to more than one determinant group on insulin, then some antibodies would be expected to cross-react with the insulin-AB_1 complex, Case 2, Fig. 1. In this case, a decrease in total insulin-antibody activity in the test antiserum (AB_2) should be noted.

To test this hypothesis a system consisting of an insoluble insulin aggregate (ICS) saturated with antibodies from a single antiserum (AB_1) was incubated with several different test antisera (AB_2).

The validity of such an experiment requires that the ICS be saturated with antibodies from the saturating antiserum (AB_1) prior to the addition of the test antiserum (AB_2), Fig. 1.

Saturation of ICS with Insulin Antibodies.—The saturation of ICS with

TABLE I

Saturation of ICS by Guinea Pig and Rabbit Antibodies

Antisera	Preincubation	First incubation	Third incubation
Guinea pig			
2B	0.220*	0.050	0.220
5B	0.410	0.110	0.410
6B	0.310	0.240	0.320
13G	0.350	0.125	0.350
Rabbit			
C5	0.365	0.120	0.360
C20	0.135	0.020	0.130
C28	0.400‡	0.370‡	0.400
C33	0.085	0.035	0.090

* Extinction observed at 580 mμ proportional to hemoglobin released by immune hemolysis for estimation of antibody concentration at a 1:80 dilution of test samples.

‡ Much greater differences in antibody concentrations were observed in these same samples at higher dilutions.

TABLE II

Specificity of Binding of Guinea Pig and Rabbit Insulin Antibodies to ICS

Antisera	Nonincubated	Incubation with CS	Incubation with ICS
Rabbit C5........................	0.345*	0.340	0.245
Rabbit C20.......................	0.165	0.160	0.075
Guinea pig 5C....................	0.325	0.330	0.260

* Extinction observed at 580 mμ proportional to hemoglobin released by immune hemolysis for estimation of antibody concentration at a 1:80 dilution of test samples.

AB_1 was tested by repeatedly incubating aliquots of AB_1 with a given amount of ICS. It was found necessary to incubate the ICS a minimum of three times with AB_1. The antibody activity in all experiments was tested at 4 different dilutions of antisera by the immune hemolysis technique. In all cases tested, the ICS (20 mg/ml of antiserum) appeared to be saturated with insulin antibodies following the second incubation with AB_1, because no decrease in antibody was ever observed in the supernatant of aliquots following the third incubation, Table I. In no case was there observed an increase in antibodies following the

second and third incubation of ICS with AB_1. It appeared reasonable to assume that the ICS was saturated with antibodies by three repeat incubations with AB_1 and any further incubation of AB_1 with ICS would not be necessary.

To determine whether insulin antibodies were non-specifically adsorbed to
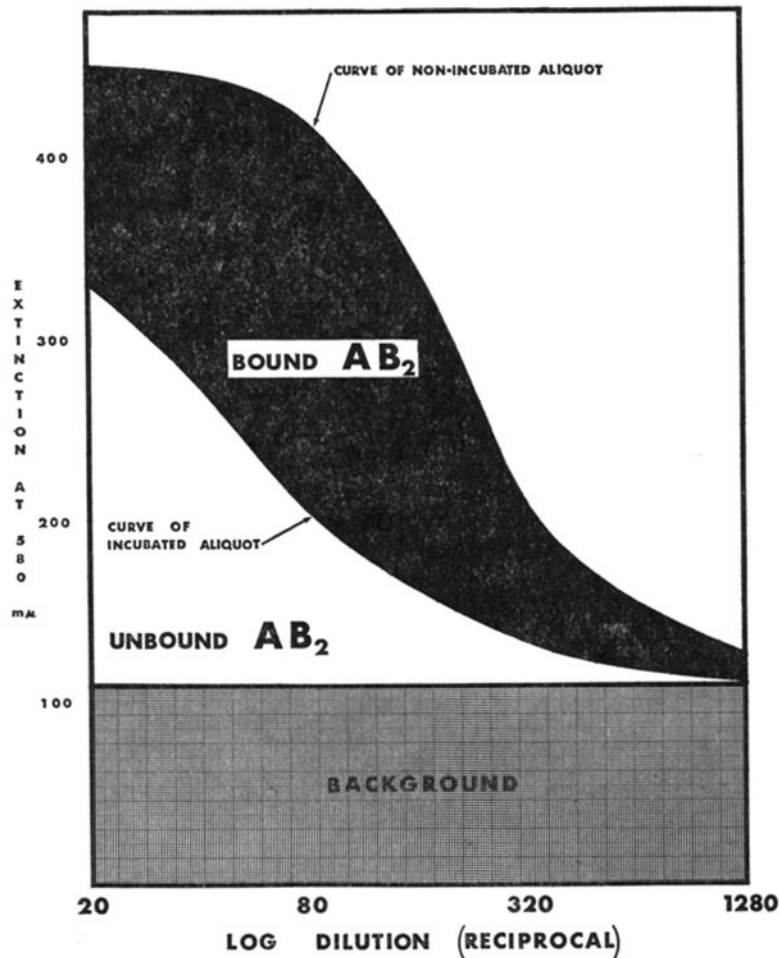
FIG. 2. Criteria for estimating binding of AB_2 to AB_1-ICS

the cellulose stroma (CS) component of the ICS, 1 ml aliquots of guinea pig 5C antiserum and two rabbit (C5 and C20) antisera were each incubated with 20 mg CS and 20 mg ICS, Table II. Antibody activity in the aliquots of insulin antisera which had been incubated with CS remained unchanged. However, a marked decrease in insulin antibody activity was noted in the same aliquots of

insulin antisera which had been incubated with ICS. See Table II and third column of Table I. Consequently, it was assumed that the ICS aggregate saturated with AB_1 could be used as a reliable model to determine whether antibodies were present in test antisera (AB_2) which could bind to portions of the insulin molecule to which the antibodies in the saturating antiserum could not bind, Fig. 1.

Quantitation of Binding of Insulin Antibodies in Test Antisera (AB_2) to AB_1-ICS.—The criteria for binding of AB_2 were established by comparing the area under the curve obtained by the hemolytic titration of non-incubated AB_2 with the area under the curve obtained by the hemolytic titration of AB_2 which

TABLE III

Reproducibility of Immune Hemolytic Titration of Antiserum

Aliquot No.*	Dilutions			
	1/20	1/80	1/320	1/1280
1	0.495‡	0.340	0.125	0.065
2	0.480	0.330	0.115	0.070
3	0.500	0.340	0.115	0.070
4	0.485	0.330	0.125	0.070
5	0.500	0.330	0.115	0.075

* Antiserum to insulin from rabbit C5.

‡ Extinction observed at 580 mμ proportional to hemoglobin released by immune hemolysis for estimation of antibody concentration.

had been incubated with AB_1-ICS aggregate. The hemolytic titration curves were a plot of the hemoglobin released against the log of the reciprocal of the dilution of the test antisera, Fig. 2. All such curves of the non-incubated AB_2 and the AB_2 which had been incubated with the AB_1-ICS were plotted on the same graph paper. The area under each of these curves was then estimated by cutting out the respective segment of graph paper and weighing them to the closest milligram with an analytical balance. The per cent binding of AB_2 was calculated from the differences in weight of graph paper representing the area under the hemolytic titration curve of non-incubated AB_2 and that of the incubated AB_2, Fig. 2. This method appears to be very accurate and very reproducible. It is our impression that the hemolytic titration curves obtained from 5 aliquots of the same antiserum simultaneously tested were so reproducible that the error due to the method of calculating relative concentration of antibody was greater than the actual observed values, Table III. It was also felt that the maximum possible differences in comparing these aliquots was considerably less than 5 per cent. Therefore, antibody binding to AB_1-ICS was considered to have taken place in those situations where incubated AB_2 demon-

strated a decrease in insulin antibodies of 10 per cent or more, when simultaneously compared to non-incubated AB_2.

Insulin Antibody Binding in Rabbit AB_2 to Guinea Pig AB_1-ICS.—Table IV summarizes the results observed in a group of experiments in which 5 different guinea pig (1B, 2B, 2C, 5B, and 5C) AB_1-ICS aggregates were prepared and 6 different rabbit AB_2 (C5, C20, C23, C28, C29, and C30) were tested.

Insulin antibodies capable of binding with portions of the insulin molecule

TABLE IV

Binding of Various Rabbit Antibodies (AB_2) to AB_1-ICS Saturated by Various Guinea Pig Antisera

AB₁ (guinea pig)	AB₂ (rabbit)	Per cent AB₂ bound
1B	C5	30.0←
	C20	0.5
	C28	0.5
2B	C5	64.0←
	C20	57.5←
	C28	0.5
2C	C28	1.0
5B	C23	50.0←
	C29	0.5
	C30	69.0←
5C	C5	36.5←

← Indicates significant binding.

to which guinea pig antibodies could *not* bind were demonstrated in 4 (C5, C20, C23, and C30) of the 6 rabbit antisera (AB_2) tested.

These results demonstrate the difficulty in attempting to predict qualitatively or quantitatively which rabbit AB_2 possesses antibodies capable of binding to specified guinea pig AB_1-ICS. In this regard, antiserum from rabbit C5 contained antibodies which bound to all three guinea pig AB_1-ICS against which it was tested. Whereas rabbit C28 produced insulin antibodies *not* capable of binding to any of three guinea pig AB_1-ICS. Rabbit C20 on the other hand, produced insulin antibodies which did *not* bind to AB_1-ICS prepared with guinea pig 1B antiserum, but 57.5 per cent of the insulin antibodies in rabbit C20 antiserum did bind to AB_1-ICS prepared with guinea pig 2B antiserum.

Antibodies produced by rabbits which bind to sites on the insulin molecule to which guinea pig antibodies cannot bind appear to possess a marked in-

dividual variability. Their demonstration apparently is dependent upon the individual guinea pig antiserum used to saturate the AB_1-ICS and also upon the individual rabbit antiserum tested. A multiplicity of antigenic determinants on the insulin molecule is therefore implicit in explaining the observed results.

Binding of Guinea Pig AB_2 to Rabbit AB_1-ICS.—The binding of AB_2 from six different guinea pig (1B, 1D, 2C, 2D, 3C, and 6B) insulin antisera was tested against four rabbit (C5, C20, C28, and C33) AB_1-ICS aggregates, Table V.

Only two of six guinea pig AB_2 contained antibodies capable of binding with

TABLE V

Binding of Various Guinea Pig Antibodies (AB_2) to AB_1-ICS Saturated by Various Rabbit Antisera

AB_1 (rabbit)	AB_2 (guinea pig)	Per cent AB_1 bound
C5	2C	<0.5
	6B	<0.5
C20	1B	2.0
	3C	6.0
C28	1B	0.5
	2C	<0.5
	3C	<0.5
	6B	<0.5
C33	1D	18.5←
	2D	69.0←

← Indicates significant binding.

only one AB_1-ICS prepared with rabbit C33 antiserum. There was a suggestion of binding of guinea pig AB_2 (3C) to AB_1-ICS prepared with rabbit C20 antiserum.

The binding of guinea pig AB_2 to rabbit AB_1-ICS was noted less frequently than the binding of rabbit AB_2 to guinea pig AB_1-ICS, Table IV. This may be due to a greater similarity between the antibodies produced by guinea pigs than those produced by rabbits, possibly because guinea pigs produce antibodies to fewer insulin determinants than do rabbits.

Binding of Rabbit AB_2 to AB_1-ICS Saturated with Rabbit Insulin Antibodies.— The insulin antibody differences observed when rabbit antisera (AB_2) were tested against guinea pig AB_1-ICS aggregates, Table IV, and *vice versa*, Table V, suggested the probability of antibody variations within each species. Insulin antibody differences between rabbits were demonstrated by binding experi-

ments with rabbit AB_2 to rabbit AB_1-ICS aggregates, Table VI. Twelve experiments were performed testing the binding of insulin antibodies (AB_2) from seven rabbit antisera to four rabbit AB_1-ICS aggregates, Table VI.

Seven of the twelve experiments demonstrate rabbit antisera which contained insulin antibodies (AB_2) capable of binding with rabbit AB_1-ICS. The relative amounts of binding antibodies varied between 30 per cent and 93 per cent, indicating that antigenicity of various portions of the insulin molecule can vary from rabbit to rabbit. A striking example of this is noted in the case where

TABLE VI

Binding of Various Rabbit Antibodies (AB_2) to AB_1-ICS Saturated by Various Rabbit Antisera

AB₁ (rabbit)	AB₂ (rabbit)	Per cent AB₂ bound
C5	C20	30.5←
	C29	3.5
	C30	75.3←
	C31	1.0
	C33	0.5
C20	C5	53.5←
C28	C5	38.5←
	C29	30.0←
	C30	0.5
	C31	0.5
C33	C5	82.3←
	C28	93.8←

← Indicates significant binding.

93 per cent of the insulin antibodies in test rabbit C28 antiserum was capable of combining with portions of the insulin molecule to which antibodies in the saturating antiserum from rabbit C33 could *not* combine, Table VI.

These results demonstrate an individual variation in the antibodies produced by rabbits when immunized with insulin. This variability is concerned with the antigenic determinant to which the antibodies are directed. It appears that rabbits produce measurable amounts of antibodies to several antigenic determinants on insulin.

Binding of Guinea Pig (AB_2) to AB_1-ICS Saturated with Guinea Pig Insulin Antibodies.—The observed variation in antibodies noted among rabbit insulin antisera was also noted among guinea pig antisera but to a lesser degree, Table VII. Twenty-one experiments were performed in which 14 different guinea pig (AB_2) antisera were tested against seven guinea pig AB_1-ICS aggregates.

Definitive binding of guinea pig AB_2 to guinea pig AB_1-ICS was noted in only three or possibly four of the experiments, Table VII, as compared to the binding of rabbit AB_2 to rabbit AB_1-ICS which was observed in seven of twelve experiments, Table VI. These results emphasize the greater degree of similarity among

TABLE VII

Binding of Various Guinea Pig Antibodies (AB_2) to AB_1-ICS Saturated by Various Guinea Pig Antisera

AB_1 (guinea pig)	AB_2 (guinea pig)	Per cent AB_2 bound
1B	2B	0.5
1C	2B	1.0
	4C	2.0
	5C	0.5
	8B	0.5
2B	1B	56.4←
	1C	1.0
2C	3B	1.0
	5B	0.5
	8B	1.0
5B	8B	3.0
6B	1D	18.0←
	2D	0.5
	3D	0.5
	4D	0.5
	5D	1.0
	6D	10.0(?)
8B	1B	2.0
	1C	18.0←
	5B	0.5
	5C	0.5

← Indicates significant binding.

insulin antibodies produced by guinea pigs and the relative marked variability of the insulin antibodies produced by rabbits.

Relationship of Binding of AB_2 to Dissociation of Antibodies from AB_1-ICS.— The binding of AB_2 to AB_1-ICS has been attributed to antibodies in the test antiserum (AB_2) directed to portions of the insulin molecule with which the antibodies in the saturating antiserum (AB_1) could not react. An alternative possibility is the dissociation from the AB_1-ICS of antibodies which have

reduced ability or are incapable of causing hemolysis of insulin-cells. Antibodies in AB_2 could react with those portions of the insulin molecule from which such antibodies had dissociated resulting in a net decrease in antibody activity of incubated AB_2.

In order to test these two possibilities, antisera were labeled (11, 12) with I^{131}, and AB_1^{I-131}-ICS aggregates were prepared. The relationship between dissociation of AB_1^{I-131} from AB_1^{I-131}-ICS to binding of antibodies in AB_2 was then tested and a typical example of the results obtained is presented in Table VIII. Rabbit C20 antiserum and guinea pig 3E antiserum were labeled with I^{131}, and AB_1^{I-131}-ICS aggregates were prepared with each of these antisera.

TABLE VIII

Relationship of Binding of AB_2 to Dissociation of Antibodies from AB^{I-131}-ICS

Aggregate	AB_2	Per cent AB_2 bound	Per cent activity precipitate	Per cent activity supernate
Rabbit C20	AO2	21.3	94.7	4.6
AB^{I-131}-ICS	BO2	87.7	94.7	4.5
	13D	<0.5	93.9	4.3
	2C	62.3	96.0	5.3
Guinea pig 3E	AO2	<0.5	87.2	8.1
AB^{I-131}-ICS	BO2	<0.5	90.0	7.9
	13D	3.8	90.2	7.1
	2C	76.5	88.9	8.7

Insulin antibody binding to the labeled AB_1^{I-131}-ICS was tested with four antisera (AB_2).

Three of the four AB_2 contained antibodies which bound to the AB_1^{I-131}-ICS prepared with labeled C20 antisera. However, only minimal differences in dissociation of I^{131} were discernible as measured by radioactivity of the incubated AB_2 (4.3 per cent to 5.3 per cent) and that remaining with the AB_1^{I-131}-ICS (93.9 per cent to 96.0 per cent). Only one of the same four AB_2 contained antibodies capable of binding to the guinea pig AB_1^{I-131}-ICS aggregate. Again, the amount of dissociation of antibodies in AB_1 varied slightly as measured by the radioactivity remaining on the aggregate and that observed in the supernatant. It appears that no relationship between dissociation of AB_1^{I-131} from ICS to binding of AB_2 to the AB_1^{I-131}-ICS was demonstrable with these methods.

Since insulin antibodies probably represent only a minimal portion of all the labeled material attached to the AB_1^{I-131}-ICS, the possibility exists that dissociation of AB_1 may be quite significant in terms of absolute amounts of insulin antibodies attached to the ICS. Therefore the dissociation of AB_1 may possibly account in part for the individual differences noted in the binding of antibodies from various AB_2 to AB_1-ICS.

If this were the case then repeated incubations of the same test antiserum with a number of aliquots of a single AB₁-ICS aggregate should cause a progressive decrease in antibody activity with each successive incubation. On the other hand if only the antibodies (in AB₂) that could react with exposed portions of insulin on the AB₁-ICS aggregate were removed, then repeat incubations of AB₂ would result in a limited decrease of antibody activity observable only after the first few incubations.

Therefore, two test antisera were repeatedly incubated with aliquots of an insoluble insulin aggregate which had been saturated with an insulin antiserum.

TABLE IX

Comparison of Binding of Insulin Antobodies between ICS and AB₁-ICS Aggregates

Test antisera (AB₂)	Aggregate	No. of incubations with fresh aggregate					
		1	2	3	4	5	6
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Rabbit C28	Insulin—cellulose stroma..	79*	64	46	35	23	12
	Rabbit C33 AB₁-ICS......	89	79	77	75	75	75
Rabbit C5	Rabbit C20 AB₁-ICS......	69	48	50			
	Guinea pig 5C AB₁-ICS...	63	48	49			

* Amount of antibody remaining in an aliquot of insulin antiserum after incubation with fresh aggregate.

In addition, one of the test antisera was repeatedly incubated with aliquots of an insoluble insulin complex which had *not* been saturated with antibodies, Table IX.

An aliquot of test antiserum (AB₂) from rabbit C28 was incubated repeatedly with six fresh aliquots of insoluble insulin (ICS). A second aliquot of the same test antiserum was incubated repeatedly with six fresh aliquots of insoluble insulin which had been saturated with antibodies from serum C33 (AB₁). A decrease in insulin antibodies was noted following each successive incubation with ICS, row 1, Table IX. On the other hand, a decrease in antibodies was noted *only* after the first and second incubations with AB₁-ICS. No significant decrease in insulin antibody activity was noted following the third, fourth, fifth, and sixth successive incubations of test antiserum with AB₁-ICS, row 2, Table IX.

In addition, aliquots of rabbit C5 antiserum (AB₂) were incubated repeatedly with three fresh aliquots of ICS which had been saturated with antibodies (AB₁) from either rabbit C20 or guinea pig 5C. Again a decrease in antibody activity was noted only after the first and second incubations and *no* decrease

was observed after the third incubation of test antiserum with AB_1-ICS, rows 3 and 4, Table IX.

The results do not appear to be compatible with the hypothesis that the individual variations noted in test antisera (AB_2) were due to dissocation of insulin antibodies from AB_1-ICS during the period of incubation with the test antisera. If dissociation of antibody does occur it probably does not result in the individual variations noted in AB_2.

It is probable, therefore, that antibodies in AB_2 which bind to AB_1-ICS aggregates are attaching to a portion of the insulin molecule to which the antibodies in AB_1 cannot bind. Since these variations in antibodies are peculiar to the individual antiserum and cannot be predicted, it is postulated that a genetic control directs antibody production only to certain antigenic determinants on the insulin molecule.

DISCUSSION

The binding of antibodies in AB_2 to AB_1-ICS was reproducible regardless of which of the three insulin preparations was used to make the ICS. Nor has it been possible to relate the antibody variations observed to any group of antisera obtained from animals immunized with one of these insulin preparations. Consequently it would be unlikely that the observed antibody variations were due to contaminants or to differences in structure between the pig and beef insulin. The immune hemolysis reaction employed in these studies has been demonstrated to possess a high degree of specificity for insulin (10). It is therefore reasonable to assume that the reactions between AB_2 and AB_1-ICS involve the insulin molecule and antibodies directed against it.

Recently, Yagi, Maier, and Pressman (13) have demonstrated two distinct classes of antibodies in the serum of guinea pigs immunized with beef insulin. One class of antibodies migrates with gamma globulin, the other with B_2 globulin, and both are similar in size (7S globulins). We repeated these experiments and could not obtain immune hemolysis with the B_2 insulin antibodies. Consequently binding experiments comparing these two classes of insulin antibodies were not possible. It is not known to what degree the antibodies of these two classes vary with respect to the determinants on insulin to which they can bind.

Direct evidence that at least two antibodies can react with an insulin molecule has been demonstrated in all situations where antibodies in AB_2 bound to AB_1-ICS aggregates. By selecting the appropriate AB_2 it is probable that *more* than two antibodies will simultaneously combine with the insulin molecule.

The probability that more than one antibody molecule can react with an insulin molecule has been inferred by the demonstration of precipitate formation with the insulin-antibody system by several independent investigators (14–18). The possibility exists, however, that the observed precipitates with antibody

and insulin resulted because of physical chemical reactions coincident with a single antibody insulin combination and not because of lattice formation. Such structural alterations in the insulin-antibody complex may account for the pattern of cells noted in the hemagglutination reaction at very high dilutions of antiserum.

Berson and Yallow (19, 20) have demonstrated by ultracentrifugation that the antibody-insulin complex sediments at a rate similar to (within experimental error) gamma globulin. They concluded therefore that only one antibody molecule reacts with an insulin molecule. Their calculation of the equilibrium constants between antibody and insulin was based on this conclusion.

The direct evidence presented in this investigation and the numerous independent observations of precipitation between antibody and insulin suggest that *more* than one antibody can react with an insulin molecule simultaneously. It is probable therefore that the equilibrium constants between antibody and insulin proposed by Berson and Yallow may be subject to change.

The present investigation does not allow the estimation of the number of determinant groups which the insulin molecule possesses. The portion of the insulin molecule toward which antibodies are produced appears to be an individual characteristic which varies from animal to animal. It is probable that the determinants to which one animal produces antibodies overlap determinants to which others produce antibodies. In this sense, it is possible that the insulin molecule has an infinite number of determinants and it may be erroneous to consider insulin as possessing finite, distinct antigenic determinants. It is probably more accurate to consider the insulin sites toward which an animal directs the antibodies it produces.

Previous studies demonstrated guinea pig antisera to be capable of neutralizing insulin whereas rabbit antisera could not (1). There is much less antibody variation among guinea pigs, Tables V and VII, than among rabbits, Tables IV and VI, possibly because guinea pigs produce antibodies to fewer determinants than do rabbits. It may be that guinea pigs produce relatively greater amounts of antibodies toward a portion of the insulin molecule which is critical for biological activity in the rat. Rabbits may produce relatively smaller amounts of such antibodies, and the method demonstrating them with intact anesthetized rat may not be sufficiently sensitive. Preliminary results indicate that it may be possible to select an occasional rabbit capable of producing antiserum which will neutralize insulin.

These individual characteristics of antibodies when insulin is the antigen may possibly be related to the manner in which the insulin molecule is transported in circulation and not involve antibody production. Evidence has been presented that circulating insulin is bound by a carrier protein (21, 22). It is possible that the external surface of such an insulin carrier complex exposed to the antibody-competent cells is the same as "self" and therefore non-antigenic.

Only those portions of the insulin molecule to which carrier protein or proteins attach would be capable of stimulating the production of antibodies.

If this postulate is correct, then there may be different binding sites between insulin and carrier protein in different animals, and the antigenic determinants would also vary in different animals. Therefore, insulin may be neutralized by antibody only in those animals which have binding sites between carrier protein and insulin differing from the animal in which the antibodies are produced. Experiments designed to test these possibilities are currently being investigated with inbred strains of guinea pigs and rabbits.

It is not known whether the variations in antibodies observed when animals are immunized with insulin will also be observed with unrelated, non-biologically active proteins. It would be presumptive, therefore, to postulate that the antibody variations noted are general and will be manifest in the antibodies engendered by all antigens.

SUMMARY

1. A method is presented for measuring the degree to which insulin antibodies in one antiserum react with an insoluble insulin complex saturated with antibodies from a different antiserum.

2. Many rabbits produce antibodies which bind to portions of the insulin molecule to which antibodies from guinea pigs or other rabbits cannot bind.

3. *Occasional* guinea pigs produce antibodies which bind to portions of the insulin molecule to which antibodies from rabbits or other guinea pigs cannot bind.

4. Studies with labeled antisera and repeated incubations of test antisera with antibody insulin complexes demonstrate the individual antibody variations to be due to antibodies directed to different determinants and not to dissociation of antibodies from the same determinant on the insulin molecule.

5. More than one antibody molecule can simultaneously bind to an insulin molecule.

6. Insulin has a multiplicity of antigenic determinants.

7. The relationship between antigenic determinants, insulin antibodies, and neutralization of insulin by antisera is discussed.

8. The determinants to which insulin antibodies are directed appear to be characteristic for the individual rabbit or guinea pig immunized. It is postulated therefore that genetic factors direct antibody production toward specific determinants when insulin is the antigen.

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