IMMUNOLOGIC STUDIES IN INSULIN RESISTANCE

II. THE PRESENCE OF A NEUTRALIZING FACTOR IN THE BLOOD EXHIBITING SOME CHARACTERISTICS OF AN ANTIBODY

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The huge doses of insulin required by some insulin-resistant patients have led many observers to postulate the presence of an insulin-neutralizing factor in the blood of such individuals. In attempts to demonstrate this factor, the usual procedure has been to inject insulin mixed with the patient's serum into laboratory animals and then to look for a decrease or absence of insulin effect. Some studies have given entirely negative results (1 to 4), possibly because of the large amounts of insulin used in carrying out the tests. However, a few workers have obtained evidence indicating some insulin-neutralizing activity (5 to 7).

The interpretation of experiments to be reported here, as well as those reported by others, is made difficult by the wide variations in response to insulin exhibited by different animals and even by the same animal at different times. Also, factors other than the injection of insulin may possibly affect the animal's response. Irritation from the injected foreign protein (human serum), the presence of high concentrations of glucose in the injected serums, and delay in the absorption of the insulin (if mixture is not injected intravenously), may all combine to interfere with insulin effect and produce the impression that neutralization has taken place.

Earlier experiments in mice with blood obtained from a case of insulin resistance, A. M., provided evidence for the presence of an insulin-neutralizing factor (8, 9). It was believed at that time that this was an antibody. Additional evidence has been obtained from a study of a series of 25 blood specimens obtained from the same patient. Tests have also been made with blood from a second insulin-resistant patient. These studies leave little doubt that an insulin-neutralizing factor was present and that this factor was an antibody for crystalline insulin.

TESTS FOR INSULIN-NEUTRALIZING ACTIVITY

Materials and methods

Adult albino mice were starved for 24 hours without bedding but with access to water. Each animal was then injected intra-abdominally with 0.5 ml. of a mixture of 2 or more of the following: U 40 crystalline insulin (Lilly), saline, human serum, and glucose. The mice were observed continuously for 90 minutes in an incubator measuring approximately 7 × 4 × 5 feet. The thermostats were set for a temperature of 35° C. to 37.5° C., but because the incubator door was opened at intervals during the tests, the temperature averaged slightly lower than this. The number of mice showing insulin effect was recorded. No animal was used more than once.

Insulin effect was considered to be present when one or more of the following symptoms appeared: convulsions, extreme irritability and overactivity, coma, loss of equilibrium, and marked weakness or paralysis of the hind legs. These symptoms disappeared quickly when a glucose solution was injected intra-abdominally. During the test, the mice were kept under continuous observation because sumptoms of insulin effect were sometimes transitory.

All dilutions of insulin were freshly made. The human serum was obtained from clotted blood and stored under sterile conditions in rubber-stoppered test tubes in the ice-box. The mixtures of insulin and serum were not incubated before the tests were done. In most instances, 5 mice were injected with each mixture. This small number was used because the amount of serum was limited. Glucose determinations on the serums from normal and insulin-resistant patients were made by the Folin micromethod, using serum instead of whole blood. Most of these determinations were made within a short period after the serums were tested in mice.

Experiments with normal human serum

The tests with normal serum were originally designed as controls for some of the experiments with serums from the 2 insulin-resistant patients. However, for purposes of presentation, tests of

a similar nature are grouped together. The dates on which the different tests were done are given in the tables. Thus tests done on the same day may be found in the tables and compared if desired.

A total of 10 tests, in which 5 mice were used in each, were carried out with 5 normal serums. The results are shown in Table I. Solutions for injection were made by mixing 2.5 ml. of human serum, with 0.5 ml. of U 40 crystalline insulin diluted 1 : 100. Each mouse was injected with 0.5 ml. which contained 0.033 units of insulin, approximately 0.4 ml. of serum and 0.1 ml. of saline. The incidence of symptoms varied, but in all tests, 2 or more of the 5 mice in each group showed evidence of insulin effect. In this group of 50 mice, 40 showed symptoms, an incidence of 80 per cent, which is below that observed in experiments done without the addition of serum. The greater resistance of the mice to insulin in the experiments shown in Table I, compared to that noted in the first report (8), may have been due to the lower temperature at which the more recent tests were carried out.

Tests in 54 mice were also done with normal serum to which sufficient glucose was added to raise the concentration to the level expected in serums obtained from insulin-resistant diabetic patients. The final concentration attained after the addition of glucose was equivalent to a blood sugar of between 240 and 300 mgm. per 100 ml. The solution containing insulin and serum was made in the proportions described above but

TABLE I

Tests with normal serum

Number of test	Serum	Date		Number of mice showing	
		Serum taken	Of test	Symptoms	No symptoms
1	A	November 1, 1941	April 8, 1942	5	0
2	B	November 7, 1941	October 27, 1942	4	1
3	A	November 8, 1941	August 13, 1942	4	1
4	A	November 12, 1941	November 6, 1942	2	3
5	C	December 6, 1941	August 26, 1942	4	1
6	C	December 6, 1941	August 27, 1942	5	0
7	D	February 2, 1943	April 20, 1943	5	0
8	D	February 2, 1943	April 27, 1943	3	2
9	D	February 2, 1943	April 29, 1943	3	2
10	E	March 24, 1942	August 11, 1942	5	0
Total (50 mice)				40	10

Incidence of symptoms = 80 per cent

TABLE II

Tests with normal serum with added glucose

Number of test	Serum	Date		Glucose content	Number of mice showing	
		Serum taken	Of test		Symptoms	No symptoms
				mgm. per 100 ml.		
1	D	February 2, 1943	April 27, 1943	240	1	4
2	D	February 2, 1943	April 29, 1943	240	2	3
3	D	February 2, 1943	May 8, 1943	240	7	0
4	D	February 2, 1943	May 11, 1943	240	3	2
5	D	February 2, 1943	May 13, 1943	240	3	2
6	D	February 2, 1943	June 4, 1943	240	2	3
7	G	June 23, 1943	July 8, 1943	300	13	9
Total (54 mice)					31	23

Incidence of symptoms = 57 per cent

with the addition of 0.3 ml. of a 2 per cent glucose solution for each 3.0 ml. of the mixture of serum, saline, and insulin. Five-tenths ml. was injected into each of 5 or more mice. Each mouse received, therefore, 0.0303 units of insulin, approximately 0.4 ml. of serum, 0.1 ml. of saline, and 0.91 mgm. of added glucose. In 7 tests done with normal serum to which glucose was added, the incidence of symptoms was again very variable (Table II). In 1 test, only 1 mouse showed symptoms. Of 54 mice tested in this way, 31 showed symptoms, an incidence of about 57 per cent.

These results indicate that under the experimental conditions and with high concentrations of glucose in the serum, an incidence of symptoms of somewhat over 50 per cent may be expected. Because of the increased volume of the injection mixture, due to the addition of glucose, the dose of insulin in these tests was 0.003 units less than the tests shown in Table I and those to be described below done with serums from the 2 insulin-resistant patients. It is probable that this slight reduction in dose was not responsible for the decreased incidence of symptoms in tests done with added glucose.

Experiments with serum from 2 insulin-resistant patients

The clinical course of the first insulin-resistant patient, from whom serums were obtained for these studies, is reported in detail elsewhere (10). This patient, A. M., was observed for about 15

months and bloods taken at intervals during this period were tested for insulin neutralizing activity. Mice were injected with mixtures of 2.5 ml. of the patient's serum, diluted or undiluted, and 0.5 ml. of U 40 insulin diluted 1 : 100, as in the tests with normal serum. No glucose was added. Each mouse received therefore 0.033 units of insulin, 0.40 ml. or less of serum, and 0.1 ml. or more of saline.

The results of 24 tests on 21 serums are shown in Table III. The serums are listed in the order in which they were taken and the dates are given, as well as the dates on which the tests were done. The concentrations of glucose in all but 4 of the serums are also shown. Five mice were used in each test, and 3 serums were tested twice. The regular alternation in the results of the tests is striking. In the first 4 tests, no mice showed

TABLE III

Results of tests with 22 serums obtained from A. M. over a period of 14 months

Number of test	Date		Concentration of glucose	Number of mice showing* symptoms	Resistance	Remarks
	Serum taken	Of test	*mgm. per 100 ml.*			
1	August 19, 1941	August 13, 1942	279	0	+	Patient receiving insulin
2	August 21, 1941	August 21, 1942		0		
3	August 28, 1941	August 13, 1943	340	0		
4	October 8, 1941	August 13, 1943		0	?	No insulin given from September 7, 1941 to February 17, 1942
5	January 21, 1942	August 13, 1943	332	1		
6	February 11, 1942	August 11, 1942	365	3		
7	February 11, 1942	August 21, 1942	365	4		
8	February 21, 1942	August 21, 1942	227	3	0	Insulin given from February 18, 1942 to March 12, 1942
9	February 27, 1942	August 11, 1942	354	0	+	
10	March 2, 1942	August 11, 1942	389	0		
11	March 11, 1942	August 21, 1942	393	0		
12	April 6, 1942	August 13, 1942	286	0	?	No insulin given from March 13, 1942 to October 29, 1942
13	May 27, 1942	August 11, 1942	469	0		
14	August 18, 1942	May 11, 1943		1		
15	August 18, 1942	April 27, 1943	244	2		
16	October 30, 1942	November 6, 1942	280	2		Insulin given from October 30, 1942 to November 15, 1942
17	November 2, 1942	November 6, 1942	319	3		
18	November 4, 1942	November 6, 1942	42	3	0	
19	November 6, 1942	June 4, 1943	275	1		
20	November 8, 1942	June 4, 1943	298	0	?	
21	November 9, 1942	May 13, 1943	305	0	+	
22	November 9, 1942	June 4, 1943		0		
23**	November 10, 1942	May 13, 1943	202	0		
24**	November 12, 1942	May 13, 1943	297	0		

* 5 mice were used in each test. ** Serum diluted 1 : 2.

symptoms and this is also true of the ninth to the thirteenth and the twentieth to the twenty-fourth tests, inclusive. On the other hand, one or more mice showed symptoms in the fifth to the eighth and the fourteenth to the nineteenth tests. In 8 of the 10 tests in which one or more mice showed symptoms, the serum contained 227 mgm. per 100 ml. of glucose or more. This indicates that the presence of high concentrations of glucose in the serums was not the determining factor in the prevention of symptoms in tests in which no symptoms occurred. The results of these tests will be discussed below in relation to the patient's clinical course.

Seven tests done with a total of 40 mice with a serum obtained on November 19, 1942, 5 days after all injections of insulin had been stopped, are shown in Table IV. This serum was

TABLE IV

Tests with serum obtained from A. M. on November 19, 1942

Number of test	Date of test	Dilution of serum	Number of mice showing		Incidence of symptoms
			Symptoms	No symptoms	
					per cent
1	April 20, 1943	1 : 2	0	5	
2	May 4, 1943		0	5	
3	May 8, 1943		0	10	
4	June 19, 1943		0	5	
Total (25 mice)			0	25	0
5	April 20, 1943	1 : 4	0	5	
6	April 29, 1943		1	4	
7	August 4, 1943		1	10	
Total (21 mice)			2	19	9.5
8	August 5, 1943	1 : 8	4	7	37

diluted 1 : 2 in 4 tests, 1 : 4 in 3 tests, and 1 : 8 in 1 test. Of 30 mice tested with serum diluted 1 : 2, none showed symptoms. Of 21 mice tested with serum diluted 1 : 4, 2 showed symptoms. Four of 11 mice receiving serum diluted 1 : 8 showed symptoms. In comparing the results of tests with undiluted normal serum and those obtained with diluted serums, account should be taken of the tendency of undiluted normal serum to decrease the incidence of symptoms. This non-specific inhibition of insulin is

TABLE V

Results of tests with serum obtained from C. S.

Number of test	Date test done	Dilution of serum	Number of mice showing		Incidence of symptoms
			Symptoms	No symptoms	
					per cent
1	August 10, 1943	Undiluted	2	9	
2	August 26, 1942	1 : 2	0	3	
3	August 27, 1942		0	5	
4	August 6, 1943		3	8	
Total (30 mice)			5	25	17
5	August 26, 1942	1 : 4	2	3	
6	August 27, 1942		3	2	
Total (10 mice)			5	5	50

decreased or absent in tests with serums diluted 1 : 2 or more. Furthermore, the "glucose effect" in such tests is also diminished.

A few tests were done on a serum (Table V) obtained from a second insulin-resistant patient whose clinical course has also been briefly described (10). Two of 11 mice tested with undiluted serum showed symptoms. Of 19 mice tested with serum diluted 1 : 2, 3 developed symptoms. Five mice out of a total of 10 showed symptoms when the serum was diluted 1 : 4. These results indicate that the insulin-neutralizing capacity of serum from this patient was less marked than that of the serum obtained from A.M. on November 19, 1942 (Table IV).

Serums from these 2 patients were also tested for their ability to protect mice from the same preparation of human insulin which was used in the insulin tolerance tests. This preparation has been described in the first part of this study. One patient, A. M., exhibited susceptibility to this preparation at a time when she was resistant to crystalline insulin. The other, C. S., was apparently resistant to human as well as crystalline insulin (10). The potency of this preparation of human insulin was approximately 25 units per ml. In the tests with mice, 0.5 ml. of this diluted 1 : 75, was added to 2.5 ml. of serum diluted 1 : 2. The estimated dose per mouse was slightly less than 0.03 units. The

TABLE VI

Serum from A. M. and C. S. tested with human insulin

Patient	Date serum taken	Date of test	Dilution of serum	Number of mice showing		Incidence of symptoms
				Symptoms	No symptoms	
						per cent
A. M.	November 19, 1942	May 5, 1943	1 : 2	1	4	
		June 19, 1943		3	2	
		June 29, 1943		8	3	
	Total (21 mice)			12	9	57
C. S.	August 23, 1942	August 5, 1943	1 : 2	1	10	
		August 6, 1943		2	9	
	Total (21 mice)			3	19	14

results of the tests are shown in Table VI and may be compared with similar tests with crystalline insulin in Tables IV and V. The results indicate that the serum obtained from A. M. on November 19, 1942, was less active in preventing symptoms in mice injected with human than with crystalline insulin. A dilution of 1 : 2 failed to protect against human insulin whereas a dilution of 1 : 2 gave solid protection, and 1 : 4 gave some protection against crystalline insulin. This result is consistent with the patient's greater susceptibility to human insulin (10). The serum of C. S. gave about equal protection against both insulins. This patient was resistant to human as well as crystalline insulin (10).

TESTS FOR SKIN SENSITIZING ANTIBODY

Earlier studies (8) indicated that the antibody responsible for passive sensitization of normal human skin was distinct from the insulin-neutralizing factor. A few further studies have been made on this point.

Four serums obtained from A. M. were diluted serially in steps of 2 and the dilutions were injected endermally in a recipient known to receive passive transfer well. The sites were tested 24 hours later with 0.02 ml. of a 1 : 10 dilution of U 40 crystalline insulin (Lilly). Serums obtained on August 19, 1941, January 21, 1942, and February 11, 1942, gave titers of 1 : 32 and the serum of October 9, 1941, a titer of 1 : 16. Therefore, little or no change in the amount of skin-sensitizing antibody in the patient's serum occurred over a period of about 6 months. Tests for insulin-neutralizing activity (Table III, tests numbers 1, 4, 5, 6, and 7) show that a decrease in this occurred during that period. Blood taken on February 21, 1942, 3 days after desensitization with insulin was begun and when responsiveness to insulin was present, caused weak sensitization of normal skin in a dilution of 1 : 4, and none in a dilution of 1 : 8. This decrease may have been caused by the desensitization. Attempts to demonstrate skin-sensitizing antibody in the serum of C. S. were unsuccessful, although insulin-neutralizing activity was present.

OTHER TESTS FOR ANTIBODY

In Table VII are shown the results of tests for precipitins by the collodion-particle method (11).

TABLE VII

Tests for precipitins in serums obtained from A. M.

Date serum obtained	Titer of precipitins	I.N.A.*	Resistance to insulin
August 25, 1941	1 : 32		+
August 27, 1941	0		+
August 28, 1941	1 : 4	+	+
September 2, 1941 A.M.	0		+
September 2, 1941 P.M.	1 : 16		+
February 21, 1942	0		0
February 27, 1942	0	+	?+
March 11, 1942	0	+	+

* Insulin neutralizing activity. See Table III, tests number 3, 9, and 11.

Crystalline insulin (Lilly) was used as the antigen. Of 8 serums tested, 3 showed precipitins. All the serums, barring that of February 21, 1942, and possibly that of February 27, 1942, were obtained from A. M. at a time when she was resistant to insulin. On the basis of other studies made on this patient (Table III), it may be assumed that 6 of these serums contained the insulin-neutralizing factor, although only 3 were tested for its presence (Table III, tests number 3, 9 and 11).

Two serums (August 25, 1941 and March 11, 1942) were tested in normal skin and conferred sensitivity to insulin on the injected sites. It is probable that most if not all of the other 6

serums listed in Table VI also contained the skin-sensitizing antibody (see above).

The serum obtained from A. M. on November 19, 1942, was tested for the presence of complement-fixing antibodies and for precipitins by the ring test. The pH of the insulin was adjusted to about 7.0 in order to avoid non-specific precipitation. No antibody was demonstrated by either method. This serum was chosen because it had been thoroughly tested in mice (Table IV) and insulin-neutralizing activity was clearly demonstrated in a titer of 1 : 2 and was probably present in a dilution of 1 : 4. No precipitins were demonstrable by the ring test in the serum obtained from C. S.

DISCUSSION

Because of the small number of animals used and the relatively low incidence of symptoms (57 per cent) in the control tests, the interpretation of a single test for insulin-neutralizing activity in serum is subject to error. On the basis of chance, it might occasionally happen that a group of 5 mice would be selected in which no animal would show symptoms, even though neutralization of insulin had not taken place. Therefore, absence of symptoms in a group of 5 mice is indicative of insulin-neutralization but cannot be taken as conclusive evidence thereof. On the other hand, the appearance of symptoms in one or more mice in a group of 5 is a reliable indication that neutralization of insulin was incomplete or absent.

Consideration of the results of the tests with serums obtained from A. M. in relation to the patient's clinical course (10) is of interest. As shown in Table III, no symptoms appeared in any of the mice tested with serums obtained on the 3 occasions when the patient was demonstrably resistant to insulin (tests number 1, 2, 3, 9, 10, 11, 21, 22, 23, and 24). The same result was obtained with serums drawn within a period of 8 weeks after resistance was demonstrated and the administration of insulin had been stopped (tests number 4, 12, and 13). In this entire group, no symptoms developed in a total of 65 mice tested. A different result was obtained with serums taken when the patient was responsive to insulin (tests number 8, 18, and 19), as well as serums taken within a period of approximately 3 months or less before responsiveness to insulin was demonstrated (tests number 5, 6, 7, 14, 15, 16, and 17). In this group of 50 mice, 23 showed symptoms, an incidence of 46 per cent. The appearance and disappearance of insulin-neutralizing activity in the patient's serum was apparently dependent on whether or not the patient received insulin. During 2 long periods of about 5 and 7 months (September, 1941 to February, 1942, and March, 1942 to November, 1942), when the patient was receiving no insulin, the neutralizing activity of the serum disappeared. At the end of these periods (February, 1942, and November, 1942), when resistance to insulin was markedly diminished or absent, the administration of insulin was followed in about 10 to 12 days by the reappearance of neutralizing activity in the serum and the development of resistance to insulin. These results are in accord with the view that the factor in the patient's serum responsible for the prevention of symptoms in mice was a neutralizing antibody for crystalline insulin.

The method used for the demonstration of neutralization of insulin did not lend itself to quantitative measurements. Rough estimates of the amount of the insulin-neutralizing factor, made by diluting the serum, showed that only small amounts were present. A dilution of 1 : 4 resulted in some loss of protection in tests with serum from A. M. (Table IV) and complete loss in tests with serum from C. S. (Table V). The smaller capacity of the second serum to neutralize insulin may have been due to a reduction in the circulating antibody to insulin, caused by the large doses of insulin which the patient was receiving daily.

The patients are compared in Table VIII with respect to the results of the tests for insulin-neutralization and with respect to their resistance to the 2 insulins, as indicated by tolerance tests (10). It is interesting that the serum of A. M. exhibited species specificity. In a dilution of 1 : 2, it failed to neutralize human insulin (Table VI) although neutralization of crystalline insulin was marked. Specificity was not demonstrated in tests with serum from C. S. The difference in the behavior of these serums may be

TABLE VIII

Comparison of A. M. and C. S. with respect to clinical features and results of tests in mice with serum diluted 1 : 2

Serum	Insulin resistance		Incidence of symptoms in mice		Remarks
	Crystalline	Human	Crystalline	Human	
			per cent		
A. M. November 19, 1942	+++	0	0	57	Serum taken after 5 days without insulin
C. S. August 23, 1942	+++	+++	17*	14	Patient receiving approximately 1000 units daily with partial control of the diabetes

* One test done with undiluted serum.

explained by assuming that an antibody of wider reactivity was developed by C. S. than by A. M. This is discussed in the first part of this study in relation to the clinical histories (10).

The relationship of precipitins and complement fixing antibodies to insulin-resistance is not clear at the present time. No correlation was observed between the presence of precipitins in the serum of A. M. and either insulin-neutralizing activity or skin-sensitizing antibody (Table VII). Goldner and Ricketts (12) described 4 patients who were allergic to insulin and whose serum contained precipitins demonstrable with the collodion particle method. Two of these patients were also resistant to insulin. One of the 2 was extremely resistant and this patient's serum contained precipitin in a titer of 1 : 80. The serum of 3 other allergic patients, one of whom was insensitive to insulin, contained no precipitins. In animal experiments (13), the development of complement-fixing antibody was not associated with the appearance of resistance to insulin.

In a previous report (8), the conclusion was reached that the factor responsible for neutralization of insulin, and the antibody demonstrable by passive transfer to normal skin (Prausnitz-Küstner reaction), were not the same. This is supported by the tests made with 4 serums from A. M., which showed that the insulin-neutralizing factor and the skin-sensitizing antibody did not vary in a parallel fashion. Furthermore, the serum of C. S. protected mice from insulin but did not sensitize human skin to insulin.

SUMMARY

A method for demonstrating neutralization of insulin by serum obtained from insulin-resistant subjects is described. The results of tests on a number of serums obtained from 2 insulin-resistant patients showed that the presence of insulin-neutralizing activity was associated with resistance to insulin. The factor responsible for neutralization of insulin in the serum of one patient exhibited species specificity, and appeared to vary independently of the skin sensitizing (allergic) antibody.

It is concluded that: (*1*) insulin resistance may occur on an immunologic basis and may be associated with the presence in the serum of a neutralizing antibody for crystalline insulin; (*2*) under certain circumstances, this antibody may exhibit species specificity; (*3*) the insulin-neutralizing and the skin-sensitizing antibodies are distinct.

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