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Research Article

IMMUNOLOGIC STUDIES IN INSULIN RESISTANCE. III. MEASUREMENT OF AN INSULIN ANTAGONIST IN THE SERUM OF AN INSULIN-RESISTANT PATIENT BY THE BLOOD SUGAR CURVE METHOD IN MICE

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The detection of an insulin antagonist in the serum of insulin resistant subjects has been attempted on a number of occasions. In certain instances, the excessive quantities of insulin used in the neutralization experiments in animals may explain the failure to obtain evidence of an insulin antagonist. In the few successful experiments which have been done, little information of a quantitative nature has been obtained. Glen and Eaton (1) and Marble, Fernald and Smith (2) were able to demonstrate decreased responsiveness to insulin in rabbits injected with serum from insulin resistant patients. This change in the experimental animal was still demonstrable a week after injection of the serum. In the same year, Banting *et al.* (3) described experiments in which the incidence of hypoglycemic convulsions was reduced in mice injected with mixtures of serum and varying amounts of insulin. The serum was obtained from a patient with schizophrenia who developed insulin resistance during insulin shock therapy. This case is especially interesting because *diabetes was not present,* suggesting that the resistance was directed against prepared insulin only. When the same serum was retested a week later in mice maintained on a high carbohydrate diet, practically no insulin neutralization could be demonstrated. Lerman (4), in studying serum of 5 patients with insulin resistance, obtained evidence for the presence of an insulin antagonist in 2. One of these serums was provided by the writer, and was obtained from a diabetic patient, A. M., the subject of this and previous reports (5, 6). A high degree of allergy to insulin, coupled with marked insulin resistance, combined to make the giving of insulin to this patient both disagreeable and ineffective. A further important feature was the presence of a relatively mild degree of diabetes, making possible prolonged periods during which insulin therapy could be omitted without immediate danger to the patient's life. A number of hospital admissions provided opportunity to show that:

a. At the end of a period of 5 months or more without insulin therapy, small doses of insulin would give rise to urticaria, constriction in the chest, and, on one occasion, collapse.

b. After desensitization with graded doses of insulin, a period of 3 to 5 days followed during which the injection of relatively small doses of insulin would be followed by a fall in blood sugar.

c. Within about 11 to 15 days of starting desensitization and treatment with insulin, a solid resistance to insulin developed.

These changes were correlated with the absence, and then the appearance, of insulin-neutralizing activity in the patient's serum. The allergic reactions appeared to be mediated by a mechanism quite independent of that giving rise to resistance (5).

The method previously used for the demonstration of insulin-neutralizing activity of the patient's serum (6) consisted in observing the incidence of hypoglycemic symptoms in mice injected with a mixture of serum and insulin. This method had a number of disadvantages which outweighed its simplicity. A relatively large dose of insulin was required in order to produce symptoms of hypoglycemia with some regularity when normal serum was used, and because of this the chance of detecting small amounts of neutralizing activity in the serums to be tested was decreased. This, in turn, led to the use of a large amount of serum

in each animal, a circumstance which in itself introduced non-specific blood-sugar raising effects (see below). These effects had to be distinguished from specific insulin neutralization, and tended to decrease the regularity of symptoms in control tests. Furthermore, the recognition of truly hypoglycemic symptoms was not always easy. Finally, reliance on the incidence of hypoglycemic symptoms as an indication of insulin effect provided no direct measure of the effect of the injected mixture on the blood sugar itself. With the present method, some of these difficulties are avoided, and the small amounts of serum required made possible a more detailed study of the behavior of the serums tested. One serum, obtained from A. M. before insulin therapy was started in the patient's final and hitherto unreported hospital admission, will be referred to below as "before" serum, and a second specimen, obtained 25 days after insulin treatment had been started, at a time when resistance had been reestablished, will be referred to as "after" serum.

CASE REPORT

(*Final hospital admission* [1])

After discharge from the Evans Memorial Hospital on 11/15/42, the patient, a 45-year-old, white female, was seen at infrequent intervals. In the fall of 1943, a diagnosis of pulmonary tuberculosis was made, and she spent several months in a sanatorium for tuberculosis. She refused to stay, however, and received no further hospital care until April, 1944, when she was readmitted to the Evans Memorial Hospital with advanced pulmonary tuberculosis. With the exception of this, examination revealed findings similar to previous admissions. No insulin had been given since November, 1942, and it was thought advisable to try insulin therapy again. The patient was intolerant of many procedures, and no attempt was made to restudy the effect of insulin therapy in detail. As shown in Table I, the administration of insulin in daily doses of between 40 and 102 units brought about a marked reduction in the 24-hour output of glucose for a period of 6 days. This was first apparent on the third day of insulin treatment, at which time the blood sugar fell from an average of 300 to 154 mgm. per 100 ml. Some degree of insulin effect was apparent up to the ninth day of therapy. Thereafter no response to insulin occurred with daily doses of 120 to 280 units. All insulin therapy was stopped on 5/20/44 after a total of 3,969 units had been given in a period of 33 days. During the treatment with insulin, the patient complained of itching and soreness at the site of the injections, and, in addition, had several attacks of urticaria while desensitization was in progress. The sequence of events following attempted therapy with insulin was similar to that following earlier attempts (5), and the presence of active tuberculosis appeared to have no influence on the patient's resistance.

TABLE I

Insulin dosage and glucose excretion of A. M. during final hospital admission

Day of month	Insulin	Glucose excretion	Blood sugar	Remarks
	units per day	*grams per day*	*mgm. per 100 ml.*	
4/12/44	0	76		
4/13/44	0	93	FBS 314	
4/14/44	0	100		
4/15/44	0	111		
4/16/44	0			
4/17/44	31	77	FBS 520	"Before" blood taken. Urticaria
4/18/44	58	53		Urticaria
4/19/44	96	20	4 PM 154	Urticaria
4/20/44	40	5.1		
4/21/44	82	tr.	FBS 352	
4/22/44	64	tr.	FBS 230	
4/23/44	130	tr.		
4/24/44	120	26		
4/25/44	220	48		
4/26/44	280	81	FBS 520	
4/27/44 to 5/20/44	120 to 280	150 to 211	425 on 5/12/44	"After" blood taken on 5/12/44

The patient refused sanatorium care, and finally left the hospital against advice. It was learned later that she died at home in the fall of 1944, and that no autopsy was done. The discharge diagnoses were: Diabetes mellitus with diabetic retinitis and peripheral neuritis; insulin allergy and resistance; chronic pyelonephritis; pulmonary tuberculosis.

MATERIALS AND METHODS

Each of 6 large white mice, starved for 18 hours, was injected intra-abdominally with 0.2 ml. of a mixture of serum, diluted or undiluted, and a dilution of insulin, after a fasting blood specimen had first been obtained. Subsequent blood samples were taken 30 minutes and 60 minutes after the injection of the mixture to be tested. For each blood sugar determination, .1 ml. of blood was drawn from a clean cut across a tail vein into a .1 ml.

[1] Previous hospital admissions have been reported (5).

micro blood sugar pipette which had been rinsed with a heparin solution containing 20 mgm. in 1 ml. of distilled water and thoroughly dried. The presence of heparin reduced the incidence of clotting, and did not interfere with the blood sugar determinations. These were done according to the technic of Folin, and the color readings were made in an Evelyn colorimeter. No animal was used more than once. The flow of blood was found to be freer when the animals were kept warm during the test. This was done with an ordinary desk lamp held at such a distance from the animals that a temperature of 27° C. to 30° C. was maintained. The performance of the test was facilitated by first drawing up a schedule in which the times at which blood samples were to be obtained and injections given were noted. A period of 5 minutes was allowed for each procedure.

Serum for testing was obtained from clotted blood, drawn with sterile precautions, and was stored in the frozen state at −10° C. The "before" and "after" serums from A. M., and serum from a case of uncomplicated diabetes, all of which contained high concentrations of glucose, were dialyzed in 10 ml. amounts in cellophane bags against 40 ml. of buffered salt solution at pH 7.4 for 18 hours at 4° C. No significant change in volume occurred.

TABLE II

Changes in the blood sugar of mice following the injection of normal serum and "before" serum from A. M., with and without insulin

Test no.	Serum	Insulin	Mouse no.	FBS	Change in blood sugar at		Mouse no.	FBS	Change in blood sugar at		Average change in blood sugar at	
					30 min.	60 min.			30 min.	60 min.	30 min.	60 min.
		units per mouse		*mgm. per 100 ml.*				*mgm. per 100 ml.*				
1	Normal	0	1	116	+4	+4	4	80	+28	+31	+24	+22
			2	95	+22	+28	5	128	+43	+35		
			3	125	+13	+5	6	102	+37	+31		
2	Normal	0	1	87	+28	+11	4	107	+3	0	+6*	+16**
			2	79		+50	5	124	−9	−1		
			3	113	+3	+20	6					
3	Uncomplicated diabetes	0	1	116	+4	+4	4	80	+28	+31	+24	+22
			2	95	+22	+28	5	128	+43	+35		
			3	125	+13	+5	6	102	+37	+31		
4	A. M. "before"	0	1	113	+38	+63	4	125	+18	+21	+35	+45
			2	92	+39	+55	5	88	+41	+48		
			3	112	+45	+42	6	132	+33	+39		
5	A. M. "after"	0	1	91	+16	+9	4	106	+27	+43	+25	+30
			2	94	+27	+30	5	81	+17	+21		
			3	89	+25	+40	6	102	+38	+40		
6	A. M. "after"	0	1	83	+42	+72	4	91	+43	+81	+38	+49
			2	108	+29	+24	5	70	+43	+40		
			3	63	+22	+22	6	88	+48	+56		
7	Normal	.005	1	97	−5	−15	4	86	−11	−10	−9	−16
			2	95	−17	−21	5	92	−5	−6		
			3	86	+10	−10	6	114	−26	−36		
8	A. M. "before"	.005	1	94	−44	−46	4	83	+3	−14	−13	−24
			2	98	−5	−24	5	91	−14	−17		
			3	91	−10	−26	6	90	−7	−16		
9	Uncomplicated diabetes	.005	1	94	+3	−12	4	81	−15	−18	−18	−26
			2	83	−20	−16	5	104	−36	−43		
			3	88	−26	−27	6	109	−15	−39		
10	Normal	.01	1	90	−19	−24	4	110	−28	−32	−18	−30
			2	97	−13	−38	5	89	−16	−31		
			3	106	−18	−22	6	92	−15	−32		
11	A. M. "before"	.01	1	97	−53	−63	4	92	−51	−48	−55	−68**
			2	110	−45	−67	5	112	−61	−79		
			3	79	−25		6	150	−99	−85		

* Average based on 4 animals.

** Average based on 5 animals.

Glucose determinations were made on the dialyzed serums, as well as other serums which were not dialyzed, and all serums tested contained between 40 and 55 mgm. glucose per 100 ml. Dialysis produced no observable change in the behavior of serums in mice, other than that attributable to the reduction in the glucose content.

RESULTS

Six tests in which mice were injected with mixtures of a serum and saline with no added insulin are shown in Table II (tests 1 to 6). Of the 35 mice tested, all but 1 showed a rise in blood sugar following injection. The rise was greatest in the first 30-minute period. Wide variations occurred in the responses of individual mice and, with the small number of animals used, the difference in the degree of the rise from test to test is hardly susceptible of interpretation. These results stand in sharp contrast to those obtained when insulin was injected with normal serum, serum from an uncomplicated case of diabetes mellitus, or "before" serum from A. M. A moderate but prompt fall in blood sugar followed the injection of .005 unit of insulin mixed with serum in 15 of 18 mice, and this fall was sustained or more marked at 60 minutes (Table II, tests 7 to 9). A greater fall occurred in mice receiving .01 unit (Table II, tests 10 and 11).

Tests with "after" serum from A. M., in which varying amounts of insulin were added to a constant volume of serum, are shown in Table III. In all tests with .06 unit or less, a rise in the average blood sugar occurred at the end of 30 minutes. Only in test 9 did a fall occur in the first 30 minutes, the amount of insulin required to cause this, .075 unit, being 15 times greater than that required when a normal or "before" serum was used. A rise in blood sugar over the fasting level was also observed at the end of 60 minutes in 2 tests with .01 unit, 1 of 2 tests with .02 unit, and 2 tests with .03 unit. The fall in test number 3 is unexplained. With .05 and .06 units, a moderate fall was seen at 60 minutes. In the test with .075 unit, the fall in the blood sugar was of about the same degree as that seen with "before" and normal serums in combination with .005 unit of insulin.

Titration of the neutralizing activity of this serum was also attempted by mixing varying dilutions of serum with a small constant dose of insulin. The results are shown in Table IV. A rise

TABLE III

Changes in the blood sugar of mice following the injection of a mixture of "after" serum with varying amounts of insulin

Test no.	Insulin	Mouse no.	FBS	Change in		Mouse no.	FBS	Change in		Average change in	
				30 min.	60 min.			30 min.	60 min.	30 min.	60 min.
	units per mouse		*mgm. per 100 ml.*				*mgm. per 100 ml.*				
1	.01	1	100	−1	−1	4	99	+20	+5	+10	+9
		2	95	+7	0	5	97	+20	+20		
		3	95	+1	+3	6	125	+13	+28		
2	.01	1	133	+3	+16	4	71	+14	+7	+10*	+9*
		2	100	+11	−3	5					
		3	88	+16	+23	6	95	+5	+1		
3	.02	1	92	−38	−41	4	126	−15	−38	+6	−13*
		2	97	0	−23	5	81	+36	+21		
		3	90	+39		6	92	+14	+4		
4	.02	1	64	+23	+15	4	82	+22	+17	+33	+25
		2	88	+46	+41	5	91	+33	+31		
		3	80	+43	+32	6	88	+30	+17		
5	.03	1	88	+22	+2	4	71	+29	+23	+20	+4
		2	81	+29	+11	5	78	+20	−2		
		3	88	+10	−7	6	97	+13	−4		
6	.03	1	92	+12	+4	4	69	+41	+25	+28	+23
		2	82	+19	+34	5	94	+32	+24		
		3	75	+41	+35	6	97	+27	+17		
7	.05	1	95	+5	−16	4	78	+2	−8	+12	−7
		2	88	+30	+8	5	76	+16	−8		
		3	82	+13	−7	6	82	+8	−10		
8	.06	1	84	+22	−13	4	95	+29	−4	+12	−12
		2	102	+22	+18	5	104	+6	−29		
		3	88	−1	−14	6	96	−8	−33		
9	.075	1	89	−14	−21	4	74	−6	−19	−9	−21
		2	77	−9	−17	5	75	+3	−16		
		3	83	−15	−24	6	97	−16	−32		

* Average based on 5 animals.

in the average blood sugar occurred at the end of 30 minutes in tests done with serum diluted 1 : 3, 1 : 6, 1 : 12 and 1 : 24. When diluted 1 : 48, a marked fall occurred in 30 minutes. At the end of 60 minutes the results in this group of tests were less regular. A rise occurred in 3 tests (Table IV, tests 1, 3 and 4) with serum diluted 1 : 3, 1 : 6, and 1 : 12, but a fall occurred in 1 test done with 1 : 6, and 1 done with 1 : 24. When compared with the results obtained with undiluted normal serum (Table II, tests 6 to 9), these 2 tests suggest that some neutralization of insulin took place. Marked variation in the behavior of individual mice is evident in all but the first and the last.

The effect of injecting .1 ml. of "after" serum 18 hours before the injection of insulin was also tried. The amounts of serum and insulin were such as to make the tests comparable to those done with mixtures of insulin and serum. With a dose of .005 unit per mouse there was a rise in blood

TABLE IV

Changes in the blood sugar of mice following the injection of a mixture of varying dilutions of "after" serum and 0.005 unit insulin

Test no.	Dilution of serum	Mouse no.	FBS	Change in		Mouse no.	FBS	Change in		Average change in	
				30 min.	60 min.			30 min.	60 min.	30 min.	60 min.
			mgm. per 100 ml.				*mgm. per 100 ml.*				
1	1:3	1	65	+25	+32	4	89	+21	+9	+25	+26
		2	105	+39	+39	5	110	+15	+35		
		3	96	+47	+43	6	95	+2	+6		
2	1:6	1	89	−12	−15	4	82	+1	+1	+6	−6
		2	75	+13	−9	5	88	−11	−14		
		3	73	+7	+6	6	91	+6	−3		
3	1:6	1	95	+13	+22	4	109	+19	+20	+7	+5
		2	105	+2	0	5	124	−16	−36		
		3	107	+21	+24	6	78	+3	−1		
4	1:12	1	124	+2	−12	4	67	+24	+26	+12	+6
		2	117	+12	−11	5	70	+18	+16		
		3	86	+7	+6	6	88	+12	+14		
5	1:24	1	92	+7	−8	4	102	+5	−5	+6	−5
		2	67	+4	−6	5	77	+16	−6		
		3	78	+13	+2	6	74	−7	−9		
6	1:48	1	93	−39	−46	4	82	−38	−43	−28	−33
		2	75	−21	−22	5	80	−19	−26		
		3	86	−29	−35	6	108	−25	−28		

sugar in 5 of 6 mice in 30 minutes, and in 4 of 6 mice at 60 minutes with an average rise of 11 mgm. per 100 ml. in 30, and of 9 mgm. per 100 ml. in 60 minutes. With .01 unit per mouse there was a rise in all 6 mice at 30 and 60 minutes. However, with .03 unit per mouse, the average change was downward at 30 and 60 minutes. Thus, as would be expected, the anti-insulin effect of the serum was diminished when a period of time was allowed to elapse between the injection of the serum and the administration of insulin. It appears, therefore, that the insulin antagonist in the "after" serum of A. M. is most active when it is injected simultaneously with insulin. This suggests that this antagonist is not the glycotropic factor of the pituitary. This hormone, when injected, produces no immediate effect on the susceptibility of the animal to insulin, but on the contrary, a period of several hours is required before interference with the action of insulin becomes demonstrable (7).

No decrease in the insulin-neutralizing effect of the serum could be demonstrated when insulin was added to the serum in 3 to 6 divided doses at 5-minute intervals in amounts totaling .03 unit per mouse and .06 unit per mouse. This was done in an unsuccessful attempt to demonstrate a Danysz phenomenon.

DISCUSSION

The performance of the test, irrespective of the injection of insulin, may be assumed to have effects on the experimental animal which, in themselves, may cause elevation or depression of the blood sugar. Unexplained falls in blood sugar of individual mice receiving serum or saline without insulin were occasionally seen in some earlier unreported experiments, but were not observed in later experiments. The marked tendency for the blood sugar to rise when serum alone was injected (Table II) may probably be explained as being due to the animal's fright and anger at being held, bled, and injected, and to the non-specific effect of injecting foreign material. The regular rise in the blood sugar of mice following the injection of serum without any added insulin casts some doubt on the significance of experiments which have been carried out in the past, allegedly demonstrating a specific blood-sugar raising effect of serum from an insulin-resistant subject (8).

The results of this study support, in a roughly quantitative manner, the conclusions reached in the earlier qualitative tests (6). An estimation of the insulin inactivating capacity of the patient's plasma volume at the time the "after" serum was drawn readily explains the resistance to insulin and, in addition, indicates that the doses given the patient during the final hospital admission were a fraction of what would have been required to induce a fall in blood sugar. The amount of insulin which, on the basis of the studies made, would be neutralized by the patient's entire plasma volume (2,500 ml.) can be estimated in a number of ways. One figure can be arrived at by using the largest dose of insulin per mouse with which neutralization of insulin was demonstrable, namely .06 unit per .1 ml. (Table III, test 8).

$$.06 \times 10 \times 2{,}500 = 1{,}500 \text{ units}$$

When calculated on the basis of tests in which dilutions of serum were made, the required dose for rapid insulin effect would be (Table IV, test 5)

$$.005 \times 24 = .12 \text{ u per } .1 \text{ ml. serum}$$

and

$$.12 \times 10 \times 2{,}500 = 3{,}000 \text{ units}$$

In tests of this kind the agreement cannot be expected to be close, but the evidence clearly indicates that something well over 1,000 units would be required in order to bring about a rapid fall in blood sugar in the patient. This reasoning is based on the extremely doubtful assumption that the insulin-neutralizing factor was present in the circulating blood alone. If an antibody was actually involved, the tissues might also have played a large part in the inactivation of the administered insulin. Furthermore, the continuous production of the antibody would serve to augment the dose of insulin required to bring the diabetes under control if the doses were spread, as is usually the case, over a period of time. Thus, the reported instances of insulin-resistant individuals requiring 3,000 to 4,000 units of insulin daily can be explained on the very mechanism that accounted for insulin resistance in A. M.

Assuming the immunologic nature of insulin resistance in A. M., the fact that relatively little insulin could be tolerated because of the high degree of allergy provided a circumstance especially favorable for the demonstration of the insulin-neutralizing antibody. Administration of doses of insulin sufficiently large to have controlled the diabetes should at the same time have brought about a marked diminution in the antibody content of the circulating blood. This may in part explain the failure of many of the attempts in the past to demonstrate insulin-neutralizing activity in the serum of resistant subjects, who, after all, can be recognized clinically only by their failure to respond to any but very large doses of insulin. Inadequate treatment of the diabetes with insulin, or better still, no treatment at all for a brief period before obtaining serum, would probably facilitate the demonstration of insulin-neutralizing activity in the serum of resistant individuals.

The ready demonstration of insulin-neutralizing activity in the serum of resistant patients will probably depend on a high degree of resistance. Thus, patients who are resistant to insulin but who require only 300 to 500 units daily will, in all likelihood, have nothing in their serums that could be demonstrated by the technic described. Study of such patients with a view to determining the cause of the resistance will probably have to depend on different methods.

Failure of the usual *in vitro* immunologic tests for the demonstration of antibody in the serum of insulin-resistant subjects is, like the failure to demonstrate the Danysz effect in the serum of A. M., no argument against the immunologic nature of the resistance. Such methods depend for their success on suitable characteristics of both the antigen and the antibody, as well as adequate concentrations of the latter. The evidence to date (6) is against the view that the precipitins or complement fixing antibody occasionally encountered in the serum of patients receiving insulin (9), or animals injected with insulin in an attempt to induce allergy or antibody formation (10, 11), are actually concerned with insulin resistance.

SUMMARY AND CONCLUSIONS

1. A method is described which adequately demonstrates interference, by serum of an insulin-resistant patient, with the blood sugar lowering effect of insulin in mice, and which permits rough quantitation of this interference.

2. Estimation, on the basis of the results of this study, of the total insulin-neutralizing activity of the patient's blood volume, readily explained the patient's resistance.

3. The mechanism giving rise to insulin resistance in the patient studied, presumably immunologic in nature, can explain the extreme instances of insulin resistance which have been occasionally reported.

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