Guinea Pig Anti-Insulin Serum

Adjuvant Effect of H. Pertussis Vaccine

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SUMMARY

After three injections at weekly intervals of insulin in a water-in-oil emulsion containing H. pertussis vaccine, guinea pigs were given the same inoculum containing no vaccine at intervals of four weeks. At the first bleeding after eight weeks, most animals yielded serum binding more than 1.0 U. insulin per milliliter; the mean binding capacities of sera from animals in four out of seven groups exceeded 4.5 U. insulin per milliliter. Over eighteen months, five groups totalling 136 animals yielded 3,000 ml. serum, the pooled sera from each group binding 2.7 to 4.8 U. insulin per milliliter. It is concluded that use of H. pertussis vaccine offers a reliable method for the production of large volumes of potent anti-insulin serum. DIABETES *17*:513-16, August, 1968.

Guinea pigs are more responsive than other experimental animals[1] or man[2,3] to the antigenic action of insulin but individual guinea pigs show wide variations in their responses. An initial injection of killed Mycobacterium butyricum followed by monthly injections of insulin in a viscous water-in-oil emulsion, for example, had a very variable effect.[4,5] Between 28 and 83 per cent of animals in any treated group failed to produce significant amounts of insulin antibodies over periods up to eighteen months. It was also noted, however, that by incorporating killed Hemophilus pertussis in the emulsion, production of insulin antibodies could be induced in previously nonproductive guinea pigs, and a more uniform and positive response could be elicited in the initial stages of immunization.[5] We now wish to report that by a standardized procedure involving the use of H. pertussis vaccine, it has proved possible to produce large volumes of very active guinea pig anti-insulin serum.

MATERIALS AND METHODS

Guinea pigs. All animals (male or female, albino, 350 to 500 gm.) were obtained from the same commercial source (Frank Edmondson, Washington, Indiana) and were kept for two to four weeks before use.

Inocula. Water-in-oil emulsions were prepared immediately before use from equal volumes of oily and aqueous phases. The *oily phase* contained heavy mineral oil (U.S.P.; 14 ml.) and lanolin (Adeps Lanae, anhydrous, U.S.P.; 6 ml.) which were thoroughly mixed at 60 to 90° C. and then cooled to room temperature. The *aqueous phase* (20 ml.) contained either (A) insulin (bovine or porcine, 20 mg.) dissolved in a solution of phenol (0.3 per cent, w/v) acidified (pH, 2.6) with hydrochloric acid; or (B) the same amount of insulin (20 mg.) dissolved in a smaller volume of acid-phenol solution (14 ml.) to which was added a standard volume (6 ml.) of H. pertussis vaccine (Fluid, U.S.P., Eli Lilly and Company, Indianapolis) immediately before emulsification. The vaccine contained an unwashed suspension of killed H. pertussis in a solution of sodium chloride preserved with merthiolate (1:10,000). Recrystallized preparations of bovine (Lot Nos. T2842, 25.2 U./mg.; PJ4609, 23.8 U./mg.) and porcine (Lot No. PJ5589, 23.9 U./mg.) insulins were used throughout. Emulsification in a Waring-type blender provided viscous inocula which, for convenience of description, will be termed *Routine* (RI) and *Pertussis* (PI) according to the aqueous phase incorporated (A or B, respectively).

Immunization procedure. At each injection, guinea pigs received a constant volume of emulsion (2.0 ml.), the dose being divided equally between subcutaneous sites high on the back and low on the anterior abdominal wall. The first three injections (PI) contained pertussis vaccine and were given at weekly intervals (0, 1 and 2 weeks). Four weeks after the last of these three injections (i.e., at six weeks) and at intervals of four weeks thereafter, they received the Routine inoculum (RI) containing no pertussis vaccine.

At eight weeks and two weeks after each subsequent injection of the routine emulsion, the guinea pigs were bled by cardiac puncture and the blood (usually 10 to 12 ml.) allowed to coagulate. Serum separated from blood drawn from each group of guinea pigs at a given

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bleeding was combined, its combined volume measured and potency assayed. At the first time of bleeding (eight weeks) and before sera of individual animals were pooled, samples of serum from each animal (0.1 ml.) were diluted in saline (0.9 ml.; 0.9 per cent w/v) for individual assay.

Insulin antibody assay. The method used to assay antibody activity in guinea pig anti-serum has been reported in detail elsewhere[6] and is illustrated in table 1. A constant small volume of serum (10 μl.) was diluted in a solution (1 per cent BSA; 1.0 ml.) of phosphate buffer (0.1M; pH 7.0) containing bovine serum albumin (1 per cent, w/v; Bovine albumin, Fraction V, Sigma Chemical Company, St. Louis, Mo.). A mixture of unlabelled (100 to 150 mU.) and I-131 labeled (0.3-0.5 mU.; 8-12 mc./mg.; Abbott Laboratories, North Chicago, Illinois) bovine insulin dissolved in the same buffered solution of albumin (0.5 ml.; 1 per cent BSA) was then added and allowed to react at room temperature (20-25° C.) for thirty minutes. At this time the reaction was stopped by addition of a suspension (1.0 ml.) of finely divided cellulose (10 per cent, w/v; MN-300, Macherey, Nagel and Company, Duren, Germany) in the phosphate buffer (0.1 M; pH 7.0). After repeated agitation during a further thirty minutes at room temperature, this final suspension was centrifuged and an aliquot (1.0 ml.) of the clear supernatant solution removed for assay of γ-radioactive content in an automatic well-type scintillation counter (Series 3000 model 5052; Packard Instrument Co., La Grange, Illinois). As shown in table 1, additional tubes containing an excess of nonprecipitating anti-insulin serum (Excess), no serum (Blank), or no serum treated later with buffer instead of the suspension of cellulose (No cellulose) were also examined. From the radioactive contents of the supernatant solutions from these reaction mixtures, the insulin binding potency of the sample was calculated using the following expression:

$$\text{Insulin bound (mU.)} = \text{Insulin added (mU.)} \times (C_S - C_{B1})/(C_{Exc} - C_{B1}).$$

The amount of insulin incorporated in the insulin mixture was sufficient to ensure that samples of serum did not bind more than 65 per cent of the total added radioactive insulin; under these conditions a linear relationship has been established between insulin binding and antibody content of a sample.[6] In most assays, an excess of nonprecipitating anti-insulin serum was not used to determine C_{Exc}, for *under the conditions used in the present assays* it has been shown[6] that $C_{Exc} = C_{NC} \times 1.09$.

TABLE 1

Composition of reaction mixtures for assay of anti-insulin serum.

(The compositions of the solutes (buffer and 1 per cent BSA), insulin mixture, and suspension of cellulose are given in the text. Radioactivity (*) was measured in equal aliquots (1.0 ml.) of supernatant solution from each reaction mixture after centrifugation and used to assess the potency of the sample by the method described in the text.)

Tube	Sample	Blank	Excess	No cellulose
Anti-insulin serum (ul.)	10	Nil	100	Nil
1 per cent BSA (ml.)	1.0	1.0	0.9	1.0
Insulin mixture (ml.)	0.5	0.5	0.5	0.5
Suspension of cellulose (ml.)	1.0	1.0	1.0	Nil
Buffer (ml.)	Nil	Nil	Nil	1.0
Radioactive content*	C_S	C_{B1}	C_{Exc}	C_{NC}

All potencies are expressed as units bovine insulin bound per milliliter serum, mean values (± S.D.) being quoted for serum from individual animals in the various groups.

RESULTS

The guinea pigs (193) were immunized in groups of seventeen to forty animals for periods of eight to fifty-two weeks with either bovine (Groups, X, Y, A and E) or porcine (Groups W, B and C) insulin. No animals were seen to develop hypoglycemia after the first injections of insulin but some (10 per cent) died during the eight weeks preceeding the first bleeding. After that, most losses were due to hemorrhage during or immediately after cardiac puncture and about one in every seven animals died during each period of four weeks. From each animal, an average of 4.3 ml. serum was obtained at each bleeding, the total yield over eighteen months being just over 3,000 ml. These and other details of the findings are summarized in table 2 and figure 1.

At the end of the first eight weeks, and after three injections containing, and one injection without H. pertussis vaccine, all seven groups of guinea pigs yielded pooled serum binding more than 1.0 U. insulin per milliliter (figure 1). Within each group (table 2), the potencies of sera obtained from individual animals varied widely (coefficients of variation, 41 to 80 per cent). In groups producing very active pools of anti-insulin serum (Groups, W, A, B and C), none of the individual animals yielded serum binding less than 1.0

TABLE 2

Insulin antibody production by seven groups of guinea pigs.

(The table shows the initial and final numbers of animals in seven groups of guinea pigs treated with bovine or porcine insulin for eight to fifty-two weeks; the mean potencies (± S.D.) of samples obtained from each group at eight weeks and the number of animals in each group producing weak (1.0 U./ml.) or very potent (7.0 U./ml.) serum; and the total volumes and mean potencies of all sera obtained from animals in each group during the periods of study.)

Group no.	X	Y	W	A	B	C	E
Animals Initial	20	40	27	32	17	20	37
Final	6	7	2	4	13	19	26
Study period (wks.)	36	52	44	40	24	8	8
Insulin (species)	Bovine	Bovine	Porcine	Bovine	Porcine	Porcine	Bovine
Initial injection	July, 1966	Sept., 1966	Jan., 1967	Feb., 1967	June, 1967	Oct., 1967	Oct., 1967
Initial bleeding (8 wks.)							
(a) Individual samples (n)	17	37	23	29	15	19	26
(b) Mean potency (U./ml.)	2.54±1.53	1.41±1.13	5.03±2.48	5.35±2.22	5.39±2.45	4.70±2.91	2.83±1.58
(c) Weak producers	2	16	0	0	0	0	1
(d) Potent producers	0	0	6	8	3	4	1
Serum obtained during study							
(a) Pooled samples (n)	8	12	10	9	5		
(b) Total volume (ml.)	407	1,195	579	644	223		
(c) Mean potency (U./ml.)	2.96	2.71	4.26	4.78	4.35		

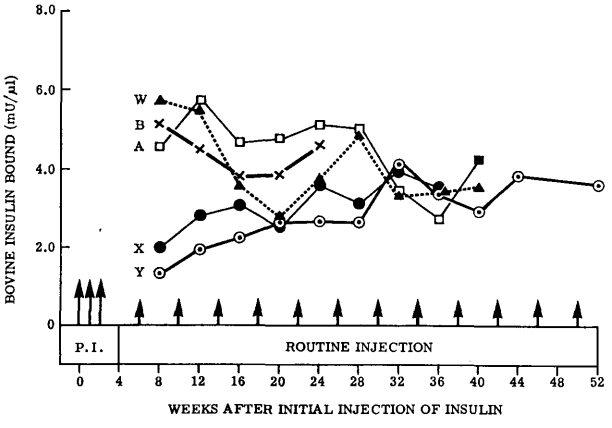

FIG. 1. Insulin-binding potencies (U./ml.) of pooled samples of serum obtained at intervals of four weeks from five groups of guinea pigs given repeated injections of bovine (Groups X, Y, and A) or porcine (Groups W and B) insulin. Inocula given at 0, 1 and 2 weeks (PI) contained H. pertussis vaccine which was omitted from subsequent injections (RI) given at six weeks and after each succeeding period of four weeks; the times of injection are given by the arrows.

U. insulin per milliliter and many (20 to 25 per cent) produced serum binding more than 7 U. insulin per milliliter. The most active sera from these latter animals bound 9.5 to 12.0 U. insulin per milliliter. Less dramatic were the initial responses seen in animals of the other three groups (X, Y and E). Of eighty individual samples from animals in these three groups, only one bound more than 7 U. insulin per milliliter; and in one group (Y) there was a high proportion (43 per cent) of animals yielding serum with little or no potency (less than 1.0 U. insulin bound/ml.).

After this first bleeding, individual animals were not examined but pooled samples were obtained every four weeks for twenty-four to fifty-two weeks from five groups of guinea pigs (X, Y, W, A and B). As shown in figure 1, the later responses of these animals were of two broad types. Those groups which had shown relatively poor initial responses (X and Y) produced increasingly active anti-insulin serum for the next two or three months and after twenty weeks consistently yielded serum binding more than 2.5 U. insulin per milliliter. The potencies of pooled sera from the other three more responsive groups of animals (W, A and B) fell or fluctuated, sometimes over a wide range (Group W), but seldom if ever reached values of less than 3 U. insulin bound per milliliter. If, within each group of animals, the sera obtained at each bleeding were pooled, it is seen (table 2) that all five groups of guinea pigs produced pooled serum binding more than 2.5 U. insulin per milliliter. On a basis of the initial number of animals used in each group and ignoring losses of animals during the studies, it can also be calculated that serum was obtained at rates of 2.2 to 2.6 ml. per bleeding per animal.

DISCUSSION

Since Moloney and Coval[7] first drew attention to some of its unique characteristics, guinea pig anti-insulin serum has been put to several practical uses. It is the only anti-insulin serum currently used to assay insulin in blood and other biological fluids,[8-10] to distinguish

between insulin and other components of blood having insulin-like activity upon isolated tissues in vitro,[11-13] to induce insulin deficiency in experimental animals,[1,7,14,15] to stain islets after conjugation with fluorescein,[16] and, very recently, to protect and estimate insulin secreted from pancreatic tissue in vitro.[17] To provide the serum needed for such purposes, sometimes in large amounts, a reliable and effective method for its production is desirable. For the study of factors likely to influence antibody production in these animals such a method is essential.

By incorporating H. pertussis vaccine in the initial inocula of insulin, better results were obtained in the present study than in those reported previously.[1,4,5] The experience had been that maximal potencies were generally of a lower order, less than 2.4 U. per milliliter, and nonresponders more frequent. As mentioned above, use of the routine adjuvant (RI) alone or after a single injection of killed Mycobacterium butyricum results in very variable responses with doubtful assurance of obtaining potent anti-insulin serum.[4,5] In animals which do not produce antibodies to insulin under this regime, however, incorporation of H. pertussis vaccine induces a prompt and lasting response.[5] With the present procedure, as shown in table 2, the incidence of weakly responsive animals was low in all but one group of guinea pigs (Y) and relatively large volumes of very potent pooled anti-insulin serum were obtained from three (W, A and B) of the five groups immunized for twenty-four weeks or more. Moreover the relative potencies of the pooled sera ultimately collected from these five groups were reflected in the mean potencies of the sera obtained from the individual animals in each group at the first time of bleeding. For example, those groups which yielded the weakest serum at the first time of bleeding (X and Y), continued to do so later and produced the weakest combined pools of serum. No definite explanation for the relatively unresponsive behavior of these latter animals is provided by the present results. No clear evidence could be found in the present studies that the responses of guinea pigs are affected by the time of year at which the first injection of insulin is given,[5] and the results obtained with bovine insulin could not be distinguished from those produced with porcine insulin.

Whatever the explanation for this lack of uniformity in immunological response, there can be no doubt as to the effectiveness of H. pertussis vaccine in stimulating the initial response. In most animals it induces a prompt effect and in the remainder a good response is ultimately achieved. It is, therefore, possible to claim that by this method large volumes of highly active serum can usually be obtained within eight to twelve weeks.

ACKNOWLEDGMENT

This study was supported by a U.S. Public Health Grant (No. PHS AM-10116). The authors wish to thank Eli Lilly and Company, Indianapolis, for the preparations of insulin; and Miss Mary Posey for technical assistance.

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