PRODUCTION OF ANTIBODIES OF HIGH BINDING AFFINITIES TO GLUCAGON IN RABBITS

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Many investigators have experienced difficulties in consistently producing high titer antisera to glucagon. Usually, several months were required for the animals to achieve high levels of antibody, with only a few of the animals responding satisfactorily to the antigenic stimulus (Grodsky et al, 1961; Unger et al, 1961; Probst and Colwell, 1966; Schopman et al, 1967). Similar problems have been reported with the bovine parathyroid hormone (Tashjian et al, 1962). At least two factors may be directly implicated in this deficiency; the small size of the glucagon molecule (the molecular weight is 3,485 (Staub et al, 1955)), and the immunochemical similarity or identity of this hormone in the different species thus far investigated, as evidenced by the applicability of beef-porcine glucagon in radioimmunoassays of endogenous glucagon in man, cow, dog and pig (Unger et al, 1961; Lawrence, 1966; Schopman et al, 1967). This preliminary communication reports on a procedure for obtaining a comparatively rapid production of rabbit antisera of high binding affinity, the duration of the response, and susceptibility to 2-mercaptoethanol treatment. The antisera produced in this manner have been successfully used for assaying human serum glucagon levels (Lawrence, 1966).

MATERIALS AND METHODS

Twenty New Zealand White male rabbits, 2.5 to 3.5 Kg, received a total of 16 injections into the thigh muscles of a 1 mg glucagon preparation for four weeks--four injections per week on alternate days--for a total of 16 mg of glucagon per rabbit. An intramuscular booster injection of 1 mg of the same

glucagon preparation was administered fifty-six days after the last injection. Food and water were given ad libitum and no untoward effects were noted in the animals, except for granuloma formation at injection sites. Intracardiac blood samples were obtained on the days indicated (Table 1) and permitted to clot for two hours at 10°C; the serum was separated and stored at -20°C without preservatives until used. Blood samples were obtained before the injections were given on the days on which the two coincided.

Glucagon for injections was prepared by suspending the hormone in a mixture of Freund's complete adjuvant (3/4 v/v) and liquefied beeswax (1/4 v/v) to a final concentration of 1 mg glucagon per 0.25 ml of the adjuvant mixture. Disposable tuberculin syringes were filled to the 0.25 ml level and stored at 10°C until used. Prior to injecting, the syringes were immersed in warm water to soften the beeswax and facilitate the injections.

Special research lots of recrystallized glucagon (kindly supplied by Dr. M. A. Root of the Lilly Research Laboratories) were employed. Such preparations have been shown to contain minimal amounts of insulin as a contaminant (Yalow and Berson, 1961). The use of commercial glucagon lots (Lilly) for immunization has shown that these preparations contain sufficient quantities of insulin to elicit anti-insulin antibodies in about 30% of the rabbits tested (13), the presence of which may complicate glucagon radioimmunoassays since some of the insulin contaminant of the glucagon used for iodination may also be iodinated. Cysteine treated glucagon was not used.

Since the glucagon-antibody complexes are of the non-precipitating type, antibody activity was detected by the binding of a trace quantity of I-131 labeled glucagon by an aliquot of the serum studied. Tests for the possible presence of anti-insulin antibodies were conducted with insulin-I-131.

Iodination of glucagon with I-131 by the chloramine-T method of Greenwood et al.(1963) resulted in preparations with activities of 500 to 650 mc/mg. The labeled glucagon was purified on a cellulose column as described by Berson

and Yalow (1961). Insulin-I-131 was purchased from Abbot Laboratories.

The titer was arbitrarily defined as the per cent of 25 μμg of glucagon-I-131 bound by 50 μl of antiserum, as determined by the Lawrence (1966) modification of Unger's method (Unger et al, 1961). Briefly, the mixture of labeled glucagon and the antiserum was permitted to react for 96 hours at 4°C, followed by the separation of the antibody bound and free radioactive glucagon fractions by descending chromatography on Ecteola ion exchange cellulose strips in 0.075 M veronal buffer, pH 8.6. The strips were then scanned on a 4-Pi chromatogram scanner. Glucagon-I-131 incubation damage never exceeded 6.6% and the proper corrections were applied.

Specificity of binding was demonstrated by the addition of different quantities of unlabeled glucagon to the reaction mixtures and noting the corresponding decrease in the binding of glucagon-I-131 (Fig. 1). These curves also serve as standards in radioimmunoassays and as screening tests in selecting antisera suitable for such assays (sensitive sera). Acceptable sera were defined as those binding at least half of the trace amount of labeled glucagon in the absence of any unlabeled glucagon in the reaction mixture, and the presence of 25 to 250 μμg of unlabeled glucagon would inhibit the binding by at least 50%.

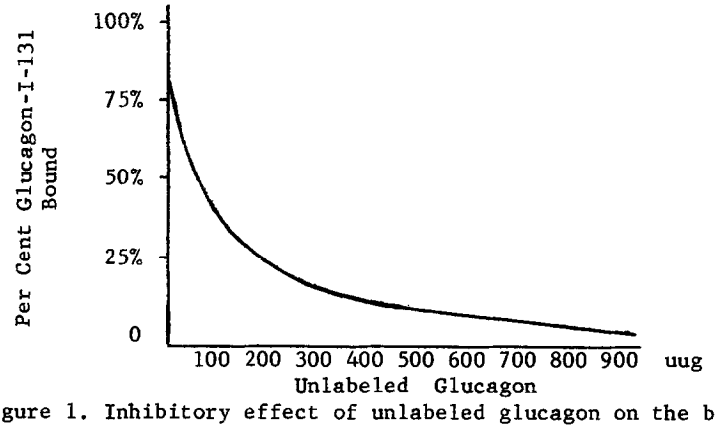

Figure 1. Inhibitory effect of unlabeled glucagon on the binding of glucagon-I-131 by antiserum.

The effect of 2-mercaptoethanol treatment (Deutsch and Morton, 1957) on the binding activity was also determined.

Detection of the possible presence of anti-insulin antibodies was performed in a similar manner, except that 10 μμg of insulin-I-131 was used and the separations were performed on Whatman 3 MM filter paper strips.

RESULTS

Table 1 summarizes the findings on the production of antisera to glucagon in rabbits by means of an intensive immunization schedule. No antibodies to insulin were formed with the use of recrystallized glucagon. All rabbits showed antibody activity 21 days after the initiation of immunization and reached peak activity seven days after the last injection; thereafter the titer decreased steadily. Fifty-six days after the last injection, a booster injection of the same antigenic preparation resulted in an anamnestic response of enhanced binding activity that reached its peak in 14 days. Treatment with 2-mercaptoethanol completely inactivated the antibody activity detected on day 21, and partially inactivated the activity observed on days 28 and 35 (significant at the 5% level as determined by the Student's "t" test). Thereafter, the binding activities between the 2-mercaptoethanol treated and untreated sera were not significant at the 5% level. The latest findings have shown that 2-mercaptoethanol treatment inactivates the antibody activity of certain 7S globulins, in addition to that of the 19S macroglobulins. Grey (1963) has postulated that the mercaptoethanol sensitive 7S globulins represent a more primitive type of immune response, in the evolutionary sense, that has been largely replaced in the mammals by resistant 7S antibodies. Interestingly, 2-mercaptoethanol sensitive 7S rabbit pancreas isoantibodies have recently been reported (Brinckerhoff and Rose, 1967).

As judged by our criteria, the maximum number of rabbits yielding antisera suitable for glucagon radioimmunoassay after the primary injections was seven out of twenty; following a secondary stimulation, the number of rabbits responding favorably doubled. Antibody activity was detectable in all rabbits six

Table 1. Antibody Titers in New Zealand White Rabbits to Glucagon.

A Day	B Per Cent Iodinated Glucagon Bound per 50 µl of Antiserum	C Per Cent Iodinated Glucagon Bound per 50 µl of Antiserum After 2-mercapto-ethanol Treatment	D Significance Between B & C at 5% Level	E Number of Sensitive Antisera
7	0	0		0
14	0	0		0
21	16.3 ± 8.5	0	+	0
28	49.4 ± 8.9	20.1 ± 4.7	+	3
35	66.3 ± 9.7	42.1 ± 5.3	+	5
42	60.8 ± 7.3	58.6 ± 8.4	-	7
49	50.3 ± 5.5	52.9 ± 6.6	-	7
56	36.6 ± 7.1	42.1 ± 9.3	-	3
84	27.5 ± 9.7	23.3 ± 6.9	-	0
91	78.0 ± 8.6	73.1 ± 7.5	-	11
98	87.9 ± 8.1	81.8 ± 9.3	-	14
105	86.4 ± 6.3	87.5 ± 7.6	-	14
112	87.9 ± 8.8	85.2 ± 5.9	-	14
140	73.1 ± 9.5	77.8 ± 7.4	-	11
168	71.7 ± 8.1	67.2 ± 6.9	-	10
196	59.3 ± 5.8	55.1 ± 6.7	-	8
252	43.6 ± 9.8	39.6 ± 7.3	-	3

A. Number of days since the first injection. Last injection given on day 28. Booster injection given on day 84.
B. and C. Mean titers of the 20 rabbit sera ± standard error.
D. Significance at the 5% level determined by Student's "t" test.
E. Number of rabbits producing antisera suitable for use in glucagon radioimmunoassay.

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months after the secondary glucagon injection.

Methods employed in this study make possible the detection of picogram quantities of binding antibody.

DISCUSSION

Despite the advances being made in the understanding of the complex factors involved in determining the antigenicity of a given substance, the determination of conditions that result in an immunologic response remains essentially an empirical process. Since it is well recognized that poorly antigenic material may induce excellent antibody formation following intense immunization, we felt that such an approach should be taken in the case of glucagon.

In our experiments we induced the formation of a granulomatous glucagon depot in a highly vascularized tissue (muscle) to ensure the continuous systematic distribution of glucagon and the resultant stimulation of the antibody synthesizing system. Beeswax was employed in our adjuvant preparation because of its long persistence in tissues (partly due to its melting point, 62 to 65°C) and non-toxicity.

Four to seven weekly intramuscular injections of glucagon, for four weeks, resulted in the type of antibody response described above; three injections yielded a weak and an irregular response and one or two weekly injections failed to stimulate antibody production. Single monthly glucagon injections (5 mg) were ineffective. Substitution of mineral oil for Freund's adjuvant had an adverse effect on antibody formation when fewer than six injections were given per week. Replacement of beeswax by other substances resulted in an inconsistent antibody response. Subcutaneous injections were far less effective. These observations indicate that frequent intramuscular injections are more effective in eliciting an antibody response to glucagon administered in our adjuvant.

Animals failing to respond to the initial glucagon injections were challenged again 30 days after the last injection. A small number of these animals responded with a weak anamnestic reaction.

Our recent studies indicate that intramuscular or subcutaneous injections (three per week) of glucagon in a mixture of either mineral oil or Freund's complete adjuvant and polyvinylpyrollidone result in a similarly rapid antibody

response; however, far fewer animals yielded sera suitable for our radioimmunoassay. We also observed that intravenous administration of glucagon sensitized sheep erythrocytes induces antisera in rabbits. Furthermore, it may be possible to regularly produce mouse ascitic antibody to glucagon, as indicated by some initial observations.

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REFERENCES

1. Berson, S.A., and Yalow, R.S., J. Clin. Invest. 40, 1803 (1961).
2. Brinckerhoff, C.E., and Rose, N.R., Proc. Soc. Exp. Biol. Med. 124, 252 (1967).
3. Deutsch, H.F., and Morton, J.I., Science 125, 600 (1957).
4. Greenwood, F.C., Hunter, W.M., and Glover, J.S., Biochem. J. 89, 114 (1963).
5. Grey, H., Proc. Soc. Exp. Biol. Med. 113, 693 (1963).
6. Grodsky, G.M., Hayashida, T., Peng, C.T., and Geschwind, I.I., ibid. 107, 491 (1961).
7. Lawrence, A.M., Proc. Natl. Acad. Sci. 55, 316 (1966).
8. Probst, G.W., and Colwell, R.W. Biochemistry 5, 1209 (1966).
9. Schopman, W., Hackeng, W.H.L., and Steendijk, C., Acta Endocrinol. 54, 527 (1967).
10. Staub, A., Sinn, L., and Behrens, O.K., J. Biol. Chem. 214, 619 (1955).
11. Tashjian, A.H., Levine, L., and Munson, P.L., Biochem. Biophys. Res. Comm. 8, 259 (1962).
12. Unger, R.H., Eisentraut, A.M., McCall, M.S., and Madison, L.L., J. Clin. Invest. 40, 1280 (1961).
13. Worobec, R., Unpublished Observations.
14. Yalow, R.S., and Berson, S.A., Proc. Soc. Exp. Biol. Med. 107, 148 (1961).