A Simplified Glucagon Immunoassay and Its Use in a Study of Incubated Pancreatic Islets* (33549)

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Several methods for the radioimmunoassay of glucagon have been described (1–9). Although, in general, these methods are sufficiently sensitive and specific for most physiologic studies, sometimes they are lengthy (3–6 days) and too cumbersome for the simultaneous analysis of many samples. The purposes of this study were to develop a simple and rapid assay method and to study the secretion of glucagon by isolated rat pancreatic islets, incubated under various experimental conditions.

Materials and Methods. Glucagon standards. A stock solution of crystalline glucagon[1] (lot 258-234B-167-1; 1 mg/ml), prepared with distilled water adjusted to pH 9.0 with NaOH, was divided into 0.2-ml portions and stored at −20°. When needed, working standards were prepared by diluting the stock solution with 0.05 *M* Veronal buffer at pH 8.0, containing 0.25% bovine serum albumin[2] (hereafter, this buffer will be called BSAVB). The BSAVB was used also for the dilution of antiglucagon serum (AGS) and for the preparation of the ^{125}I-labeled glucagon solutions.

Antiglucagon sera. Antiglucagon sera were prepared according to the method of Assan *et al.* (10), with slight modifications (11) and, more recently, according to the method of Worobec *et al.* (8), using polyvinyl pyrrolidone instead of bees wax. With this method, after 13–16 injections, 8 rabbits out of 15 produced sera with a binding capacity sufficient to warrant further exploration. The suitability of a serum was determined in the following manner: 0.1 ml of serum was incubated overnight at 4° with 50 pg of labeled and 5 ng of nonlabeled glucagon, in 1.1 ml of BSAVB containing 1000 KIU of Trasylol.[3] After incubation, the free (*F*) and antibody-bound (*B*) glucagon were separated by the addition of 0.5 ml of a 10% cellulose suspension[4], a technique previously used for the separation of insulin (12–14). Sera giving a $(B/B + F)$ ratio greater than 60%, were used to prepare standard curves. The selection of the serum and of the optimum amount to be used in the assay (in general 1–8 μl) was based on the slope of the standard curve, as determined for each batch of serum. Glucagon was labeled with ^{125}iodine using the method of Greenwood and Hunter (15) and purified by means of a column of Whatman CF 11 cellulose powder (12). The specific activity of labeled glucagon varied from 198 to 437 mCi/mg. Immunologically inactive or damaged glucagon ranged between 3 and 5%, at the time of iodination.

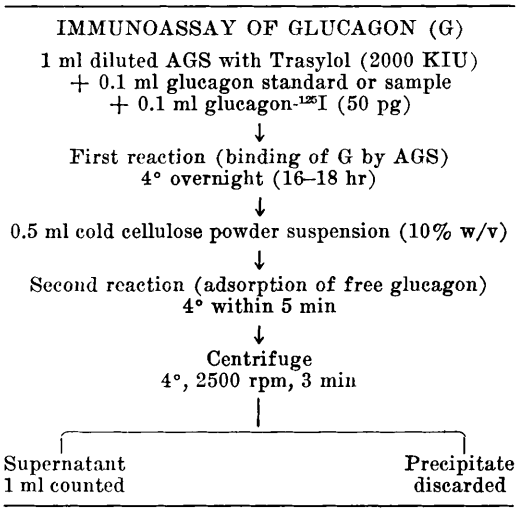

FIG. 1. Glucagon immunoassay: flow sheet.

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[1] Gift of Dr. Mary Root, Lilly Research Laboratories, Indianapolis, Indiana.

[2] Fraction V, Sigma Chemical Company, St. Louis. Bovine serum albumin from other sources caused a flattening of the standard curve.

[3] FBA Pharmaceuticals, Inc., New York, New York.

[4] Cellulose powder MN 300, Macherey, Nagel and Co., Düren, Germany.

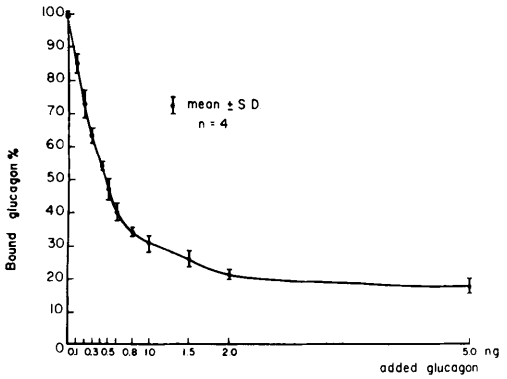

FIG. 2. Glucagon immunoassay: standard curve (rabbit antiserum).

Assay procedure (Fig. 1). To 1 ml of BSAVB, containing a suitable amount of AGS and 2000 KIU of Trasylol, add 0.1 ml of sample or of standard glucagon solution (0–2 ng) and 0.1 ml of glucagon-^{125}I solution, containing 50 pg of labeled hormone. In addition, prepare a blank containing no AGS (BL). Mix all tubes well and incubate overnight (16–18 hr) at 4°. In the morning, add 0.5 ml of a cold suspension of powdered cellulose MN 300 (10% w/v in 0.05 *M* Veronal buffer, pH 8.0, stirred continuously) to each tube. After thorough mixing with a Vortex mixer, spin the tubes at 2,500 rpm for 3 min in a refrigerated centrifuge and transfer aliquots of the supernatant fluid (usually 1 ml) to the tubes of an automatic gamma counter. Calculate the results using the formula: $(C_S - C_{BL}/C_0 - C_{BL}) \times 100$, where C_S = counts in the supernatant aliquot of the standard or the sample; C_{BL} = counts in the aliquot of the blank; C_0 = counts in the aliquot of a standard to which no unlabeled glucagon was added. A standard curve may be obtained assuming the amount of bound glucagon in the absence of added unlabeled glucagon to be 100% (Fig. 2). The decrease in this value obtained with 0.1 ng of unlabeled glucagon was constant and reproducible, however, with our best sera, as little as 30 pg of unlabeled glucagon could be detected with confidence. When the percentage of bound radioactivity ($B/B + F$) or the B/F ration was desired, the entire supernatant was transferred to another tube, the cellulose sediment was resuspended in BSAVB and respun and the radioactivities of the combined supernatant fluids and of the washed sediment were measured. The percentage of bound radioactivity was considered equal to $(B - BL/B - BL + F) \times 100$, where B = counts in the combined supernatant fluids of the samples; BL = counts in the combined supernatant fluids of the blank containing no AGS; and F = counts in the washed cellulose. This percentage was used to express the titer of the serum.

Blood collection. Blood was obtained from the antecubital vein, allowed to clot at room temperature for 30 min and centrifuged. After the addition of Trasylol (1000 KIU/ml), the serum was mixed well and stored at −20°.

Isolated pancreatic islets. Rat pancreatic islets were obtained using the method of Lacy and Kostianovsky (16), but without staining with neutral red, a substance found to be toxic for the A cells (17). Ten islets were incubated in 2 ml of Krebs–Henseleit buffer with Trasylol (4000 KIU) and with or without glucose in an atmosphere of 95% O_2 and 5% CO_2, at 37°, using a Dubnoff shaking incubator. Aliquots of incubation medium (50–100 μl) were taken at various time intervals for the assay of glucagon.

In some experiments, after 10 min of incubation, the tubes were immersed in ice water and the glucose concentration of the medium was changed by removing an aliquot of incubation medium and replacing it with an equal volume of fresh buffer containing either no glucose or an amount of glucose calculated to give the desired new concentration. The actual concentration was determined at the end of the experiment. Control replacements were done without changing the concentration of glucose. These manipulations lasted about 10 min after which the incubation at 37° was resumed.

Results. The optimum incubation time for the binding of glucagon by AGS (first reaction) differed slightly from antiserum to antiserum, however, varying the time from 16 to 72 hr did not modify significantly the slope of the standard curve (Fig. 3). Therefore, for practical reasons, a 16–18 hr over-

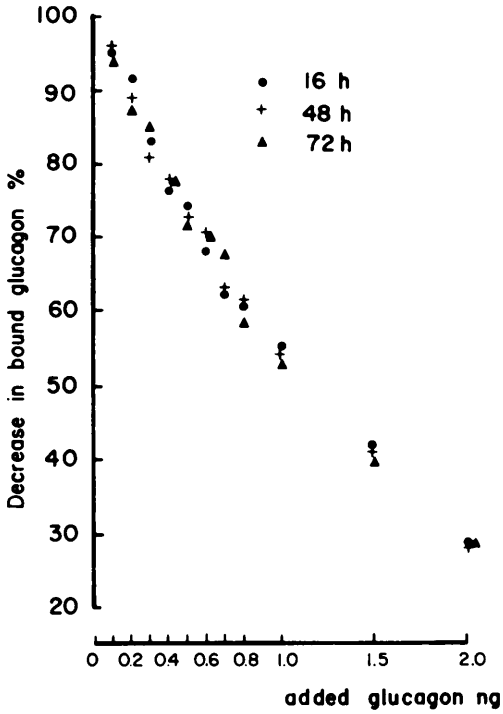

FIG. 3. Glucagon immunoassay: effect of incubation time on the standard curve (guinea pig antiserum).

night incubation was used. The optimum pH for the first reaction was found to vary between 7.5–8.0 (Fig. 4). We selected pH 8.0

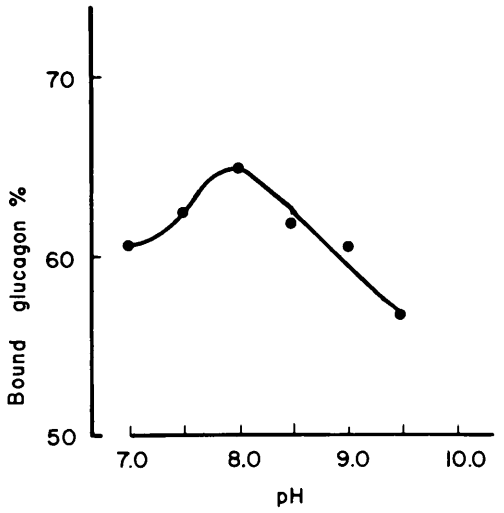

FIG. 4. Glucagon immunoassay: effect of pH on the binding of glucagon by AGS; phosphate or glycine buffer.

because human serum has a tendency to become alkaline during storage and because, when small quantities of cellulose are used, the adsorption of free glucagon (second reaction) decreased at a pH higher than 8.0 (Fig. 5). Figure 6 shows the effect of serum on the adsorption of glucagon by cellulose powder. As shown, in the presence of 0.2 ml of human serum and of 1 ng of cold glucagon, the adsorption by 50 mg of cellulose powder was almost complete. More cellulose was required when more than 0.2 ml of serum were used. However, since 1 ng of glucagon is an amount greater than that which can be expected in 0.2 ml of serum, 50 mg of cellulose were used in the assay system. Figure 7

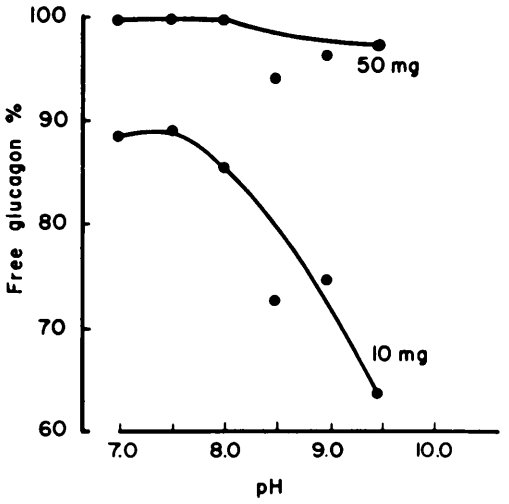

FIG. 5. Glucagon immunoassay: effect of pH on the binding of free glucagon by cellulose powder.

shows that when the adsorption time was greater than 5 min, there was a decrease in the amount of antibody-bound glucagon, perhaps because the removal of all free glucagon from the reaction system by powdered cellulose caused a change in the previously reached equilibrium between glucagon and its antibodies. Accordingly, this step was carried out within 5 min or as rapidly as possible. An automatic pipette[5] and a Vortex mixer are useful aids for rapid delivery and quick and complete mixing. Concentrations

[5] Becton, Dickerson and Co., Rutherford, New Jersey.

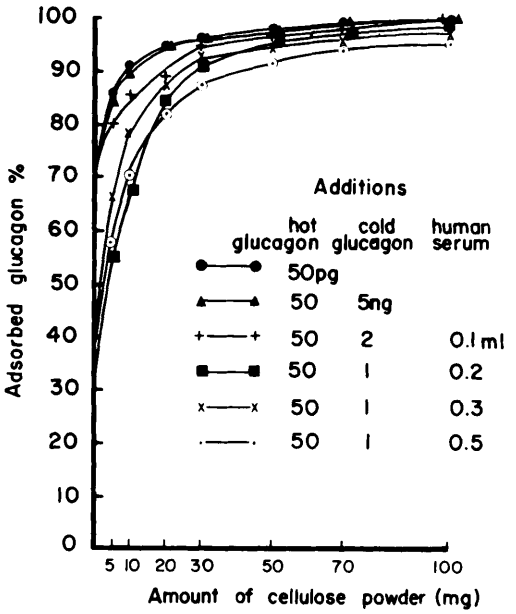

FIG. 6. Glucagon immunoassay: adsorption of glucagon to different amounts of cellulose powder.

of bovine serum albumin greater than 0.25% in the first reaction mixture decreased glucagon binding. Using 0.1–0.2 ml of serum, we often found that the radioactivity of the supernatant was higher in tubes containing serum samples than in "standard" tubes containing no added unlabeled glucagon. Therefore a control tube containing the serum sample and all other components of the reaction mixture, except AGS, was added to the assay system. Increasing the temperature of incubation is not desirable as this increased the variability of the result. The effectiveness of Trasylol in preventing the enzymatic destruction of glucagon during storage of the serum samples and during incubation (11, 18, 19) was confirmed, although, in our experience, the addition of Trasylol to the blood collection tubes (9) offered no advantage (Table I). Table II shows that, under the conditions described, the recovery of added glucagon was satisfactory when 2000 KIU of Trasylol were added to 0.1 ml of serum. With 1000 KIU of inhibitor the recovery varied from 30 to 74%. Using the method described above,

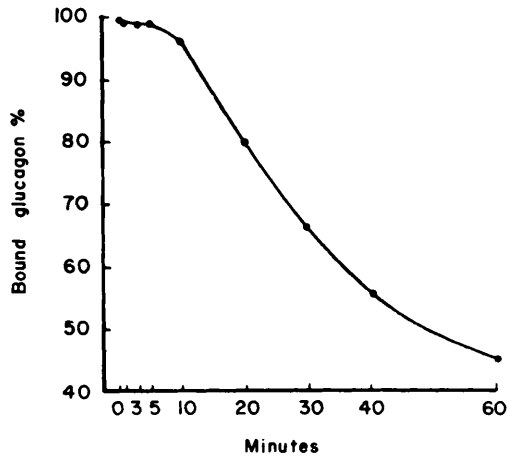

FIG. 7. Glucagon immunoassay: effect of separation time on the amount of bound glucagon.

TABLE I. Glucagon Immunoassay: Effect of Trasylol (500 U/ml of blood).[a]

Condition[b]	Glucagon (ng/ml; av ± SE)	p
A	1.5 ± 0.1 (4)	NS
B	1.4 ± 0.1 (4)	<0.025
C	0.8 ± 0.1 (4)	
A'	0.8 ± 0.1 (4)	

[a] In all cases the blood was allowed to clot for 30 min at room temperature, then centrifuged. All samples were assayed after being kept at —20° for about 5 hr. One thousand additional units of Trasylol were added to the assay system, except in sample A'.

[b] A and A': The blood was collected into a Trasylol-containing syringe; B: Trasylol was added after separation of the serum; and C: no Trasylol was added.

TABLE II. Glucagon Immunoassay: Recovery of Glucagon Added to Serum.[a]

Glucagon (ng/0.1 ml; av ± SD)		
Added	Found	Recovery (%)
0	0.07 ± 0.01 (4)	
0.1	0.16 ± 0.02 (4)	90
0.2	0.22 ± 0.01 (4)	75
0.3	0.34 ± 0.02 (4)	90
0.4	0.42 ± 0.02 (4)	88
0.5	0.57 ± 0.06 (4)	100

[a] Each tube contained 2000 U of Trasylol; no. of experiments in parentheses.

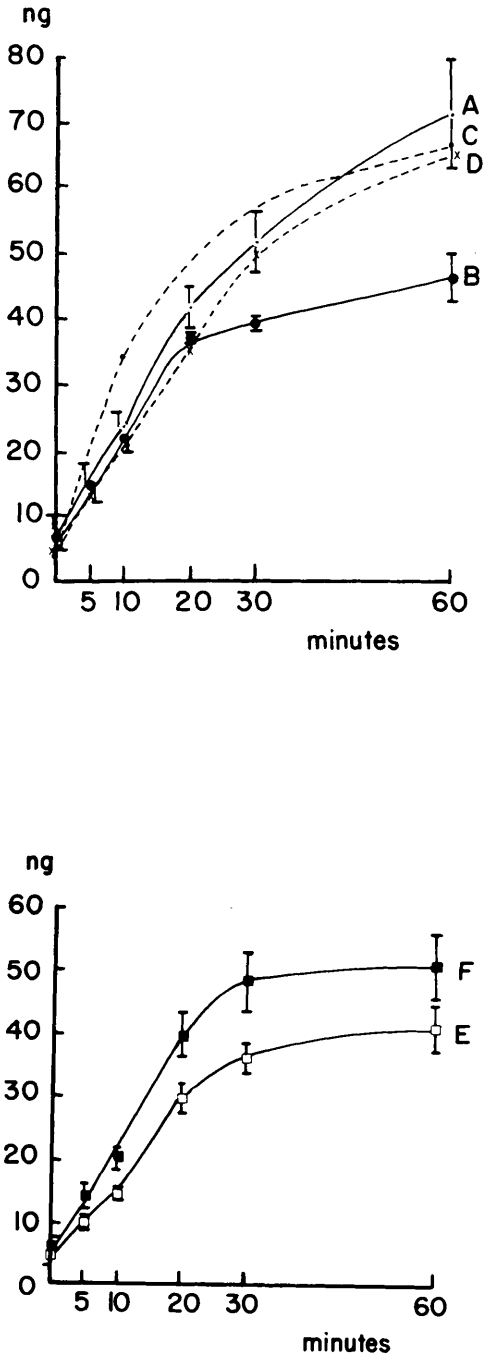

FIG. 8. Glucagon secretion by isolated islets: each flask contained 10 islets incubated in buffer with the following concentrations of glucose (mg/100 ml): (A) 30 (6); (B) 30 changed to 300 after 10 min of incubation (5); (C) no glucose (1); (D) 100 (4); (E) 300 (12); (F) 300 changed to 30 after 10 min of incubation (11). Average values ± SE; number of experiments in parentheses.

the average serum glucagon concentration in 7 normal fasting human subjects was found to vary between 0.72 and 1.68 ng/ml (av 1.05 ± 0.15 SE).

Secretion of glucagon by isolated rat islets. Figure 8 shows that islets incubated in a low-glucose medium secrete 71.8 ± 8.4 (SE) ng of glucagon/10 islets/hr, whereas islets incubated in a high-glucose medium secrete only 41.6 ± 4.0 ng/10 islets/hr ($p<0.005$). When the glucose concentration was 100 mg/100 ml, the rate of glucagon secretion was 65.9 ± 13.5 ng/10 islets/hr. This rate of secretion did not differ significantly from that obtained in the low-glucose medium, but was significantly higher ($p<0.025$) than that obtained in the high-glucose medium. Figure 8 also shows that when the concentration of glucose in the medium was increased from 30 to 300 mg/100 ml, the secretion of glucagon was suppressed significantly ($p<0.05$ at 30 min, $p<0.005$ at 60 min). On the other hand, when the concentration of glucose in the medium was decreased from 300 to 30 mg/100 ml, the secretion of glucagon tended to increase ($p<0.05$ at 30 min).

Discussion. Fine cellulose powder, previously used for insulin (12, 13) offers a large adsorbing surface, shortens the time required for the separation of free and antibody-bound hormones and therefore decreases hormone damage during the procedure. Using methods similar to the one described in this paper, Wright and Malaisse (13) found that the best time for the separation of free from antibody-bound insulin was 30 min, while Zaharko and Beck (14) recommend a 2-min separation time. Our results indicate that in the case of glucagon, the separation time should not be greater than 5 min. The characteristics of insulin and glucagon and of the antigen–antibody reaction may account for this difference. The separation is influenced also by the amount of serum present in the system and by the type of cellulose powder (13). Thus, for example, we found that Whatman cellulose powder did not give satis-

factory results and that the counts in serum samples and in buffer containing the same amount of 7% bovine serum albumin were higher than in "standards" containing no added glucagon. The reasons for this phenomenon are not clear: it could not be overcome by increasing the amount of cellulose or of Trasylol, suggesting that it might have been due to nonspecific binding of glucagon by serum proteins, rather than to inadequate separation of free from bound glucagon or to glucagon damage. Whatever the reason, this difficulty was overcome by the use of control tubes containing serum, but not AGS. The effect of Trasylol in retarding the proteolytic destruction of glucagon *in vitro* observed by other workers (9, 11, 18, 19) was confirmed. For this purpose, it was not necessary to collect the blood sample into a Trasylol-containing syringe, provided the inhibitor was added to the serum immediately after separation.

The isolated islets are ideal for the study of glucagon secretion because they produce pancreatic glucagon presumably uncontaminated by gut glucagon and by proteolytic enzymes. The results obtained in these experiments indicate that the secretion of glucagon is stimulated when the glucose concentration of the medium is low and suppressed when it is high, confirming results obtained *in vivo* (20–22) and suggesting that pancreatic glucagon does not play a role in the rise of serum IRG observed after an oral glucose load (3, 5, 11, 23). This rise is now considered to be the result of an increased release of gut glucagon (23, 24). On the other hand, the decreased serum IRG levels noted after an intravenous glucose injection (11) may well be a reflection of depressed glucagon secretion from the pancreas.

While this paper was being written, Vance *et al.* (25) published the results of similar studies. However, in their system, the amount of glucagon secreted by islets incubated at low glucose concentration, was about 1.5 ng/10 islets/30 min as compared to a secretion of about 70 ng/10 islets/hr obtained in our system. The reasons for this large difference are not clear.

Summary. This paper describes a rapid and sensitive radio-immunoassay for glucagon, in which a powdered cellulose suspension is used for the separation of free from antibody-bound glucagon. One hundred samples can be assayed conveniently in 2 days. The method was applied to the assay of glucagon in serum and in incubation media. Isolated rat pancreatic islets incubated in Krebs–Henseleit buffer containing glucose at a concentration of 300 mg/100 ml secreted glucagon at an average rate of 42 ng/10 islets/hr. When the concentration of glucose was 30 mg/100 ml the average rate of glucagon secretion was 72 ng/10 islets/hr ($p<0.005$).

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Differences in the Distribution of Antigen Reactive Cells in the Lymphoid Tissues of the Rat and Mouse (33550)

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Although many investigators studied the distribution of antibody producing cells in the lymphoid tissues of rodents during the primary antibody response (1–5), few studied the distribution of antigen reactive cells (6–8). Recent reports showed that the distribution of the latter cells in unimmunized animals varies within the same species depending upon the antigen–antibody system studied. For instance, studies of the primary antibody response of rats to sheep erythrocytes (SRBC) show that the concentration of antigen reactive cells in the thoracic duct lymph is greater than that in the spleen (6). On the other hand, studies of the primary response of rats to alum precipitated tetanus toxoid (TTAP) show that the relative concentrations are reversed (7).

The object of the present investigation was to compare the distribution of antigen reactive cells in the lymphoid tissues of the rat and mouse using a single antigen–antibody system. In particular, we compared the ability of syngeneic thoracic duct cells and spleen cells to restore the primary antibody response of sublethally irradiated rats and mice to TTAP. The experimental results show that there are considerable differences in the distribution of antigen reactive cells in the two rodent species.

Materials and Methods. Experimental animals. Inbred Lewis rats obtained from Microbiological Associates, Inc., Walkersville, Md. and (C57BL/Ka × C3HF/Lw)F_1 mice maintained in the Laboratory of Biology, National Cancer Institute, Bethesda, Md. were used.

Immunization and antibody titration. Immunization of rats to TTAP (Eli Lilly Co., Indianapolis, Indiana) was achieved by a single intraperitoneal (0.5 ml, 7.5 Lf) and subcutaneous (0.5 ml, 7.5 Lf) injection of 15 Lf toxoid. Mice were similarly immunized with 7.5 Lf (total) toxoid. Antibody titrations were performed in microtiter agglutination plates (Cooke Engineering Co., Alexandria, Va.) using a previously described modification (7) of the tanned red cell hemagglutination technique of Stavitsky (9).

Irradiation. Whole body X-irradiation was performed with two Westinghouse Quadrocondex units (15 mA; 200 kV, 54 cm SAD; 0.25 mm Cu + 0.55 Al Filtration; dose rate

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