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Enhancement of the Immune Response in Rabbits by Administration of Immunoglobulin-G

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Summary. Enhancement of the antibody response of rabbits to diphtheria toxoid (DT) and bovine serum albumin (BSA) was obtained by administration of IgG from either normal rabbit serum or serum of rabbits treated with Freund's complete adjuvant (FCA). It was shown that the concentration of IgG was greater in the serum of adjuvant treated rabbits than in normal rabbit serum, and that, in the case of BSA, greater enhancement of antibody formation was provided by the IgG derived from a volume of serum from adjuvant-treated rabbits than by the IgG from an equal volume of normal serum. Interpretation of these results within the framework of the natural selection model of antibody production provides a mechanism of action for Freund's adjuvant. Attempts to specifically remove the adjuvant effect of IgG for BSA by absorption with insoluble, polymerized BSA were unsuccessful. However, specific anti-BSA antibody was recovered from normal IgG by elution from the immunosorbent. This material is thought to represent natural antibody of the IgG class.

INTRODUCTION

We reported earlier (Dawe, Segre and Myers, 1965) that the antibody-enhancing activity of Freund's complete adjuvant was passively transferred, in part, by the serum from rabbits injected with the adjuvant alone. The concentration of globulin in the serum of adjuvant-treated rabbits increased progressively from the second to the ninth week after adjuvant injection. A correlation was found between the ability of the serum of adjuvant-treated rabbits to enhance the immune response of recipient rabbits, and the globulin concentration of the donor serum.

On the basis of these findings we proposed that the antibody-enhancing activity of serum from adjuvant-treated rabbits is due to its globulin content. We now report that purified immunoglobulin G from the serum of adjuvant-treated and normal rabbits enhances the response of recipient rabbits to diphtheria toxoid and bovine serum albumin.

MATERIALS AND METHODS

Antigens

Two presumably unrelated antigens were used in the experiments. Fluid diphtheria toxoid (DT), containing 50 Lf units/ml, 1300 Lf units/mg nitrogen, was obtained from Eli Lilly Co., Indianapolis, Ind. The dose injected, 1 ml, was previously shown (Dawe *et al.*, 1965) not to elicit a detectable primary response in the rabbit when used alone, but to induce a marked response when emulsified in Freund's complete adjuvant (FCA).

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Bovine serum albumin (BSA) was fraction V, Nutritional Biochemicals Corp., Cleveland, Ohio. One ml of a 1 per cent solution was used as an antigenic stimulus. This dose also failed to elicit a detectable primary response when used alone, but induced a marked antibody response when emulsified in FCA.

Preparation of IgG

Healthy adult New Zealand white rabbits were injected with 2 ml of a 1 : 1 mixture of 0·15 M NaCl and FCA. The injections were made subcutaneously at 4–6 sites in the back. Serum samples were collected at weekly intervals and tested for the presence of antibodies to DT and BSA and IgG concentration.

At 7 weeks the rabbits were exsanguinated by cardiac puncture and the serum was collected. The serum was stored at −20° until fractionated.

A crude globulin fraction was prepared from the serum of the donor rabbits by 3 successive precipitations with $(NH_4)_2SO_4$ at 50 per cent saturation, and was dialysed against 0·0175 M phosphate buffer, pH 6·8, at 4°.

The crude globulin was then chromatographed on DEAE-cellulose and the IgG was eluted with 0·0175 M phosphate buffer pH 6·8. The first protein peak was collected, concentrated by pervaporation or pressure dialysis and tested for purity by immunoelectrophoresis. If the preparations were not pure IgG they were recycled through a DEAE-cellulose column. The concentration of globulin was determined from the optical density at 280 mμ using the extinction coefficient of 1·5 density units/1 mg/ml.

Treatment groups

Each of two rabbits (Group A) received by intraperitoneal injection 1 g of IgG, prepared from the serum of the adjuvant treated rabbits, mixed with 1 ml of DT and 1 ml of BSA. The IgG-antigen mixture was placed in a water bath at 37° for 30 minutes before injection.

Two rabbits (Group B) were injected with 1 g of IgG from normal rabbit serum mixed with 1 ml of DT and 1 ml of BSA. Two rabbits (Group C) received 0·5 g of IgG from normal rabbit serum mixed with 1 ml of DT and 1 ml of BSA. Two additional rabbits (Group D) were injected with the IgG isolated from an amount of normal rabbit serum approximately equivalent to the amount of serum of the adjuvant treated donor rabbits which yielded 1 g of IgG. This IgG (350 mg) was also mixed with DT and BSA.

Two rabbits (Group E) were injected with 1 g of IgG derived from the serum of adjuvant-treated rabbits, and two rabbits (Group F) with 1 g IgG from serum of normal rabbits, absorbed with insoluble BSA. These materials were also mixed with the antigens.

Controls consisted of three rabbits (Group G) injected with 1 ml of DT, and of three rabbits (Group H) injected with 1 ml of BSA.

Serum samples were collected at weekly intervals and tested for the presence of antibodies using the passive haemagglutination (HA) test of Boyden (1951) as modified by Stavitsky (1954). Titres were expressed as the number of tubes showing agglutination in a series of two-fold serum dilutions. The first tube contained a 1 : 10 dilution of serum.

Treatment of rabbit IgG with an immunosorbent prepared from ethylene maleic anhydride and BSA

IgG prepared from serum of rabbits treated with FCA and from normal rabbit serum was treated with an immunosorbent made from ethylene maleic anhydride (EMA) and BSA according to the procedure described by Centeno and Sehon (1966). In this procedure, a 0·5 per cent solution of BSA in 0·15 M phosphate buffer, pH 6·1, was cooled to 0°

in an ice bath, then a 0·6 per cent EMA solution in anhydrous acetone was added dropwise. The resulting insoluble EMA-BSA conjugate was washed 3 times with 0·15 M NaCl, resuspended in phosphate buffer pH 6·1 and stirred for 4 hours at 4°. After this the EMA-BSA conjugate was washed once with pH 3·0 glycine-HCl-saline buffer and several times with 0·15 M NaCl.

One gram of IgG from the serum of either the FCA-treated rabbits or normal rabbits was mixed with the amount of immunosorbent produced by mixing 20 ml of EMA solution with 20 ml of BSA solution. The mixture of IgG and the immunosorbent was incubated in a water bath at 37° for 15 minutes with occasional gentle stirring. The immunosorbent was separated by centrifugation and the supernatant fluid collected and its protein concentration determined. This material was used in transfer experiments as previously described.

After the immunosorbent had been used for the absorption of the rabbit IgG preparations, it was treated to recover absorbed antibodies. The immunosorbent was treated in succession with glycine-HCl-saline buffer of pH 3·0, 2·5, and 2·0. The immunosorbent was suspended in each buffer for 2–5 minutes, sedimented by centrifugation and the supernatant recovered. The supernatant was immediately neutralized with 1 N NaOH to pH 7·8. After the last low pH treatment the immunosorbent was washed twice with 0·15 M NaCl and the supernatant fluids were saved. The neutralized supernatant fluids recovered from the low pH treatment and the supernatant from the two washings were pooled and concentrated by pressure dialysis to approximately 10 ml. The material was then tested for the presence of anti-BSA antibodies.

Determination of IgG concentrations of serum

The IgG concentration of serum from rabbits injected with FCA was determined by the single radial diffusion technique described by Mancini, Carbonara and Heremans (1965), slightly modified.

Anti-rabbit IgG was prepared in guinea pigs. This antiserum was absorbed with $F(ab')_2$ from IgG (Nisonoff, 1964) conjugated to EMA. After absorption the antiserum reacted only with rabbit IgG in double diffusion and immunoelectrophoresis tests.

The guinea pig anti-rabbit IgG serum was diluted 1 : 5 with 0·15 M NaCl and mixed with an equal volume of 3 per cent Difco Noble Agar at 56°. The agar-antiserum mixture was injected between two glass plates held apart by a U-shaped brass template 1 mm thick. After the agar solidified, the top plate was removed and holes were cut in the agar using a blunt 12 gauge needle attached to a vacuum source. The holes were 12 mm apart. A microlitre syringe was used to deliver 2 μl of either standard IgG solutions or the serum to be tested into the holes in the agar. The plates were then placed in a moist chamber for 36–48 hours, to allow the development of the precipitin rings. Upon removal from the chamber, the plates were submerged in 0·15 M NaCl solution for 8–12 hours and then dried at 37° with a piece of filter paper on the agar surface. The plates were fixed in 2 per cent acetic acid for 5 minutes and then stained for 15 minutes in 0·5 per cent azocarmine B in methanol-acetic acid (9 : 1). The excess stain was removed by washing in methanol-acetic acid. The plates were dried, placed in a photographic enlarger and the diameter of the images of the precipitin rings was measured at a constant enlargement. A standard curve was constructed on semi-log paper by plotting the concentrations of IgG on the logarithmic scale and the diameter of the images of the precipitin rings on the linear scale. The concentration of the unknown solutions was determined from the curve.

RESULTS

PREPARATION OF IgG BY CHROMATOGRAPHY ON DEAE-CELLULOSE

Table 1 summarizes the results of the isolation of IgG from the serum of the adjuvant-treated donor rabbits. The recovery of IgG from the serum samples ranged from 21·6 to 47·3 per cent with a mean of 33·3 per cent. The mean volume of serum recovered from an individual rabbit was 50·9 ml. The mean per cent recovery of IgG for normal rabbit serum was 25·4 per cent. The IgG content of normal serum was approximately half that of serum from adjuvant-treated rabbits.

All the serum samples were tested for antibodies against DT and BSA using passive haemagglutination, and were found to be negative.

TABLE 1

RECOVERY OF IgG FROM THE SERUM OF RABBITS INJECTED WITH FREUND'S COMPLETE ADJUVANT AND FROM NORMAL RABBIT SERUM

Rabbit No.	IgG concentration* (mg/ml)	Volume of serum (ml)	Total IgG in serum (mg)	IgG recovered (mg)	Per cent recovery
70	15·5	54	837	396	47·3
71	15·5	32	496	201·5	40·6
72	34·0	47	1598	426	26·6
73	39·5	49	1935·5	450	23·2
74	16·2	57	923·4	383	41·5
75	21·5	37	795·5	172	21·6
76	15·5	61	945·5	361·4	38·2
77	29·0	47	1363	416·25	30·5
78	25·0	65	1625	497·2	30·5
79	16·2	38	615·6	178·9	29·1
80	30·0	66	1980	589	29·7
81	18·0	58	1044	427	40·9
			Group means		
	23·0	50·9	1179·9	374·9	33·3
NRS† Lot 1	10·5	200	2100	588	28·0
NRS Lot 2	9·8	200	1960	468	22·8
			Group means		
	10·1	200	2030	528	25·4

* Determined by the radial diffusion method.
† Normal rabbit serum.

EFFECT OF IgG ON THE ANTIBODY RESPONSE OF RABBITS INJECTED WITH DT AND BSA

The antibody response of the recipient rabbits is summarized in Figs. 1 and 2. In general, administration of IgG appeared to enhance the antibody response of the rabbits to both DT and BSA. In order to compare the antibody responses for the entire observation period, the areas under the antibody response curves illustrated in Figs. 1 and 2 were computed for each group. The data so obtained were subjected to analysis of variance. Comparisons were made between pairs of groups, using all possible pairing combinations. Cases where two or more treatment groups had responses not significantly different from each other were considered as one group and tested against the control group.

There were no significant differences among the four treatment groups (Groups A, B, C, and D) response to DT. The DT response of the four treatment groups combined, however, was significantly ($P<0·05$) greater than the response of the control group (Group G),

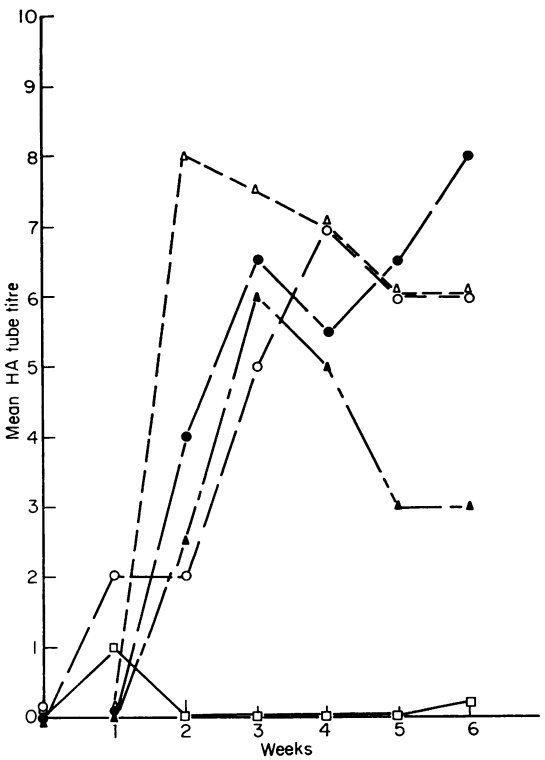

FIG. 1.

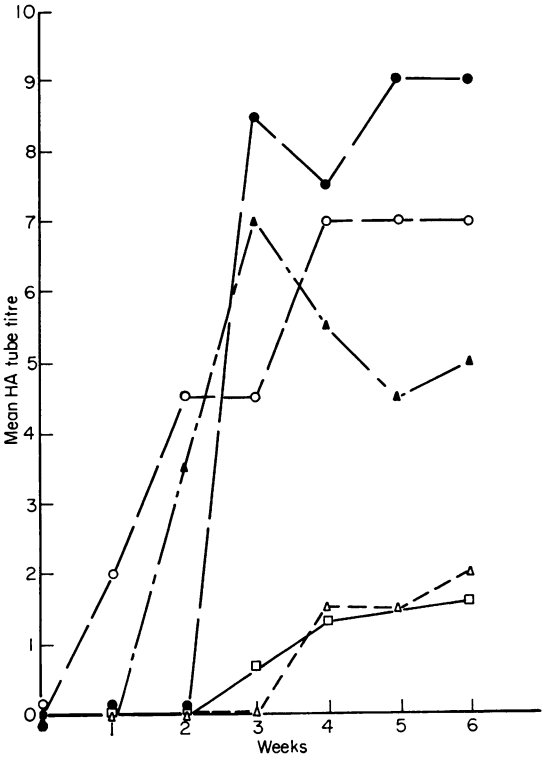

FIG. 2.

FIG. 1. Antibody responses of rabbits injected with diphtheria toxoid plus IgG. Group A: 1 g of IgG from the serum of adjuvant-treated rabbits (●—●); Group B: 1 g of IgG from normal rabbit serum (○—○); Group C: 0·5 g of IgG from normal rabbit serum (▲—▲); Group D: 0·350 g of IgG from a volume of normal rabbit serum approximately equivalent to the volume of adjuvant treated donor serum used in Group A (△—△); Group G: diphtheria toxoid only (□—□).

FIG. 2. Antibody responses of rabbits injected with BSA plus IgG. Groups and symbols as in Fig. 1. Group H: BSA only (□—□).

which received DT only. The response of the two groups which received one gram of IgG (Groups A and B) combined was also significantly ($P<0·05$) greater than the response of the control group.

The response to BSA followed a similar pattern. There were no significant differences among the responses of groups A, B and C. The response of the rabbits which received 0·350 g of IgG (Group D) was not different from the response of control rabbits (Group H). However, the response of groups A, B, C and D combined was significantly greater ($P<0·05$) than the response of the control group. Groups A and B combined and groups A, B and C combined had a significantly ($P<0·05$) greater response than either group D or the control group H.

EFFECT OF SPECIFICALLY ABSORBED IgG ON THE ANTIBODY RESPONSE OF RABBITS INJECTED WITH DT AND BSA

The antibody response of the recipient rabbits is illustrated in Figs. 3 and 4. It is apparent that treatment of IgG from both adjuvant-treated and normal rabbits with a

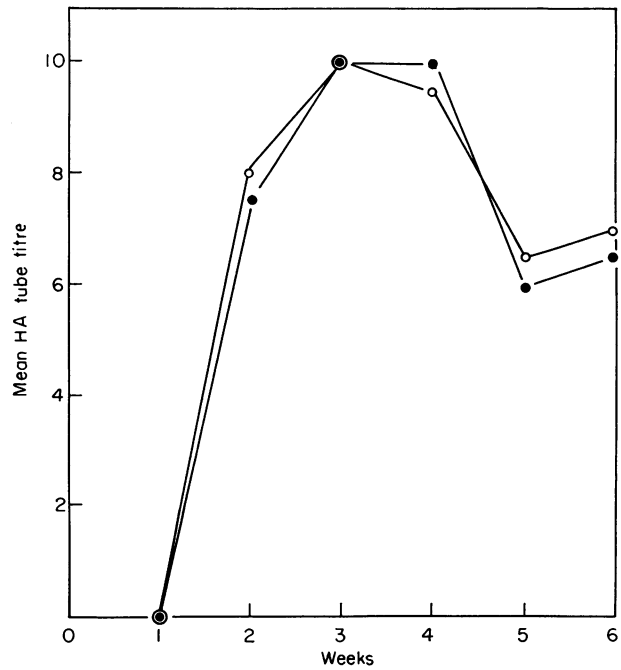

FIG. 3. Antibody responses of rabbits injected with diphtheria toxoid plus IgG absorbed with BSA immunosorbent. Group E: 1 g of IgG from the serum of adjuvant-treated rabbits (●—●); Group F: 1 g of IgG from normal rabbit serum (○—○).

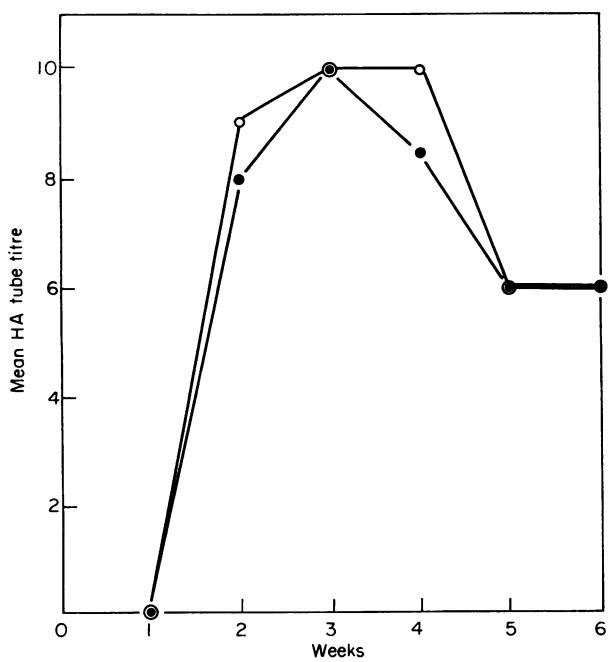

FIG. 4. Antibody responses of rabbits injected with BSA plus IgG absorbed with BSA immunosorbent. Groups and symbols as in Fig. 3.

BSA immunosorbent did not affect its ability to enhance the response of recipient rabbits to either DT or BSA.

RECOVERY OF BSA ANTIBODIES FROM THE IMMUNOSORBENT USED TO ABSORB RABBIT IgG

Both IgG from normal rabbit serum and IgG from the serum of adjuvant-treated rabbits were treated with an immunosorbent prepared by conjugating BSA and EMA. A total of 3992 mg of IgG was treated in four approximately equal batches. The material recovered from the immunosorbent after treatment of the IgG was pooled, concentrated and found to contain 184 mg of protein. This recovery represents 4·6 per cent of the protein treated with the immunosorbent.

When the material recovered from the immunosorbent was tested by HA using BSA-coated tanned red blood cells, its titre was 1 : 8192. In an inhibition test in which the serum dilutions were mixed with an equal volume of 0·1 per cent BSA prior to the addition of the BSA-coated tanned red blood cells, the titre was 1 : 16. When this material was tested against BSA by immunodiffusion and immunoelectrophoresis procedures, lines of precipitation did not develop.

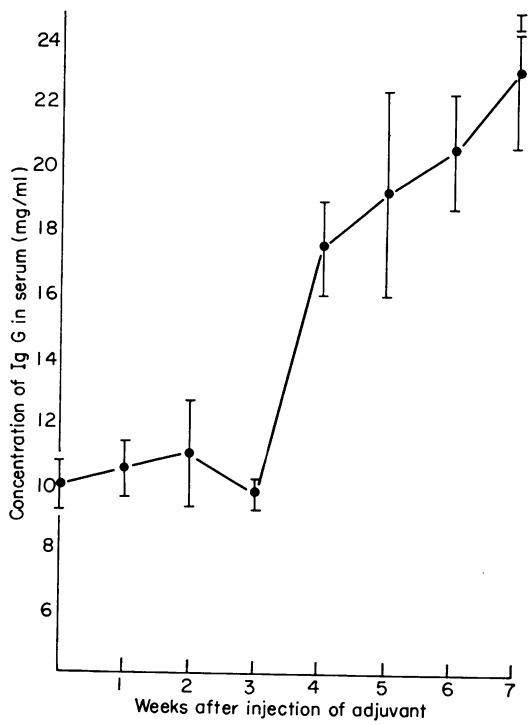

FIG. 5. Concentration of IgG in the serum of rabbits injected with Freund's complete adjuvant. Vertical bars indicate the standard error of the mean.

DETERMINATION OF THE IgG CONCENTRATION IN SERUM FROM RABBITS INJECTED WITH FREUND'S COMPLETE ADJUVANT

The results of the determination are summarized in Table 2 and Fig. 5. The mean concentration of IgG in serum samples ranged from 10 mg/ml before injection to 23 mg/ml

TABLE 2

CONCENTRATION OF IgG IN THE SERUM OF RABBITS INJECTED WITH FREUND'S COMPLETE ADJUVANT

Rabbit No.	Concentration of IgG in mg/ml of serum at weeks post injection							
	0	1	2	3	4	5	6	7
70	8	8·6	7·9	7·4	11	13·3	15	15·5
71	12·2	12·5	11·1	11·1	16·5	16·5	18·3	15·5
72	13	13	10	11·6	25	34	30·1	34
73	10·5	11·1	20·3	–*	17·5	24·5	23	39·5
74	7·4	8	13·6	–*	13	16·6	24·6	16·2
75	7·2	8·6	13·6	–*	13	14·6	11·8	21·5
76	18·5	18·5	11·5	11·6	27·7	21	17·5	15·5
77	10·5	11·5	10·6	10	19·5	18·5	23	29
78	9	10	10·4	8·6	15·5	19·5	24	25
79	8	7·1	6·8	8·8	–*	11·1	14·6	16·2
80	7·5	8·2	6·8	8·7	14·2	16·2	24·3	30
81	8	8·8	8·8	9·8	19·5	24	29·9	18
Mean	10	10·5	11	9·7	17·5	19·2	20·5	23
Standard deviation	±3·3	±3·14	±3·7	±1·5	±5·2	±11·3	±6·1	±8·4
Standard error of the mean	±0·95	±0·91	±1·07	±0·5	±1·57	±3·26	±1·76	±2·43

*Sample lost in processing.

at 7 weeks after injection. There was a slight increase in IgG concentration in the first 2 weeks following injection of the adjuvant, then a drop during the third week. During the fourth week there was a sharp rise in IgG concentration followed by steady increase to the seventh week after injection. The variation in the concentration of IgG among individual rabbits increased with time. This increase was reflected by increase in the standard error of the mean. At 7 weeks post injection the concentration of IgG in the serum samples varied from 15·5 mg/ml to 39·5 mg/ml. Some of the rabbits had higher IgG concentrations at 5 or 6 weeks after injection than at 7 weeks. One rabbit with a peak IgG concentration at 4 weeks post injection had an IgG concentration at 7 weeks which was lower than that in the pre-injection serum sample.

DISCUSSION

Enhancement of the antibody response to DT and BSA was obtained in rabbits given IgG derived from either normal rabbit serum or from the serum of rabbits treated with FCA. This latter finding has to be considered in conjunction with the previously reported passive transfer of adjuvant action by whole serum of rabbits treated with the adjuvant (Dawe *et al.*, 1965) as well as with the increase in concentration of IgG resulting from adjuvant treatment of the donor rabbits (Table 2, Fig. 5). Taken together, the results indicate that FCA induces an increase in concentration of IgG which, in turn, mediates part of the antibody-enhancing activity of the adjuvant. Other mechanisms of action, such as the ability of the adjuvant to retain the antigen at the site of injection (Freund, 1947, 1951; Herdegen, Halbert and Mudd, 1947; Talmage and Dixon, 1953) and to stimulate proliferation of cells related to antibody synthesis (White, Coons and Connolly, 1955; Svet-Moldavsky and Raffkina, 1963), are not eliminated from consideration. The action of adjuvant is probably brought about through several mechanisms.

In line with the view of Eisen and Karush (1964) that the concentration of circulating natural antibody regulates the magnitude of the antibody response, the effect of IgG was ascribed to its content of natural antibodies, specific for the antigens used. However, when IgG treated with a BSA immunosorbent was used, the antibody response of the recipient rabbits to BSA was as great as their response to DT (Figs. 3 and 4). The antibody response of these rabbits was also as great as the response of those which received unabsorbed IgG (Figs. 1 and 2). However, it was possible to demonstrate specific antibody activity against BSA in the material recovered from the immunosorbent after treatment of the IgG with it. This result suggests that there were natural anti-BSA antibodies in the IgG preparations, and indeed, that the immunosorbent removed at least part of them. These findings seem to contradict themselves since it appears that specific natural antibody can be removed from the globulin without having an apparent effect on the ability of the globulin to enhance the antibody response of recipient animals to the corresponding antigen.

Additional experimentation will be necessary to attempt resolution of this contradiction. Determination of the class of antibody made by the recipient rabbits following administration of BSA-absorbed or untreated IgG may reveal differences in response that were not detected in the present work. The single absorption of the IgG preparation with insoluble BSA may not have been sufficient to remove all natural antibody specific for BSA. Finally, the possible presence of bacterial endotoxin, a known adjuvant of immunity, in the absorbed IgG, may have been a source of error.

There is no reason to believe that FCA would cause any change in the relative concentration of various specificities of natural antibody molecules. Rather, it would increase the concentration of all natural antibodies in proportion to their concentration in normal serum. Consequently, it would be expected that equal quantities of IgG, whether from adjuvant treated or normal rabbits, would have the same effect on the antibody response of recipient rabbits. The data obtained in these studies indicate that indeed this is the case (Figs. 1 and 2). A decrease in the dose of IgG administered to the recipient rabbits resulted in a lowered response to BSA (compare groups A, B and C with group D in Fig. 2). However, this phenomenon was not demonstrated in the case of the response to DT.

It has been reported that injection of FCA resulted in increase of the gamma globulin concentration of the serum (Dawe *et al.*, 1965; Humphrey, 1963; Shepel and Klugerman, 1963; Silverstein, Thorbeche, Kraner, and Lukes, 1963). In this study, it was found that the concentration of IgG in the serum of rabbits injected with FCA increased from 10 mg/ml to 23 mg/ml at 7 weeks post injection, giving a total increase of 13 mg per ml (Table 2, Fig. 5). The concentration of IgG in the serum of the adjuvant treated rabbits increased only slightly during the first 3 weeks after injection, then sharply during the fourth week and continued to rise rapidly up the seventh week post injection (Fig. 5). These results agree with the results reported by Humphrey (1963). He found that the greatest increase in gamma globulin concentration occurred, in rabbits injected with the adjuvant, between the fourth and seventh week after injection. He also reported that serum gamma globulin concentration of the adjuvant treated rabbits was increased by as much as 10 mg/ml 7 weeks after injection.

The increase in gamma globulin in the serum of the adjuvant treated donors was to 230 per cent. Dawe *et al.* (1965) reported a 200·5 per cent increase in the gamma globulin concentration of serum of rabbits treated with FCA. They were measuring increases in total globulin, whereas in the present experiment the increase in the concentration of IgG was determined specifically. This similarity of responses in the two experiments suggests that the adjuvant may exert its effect largely on the IgG concentration.

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