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Specific Inhibition of Antibody Production

I. PROTEIN-OVERLOADING PARALYSIS

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Summary. A state of protein-overloading paralysis has been induced to bovine γ globulin (BGG) in adult CBA mice. Paralysis was demonstrated by a lack of immune elimination of ^{131}I labelled BGG after prior challenge with Freund's adjuvant containing BGG. The degree of paralysis was found to diminish when the time between the paralysing injection and challenge injection was increased. This loss of the paralysed state could be prevented by injections of antigen whilst the mice were still paralysed.

The superficial nature of the differences between paralysis (unresponsiveness induced in adults) and tolerance (unresponsiveness induced in neonatals) to BGG in CBA mice is discussed. The possibility that suppression of antibody production in previously immunized animals given a massive dose of antigen, is a distinct phenomenon from the induction of paralysis/tolerance in animals not previously immunized, is briefly discussed.

INTRODUCTION

Specific immunological unresponsive states have recently been classified and defined by Medawar (1961), whose basic definitions are used in this paper. The term *paralysis* is used to describe a state of unresponsiveness induced by the injection of antigen into adult (immunologically mature) animals. The validity of the separation of *tolerance* (unresponsiveness induced in neonatal animals) from *paralysis*, when soluble protein antigens are used, as in the system to be described here, will be discussed in a subsequent paper.

Specific protein-overloading paralysis has been induced in adult rabbits by Dixon and Maurer (1955b). They showed that very large doses of antigen were required to suppress a subsequent immune response and that unresponsiveness appeared to persist only for as long as antigen was detectable in the circulation. Earlier, Johnson, Watson and Cromartie (1954), using massive doses of bovine serum albumin (BSA), in rabbits, were able to inhibit partially a subsequent immune response to BSA. More recently, Sercarz and Coons (1959) paralysed mice with massive doses of the same antigen. Paralysis induced in mice to pneumococcal polysaccharide (Felton, 1949) appears to differ from protein-overloading paralysis on the basis of the doses of antigen used and the time for which the induced state persists. For instance, depending on the type of pneumococcal polysaccharide used, a paralysing dose may be as small as 5.0 μg.; an immunizing dose being 0.5 μg. The doses of protein used by Dixon and Maurer were immense by comparison.

This paper describes the induction of protein-overloading paralysis to bovine γ globulin (BGG) in CBA mice. The antigen-elimination technique has been used to detect

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an immune response to BGG. It has previously been shown that the technique is suitable for detecting a state where mice were immune to one fraction of BGG and unresponsive (tolerant) to another (Dresser, 1961). Experiments have been carried out to determine the effect of varying the amount of BGG in the single paralysis-inducing injection; the effect of the addition of Freund's adjuvant at the time of the paralysing injection; and the effect of prior immunization. An experiment to determine the effect of varying the interval between the paralysing injection of BGG and challenge with Freund's adjuvant containing BGG is also described.

MATERIALS AND METHODS

CBA mice were used and were not less than 3 months old when given the paralysing injection of antigen. The antigen used was bovine γ globulin (BGG) (Armour & Co., BGG batch No. BF 0270). Mouse globulin (MGs) was prepared by ammonium-sulphate fractionation of outbred mouse serum at 4° (Eisen and Pressman, 1950). After determination of the concentration of protein, using a 'Unicam' spectrophotometer at 280 mμ., the MGs solution was stored at −20°. BGG was stored in a desiccator at 2° and solutions (w/v) were made up in 0.9 per cent NaCl (saline). MGs and BGG were labelled with ^{131}I by the method of Wormall, following a procedure which has previously been described (Dresser, 1960).

The paralysis-inducing injection was a single intraperitoneal (ip.) injection of a 10 per cent solution (w/v) of BGG in saline. The injected mice and uninjected controls were challenged 2 months later (in one experiment 3 months) by an injection of Freund's adjuvant, containing 8 mg. BGG in the amount of adjuvant (Freund's BGG) given to each mouse (Dresser, 1960). In some experiments Freund's adjuvant, made with saline in place of the antigen solution (Freund's saline) was injected at the same time as the paralysing injection. The result of the challenge was measured 1 month later by the antigen-elimination technique, the mice being injected (ip.) with 2 mg. of BGG-^{131}I. The course of the elimination of the labelled antigen was then followed at 2-day intervals, until 12 days later, by bleeding for 0.05 ml. of whole blood; this is a modification of the previously described procedure (Dresser, 1960). The blood issuing from a small cut in the ventral tail artery was measured with a micro (constriction) pipette previously rinsed in a 5 per cent sodium citrate solution. The sample of whole blood was placed on a dished planchette together with two drops of PVA solution (a saturated solution of polyvinyl alcohol in 85 per cent 'teepol' and 15 per cent ethanol). No difference in the elimination curves has been observed between the results obtained from this technique using whole blood and from the former use of serum. An advantage of the present technique is the greater accuracy of the estimation of the volume of blood removed at each bleeding, which is necessary when the half-lives of injected proteins are being estimated and corrections for the effect of bleeding have to be made.

The Ouchterlony gel-diffusion tests have been carried out using a procedure which has already been described (Dresser, 1961).

RESULTS

The different antigen-elimination responses obtained in tolerance experiments have been arbitrarily categorized (Dresser, 1961). To avoid duplication the same categories are

used in this paper. There are five categories ranging from a complete absence of response (TT) to a full immune response (+++). The category TT represents the exponential elimination of 2 mg. BGG-^{131}I at the same rate as in mice not previously treated in any way.

The effect of varying the amount of BGG in the paralysing injection is shown in Table 1.

TABLE 1

THE EFFECT OF THE SIZE OF THE PARALYSING INJECTION ON 3-MONTH-OLD MICE

Sex	*Size of paralysing injection*	*Category of response*				
		TT	*T*	+	++	+++
♀	70 mg.	—	—	—	3/5	2/5
♀	150 mg.	—	—	4/5	1/5	—
♂♀	150 mg.	3/8	4/8	—	1/8	—
♂	300 mg.	1/5	2/5	1/5	1/5	—
♂	450 mg.	—	3/5	1/5	1/5	—

It can be seen that an increase in the amount of BGG injected increases the degree of paralysis obtained when mice are challenged 2 months later. The apparent ineffectiveness of the dose of 450 mg. BGG when compared with 150 or 300 mg. BGG will be discussed.

Table 2 illustrates the results obtained when mice were injected subcutaneously with

TABLE 2

THE EFFECT OF ADJUVANT AND PREVIOUS IMMUNIZATION ON THE INDUCTION OF PARALYSIS IN MALE MICE

Age when paralysed	*Treatment*	*Size of paralysing injection*	*Category of response*				
			TT	*T*	+	++	+++
3 months	Freund's saline (1 hour previous)	150 mg.	—	—	—	—	5/5
,,	,,	300 mg.	—	—	—	1/5	4/5
,,	,,	450 mg.	—	—	1/5	3/5	1/5
7 months	Freund's BGG (4 months previous)	300 mg.	—	—	—	—	2/2
,,	,,	450 mg.	—	—	—	1/4	3/4

Freund's saline within a few minutes of the paralysing injection of antigen. This table also illustrates the effect of immunization with Freund's BGG 4 months before the paralysing injection. The adjuvant injection considerably reduces the paralysing effect of even the largest dose used (450 mg.; about 16 mg. BGG/g. body weight). Furthermore, prior immunization seems to completely prevent this dose from paralysing, at least for sufficiently long for the effect to be detected after rechallenge with Freund's BGG 2 months afterwards and subsequent testing of the result of the rechallenge by the antigen-elimination test. Fig. 1 illustrates the effect of BGG (60 mg. and 3 mg.) injected into mice immunized with Freund's BGG 2 months previously. The labelled BGG was injected 18 days after the injection of unlabelled BGG; in this experiment there was no rechallenge

with Freund's BGG. The result of the larger dose of BGG is a decrease in the rate of elimination of the labelled antigen. The half-lives of the labelled BGG in the two least responsive mice in this group are about 2.2 and 1.4 days respectively.

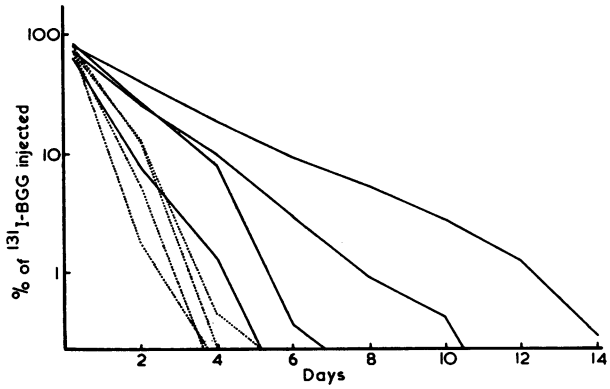


FIG. 1. Graph illustrating the elimination of 2 mg. of labelled BGG from the circulation of CBA mice. All the mice were immunized with Freund's BGG 2.5 months before the injection of labelled antigen. Eighteen days before the injection of labelled antigen one group (solid lines) was injected intravenously with 60 mg. BGG, and the other group (dotted lines) with 3 mg. BGG.

The effect of varying the time between a paralysing dose of 150 mg. (about 6 mg./g. body weight in this experiment) and challenge with Freund's BGG is shown in Fig. 2. The group challenged 2 months after the paralysing injection showed a greater degree

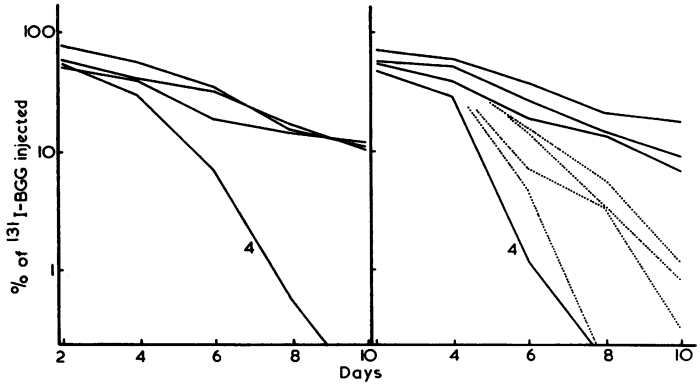
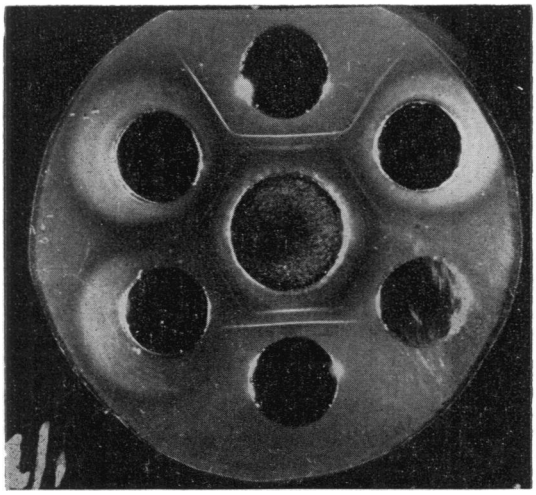
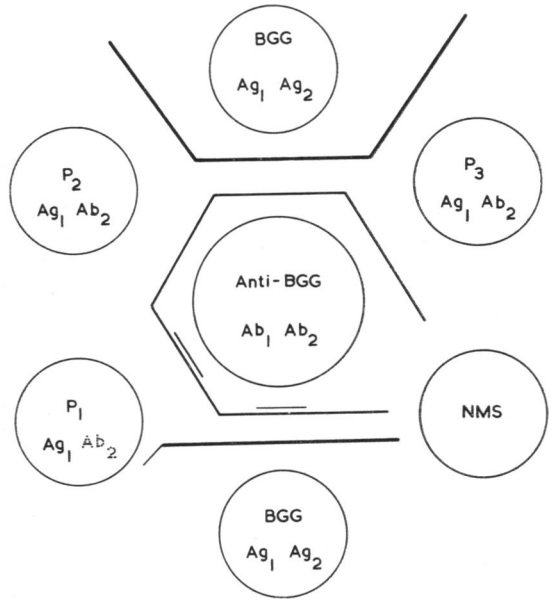


FIG. 2. This graph illustrates the effect of the time interval between the paralysing injection and challenge with Freund's BGG. The left-hand graph illustrates the elimination of labelled BGG in four mice injected with 150 mg. BGG 2 months before challenge. In the right-hand graph the dotted lines show the elimination of labelled BGG in four mice paralysed 3 months before challenge with Freund's BGG. The solid lines show the effect of retesting, after a further month had elapsed, the mice whose previous elimination curves are illustrated in the left-hand graph. Mouse No. 4 showed an immune response on both occasions.

of paralysis than the group challenged after 3 months. Furthermore, the group challenged at 2 months was still paralysed when retested with the 3-month group; the single exception in this group showed an immune response on both occasions.

Figs. 3 and 4 illustrate an Ouchterlony gel-diffusion test carried out with serum from mice injected with 150 mg. BGG and subsequently judged to be paralysed (TT) by the antigen-elimination test. The result is similar to that obtained with serum from tolerant mice (Dresser, 1961). The serum from the paralysed mice appears to contain one fraction of BGG together with antibodies to other fraction(s) of BGG. Antibody was detected by





FIGS. 3, 4. Photograph and diagram of gel-diffusion experiment. P_1, P_2 and P_3 are sera from mice judged to be paralysed by the antigen-elimination technique; BGG is a 1 per cent BGG solution; NMS is normal CBA mouse serum. Ag_1 is the paralysing component of BGG and Ag_2 is the other major component. Ab_1 and Ab_2 are the respective antibodies. Anti-BGG is an outbred mouse antiserum. P_1 seems to contain very little if any Ab_2. A faint third line has formed against one of the BGG wells and against P_1 and very faintly against P_2. This may be due to a minor component.

precipitation (ring tests) in the same sera. Less clear-cut results were obtained with sera from the mice injected with 450 mg. BGG and subsequently challenged with Freund's BGG. Both ring and gel-diffusion tests failed to detect antibody to BGG, but only one component of the antigen was detected in these sera. P_1 in Fig. 4 shows a similar phenomenon, in which, however, the antibody to the 'non-paralysing' component of BGG is in low concentration rather than absent.

The effect of a large quantity of unlabelled BGG on the rate of elimination from the circulation of small amounts of labelled BGG and MGs, injected at the same time, is shown in Table 3. When compared with controls, mice injected with 100 mg. BGG showed a significantly increased rate of removal of the labelled proteins. The effect is non-specific.

DISCUSSION

It has been shown (Table 1) that the degree of paralysis is related to the size of the paralysing dose of antigen. Furthermore, the degree of paralysis decreases with time unless

further injections of antigen are made before the loss of the paralysed state (Fig. 2). Ouchterlony gel-diffusion tests have shown that mice injected with 150 mg. BGG and judged to be paralysed by the antigen-elimination test, have antibodies to some components of BGG in their serum together with one antigenic component of BGG that has not been eliminated. When higher paralysing doses of BGG are used the Ouchterlony tests, although giving equivocal results in part, did show that very little antibody was present in the sera of these mice, which probably indicates a degree of paralysis to at least the other major component of BGG. Paralysis to one component of BGG and immunity to another, confirms previous results which illustrated the specificity of the tolerance phenomenon.

TABLE 3

THE EFFECT OF MASSIVE DOSES OF BGG ON THE RATE OF ELIMINATION OF 2 MG. LABELLED BGG AND LABELLED MOUSE GLOBULIN IN MALE MICE

^{131}I *labelled protein*	*Age*	*Size of BGG injection*	*Half-life ± S.D. (days)*	*'t' test P.*
BGG (2 mg.)	3 months	Nil (control)	4.16 ± 0.23	—
,, ,,	,,	10 mg.	3.13 ± 0.58	<0.2
,, ,,	,,	100 mg.	2.60 ± 0.44	<0.01
,, ,,	4½ months	Nil (control)	4.47 ± 0.35	—
,, ,,	,,	100 mg.	2.36 ± 0.16	<0.001
MGs ,,	3 months	Nil (control)	8.05 ± 1.39	—
,, ,,	,,	100 mg.	4.43 ± 1.32	<0.1

The evidence discussed so far is basically very similar to that discussed in relation to tolerance to BGG seen in CBA mice (Dresser, 1961). An apparent difference is that between the length of time for which tolerance persists and that for which paralysis persists (Fig. 2), the latter being considerably shorter. The single tolerance-inducing dose of antigen was 8 mg./g. body weight and the paralysing dose was about 6 mg./g. body weight. The difference in the length of time for which these two unresponsive states persist is at least twofold. Table 3 shows that massive doses of serum protein increase the rate of removal of labelled proteins from the circulation, confirming the results of Dixon and Maurer (1955a) in rabbits. An approximate estimation was previously made of the number of molecules of BGG present at the time of the loss of the state of tolerance by a mouse. In view of the results summarized in Table 3 the previous assumption that BGG has a half-life of 4 days has been modified to 3 days to fit the circumstances of these experiments which involve massive doses of BGG. It is estimated that 2 months after a paralysing injection of 150 mg. there would be 10^{11}–10^{12} molecules present (mouse paralysed) and at 3 months about 10^{7}–10^{8} molecules present (mouse partially immune). This result is similar to that estimated for the tolerance experiments; namely 10^{9}–10^{10} whilst tolerant and 10^{6}–10^{7} when capable of giving a small immune response.

In Table 1 it can be seen that mice given a paralysing injection of 450 mg. of BGG were apparently less paralysed than one of the groups of mice injected with 150 mg. BGG. Ouchterlony tests indicated that these mice were probably not strongly immune to any

fraction of BGG. The results summarized in Table 3 may explain the result obtained; the large dose of antigen may increase the elimination rate of the labelled protein even 3 months later. However, the negative result obtained in the Ouchterlony test is not conclusive and the faster elimination rates may be due to a very small immune response. Mann and Welker (1949) have shown that large doses of protein may act as an adjuvant, so that the results obtained in this experiment (Table 1) may in fact illustrate a phenomenon similar to that which is shown more clearly in Table 2.

The results illustrated in Fig. 1 may indicate that previously immunized animals can be at least partially paralysed. At first sight, however, it might appear that the slow climination of labelled antigen in the group receiving the large dose of antigen could be due to antibody being 'mopped up' by the large pool of antigen present. However, the half-life of the labelled BGG in the least responsive mouse is about 2.2 days between 18 and 30 days after the large dose of unlabelled BGG. The results summarized in Table 3 show that this half-life for BGG was probably not longer than 2.2 days during the period immediately after the injection of the large dose of BGG. If it is assumed that the CGG injected in the large dose had a half-life of $2\frac{1}{2}$ days, then 18 days later (the time when 2 mg. of labelled BGG was injected) there would be about 0.5 mg. of BGG left in the circulation of each mouse. It therefore follows that the amount of BGG in the circulation of the two groups of mice immediately after the injection of 2 mg. labelled BGG could not exceed 0.2 fold. The slow elimination of antigen could hardly therefore be due to the presence of a large amount of antigen in the circulation. Comparison with passive immunization experiments shows that the antigen-elimination rates obtained indicate a more than twofold difference in the rate of antibody production. It therefore seems likely that the large dose of BGG injected into the previously immunized mice greatly slows the subsequent rate of antibody production. The degree of paralysis obtained with the dose employed is obviously very short-lived, as can be seen in Table 2.

The presence of Freund's adjuvant, which does not contain any antigen, seems to markedly influence the degree of paralysis obtained after single massive doses of antigen. This is illustrated in Table 2. The adjuvant may act by increasing a tendency to give an immune response after challenge 2 months later, simply by the stimulation of the production of antibody by a few cells, which would speed the removal of antigen from the animal and allow an earlier loss of the state of partial paralysis. This hypothesis is difficult to reconcile with the evidence that previously immunized animals can at least be partially paralysed, although compatible with the evidence that the break from such a paralysed state is very greatly speeded when compared with paralysis induced in animals not previously immunized. It is likely that at least one component of BGG requires an adjuvant to be present for the stimulation of an immune response (Dresser, 1960). A more attractive hypothesis is that the adjuvant may act directly on all immunologically competent cells determining that the antigen will tend to induce immunity, whereas in the absence of adjuvant the tendency may be for the antigen to induce a state of paralysis. It seems to follow, if this latter hypothesis is to remain tenable, that the minimum time necessary for the inductive phase of antibody production is shorter than the time necessary for the induction of paralysis/tolerance. The results reported in this paper show that once a state of paralysis has been induced, immunity can not be induced even in the presence of an adjuvant. Inhibition of antibody production by high concentrations of antigen in cells already induced to produce antibodies may not therefore be the same phenomenon as the induction of a state of paralysis/tolerance.

In conclusion, it can be stated that protein-overloading paralysis and tolerance to BGG are similar phenomena. The results of experiments with both types of unresponsiveness being comparable when similar doses, reckoned on the basis of size to body weight, are used.

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