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Rate of Antigen Catabolism and Immunogenicity of [^{131}I]BGG in Mice

I. ACTION OF MYCOBACTERIAL ADJUVANTS

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Summary. Two series of experiments are described in which elimination of [^{131}I]BGG is observed in CBA mice treated with mycobacterial adjuvants. Neither mycobacteria in oil nor Wax D in oil induced immune-type elimination in the first series where control mice catabolized the antigen relatively slowly (mean half-lives of 4·38 and 5·0 days). In a second series where control mice showed faster catabolism (mean half-lives 3·82 and 2·96 days) the adjuvants did induce immune elimination. It was found also that such adjuvants invariably accelerated the elimination rate in the pre-immune period of elimination.

Porton White mice eliminated the antigen rapidly and even mice not given adjuvant frequently gave a secondary immune response on a further exposure to antigen.

The results suggest that increases in the rate of antigen catabolism contribute to adjuvant activity in this model.

INTRODUCTION

The experiments described here were initially set up to examine the antigen elimination model described by Dresser (1960) in assessing adjuvant activity of wax fractions derived from strains of *Mycobacterium tuberculosis*. Useful comparison, however, between adjuvant substances could not be made with the model because of the varying effectiveness of substances on repeated testing. The observations suggested that the rate of antigen breakdown might be contributing to this and might indeed be related to the mode of action of the adjuvant itself.

In the experimental model a trace-labelled purified protein antigen, bovine γ-globulin (BGG) is given intravenously to mice in which it is catabolized as host protein. Detectable amounts of the antigen are still in the bloodstream when antibody is first released from the lymphoid tissues. When this happens on the seventh day or later after the administration of antigen (Fig. 1), there is a sharp acceleration of elimination due to the formation of antigen–antibody complexes and their rapid phagocytosis and breakdown. (The period of elimination as observed by whole body counting up to this point of acceleration is referred to here as the 'pre-immune phase'.) If aggregated material has been removed by

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previous centrifugation, antibody formation is not initiated unless another stimulus in the form of a suspension of mycobacteria-in-oil with or without added antigen is injected *subcutaneously* (Dresser, 1960, 1961). The various substances to be examined for adjuvant activity can be tested for their ability to give this immunogenic stimulus after injection

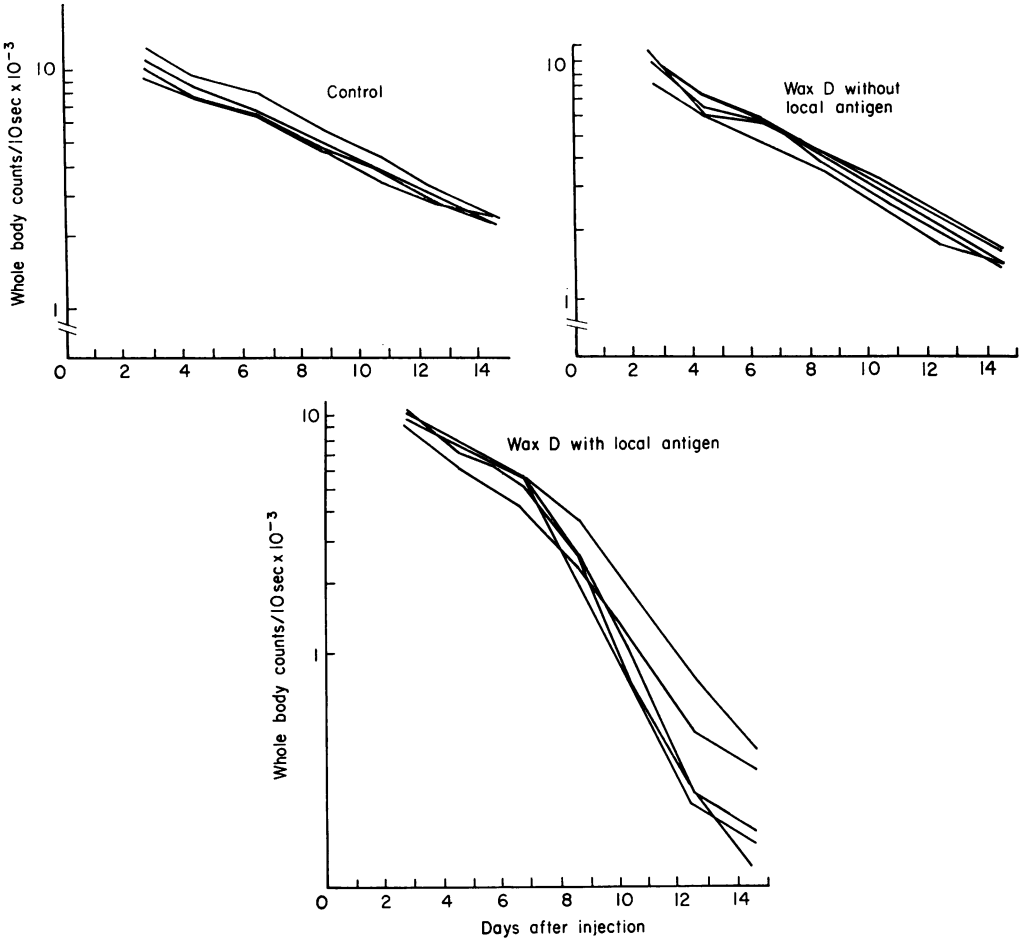

FIG. 1. Influence of a subcutaneous adjuvant with and without local antigen on the elimination of 1 mg i.v. dose of [^{131}I]BGG. Top left, control group; top right, each mouse received 300 μg Wax D peptidoglycolipid fraction WL52 in 0·2 ml Bayol 55 subdivided for injection into four sites; bottom, each mouse received 300 μg Wax D peptidoglycolipid fraction WL52 and 100 μg BGG in 0·2 ml emulsion (saline, Bayol 55, and Arlacel A) similarly subdivided.

into subcutaneous sites. Some mice not showing the immune-type elimination on the primary exposure to antigen may have become latently immunized and will show an obviously immune-type elimination on secondary exposure. Others not responding on the first exposure may, however, have become immunologically paralysed specifically to BGG: they will not give an immune response on a second exposure even if attempts are made to encourage antibody formation by presenting the antigen in a highly immunogenic manner in complete Freund-type adjuvant (Dresser, 1961).

MATERIALS AND METHODS

Animals

For most experiments CBA-strain male mice were used, 3–5 months old, 22–25 g in weight. In one experiment, 4–5 month-old Porton White male mice were used.

Antigen

Bovine γ-globulin (BGG), ethanol fractionated (Cohn fraction II) batch No. HL2970. The BGG was trace-labelled by the direct oxidation method of Hunter and Greenwood (1962) with iodine-131 (IBS3 from the Radiochemical Centre, Amersham, England). Amounts of 100 mg were labelled so that about 7 μCi of ^{131}I were attached to each 1 mg dose for intravenous injection. After radioiodination the antigen solution was spun for 30 minutes at 30,000 ***g*** in a 3 × 10 ml swing-out head of an MSE Super Speed 50 ultracentrifuge to remove aggregates. The contents of the upper two-thirds of the tubes were removed, and the protein content determined from the optical density of a diluted aliquot in ultraviolet spectrophotometer at 280 mμ. Each mouse received 0·5 ml of a 2 mg/ml solution of the BGG in 0·15 M NaCl intravenously.

Immunological adjuvants

The mycobacterial component of the adjuvants used was either the heat-killed cells of *M. tuberculosis* var. *hominis*, Weybridge strain C or a purified peptidoglycolipid fraction of Wax D (WL52) *M. tuberculosis*, strain Canetti (White, Jollés, Samour and Lederer, 1964).

Two types of mixture were used:

(1) (a) Suspension in oil of *M. tuberculosis*, 1·5 mg/ml of Bayol 55.
(b) Solution in oil of Wax D peptidoglycolipid WL52, 1·5 mg/ml Bayol 55.

(2) Water-in-oil emulsions with BGG antigen in Freund's complete adjuvants (CFA).

These were prepared by suspending the mycobacteria in oil (2·5 mg/ml) or dissolving the Wax D fraction in oil (2·5 mg/ml). The oil was mixed with Arlacel A (mannide monooleate) and BGG solution (2·5 mg/ml) in the proportions of 3 : 1 : 1 respectively: the mixture was emulsified by frequently repeated withdrawal and expression of the mixture through a fine needle until a uniform white emusion of thick consistency was produced.

In one experiment heat-killed *Corynebacterium rubrum* or *M. phlei* substituted for *M. tuberculosis* in the two types of preparation.

Conditions of experiments and injection procedure

Before the experiments the animals had been fed Diet 41 (Wm. Pearson Ltd, Glasgow). This was continued throughout the experiments. For drinking water 0·075 M NaCl with 0·01 per cent potassium iodide was given.

The adjuvants were given subcutaneously into the footpads of the hind-limbs and into the anterior surface of the forelimbs, the total dose 0·2 ml being subdivided for this purpose. With the CFA each animal received 300 μg of bacteria or Wax D peptidoglycolipid fraction and 100 μg of antigen. The same quantities of bacteria or wax were in the 0·2-ml volumes of the oil suspensions or solutions.

The first series of experiments were performed during early summer. The mouse room had a southern exposure and at times the room became warm (about 27°). The later series of experiments were performed at ambient temperatures of 18–20°.

Determination of antigen elimination by whole-body counting

The radioactivity in each animal was determined every second day from the 2nd to the 14th day by placing the mouse in a centred container within a large Nuclear Enterprises plastic well scintillation counter. The counts corrected for decay were then plotted on semilog paper and the rates of elimination (as half-lives in days) obtained graphically from the plot.

RESULTS

EFFECT ON BGG ELIMINATION OF CFA AND MYCOBACTERIA-IN-OIL GIVEN AT DIFFERENT TIMES BEFORE INTRAVENOUS ANTIGEN

CBA mice in groups of five were injected subcutaneously with 0·2 ml mycobacteria-in-oil 10 days before, 4 days before, or on the day of i.v. injection of [^{131}I]BGG: a group without any subcutaneous injection acted as a control group. In addition a further group of five mice were given antigen in CFA subcutaneously on the day of i.v. antigen. After 2 months all except the last group were tested by reinjection of 1 mg [^{131}I]BGG intravenously and antigen in CFA subcutaneously.

In the two groups given adjuvant on the day of intravenous antigen injection, antigen elimination was significantly faster during the first 8 days (mean half-lives 2·83 and 2·96 days, $P<0·01$ by Student's *t*-test) than the control group without adjuvant (mean half-life 4·38 days). Only those mice receiving antigen in CFA showed a primary immune elimination. The others showed an immune paralysis towards BGG when subsequently tested with CFA in the elimination experiment 2 months later (Table 1).

TABLE 1

RATES OF ELIMINATION OF [^{131}I]BGG AND IMMUNE RESPONSES IN TWO STRAINS OF MICE AFTER VARIOUS TREATMENTS

Treatment	Mean half-life of elimination up to day 8 (in days ± SD)	Primary immune responses	Secondary immune responses
CBA mice			
Nil	4·38 ± 0·66	0/5	0/5*
M. tuberculosis in oil 10 days before, s.c.	4·14 ± 0·456	0/5	0/5*
M. tuberculosis in oil 4 days before, s.c.	4·03 ± 0·17	0/5	0/5*
M. tuberculosis in oil on day of i.v. BGG, s.c.	2·83 ± 0·14	0/4†	0/4*
M. tuberculosis in CFA with BGG, s.c. on day of i.v. BGG	2·96 ± 0·252	5/5	NT
CBA mice			
Nil	5·0 ± 0·134	0/5	NT
Wax D in oil on day of i.v. BGG	4·12 ± 0·277	0/5	NT
Wax D in oil with Bayol 55, Arlacel A and BGG on day of i.v. BGG	4·18 ± 0·392	5/5	NT
Porton White Mice			
Nil	1·98 ± 0·163	–	3/5
M. tuberculosis in oil 10 days before, s.c.	1·54 ± 0·05	–	2/5
M. tuberculosis in oil 4 days before, s.c.	1·34 ± 0·314	–	3/5
M. tuberculosis in oil on day of i.v. BGG, s.c.	1·6 ± 0·1	–	4/5

NT, not tested.
* Stimulated with Freund's adjuvant containing BGG.
† One death in this group.

EFFECT ON [^{131}I]BGG ELIMINATION OF SUBSTITUTION OF A WAX D FRACTION FOR *M. tuberculosis* IN THE ADJUVANT MIXTURES

Two groups of five mice were given subcutaneous injections of either Wax D fraction WL52 in oil or antigen in CFA with the same Wax D fraction in the oil phase. The elimination rates of antigen given on the same day to these animals and also to control animals are shown in Table 1 and Fig. 1. The elimination rates in both groups receiving the Wax D fraction are significantly faster (mean half lives 4·12 and 4·18 days) than the control group (mean half life 5·0 days $P<0·01$ by Student's *t*-test). Only that group receiving local BGG in adjuvant showed a primary immune elimination.

[^{131}I]BGG ELIMINATION IN PORTON WHITE MICE WITH OBSERVATIONS OF ADJUVANT ACTION

Porton White mice in three groups of five were injected subcutaneously with 0·2 ml mycobacteria-in-oil, 10 days before, 4 days before and on the day of i.v. injection of [^{131}I]BGG, respectively. These mice as well as a control group of five mice were injected with 1 mg [^{131}I]BGG intravenously and elimination observed for 14 days.

There was a significant difference between each mycobacterial group ($P<0·01$, *t*-test) and the control group in their rates of elimination over the first 8 days (Table 1). Because of the short half-life of the antigen ($1·98\pm0·163$ days) it was not possible to detect any superimposed acceleration of its removal by antibody of the primary response.

The animals were therefore injected intravenously with an indicator dose of antigen after a further 2 months, to reveal any secondary responses in which the antigen is eliminated very rapidly (von Dungern, 1903; Dresser, 1960). Several of the animals in the control group (three out of five) as well as many test mice showed a secondary response (Table 1).

The results in the CBA mice differed from those of Dresser (1960) in that neither the mycobacteria nor the wax had been able to stimulate an immune response unless placed in the tissues with local antigen in the water-in-oil emulsion.

There was an interval of 6 months before a second series of experiments of this type was carried out.

EFFECT ON [^{131}I]BGG ELIMINATION OF SUBSTITUTION OF *C. rubrum* OR *M. phlei* FOR *M. tuberculosis* IN THE ADJUVANT MIXTURES

Two other bacterial species, *C. rubrum* and *M. phlei*, in addition to *M. tuberculosis*, were used separately in adjuvant mixtures. Groups of five mice were given a suspension in oil of one of the species or were given the CFA preparation. Along with the control groups, these mice were injected intravenously with 1 mg [^{131}I]BGG.

All groups receiving adjuvant showed an obvious primary immune elimination (Table 2, Fig. 2). All elimination rates in the preimmune phase were accelerated significantly over the control group ($P<0·01$ in all groups except the *M. tuberculosis* in oil group where $P<0·05$, by the *t*-test).

FURTHER EXPERIMENTS WITH *M. tuberculosis* AND WAX D IN THE ADJUVANT MIXTURES

Further groups were set up with *M. tuberculosis* or Wax D in the two types of adjuvant preparation. Primary immune responses were obtained in all adjuvant treated mice (Table

2). Control untreated mice showed a mean half-life in the first eight days of 2·96 days, the elimination being significantly slower than in all groups except the Wax D in oil group ($P<0·01$ by the t-test.)

TABLE 2

RATES OF ELIMINATION OF [^{131}I]BGG AND IMMUNE RESPONSES IN CBA MICE AFTER VARIOUS TREATMENTS

Treatment (by subcutaneous injection)	Elimination rate (half-life in days) up to day 8	Primary immune responses
Nil	3·82±0·178	0/5
CFA with *M. tuberculosis*	2·58±0·16	5/5
M. tuberculosis in Bayol 55	3·16±0·434	5/5
CFA with *C. rubrum*	2·3 ±0·155	5/5
C. rubrum in Bayol 55	2·5 ±0·18	5/5
CFA with *M. phlei*	2·4 ±0·29	3/3
M. phlei in Bayol 55	2·58±0·37	4/4
Nil	2·96±0·09	0/5
CFA with *M. tuberculosis*	2·54±0·167	5/5
M. tuberculosis in Bayol 55	2·26±0·27	5/5
CFA with Wax D	2·38±0·13	5/5
Wax D in Bayol 55	2·72±0·23	5/5

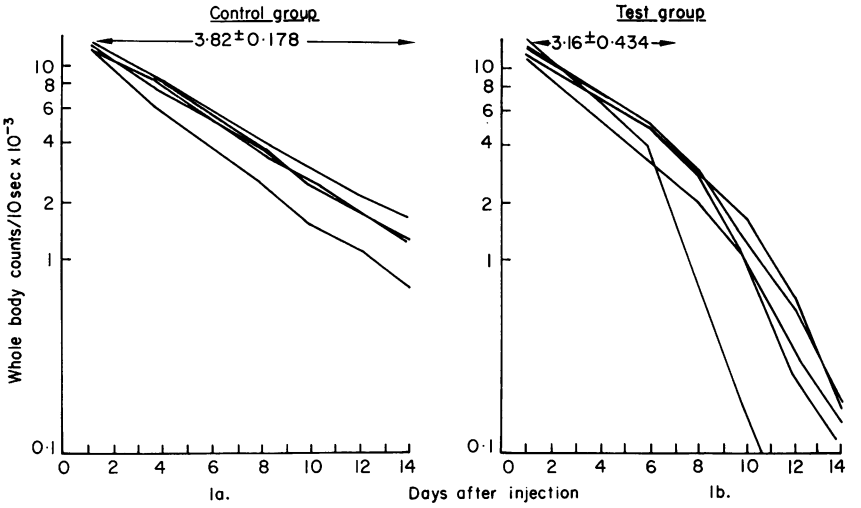

FIG. 2. Influence of a subcutaneous adjuvant without local antigen on the elimination of a 1 mg i.v. dose of [^{131}I]BGG. The mice in the test group received 300 μg of killed *M. tuberculosis* var. *hominis* in 0·2 ml Bayol 55 subdivided for subcutaneous injection into four sites.

DISCUSSION

The presence of antigen at the site of adjuvant injection

The later experiments agreed with those of Dresser (1960) in that mycobacterial adjuvants without local antigen were able to produce a demonstrable immune elimination of the intravenously injected antigen. Nevertheless if circumstances are unfavourable to the establishment of the responses as they appear to have been in the earlier series, only the

CFA with a high local concentration of antigen induced antibody formation. The adjuvant mixtures lacking emulsified antigen must therefore be regarded as weaker adjuvants. This would be in accordance with the findings of Farr and Dixon (1960) who demonstrated the importance of local concentration of antigen as opposed to total amount of antigen in stimulating an antibody response.

Substitutes for M. tuberculosis

Corynebacterium rubrum, *M. phlei* and the peptidoglycolipid Wax D fraction, WL52, satisfactorily substituted for *M. tuberculosis* in these experiments. These agents have previously been shown to have adjuvant effects in other antigen systems such as the induction of acute encephalitis (White and Marshall, 1958; White *et al.*, 1964; Shaw, Alvord, Fahlberg and Kies, 1964).

In the present experiments not only did they produce an antibody response to BGG when they were themselves injected in an oily medium without local antigen but they produced also the accelerated catabolism of protein in the preimmune phase noted in the experiments with *M. tuberculosis*.

Acceleration of antigen catabolism in the preimmune phase of elimination

There was a contradiction between the results in the early and late series of experiments in the CBA mice; in the one case the mice had not produced antibody unless adjuvant with local antigen had been used; in the other local antigen was not necessary.

The more rapid rate of protein catabolism in the later experiments may have been decisive in changing the experience with the adjuvants lacking emulsified antigen. The elimination rates were faster in the control groups of the second series of experiments than in the first and the adjuvants themselves further increased the catabolic rate of the antigen in the pre-immune phase. There was also some suggestion from the experiments with the Porton White mice that very rapid catabolism of antigen predisposed to or was related to an immune response. There is no immediate obvious explanation for the faster catabolism of the CBA mice in the later series of experiments. The animals were of the same age, sex, and strain and were on the same diet: the antigen was iodinated to the same extent by the same procedure. The only apparent difference in experimental conditions lay in the summer and winter ambient temperatures which may have affected the metabolic rates of the mice.

Sewell (1960) has reported that phagocytosis of carbon particles from the bloodstream is greater in summer months. Although this might apparently contrast with the findings here where the greater immunogenicity was achieved in the faster metabolic conditions of the winter months, the dissociation of immunogenicity and rate of carbon particle uptake would be in keeping with previous observations in mice stimulated with mycobacteria (Biozzi, Benacerraf, Grumbach, Halpern, Levaditi and Rist, 1954; Dresser, 1960).

The results suggest that increases in the rate of antigen catabolism contribute to immunogenicity in this model. This proposition will be examined in more detail in a further communication (Stark, 1970).

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