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Studies on the adjuvant action of beryllium

II. SYSTEMIC EFFECTS WITH PARTICULAR REFERENCE TO SECRETORY IMMUNITY

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Summary. When 5 mg doses of beryllium hydroxide, $Be(OH)_2$, were injected intravenously (i.v.) into rats, much of the injected material was retained in the lungs.

When the injection of $Be(OH)_2$ was accompanied by particulate antigens such as killed *B. abortus* organisms or SRBC, immune responses took place in the intra-thoracic lymph nodes (ITLN) so that antibodies of the IgA class were generated and endowed the bile with significant agglutinating activity. This did not happen when the antigens were injected without the beryllium adjuvant. Similarly, a soluble antigen, bovine serum albumin (BSA) only gave rise to significant amounts of biliary and serum antibodies after i.v. injection if $Be(OH)_2$ was used too. The highest titres occurred when the antigen followed the adjuvant after an interval of a few seconds but substantial titres still resulted if the injection of antigen was delayed for 24 hr.

The amounts of biliary antibodies generated by the parenteral injection of antigen and adjuvant were comparable to those that occurred after the same materials were injected directly into the GALT. The adjuvant action of $Be(OH)_2$ was able to induce in athymic (nude) rats the production of measurable amounts of both biliary and serum antibodies to SRBC.

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INTRODUCTION

The experiments described in the previous paper showed that a strong adjuvant effect could be obtained when small amounts of $Be(OH)_2$ were introduced into lymph nodes. We wanted to exploit this finding in the context of secretory immunity but we did not want to inject the material directly into the lymph nodes of the GALT, because such a route of immunization is not a practical proposition except under special, laboratory conditions. However, we reasoned that if a dose of $Be(OH)_2$ were given intravenously (i.v.), its particulate nature would lead to its retention in the capillary beds of the lungs, and that some of it might be removed by the local lymphatics and deposited in the intra-thoracic lymph nodes (ITLN). If this were to happen at the same time as antigen was injected i.v. an augmented response might then take place in the ITLN. It is known that the ITLN of rats are capable of generating substantial amounts of antibodies of the IgA class, as well as conventional IgG and IgM antibodies (Spencer, Gyure & Hall, 1983). The possibility of thus inducing a secretory antibody response by the parenteral presentation of antigen and adjuvant seemed worth putting to the test.

MATERIALS AND METHODS

Animals

Inbred male Wistar rats (RT1^u), approximately 14 weeks of age, were taken from the Institute's barrier-maintained colony, as required. Athymic 'nude' (rnu/rnu) rats from the Rowett strain back-crossed to CBi Lister Hooded (RT1^c rats) were bred and maintained in isolators on site, and were transferred to the open animal house before use.

Surgical procedures

The techniques for immunizing the GALT by injecting the Peyer's patches, and for collecting bile have been described (Hall *et al.*, 1979; Andrew & Hall, 1982).

Antigens and adjuvant

Killed *Brucella abortus* organisms and sheep red blood cells (SRBC) were chosen as particulate antigens, and used as described (Andrew & Hall, 1982). Bovine serum albumin (BSA, Sigma) was used as a soluble antigen. The $Be(OH)_2$ suspension was prepared and, where necessary labelled with ^7Be, as described in the previous paper.

Antibody assay

Humoral antibodies to the particulate antigens were titrated by standard agglutination methods, and lymphoid cells making antibodies to SRBC were detected by a conventional plaque assay (Cunningham & Szenberg, 1968).

Antibodies to BSA were measured by a solid-phase radioimmunoassay (Rose, Peppard & Hobbs, 1984). The BSA was allowed to bind to polyvinyl microtitre plates which, after washing were exposed to doubling dilutions of the bile or blood serum under test. After the specific antibody had bound to the BSA, and excess material had been washed away, the immunoglobulin which had bound to its antigen was detected and measured in terms of the binding of ^{125}I-labelled, affinity-purified, rabbit anti-rat $F(ab')_2$ reagent. The results were plotted graphically and an example is shown in that form in the results section. For ease of presentation, each graph was reduced arbitarily to a single numerical result by drawing a vertical (dotted) line in the manner shown, and noting the ordinate value of the intercept between the vertical and the line of best fit through the plotted results. Although relatively time-comsuming, this method was found to be reproducible and reliable. Variants of Farr's test, in which the antibody and bound antigen are precipitated with ammonium sulphate, were found suitable for the assay of serum antibodies and gave directly a single numerical result, but they were inapplicable to bile. We found that the presence of bile salts interfered with the precipitation step in an uncontrollable manner, and so far we have been unable to overcome this difficulty.

The evidence that, for practical purposes, the antibodies in the bile of rats are almost entirely of the IgA class has been presented previously (Hall *et al.*, 1979; Andrew & Hall, 1982; Spencer *et al.*, 1983; Denham *et al.*, 1984).

Short-term culture of lymphoid cells

Cell suspensions were prepared from lymph nodes or spleens as described (Spencer *et al.*, 1983) and incubated in medium for 3 hr at 37° before harvesting and processing the supernatants (Hall, Hopkins & Orlans, 1977).

RESULTS

It was necessary first to show that much of an i.v. dose of $Be(OH)_2$ did, in fact, become deposited in the lungs. Four rats received injections of either the insoluble $^7Be(OH)_2$ or the soluble $^7BeSO_4$, and were killed either 2 or 24 hr afterwards. In each case, a dose of 5 mg of the beryllium salt was given. The results are shown in Table 1. It can be seen that over half of the injected dose of $Be(OH)_2$ was deposited in the lungs within 2 hr and was still there at 24 hr.

Table 1. The percentage of injected radioactivity in various tissues of rats 2 and 24 hr after the i.v. injection of radioactive ^7Be in soluble or particulate form.

	Percentage of injected radioactivity			
	Soluble $BeSO_4$		Particulate $Be(OH)_2$	
	2 hr	24 hr	2 hr	24 hr
Spleen	1·7	2·8	1·4	4·5
Gut	2·8	ND	0·4	0·3
Liver	24·8	54·3	23·8	14·9
Lung	4·0	1·1	58·4	54·4

Adjuvant effect of $Be(OH)_2$ particulate antigens

In these experiments rats received i.v. doses of either 10^9 SRBC or 10^9 *B. abortus* organisms. Half the rats

received the antigen alone, and half received the antigen in combination with 5 mg $Be(OH)_2$. Seven days after the injections, the blood serum and bile of the rats were collected and assayed for specific agglutinins. The results are shown in Tables 2 and 3. In the responses to SRBC the titres of both serum and biliary antibodies were increased substantially in two out of the three rats that received $Be(OH)_2$. In the responses to *B. abortus* no biliary antibody was detected at all unless the $Be(OH)_2$ adjuvant had been used. These preliminary experiments indicated that our original predictions seemed to have been correct. However, although they are convenient, particulate antigens of the types used are intrinsically immunogenic, and therefore are not the best subjects for the demonstration of adjuvant effects; but before abandoning them, we used the plaque assay to show that, under the conditions of these experiments, antibody-forming cells were, in fact, generated in ITLN (Table 4).

Table 2. Titres of agglutinins against SRBC in samples of bile and blood serum of rats 7 days after the i.v. injection of 10^9 SRBC in saline or 10^9 SRBC with 5 mg $Be(OH)_2$

	Rat No.	Anti-SRBC agglutination titres	
		Serum	Bile
SRBC	1	1/64	1/8
+saline	2	1/256	1/16
SRBC	3	1/64	1/16
+$Be(OH)_2$	4	1/4096	1/512
	5	1/65,536	1/128

Table 3. Titres of anti-*Brucella* agglutinins in samples of bile and blood serum of rats 7 days after the i.v. injection of 10^9 *Brucella* organisms in saline or 10^9 *Brucella* organisms with 5 mg $Be(OH)_2$

	Rat No.	Anti-*Brucella* agglutination titres	
		Serum	Bile
Brucella	1	1/256	0
+saline	2	1/1024	0
	3	1/1024	0
Brucella	4	1/512	1/128
+$Be(OH)_2$	5	1/4096	1/128
	6	1/512	1/256

Table 4. The distribution in the lymphoid tissue of rats of direct anti SRBC PFC 7 days after the i.v. injection of 10^9 SRBC in saline or 10^9 SRBC with 5 mg $Be(OH)_2$

	Rat No.	PFC per 10^6 lymphocytes		
		MLN	ITLN	Spleen
SRBC	1	0	13	83
+saline	2	0	5	8
SRBC	3	0	179	64
+$Be(OH)_2$	4	0	63	63

Adjuvant effect of $Be(OH)_2$ with soluble antigen

A dose of 5 mg BSA dissolved in 0·2 ml of saline was injected i.v. into each of six rats; three rats then received immediately a 5 mg i.v. dose of $Be(OH)_2$, and three received 0·5 ml saline. Seven days later samples of serum and bile were collected from each rat and the antibody activities of the samples were measured by radioimmunoassay. The results are shown graphically in Fig. 1. The rats which had received the $Be(OH)_2$ developed substantial amounts of antibody in their blood serum and significant amounts in their bile. The control rats which had received saline instead of adjuvant had only small amounts of antibody in either fluid.

The experiment with $Be(OH)_2$ was repeated, but on the seventh day the three rats were killed. Cell suspensions were prepared from the mesenteric lymph nodes (MLN), the ITLNs and the spleens in such a way that the volumes and concentrations of cells in each suspension were equal. After 3 hr of culture at 37° the supernatants from each culture were harvested and pooled according to the origin of the cells used for culture. The pooled supernatants were submitted to radioimmunoassay for antibody activity against BSA. The results are shown in Table 5. Although the BSA must have become widely systemized, most antibody was synthesised by cells from the spleen and the ITLN, and the MLN made relatively little.

Timing of antigen dose in relation to $Be(OH)_2$ adjuvant

In the experiment described above the antigen and adjuvant were injected separately, albeit at intervals of only a few seconds. Separate injections were employed in order to avoid the possibility of the soluble antigen binding to the $Be(OH)_2$ particles *in vitro*. However, in

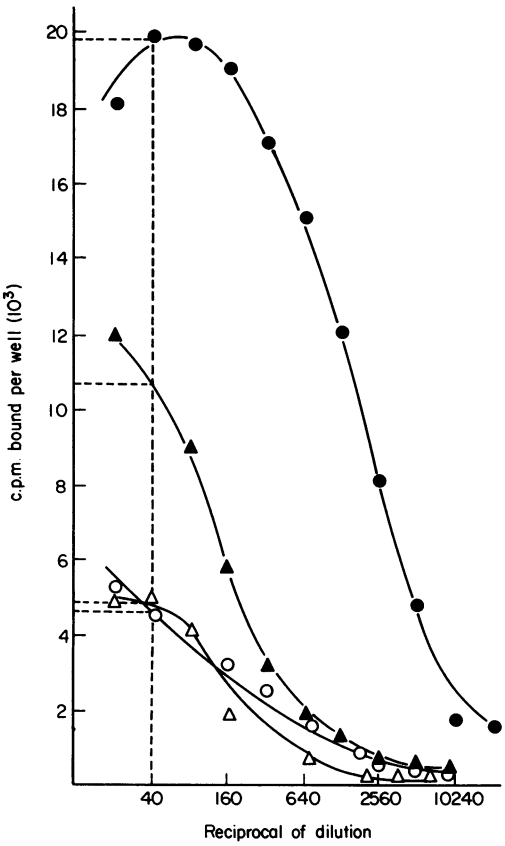

Figure 1. Titration curves from radioimmunoassays of antibodies to BSA showing counts per minute (c.p.m.) of ^{125}I-rabbit anti-rat F(ab')$_2$ bound by doubling dilutions of blood or bile from rats immunized i.v. 7 days previously. Serum from rats immunized with BSA + Be(OH)$_2$ (●——●); bile from rats immunized with BSA + Be(OH)$_2$ (▲——▲); serum from rats immunized with BSA alone (○——○); bile from rats immunized with BSA alone (△——△). The *dotted lines* indicate the method of deriving a single numerical result from each curve (see 'Methods').

order to show for certain that a preformed antigen–Be(OH)$_2$ adduct could not account for the bulk of the adjuvant effect, a time interval of 24 hr between the injection of antigen and adjuvant, or vice versa, was employed. The results of these procedures are shown in Table 6. When Be(OH)$_2$ was given 24 hr before the antigen the amount of antibody formed was only about two dilutions less than when the injections were separated by a few seconds. Be(OH)$_2$ given 24 hr after the antigen still had a significant adjuvant effect but one that was obviously less than giving it before or with the antigen.

Table 5. Radioimmunoassay of antibodies to BSA in the supernatant fluids of cultures prepared from lymphoid cells collected from rats 7 days after they had been given an i.v. injection of BSA and Be (OH)$_2$

Source of lymphoid cells	c.p.m. of ^{125}I-anti-F(ab')$_2$ bound
Intra thoracic lymph nodes	7500
Mesenteric lymph nodes	2600
Spleen	7500

Table 6. Radioimmunoassay of antibodies to BSA in the blood serum of rats 7 days after they had been given an i.v. injection of one of various combinations of BSA and Be(OH)$_2$

Immunization protocol	c.p.m. of ^{125}I-anti-F(ab')$_2$ bound
BSA and Be(OH)$_2$ injected together	24,600
Be(OH)$_2$ injected 24 hr before BSA	21,800
Be(OH)$_2$ injected 24 hr after BSA	7400
BSA injected without adjuvant	2400

Local versus systemic use of Be(OH)$_2$ adjuvant in secretory immunity

The previous experiments showed that the production of some biliary (IgA) antibodies could be induced by the i.v. injection of antigens together with Be(OH)$_2$ but did not measure the efficiency of the process. Therefore, we compared the amount of biliary antibody formed in response to an i.v. dose of 5 mg BSA and 5 mg Be(OH)$_2$ with the amount formed when the same materials were injected directly into the GALT via the Peyer's patches. Three rats received i.v. injections and three rats received injections into the Peyer's patches; each group was controlled by three rats which received the antigen in saline, without adjuvant. Bile was collected from all the rats between the 6th and 8th day after the injections; equal volumes of bile from each rat in a group were pooled, and the pooled bile from each group was submitted to radioimmunoassay for specific antibodies. The results are shown in Table 7. The rats immunized with antigen and adjuvant either by the i.v. or the GALT route produced virtually identical amounts of biliary antibody, whilst the controls, which received no adjuvant produced only small amounts.

Adjuvant effect of $Be(OH)_2$ in athymic (nude) rats

Because of the relatively powerful adjuvant effect of $Be(OH)_2$, we thought that it might be interesting to see if it could overcome the anergy which athymic (nude) rats display in response to thymus-dependent antigens such as SRBC (Andrew & Hall, 1982). Accordingly, six such rats were given the standard i.v. doses of 10^9 SRBC and 5 mg $Be(OH)_2$, while five control nude rats received 0·05 ml SRBC alone. Blood serum and bile were collected from all rats on day 7 and titrated for specific agglutinins. The results are shown in Table 8. Two of the control rats developed measurable, though lowish, titres of serum antibody, and the bile of one of them also contained some antibody activity. However, all of the nude rats which had received $Be(OH)_2$ had unequivocal antibody activity in the blood, and all but one had similar amounts in the bile.

Table 7. Radioimmunoassays of antibodies (presumptively of the IgA class) to BSA in the bile of rats 7 days after they had been immunized with BSA and $Be(OH)_2$ by various routes

Route and type of immunization	c.p.m. of ^{125}I-anti-F(ab')$_2$ bound
Immunized in Peyer's Patches with BSA + $Be(OH)_2$	11,800
Immunized in Peyer's Patches with BSA without adjuvant	4000
Immunized by i.v. injection of BSA + $Be(OH)_2$	12,200
Immunized by i.v. injection of BSA without adjuvant	2100

Table 8. The titres of agglutinins to SRBC in the blood serum and bile of nude rats 7 days after an i.v. injection of 10^9 SRBC in saline or 10^9 SRBC with 5 mg $Be(OH)_2$

	Rat No.	Bile titre	Serum titre
SRBC + saline	1	0	1/8
	2	1/2	1/8
	3	0	0
	4	0	0
	5	0	0
SRBC + $Be(OH)_2$	6	1/64	1/16
	7	1/32	1/32
	8	1/128	1/32
	9	0	1/8
	10	1/16	1/64
	11	1/32	1/32

DISCUSSION

Although we have shown that beryllium can be used to potentiate secretory immune responses, in rats it must be emphasized that the i.v. use of $Be(OH)_2$ can have lethal consequences, especially in other species. Given at our standard i.v. dose of about 20 mg/kg body weight it often killed both mice and sheep. Sometimes death was immediate, due apparently to embolic phenomena, but more usually it occurred a day or two later. The exact cause of death at these times was not ascertained precisely but pneumonitis must have been a major factor.

Of course, the whole point of the experiment was to deliver $Be(OH)_2$ via the lung, to the ITLN but the injection of a relatively large amount of insoluble material is a crude way of going about it, and cannot itself be the basis of an acceptable means of immunization. However, now that the possibilities have been demonstrated, other ways of using beryllium, such as introducing beryllium salts into liposomes etc., can be explored. Alternatively, if the exact mechanism of the adjuvant action of beryllium were to be understood, it might become possible to exploit it without the risk of serious toxicity. The results described in the preceding paper inclined us to the view that the adjuvant effects were likely to be caused by a lymphokine-like factor released from intra-nodal macrophages. Whether such factors will turn out to be conventional lymphokines or conjugates of beryllium and some macrophage-derived proteins, must, for the moment, remain a matter for speculation. Whatever their nature, it seems that their production is unlikely to involve T cells, because some adjuvant activity was demonstrable in athymic rats. Indeed, it seems that the adjuvant effect of beryllium can, to some extent replace T cells, as can LPS in mice (Sjoberg, Anderson & Moller, 1972). However, even with the beryllium adjuvant the titres of haemagglutinins to SRBC made by the athymic rats were much lower than those observed in euthymic rats (Andrew & Hall, 1982).

In our experience, the best way of inducing a rapid, primary secretory immune response in laboratory rats has been to inject antigens directly into the GALT (Hall *et al.*, 1979) and it is against this standard that

alternative routes of antigen administration must be judged. When adjuvants are not employed, the i.v. route is not very successful in inducing the formation of secretory antibodies of the IgA class (Andrew & Hall, 1982); however, the present experiments show that the i.v. injection of $Be(OH)_2$ alters this so that titres of antibody approaching those attained after direct injection of the GALT, can be induced by a single parenteral injection. This might turn out to be useful. It is often difficult to immunize rats against the non-toxic antigens of intestinal pathogens by the oral route, unless several sequential doses are given (e.g. Lycke, Lindholm & Holmgren, 1983), and in order to prime rats for a significant response to cholera toxin Pierce & Gowans (1975) found it necessary to give the antigen intraperitoneally (i.p.) in Freund's complete adjuvant (FCA). A similar protocol has been used in sheep (Beh, Husband & Lascelles, 1979; Beh & Lascelles, 1981); but the rationale of this procedure is complex. According to the standard text (Yoffey & Courtice, 1970) antigenic material placed in the peritoneal cavity drains mainly to the ITLN, and this certainly happens in rats (Spencer *et al.*, 1983). On the other hand, Beh and others (Beh *et al.*, 1979; Beh & Lascelles, 1981) reported that the mesenteric, coeliac and hepatic nodes were the ones that reacted when antigen in FCA was injected i.p. into sheep. Perhaps the sterile peritonitis induced by the adjuvant caused the visceral peritoneum to become 'leaky' and allow the antigen to penetrate to the intra-abdominal nodes from which it is normally excluded. It is significant that i.p. injections of antigen without adjuvant often fail to induce significant amounts of antibodies of the IgA class (Andrew & Hall, 1982).

At the moment, then, the search for a simple means of producing a powerful secretory immune response, using a single dose of antigen, still has some way to go. The oral route requires repeated dosage, is often ineffective, and may even induce tolerance (Tomasi, 1980). The parenteral routes necessitate the use of powerful adjuvants which are more or less damaging or toxic. In the case of beryllium, there are grounds for hoping that it may be possible to separate its adjuvant action from serious toxic effects. Similarly, the use of muramyl dipeptide may avoid the unwanted effects of FCA (Taubman *et al.*, 1983).

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