STUDIES ON IMMUNITY IN ANTHRAX

V. IMMUNIZING ACTIVITY OF ALUM-PRECIPITATED PROTECTIVE ANTIGEN

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Elaboration of the protective antigen of *Bacillus anthracis* in chemically-defined, non-protein media, and studies on the influence of cultural conditions on elaboration of the antigen, have been described in previous reports (1, 2). Preliminary evidence that the protective antigen in culture filtrates could be concentrated, stabilized, and partially purified by precipitation with alum has been briefly summarized (1). The present report presents further experimental studies of the immunizing activity and related properties of the alum-precipitated antigen, and preliminary observations on its use in the immunization of man.

MATERIALS AND METHODS

Antigen-containing filtrates. Composition of the chemically-defined media and methods for production of antigen-containing culture filtrates have been described previously (1, 2). Spore suspensions of non-proteolytic mutants of the Vollum strain of *B. anthracis* were used as inoculum (3). In the earlier preparations, as indicated, the NP-A strain was used; in later work the R1-NP strain, a non-proteolytic, non-encapsulated double mutant was substituted (2).

Alum-precipitated antigen. Culture filtrates were precipitated with alum immediately after filtration by addition of 0.1% aluminum potassium sulfate (in terms of the anhydrous salt) followed by adjustment to pH 5.9 with dilute hydrochloric acid. Under these conditions essentially complete precipitation of protective antigen was obtained. Alum and hydrochloric acid solutions were sterilized by filtration, and the manipulations of precipitation and washing were carried out aseptically. The precipitate settled rapidly, and after several hours or overnight at 4°C most of the supernatant could be drawn off. The remainder was removed after centrifugation at moderate speed, and the precipitate was washed by suspension in sterile 0.9% sodium chloride solution, hereafter referred to as saline. After a second centrifugation, the precipitate was resuspended in saline to a volume 1/10 that of the culture filtrate, and merthiolate was added to a concentration of 1—10,000. This product is referred to as stock antigen.

Stock antigen preparations to be used for human immunization were tested for sterility in nutrient broth and in Brewer thioglycollate medium (4), at 37°C and at room temperature. Safety tests were performed by subcutaneous injection of 5 ml into each of four guinea pigs, and by intraperitoneal injection of 1 ml into eight or more mice.

Assay of antigenic potency. For standard assays, the rabbit test used in previous work was employed (1, 3). Animals were given five intracutaneous injections of 0.5 ml of the appropriate dilution of stock antigen on alternate days, and challenged 7 days after the last dose by intracutaneous injection of 10,000 spores of the Vollum strain of *B. anthracis*. One standard spore suspension was used for all intracutaneous challenge injections. The intracutaneous LD_{50} of this preparation for rabbits was approximately 100 spores. Although it was observed that effective immunity could be induced with a smaller number of immunizing injections, the schedule described was used throughout for consistency and to allow comparison with previous observations.

Immunogenicity tests in guinea pigs and monkeys were carried out as described below.

RESULTS

Immunizing activity of alum-precipitated antigen. Alum-precipitated antigens were prepared from antigen-containing filtrates of cultures grown in the four successive modifications of the chemically-defined media for production of antigen (2).

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TABLE I

Activity of preparations of alum-precipitated antigen in the immunization of rabbits

Preparation	Culture Medium and Strain Used	Storage of Antigen at 4°C Before Injection	Antigenic Potency as Indicated by Survival Ratios* in Rabbits Injected with Dilutions Indicated			
			1:10	1:25 or 1:30	1:62	1:90
		months				
75	528; NP-A	0-2	6/6†	2/2		
		3	3/3	3/3		
		8		2/2	1/3 (4,7)	
		25	2/2	2/3(4)		
77	528; NP-A	0-1	2/2	6/6	5/6(3)	
		6		2/2	1/3(7,10)	
		10		1/3(4,7)	2/3(4)	
		23	3/3	2/3(5)		
85	528; R1-NP	0	2/3(4)	3/3	0/3 (3,4,4)	
92	528; NP-A	0	3/3	3/3		0/3(3,4,7)
		0	3/3	1/3(3,6)		
93-1	555; R1-NP‡	0		3/3		1/3(3,5)
		14	3/3	2/3(5)		0/3(3,3,4)
98	555; R1-NP	0		3/3		3/3
100	599; R1-NP	0		2/3(5)		3/3
		10	2/2	3/5(4,5)		0/3(3,4,8)
101	599; R1-NP	0	2/3(12)	3/3		3/3
138	599; R1-NP	0	3/3	3/3		0/3(4,4,4)
188	687; R1-NP	0	3/3	1/3(4,5)		
		4	3/3	1/3(3,4)		
190	599; R1-NP§	0	2/3(5)	3/3		3/3
197	599; R1-NP	0	3/3	3/3		3/3

Unimmunized control animals

0/19 (2,2,3,3,3,3,3,3,3,3,3,3,4,4,4,4,5,5,5)

* Survival ratios are recorded as the number of immunized animals surviving divided by the number of animals challenged. Numbers in parentheses refer to the days after challenge on which deaths occurred.

† Preparation also protected 11/11 at lower dilutions or undiluted.

‡ Preparation was alum-precipitated and held at pH 6.5.

§ Culture volume of this preparation was 30 liters.

The volume of the original filtrate used in the different preparations ranged from 1 to 30 liters. The immunizing potencies were estimated in rabbits; typical results of initial assay are given in Table I.

It may be noted that at a 1-10 dilution the antigen preparations immunized nearly all of the rabbits. Most of the preparations gave complete or nearly complete protection at 1-25 or 1-30 dilution, and many produced significant immunity at a dilution of 1-62 or 1-90. All of the four media and the two strains proved satisfactory for production of alum-precipitated antigen. It would appear from all of the results, some of which have not been included in the table, that particularly consistent and potent preparations were obtained with 599 medium and the R1-NP strain. There are indications that the 687 medium yielded somewhat less active alum-precipitated products than the other media. Although no precise statement can be made, the immunizing potencies of the alum-precipitated products appeared to be roughly equivalent to the potencies of the culture filtrates from which they were prepared.

Exploratory experiments on the immunization of guinea pigs with the alum-precipitated antigen indicated that the amount of the antigen required was considerably larger than for rabbits. A total of 2 to 4 ml of stock antigen, injected intraperitoneally or subcutaneously in two or three doses, protected 11 of 14 animals against a challenge dose of 1000 spores injected intracutaneously. The challenge dose represented approximately 200 LD_{50}. A single subcutaneous dose of 2 ml of stock antigen protected five of seven animals against challenge 2 weeks after immunization. Although the optimum procedure for immunization of guinea pigs has not been determined, the results demonstrate the immunizing potency of the alum-precipitated antigen in this species.

Toxicity tests. Seven preparations of stock antigen were tested for possible toxicity by subcutaneous injection of 5 ml into each of four guinea pigs, and by intraperitoneal injection of 1 ml into each of eight or more mice. All of the 28 guinea pigs and all but one of the total of 92 mice survived 21 days. The antigen was also well tolerated by the large number of rabbits that received the antigen during the immunization studies. To test for the occurrence of more subtle toxic reactions, 25 rabbits were given five intracutaneous injections on alternate days of 0.5 ml of a 1-10 dilution of preparation 92. The animals were sacrificed 23 days after the final injection. Complete autopsies in which all organs

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were examined grossly and microscopically revealed no specific lesions[1].

1 The authors are indebted to Dr. Joseph Victor and colleagues for these studies, and for other post-mortem examinations.

Stability of alum-precipitated antigen. Gladstone (5) demonstrated that the protective antigen activity of filtrates of cultures in plasma was relatively stable when stored in the refrigerator at a pH in the neutral region. The activity of filtrates from the chemically-defined medium, however, was rapidly lost under these conditions (1). Certain preparations of alum-precipitated antigen were titrated for antigenic activity in rabbits after storage for various intervals in the refrigerator at approximately 4°C; the results are given in Table I. It would appear that although the antigens lost approximately half their initial activity within a year, they retained appreciable immunizing potency after storage up to 2 years.

Duration of immunity. Rabbits were immunized with the alum-precipitated antigen in the usual manner and challenged in groups at intervals thereafter. Immunization procedures and experimental results are summarized in Table II. It may be noted that the immunity was sufficiently effective to protect a significant number of animals 8 and 16 weeks after immunization. None of six animals survived when challenged 23 weeks after immunization, although there appeared to be an increase in survival time. **(3)**

TABLE II

Duration of immunity induced by protective antigen in rabbits and monkeys.

Animal	Number of Immunizing Injections	Total Volume Stock Antigen Injected	Interval Between Immunization and Challenge	Survival Ratio*
		ml	*weeks*	
Rabbits	5	.62	1	3/3
			14	2/3 (5)
	5	.25	5	3/4 (7)
			8	3/4 (5)
			16	2/4 (4,8)
			23	0/6 (4,4,4,5,6,8)
	none	0	—	0/4 (3,3,4,4)
Monkeys *(M. rhesus)*	2	1.5	2	3/3
			16	2/3 (8)†
			30	3/3
			58	3/3
	none	0	—	0/4 (3,3,5,11)

2 weeks. Stock preparation 77 was used, 0.5 ml in the first injection and 1.0 ml in the second. The monkeys in groups of three were challenged at intervals thereafter by intracutaneous injection of between 50,000 and 100,000 spores. The results are given in Table II. The challenge dose was fatal to all of four unimmunized control monkeys; autopsies revealed pathological and cultural evidence of generalized anthrax. All except one of the 12 immunized animals survived challenge, although the final group was challenged more than a year after immunization. No evidence of infection was found in the latter group of three animals at autopsy 6 weeks after challenge. It would appear that the alum-precipitated antigen produced a significant immunity of considerable duration.

To test for possible reactions to a booster immunizing injection, the five surviving monkeys of the second and third groups were injected with 0.5 ml of stock antigen 3 months after challenge. No significant local reactions to the injections were observed, and no lesions attributable to the treatment were detected at autopsy 3 weeks later.

Immunization of monkeys against respiratory challenge. Eight animals were immunized by subcutaneous injection of two 1.0 ml doses of preparation 138 at an interval of 16 days. Sixteen days after the second injection four of the immunized animals and four controls were challenged by inhalation of an aerosol of spores of

* Survival ratios are recorded as the number of immunized animals surviving divided by the number of animals challenged. Numbers in the parenthesis refer to the days after challenge on which deaths occurred.

† Autopsy findings were typical of anthrax.

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B. anthracis. The calculated dose per animal ranged from 39,000 to 82,000 spores. The four controls died with generalized anthrax between 3 and 7 days after challenge. Three of the four immunized monkeys survived; the fourth animal died with anthrax meningitis 17 days after challenge. The other four immunized animals and two normal controls were challenged 34 days after immunization by inhalation of 890,000 to 3,000,000 spores. The control animals died promptly of generalized anthrax, whereas the immunized monkeys survived.

Immunization of man. After the safety and effectiveness of the antigen had been demonstrated in animals, several volunteers from the research staff were injected subcutaneously with small doses of the antigen without ill effects. A group of 55 volunteers were then given two injections of 0.5 ml of stock antigen at an interval of 2 weeks. No significant systemic reactions were noted, and in only three persons was tenderness detectable at the site of injection after 24 hours.

The immunization program was then extended to a larger group. Three subcutaneous injections of 0.5 ml of stock antigen at intervals of 2 weeks were given as the initial series. A booster dose of 0.25 ml was given after 6 months. In 660 persons who received 1,936 injections in the initial series, the incidence of systemic reactions was 0.7% and of significant local reactions was 2.4%. In 445 booster injections the corresponding incidences were 1.3% and 2.7%.

The systemic reactions consisted of mild, generalized muscular aching, slight headache, and mild to moderate malaise of 1 to 2 days duration. The more severe febrile reactions that occasionally follow administration of diphtheria toxoid, typhoid-paratyphoid, plague, and rickettsial vaccines were not observed.

The significant local reactions at the site of injection were characteristic but varied in intensity. Painless, brawny swelling 5 to 10 cm in diameter appeared 12 to 18 hours after injection and reached its maximum extent on the second day, when the upper arm was significantly enlarged. In some instances, the swelling extended to the mid-forearm. Local pruritus was a frequent complaint and was relieved by antihistaminic drugs. The swelling began to decrease between 2 and 3 days after injection and disappeared completely by the

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TABLE III

Frequency of significant local reactions in man following injection of alum-precipitated protective antigen

Antigen Lot No.	Initial Series						Boosters	
	1st Injection		2nd Injection		3rd Injection			
	Persons	Reactions	Persons	Reactions	Persons	Reactions	Persons	Reactions
		%		%		%		%
87	151	0	122	0	24	0		
96	346	0	343	0.6	230	1.3		
101	6	0	25	4.0	125	6.4		
138	125	0	129	0	129	0.7	322	2.7
181	32	0	31	3.0	29	0	123	2.4
Total	660	0	650	0.6	537	2.2	445	2.6

fifth day. No systemic symptoms were associated with the local reactions. In our experience these reactions differ slightly from the local reactions usually produced by bacterial, viral, and rickettsial vaccines.

Table III records the incidence of significant local reactions according to the lot of antigen and according to the injection number, i.e., first, second, third, or booster injection. It may be noted that local reactions were never observed following the initial injection, and that the incidence increased with the number of previous injections. The preparations of antigen apparently differed with respect to the frequency of production of reactions. Attempts to reproduce the local reaction in animals are in progress.

DISCUSSION

The alum-precipitated protective antigen produced significant immunity in a number of species, and the immunity was effective for a considerable period. No toxicity was detected when the antigen was injected into laboratory animals, and the material was well tolerated in a trial in man

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that involved more than 2,000 injections. The results of this trial indicated that the clinical acceptability of the product compared favorably with numerous vaccines in common use. Local and general reactions were relatively infrequent and of short duration. None of the local reactions occurred following the initial injection, suggesting that these effects had an allergic basis. Further investigation will be required to determine whether the reactions represented a response to the protective antigen itself, or to a constituent that could be eliminated by appropriate fractionation.

Assessment of the antigen in animals of economic importance is in progress and will be reported subsequently; preliminary results suggest that the antigen produces effective immunity in cattle. Inasmuch as anthrax is uncommon in man, and no serological method is available at present for measurement of the immune response, it will be difficult to estimate the effectiveness of the antigen in human immunization.

The methods for production of the antigen are readily adaptable to production on a practical scale, and the stability of the product would appear to be adequate for practical use. It is probable that additional investigation will allow further simplification and improvement of the methods for production of the antigen. Although preliminary experiments involving variation of the pH of alum-precipitation or increase in the concentration of alum have given no consistent increase in potency, it is possible that further study of these or related variables will improve the stability or acceptability of the product. If the storage stability were considered inadequate, lyophilization of the alum-precipitated product would be feasible (1).

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SUMMARY

Methods have been presented for alum-precipitation of the protective antigen of *Bacillus anthracis*. The product induced effective immunity in rabbits, guinea pigs, and monkeys. Significant immunity persisted for more than three months in rabbits and for more than 14 months in monkeys. Immunity in monkeys was effective against both intracutaneous and respiratory challenge. Alum-precipitated antigen deteriorated slowly when stored at 4°C, but considerable potency remained after storage for approximately two years. The product was devoid of toxicity when tested in mice, guinea pigs, rabbits, and monkeys. Injection of the antigen into man was well tolerated; no serious reactions were encountered and moderate local reactions were infrequent.

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