THE USE OF ANTHRAX ANTIGEN TO IMMUNISE MAN AND MONKEY

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The production in vitro of a non-toxic antigenic material present in sterile filtrates of cultures of *Bacillus anthracis* has been described by Wright et al. (1954) and Belton and Strange (1954). This material effectively immunised rabbits and monkeys against subsequent intracutaneous and inhalation challenge with multiple lethal doses of anthrax spores. It has since been determined that the material prepared by the method of Belton and Strange (1954) may be stored for at least two and a half years at 0-4°C with no loss of activity (F. C. Belton unpublished).

The objects of the present studies were: (1) to test the reaction of man to repeated injection of an alum precipitate of the antigen as prepared by Belton and Strange (1954), and (2) to observe in monkeys the duration of immunity induced by two subcutaneous injections of the antigen.

Materials and Methods

Preparation of Antigen and Preliminary Testing

Vaccine was prepared by the method described by Belton and Strange (1954). The product was tested for sterility according to the Therapeutic Substances Act regulations at three stages—after filtration, after alum precipitation, and after concentration. Merthiolate 1 in 5000 was added after the first sterility test.

The antigenic activity was assessed by active immunisation tests in groups of 10 rabbits. Dilutions of the material up to 1 in 300 were given subcutaneously in two doses of 1.25 ml per dose with a ten-day interval between doses. Seven days after the last dose the rabbits were challenged intradermally with about 250 lethal doses of anthrax spores. About half the rabbits given a 1 in 300 dilution of antigen were protected. Higher concentrations of vaccine usually gave full protection. Control rabbits were invariably all dead by the fifth day after challenge.

Before injecting humans with the material, it was tested for the absence of toxicity by the methods described by Belton and Strange (1954). In addition, rabbits received 50 ml amounts of filtrate intravenously. None died or showed any deviation from the normal.

Six batches of antigen were used over the test periods, and there was no evidence that they varied in potency or in tendency to produce reactions.

Immunisation Procedure for Monkey and Man

The immunity response in monkeys was similar to that in rabbits, good protection being produced when the vaccine was diluted 1 in 100 and given subcutaneously in two doses of 1.25 ml each with an interval of ten days between doses (Belton and Strange 1954).

The schedule for immunising humans was based on that found to be effective for monkeys, but the vaccine was diluted 1 in 4 and the dose reduced to 1 ml (i.e., the antigen content per dose was increased 20 times), and an annual booster dose of 1 ml was given; this procedure was selected in a purely arbitrary manner. As all the experimental work had been based on subcutaneous injection of vaccine, this method was continued even though the inoculum contained alum. The deltoid region was chosen as the site of inoculation.

Method of Challenge of Monkeys with Virulent Anthrax Bacilli

Monkeys were challenged by the inhalation route. The dose of the strain M.36 that was given contained approximately 10-15 LD.50. Controls given no vaccine were usually all dead by the sixth day. Surviving test monkeys were retained for twelve months.

Methods of Recording Reactions

Reactions in man were recorded by a code devised to permit of ready analysis. In this code principal symptoms were indicated by an initial letter, followed by a numeral, indicating the number of days for which each symptom had been observed to persist:

O	No reaction of any kind
P	Local pain
R	Local erythema
S	Local swelling
L	Lymphadenopathy and/or lymphangitis
T	Pyrexia
U	Urticaria
P_1	Local pain or tenderness for twenty-four hours or less
P_2, S_2, R_2 &c.	Local pain, swelling, and redness for two days or less

A further reaction type in the form of a small painless, persistent nodule was often observed; but, as these nodules never progressed to abscesses, always developed late, and bore no relation to the occurrence of other signs, records were discontinued.

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Method of Titrating Antibody Level in Immune Serum

The antibody level in immune serum was assessed by the method described by Belton and Henderson (1956). Graded dilutions of immune serum are added to constant amounts of the toxin described by Smith et al, (1954), which produces a characteristic skin lesion; aliquots of these mixtures are later injected intracutaneously into the shaved skin of the rabbit, and the end point of skin lesion production is recorded next day.

Results

REACTIONS IN MAN

General Reactions

The vaccine has been administered to human volunteers in this Establishment over a period of four years. In Table I, the number of inoculations given is analysed according to the nature of the injection (first, second, or booster doses) and to the type of reaction that followed. It is seen that 373 persons received 1,057 inoculations, 369 of them receiving two or more. The symptom P might well be discounted as largely subjective and liable to individual variation; if so, the number of inoculations unaccompanied by objective reactions of any kind was 823 (78%). Pain or tenderness was always very mild and usually of brief duration, persisting for more than twenty-four hours in only 27 cases. It is noteworthy, however, that there was a sharp rise in the number of cases of pain resulting from the second dose of vaccine.

TABLE I—NUMBER AND TYPE OF REACTIONS IN MAN

Reaction	Injection 1st	2nd	Booster	Total
O*	229 (61.4%)	116 (31.0%)	83 (26.3%)	428 (40.5%)
P	119 (32.0%)	167 (45.0%)	109 (34.6%)	395 (37.4%)
R, S, L, T, & U	24 (6.4%)	76 (23.0%)	116 (36.5%)	216 (20.4%)
Undetermined	1 (0.3%)	10 (3.0%)	7 (2.3%)	18 (1.7%)
Total	373	369	315	1,057

* Terms defined in section on Methods.

In Table II the reactions experienced by 83 persons who received two or more booster doses are recorded. It is seen that there was a strong tendency for reactions to increase in number and duration with successive doses, but the trend was most pronounced in the mild reactions. Erythema (R) was almost always accompanied by local tenderness and/or swelling, but for the sake of brevity erythema has been chosen as the indicator and the other symptoms have been omitted. Mild axillary lymphadenopathy was discovered twice: in one case accompanied by pain and swelling at the site of inoculation; in the other by erythema. None of the reactions caused any incapacity.

The trend towards increase in numbers of individuals reacting on repeated injection is not serious. Furthermore, we have clear evidence that reactions may be observed after first, second, or a booster dose, but on subsequent injections there may be no response.

TABLE II—ANALYSIS OF REACTIONS TO BOOSTER DOSES

Reaction	Injection 1st	2nd	Booster 1	2	3	4	5	
O*	64	46	32	24	4	1	1	
P_1	17	19	32	27	3	--	--	79.4%
P_2	--	4	3	--	--	--	--	
P_2S_2	1	--	--	--	--	--	--	
P_2, S, L	--	1	--	--	--	--	--	
P_1, R, S, U	--	--	--	--	1	--	--	
R_1	1	4	5	19	6	--	--	19.3%
R_2	--	2	7	7	1	--	--	
R_2	--	4	3	4	--	--	--	
R_1	--	--	1	1	--	--	--	
Undetermined	--	3	--	1	--	--	--	1.1%
Total	83	83	83	83	15	1	1	

* Terms defined in section on Methods.

Clinical Observations

The objective reactions were divisible into the following clinical types:

(1) Very mild tenderness, redness and swelling in various combinations at the site of inoculation, which were of gradual onset and usually of brief duration.

(2) Similar but more severe reactions, usually following the second injection or a booster.

(3) Similar to the second type but accompanied by almost equally severe local reactions at the site of a previous inoculation, which had in many cases been given in the opposite arm. Some of these cases appeared to respond to antihistamines.

(4) Immediate reaction at the site of inoculation, followed by generalized urticaria. Two such cases occurred, both rapidly controlled by antihistamines.

There were three cases of mild pyrexia, one of which was of doubtful nature. None of these cases was sufficiently severe or prolonged to cause absence from work.

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Nature of Response

In view of the tendency for occasional development of allergies, an experiment was made to determine the nature of the allergen.

Three inocula were prepared as follows for intradermal injection into volunteers who had already been immunised:

(1) Alum-precipitated culture-medium containing no anthrax antigen was diluted 100 times, and 0.1 ml (i.e., 1/1000th of the dose of alum-precipitated medium contained in the normal immunising dose) was given into the skin of the flexor surface of the right arm.

(2) Purified anthrax antigen (Strange and Belton 1954) 0.1 ml, containing 0-05 µg (i.e., 1/100th of the antigen content of the normal immunising shot), was given into the skin of the flexor surface of the left forearm, immediately distal to the flexure of the elbow.

(3) Alum-precipitated anthrax antigen, as used in the normal immunisation procedure, diluted 1 in 100, 0.1 ml (i.e., 1/1000th of the normal immunising dose) was given into the skin a few inches distal to the second test injection.

TABLE III—PREVIOUS RESPONSE OF VOLUNTEERS FOR INTRADERMAL TEST FOR ALLERGY

Case no.	Reactions following initial* injections		Reactions following booster shots				
	1	2	B1	B2	B3	B4	B5
1	O	O	O	O	R_1S_1	O	O
2	O	O	P_1	O	$P_1R_1S_1U$	--	--
3	P_1*	P_1R_2	$P_1R_2S_2U$	--	--	--	--
4	O	P_2R_2	$P_1,R_2S_2L_2T_2$	--	--	--	--
5	O	R_2	P_1	S_2R_1	--	--	--
6	P_2	P_2	P_1R	$P_2R_2S_2$	--	--	--
7	O	P_2	$P_2R_2S_1$	$P_2R_2S_2$	--	--	--
8	O	O	O	O	--	--	--
9	O	O	O	O	--	--	--
10	O	O	O	O	--	--	--
11	P_1	P_2	O	P_2	--	--	--

* Terms defined in section on Methods

Eleven volunteers were chosen, who provided the widest possible range of reaction types, and these are detailed in Table III.

Of these volunteers, cases 2 and 3 had had immediate urticarial reactions following their last booster doses. Case 3 is also of interest in that she developed the highest toxin-neutralising titre hitherto recorded (see below). Case 4 had a mild febrile reaction after a booster dose, and also developed simultaneous flares at the sites of the two initial doses given twelve months previously. Case 11 is the only individual hitherto tested who has not produced demonstrable toxin-neutralising antibodies.

Where reactions followed the intradermal tests, urticarial weals developed at the site of inoculation within two minutes and persisted for three to six hours. No flares developed in the deltoid region, and there was no generalised urticaria. The weals could not be measured with accuracy, but some impression of their relative sizes is recorded in Table IV. The largest (++++) measured about 5 cm and the smallest (+) about 1 cm.

TABLE IV—INTRADERMAL REACTIONS IN VOLUNTEERS

Case no. (as in Table III)	Inoculation		
	Medium	Purified antigen	Alum-antigen
1	—	—	—
2	—	+ + + +	+ + +
3	—	+ + + +	+ + +
4	—	+ + +	+ +
5	—	+ + +	+ +
6	—	+ + +	+ +
7	—	+ + +	+ +
8	—	+	+
9	—	—	—
10	—	—	—
11	—	Trace	Trace

+ to ++++ = degrees of reaction (see text).

From these various observations it is clear that the intradermal reactions were associated with anthrax antigen and not the medium, and were related in both distribution and severity to the inoculation reactions experienced by the volunteers.

A response to antihistamines had been observed in some cases of inoculation reaction. Cases 2 and 5 (Table IV) were retested the following week an hour after receiving an oral dose of 0.05 g of tripelennamine hydrochloride (‘Pryibenzamine,’ Ciba). One case gave a characteristic immediate urticarial reaction and the other had a mild reaction of gradual onset. In both cases the subsequent intradermal reactions were much less severe than in the first instance and persisted for a relatively short period. This suggests that reactions to anthrax antigen, whether urticarial or not, are allergic, the severity and time of onset depending on the idiosyncrasy of the recipient. In the test series, past medical histories of allergy were known in cases 2 and 3 (Table IV), both of whom developed urticaria immediately after receiving respectively their third and first booster shots. No explanation, however, can be offered for the later onset

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of the more usual type of reaction in the non-urticarial subjects.

Though no severe anaphylactic reactions have yet been observed, it is clearly wise to proceed with caution when administering booster shots to persona with histories of allergic states.

Some delayed reactions were observed on the 3rd day after the test. In cases 3 and 4 (Table IV) these consisted of a local minor flare at the sites of the intradermal injection of both the purified and the alum-precipitated anthrax antigen. In cases 1 and 5-7 the reaction took the form of a small pale painless nodule at the site of injection of the alum-antigen only. The significance of these minor reactions is not understood and seems to bear little relation to the rest of the picture.

Antibody Response

The antibody response to the vaccine was tested in 91 persons. The tests were made at various stages of the immunisation schedule, and some persons were tested more than once. The following conclusions were drawn:

(1) Of 32 persons tested after the initial two doses, 1 produced a response; but as tests were made at intervals of up to a year after the second dose, probably the serum antibodies had decreased.
(2) Of 28 persons tested during the twelve months following the first booster dose, 2 did not respond.
(3) Of 20 persons tested before and after the first booster dose, 18 produced a demonstrable response. Of the 2 who did not respond, 1 again did not respond after a second booster.
(4) Of 30 persons tested after but not before a second booster dose, only 1 did not respond and he produced a response after a third booster.
(5) One individual was tested after the fourth and fifth booster doses and responded on both occasions.
(6) In 17 cases, a fall in titre of about half in twelve months was demonstrated.

From these results it is clear that the initial two doses were inadequate to produce a lasting antibody response, but that a booster dose produced a demonstrable response in all but 1 case, but this response decreases by about half in one year.

Three cases of special interest were observed among personnel actively engaged in work on *B. anthracis*. Of these, two showed no fall in titre during the year following a fourth booster dose, and the third showed an exceptionally high titre after a first booster. We suggest that the titres were maintained or enhanced as a result of repeated minor exposures to the organism. Specific support for this hypothesis was obtained in experiments on monkeys (see below). In spite of the considerable amount of work done on anthrax in these laboratories, with a consequent minor degree of contamination, there have been no cases of infection since the immunisation procedures were introduced.

On comparing the results of the toxin neutralisation tests with inoculation reactions, no evidence was found that there was any relation between the observed antibody titre and the severity of reaction, apart from the general tendency for both to increase with booster doses.

RESPONSE IN MONKEYS

Experiment for Testing Duration of Immunity

30 *Macacus rhesus* monkeys, each weighing 3-5 kg, were immunised with two doses of a vaccine that had been used for immunising man, and quantitatively the same amounts were given. The monkeys were divided by random sampling into three groups of 10. The first of these groups was challenged seven days after the second dose of vaccine. The second and third groups were challenged respectively one and two years later. An equal number of unimmunised controls were included in each inhalation challenge.

Result of Challenge by Respiratory Route

All the monkeys in the first two immunised groups survived; all the controls were dead by the sixth day. In the group challenged two years after immunisation, 1 monkey out of a group of 7 survivors died and 1 control survived. (3 monkeys in this third immunised group died of other causes in the two years).

Test of Immunity Level

Assessment of the serum before challenged showed a steady decrease in titre in two years; as might be expected, there was some variation between individuals in this respect. Unfortunately no figures are available for the immunity level immediately following vaccination, but at the end of a year all the unchallenged monkeys gave a serum that neutralised the test dose of toxin at a 1 in 3 dilution or higher. Tested after eighteen months, the same monkeys showed neutralisation of the test dose of toxin with undiluted serum but nothing better. After two years no detectable antibody was present.

Serum obtained after challenge showed a dramatic rise in antibody titre, the test dose of toxin being inhibited by a dilution of at least 1 in 256 of each serum. The most probable explanation of this rise is that a degree of invasion of the immunised host did take place in such a manner that a sharp secondary immunity stimulus was given. There was an interesting increase in immune response in 1 monkey which was sick for three days following the third challenge; a month after challenge this monkey's serum was active at a dilution of 1 in 512. The surviving control monkey in this experiment was similarly sick and also developed an antibody titre of

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between 1 in 256 and 1 in 512. These facts indicate that the illnesses were due to specific but transitory infection.

The limits of the rabbit skin test are clearly shown in the results of the last challenge. No immunity was demonstrable in the serum before challenge, but 6 of 7 monkeys survived. It therefore seems safe to assume that, if the capacity for neutralising toxin is detectable in an immunised monkey's serum, it is highly probable that the monkey will be immune to exposure to *B. anthracis* by the respiratory route. By the same reasoning one might conclude that, if human serum shows similar capacity, the individual will be amply protected against chance infection.

Summary

1,057 doses of alum-precipitated anthrax antigen were administered to 373 people by the subcutaneous route.

Reactions were mild but tended to increase in frequency but not in severity with successive doses given in the form of an "annual booster." The incidence of febrile reactions was very low (0.19%). There was no incapacitation for work.

A reaction after one dose did not necessarily predispose to subsequent reactions.

There is no evidence of any connection between the severity of reaction and the site of inoculation relative to previous injections.

Intradermal tests in immunised people suggested that the reactions were allergic and were associated with the antigen and not with impurities in the vaccine. Antibodies neutralising anthrax toxin can be demonstrated in the blood of immunised people; only 1 exception to this was found in 91 persons examined.

No relationship was demonstrated between the antibody level and reaction to inoculation.

Experiments on monkeys suggested that the immunisation procedure used for man was effective, particularly when demonstrable circulating antibody was present.

Experiments on monkeys showed that two injections of antigen protected for at least one and probably nearer two years.

There is evidence that the antigen remains fully effective for man and animals on storage for at least two and a half years.

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