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Immunization of man with salmonella vaccine and tetanus–diphtheria vaccine. Dose-response relationship, secondary response, and competition of antigens

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INTRODUCTION

During an investigation into intracutaneous typhoid immunization (Clasener & Beunders, 1967) we encountered the problem of the dose-response relationship in the immunization of man with typhoid vaccines. Since the literature (see Stevens, 1956) contained no relevant data on the combination of antigen, antigen dose, and species we were interested in, an experiment seemed necessary. In this work, the standard immunization schedule for military recruits was changed only in that some groups were given less than the normal amount of salmonella antigen. The diphtheria and tetanus immunization, given simultaneously in this schedule, was the same for all test groups. This also offered an opportunity to determine the influence of the different doses of salmonella vaccine on the antibody response to tetanus–diphtheria immunization.

If an organism is stimulated by several antigens simultaneously, the production of antibody for one antigen may be influenced by the stimulation with the other antigens, this influence being called synergy if positive and competition if negative. Both kinds of influence undoubtedly exist (Adler, 1959; Johnson, 1964), but are dependent on many variables such as the kind of antigen, the absolute and relative amounts of antigen, previous contact with antigens, species, etc. (Adler, 1964). Immunization procedures in man are not always well founded in this respect (see Barr & Llewellyn-Jones, 1953).

MATERIALS AND METHODS

Subjects

About 200 healthy 20-year-old males were randomly divided into four groups.

Antigens and dosage

Tetanus–diphtheria (TD) vaccine contained tetanus toxoid 10 Lf/ml. and diphtheria toxoid 5 Lf/ml., adsorbed with aluminium phosphate. Typhoid-paratyphoid (TAB) vaccine contained *Salmonella typhi* 1000×10^6/ml., *S. paratyphi A* 750×10^6/ml. and *S. paratyphi B* 750×10^6/ml. The vaccine was killed and preserved with acetone.

The same batch of each vaccine was used throughout the experiment. The vac-

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cines were standard commercial preparations of the National Institute of Public Health, Utrecht.

Immunization schedule

Table 1 shows the detailed schedule. It is seen that the doses of TAB differed for the four groups, but all groups received the same dose of TD. The two vaccines were given in opposite arms.

Military recruitment in The Netherlands is normally the first occasion for vaccination against typhoid, so the TAB vaccination was certainly a primary one for all subjects; the TD vaccination might have been secondary for some.

Table 1. *Immunization schedule in ml. for the two vaccines*

	5. ii. 65		4. iii. 65	
Group	TD*	TAB†	TD*	TAB†
1	0·5	—	0·5	—
2	0·5	0·1	0·5	0·1
3	0·5	0·2	0·5	0·2
4	0·5	1·0	0·5	1·0

* Given intramuscularly. † Given subcutaneously.

Sera

Blood was taken by venepuncture at the time of the first and second injections, and 3 weeks after the second injection. Sera were stored at $-20°$ C.

Antibody titrations

Antibodies for the salmonella antigens were measured by the bacterial agglutination method. Antibodies for tetanus and diphtheria antigens were measured by the passive haemagglutination technique (Stavitsky, 1954; Tasman, van Ramshorst & Smith, 1960).

Bacterial agglutinations

Twofold serum dilution series beginning with 1/10 were made in volumes of 0·5 ml. in 0·9 % NaCl solution. Round-bottomed test tubes (50 × 7 mm.) were used. To these serum dilutions, equal volumes (0·5 ml.) of killed bacterial suspensions were added, resulting in serial twofold serum dilutions beginning with 1/20.

The bacterial suspensions were standard commercial preparations for the determination of O- and H-agglutinins, obtained from the National Institute of Public Health, Utrecht, The Netherlands. After incubation overnight at room temperature, covered with a plastic sheet to prevent evaporation, readings were made under a strong light from above against a black background with a binocular headloupe giving a 2·25 magnification.

All sera taken on the three dates from all subjects who had received TAB vaccine were titrated on the same day against the same batch of antigen for each of the antigens used. Sera of group 1 were not titrated for salmonella agglutinins.

The batch of *Salmonella paratyphi A* O antigen was not suitable for use.

Scoring

The number of the last tube showing agglutination, as compared to the control tube, was taken as the value of the serum. This is the same as taking the $\log_2$ of 1/10 of the reciprocal of the serum dilution. The sera showing no agglutination in the first dilution were assigned a value of 0.

Haemagglutination

Erythrocytes

Sheep blood was collected in equal volumes of Alsever solution. After centrifugation, the red cells were washed three times in saline buffered at pH 7·4 by mixing equal volumes of 0·15 M-NaCl and 0·15 M phosphate buffer (pH 7·4). The packed cells were stored at 4° C. for 3 days with 1/10,000 merthiolate, washed once to be sure there was no lysis, and then used for adsorption or sensitization.

Adsorption

To remove any sheep red-cell antibodies, all sera were adsorbed with sheep red cells. Four volumes of a 25 % suspension of sheep red cells were incubated overnight at 4° C. with 1 vol. of heat-inactivated serum. After centrifugation, the resulting 1/4 serum dilution was stored at −20° C.

Sensitization

Equal volumes of a 2 % red-cell suspension and a 1/40,000 solution of tannin (BDH) in saline were mixed at room temperature for 10 min. After centrifugation and one washing with 0·15 M-NaCl, the cells were resuspended in 0·15 M-NaCl to the original volume. This tanned 2 % red-cell suspension was then mixed with an equal volume of toxoid solution containing 30 Lf diphtheria or tetanus toxoid per ml. of saline buffered at pH 6·4 by mixing equal volumes of 0·15 M-NaCl and 0·15 M phosphate buffer (pH 6·4). After centrifugation, the sensitized cells were washed three times and resuspended in 1 % rabbit serum in saline. Rabbit blood was obtained by cardiac puncture. After being held for one hour at 37° C. and overnight at 4° C., serum was pipetted off and stored at −20° C. On the day of use, the rabbit serum was heat-inactivated and diluted 1/100. The 2 % suspension of sensitized cells in 1 % rabbit serum was kept at 4° C. for 3 days, within which period all sera were titrated. All sera taken on the three dates were titrated with the same batch of sensitized cells. All sera taken on the same date were titrated on one day. This was done to exclude, as far as possible, variations in the properties of the antigen and daily variations in the titration of the sera to be compared.

Dilutions

Twofold serum dilution series beginning with 1/10 were made in volumes of 0·5 ml. in 1 % rabbit serum. Plastic plates with round-bottomed cups 15 mm. in diameter were used. To these serum dilutions the sensitized red cells were added as equal volumes (0·5 ml.) of a 1/10 dilution, in 1 % rabbit-serum, of the 2 % suspension. Readings were made after overnight incubation at room temperature.

Scoring

The number of the last cup in which the maximal reaction was seen, was taken as the value of the serum. With a serum dilution of 1/20 in the first cup, this way of scoring amounts to taking $\log_2$ of 1/10 of the reciprocal of the serum dilution. Sera negative in the first dilution were assigned a value of 0. The logarithmic transformation makes the resulting frequency distributions symmetrical instead of skewed; the other transformations simplify the calculations.

Control sera containing 10 AU diphtheria or tetanus antitoxin per ml. scored 9. So with this system we could measure a minimum antibody content of 0·04 AU per ml.

RESULTS

Results of the bacterial agglutinations

These are shown in Table 2. As can be seen in the graph (Fig. 1) constructed from the data in this table, the dose-response correlation can be roughly described with the formula of Smith & St John-Brooks (1912): log dose $= k + 1/n$ log titre.

Table 2. *Means and standard deviations (sd) of the 'titres' (transformation see text) of the agglutinating bacterial antibodies before and after vaccination with various doses of TAB vaccine*

		Group 2 (0·1 ml.) 44 subjects		Group 3 (0·2 ml.) 36 subjects		Group 4 (1·0 ml.) 43 subjects	
Antigen	Date	Mean	*sd*	Mean	*sd*	Mean	*sd*
TO	5. ii. 65	1·6	1·4	1·5	1·3	1·0	1·4
	4. iii. 65	2·8	1·2	3·5	1·1	4·9	1·0
	26. iii. 65	2·8	1·2	3·1	1·1	4·3	1·0
BO	5. ii. 65	1·8	1·2	1·6	1·0	1·0	1·2
	4. iii. 65	2·4	1·1	2·5	1·0	3·5	1·1
	26. iii. 65	2·1	1·3	2·5	1·0	3·0	1·0
TH	5. ii. 65	0·0	—	0·0	—	0·0	—
	4. iii. 65	4·4	2·0	4·9	1·6	5·8	1·3
	26. iii. 65	3·8	1·9	4·7	2·1	5·3	1·2
AH	5. ii. 65	0·0	—	0·0	—	0·0	—
	4. iii. 65	5·3	1·4	5·8	1·2	6·3	1·0
	26. iii. 65	4·7	1·4	5·1	1·1	6·3	1·1
BH	5. ii. 65	0·0	—	0·0	—	0·0	—
	4. iii. 65	3·9	1·3	4·9	1·4	5·7	1·1
	26. iii. 65	3·7	1·0	4·5	1·5	6·0	1·0

Some antibody against TO- and BO-salmonella antigens was found in the pre-immunization sera taken on 5 Feb. 1965.

No enhanced secondary response was seen after the second TAB injections as judged from a comparison of the values reached on 4 Mar. 1965 (4 weeks after the first TAB injection) and 26 Mar. 1965 (3 weeks after the second TAB injection).

Results of the toxoid haemagglutination tests

The influence of the primary TAB immunization on the tetanus-diphtheria immunization may depend on whether the latter was primary or secondary. Since the system used was not very sensitive, absence of antibodies in the sera taken before

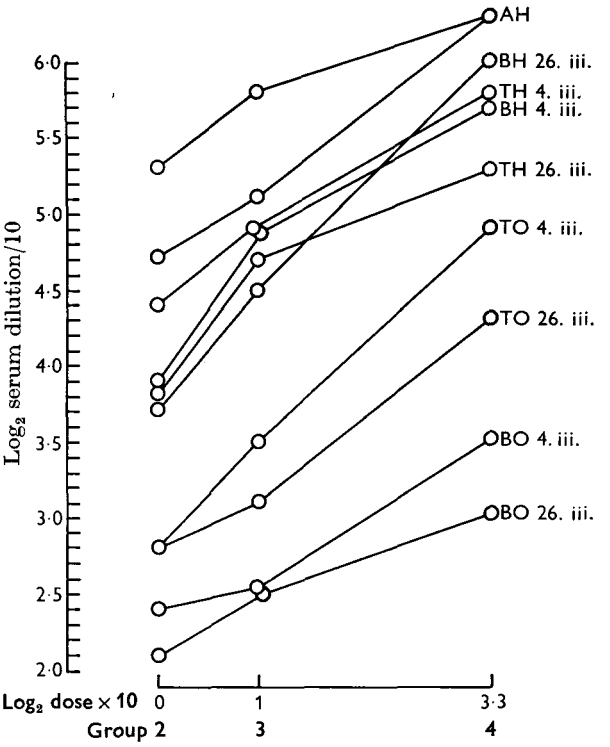

Fig. 1. Antigen-dose/antibody-response relationship for the three groups shown in Table 2.

immunization would not exclude previous contact with the antigen. From the bimodality of the frequency distribution of the titres of the four groups together (left part of Tables 3 and 4) it seemed justifiable to assume that absence of antibodies in the sera taken 4 weeks after the first injection indicated a primary response. On the basis of this definition of primary response, the bimodal frequency distribution of tetanus antibody titres of the sera taken 3 weeks after the second injections (26 Mar. 1965), could be nicely split, resulting in four classes. The class of persons who reacted with a secondary response to both diphtheria and tetanus antigens had probably been immunized previously (Table 5). Persons who had a secondary response to the diphtheria antigen and a primary response to the tetanus antigen had probably had their contact with the diphtheria antigen by infection, because in childhood tetanus and diphtheria vaccine are usually given together (Table 6). Only a few subjects showed a primary response to both tetanus and diphtheria antigens (Table 7). None showed a primary response to diphtheria antigen

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Table 3. *Classification of the total population according to the tetanus antitoxin level on 4 March 1965*

Values of the sera expressed as the number of the last positive cup	Frequency distribution of all sera of the 4 groups			Frequency distribution of the sera of all subjects of the 4 groups whose tetanus vaccination was probably:					
				Secondary			Primary		
	5. ii. 65	4. iii. 65	26. iii. 65	5. ii. 65	4. iii. 65	26. iii. 65	5. ii. 65	4. iii. 65	26. iii. 65
0	119	84	5	35	—	—	84	84	5
1	13	—	—	13	—	—	—	—	—
2	12	2	1	12	2	—	—	—	1
3	9	3	6	9	3	1	—	—	5
4	4	4	27	4	4	—	—	—	27
5	5	1	28	5	1	2	—	—	26
6	7	14	16	7	14	2	—	—	14
7	1	18	7	1	18	4	—	—	3
8	—	23	9	—	23	7	—	—	2
9	—	12	14	—	12	13	—	—	1
10	—	6	15	—	6	15	—	—	—
11	—	2	10	—	2	10	—	—	—
12	—	1	19	—	1	19	—	—	—
13	—	—	7	—	—	7	—	—	—
14	—	—	1	—	—	1	—	—	—
15	—	—	—	—	—	—	—	—	—
16	—	—	5	—	—	5	—	—	—
Total	170	170	170	86	86	86	84	84	84

Table 4. *Classification of the total population according to the diphtheria antitoxin level on 4 March 1962*

Values of the sera expressed as the number of the last positive cup	Frequency distribution of all sera of the 4 groups			Frequency distribution of the sera of all subjects of the 4 groups whose diphtheria vaccination was probably:					
				Secondary			Primary		
	5. ii. 65	4. iii. 65	26. iii. 65	5. ii. 65	4. iii. 65	26. iii. 65	5. ii. 65	4. iii. 65	26. iii. 65
0	88	16	13	72	—	—	16	16	13
1	3	—	—	3	—	—	—	—	—
2	10	3	1	10	3	—	—	—	1
3	16	1	1	16	1	—	—	—	1
4	23	8	3	23	8	3	—	—	—
5	15	14	9	15	14	9	—	—	—
6	10	31	8	10	31	8	—	—	—
7	1	36	11	1	36	10	—	—	1
8	3	48	17	3	48	17	—	—	—
9	1	7	24	1	7	24	—	—	—
10	—	4	23	—	4	23	—	—	—
11	—	1	11	—	1	11	—	—	—
12	—	1	20	—	1	20	—	—	—
13	—	—	1	—	—	1	—	—	—
14	—	—	4	—	—	4	—	—	—
15	—	—	1	—	—	1	—	—	—
16	—	—	23	—	—	23	—	—	—
Total	170	170	170	154	154	154	16	16	16

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together with a secondary response to the tetanus antigen. Some values of groups 1 and 4 were compared by means of Student's test. Values of t are given in Tables 5 and 6. These differences are not significant at the 1% level. Furthermore, no significant differences were found for the total values (not given) of each group or

Table 5. *Number (n), mean (m) of the antitoxin value and standard deviation (sd) of the class of subjects of each group for whom the tetanus and diphtheria immunization were probably secondary*

	5. ii. 65			4. iii. 65			26. iii. 65		
Group	*n*	*m*	*sd*	*n*	*m*	*sd*	*n*	*m*	*sd*
				Diphtheria					
1	28	2·7	2·2	*28	7·4	1·4	28	10·4	3·8
2	21	2·7	2·8	21	6·7	1·7	21	9·9	3·2
3	17	3·0	2·3	17	6·8	1·5	17	10·2	2·7
4	20	2·6	2·9	*20	6·6	1·1	20	10·6	3·3
				Tetanus					
1	28	1·5	2·1	28	7·3	2·2	†28	10·5	2·8
2	21	2·0	2·0	21	7·3	1·8	21	10·3	2·1
3	17	1·7	2·3	17	6·8	1·9	17	9·8	3·2
4	20	2·1	1·8	20	7·8	1·7	†20	11·1	1·7

* $t = 2{\cdot}24$. † $t = 0{\cdot}87$.

Table 6. *Number (n), mean (m) of the antitoxin value and standard deviation (sd) of the class of subjects of each group for whom the diphtheria immunization was probably a secondary one and the tetanus immunization probably a primary one*

	5. ii. 65			4. iii. 65			26. iii. 65		
Group	*n*	*m*	*sd*	*n*	*m*	*sd*	*n*	*m*	*sd*
				Diphtheria					
1	16	1·2	1·7	*16	7·6	1·5	†16	11·5	3·7
2	19	1·3	2·1	19	7·1	2·0	19	10·4	3·0
3	15	1·1	1·7	15	5·7	2·2	15	8·5	3·4
4	18	2·3	2·4	*18	6·8	1·3	†18	9·9	2·7
				Tetanus					
1	16	0·0	—	16	0·0	—	‡16	5·4	1·3
2	19	0·0	—	19	0·0	—	19	4·9	1·7
3	15	0·0	—	15	0·0	—	15	3·8	1·6
4	18	0·0	—	18	0·0	—	‡18	4·2	1·9

* $t = 1{\cdot}67$. † $t = 1{\cdot}38$. ‡ $t = 2{\cdot}10$.

for the number of subjects in each group located in the same class of response type. Comparison of the diphtheria antitoxin values in Tables 5 and 6 shows clearly that the secondary response to diphtheria vaccination is not influenced by the factor of whether the tetanus immunization is a primary or a secondary one.

Table 7. *Number (n), mean (m) of the antitoxin value of the class of subjects of each group for whom the tetanus and diphtheria immunization were probably primary*

	5. ii. 65		4. iii. 65		26. iii. 65	
Group	*n*	*m*	*n*	*m*	*n*	*m*
			Diphtheria			
1	3	0·0	3	0·0	3	0·0
2	4	0·0	4	0·0	4	0·0
3	4	0·0	4	0·0	4	2·5
4	5	0·0	5	0·0	5	0·4
			Tetanus			
1	3	0·0	3	0·0	3	4·7
2	4	0·0	4	0·0	4	5·3
3	4	0·0	4	0·0	4	4·8
4	5	0·0	5	0·0	5	3·8

DISCUSSION

Salmonella antibodies

Intracutaneous TAB immunization of man with 0·2 ml. doses results in agglutinating antibody titres as high as those obtained with 1·0 ml. doses given subcutaneously (Clasener & Beunders, 1967). The dose-response correlation found in this study demonstrates that this result is dependent on the intracutaneous route: when 0·2 ml. doses are given subcutaneously, the titres are considerably lower than when 1·0 ml. doses are given subcutaneously.

Since TAB immunization in The Netherlands is not normally given before military service, the antibodies in the prevaccination sera against the TO- and BO-antigens probably resulted from natural contact.

Whether the response after a second antigenic stimulation will be much higher than after the primary stimulation depends on several factors. Besides the dose of primary stimulation (Uhr & Finkelstein, 1963) and time interval (Fecsik, Butler & Coons, 1964), the most important factor is probably the kind of antigen. With particulate microbial antigens there is no difference between primary and secondary response such as is found with toxoids (Burnet & Fenner, 1949; Bauer, Mathies & Stavitsky, 1963). The absence of a secondary response to salmonella vaccine probably also explains the fact that in our first experiment (Clasener & Beunders, 1967) the group that received three injections did not produce more agglutinating antibodies than the group that received two injections. It seems very important in this connexion that in field trials the protection after one injection of typhoid vaccine was found to be as high as that after two injections (Yugoslav Typhoid Commission, 1964; Ashcroft, Ritchie & Nicolson, 1964).

Tetanus and diphtheria antibodies

Although the haemagglutination technique does not recognize one possibly important property of antitoxins, namely avidity, the correlation between haemagglutination titres and toxin-neutralizing antibodies seems sufficiently well estab-

lished to give biological significance to the results (Scheibel, 1956; Tasman *et al.* 1960; Surjan & Nyerges, 1962*a*, *b*).

Whereas the titres of salmonella agglutinating antibodies were divergent for the four groups, correlating with the different doses, haemagglutinating antibody titres against diphtheria and tetanus were essentially the same for the four groups. It appears therefore that, in men of this age, immunization with salmonella antigens has no influence on a simultaneous primary or secondary immunization against diphtheria or tetanus.

SUMMARY

Immunization of men with various amounts of salmonella antigens gave a linear relationship between the logarithm of the dose and the logarithm of the titres of the antibodies produced. No secondary response was observed for any of the doses used. These doses of salmonella vaccine did not interfere with or stimulate simultaneous primary or secondary vaccination with tetanus and diphtheria vaccine.

This experiment would not have been possible without the generous cooperation of Colonel B. J. W. Beunders, M.D., Head of the Health Department of the Dutch Army and Airforce, and his collaborators. I am also greatly indebted to Prof. Dr J. E. Dinger for his stimulation and help. Discussion with Dr A. Tasman on the design of the experiment, with Dr G. L. Smit on the haemagglutination method and Dr H. A. Valkenburg on the statistical aspects of the study are gratefully acknowledged. Dr J. D. van Ramshorst kindly provided the diphtheria and tetanus toxoids and the control sera for the haemagglutination reaction.

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