J. Hyg., Camb. (1963), **61**, 43
Printed in Great Britain

Chrome typhoid vaccine

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(*Received* 14 *August* 1962)

INTRODUCTION

Heat-killed and phenol-preserved typhoid vaccines have been in general use since their introduction by Almroth Wright, then Professor of Pathology at the Army Medical School, at the end of the last century (Wright & Semple, 1897). Details of the first two anti-typhoid inoculations in humans, published in the medical literature are recorded in the issue of the *Lancet* for 19 September 1896 (Wright, 1896).

Since then much research has been done by those interested in this field, to promote vaccines which would give protection and less side-effects; the earlier vaccines are stated to have caused ferocious reactions on occasions (Harrison, 1953).

The ideal vaccine is one that confers full life-long protection to a vaccinated individual, against a particular disease, causes no harmful local or systemic reaction upon its exhibition and administration, and is given in one small single dose, preferably by mouth.

Such ideals have not been realized as yet, but many methods and techniques have been tried, and various vaccines have been proposed, in all parts of the world. To mention only a few:

(i) Alcohol-killed and alcohol-preserved vaccine in Britain (Felix, 1941; Felix, Rainsford & Stokes, 1941).

(ii) Endotoxoid vaccine in South Africa (Grasset, 1951, numerous publications).

(iii) Acetone-dried vaccine in the U.S.A. (Landy, Gaines, Seal & Whiteside, 1954).

(iv) Other vaccines utilizing antigenic extracts such as that prepared in France (Boivin & Mesrobeanu, 1938, numerous publications; Kourilsky, Kourilsky & Boivin, 1939); in Britain (Topley, Raistrick, Wilson, Stacey, Challinor & Clark, 1937; Henderson & Morgan, 1938); and an aluminium hydroxide absorbed vaccine in Hungary (Kraus, Joo & Rethy, 1956).

In Japan, Ando & Shimojo (1957) quote the use of calcium chloride treated vaccines by Kishida, concentrated Ringer's solution by Hirano and Anzai, and alcohol-acetone vaccines by Ogunuki; Ando and other workers have advocated a vaccine in which the bacilli are killed by formalin and treated with chrome salts.

In this laboratory, experimental chrome vaccines were prepared and tested *in vitro* by agglutination reactions, and *in vivo* by active and passive mouse tests and the production of antibodies (agglutinins) in rabbits.

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In vitro *experiments*

Organisms used: (*a*) *Salmonella typhi* Ty 2; (*b*) *Salmonella typhi* T 15; (*c*) *Salmonella typhi* T 18.

These organisms constitute the typhoid component of the current British Army typhoid-paratyphoid vaccines. The Ty 2 is a classical strain (Weil & Felix, 1920); the other two are 'wild' strains received at these laboratories, and which have been selected on account of their all-round excellent properties for vaccine production. The organisms were cultured on trypsinized-meat broth agar and harvested in physiological saline.

Agglutinatable suspensions. (*a*) Each harvest was divided in three lots, (i) , (ii) and (iii), and treated in one of the following ways: (i) Formalin and chrome alum ($KCr(SO_4)_2.12H_2O$) were added to give final concentrations of 1 % formalin and 0·02 % chrome alum on the lines laid down by Ando (1953). (ii) Placed in a water-bath at 54° C. for 1 hr., and, after cooling, 5 % phenol was added to give a final concentration of 1 % phenol. (iii) The third lot was left untreated.

These concentrated suspensions were then washed in saline, and suspended to give a final concentration of 8000 million organisms per ml.

(*b*) In a second agglutination experiment, a harvest of *Salm. typhi* Ty 2 was divided in two lots and treated as in (*a*) (i) and (iii) above, respectively. These concentrated suspensions were diluted to 8000 million organisms per ml, and aliquot lots were subjected to heat for varying periods of time, namely 0, 10, 20, 25, 30, 35 and 40 min. in a water-bath at 60° C. and in free steam at 100° C.

Technique of agglutination. Tests were performed in round-bottomed test tubes. Mixtures of agglutinable suspensions and doubling dilutions of serum were incubated at 37° C. for 2 hr., kept in a refrigerator overnight, and read after standing for 2 hr. at room temperature (Felix & Bensted, 1954).

In vivo *experiments*

(1) The *in vitro* experiments were followed up by mouse protection tests and immunization of rabbits. For this purpose a vaccine was prepared with *Salm. typhi* Ty 2, a strain which is known to have a high content of Vi-antigen. The organism was killed by formalin (1 %) and treated with chrome alum (0·02 %). This vaccine, in a concentration of 2000 million organisms per ml. in 0·01 % merthiolate, was divided in two portions: while one portion was stored in a refrigerator at a temperature of 4–6° C., the other half was kept at a temperature of 41° C. for 1½ months to study the effects of improper and unfavourable storage conditions on this vaccine (accelerated ageing, Landy, 1953; Henderson, Peacock & Rickley, 1951).

(2) *Active mouse protection tests.* Groups of mice were immunized by subcutaneous graded doses of the chrome vaccine, namely 40, 80, 160, 320 and 640 million organisms per mouse; at the same time groups of mice were injected with a cholera vaccine, to serve as controls for non-specific immunity. The mice were challenged by an intraperitoneal injection of 80 million Ty 2 organisms, 10 days after immunization.

(3) *Antibody production in rabbits.* Six rabbits were immunized by three intravenous injections of 500 million, 1000 million and 2500 million organisms given on day 1, day 8 and day 28. Blood was collected on day 15 and day 28.

(4) *Passive mouse protection tests.* Groups of mice were passively protected by graded doses of pooled rabbit sera, viz. 0·05, 0·2 and 0·4 ml., injected intravenously. Two to three hours after passive immunization the mice were challenged by an intraperitoneal injection of 320 million *Salm. typhi* Ty 2 organisms.

(5) *Laboratory animals.* The mice (Strong A albino) and the rabbits (Porton coloured) were supplied by Allington Farm, Porton.

RESULTS

(1) The results of the agglutination reactions of the chrome suspension, compared with an untreated suspension and a phenolized suspension, are reproduced in Table 1. It is seen that, while heat-phenol damaged the Vi-antigen, the formalin-chrome treatment preserved this antigen to a marked degree.

(2) In the second experiment a clearer picture was obtained of the effects of

Table 1. *The comparative agglutination titres of* Salmonella typhi *Ty* 2, *T* 15 *and T* 18 *suspensions—untreated; heat-killed and phenolized; formalin-killed and chrome treated*

	Untreated suspension			Heat-phenol suspension			Formalin-chrome suspension			Serum controls	
Serum	Ty 2	T 15	T 18	Ty 2	T 15	T 18	Ty 2	T 15	T 18	Standard Vi susp.	Standard TO susp.
					(*a*) Not subjected to heat						
Vi	160	160	160	Trace	0	0	640	160	640	640	—
TO	0	320	160	640	640	320	0	640	320	—	640
Saline control	0	0	0	0	0	0	0	0	0	0	0
					(*b*) Steamed for 2 hr.						
TO	640	640	640	640	640	640	640	640	640	—	640
Saline control	0	0	0	0	0	0	0	0	0	—	0

Table 2. *The effect of heat on the agglutinability of formalin-chrome suspensions and untreated suspensions of* Salmonella typhi *Ty* 2

A. Formalin-chrome suspensions

	Heated at 60° C. for (min.)							Steamed at 100° C. for (min.)				
Serum	0	10	20	25	30	35	40	10	20	25	30	35
Vi	640	640	640	640	640	640	640	640	640	trace	trace	0
TO	0	0	10	20	40	40	40	320	640	640	640	640

B. Untreated suspensions.

	Steamed at 100° C. for (min.)						Serum controls	
Serum	0	10	20	25	30	35	Standard Vi susp.	Standard TO susp.
Vi	320	0	0	0	0	0	640	—
TO	160	320	640	640	640	640	—	640

https://doi.org/10.1017/S0022172400020726 Published online by Cambridge University Press

heat on the Vi-antigen. It is seen from Table 2 that while heat at 60° C. for up to 40 min. (the limits of the experiment) had no destructive effect on the Vi-agglutinability and O-inagglutinability of the chrome treated suspension, steaming at 100° C. progressively damaged the Vi-antigen, at least as regards its agglutinable properties.

(3) The titre of antibodies (agglutinins) produced in rabbits showed efficient Vi-antigenicity when the chrome suspension stored at 4° C. was injected, but the suspension kept at 41° C. did not evoke Vi-antibodies. Results are reproduced in Table 3.

Table 3. *The titres of TVi and TO antibodies (agglutinins) in sera of rabbits, immunized with two or three intravenous doses of (a) chrome vaccine stored at* 4° C. *of (b) chrome vaccine stored at* 41° C.

	(*a*) Chrome vaccine: 4° C.			(*b*) Chrome vaccine: 41° C.		
	Rabbit	2nd bleed	3rd bleed	Rabbit	2nd bleed	3rd bleed
Vi titres	1	100	300	4	0	0
	2	200	300	5	0	0
	3	80	400	6	0	0
O titres in thousands	1	1·5	4·0	4	2·0	1·5
	2	2·0	1·5	5	1·5	1·5
	3	2·0	2·0	6	1·5	1·0

Readings: figures represent reciprocals of titres, standardized against TVi horse XII and TO horse sera supplied by the Central Public Health Laboratory, Colindale.

Table 4. *Passive mouse-protection tests. Number of mice surviving an intraperitoneal challenge by* 320 *million* Salmonella typhi *T* 15 *organisms, after passive immunization by graded doses of immune serum*

Serum	Chrome vaccine: 4° C.	Chrome vaccine: 41° C.
0·05 ml.	0	0
0·2 ml.	8	0
0·4 ml.	7	0

Challenge organism virulence control: 40 million organisms per mouse = 6; 80 million organisms per mouse = 2; 160 million organisms per mouse = 0.

Serum control: mice injected intravenously with 0·5 ml. 'normal' rabbit serum, not challenged = 10; mice injected intravenously with 0·5 ml. 'normal' rabbit serum, challenged as for test mice = 0.

Notes. (i) Figures for mice represent survivors from 10 mice tested in each group. (ii) 'Normal' rabbit serum refers to pooled rabbit serum free from TVi, TO and TH agglutinins.

(4) Table 4 shows the results of the passive mouse protection tests. While the serum containing Vi-antibodies (produced by the intravenous inoculation of rabbits with the chrome vaccine stored at 4° C.), gave good protection to the mice, the serum lacking the Vi-antibodies (from rabbits immunized with the chrome vaccine kept at 41° C.) failed to give protection to the test mice.

(5) The results obtained in the active mouse protection tests followed the same pattern of observations recorded in the previous two *in vivo* tests, namely that

storage of the chrome vaccine at 41° C. markedly affects its immunizing properties as seen by comparing the numbers of vaccinated mice, immunized by the chrome vaccines, surviving the challenge dose (see Table 5).

Table 5. *Active mouse-protection test. The protection afforded to mice, immunized subcutaneously by a single dose of a chrome typhoid vaccine, against an intraperitoneal challenge by* 80 *million* Salmonella typhi *Ty* 2 *organisms in saline*

Immunizing dose in millions per mouse ...	40	80	160	320	640
Chrome vaccine at 4° C.	3	3	3	6	7
Chrome vaccine at 41° C.	0	0	1	1	0
Cholera vaccine (control)	1	0	2	1	0

Challenge organism virulence control: 5 million organisms per mouse = 9; 10 million organisms per mouse = 7; 20 million organisms per mouse = 5; 40 million organisms per mouse = 1; 80 million organisms per mouse = 0.

Notes. (i) Figures for mice represent survivors from 10 mice tested in each group. (ii) Cholera vaccine was used as a control for non-specific immunity.

DISCUSSION

The chrome enteric vaccine is based on the conception that the Vi-antigen of salmonella organisms is the important immunogenic agent, and therefore any treatment in the preparation of a typhoid vaccine which tends to preserve this antigen is to be recommended.

The time-honoured method of typhoid-paratyphoid vaccine production in this country is to kill the organisms by heat and preserve the vaccine with 0·5 % phenol; it has been known, for many years now, that both heat and phenol alter or damage the Vi-antigen.

Ando & Nakamura (1950) consider the Vi-antigen as consisting of two parts, one heat-stable and the other heat-labile; the Vi-agglutinability, O-inhibition and also immunizing power (active immunity in mice and Vi- and protective antibody production in rabbits) are due to the presence of the labile part, as originally described by Felix, while the stable portion causes precipitation and feeble production of Vi- and protective antibody in rabbits.

It has been found by Japanese workers that, if typhoid bacilli are killed by formalin and treated with chrome salts, the Vi-antigen is stabilized, and, furthermore, it is claimed that a decrease in toxicity for animals occurs without impairment of the Vi- or O-antigens. It is suggested that the chrome acts as it does in the process of tanning of leather, and it combines with the surface antigen, i.e. the Vi-antigen, and produces a less soluble and a more stable structure; the antigenicity of such stable chrome vaccine with decreased toxicity was found to be very marked in animal and human experiments (Ando, Shimojo & Tadokoro, 1952).

In the series of experiments done at this laboratory the experimental chrome vaccines were tested, as stated in the introduction, by (i) *in vitro* tests, i.e. agglutination tests, and (2) *in vivo* tests, i.e. production of antibodies in rabbits, and mouse protection tests, both active and passive.

In the agglutination tests the stabilizing effect of formalin and chrome alun treatment on the Vi-antigen is shown by the O-inagglutinability of these prepara tions, when compared to a heat-phenol treated suspension (Table 1), and afte subjection to various degrees of heat (Table 2). Formalin by itself confers no such heat-resistance on the Vi-antigen.

When the rabbits were injected with the experimental chrome vaccine (which had been properly stored at refrigerator temperatures of 4–6° C.), a good rise o: Vi-antibodies was obtained, accompanying a similar high rise of O-antibodies. The estimation of circulating Vi- and O-antibodies in rabbits immunized with different typhoid vaccines has been practised for many years, and has proved to be a reliable and sensitive method of detecting any damage that may have been done to the Vi-antigen in the course of preparation of the vaccine (Felix, 1951).

Suspensions of bacteria killed by formalin, without chrome alum treatment, give rise to considerable amounts of Vi-antibodies; so it is impossible to say if treatment of the formol-killed vaccine used in this study with chrome alum had any effect *per se* on the production of Vi-antibodies as seen in Table 3 (Felix, Bhatnagar & Pitt, 1934; Kaufmann, 1935; Bensted, 1940). However, the antibodies produced by formol vaccines are somehow deficient in protective powers, as characterized by a reduced power of promoting phagocytosis and of protecting against infection with virulent strains of the typhoid bacillus (Felix & Bhatnagar, 1935; Felix & Petrie, 1938); though Ando (1953) and Nakamura found no difference between antisera produced by using living bacilli or formalin-killed bacilli. Treatment of the experimental formalin-killed vaccine with chrome alum gave rise in rabbits to antibodies which appear to have adequate protective powers (see Table 4) when tested by the passive mouse protection test.

In the active mouse protection test, carried out with a constant challenge and graded immunizing doses (Batson, 1949), good protection was obtained by vaccination with one subcutaneous injection of the chrome vaccine stored at 4–6° C. As regards mouse-protection tests, it was considered by Felix (1941) that active immunization experiments in mice do not disclose those great differences in the antigenic value of various preparations of the Vi-antigen, which are so clearly demonstrated by using the serum of vaccinated rabbits or horses in passive protection experiments with mice, or even by *in vitro* laboratory tests. Ando & Nakamura (1950) disagree with this opinion and state, 'In our experiments the mouse-method without using mucin, if ED 50 (the average immunizing dose) is determined against a certain challenge dose, is shown to be sensitive enough for determining the immunizing power of vaccines. This method distinctly shows that the Vi-antigen carries out a function that the O-antigen cannot fulfil.'

It will be noted that improper storage of the experimental chrome vaccine at 41° C affected the Vi-antigen markedly, as shown by all the *in vivo* tests; but this was a severe and artificial stability test, which does not contradict the other good qualities of the chrome vaccine—namely its Vi-antigen stability and its protective properties in both active and passive mouse protection tests.

The conclusions to be drawn from this series of tests, however, have to be considered against the results of the field trials in Yugoslavia and the laboratory

assays of the vaccines used in that trial (Cvjetanovic, 1957; Yugoslavia Typhoid Commission, 1957; Edsall, Carlson, Foomal & Benenson, 1959; Standfast, 1960), in which the phenolized vaccine gave better protection to vaccinated persons than the alcoholized vaccine, notwithstanding the generally recognized preserving effect of alcohol treatment on the Vi-antigen of typhoid suspensions on one hand and the damaging effect of heat and phenol on the other (Felix, Rainsford & Stokes, 1941; Bensted, 1940; Climie, 1942; Felix & Anderson, 1951).

The Japanese workers have conducted human trials on numerous occasions with satisfactory results, and have demonstrated the rise of Vi-antibodies which was effected by the chrome vaccine, as compared with the usual lack of these antibodies when heat-killed vaccines are used. They considered the possession of the Vi-antigen and the production of Vi-antibodies to be of much importance in gauging the efficacy of typhoid vaccines, which is a reasonable view based on the observed facts that the Vi-antibody is certainly found in patients and carriers of typhoid bacilli, and bacilli isolated from patients contain this antigen (Bhatnagar, 1938; Bhatnagar, Speechley & Singh, 1938; Felix, Kirkorian & Reitler, 1935). Kaneko, Hajashi, Hiraj & Ando (1953) compared a chrome vaccine with a heated typhoid-paratyphoid vaccine and stated, 'The Vi-antibody in sera may be considered insufficient to be the direct proof of the protective immunity against the typhoid fever, but its presence should be related to some extent to the typhoid immunization and it is supposed that the chrome vaccine is the superior one for the typhoid immunization conferring higher Vi serum titres.'

The final judgement on the superiority, or even the efficacy, of the chrome vaccine will have to rest on full-scale field trials—Perry, Findlay, & Bensted (1934) remarked many years ago: 'A field trial in endemic areas of the disease would of course supply the most satisfactory evidence of the immunising power of the typhoid vaccine.'

SUMMARY

1. Experimental typhoid vaccines, treated by 1 % formalin and 0·02 % chrome alum ($KCr(SO_4)_2.12H_2O$), as suggested by Japanese workers were prepared and tested by the usual *in vitro* and *in vivo* tests.

2. Agglutination tests, antibody production in rabbits, active and passive mouse protection tests confirm the stability of the Vi antigen of the vaccine, if properly stored, and the good protection afforded to laboratory animals in both the active and passive mouse protection tests.

3. It is suggested that only a full-scale field trial in a typhoid endemic area can give the answer as to the real efficacy and/or superiority of the chrome vaccine over other typhoid vaccines.

I would like to record my debt to Colonel M. H. P. Sayers, M.D., O.B.E., formerly Officer Commanding, the David Bruce Laboratories, for initiating me in the techniques of vaccine production and research, and to the staff of the Vaccine Department, and Animal Houses of these laboratories, particularly Mr A. Parkhurst, F.I.M.L.T., for technical assistance.

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