

## IMMUNIZATION TO Vi AND O ANTIGENS OF *SALMONELLA TYPHI* USING A STABLE ANTIGEN IN OIL

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It has been shown by a number of workers that saline suspensions of *Salmonella typhi* lose their Vi activity rapidly, while dried organisms retain the ability to stimulate antibody production and to react with Vi antibody. Chu & Hoyt (1954) found that the Vi haptene, although very soluble in water, retains its antigenic specificity even when autoclaved for 30 min. at 20 lb. pressure. A vaccine prepared by harvesting Vi-positive organisms in alcohol, which were then dried and resuspended in peanut oil, was found to produce high antibody titres against both O and Vi antigens when a single injection was given to rabbits. These observations have formed the basis for the preparation of a vaccine, whose properties are described below.

### METHODS

*S. typhi*, strain Ty 2, was grown in Blake bottles on nutrient agar. The organisms were shown to have O, H and Vi antigens. After 18 hr. incubation, the growth was harvested by washing the surface of the agar with 20 ml. of absolute ethyl alcohol. The alcohol suspension was transferred to a 250 ml. centrifuge bottle and diluted with 200 ml. absolute ethyl alcohol. The bottle was stoppered, shaken vigorously to wash the bacteria and centrifuged. The supernatant alcohol was discarded and the washing was repeated once. The bacterial sediment was placed in the 37° C. incubator to dry overnight. The dried sediment was ground to a fine powder by means of a glass stirring rod attached to a small laboratory stirrer, under aseptic conditions. A loopful of the dry powder was cultured to test for the persistence of living organisms. When the cultures indicated that the organisms were dead, the powder was weighed on an analytical balance; 20 mg. of powdered bacteria were added to 40 ml. peanut oil of a pharmaceutical grade. The dried bacteria were suspended in the oil by grinding in a Pyrex test-tube using a glass stirring rod. When the organisms were evenly suspended the oil emulsion was transferred to vaccine bottles and autoclaved at 15 lb. for 30 min. The oil vaccine was tested for sterility and used for inoculation of animals.

A saline vaccine was prepared by suspending 20 mg. dried organisms in 40 ml. sterile saline. The vaccine was prepared at the time of administration and was not heated before use.

### IMMUNIZATION OF ANIMALS

Rabbits weighing 2–2.5 kg. were subjected to a preliminary bleeding before immunization. Those which showed no antibodies against Vi antigen of Ballerup (XXIX, Vi) and O antigen of *S. typhi*, strain O-901, were used; the vaccine was

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injected into the subcutaneous tissue of the right leg of each rabbit, the immunization consisting of a single injection. Three groups of animals were prepared:

Group I. Seven rabbits injected with 2 ml. of dried bacteria in oil.

Group II. Five rabbits injected with 2 ml. of dried bacteria resuspended in saline.

Group III. Two rabbits injected with 2 ml. of commercial typhoid-paratyphoid vaccine.

At the recorded intervals the animals were bled, and agglutination tests were performed on serial dilutions of pooled rabbit sera. The Vi antibody was determined by slide agglutination against the Ballerup organism (XXIX, Vi). The O antibody was measured in the tube agglutination tests against *S. typhi*, strain O-901.

Active and passive protection tests were performed in mice; details are given below.

RESULTS

(1) Active immunization

Tables 1 and 2 show that a single injection of dried organisms suspended in peanut oil results in the formation of both Vi and O antibodies in the rabbits. By com-

Table 1. O-antibody response to injection of *Salmonella typhi*, strain Ty 2

Immunizing antigen suspended in	Titre of pooled sera against <i>S. typhi</i> , O-901				
	Days after injection				
	5	10	30	240	720
Peanut oil	320	1280	1280	1024	64
Saline	160	320	160	0	0
Saline (commercial)	320	320	160	0	0

The values represent the highest dilution giving a positive reaction.

Table 2. Vi-antibody response to injection of *Salmonella typhi*, strain Ty 2

Immunizing antigen suspended in	Titre of pooled sera against Ballerup organism				
	Days after injection				
	5	10	30	240	720
Peanut oil	80	320	160	80	80
Saline	0	0	0	0	0
Saline (commercial)	0	0	0	0	0

The values represent the highest dilution giving a positive reaction.

parison, the saline suspensions were inferior both as to the level of antibody produced and as to the length of time during which the titre remained elevated. As was anticipated, no Vi antibodies were formed by the animals which received the saline suspensions. The results indicate that a single injection of dried organisms suspended in peanut oil is effective in producing immunity, in so far as the antibody titre is an indication of resistance.

(2) *Passive protection*

As further evidence of immunization, mouse protection tests were performed in the following manner. Undiluted rabbit serum, pooled from the animals used for immunization in Group I (above) was injected intra-abdominally into the mice of the experimental group. Control animals received normal rabbit serum shown to possess no agglutinins against *S. typhi*. Each mouse received 1 ml. of serum. One and a half hours after the animals had received the serum, they were inoculated with the test dose of bacteria (approximately  $1 \times 10^9$  organisms). This was prepared by suspending the growth of *S. typhi*, strain Ty 2, which developed on an agar slant after 18 hr. incubation, in 15 ml. sterile saline. The organisms were washed off the slant and the suspension was used immediately. Each mouse received 0.5 ml. intraperitoneally. The results are described in Table 3.

These results show that the immune serum does in fact protect against death due to injection of virulent *S. typhi*.

Table 3

Time observed (hr.)	Control group 20 mice	Experimental group 20 mice
6	Movement sluggish, fur ruffled	Active
16	16 animals dead 4 ill	4 animals ill, remainder active
20	20 dead	1 animal died, others remained alive and active. No further deaths were observed in this group

Table 4

Group	No. of mice	No. of survivors	% of survivors
Vaccine in oil	60	57	95
Vaccine in saline (commercial)	20	11	55
Control	20	5	25

(3) *Active protection tests*

To obtain further evidence of the superior immunizing properties of the oil vaccine active immunization of mice was followed by injection of a challenge dose of *S. typhi*, strain Ty 2. The mice to be immunized were divided into two groups; each mouse in one group received 0.5 ml. of oil suspension prepared as described under the section on methods. The second group received 0.5 ml. of a commercial saline vaccine. A control group of 20 animals received 0.5 ml. of peanut oil without bacteria. 18 days later each mouse received a challenge dose of approximately  $1 \times 10^9$  organisms prepared by suspending an 18-hour nutrient agar slant culture of *S. typhi*, strain Ty 2, in saline. The challenge dose was contained in 0.5 ml. Control mice were of the same age and weight range as the experimental groups. The results of the test are shown in Table 4.

The results support the evidence obtained through study of serum antibody in the rabbits and the passive protection given by the immune serum to mice, and indicate that the oil vaccine is an effective agent in developing immunity to *S. typhi*. They also substantiate the conclusion of Felix (1951), who feels that the antibody titre developed during immunization with *S. typhi* is a satisfactory measure of the efficiency of the vaccine.

#### DISCUSSION

From a practical standpoint, the oil vaccine has certain other advantages which can be observed in the data presented above. The administration of a single injection produces an antibody response which reaches a higher titre and persists much longer than that following the administration of a saline vaccine. This is undoubtedly due to the gradual release of bacteria from the oil reservoir in the tissues providing a prolonged antigenic stimulus. Freund (1951) has, of course, developed this point of view extensively; however, he feels that an oil-in-water emulsion is essential to the preparation of a satisfactory adjuvant. This may be true when a mineral oil is used as the base of the emulsion; however, when a vegetable oil is used the situation is changed, since the vegetable oil is susceptible to metabolism by the body and eventually is broken down, during which process the bacterial cells are exposed to the action of the tissues. If the organisms are incorporated in an inert mineral oil, they are protected from contact with the tissues.

This aspect of the vegetable oil eliminates another objection to the use of oil as a vehicle for the vaccine. Since the vegetable oils are slowly metabolized, they are not prone to produce sterile abscesses or paraffinomas such as frequently result from the injection of paraffin-base oils. Although there has been some disagreement as to the relationship between the Vi antigen and virulence of salmonella strains, there can be no doubt that it is an integral part of the antigenic structure of *S. typhi*, and as such it should be represented in any vaccine intended to immunize against this organism. We have shown in this and the preceding paper (Chu, Hoyt and Pickett, 1956) that the conditions under which the saline suspension is prepared favour the extraction of the Vi antigen into the liquid phase, where it becomes inactive antigenically. On the other hand, the antigenic properties of the Vi antigen are protected and preserved by the procedures described above, whereby the organisms are handled in alcohol rather than in water, and are finally suspended in a vegetable oil; such a suspension is able to provoke a substantial production of antibody against both O and Vi antigens. Moreover, the immune serum produced by use of the oil vaccine is able to protect mice passively, and mice actively immunized resist the challenge dose better than those immunized by the conventional saline vaccine. Antigenically, the oil vaccine appears to be superior to the saline vaccine from both theoretical and experimental points of view. It protects the Vi antigen (or antigens) and prolongs the effective period of immunization.

These observations suggest that a vaccine prepared against salmonella strains which contain the full antigenic complement can be prepared by avoiding undue

contact with water and making the final suspension in an injectable vegetable oil, such as peanut or sesame oil. It is not permissible to translate information directly from experimental infections in mice to naturally infected humans, yet it seems possible that a single injection of such a vaccine may result in protection which will at least equal that conferred by the series of injections of the saline vaccines now in use. The reports of Felix (1941), in which he describes the presence of Vi antigens in *S. paratyphi A* and *S. paratyphi B*, would make it seem advisable to apply the same treatment to these organisms in preparing a prophylactic antigen.

#### SUMMARY

A method is described for the preparation of a vaccine, consisting of a suspension of bacterial cells in a vegetable oil, which retains the antigenic activity of both Vi and O antigens of *S. typhi*. The ability of such a vaccine to induce active and passive protection in mice is demonstrated.

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