SOLUBILITY OF Vi ANTIGEN OF *SALMONELLA TYPHI*

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In 1953 Felix & Bhatnagar (1955) showed that the heat-killed phenolized vaccine in use at that time failed to stimulate production of the Vi antibody. The important role of the Vi antigen in the problem of immunity to typhoid fever has been generally recognized, and several procedures have been suggested in an effort to preserve the Vi antigen intact. These have been based on the concept of the Vi antigen as a heat-labile component of very limited stability. Felix (1941) devised a vaccine giving rise to both O and Vi antibodies; the bacteria were harvested from agar cultures in 75 % alcohol, suspended in a solution of 25 % alcohol in saline and stored at 1–2° C. Rainsford (1942) showed that this vaccine rapidly lost Vi antigenicity, as measured by the ability to agglutinate with Vi antibodies, when stored at 25° C. He suggested the preservation of Vi antigen in a 32 % NaCl solution, or by drying from an acetone suspension. Ether has been used by Gohar & Elian (1942) for a similar purpose as has 34 % sucrose (Loureiro; see Felix & Anderson, 1951). These methods have been reviewed by Felix (1951).

Peluffo (1941), on the other hand, followed the disappearance of the Vi antigen of *Salmonella typhi*, using organisms suspended in glycerine, in alcohol and in acetone, and found that they remained agglutinable by Vi antisera for as long as 8 months. The Vi antigen of dehydrated organisms survived heating at 150° C. for 1 hr. Such organisms, when injected into animals, gave rise to Vi antibodies as well as to O and H, and the immune serum was found to protect mice against living virulent organisms. No details of the protection tests were recorded. He concluded that the Vi antigen is actually more stable than had been supposed, and that the thermolability described by other workers was due to the use of aqueous suspensions in their studies.

In 1953 Landy (1953) emphasized the need for a stable Vi antigen in the development of immunity to typhoid fever. He described the preparation and standardization of a dehydrated vaccine to be reconstituted with water at the time of use. Such a product remains active when kept dry even though not refrigerated. However, once reconstituted, it requires refrigeration and becomes subject to loss of antigenicity just as any other saline-suspended vaccine.

In his studies on different *Salmonella* species, Felix (1952) concluded that Vi antigens in aqueous suspensions are all inactivated by dilute alkali and by heat; inactivation by dilute acid was irregular and the TVi antigen was found not to be destroyed by dilute acid treatment. Kauffmann (1951), on the other hand, reported the Vi antigen to be thermolabile but to be destroyed by HCl. Numerous

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other studies have been made with the general agreement that TVi antigen is stable when dry but unstable in aqueous suspension. Chu & Hoyt (1954) showed that the TVi antigen is in reality quite heat-stable in solution, and retains its immunologic identity after autoclaving for 30 min. at 20 lb. pressure. The loss of activity of Vi-positive organisms, it was suggested, is due to the solution of the Vi antigen in the water, rather than to its destruction.

Since there has been disagreement regarding the physical and chemical properties of the Vi antigen we have carried out the studies reported here with the object in mind of learning the conditions which might promote the development of a more useful vaccine retaining the full antigenic structure of *S. typhi* under as wide a range of environmental conditions as possible.

I. METHODS AND MATERIALS

S. typhi, strain Ty 2, was used as a source of Vi antigen. Cultures were grown on nutrient agar. In testing for soluble Vi antigen, the haemagglutination reaction described by Spaun (1951) was used. The bacteria were extracted without previous freezing or other treatment designed to increase the solubility of the Vi antigen. In testing for persistence of Vi antigen in bacterial cells after treatment, a Vi antiserum prepared against the Ballerup organism (XXIX, Vi) was used.

II. SOLUBILITY OF TVi ANTIGEN IN VARIOUS SOLVENTS

To distinguish between simple extraction of Vi antigen by a liquid and destruction of the antigen, it is necessary to treat Vi-positive bacterial suspensions of *S. typhi* and test both extract and sediment for Vi activity, the extract by means of the coating technique and the sediment by direct application of Vi antiserum. Previous reports have been interested only in the behaviour of the bacterial cells following treatment, with the result that extraction of the Vi antigen may have been confused with destruction. In investigating the action of the solvents listed below, strain Ty 2 was grown on nutrient agar slants for 24 hr. at 37° C. To each tube, 5 ml. of one of the various solvents were added and the surface growth was suspended. After extraction for 72 hr., the suspension was transferred to a centrifuge tube and the bacteria were sedimented. The supernatant was removed and evaporated off in a hot-air oven at 90° C., and the residue was mixed with 5 ml. of 0·9 % NaCl solution. Washed rabbit red cells were added to make a concentration of 5 % and the suspension was incubated for 30 min. at 37° C. The sedimented bacteria were resuspended in 0·5 ml. of 0·9 % NaCl solution. In the case of the water, saline and phenol-saline extraction, the organisms were extracted for 72 hr., boiled for 15 min. and centrifuged. The supernatant liquid was used to coat the rabbit red cells without additional treatment. In the NaOH extraction the organisms were treated with 5 ml. N/1-NaOH. After 72 hr. the mixture was neutralized with HCl and treated as with the saline extraction. Bacteria and red cells were tested for the presence of Vi antigen by means of slide agglutination using Ballerup (XXIX, Vi) antiserum. Results are shown in Table 1.

Agglutination of coated cells means that the TVi antigen has been transferred

to the rabbit red cells, while agglutination of the bacterial sediment indicates that the TVi antigen has been retained. The results show that the aqueous solutions (water, saline, phenol-saline) remove the Vi antigen to such an extent that the bacterial cells are no longer able to react with the Vi antiserum. The extracts are able to coat the red cells, indicating that the antigen retains its specificity. The organic solvents, on the other hand, contained no Vi antigen after the extraction period, and the bacteria remained agglutinable. The NaOH extraction was the only one which appeared to destroy the Vi antigen, since neither red cell nor bacterium was able to react following the treatment.

III. SOLUBILITY OF TVi ANTIGEN IN ALCOHOL-WATER MIXTURES

Since the alcohol vaccine of Felix (1941) has been found effective in immunizing against the TVi antigen, we felt it might be useful to investigate the solubility of the TVi antigen in different alcohol-water mixtures. Accordingly, 24 hr. nutrient agar slant cultures of *S. typhi* Ty 2 were prepared. Dilutions of alcohol in water were made according to Table 2. 10 ml. of each mixture were added to an agar slant to wash off the bacteria, which were then transferred to a test-tube and allowed to extract for 1 hr. in a 56° C. water-bath. After centrifugation, the sediment was resuspended in 1 ml. of 0·9 % NaCl solution and tested by slide agglutination. The alcohol-water mixture was removed by heating in a hot oven at 90° C. and the residue was redissolved by adding 1 ml. of a 5 % suspension of washed rabbit erythrocytes. After 15 min. at 37° C. the cells were tested by slide agglutination.

Reactions of coated red cells and sediments are shown in Table 2.

The readings show that in alcohol concentrations above 70 % little or no TVi antigen is dissolved. However, concentrations of alcohol weaker than 30 % show nearly complete removal of the TVi antigen, as shown by slide agglutination tests. From this we may conclude that the 25 % alcohol vaccine, though permitting the retention of some of the TVi, may not be the best preparation for inducing active immunity to this antigen.

IV. SOLUBILITY OF TVi ANTIGEN IN SALINE

The disappearance of TVi antigen from the bacterial cell appears to be due to its ready solubility in water, but the limits of solubility have not been tested. To clarify this phase of the problem, 24 hr. nutrient agar slant cultures of Ty 2 were extracted with different amounts of 0·9 % NaCl solution. After suspending the growth from one slant in the indicated volume of saline (Table 3), the suspension was removed and boiled for 30 min. After cooling, the suspensions were tested by slide agglutination, using Ballerup (XXIX, Vi) antiserum.

It appears that the saturation point was reached at about the ratio of 2·0 ml. saline per agar slant. As further evidence of this, 24 hr. agar slant cultures of Ty 2 were harvested in saline, using 1·0 ml. per slant. The suspension was boiled for 30 min., after which a single 10·0 ml. intramuscular injection was given to each of two rabbits previously shown to be lacking in Vi antibodies. Twelve days later the

Table 1. *Response of T Vi antigen to various solvents*

	Reaction with Vi antiserum after extraction by									
Antigen	Water	Saline	Phenol saline	N/1-NaOH	Acetone	Alcohol	Chloro-form	Carbon tetra-chloride	Diethyl ether	Benzene
Coated rbc	+	+	+	−	−	−	−	−	−	−
Bacterial sediments	−	−	−	−	+	+	+	+	+	+

Table 2. *Extraction of T Vi antigen by alcohol-water mixtures*

	Reaction after extraction with alcohol-water mixtures (% alcohol by volume)									
Antigens	10	20	30	40	50	60	70	80	90	100
Coated rbc	4+	4+	3+	3+	2+	1+	−	−	−	−
Bacterial suspension	−	−	±	±	1+	2+	3+	4+	4+	4+

Table 3. *Saturation of T Vi antigen in 0·9 % NaCl solution*

	Agar slant suspensions extracted by saline (0·9) volume of						
	0·5 ml.	1·0 ml.	1·5 ml.	2·0 ml.	3·0 ml.	4·0 ml.	5·0 ml.
Reaction of antigen with Vi antiserum	3+	3+	2+	1+	−	−	−

animals were bled; the serum was used in slide agglutination tests against *S. typhi* 0901 (IX, XII) and Ballerup (XXIX, Vi). Agglutination against *S. typhi* 0901 was read as 4+, and against Ballerup as 3+. Serum from animals immunized with dilute suspensions of the Vi-positive Ty 2 agglutinated only *S. typhi* 0901 but not Ballerup, showing that no Vi antibodies are formed when a dilute suspension is employed as the vaccine.

V. ANTIBODY RESPONSE TO Vi AND O ANTIGENS IN OIL VEHICLES

These observations support the conclusions of Peluffo and of Felix, who related the loss of Vi activity to the use of aqueous suspensions, and suggest that a complete antigen against a Vi-positive organism should be prepared without exposing the bacteria to unnecessary extraction by water. Accordingly, nutrient agar slants were inoculated with *S. typhi* Ty 2 and incubated for 18 hr. At this time the organisms were harvested by washing the surface of the slants with 5 ml. absolute alcohol. The suspension was transferred to a centrifuge bottle and diluted with 25 ml. absolute alcohol. The tube was centrifuged and the supernatant discarded. After a second washing in absolute alcohol the bacterial sediment was dried overnight in the 37° C. incubator. Aliquots of the dried antigen were then resuspended in four different vehicles, as follows:

(1) Dry bacteria + light mineral oil.
(2) Saline suspension of bacteria + mineral oil.
(3) Saline suspension of bacteria + peanut oil.
(4) Dry bacteria + peanut oil.

These vehicles were chosen for the following reasons. The available evidence indicates that the Vi antigen would be preserved best in a non-aqueous vehicle. The studies of Freund suggested the use of an oil, since he has shown that emulsions of bacterial suspensions in mineral oil give rise to high antibody titres. However, in his review on the subject (Freund, 1951), he states that (*a*) it is essential to administer the antigen in a water-in-oil emulsion, and (*b*) no oil of plant origin has been found as a substitute for mineral oil. Mudd, Felton & Smolens (1948) found that seven of ten adults injected with such a vaccine developed nodules at the injection sites which persisted for several months, many of which became painful and were the focal points of inflammatory reactions on re-inoculation or skin testing. We therefore decided to try the four vehicles listed above to learn whether a vegetable oil could be adapted to the procedure. Vegetable oils are slowly metabolized and disappear from the site of inoculation and so would be free from the objections demonstrated by Mudd *et al.*

The four preparations were made up to contain about 1×10^7 organisms per ml. In preparations 1 and 4 the dry bacteria were suspended in the oil by careful grinding in a test-tube, using a glass stirring rod. Since they were quite dry they dispersed in the oil readily and made a smooth suspension. In preparations 2 and 3 the bacteria were first suspended in the saline solution and the suspension was emulsified in an equal volume of oil by mixing with a small motor-driven stirring blade. The suspensions were sterilized by placing them in a boiling water-

bath for 30 min., after which they were tested for the presence of viable organisms. Two rabbits were inoculated with each preparation; each rabbit received a single injection, intramuscularly, of 5 ml. The rabbits were bled after 10 days and after 30 days. Agglutination tests against O and Vi antigens were carried out; the results are shown in Table 4. It is seen that the antibody response in the animals immunized with the bacterial suspension in peanut oil comes about sooner and reaches a higher level at the end of 30 days, with respect to both the O and Vi antigens. The animals receiving mineral-oil vaccine developed antibodies against both O and Vi antigens, but more slowly and in lower titre. The animals immunized against the vaccines which contained saline in addition to the oil showed no antibodies against the Vi antigen.

Table 4. *Antibody response to* Salmonella typhi *in various vehicles*

	Antibody activity of serum			
	10 days—slide agglutination		30 days—titre of tube agglutination test	
S. typhi suspended in	O	Vi	O	Vi
Group 1, mineral oil	−	−	1:80	1:5
Group 2, mineral oil, saline	+	−	1:160	−
Group 3, peanut oil, saline	−	+	1:640	−
Group 4, peanut oil	+	+	1:1280	1:160

SUMMARY

The observations reported here show that the T Vi antigen is effective in stimulating antibodies when it is attached to the cells. It is also clear that the Vi antigen is readily removed in aqueous solutions, but in such organic solvents as acetone, chloroform, ether, alcohol, carbon tetrachloride and benzene it is retained by the cell. In alcohol-water mixtures the Vi antigen is progressively more soluble in those solutions where the alcohol concentration is less than 70 %. However, its solubility in water is limited, and by making progressively heavier suspensions it was found that a point is reached at which Vi antigen is retained by the cell, the suspending solution apparently being saturated. Such an aqueous suspension is able to stimulate the production of Vi antibody.

The administration of dried organisms in peanut oil resulted in the production of antibodies against both O and Vi antigens. This was in contradistinction to the results obtained when mineral oil, mineral oil + saline, or peanut oil + saline were used: in these cases the O antibody titre was lower and Vi antibodies were produced in low titre or not at all. It seems clear that the presence of water in the vaccine has the effect of removing the antigenicity of the Vi hapten; water-free, vegetable-oil vaccine is suggested by these studies as an effective agent for immunizing against all of the antigens of *Salmonella typhi*. The use of such a vaccine in producing active and passive immunity in animals is discussed in the paper which follows.

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(*MS. received for publication* 22. VI. 56)