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Vi Antigen from *Salmonella typhosa* and Immunity Against Typhoid Fever

I. Isolation and Immunologic Properties in Animals

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The role of Vi antigen in human immunity against typhoid fever has been debated for decades. Circumstantial evidence indicates that Vi antigen may play a role in pathogenicity and immunity. A Vi preparation was isolated from *Salmonella typhosa*, the causative organism in human typhoid fever, by a mild precipitation method. It was significantly more potent in animal studies than preparations from *Citrobacter* used in the past for human study and less toxic than conventional typhoid vaccines. With this antigen, the role of Vi antigen in human protection is now being investigated.

Although the spread of typhoid fever can be controlled by sanitation, vaccination is the method used for controlling this disease in many parts of the world. Field trials (1954-1967) in Yugoslavia, Guyana, Poland, and the U.S.S.R. (5) have shown that inactivated cellular vaccines offered significant protection, and the acetone-inactivated vaccine K was more protective than the heat-phenol-inactivated vaccine L. Unfortunately, adverse reactions due to endotoxin or other toxic substances are frequent in vaccines.

Many attempts have been made to identify and isolate protective antigens from the typhoid bacillus. However, no single component has been identified as protective in human immunity. Despite its importance in animals (7, 12, 22), the role of Vi antigen in human immunity remains to be ascertained. As a result of our study of laboratory assay methods for control of typhoid vaccines, we isolated a Vi antigen from *Salmonella typhosa* by precipitation with hexadecyltrimethylammonium bromide (Cetavlon) (1, 21), and studied its immunological properties in animals and tissue cultures. It appeared to be more potent than preparations used in the past in human studies (9). The isolation procedures and some immunological properties of this Vi preparation are described here. Study of this antigen in human volunteers is in progress.

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MATERIALS AND METHODS

Culture. Bacterial cultures were grown on veal infusion agar (Difco Laboratories, Detroit, Mich.) at 37 C overnight. *S. typhosa* strain Ty 2 was treated with acetone as previously described (22). *S. typhosa* 0901 (a Vi-negative strain) was harvested, washed three times, and pasteurized in saline before being used for absorption experiments. *S. typhosa* M 4702 and E-2322-72 were multiple-antibiotic-resistant strains isolated from recent epidemics and obtained from the Center for Disease Control, Atlanta, Ga.

Vaccines. The U.S. Standard Typhoid Vaccine lot 6A, acetone-inactivated and dried, and typhoid vaccine L, heat-phenol-killed and dried, were prepared from *S. typhosa* strain Ty 2 and standardized by bacterial counts (6, 22).

Isolation of Vi antigen preparations. The procedure for isolation of Vi antigen (Vi-Ty 2) from *S. typhosa* Ty 2 is given in Fig. 1.

We designated the Vi preparation from M. Webster, National Heart and Lung Institute, Bethesda, Md., as Vi-W_1, and the other Vi preparation from S. Berman of Walter Reed Army Institute of Research, Washington, D.C., as Vi-W_2. Both Vi-W_1 and Vi-W_2 were isolated from *Citrobacter* 5396/38 by the method of Webster et al. (19).

Biological assays. Male mice of the N:NIH (SW) strain were obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md.

The active mouse protection test was performed as described previously (22). Toxicity was assayed by the actinomycin D enhancement test in mice (23).

For lymphocyte transformation assay, mice weighing 16 to 18 g each were immunized intraperitoneally with 10 μg of a Vi preparation or with 10^7 cells of a conventional typhoid vaccine in 1 ml of saline. Control mice were given 1 ml of saline. Three to four

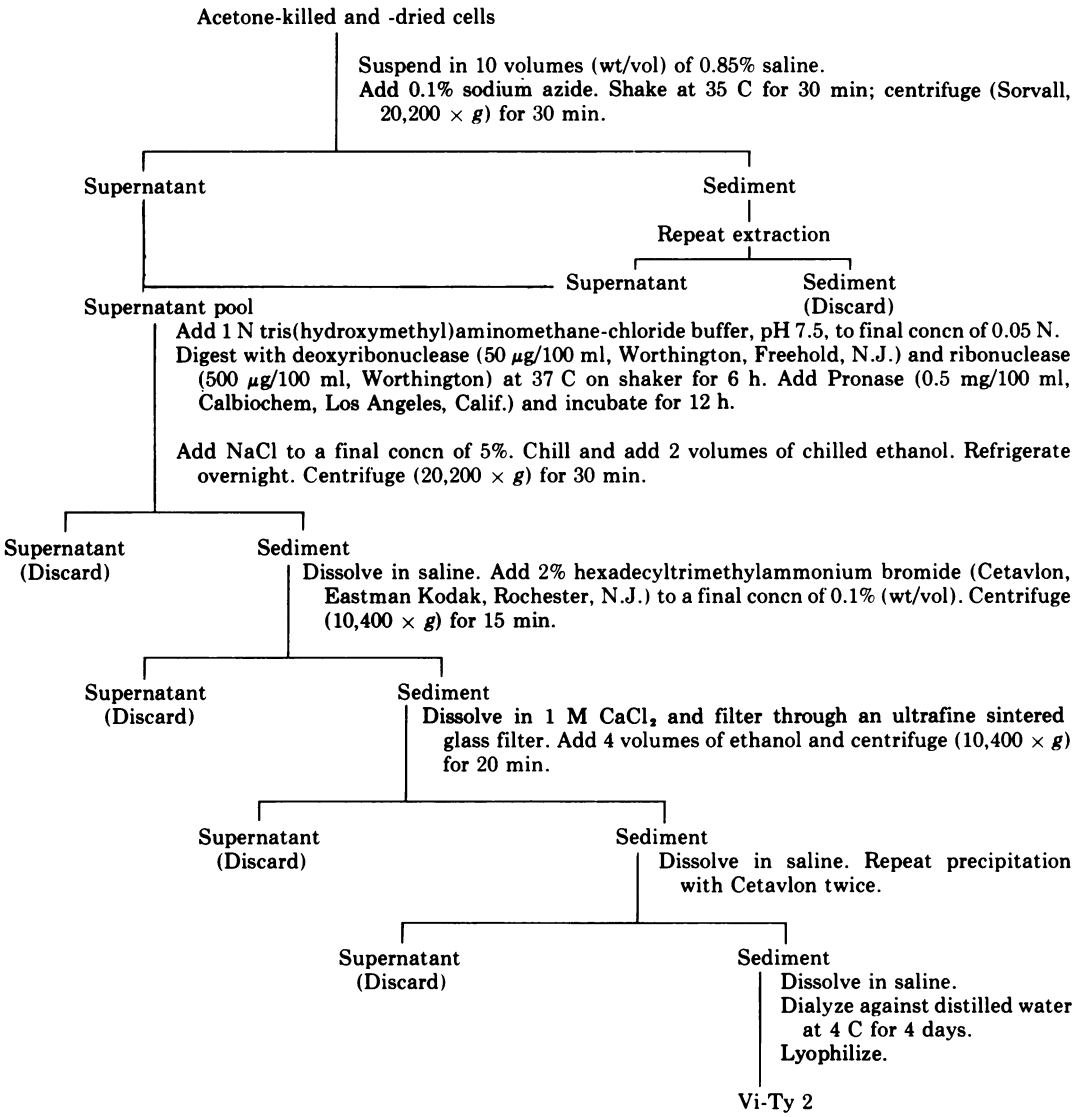

FIG. 1. *Isolation of Vi-Ty 2.*

weeks later, spleens were removed into RPMI 1640 medium. Lymphoid cells were teased from each spleen, filtered through a gauze pad, and washed twice in RPMI 1640 medium by centrifugation. A total of 3×10^6 to 5×10^6 cells, as determined by microscopic count, were suspended in glass tubes in 1 ml of RPMI 1640 medium supplemented with 73.5 μg of neomycin, 300 μg of glutamine, and 5% heat-inactivated normal human serum. Cell cultures were stimulated by the addition of 5 μg of a Vi preparation or Vi and 0.15 μg of lipopolysaccharide in 0.1 ml of RPMI 1640 medium and were incubated for 48 h at 37 C in a humidified atmosphere of 5% CO_2 and 95% air. Phytohemagglutinin (lot 575122, Difco) was used as positive control. A 1-μCi portion of tritiated thymidine (specific activity 5 Ci/mmol, Schwarz/Mann, Orangeburg, N.Y.) in 0.1 ml of RPMI 1640 medium was added to each tube, and incubation continued for 18 h. Pulse was terminated by adding 3 ml of chilled RPMI 1640, or Hanks medium. The cell suspension was vortexed and filtered through a 25-mm membrane filter disk (type HA, 0.45 μm, Millipore Corp., Bedford, Mass.). The disks were washed three times with 5% trichloroacetic acid and twice with 95% ethanol. They were then placed in glass scintillation vials containing 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) and counted in a Packard Tri-Carb liquid scintillation counter. Each test was done in triplicate.

Serological tests. The method of Landy and Lamb

(13) was used for the hemagglutination test, and the gel-diffusion test was previously described (24).

Bactericidal assay was done with Vi antiserum from a goat immunized with Vi-Ty 2 in Freund complete adjuvant (Difco). Growth from a 4-h veal infusion agar slant was suspended in chilled sterile saline and diluted to about 10^7 organisms per ml. A 0.1-ml portion of the suspension was added to tubes containing 0.5 ml of inactivated serum (56 C for 30 min) and 0.5 ml of absorbed guinea pig complement. Complement control (without antiserum), serum control (without complement), and cell control (without complement or antiserum) were run simultaneously. After incubation at 37 C for 50 min, 3 ml of chilled sterile saline was added to each tube, and a 20-μliter sample was then pipetted into 10 ml of chilled saline. A 0.2-ml portion was plated on veal infusion agar, and colonies were counted after overnight incubation at 37 C. In a series of serum dilutions, the dilution of the last plate showing more than 80% reduction in colony count as compared with the cell control plate was taken as the end point.

Complement was routinely absorbed with pasteurized cells of *S. typhosa* 0901 at 0 C for 1 h before use. For absorption of goat serum, pasteurized cells of *S. typhosa* 0901 were incubated with undiluted serum overnight at 4 C, and the cells were removed by centrifugation. Complement and sera were sterilized by filtration (Millipore HA, 0.22 μm pore size).

Statistical methods. Mean lethal dose (LD_{50}) and mean effective dose (ED_{50}) values were calculated by the logit method (3). Weighted means, relative toxicity, and potency were calculated by the procedure of Bliss and White (4). Results of lymphocyte transformation were compared by Student's *t* test.

RESULTS

Isolation of Vi antigen. By repeated precipitation with Cetavlon, Vi antigen (Vi-Ty 2) was isolated from *S. typhosa*. Immunodiffusion with antiserum against the homologous strain revealed a single, sharp and narrow band. In contrast, Vi-W_1 and Vi-W_2 yielded a blurred, wide band indicating degradation of the antigen. Vi-Ty 2 sensitized sheep erythrocytes and gave positive hemagglutination in Vi antisera against *Citrobacter* 5396/38, *Paracolobactrum ballerup*, and *S. typhosa* Ty 2, respectively. No reaction of the sensitized red cells was observed with anti-O serum of *S. typhosa*, indicating the specificity of the antigen.

Endotoxin properties of the purified polysaccharide. Determination of endotoxin in actinomycin D-treated mice (23) indicated that the Vi-Ty 2 preparation contained about 3.6% endotoxin as referenced to lipopolysaccharide from *S. typhosa* 0901 (Difco, control 542510) (Table 1). Comparing Vi-Ty 2 with the whole-cell, acetone-inactivated tyhpoid vaccine 6A, it was found that Vi-Ty 2 was 1/26th as reactive as vaccine 6A relative to estimated human doses of 50 μg of Vi-Ty 2 and 5×10^8 cells of vaccine 6A (Table 2).

Protective activities in mice. Table 3 compares the protective activity of Vi-Ty 2 with Vi-W_1 and Vi-W_2. Vi-Ty 2 was eight times more

TABLE 1. *Determination of endotoxicity of Vi-Ty 2 in actinomycin D-treated mice*[a]

Preparation	LD_{50} (μg)[b]	Endotoxicity relative to *S. typhosa* lipopolysaccharide as 100%
Vi-Ty 2	1.83 (0.87, 3.82)	3.63 (1.38, 9.62)
S. typhosa 0901[c] lipopolysaccharide	0.067 (0.033, 0.134)	

[a] Adult N:NIH (SW) male mice treated with 12.5 μg of actinomycin D were used. Survival was observed for 3 days.
[b] The 90%-confidence limits are in parentheses.
[c] Difco, control 542510.

TABLE 2. *Comparison of toxicity of typhoid vaccine 6A and Vi-Ty 2 in actinomycin D-treated mice*

Dosage	Vaccine[a]	
	6A ($\times 10^8$ bacteria)	Vi-Ty 2 (μg)
LD_{50}	1.33 (0.76, 2.34)	3.45 (1.99, 5.98)
LD_{50} per human dose[b]	375.94 (213.68, 657.89)	14.49 (8.36, 25.13)
Toxicity per human dose relative to Vi-Ty 2	25.82 (11.76, 56.68)	

[a] The 90% confidence limits are in parentheses.
[b] Human dose: vaccine 6A = 5×10^8 bacteria; Vi-Ty 2 = 50 μg.

TABLE 3. *Comparison of protective activity of Vi-Ty 2 with Vi-W_1 and Vi-W_2*

Antigen	ED_{50} (μg)[a]	Relative potency
Vi-W_1	0.0168 (0.0103, 0.0274)	
Vi-Ty 2	0.0021 (0.0014, 0.0031)	7.92 (4.14, 15.17)
Vi-W_2	0.58 (0.28, 1.18)	
Vi-Ty 2	0.0029 (0.0015, 0.0055)	199.30 (77.23, 514.32)

[a] Weighted geometric mean of two tests; 90% confidence limits in parentheses.

protective than Vi-W_1 and 199 times more protective than Vi-W_2 against a challenge dose of approximately 250 LD_{50} of *S. typhosa* Ty 2.

Based on the estimated human doses, Vi-Ty 2 was nine times more potent in protecting mice than was vaccine 6A (Table 4).

Bactericidal activity of Vi antibody. Serum from a goat immunized with Vi-Ty2 showed bactericidal titer of 1,024 against the homologous strain *S. typhosa* Ty 2 as well as strains M 4702 and E-2322-72 which had been isolated from recent epidemics and shown to be resistant to a variety of antibiotics. After absorption of the serum with *S. typhosa* strain 0901, the bactericidal titer remained practically unchanged. *Citrobacter* 5396/38 was resistant to the bactericidal activity of both unabsorbed and absorbed sera.

Stimulation of lymphoid cells by Vi antigen preparations. Vi-Ty 2 was significantly more active than Vi-W_1 in stimulating transformation of spleen cells from immunized mice (Table 5). Both Vi preparations were slightly mitogenic in cell cultures from control animals which had been given only saline. In control cells, Vi-Ty 2 was consistently but not significantly more active than Vi-W_1. Except in cells from mice immunized with vaccine L, the activities of Vi-W_1 in immune cells were not different from those in cells from control animals.

Experiments were done to assess whether the difference in stimulatory activities between Vi-Ty 2 and Vi-W_1 was due to the contaminating endotoxin in Vi-Ty 2 by mixing lipopolysaccharide from *S. typhosa* 0901 with Vi-W_1 as stimulatory agent. It was shown that the stronger stimulatory activities of Vi-Ty 2 were not caused by endotoxin, because the activities of the mixture of Vi-W_1 and lipopolysaccharide were not significantly different from those of Vi-W_1.

DISCUSSION

Inactivated cellular typhoid vaccines afford significant protection, but no single component of the vaccines has been identified as a protective factor. The ability of vaccines employed in the W.H.O. field trials to stimulate antibodies in animals or man against the O and Vi antigens did not correlate with effectiveness in man (2). Only the ability to stimulate H antibody appeared to correlate with efficacy in the field. However, H antibody was not protective in mice (16), and no immunity was observed in chimpanzees having high H antibody titers induced by vaccination with a rough strain of *S. typhosa* (17). This correlation, therefore, appears to be spurious and may indicate the involvement of other antigens of similar lability (11).

The ability of the O antigen to protect man was tested in a field trial in Poland by using a Westphal-type lipopolysaccharide stimulating

TABLE 5. *Stimulation of mouse spleen cells by Vi antigens*

Immunization of mice	Stimulatory agent of spleen cell cultures	Activity	
		Animal 1	Animal 2
Vi-Ty 2	Vi-Ty 2	10.63	8.70
	Vi-W_1	3.70	2.21
Vi-W_1	Vi-Ty 2	9.70	10.62
	Vi-W_1	4.33	5.61
Vaccine 6A	Vi-Ty 2	9.80	10.08
	Vi-W_1	4.63	4.21
	Vi-W_1 and LPS	3.78	5.11
Vaccine L	Vi-Ty 2	9.83	10.80
	Vi-W_1	6.25	6.98
	Vi-W_1 and LPS	7.40	7.61
Saline	Vi-Ty 2	4.37	4.53
	Vi-W_1	3.56	3.0
	Vi-W_1 and LPS	3.53	3.92

[a] Ratio of counts/min of stimulated to unstimulated spleen cell cultures from same animal. Averages of three tests were used in calculation. With cell cultures from immunized mice, the activities of Vi-Ty 2 are significantly different from Vi-W_1 at 95% confidence level. The activities of Vi-W_1 and Vi-W_1 plus lipopolysaccharide (LPS) are not significantly different.

TABLE 4. *Comparison of protective activity of typhoid vaccine 6A and Vi-Ty 2*

Dosage	Vaccine	
	6A ($\times 10^5$ bacteria)	Vi-Ty 2 (μg)
ED_{50}[a]	1.89 (1.1, 3.23)	0.0021 (0.0013, 0.0034)
ED_{50} per human dose	2,645.50 (1,547.99, 4545.45)	23,809.52 (14,705.90, 38,461.50)
Potency of one human dose relative to vaccine 6A		8.89 (4.32, 18.31)

[a] Weighted geometric mean of two tests; 90% confidence limits in parentheses.

only O antibody. This preparation was without protective effectiveness (11).

Despite its importance as demonstrated in animals (7), the role of the Vi antigen in human immunity has not been clearly delineated. Besides the lack of correlation between circulating Vi antibody and immunity (2), no significant protection was obtained in human volunteers immunized with purified Vi antigen from *Citrobacter* (9). However, there is evidence which argues for a role of Vi antigen in human protection: (i) Vi antigen is characteristic of virulent strains of *S. typhosa* as first demonstrated in mice by Felix and Pitt (7) and recently by Hornick and associates (9) in human volunteers. It may be speculated that it plays a role in attachment and colonization of mucosal surfaces as does the K88 antigen of *Escherichia coli* (10). Local intestinal secretory immunity to Vi could be a critical factor in protection, perhaps by preventing mucosal attachment, as already shown for immunoglobulin A (20). (ii) The mouse assay system which reflects field trial results for the acetone-inactivated and the heat-phenol-inactivated vaccines (15) is highly dependent on Vi antigen, as emphasized by Landy (12) and demonstrated directly by Wong et al. (22). The acetone-inactivated vaccine used in the field trials was superior to the heat-phenol-inactivated vaccine in human and animal protection (5, 15). It was shown that the major difference between these two vaccines was in their Vi antigen. Acetone fixed the Vi tightly to the bacteria, whereas the Vi in the heat-phenol-inactivated vaccine dissolved away (Wong et al., in press). This difference could be responsible for the difference in protection of man with these two vaccines. (iii) The duration of persistent immunity to a single immunizing dose of typhoid vaccine can be as long as 7 years (11). This is reminiscent of the response to other highly polymerized polysaccharide antigens, such as pneumococcal polysaccharide, and supports the contribution of Vi polysaccharide immunity to protection. (iv) Much experimental evidence in animals indicates that cellular mechanisms play a major role in immunity to salmonellae (14, 18). The possible role of Vi antigen in influencing such mechanisms is largely unknown because work in the past on typhoid immunity has concentrated mainly on circulating antibody. The "quality" as well as the quantity of Vi antigen or the manner of its presentation may influence immune responses in a manner that may or may not be directly reflected in circulating antibody level.

The Vi antigen isolated by this method was eight times more protective than Vi-W_1 and 199 times more protective than the Vi-W_2 preparation which had failed to provide significant protection in human volunteers (9). Both Vi-W_1 and Vi-W_2 were not from *S. typhosa* but were isolated from *Citrobacter* 5396/38 by the method of Webster et al. (19), which involved hot-acid hydrolysis for removal of endotoxin. The isolation method described here was quite mild in comparison and appeared to preserve protective activity better. It was also found that Vi-W_1 and Vi-W_2 behaved like a degraded antigen in immunodiffusion in agar gel in contrast to the Vi-Ty 2. Comparing Vi-Ty 2 with the whole-cell, acetone-inactivated vaccine 6A, it was found that Vi-Ty 2 was nine times more potent in protecting mice relative to the estimated human doses of 50 μg of Vi antigen and 5 $\times$ 10^8 cells of vaccine 6A. In assaying for endotoxin in actinomycin D-treated mice (23), Vi-Ty 2 was 1/26th as reactive as vaccine 6A.

It was demonstrated here that Vi antibody possessed bactericidal activity against different strains of *S. typhosa*. Although there is a correlation of bactericidal activity to protection against some gram-negative organisms, in particular the meningococci (8), the role of bactericidal antibody in typhoid fever is not clear. However, it is conceivable that it may have some protective value, especially when the organism invades the blood stream.

Vi-Ty 2 was about three times more active than Vi-W_1 in stimulating transformation of mouse lymphoid cells. It is unlikely that this was due to the slight contamination of endotoxin in the Vi-Ty 2 preparation. The significance of this difference as related to cellular immunity or humoral antibody responses remains to be clarified.

These experiments indicate that the Vi-Ty 2 preparation from *S. typhosa* was substantially reduced in endotoxin content from conventional cellular vaccine, and, in the mouse protection system, was enhanced in its protective effect. Study of this antigen in human volunteers is in progress with emphasis on the cellular immune system, as well as antibody response, particularly intestinal antibody. It is hoped that with a potent antigen preparation from the causative pathogen, the role of the Vi antigen in human typhoid immunity will be better defined.

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