

VI-NEGATIVE STRAINS OF *SALMONELLA TYPHOSA*: ATTEMPTS TO INDUCE W-V REVERSION AND THE USE OF NON-VI STRAINS IN EVALUATING TYPHOID VACCINES

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ABSTRACT

TULLY, JOSEPH G. (Walter Reed Army Institute of Research, Washington, D.C.) AND JULIUS A. CURRIE. Vi-negative strains of *Salmonella typhosa*: attempts to induce W-V reversion and the use of non-Vi strains in evaluating typhoid vaccines. *J. Bacteriol.* **84**:747-753. 1962.—Repeated attempts have been made to detect reversion of several W-form *Salmonella typhosa* strains to Vi antigen-containing cultures. Passage of the O-901, H-901, and Ty2W cultures through various mouse strains did not result in the recovery of V-form typhoid bacilli. Rabbits immunized repeatedly with non-Vi strains of typhoid bacilli, or with W cultures successively passed through mice, did not respond with Vi antibody formation. Attempts also were made to detect reversion of non-Vi strains passed in broth and plate cultures to which heat-killed Vi antigen-containing strains had been added. No evidence was obtained that the selection of Vi-containing cultures had been enhanced. The non-Vi typhoid strains employed in this study thus appeared to be stable W-form cultures. Viable-cell vaccines and acetone-killed and dried (AKD) vaccines of V- and W-form *S. typhosa* were compared in active mouse protection tests against intraperitoneal and intracerebral challenges with V- and W-form typhoid strains. Only Vi-containing cultures effectively protected mice against virulent V-form challenges, regardless of the route utilized. Greater quantities of both viable-cell and AKD vaccines prepared from non-Vi strains were required for protection against V- as well as W-form *S. typhosa* challenges.

(1934) previous study on Vi antigen, were used to develop the concept of V-W variation in Vi and O antigen relationships in strains of *Salmonella typhosa*. These studies showed that most freshly isolated strains of typhoid bacilli were mixtures of V and W forms, the pure V forms possessing sufficient Vi antigen to block agglutination of viable organisms in an O antiserum, while the pure W forms lacked Vi antigen and agglutinated only in O antiserum. However, the introduction of these symbols into the serological classification of *S. typhosa* lost some significance when strains possessing Vi antigen but either devoid of or showing decreased amounts of O antigen (Ty6S, Vi I, etc.) were described (Felix and Pitt, 1935, 1951).

The transition of V to W forms has been commonly observed to occur in the laboratory, and pure, stable V-form strains, containing sufficient Vi antigen for O inagglutinability and exhibiting high virulence for mice (Ty2V, 2593), are relatively rare. In most instances, the selection of non-Vi colonies from mixed V- and W-form *S. typhosa* strains provides stable W forms which maintain this state during continuous subcultivation. Some freshly isolated strains, however, continue to develop both V and W colonies; from such strains it is difficult to isolate pure, stable V or W cultures.

Kauffmann (1936) observed that the H-901 strain, a W-form culture, could give rise to Vi forms after passage in mice. However, he was successful in recovering from a total of 28 mice inoculated intraperitoneally only one Vi colony from a single mouse. This reversion of the H-901 culture was not observed again in a number of other mouse passages. It is of special interest, as pointed out by Felix and Pitt (1951), that the H-901 strain was isolated at the same time and place (Cherson, Russia, 1918) as the well-known Ty2 strain. The H-901 strain was found at that time to be sensitive to O agglutinins while the

The observations recorded by Kauffmann (1935), besides confirming Felix and Pitt's

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Ty2 strain was resistant. Thus, 17 years later, Kauffmann recovered a single Vi colony from the originally Vi-negative H-901 strain. It also should be noted that Kauffmann's "rejuvenated" H-901 strain and the standard Ty2V strain, when typed with Vi phages by Craigie and Yen (1938), were found to belong to the same phage type, E₁.

Standfast (1960a) recently reported on the reversion of several W-form strains of *S. typhosa* to Vi-containing cultures. He reported success in recovering Vi cultures, in some cases by a single mouse passage, from three W-form variants of the Ty2 strain and from the O-901 strain. Schmidt, Lenk, and Herz (1959) also recently published the results of attempts to isolate Vi-positive strains from Vi-negative cultures of *S. typhosa*. They reported the recovery of 3 Vi-positive strains out of 16 Vi-negative strains inoculated intraperitoneally into mice. Only 1 Vi strain was recovered from 12 negative strains serially passed in broth containing typhoid O antiserum.

In an attempt to avoid a mouse protection test based on Vi antigen, or involving the use of a possible W-V form challenge strain (O-901), Standfast (1960b) and Fisek, Gören, and Akyay (1959) reported on typhoid vaccine evaluations with a non-Vi challenge strain (T5501) of *S. typhosa*.

The present study is concerned with the stability of Vi-negative strains and with attempts to induce W-V reversion in such cultures. Experiments were also performed to evaluate the use of non-Vi challenge strains of *S. typhosa* in active mouse protection tests with typhoid vaccines.

MATERIALS AND METHODS

Cultures. The following Vi-negative *S. typhosa* strains were used in this study: H-901, a motile, smooth culture isolated by Weil and Felix (1920); O-901 (WRAIR), a nonmotile variant of H-901, isolated by Felix in 1925 (Felix, 1930) and maintained at this Institute for many years; O-901 (Lister), a nonmotile variant of H-901 maintained at The Lister Institute and received in 1961 from A. F. B. Standfast; Ty2W, a motile, non-Vi variant of the Ty2V strain recovered from a meat-extract stab culture in this department in 1956; T5501, a motile, non-Vi strain received in 1957 from A. F. B. Standfast at The Lister Institute.

Several Vi strains of *S. typhosa* were also employed for comparison. They were the following: Ty2V, an O-inagglutinable V form isolated by Weil and Felix (Felix and Pitt, 1951); 2593 (Illinois Carrier), an O-inagglutinable V form obtained from H. J. Shaughnessy of the Illinois State Department of Health (Edsall et al., 1960); Vi I (Bhatnagar), a V-form culture containing only trace quantities of O and H antigens (Bhatnagar, Speechly, and Singh, 1938).

In addition, a Vi-containing *Escherichia coli* strain (5396/38) and a non-Vi, heterologous O antigen-containing strain of *S. montevideo* (6,7:g,m,s) were also employed in vaccine tests.

All the above strains were maintained on semi-solid meat-extract agar at 4 C and passed twice on the solid form of this medium prior to virulence and vaccine studies. Colonial characteristics of each strain were determined by examination of the culture on the above medium (Landy, 1950). For all virulence tests, vaccine preparations, etc., 5-hr veal infusion agar cultures at 37 C were employed.

Virulence tests. Procedures for intraperitoneal and intracerebral virulence tests were identical to those previously described (Tully and Gaines, 1961a; Gaines and Tully, 1961), with the exception that only C₃H/He mice (obtained from Microbiological Associates, Bethesda, Md.) were employed. Table 1 lists the average intracerebral and intraperitoneal LD₅₀ values for several *S. typhosa* cultures in this mouse strain. Serological characteristics of each challenge strain were determined at the time of mouse inoculation, using standard tube agglutination tests as noted below.

Protection tests. Active mouse protection tests were carried out with either acetone-killed and dried vaccines (AKD), as previously described (Tully and Gaines, 1961a, b), or with living vaccines. In the case of viable vaccines, survivors at 6 days after intraperitoneal challenge were rechallenged with approximately 10 LD₅₀ doses of either V- or W-form *S. typhosa* by the intraperitoneal route or 75 to 100 LD₅₀ doses by the intracerebral route. All ED₅₀ values were calculated by the method of Reed and Muench (1938).

Serological procedures. Slide agglutination tests were employed for preliminary examination of *S. typhosa* cultures recovered from mouse

TABLE 1. Virulence of *V*- and *W*-form *Salmonella typhosa* strains in *C₃H* mice

<i>S. typhosa</i> strain	Antigens in strain	Average LD ₅₀ (no. bacilli)	
		Intracerebral	Intraperitoneal
<i>V form</i>			
Ty2V.....	Vi, O, H	2.9 × 10 ²	3.8 × 10 ⁶
2593.....	Vi, O, H	2.5 × 10 ²	6.7 × 10 ⁶
Vi I.....	Vi	1.0 × 10 ⁶	2.06 × 10 ⁷
<i>W form</i>			
O-901 (WRAIR)	O	3.7 × 10 ⁴	5.06 × 10 ⁸
O-901 (Lister)	O	4.3 × 10 ⁴	3.11 × 10 ⁸
H-901.....	O, H	1.3 × 10 ⁴	3.26 × 10 ⁸
Ty2W.....	O, H	1.1 × 10 ⁴	1.89 × 10 ⁸
T5501.....	O, H	2.4 × 10 ⁴	2.09 × 10 ⁸

TABLE 2. Agglutination of *Salmonella typhosa* strains in *Vi* and *O* antisera

<i>S. typhosa</i> strain	Rabbit <i>Vi</i> antiserum ^a		Rabbit <i>O</i> antiserum ^b	
	Viable antigen	Boiled antigen	Viable antigen	Boiled antigen
<i>V form</i>				
Ty2V.....	640 ^c	10	80	2,560
2593.....	640	10	80	2,560
Vi I.....	320	Neg ^d	Neg	Neg
<i>W form</i>				
Ty2W.....	Neg	Neg	2,560	2,560
O-901.....	Neg	Neg	2,560	1,280
H-901.....	Neg	Neg	1,280	1,280
T5501.....	Neg	Neg	2,560	2,560

^a Prepared against *Paracolobactrum ballerup*.

^b Prepared against *S. typhosa* O-901.

^c Reciprocal of agglutinin titer.

^d Less than 1:10.

passage as well as those isolated after repeated cultural transfer. For these tests, rabbit antisera to *Paracolobactrum ballerup* (*P. intermedium*) and *S. typhosa* O-901 were used in determination of *Vi* and *O* antigens, respectively. Tube agglutination tests, employing the same *Vi* and *O* antisera, also were used for testing isolates as well as for confirming the serological characteristics of selected cultures prior to mouse passage. The results of agglutination tests carried out with both viable and heat-killed antigen preparations are presented in Table 2. For more detailed analysis of the antigenic structure of those typhoid cultures used in mouse and cultural passage, rabbits were immunized with increasing amounts of viable cell preparations and antisera recovered 7 days after final intravenous inoculation. The *Vi* hemagglutination procedure of Landy and Lamb (1953) was used to examine these antisera for presence of *Vi* antibody.

RESULTS

Mouse passage of W-form S. typhosa. Preliminary trials were first performed on *C₃H* mice by utilizing an intraperitoneal passage of the two O-901 strains and the H-901 strain of *S. typhosa*; 24 hr after inoculation with from 10⁴ to 10⁸ organisms of these strains, the spleens of individual mice were ground in a sterile mortar and each mixture was streaked onto at least two meat-extract agar plates and incubated overnight at 37 C. At least five colonies were selected

from each plate and transferred to meat-extract agar slants. The growth from these slants was then examined in slide and tube agglutination tests with *Vi* and *O* antisera. Appropriate controls consisting of colonies recovered from spleens of mice receiving the Ty2V strain also were examined at the same time and in the same manner. Specificity of *Vi* slide agglutination results was determined by heating antigen suspensions to 100 C and observing agglutination of the Ty2V antigen in the presence of *O* antiserum.

No evidence of any change in the serological properties of the O-901 or H-901 strains was obtained. However, four O-901 cultures which showed some slight colonial changes were recovered from mice. These strains, along with the original O-901 (Lister) strain, were tested in *Vi*-phage cultures through the kindness of P. R. Edwards of the Communicable Disease Center, Atlanta, Ga. All were reported as *W* forms. Variations in time interval between intraperitoneal inoculation and autopsy of mice did not show presence of colonies containing *Vi* antigen. Additional trials were carried out with different strains of mice (BALB/c, Cinnamon, Bagg); other routes of inoculation (subcutaneous, intracerebral); and with other *W*-form strains of typhoid bacilli (Ty2W, T5501). All results were negative.

Subsequent attempts were then made to perform repeated intraperitoneal passage of non-

Vi strains of *S. typhosa* in mice. Continuous mouse passages of both the O-901 (WRAIR) and O-901 (Lister) strains were maintained for approximately 3 days before the number of organisms declined to a point where they could not be recovered from spleens. At no time were Vi-positive colonies detected in cultures recovered from these mice.

Additional attempts were made to detect Vi antibody in rabbits after immunization with mouse-passed cultures of the O-901 (Lister) strain. Approximately 5×10^7 cells of this strain were inoculated intraperitoneally into Cinnamon mice. After 48 hr, the spleens of mice were removed, ground in a sterile mortar with a small amount of saline, and 0.1 ml was streaked onto two meat-extract plates. The resulting growth was examined for colonial characteristics, the plate was then harvested, and 10^9 cells/ml were prepared for intravenous inoculation into two rabbits. Slide agglutination tests were performed on this antigen. Approximately 5×10^7 cells of this preparation were again administered (ip) to mice. This passage procedure was continued for at least 2 weeks, the rabbits each receiving increasing doses of the viable cells prior to subsequent mouse passage. Rabbits were bled at various intervals, and high-titer O antisera were obtained without any evidence of the development of Vi antibody, as measured by standard tube agglutination and hemagglutination techniques.

Several trials also were made in attempts to detect Vi antibody in mice after intraperitoneal inoculation with viable cells of the H-901 and O-901 strains. Again, no Vi antibody was found in pooled mouse antisera to repeated inoculations with 5×10^7 viable cells of each of these strains, although a single inoculation with as few as 10^5 cells of Ty2V will give Vi hemagglutinin titers from 1:30 to 1:60 in comparable mouse pools.

Cultural passage of W-form S. typhosa. The method described by Felsenfeld (1954) was first employed in attempts to obtain W-V reversion by cultural adaptation. The two O-901 strains and the H-901 strain were serially passed in Tryptose Broth (Difco) containing 10^6 cells/ml of heat-killed Vi I (Bhatnagar) strain. At intervals of 18 to 20 hr, single loops of the broth cultures were plated out on Tryptose and meat-extract agar plates and subcultures made to

tubes containing broth and freshly prepared heat-killed V-form cells. Growth on the plates was examined by oblique light; any variant colonies, as well as at least five other colonies per plate, were transferred to meat-extract agar stabs for use as antigen in slide agglutination tests. Some colonial changes were observed with both O-901 and H-901 strains, but when these were tested in slide agglutination procedures they appeared to have lost some of their O antigen. Slight agglutination was noted in both Vi and O antisera as well as in the saline control. Several of these colonial variants were used to immunize rabbits, and the resulting antisera were found to contain only antibodies to the O component. At no time during the 14-day serial passage of W-form *S. typhosa* strains in broth cultures containing heat-killed Vi I cells were we successful in recovering either pure Vi variants or mixtures of W-V form bacilli.

Several attempts were made to repeat a plating procedure (Booy and Wolff, 1952) reportedly successful in producing Vi forms from H-901. Large quantities of *P. ballerup* cells were grown on a synthetic medium, and the mixture was heated to 56 C for 2 hr. The wet-packed cells were added to melted nutrient agar, plates poured, and the H-901 strain streaked onto the surface. From 80 to 100 colonies were picked from these plates, transferred to Nutrient Agar (Difco) slants, and tested in slide agglutination procedures with Vi and O antisera. Several Vi-containing colonies were recovered but, on more thorough check, were found to be *P. ballerup* cultures that were not killed by the heating procedure. All other isolated colonies conformed to the serological characteristics of the H-901 strain.

Viable vaccines in protection tests. Viable vaccines prepared from both V- and W-form *S. typhosa* strains were compared in active mouse protection tests against intracerebral and intraperitoneal challenges with the Ty2V and T5501 strains. The results (Table 3) show that significant protection against a virulent Ty2V challenge was offered only by viable-cell vaccines containing Vi antigen. When immunized mice were challenged with the T5501 strain of *S. typhosa*, no great differences were noted between the V- and W-form viable-cell vaccines. The number of non-Vi typhoid bacilli required for protection against the Ty2V challenge was approx-

TABLE 3. Comparison of V- and W-form viable *Salmonella typhosa* vaccines in mouse protection tests against V- and W-form challenges

Viable <i>S. typhosa</i> vaccine	Antigens in vaccine	ED ₅₀ (no. bacilli) against <i>S. typhosa</i> challenge with strain:			
		Ty2V		T5501	
		Intracerebral (3 × 10 ⁴) ^a	Intraperitoneal (3 × 10 ⁷)	Intracerebral (2 × 10 ⁶)	Intraperitoneal (1 × 10 ⁹)
<i>V form</i>					
Ty2V.....	O, H, Vi (9,12:d)	27,300	2,900	1.67 × 10 ⁵	3.96 × 10 ⁵
2593.....	O, H, Vi (9,12:d)	16,100	1,450	1.11 × 10 ⁵	2.21 × 10 ⁵
<i>W form</i>					
Ty2W.....	O, H (9,12:d)	1.82 × 10 ⁷	1.58 × 10 ⁶	3.61 × 10 ⁶	2.07 × 10 ⁵
T5501.....	O, H (9,12:d)	9.8 × 10 ⁷	3.68 × 10 ⁶	5.99 × 10 ⁶	2.88 × 10 ⁵

^a Number of organisms in challenge.

TABLE 4. Comparison of V- and W-form acetone-killed and dried (AKD) *Salmonella typhosa* vaccines in mouse protection tests against V- and W-form challenges

AKD vaccine	Antigens in vaccine	ED ₅₀ values (ml) against <i>S. typhosa</i> challenge with strain:			
		Ty2V		T5501	
		Intracerebral (3 × 10 ⁴) ^a	Intraperitoneal (3 × 10 ⁷)	Intracerebral (2 × 10 ⁶)	Intraperitoneal (1 × 10 ⁹)
<i>V form</i>					
Ty2V.....	O, H, Vi (9,12:d)	.0006	.00010	.145	.218
2593.....	O, H, Vi (9,12:d)	.0004	.00005	> .400	.200
Vi I.....	Vi	.005	.00033	.331	.269
<i>W form</i>					
Ty2W.....	O, H (9,12:d)	.116	.056	.100	.244
O-901.....	O (9,12)	.314	.011	.203	.182
T5501.....	O, H (9,12:d)	> .50	> .10	.100	.191
<i>Misc.</i>					
<i>E. coli</i> 5396/38.....	Vi	.0077	.00001	> .40	.180
<i>S. montevideo</i>	O, H (6,7:g,m,s)	> .50	.50	> .40	.200

^a Number of organisms in challenge.

imately equivalent to the number of V or W bacilli found necessary for protection against T5501 challenge, regardless of the route employed.

AKD vaccines in protection tests. The results of active protection tests with AKD vaccines

against V- and W-form *S. typhosa* challenges are presented in Table 4. Excellent protection against the Ty2V challenge was again found with those vaccines containing Vi antigen. Greater protection was also evident when the intraperitoneal method was used. However, these comparisons

must take into consideration differences in the number of organisms employed for challenge in the two procedures. Vaccines prepared from non-Vi strains of *S. typhosa* again failed to offer effective protection against the Ty2V challenge. The results with the control vaccines add support to the well-known protective capacity of Vi antigen in the mouse test. The Vi-containing *E. coli* strain protected mice against the virulent Ty2V strain, while the *S. montevideo* strain, containing O and H antigens different from those in typhoid strains, showed no protection against this same Ty2V challenge.

Mice immunized with AKD vaccines and challenged with the T5501 strain of *S. typhosa* provided verification of the observations recorded for viable-cell vaccines against a W-form challenge. Regardless of the route employed for challenge, little protection was evident with either Vi-containing vaccines or vaccines prepared from strains of *Salmonella* possessing only O or H antigens. It was not possible to distinguish V- or W-form typhoid vaccines on the basis of the T5501 challenge by either route.

DISCUSSION

The early work of Felix and Pitt (1934), subsequently verified by many workers, showed that most virulent strains of typhoid bacilli contain an abundance of Vi and O antigens. The presence of only one of these antigens in a strain is sufficient to decrease its virulence for mice, as measured either by the intraperitoneal (Felix and Pitt, 1951) or the more sensitive intracerebral route (Gaines and Tully, 1961). The most widely known Vi-negative strains of *S. typhosa*, H-901 and O-901, have been used extensively in serological procedures, primarily because of their antigenic stability. Felix and Pitt (1951) described the history and background of these two W-form typhoid strains and indicated that, except for the report of Kauffmann, it has not been possible to induce reversion to the V form in these strains.

Studies in our laboratory (Gaines, Tully, and Tigertt, 1961; Tully and Gaines, 1961a) involved examination of variants recovered from the brains of mice after intracerebral inoculation with selected *S. typhosa* strains. In studies where purified Vi antigen had been added to an intracerebral inoculum of the Ty2W strain, we were unsuccessful in recovering from mouse brain

typhoid strains which had reverted to the V form; all cultures conformed to the serological characteristics of the original Ty2W strain. Colonial variants were recovered from brains of mice receiving the H-901 strain but, upon detailed serological study, these isolates were found to have lost some of their H antigen. No evidence was obtained for the presence of Vi antigen in these variants. Additional attempts were made to induce W-V reversion in non-Vi strains of *S. typhosa* after passage in chimpanzees (Tully, Gaines, and Tigertt, 1962). Cultures of the O-901 strain, recovered from the blood and stools of two chimpanzees, were found to be antigenically identical with the original infecting culture, and still maintained the same intracerebral and intraperitoneal virulence LD₅₀ for mice as the original strain. This was also found to be true with cultures recovered from chimpanzees challenged with a variant of the Ty2W strain.

The present investigations have once again been unsuccessful in recovering Vi antigen strains from W-form *S. typhosa* cultures. Large numbers of mice have been given non-Vi typhoid strains by different routes and at various time intervals without any discernible evidence of W-V reversion. Only limited phage typing of recovered strains was carried out. However, in our laboratory, successful methods for V-form *S. typhosa* detection have included serological procedures performed on cultures recovered from mice, the immunization of mice and rabbits with these strains, and virulence tests.

The failure to obtain evidence for induction of V forms in non-Vi typhoid strains by passage in broth cultures containing Vi antigen may possibly result from changes in the characteristics of the O-901 and H-901 strains after repeated laboratory passage or changes in the state of Vi antigen on our heat-killed Vi I (Bhatnagar) cells. However, Vi antigen from *P. ballerup* cells was also ineffective in transforming these two strains.

Mouse protection tests with viable and AKD typhoid vaccines confirmed results of a previous study (Tully and Gaines, 1961a). Mice can be protected against a virulent challenge with non-Vi typhoid vaccines but, as shown by this study and the findings of Standfast (1960a), very large numbers of the W-form cells are required. There appears to be significant protection against a V-form challenge only when vaccines, either viable or AKD, are prepared from *S. typhosa*

strains possessing Vi antigen. No distinct separation of V- and W-form vaccines was evident when the W-form challenge strain (T5501) was employed, and a vaccine prepared from *S. montevideo* was just as effective in protecting mice against this challenge as was a *S. typhosa* Ty2V vaccine.

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