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Immunogenicity of the VI antigen

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Thesis

IMMUNOGENICITY OF THE VI ANTIGEN

by

MARGARET ANN JOHNS

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Approved

by

First Reader . . . E. E. Baker . . . .
Professor of Microbiology

Second Reader . . Matthew A. Derow . . . .
Assistant Professor of Microbiology

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Purpose of this Investigation

Several bacterial polysaccharides, in particular the capsular polysaccharides of Diplococcus pneumoniae which act as haptens in rabbits, have proven to be antigenic in mice and man. Some haptens have been made antigenic by methods of increasing particle size or by mixing with adjuvants (45). Antisera against purified Vi antigens would prove a valuable tool for studying the effects of purification on the antigenic molecule. These antisera would be especially helpful in studying the effects of deacetylating the antigen since there are some indications that there are serologic differences in purified Vi antigens after deacetylation (74). The purpose of this investigation was to attempt to produce such antisera in rabbits by using various methods to increase the antigenicity of the Vi molecule and to study these sera for serologic differences. Mice were also immunized with purified Vi antigen in order to compare the response of mice and rabbits to the same purified Vi antigen preparations.

Discovery of Vi Antigen

In 1934, Felix and Pitt observed a new antigen in some strains of Salmonella typhosa which had been freshly isolated from the blood of typhoid patients (24). Until then, typhoid organisms had been known to contain at least 2 heat stable O components in the somatic fraction and one specific H antigen in the flagellar fraction (3). Strains possessing this new antigen were inagglutinable in O antiserum and appeared to be more virulent for mice than strains lacking it (23,24). For this reason, Felix and Pitt called this new antigen "Vi" or virulence antigen. The discovery of Vi antigen and the possibility that it might be the virulence antigen led to studies of its role in severity of attack and immunity to typhoid fever.

Vi antigens which were serologically similar have been found in almost all freshly isolated strains of Salmonella typhosa (24), in strains of Salmonella hirschfeldii (S. paratyphi C) (61), in Paracolobactrum ballerup (42), and in Escherichia coli (40).

Giovanardi (33) was the first to observe that, on solid culture media, Vi-containing colonies were more opaque than non-Vi-containing colonies. Kauffman (39) called colonies containing Vi antigen V forms (viel) and those which had lost Vi antigen W forms (wenig). In his terminology, V-W forms were those organisms which still possessed Vi antigen but showed some O agglutinability and V-W degradation was the process by which bacteria lost Vi antigen. Kauffman believed that even W forms retained Vi potentialities and under certain conditions might regain Vi antigen. Biochemical reactions of Vi-containing strains were found to be no different from those of strains lacking Vi antigen (3).

Relationship of Vi Antigen to Virulence

Felix and Pitt (23,24), in work with mice, observed that the presence of Vi antigen in S. typhosa rendered the bacteria resistant to the action of O antibodies in vivo as well as in vitro. This antigen also increased the ability of the bacteria to invade their host. Other workers (40,21,2) substantiated a qualitative relationship between the presence of Vi antigen in smooth (containing O antigen) typhoid cultures and their ability to kill mice injected intraabdominally with a standard dose of one-hundred million organisms. It was generally agreed (3), however, that virulence was not a function of Vi antigen alone but of the combined action of Vi and O antigens. Organisms possessing either antigen alone or both antigens in unequal proportions were less virulent than organisms having more nearly equal amounts of both (24,28).

The mechanism by which Vi antigen enhanced the ability of typhoid organisms to kill mice was not entirely known. The increased invasive power of the organisms postulated by Felix and Pitt (23) had not actually been demonstrated. In fact, Ørskov and Kauffman (59) found no difference between the invasive ability of Vi-containing and non-Vi-containing strains or in the survival of mice when they were given sublethal doses of these strains. They found that both V and Ẅ forms of the Watson Strain of S. typhosa remained in the spleens of inoculated mice for up to seven days after inoculation of one million organisms intraabdominally or intravenously even though the animals remained healthy. H-901 strain and the V variant of H-901 described by Kauffman (39), were found in the spleens for at least thirteen days. Grasset and Lewin (35) found that there was even less invasion of the blood stream by Vi strains* than by non-Vi strains

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*For brevity Vi-containing strains, cultures etc. will be referred to as Vi strains, cultures etc. and strains lacking Vi referred to as non-Vi strains throughout the rest of this paper.

when larger doses of the organisms were given. Ørskov and Kauffman and Grasset and Lewin postulated a greater toxicity for Vi forms than for non-Vi forms. They assumed that larger doses so weakened the injected animals that their normal inhibitory mechanisms were overwhelmed. **Felix and Pitt,** (24) however, demonstrated that killed suspensions of virulent organisms were no more toxic for mice than killed non-virulent organisms. Topley and coworkers (69) observed that alcohol precipitates of acetone dried extracts were no more toxic when derived from Vi cultures (Rawlings strain) than from Non-Vi cultures (H-901 strain). Smith (63) found that the toxicity of aqueous extracts for mice and rabbits did not depend upon their content of Vi antigen. She observed that Vi and non-Vi strains were equally toxic for mice; 0.5 ml of either type of suspension killed mice uniformly. The toxicity of these suspensions for rabbits was more variable but averaged 0.5 ml/kg regardless of the strain used. The toxic effect was not altered by boiling the extracts for one hour.

Almon (3) postulated that faster multiplication of Vi organisms than of non-Vi organisms might be responsible for their greater virulence, actual death then being caused by intoxication. In support of this theory, Bhatnagar (11) has reported that Vi organisms are less easily phagocytized than non-Vi forms.

In 1951, Batson, Landy and Brown (9) defined virulence as the result of a combination of factors pertaining to the organisms, the host, and the conditions under which the pathological state was established. They then compared three methods for measuring virulence of two Vi and two non-Vi strains of <u>S. typhosa in</u> mice:

1) intracerebral inoculation of organisms
2) intraabdominal inoculation of organisms in saline
3) intraabdominal inoculation of organisms in mucin

Differences in virulence among strains based only on the possession of Vi antigen could not be demonstrated when the intracerebral route of inoculation was used. In fact, one of their Vi strains was non-virulent when inoculated in this manner. There was, however, a difference in behavior of the two types of strains when the "intraperitoneal saline" method was used, which became more marked when mucin was added to the organisms injected. In view of these results, they concluded that the differences in virulence of the strains was independent of the presence or absence of Vi antigen but rather dependent on the method of testing for virulence. This supported Kauffman's view that Vi antigen alone was not responsible for virulence (40). A report in 1961 by Gaines and Tully also supported this view (32). They observed that intracerebral inoculation was a more sensitive method than intraabdominal injection in assaying for differences in virulence among strains of S. typhosa. They postulated that the reason for this was that immediate contact with phagocytic cells in the peritoneal cavity eliminated many of the organisms injected there. If Bhatnagar's observations were correct, this would explain why Vi forms appeared to be more virulent upon intraabdominal injection although by intracerebral injection virulence was independent of the presence of Vi antigen. In fact, in 1951 Felix and Pitt (28) had admitted that the protective effect of Vi antigen was probably more important than any invasive ability the antigen might confer upon the organism.

Immunogenicity of Vi Antigen

Felix and Pitt were able to demonstrate Vi antibody by agglutination and absorption tests in sera from mice immunized with virulent (Vi) organisms (24). The *in vitro* titer of Vi antibody in their results was low as compared to the titer of O antibody but immunized mice were protected against challenge with virulent *S. typhosa*. They also were able to protect mice with rabbit antisera in which Vi antibodies could not be demonstrated by agglutination techniques.

Smith was the first to perform a systematic study of immunization of rabbits with Vi and non-Vi strains (63). She vaccinated rabbits with water extracts of several strains of *S. typhosa*. The resulting titers of H and O antibodies were fairly consistent among the strains but there was great irregularity of Vi antibody production. Not all rabbits immunized with the same strain produced antibodies and no Vi antibodies were produced by animals immunized with either the Ty2 or Watson strains. In all cases the titers of H and O antibodies were much higher than the titers of Vi antibody. Stuart and Kennedy (67) obtained similar results on Vi antibody production in rabbits. Špaun (65) in 1954 showed that the quantity of Vi in a strain has an influence on the titers obtained. He found that the cultures DII and Watson showed the smallest Vi content by quantitative antigen analysis. These strains also gave the lowest antibody titers in rabbits immunized with heat treated cultures.

Significance of Vi Antigen in Typhoid Vaccines

During the years following the discovery of Vi antigen and its relationship to virulence for mice, the persisting concept was that the organisms used for vaccine production had to be highly virulent (contain Vi antigen) and recently isolated for best immunizing effects. There was, however, no general agreement as to the immunogenic significance of Vi antigen other than that Vi antibodies protected mice against challenge with virulent S. typhosa. Since Vi antigen was thought to be heat labile at that time and phenol destroyed it in culture (24) questions arose as to the protective potency of the heat-killed, phenol-preserved vaccines used for human immunization.

In 1941, Felix introduced an alcohol-killed and alcohol-preserved typhoid vaccine (27). He found more Vi antibody in sera from human beings immunized with this alcohol vaccine than those immunized with heat-killed, phenol-preserved vaccine. In 1947, Drysdale (20) compared heat-killed, phenol-preserved vaccine and alcohol killed and preserved vaccine made at the same time from a culture of S. typhosa, strain Ty2. Vi agglutination titers of sera from immunized human beings and rabbits were higher for the alcohol treated vaccine. Mouse protection tests demonstrated no difference between the two vaccines.

In the United States, the significance of Felix's discovery was slow to be realized. Siler et al (62), in 1941 published a monograph on typhoid immunization in which stress was put on the virulence of a typhoid strain to be used in heat killed vaccine without mention of the significance of Vi antigen. They failed to investigate various strains for comparative Vi antigen content. In 1943, Longfellow and Luippold (52) discovered that B. ballerup and E. coli strains containing Vi antigen could be used for a vaccine to protect mice against typhoid infection. In 1944 and 1946, Luippold (53,54) suggested improving the United States Army typhoid vaccine by adding E. coli Vi antigen extract to the vaccine. Spaun (66) attributed this rather indirect discovery of the significance of Vi antigen to the fact that the typhoid strain 58 (Panama carrier) used by the United States Army for vaccine production was low in Vi antigen content and Vi antibody was hard to detect in immune sera. The E. coli strain 5396/38, on the other hand, had a much greater amount of Vi than even strain Ty$_2$, the most virulent strain for human beings.

In 1950, Batson, Landy and Brown (10) showed that four typhoid strains from the same source (Panama carrier), two virulent and two avirulent, all protected mice against infection with virulent organisms (intracerebral challenge) disproving a direct relationship between virulence and immunogenic activity when intracerebral inoculation was performed. This raised the question of the need for Vi antigen in a vaccine. In 1953 and 1957, however, Landy (47,49) showed that vaccines in which Vi antigen had been retained were better immunizing agents. He performed mouse protection tests on mice that had been immunized with one of three vaccines;

1) an O variant of S. typhosa, strain 58
2) a heat-killed, phenol-preserved vaccine from a strain containing both Vi and O antigens
3) an acetone dried vaccine containing both Vi and O antigens

He found that the acetone dried preparation gave best results, heat-phenol preparation fair, and the O preparation very little if any protection. The heat-phenol vaccine gave some passive protection without demonstrable Vi hemagglutinins. This protection was due to Vi antibody because sera absorbed with Vi-coated erythrocytes no longer protected mice. This supported Drysdale's earlier observation that heat-killed and alcohol-killed vaccines protected mice to an equal degree.

The results of these several investigations seemed to support the view that phenol altered Vi antigen somehow so that a great deal of its immunogenicity was lost. Vaccines prepared with organic solvents seemed to give the best results. Alcohol-killed and preserved vaccines yielded the highest Vi antibody titers, but potency of the vaccine was rapidly lost at room temperature (27). Formalinized vaccines were also excellent but acetone dehydrated vaccine seemed to offer the most stable preparation since it could be stored in a dehydrated state and reconstituted with water prior to use (49). None of the vaccines have been adequately proven in human field trials as the conditions of the few trials made have not been satisfactorily controlled (66).

Purification of Vi Antigen

Chemical separation of Vi antigen from the rest of the bacterium was attempted shortly after the initial discovery of its significance in virulence for and protection of mice. The purpose for this line of investigation was two fold; first, the production of non-toxic protective vaccines for human immunization and second, determination of the chemical and biological properties of the antigen without interference from other cellular components. Purified preparations perhaps would settle the controversy over the role of Vi antigen in virulence and immunity to infection.

Early methods of purification were rather harsh, probably injuring the antigen chemically and thus immunogenically. Boivin and Mesrobeanu (12,13) prepared a Vi antigen fraction from protein free trichloroacetic acid extracts by uranyl salt fractionation. Since Freeman and Anderson (29) couldn't repeat this separation from an extract containing both Vi and O antigens, they concluded that Boivin and Mesrobeanu's preparation was not free from O antigen contamination. Henderson and Morgan (37) obtained a

Vi preparation, at least partially purified, by anhydrous diethylene glycol extraction of rough forms (lacking or having only traces of O antigen) of various strains of S. typhosa. This preparation protected mice against challenge with S. typhosa, stimulated Vi antibody production in rabbits, and reacted with Vi antisera in precipitin and complement-fixation tests. They observed that sera made with this extract didn't protect mice to the same degree that sera made from whole organisms did. Also, as stated before, heat-killed suspensions of typhoid organisms didn't produce detectable antibodies in large amounts but were immunogenic in mice. Because of these findings, Craigie and Brandon (17) assumed that Vi was a complex antigen, designating the agglutinogenic portion as V and the entire complex as Vi. They felt that the entire complex was necessary for immunization but only the V portion for serologic and protective reactions to occur. Henderson and Morgan (37) however, claimed that Vi was a single compound altered physically, chemically, and antigenically by the purification process making the extract less potent than whole organisms in inducing antibody formation. They also considered the possibility that Vi might be a single compound possessing two or more antigenic radicals.

Ashida (4) in 1949 prepared a purified antigen from S. typhosa, strain Ty_2. He called his preparation Vi Specific Substance (Vi.S.S.). The method of purification involved extraction of the antigen with 0.1 N NaOH for four hours, probably removing O-acetyl groups. This preparation was found to react in vitro but not to be antigenic in vivo. Vi.S.S. precipitated with anti-Vi sera made with Vi-containing organisms but would not precipitate with antisera against O organisms, showing the preparation to be free from detectable O antigen. Immunization of rabbits using three 0.5 mg injections of Vi.S.S. induced no detectable antibody formation. Ashida, therefore, decided that Vi.S.S. was a hapten. Landsteiner has defined a hapten as a non-protein substance which, although reactive *in vitro*, induces no or only a silght antibody response. It has been mentioned above that Vi antibody production in rabbits was poorer than in mice but Ashida performed no experiments in mice to compare with his experiments in rabbits.

In 1952, Webster, Landy and Freeman (70) obtained a Vi preparation from *E. coli* by ethanol-salt fractionation of water extracts of agar-grown bacilli followed by hydrolysis with 1 M acetic acid at 100 C for 24 hours. to remove O antigen. By serologic tests this preparation proved free from O antigen. It was antigenic for mice and non-toxic in doses up to and including 5 mg. In contrast to Ashida's results, this preparation was also antigenic for rabbits. However, larger amounts of purified antigen than of acetone dried V form bacilli were required to induce antibody formation. Human immunizations were also performed with this preparation. As little as 10 µg of this purified Vi (from *E. coli*) induced antibody formation in man comparable to that induced by three injections of 0.5 ml (400 µg total) of acetone-killed and dried *S. typhosa,* strain Ty2. The response to 40 µg in human subjects was comparable to Vi antibody levels observed in patients recovering from typhoid fever. Webster, *et al* (70) also prepared antigens from *S. typhosa,* strain Ty_2 and *P. ballerup*. The *E. coli* preparation proved to be most effective in immunizing rabbits against typhoid infection, *P. ballerup* next, and *S. typhosa*, strain Ty_2 least effective.

Both Ashida and Webster, *et al* succeeded in destroying the toxicity of their preparations but used harsh methods to do so. As stated before, the alkali extraction of Ashida probably removed all the O-acetyl groups of the antigen. Acid treatment could have degraded a portion of the antigen. Baker, Whiteside, Basch and Derow (7) in 1959 perfected a method for extraction of Vi antigen without using acid, alkali, or heat. It was felt that this preparation was more like that present in the living organisms than those prepared by more drastic means. Briefly, the method of purification consisted of alcohol fractionation of a saline suspension of acetone-dried organisms and treatment with cetyltrimethylammonium bromide in instances where O antigen contamination was still evident (7). Antigens were prepared in this manner from <u>P. ballerup</u>, <u>E. coli</u>, <u>S. typhosa</u>, strain Ty_2, and <u>S. typhosa</u>, strain 61. These preparations were shown to be free from O antigen by a modification of the agar diffusion technique of Ouchterlony (7). This material was not antigenic for rabbits given a total of 3.7 mg in six injections and only slightly antigenic in rabbits given 8.75 mg in twelve injections. Crude O antigen-containing preparations had been found to be good immunizing agents so attempts were made to immunize rabbits with purified Vi antigen mixed with O antigen without success. Also, rabbits were inoculated with Vi mixed with acetone killed and dried <u>P. ballerup</u> organisms with no success. Large doses, totaling 90 mg, did not induce antibody formation to an appreciable extent. On the other hand, these Vi antigen preparations immunized mice against challenge with living <u>S. typhosa</u> Ty_2. <u>E. coli</u> appeared to be more effective than the other preparations. When experiments were repeated using another strain of mice however, all preparations seemed to be equally effective. Purified Vi antigen in amounts as low as 0.5 µg also induced Vi antibody formation in man. (8)

Chemical Characterization of Purified Vi Antigen

Webster, Clark and Freeman (71), and Clark, McLaughlin and Webster (15), found that the acid hydrolysis product of purified Vi antigens of *S. typhosa* *P. ballerup*, and *E. coli* was an aminohexuronic acid hydrochloride containing two molecules of water of crystalization. From analytical results, they determined that Vi antigen was a polymer of N-acetylaminohexuronic acid units in alpha glycosidic linkage. These findings were confirmed by Heyns, *et al* (38) and the aminohexuronic acid shown to be aminogalacturonic acid by synthesis. Baker, *et al* (7) found that in addition to the N-acetyl groups there were also O-acetyl groups attached to carbon atoms.

MATERIALS AND METHODS

Extraction and Purification of Vi Antigen

Vi antigens prepared from Salmonella typhosa, strain Ty_2, Paracolobactrum ballerup, strain 481, and Escherichia coli, strain 136 were used in this study. Deacetylated Vi antigen derived from P. ballerup, strain 481 was also used.

Vi antigens were prepared from the various organisms by the method of Baker, et al (7). The medium for culturing the organisms consisted of 15 liters of one per cent casamino acids (Difco) broth (pH 7.2) autoclaved in a 5 gallon Pyrex jug. In the afternoon, the medium was inoculated with 5 ml of a young broth culture of the organisms to be grown. At the time of inoculation, 5 ml of phenol red and the dialysate from 5 g of Brewer's yeast sterilized by filtration were added to the jug. One to two grams of DL-tryptophane were also added to the medium for cultivation of Salmonella typhosa. The next morning the bottle was fitted with a stirring mechanism and an aeration tube. Two hundred grams of sterile dextrose and a few drops of tributylphosphate (to prevent foaming) were added and the organism grown for 6 hours at 30-34 C with constant stirring and aeration. As acid was produced by the growing bacteria (indicated by the phenol red solution), 5 per cent NaOH was added drop by drop to neutralize the culture. When the growing period was completed, 1.5 volumes of technical grade acetone were added to precipitate the organisms. After thorough stirring, the preparation was allowed to remain overnight at room temperature to complete the precipitation. The next morning the precipitated organisms were collected by centrifugation and dehydrated with acetone. Excess acetone was removed by drying in air.

Vi antigen was extracted from 100 g of organisms prepared in this manner. The dried organisms were suspended in 0.15 M NaCl. After two hours, two volumes of 95 per cent alcohol were added. The alcohol precipitate, composed of bacilli plus Vi and O antigens, was collected in centrifuge tubes and extracted with 60 per cent ethanol in 0.15 M NaCl at 37 C for 18-24 hours. This step was repeated several times. The extracts were combined and the precipitate collected by centrifugation at 0 C. It was then redissolved in 60 per cent ethanol and precipitated at 0 C. The resulting precipitate contained Vi antigen with some O antigen contamination in preparations obtained from E. coli and S. typhosa, strain Ty_2. In order to purify the Vi antigen, the preparation was dissolved in 0.15 M NaCl and the pH adjusted to 7. Then a 2 percent solution of cetyltrimethylammonium bromide (cetavlon), a detergent, was added until a precipitate formed. The precipitate was collected by centrifugation and washed 3 or 4 times with 0.15 M NaCl to remove the detergent. The precipitate was then dissolved in a small amount of 0.15 M NaCl and three volumes of alcohol were added. The resulting precipitate was collected by centrifugation and dissolved again in 60 per cent alcohol at 37 C, then refrigerated overnight. The preparation was electrodialyzed to remove the remaining detergent, neutralized with sodium acetate to convert all of the Vi to the sodium salt, dialyzed again, and lyophilized.

Vi antigen was deacetylated in the following manner. P. ballerup Vi antigen was dissolved in 0.1 N NaOH and, after two hours at room temperature, the mixture was neutralized with acetic acid. The sodium acetate formed was removed by dialysis and the antigen was lyophilized.

For stock Vi solutions, the Vi antigen was dissolved in 0.15 M NaCl to give a concentration of 1 mg/ml. Enough Vi solution was prepared to perform the current experiment and new stock solution made for the next experiment. The stock solution was frozen for the duration of an experiment.

Animals Used

Carworth Farms CF 1 albino mice were used for immunization. The animals were injected intraabdominally with 0.1-ml amounts of Vi antigen. Albino rabbits weighing 2-3 kg were used for the rabbit experiments. Unless otherwise specified the rabbits were injected in the marginal vein of the left ear. Trial bleedings were taken from the marginal vein of the right ear. All rabbits were bled before injection and the sera saved to test with the trial and final bleedings after immunization.

Immunizing Agents

Sensitized rabbit erythrocytes: Ten ml of blood were obtained from the marginal ear veins of rabbits and collected in tubes containing an equal volume of sterile ACD (modified Alsever's) solution to prevent clotting.

ACD solution:	Sodium chloride	.42 %
	Glucose	2.05 %
	Sodium citrate	.80 %
	Citric acid	.005%

autoclaved at 15 pounds pressure for 15 min.

The blood samples were centrifuged at 1500 RPM for 5 minutes and the erythrocytes saved. The erythrocytes from each rabbit were ceated with P. ballerup Vi antigen according to a modification of the method of Landy and Lamb (48). This method consisted of the following: Erythrocytes were washed 3 times in 10 volumes of 0.15 M NaCl and the supernatants were discarded. The cells were then resuspended in 10 volumes of 0.15 M NaCl and centrifuged at 1500 RPM for 10 minutes. The supernatants were again discarded and a 10 per cent suspension of cells made in 0.15 M NaCl. An equal volume of a 1 mg/ml Vi antigen solution was added, the contents mixed and incubated in a water bath at 37 C for 2 hours with shaking every 30 minutes. Landy and Lamb (48) used only 10 ug/ml of Vi antigen solution but in this case maximum absorption was sought by the presence of a large amount of antigen. Unabsorbed Vi was removed by washing the suspension three times with 10 volumes of 0.15 M NaCl and centrifuging in conical tubes at 2000 RPM for 5 minutes the first 2 times, and for 10 minutes the third time. The supernatant was discarded and the packed cells made up to a 5 per cent suspension in one experiment and a 10 per cent suspension in another. To confirm that absorption had occurred, a standard hemagglutination test (described below) was performed with the cells using a known Vi antiserum. Rabbits were injected intravenously with 1-ml amounts of the coated cells. Freshly sensitized erythrocytes were used for every 2 injections since they disintegrate rapidly after 48 hours (48). Three rabbits were given injections of a 5 per cent suspension of sensitized erythrocytes every other day until 6 injections had been administered. One month later, 3 booster injections were given every other day. One week later the rabbits were bled by cardiac puncture and the sera tested by hemagglutination. In the second experiment, 3 rabbits were given 6 injections of a 10 per cent suspension of sensitized erythrocytes in the same manner. One week later, the rabbits were bled from the ear and another series of 6 injections given. One week later, the rabbits were bled by cardiac puncture and the sera tested by hemagglutination.

Vi antigen in adjuvant: Vi antigen emulsified in Freund's complete adjuvant was used to immunize rabbits. Freund's complete adjuvant consists of Arlacel A, Bayol F and killed *Mycobacterium butyricum.* Arlacel A and Bayol F in a proportion of 1.5 to 8.5 vol/vol was autoclaved at 15 lb pressure for 20 minutes and stored in screw cap test tubes. *M. butyricum* was grown in meat extract broth for 3 days, boiled at 100 C for 20 minutes, the cells removed from the broth by filtration, dried in a 37 C incubator and stored in a desiccator until used. Five mg *M. butyricum* per 10 ml adjuvant were added before use. For injection, 0.2 ml (1 mg/ml) *P. ballerup* Vi were added to 0.2 ml complete adjuvant and the mixture emulsified by drawing it in and out of a syringe several times. Two-tenths ml of the emulsion was administered to each of 3 rabbits by injection of 0.05 ml into each toe pad of the right hind foot. One month later, boosters of Vi antigen (1 mg/ml) were administered intravenously in 1-ml amounts.

Specific precipitate in adjuvant: A preliminary precipitin titration was set up with *P. ballerup* Vi and *P. ballerup* antiserum obtained by immunization of rabbits with whole organisms. The equivalence zone was found to occur at a Vi concentration of 1 mg/ml and an antiserum dilution of 1:2. The supernatant was tested for both excess antigen and antibody. Two ml of 1 mg/ml Vi solution and 2 ml of a 1:2 antiserum dilution were then mixed in a plastic centrifuge tube and allowed to stand overnight in the refrigerator. All the materials were prepared and handled under sterile conditions. The next morning the precipitate which had formed was washed 5 times in sterile 0.15 M NaCl. The suspension was then emulsified in an equal volume of sterile Freund's complete adjuvant. Rabbits to be immunized were prepared beforehand by shaving the paws and back. The material was injected into the foot pads of both hind feet and intracutaneously into the back with a 5-ml syringe and a 20 guage needle. Each of 3 rabbits received 0.5 ml of the emulsion corresponding to 500 µg of Vi antigen. After one month, boosters of 0.5 ml specific precipitate were given intravenously.

Specific precipitates of S. typhosa, strain Ty$_2$ water extract and antiserum: Specific precipitates containing both O and Vi antigens were prepared for intravenous inoculation of rabbits as follows: Preliminary titrations of *S. typhosa*, strain O-901 antiserum and of *P. ballerup* antiserum with *S. typhosa*, strain Ty$_2$ water extract were performed to determine the equivalence zone of the antigen-antibody reactions. In the *S. typhosa*, strain Ty$_2$ water extract-*P. ballerup* antiserum system at equivalence the water extract was diluted to 0.5 mg/ml and the antiserum diluted 1:4. In the other system, the water extract was diluted to 0.75 mg/ml and the antiserum undiluted. *S. typhosa*, strain Ty$_2$ water extract of the proper dilution was mixed with an equal volume of undiluted *S. typhosa* O-901 antiserum and with an equal volume of *P. ballerup* antiserum diluted 1:4 in sterile centrifuge tubes. The tubes were refrigerated overnight at 4 C. The precipitates were then washed 5 times with sterile salt solution and finally suspended in 5 ml of sterile saline solution for inoculation. A portion of both precipitates was incubated with trypsin to remove antibody from the complex and tested by the agar gel precipitin test (to be discribed later) in order to determine which antigens were present in the precipitate. Each specific precipitate was administered intravenously to 2 rabbits. Five injections (2 a week) consisting of 0.2 ml, 0.3 ml, 0.5 ml, 0.5 ml, and 0.5 ml respectively were given. The injections corresponded to approximately 100 µg, 150 µg, 250 µg, 250 µg, and 250 µg of *S. typhosa* Ty$_2$ water extract. The amounts could only be estimated because the precipitates were in suspension and the exact amount injected each time could not be completely controlled. At the same time, 2 rabbits were given S. typhosa, strain Ty_2 water extract in the comparable amounts listed above. Four rabbits were immunized with the amounts normally used for immunization (200 µg, 300 µg, 500 µg, 800 µg, and 1 mg) in order to compare antibody titers.

In another experiment, larger amounts of the specific precipitates were administered, 0.3 ml, 0.4 ml, 0.5 ml, 1 ml and 1.5 ml respectively. Because the S. typhosa 0-901 antiserum-water extract specific precipitate was toxic in these amounts, injections of 0.1 ml, 0.2 ml, 0.3 ml, 0.6 ml, and 1 ml were administered.

Immunization with purified Vi antigen: To serve as controls, 3 rabbits were immunized with purified P. ballerup Vi antigen. One mg of Vi antigen was administered intravenously every other day for a total of 6 injections. One week later, the rabbits were bled from the ear and another series of 6 injections was given. E. coli and S. typhosa, strain Ty_2 Vi antigens were also administered in this manner, a total of 20 mg of S. typhosa, strain Ty_2 Vi antigen being given.

Collection of Antisera

Mice were bled by decapitation, the blood was collected in test tubes, allowed to clot and stand overnight at 4 C. The serum was collected after centrifugation the following morning. Mouse serum was not stored but tested immediately.

A preliminary bleeding of 10 ml was performed on each rabbit before immunization to check for previous antibody titer. In many cases trial bleedings were made during the immunization process. Final bleedings were performed over a period of three days, since it has been observed that a maximum amount of serum can be obtained if a 50-ml bleeding is performed on each of the two days prior to final bleeding from the heart. The bloods were allowed to clot and were stored overnight at 4 C. Serum was collected after centrifugation of the blood, stored at 4 C and preserved by addition of merthiolate to a final dilution of 1:10,000.

Methods of Testing Antisera

Hemagglutination: The major method for testing sera was Vi hemagglutination. The method of Landy and Lamb (48) as described previously was employed with the following modifications: A 10 per cent saline suspension of horse erythrocytes obtained from The Commonwealth of Massachusetts Department of Public Health, Biologic Laboratories, Boston, Massachusetts was sensitized with an equal volume of 100 µg/ml Vi antigen. The final suspension of erythrocytes for the tests was 0.5 rather than 1 per cent because agglutination was easier to read at this dilution. Five-tenths-ml amounts of 2-fold serial dilutions of sera in salt solution were prepared in Wassermann tubes and 0.5 ml of sensitized erythrocytes (0.5 per cent) were added to each. The tubes were shaken and incubated for 2 hours at 37 C. The titer was determined by the highest dilution of serum showing agglutination of erythrocytes. Both normal and known immune sera were run at the same time to serve as sensitization controls. Controls of sensitized cells in salt solution and unsensitized cells in salt solution were also run to check on spontaneous agglutination of cells.

In some cases, sera were tested in albumin as well as in salt solution to test for incomplete antibody formation. This was done by diluting sera in 15 per cent bovine serum albumin rather than in salt solution. Cells were also sensitized with S. typhosa 0-901 water extract to test for O antibodies in the rabbit sera.

Bacterial agglutination: Sera found to have antibodies by hemagglutination were further tested by other means. Bacterial agglutinations were performed with P. ballerup organisms. Serum dilutions were made as described above, but 0.5 ml of a once-washed suspension of acetone-killed and dried organisms was added to each tube rather than sensitized erythrocytes. The bacterial suspensions were standardized at 35 per cent transmission with a Baush and Lomb Spectrophotmeter at 600mμ. The tubes were incubated for 2 hours at 52 C. Bacterial agglutination tests for O antibody were also performed with organisms that had been boiled for 2 hours and washed 3 times with salt solution to remove the soluble Vi antigen. Controls of salt solution with serum and salt solution with organisms were also run.

Precipitin tests: Precipitin tests were performed on hemagglutination positive sera using 0.2-ml amounts of Vi dilutions of 1 mg/ml, 0.2 mg/ml, 0.04 mg/ml and 0.008 mg/ml with 0.2 ml undiluted serum as described by Edwards and Ewing (22).

Agar Gel diffusion: Ouchterlony plates were prepared with sera showing hemagglutinative reactions. The sera were compared against known positive Vi antisera by this method. The plates were prepared in the following manner: One ml of 1 per cent Noble agar (Difco) containing 0.15 M NaCl and merthiolate (1:10,000) was placed in the bottom of a petri dish 60 mm in diameter. When this layer had hardened, a 4 well mold was placed on top and 3 ml of agar added. After the agar hardened the mold was removed carefully so as not to disrupt the wells formed. The wells were filled with 0.02 ml of antigen or antiserum from a 1-ml syringe and the reactants allowed to diffuse into the surrounding medium while incubated in a moist chamber at 30 C. Zones of precipitate usually formed within a day or two at places where homologous antigen and antibody met. Plates were observed for one week before being discarded.

Concentration of sera: Some of the sera were concentrated by adding saturated ammonium sulfate to a 40 per cent concentration. These sera were then allowed to precipitate overnight or until all the precipitates settled. After centrifugation the precipitates were dialyzed against distilled water for 2 to 3 hours and then against salt solution overnight.

Absorption of sera: Sera were absorbed in the following manner: 100 mg of acetone-dried organisms were washed once with salt solution and added to 1 ml of serum in a plastic centrifuge tube. The suspension was stirred for 1 hour then placed in the refrigerator overnight. The organisms were then removed by centrifugation and the sera tested for antibody by hemagglutination.

RESULTS

Mouse Experiments

Eighteen Albino Carworth Farms mice were immunized intraabdominally with 0.6 mg P. ballerup Vi antigen administered in 6 injections of 0.1 ml each. Injections were given every other day. Six days after the last injection the mice were exsanguinated by decapitation and the sera pooled. Fourteen mice were immunized in the same manner with S. typhosa, strain Ty$_2$ Vi antigen. The two pools of serum were tested by hemagglutination (using 10 ug/ml Vi for sensitization of erythrocytes) in serial two-fold dilutions from 1:2 to 1:64. No Vi antibody was detected where as a known positive P. ballerup antiserum had a titer of 1:1600.

Because no antibody was observed in the experiment above another group of mice was immunized with smaller amounts of P. ballerup Vi antigen ranging from 1µg to 6 µg in order to avoid the possibility of immunoparalysis. Doses were administered intraabdominally in 1 µg amounts so that the first group of two received only one injection while the last group received 6 injections. Six days after the final injection each group was exsanguinated and the sera saved until all 6 groups had been collected. The hemagglutination test was repeated exactly as before with the same results. Test sera had no demonstrable antibody where as the control serum showed a titer of 1:1600.

As this time it was decided to use 100 µg/ml Vi antigen for sensitizing the erythrocytes. To obtain more serum, groups of 3 mice were immunized in the same manner with 1 µg to 6 µg amounts. The results of this experiment are summarized in Table I. Titers showed an increase from 1:4 to 1:16 with increased dosage. There was sufficient sera from the groups immunized with 5 and 6 µg to perform the hemagglutination test in albumin also. A rise in titer of 2 tubes (to 1:64) was observed with the 5 µg antiserum and a rise of 1 tube (to 1:32) with the 6 µg antiserum.

Rabbit Experiments

Vi sensitized rabbit erythrocytes: Three rabbits were given 1 ml of a 5 percent suspension of Vi coated erythrocytes every other day until 6 injections had been administered. After 1 month, 3 booster injections were administered, one every other day. One week after the last booster the rabbits were exsanguinated by cardiac puncture. By the hemagglutination technique, these rabbit sera showed antibody titers of 1:8, 1:16, and 1:4 respectively.

Because the antibody titers were so low the experiment was repeated using a 10 per cent suspension of sensitized erythrocytes. The animals were bled one week after the first 6 injections and another series of 6 injections administered. One week later, the rabbits were bled again. No antibody was observed in any of these sera. The results of these experiments are tabulated in Table II.

Vi antigen in adjuvant: Three rabbits were given toe pad injections of 0.1 ml P. ballerup Vi antigen emulsified in an equal volume of Freund's complete adjuvant. One month after the injection, 1-ml boosters of P. ballerup Vi (1 mg/ml) were administered intravenously every other day. Two rabbits received 3 booster injections; 1 received 6 booster injections. The results are shown in Table III. One of the sera from a rabbit which had received 3 boosters showed an antibody titer of 1:64; the other 2 contained no antibody.

TABLE I

IMMUNIZATION OF MICE WITH P. BALLERUP VI ANTIGEN

Total Dosage (ug)	Number Of Injections	Hemagglutination(1)	
		in 0.15 M NaCl	in Albumin
1	1	1:4	-(2)
2	2	1:4	-(2)
3	3	1:8	-(2)
4	4	1:16	-(2)
5	5	1:16	1:64
6	6	1:16	1:64

(1) Test performed on pooled sera of three mice.

(2) Insufficient serum to perform test.

Control: Sensitized cells vs positive control serum - 1:3200.

TABLE II

IMMUNIZATION OF RABBITS WITH VI COATED ERYTHROCYTES

Rabbit No.	Immunized With	Number Of Injections	Total Amount Administered	Vi Hemagglutination						
				1:2	1:4	1:8	1:16	1:32	1:64	Cont.
97	5% suspension of Vi coated erythrocytes	9	9 ml	+	+	+	-	-	-	-
98	"	9	9 ml	+	+	+	+	-	-	-
99	"	9	9 ml	+	+	-	-	-	-	-
137	10% suspension of Vi coated erythrocytes	6	6 ml	-	-	-	-	-	-	-
		12	12 ml	-	-	-	-	-	-	-
138	"	6	6 ml	-	-	-	-	-	-	-
		12	12 ml	-	-	-	-	-	-	-
139	"	6	6 ml	-	-	-	-	-	-	-
		12	12 ml	-	-	-	-	-	-	-
Normal Rabbit Serum	---	---	---	-	-	-	-	-	-	-
Standard *P. ballerup* Antiserum	Acetone killed and dried whole organisms			positive 1:32000						

TABLE III

IMMUNIZATION OF RABBITS WITH P. *BALLERUP* VI ANTIGEN IN ADJUVANT

Rabbit No.	Dose Administered	Number of Boosters 1 mg Vi/ml	Hemagglutination								
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Saline Control
112	0.2 ml total 0.1 ml Vi 0.1 ml adjuvant	3	-	-	-	-	-	-	-	-	-
114	0.2 ml total 0.1 ml Vi 0.1 ml adjuvant	3	+	+	+	+	+	+	-	-	-
166	0.2 ml total 0.1 ml Vi 0.1 ml adjuvant	6	-	-	-	-	-	-	-	-	-
Normal Rabbit Serum	--	-	-	-	-	-	-	-	-	-	-
Standard *P. ballerup* Antiserum	Acetone killed and dried organisms	-	positive 1:12,800								

Specific precipitate in adjuvant: A specific precipitate of *P. ballerup* Vi antigen and *P. ballerup* antiserum was prepared and emulsified in Freund's adjuvant as described previously. Each of 3 rabbits was given 0.5 ml of the emulsion by toe pad and intracutaneous injection. Each rabbit received 0.5 mg of Vi antigen by this method. Necrotic lesions were observed at the sites of injection a few days later. After 1 month, intravenous booster injections of 0.5 ml of the specific precipitate (containing approximately 0.5 mg Vi antigen) were administered 1 week apart. Before the second booster and 1 week later the rabbits were bled and the sera tested for Vi antibody by hemagglutination. No antibodies were produced.

Specific precipitates of water extract and antiserum: Since it has been observed that whole organisms and crude extracts induce Vi antibody production in rabbits (72), it was thought that a Vi-O complex might be responsible for immunogenicity. In order to test this hypothesis, 2 rabbits were given 5 injections of the specific precipitate of *S. typhosa*, strain Ty2 water extract and *S. typhosa*, strain O-901 antiserum. Two rabbits were also given 5 injections of the specific precipitate of *S. typhosa*, strain Ty2 water extract and *P. ballerup* antiserum. At the same time, 2 rabbits were given *S. typhosa*, strain Ty_2 water extract in amounts comparable to that in the specific precipitate and 4 rabbits were immunized with the amounts normally used for immunization in order to compare antibody titers. Two of these rabbits died during the course of immunization.

Agar gel precipitin tests were made on the specific precipitates after trypsinization to remove antibody from the complex. The *S. typhosa*, strain Ty_2 water extract-*P. ballerup* antiserum specific precipitate contained both Vi and O antigens. In contrast only O antigen was detected in the *S. typhosa*, strain Ty_2 water extract-*S. typhosa*, strain O-901 antiserum specific precipitate. Sera obtained from the 2 rabbits immunized with the water extract-P. ballerup antiserum specific precipitate contained antibody in high titer to S. typhosa, strain 0-901. O antibody was also detected by agar gel precipitin. These rabbits responded poorly to Vi antigen since antibody could be detected only by hemagglutination after 24 hours. Rabbits immunized with the water extract-S. typhosa, strain 0-901 antiserum specific precipitate produced antibody against both Vi and O antigens. Hemagglutination titers were evident after 2 hours and agglutination of P. ballerup organisms occurred in low titer. However, Vi antibody was not demonstrated by agar gel precipitin tests. O antibody was shown in high titer by both agglutination with S. typhosa, strain 0-901 and by agar gel precipitin tests. The results of this experiment are summarized in Table IV.

Because the response to Vi antigen was poor and Vi antigen was not demonstrated by agar gel precipitin in one of the original specific precipitates, it was thought that the amount of Vi in the preparation might be too low. For this reason, 2 more rabbits were immunized, 1 with the specific precipitate of S. typhosa, strain Ty_2 water extract and S. typhosa, strain 0-901 antiserum and 1 with the specific precipitate of S. typhosa, strain Ty_2 water extract and P. ballerup antiserum. This time larger amounts were administered; 0.3 ml, 0.4 ml, 0.5 ml, 1 ml and 1.5 ml respectively. The water extract-0-901 antiserum specific precipitate was toxic for 2 rabbits when administered in these amounts, consequently it was administered in the following amounts: 0.1 ml, 0.2 ml, 0.3 ml, 0.6 ml, and 1 ml respectively. When these larger amounts of specific precipitate were administered, the results were similar to those of the previous

TABLE IV

IMMUNIZATION OF RABBITS WITH SPECIFIC PRECIPITATES

Rabbit No.	Immunized With	Hemagglutination (Vi)	Agglutination With *P. ballerup* Organisms	Agglutination With *S. typhosa*, 0-901
183	Specific precipitate of *S. typhosa*, Ty2 water extract and *P. ballerup* antiserum	1:64 overnight	0	1:512
184	"	1:128 overnight	0	1:512
185	Specific precipitate of *S. typhosa*, Ty2 water extract and *S. typhosa*, 0-901 antiserum	1:256	1:4	1:1024
186	"	1:32	1:4	1:512
187	1 mg *S. typhosa*, Ty2 water extract - 5 injections	1:2048	1:128	at least 1:1024
189	2.62 mg *S. typhosa*, Ty2 water extract 5 injections	1:4096	1:256	at least 1:1024
190	"	1:64	0	at least 1:1024
191	"	0	1:16	at least 1:1024
Standard *P. ballerup* Antiserum	Acetone killed and dried whole organisms	1:6400	--	--
Normal Rabbit Serum	"	--	--	--

TABLE V

IMMUNIZATION OF RABBITS WITH LARGER DOSES OF SPECIFIC PRECIPITATE

Rabbit No.	Immunization With	Hemagglutination	Agglutination With *P. ballerup* Organisms	Agglutination With *S. typhosa*, 0-901 Organisms
220	Specific precipitate of *S. typhosa*, Ty2 water extract and *P. ballerup* antiserum	1:128	1:32	1:512
233	Specific precipitate of *S. typhosa*, Ty2 water extract and *S. typhosa*, 0-901 antiserum	1:4	0	1:512
Standard *P. ballerup* Antiserum	Acetone killed and dried whole organisms	1:3200	--	--
Normal Rabbit Serum	---	--	--	--

experiment (Table V) except that the serum from the rabbit immunized with S. typhosa, strain Ty_2 water extract-S. typhosa, strain 0-901 antiserum did not produce Vi antibodies to a significant degree, the hemagglutination titer being 1:4.

Intravenous injections of purified Vi antigen: As controls for the previous experiments, 3 rabbits were immunized with P. ballerup Vi antigen. One mg of Vi antigen was administered intravenously every other day for a total of 6 injections. One week after the last injection the rabbits were bled for 10 ml and another series of 6 injections was administered. One week after the second series, the rabbits were exsanguinated. The 6 injection sera showed titers of 1:256, 1:32 and 0 respectively. A significant rise in titer was observed after 12 injections; 1:1024, 1:512 and 1:64 respectively.

The experiment was repeated using E. coli and S. typhosa, strain Ty_2 Vi as well as P. ballerup deacetylated Vi antigen. Of the 9 rabbits immunized, 1 given E. coli Vi antigen showed a serum antibody titer of 1:512 in the 12 injection serum but no antibody in the 6 injection serum. None of the other rabbit sera showed the presence of Vi antibody. Three more rabbits were immunized with S. typhosa, strain Ty_2 Vi antigen in hopes of obtaining an antiserum to this particular Vi antigen. These last 3 rabbits could not be induced to produce Vi antibody even after a total of 20 mg S. typhosa, strain Ty_2 Vi antigen were administered. The results are tabulated in Table VI.

Further testing of sera: The 4 sera having positive Vi hemagglutination titers were tested by bacterial agglutination with a once-washed suspension of P. ballerup acetone dried organisms. No antibody was observed.

TABLE VI

IMMUNIZATION OF
RABBITS WITH PURIFIED VI ANTIGEN

Rabbit No.	Immunized With	Total Dosage Of Vi*	Vi Hemagglutination										
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Cont.
140	P. ballerup Vi	6 mg	+	+	+	+	+	+	+	+	-	-	-
		12 "	+	+	+	+	+	+	+	+	+	+	-
141	P. ballerup Vi	6 "	+	+	+	+	±	-	-	-	-	-	-
		12 "	+	+	+	+	+	+	+	+	+	-	-
142	P. ballerup Vi	6 "	-	-	-	-	-	-	-	-	-	-	-
		12 "	+	+	+	+	+	+	-	-	-	-	-
153	E. coli Vi	6 "	-	-	-	-	-	-	-	-	-	-	-
		12 "	+	+	+	+	+	+	+	+	+	-	-
154	E. coli Vi	6 "	-	-	-	-	-	-	-	-	-	-	-
		12 "	-	-	-	-	-	-	-	-	-	-	-
155	E. coli Vi	6 "	-	-	-	-	-	-	-	-	-	-	-
		12 "	-	-	-	-	-	-	-	-	-	-	-
159	S. typhosa, Ty2 Vi	6 "	-	-	-	-	-	-	-	-	-	-	-
		12 "	-	-	-	-	-	-	-	-	-	-	-
160	S. typhosa, Ty2 Vi	6 "	-	-	-	-	-	-	-	-	-	-	-
		12 "	-	-	-	-	-	-	-	-	-	-	-
161	S. typhosa, Ty2 Vi	6 "	-	-	-	-	-	-	-	-	-	-	-
		12 "	-	-	-	-	-	-	-	-	-	-	-
174	S. typhosa, Ty2 Vi	6 "	-	-	-	-	-	-	-	-	-	-	-
		12 "	-	-	-	-	-	-	-	-	-	-	-
175	S. typhosa, Ty2 Vi	6 "	-	-	-	-	-	-	-	-	-	-	-
		12 "	-	-	-	-	-	-	-	-	-	-	-
176	S. typhosa, Ty2 Vi	6 "	-	-	-	-	-	-	-	-	-	-	-
		12 "	-	-	-	-	-	-	-	-	-	-	-
Normal Rabbit Serum	---	-	-	-	-	-	-	-	-	-	-	-	-
Standard P. ballerup Antiserum	Acetone killed and dried organisms	-	Positive 1:3200										

*Individual doses equal 1 mg/injection.

The P.ballerup antisera were also tested with P. ballerup organisms which had been boiled for 2 hours and washed to remove soluble Vi antigen. No antibody was detected. The E. coli antiserum was tested in the same way with boiled E. coli organisms. No antibody was observed.

No antibody was observed by the hemagglutination technique when the erythrocytes were sensitized with O antigen although a known O antiserum had a titer of 1:12,800.

Concentration of 2 of the sera by the ammonium sulfate method outlined under Materials and Methods increased the titers by 2 tubes. One titer increased from 1:1024 to 1:4096 and the other from 1:512 to 1:2048. Antibody could not be detected by any method other than hemagglutination in the concentrated sera.

Absorption of the sera with both homologous and heterologous organisms removed all the Vi antibody as detected by hemagglutination.

Hemagglutination tests performed wth erythrocytes sensitized with heterologous Vi antigen showed no significant difference in titer from tests performed with erythrocytes sensitized with homologous Vi antigen. The results of this test are summarized in Table VII.

TABLE VII

HEMAGGLUTINATION OF POSITIVE SERA WITH ERYTHROCYTES
SENSITIZED WITH HOMOLOGOUS AND HETEROLOGOUS VI ANTIGENS

Rabbit No.	Immunized With	Erythrocytes Sensitized With	Hemagglutination										
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Cont.
140	P. ballerup Vi	P. ballerup Vi	+	+	+	+	+	+	+	+	+	+	-
		S. typhosa, Ty2 Vi	+	+	+	+	+	+	+	+	+-	-	-
		E. coli Vi	+	+	+	+	+	+	+	+	-	-	-
141	P. ballerup Vi	P. ballerup Vi	+	+	+	+	+	+	+	+	+	-	-
		S. typhosa, Ty2 Vi	+	+	+	+	+	+	+	+	+	-	-
		E. coli Vi	+	+	+	+	+	+	+	+	+-	-	-
142	P. ballerup Vi	P. ballerup Vi	+	+	+	+	+	+	-	-	-	-	-
		S. typhosa, Ty2 Vi	+	+	+	+	+	-	-	-	-	-	-
		E. coli Vi	+	+	+	+	+	+	-	-	-	-	-
153	E. coli Vi	P. ballerup Vi	+	+	+	+	+	+	+	+	-	-	-
		S. typhosa, Ty2 Vi	+	+	+	+	+	+-	+-	-	-	-	-
		E. coli Vi	+	+	+	+	+	+	+	+	+	-	-
Normal Rabbit Serum	--	P. ballerup Vi	-	-	-	-	-	-	-	-	-	-	-
		S. typhosa, Ty2 Vi	-	-	-	-	-	-	-	-	-	-	-
		E. coli Vi	-	-	-	-	-	-	-	-	-	-	-
Standard P. ballerup Antiserum	P. ballerup whole organisms	P. ballerup Vi	positive 1:64000										
Standard S. typhosa, Ty2 Antiserum	S. typhosa, Ty2 whole organisms	S. typhosa, Ty2 Vi	positive 1:4000										
Standard E. coli Antiserum	E. coli whole organisms	E. coli Vi	positive 1:32000										

DISCUSSION

Although rabbits can be immunized against Vi antigen using whole organisms or crude extracts, (72) attempts to immunize them with purified extracts have failed with one exception. Landy and Webster (46) were able to immunize rabbits with their purified preparation but the response was less than that obtained when whole organisms were used for immunization. Mice and man, however, respond very well to the purified antigens as well as to whole organisms.

In this investigation, mice produced antibody to amounts of Vi as low as 1 μg when sera were tested by the hemagglutination technique. The titers were lower, however, than those observed by Gaines, Currie and Tully (31) who immunized BALB/c mice (Bagg strain variant) with the E. coli Vi preparation of Landy and Webster. They also found that Cinnamon mice injected in the same manner did not produce antibody detectable by hemagglutination in salt solution. Immunized mice were protected to the same degree as BALB/c mice against challenge with virulent S. typhosa. They found that antibody could be detected when sera were diluted in albumin rather then salt solution for the hemagglutination tests. It was suggested that this result might be explained by the formation of incomplete antibodies which were not detected by hemagglutination performed in salt solution. The Carworth Farms strain of mice used in this investigation appears to be intermediate between BALB/c and Cinnamon mice in that saline antibody was present but lower than that of BALB/c mice. Incomplete antibody might have been present also since the sera from mice immunized with 5 and 6 μg showed higher titers in albumin than in salt solution. Rabbit sera tested for this incomplete antibody formation failed to show higher titers in albumin than in salt solution. The reason mice immunized with 0.6 mg Vi antigen did not produce antibody might have been that they were immunologically paralyzed by too large a dose of Vi antigen. Another explanation could be that the erythrocytes were sensitized with Vi antigen diluted to 10 µg/ml. Through experience, it has been observed that 100 µg/ml sensitized erythrocytes to a greater degree, increasing the sensitivity of the test since more Vi antigen was coated on the erythrocytes and available for the reaction.

Attempts to make purified Vi antigen antigenic for rabbits by the various manipulations dicussed (Vi sensitized erythrocytes, Vi antigen emulsified in adjuvant, Specific precipitate in adjuvant and Specific precipitate containing both Vi and O antigens) were less successful than the use of intravenous inoculation of purified Vi antigen alone. The Vi antibodies produced against *P. ballerup* and *E. coli* appeared to be the same serologically. Landy and Webster (46) observed that Vi antigen prepared from *E. coli* was a better immunizing agent in both rabbits and mice than that from *P. ballerup* or *S. typhosa*, strain Ty_2. Baker and Whiteside (72) obtained similar results in mice except that they found *P. ballerup* and *S. typhosa*, strain Ty_2 preparations to be equally potent in mice. Landy and Webster (46) found that *P. ballerup* Vi was 9 times as potent as *S. typhosa* Ty2 Vi in mice. When another strain of mice was tested using the preparation of Baker, *et al* (72) all Vi preparations seemed equally potent in inducing the formation of protective antibodies. The results of rabbit immunizations undertaken for this investigation seem to indicate that *P. ballerup* Vi is the most potent, *E. coli* Vi next and

S. typhosa, strain Ty_2 Vi least potent. When Smith (63) immunized rabbits with water extracts, she also observed no antibody production with S. typhosa strain Ty2. It must be remembered, however, that the reaction in rabbits is variable and especially since a poor antigen was used, no conclusions can be drawn as to the relative potency of the various preparations unless more rabbits are immunized. The failure of S. typhosa, strain Ty_2 water extract (63) or purified Vi antigen to induce antibody formation could indicate a difference among the Vi antigens. Antisera, however, agglutinated erythrocytes sensitized with heterologous Vi antigen to the same degree as they agglutinated erythrocytes sensitized with homologous Vi antigen. Also, sera absorbed with heterologous Vi containing organisms showed no reaction with erythrocytes sensitized with homologous Vi antigen. Baker and Whiteside (74), however, have reported possible serological differences in Vi antigen after deacetylation.

Vi antibody produced in rabbits against purified preparations was demonstrable only by the hemagglutination technique. This observation agrees with those of Spaun (64), and of Landy and Lamb (48) concerning the sensitivity of the hemagglutination technique. In their early experiments, Felix and Pitt (24) found that sera from immunized rabbits protected mice against challenge although no antibodies could be demonstrated by bacterial agglutination. It can be questioned whether or not these sera might have been positive by the hemagglutination technique. This technique has been shown by Spaun (64) and by Landy and Lamb (48) to be far more sensitive and reproducible than the bacterial agglutination tests used by Felix and Pitt. It may be possible that Vi antibody levels too low to be detected by serological means offer good protection against Vi containing typhoid organisms at least in mice. Interestingly, Felix and Pitt used mainly the typhoid strains Ty_2 and Watson shown by Spaun (65) to have the lowest Vi antigen content by quantitative measurements. Spaun used the agglutinin binding technique for his quantitative determinations. Since the results of this method are variable, Spaun's results may be subject to some error. However, S. typhosa Ty_2 was the strain reported above to be least immunogenic in the rabbits immunized for this and other investigations (63,46).

The success of Chu and Hoyt (14) in immunizing rabbits with Vi-coated erythrocytes was probably because of the use of a Vi preparation partially contaminated with other cellular antigens. They used an extract from boiled organisms treated with HCl which may not have destroyed other cellular antigens and may have degraded some of the Vi antigen. It has been shown that partially purified Vi antigen induces Vi antibody response in rabbits (72). The response to erythrocytes coated with purified Vi antigen was insignificant when compared to immunization with purified Vi antigen alone.

The use of adjuvant to enhance antibody production failed. This may have been because of the technique of administration, or as a result of too small a dose of Vi antigen, or because adjuvant isn't effective with polysaccharide haptens. Leskowitz (50) was successful with comparable amounts of bovine serum albumin in adjuvant but proteins are generally better immunizing agents than polysaccharides.

Because Vi in whole organisms and in crude extracts was antigenic for rabbits it was thought that Vi might exist in the natural state complexed with O antigen. The specific precipitate experiments were performed with this in mind. If a complex did exist then a specific precipitate from the water extract and 0-901 antiserum or water extract and *P. ballerup* antiserum should contain both Vi and 0 antigens. The results of the experiments reported here suggest that a complex may exist. The specific precipitate of *S. typhosa*, strain Ty$_2$ water extract and *P. ballerup* antiserum contained 0 antigen which induced good 0 antibody production in rabbits immunized with this specific precipitate. The specific precipitate of *S. typhosa*, strain Ty$_2$ water extract and *S. typhosa*, strain 0-901 antiserum did not show any Vi antigen in the agar gel precipitin test but sera from rabbits immunized with this specific precipitate showed the presence of Vi antibody by hemagglutination. It must be remembered, however, that Vi antibody in sera of rabbits immunized with purified Vi antigen was not demontrated by agar gell diffusion either. The rabbits immunized with the water extract-ballerup specific precipitate didn't produce as much antibody as the rabbits immunized with 0-901 antiserum-water extract specific precipitate. The whole problem may be one of relative concentration of the 2 antigens and also of the variability of the response of rabbits. The second series of immunizations may demonstrate this since this time the rabbit given more water extract-0901 antiserum didn't produce Vi antibodies although those receiving a smaller amount did. The rabbit given the other preparation produced no higher titer of Vi antibody than those given purified Vi antigen alone.

Vi antigen appears to be immunogenically similar to the capsular polysaccharides of *Diplococcus pneumoniae*. The acetyl polysaccharide isolated from type I pneumococcus was also a hapten in rabbits and contained uronic acid (6). Avery and Goebel (5,34) were able to make pneumococcal polysaccharide antigenic by forming a specific precipitate but they chemically altered the antigen first. This was not attempted with Vi antigen since the natural state was being maintained as nearly as possible. A specific precipitate of Vi and P. ballerup antibody administered in adjuvant was unsuccessful in immunizing rabbits. Leskowitz was successful in immunizing rabbits with specific precipitates in adjuvant but he was using a protein antigen which is a very good antigen when compared with polysaccharides.

Because of the failure to produce Vi antisera in rabbits, it appears that the only practical and the easiest way to obtain antisera for serologic studies is to immunize Human beings. Purified Vi antigen has been observed to be an excellent antigen in man as well as in the mouse. The significance of this is not known. It has been shown that the antibody formed in mice is protective against Vi-containing typhoid strains but earlier experiments showed that Vi antibody was not as effective passively in mice against organisms lacking Vi antigen (24,40). A very important fact to be remembered when making any comparison is that typhoid fever is exclusively a natural disease of man. Experimental animals do not react in the same way to laboratory infection as man does to the natural infection. The role of Vi antigen in human typhoid disease can only be settled by experiments on human volunteers who are willing to be challenged after immunization with Vi preparations. Wide spread field trials would not be effective since increased hygiene and sanitation makes typhoid epidemics highly unlikely under normal conditions.

SUMMARY

1. Mice responded to intraabdominal injections of 1 to 6 µg of purified Vi antigen with increasing antibody formation as the dosage increased. No detectable antibody was produced in mice immunized with 0.6 mg of Vi antigen. The Vi antibody titers of mouse sera were higher in albumin than in saline showing the possibility of incomplete antibody formation.

2. Rabbits were immunized with the following preparations:
   1) Vi sensitized rabbit erythrocytes
   2) Vi antigen in adjuvant
   3) The specific precipitate of Vi antigen and *P. ballerup* antiserum in adjuvant
   4) The specific precipitate of *S. typhosa*, strain Ty_2 water extract and *S. typhosa*, strain 0-901 antiserum or of *S. typhosa*, strain Ty_2 water extract and *P. ballerup* antiserum.
   5) Purified Vi antigen

The purified preparation alone was the only successful immunizing agent.

3. The Vi antibody in sera from rabbits immunized with purified Vi antigen could be detected only by hemagglutination, demonstrating that this test is more sensitive than other conventional tests.

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IMMUNOGENICITY OF THE VI ANTIGEN

Abstract of a Dissertation

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by

MARGARET ANN JOHNS

(B.S., Simmons College, 1960)

Department: Medical Sciences
Field of Specialization: Microbiology
Major Instructor: Professor Edgar E. Baker

Since the discovery of Vi antigen by Felix and Pitt in 1934 (The Lancet, 2:186, 1934), investigators have attempted to isolate, purify and characterize this antigen. Antisera against purified Vi antigens would prove a valuable tool for studying the effects of purification on the antigenic molecule. Purified Vi antigen, however, has proven to be a hapten in rabbits with one exception. Landy and Webster's Vi preparation was immunogenic for rabbits as well as for mice (J. Immunol., 69:143, 1952) but the preparations of Ashida (Japan. J. Exper. Med., 20:181, 1949), and of Baker, et al (J. Immunol.,83:687, 1959) were non-immunogenic although Baker's preparation immunized mice and man. Landy and Webster, however, observed that more of their preparation than of whole organisms was necessary to immunize rabbits to an equal degree.

In this investigation, attempts were made to obtain Vi antisera for serologic studies by immunizing rabbits with Vi antigen using methods employed with haptens. Vi antigens prepared from Paracolobactrum ballerup, strain 481, Escherichia coli, strain 136, and Salmonella typhosa, strain Ty_2 were used for this investigation. The method used for testing sera was a modification of the hemagglutination technique used by Landy and Lamb (Proc. Soc. Exp. Biol. Med., 82:593, 1953).

Rabbits were immunized with Vi coated rabbit erythrocytes in 5 and 10 per cent saline suspensions. Very low antibody titers (1:8, 1:16, and 1:4) were obtained in rabbits immunized with the 5 per cent suspension and none in rabbits immunized with 10 per cent suspension.

Three rabbits were given toe pad injections of Vi antigen emulsified in Freund's complete adjuvant. One rabbit produced Vi antibody in low titer (1:64); the other two produced no antibody.

Another group of rabbits was given the specific precipitate of Vi antigen and *P. ballerup* antiserum emulsified in adjuvant. Boosters of the specific precipitate were given intravenously one month later. No antibodies were produced by any of the rabbits injected.

Because it had been observed that whole organisms or crude Vi extracts still containing O antigen induced Vi antibody production in rabbits (J. Immunol., 83:687, 1959), it was thought that Vi antigen might exist complexed with O antigen in the natural state. In order to test the hypothesis that this complex might be the immunogenic factor, rabbits were immunized with the specific precipitate of *S. typhosa*, strain Ty$_2$ water extract and *S. typhosa*, strain O-901 antiserum or with the specific precipitate of the water extract and *P. ballerup* antiserum. If a complex did exist, then the specific precipitates should contain both Vi and O antigens. Agar gel precipitin tests were set up for the specific precipitates after trypsinization (to remove antibody) in order to determine which antigens were present in the complex. The *S. typhosa*, strain Ty$_2$ water extract-*P. ballerup* antiserum specific precipitate contained both Vi and O antigens. In contrast, only O antigen was detected in the *S. typhosa*, strain Ty$_2$ water extract-*S. typhosa*, strain O-901 antiserum specific precipitate. However, rabbits immunized with both specific precipitates produced Vi antibodies as well as O antibodies showing that a complex might exist. The response to Vi antigen was poor, so 2 more rabbits were immunized with larger amounts of the specific precipitates; one with each specific precipitate. The results were similar except that the rabbit immunized with the water extract-O-901 antiserum specific precipitate did not produce Vi antibodies to a significant degree, the hemagglutination titer being 1:4.

The problem may be one of relative concentration of the two antigens in the specific precipitates and also of the variability of the response of rabbits.

Rabbits were immunized with purified P. ballerup Vi antigen to serve as a control for the immunization described above. A series of six 1 mg injections were administered. Five days later the rabbits were bled for 10 ml and another series of six injections given. The six injection sera showed titers of 1:256, 1:32 and 0 respectively. A significant rise in titer was observed after twelve injections; 1:1024, 1:512 and 1:64 respectively. Because the best results were obtained with purified P. ballerup Vi antigen alone, rabbits were also immunized with E. coli and S. typhosa, strain Ty_2 Vi antigens. One rabbit produced antibodies against E. coli Vi (titer 1:512). Rabbits injected with S. typhosa, strain Ty_2 Vi antigen could not be induced to produce antibodies by injections of up to and including 20 mg of Vi antigen. These results may indicate that P. ballerup Vi antigen is most potent in inducing antibody response in rabbits, E. coli Vi antigen next, and S. typhosa, strain Ty_2 Vi antigen least potent. When Smith (J. Infect. Dis., 63:21, 1938) immunized rabbits with water extracts, she also observed no antibody production with S. typhosa, strain Ty_2 water extract. It must be remembered, however, that the reaction in rabbits is variable and especially since a poor antigen was used, no conclusions can be drawn as to the relative potency of the various preparations unless more rabbits are immunized.

Although the results of rabbit immunizations indicate that there might be an immunogenic difference among Vi antigens, these antisera agglutinated erythrocytes sensitized with heterologous Vi antigen to the same degree as they agglutinated erythrocytes sensitized with homologous Vi antigen. Also, sera absorbed with heterologous Vi-containing organisms did not agglutinate erythrocytes sensitized with homologous Vi antigen, showing that all the Vi antibody had been removed.

Besides hemagglutination, other methods were used for testing the antisera; bacterial agglutination, tube precipitin, and agar gel precipitin tests were performed. The Vi antibody produced by immunization with purified antigen was demonstrable only by the hemagglutination technique. This supported the observations of Spaun (Acta path. microb. scand., 29:416, 19 51) and of Landy and Lamb (Proc. Soc. Exp. Biol. Med., 82:593, 1953) who found that the hemagglutination technique was far more sensitive and reproducible than the bacterial agglutination tests used by other workers.

Because of the failure to produce Vi antisera in rabbits, it appears that the only practical and the easiest way to obtain antisera for serologic studies is to immunize human beings. Vi antigen is an excellent antigen in man. The significance of this is not known, nor is the role of Vi antigen in human infection understood. Comparisons between laboratory animals and man cannot be made since typhoid fever is a natural disease only of man. The role of Vi antigen can be understood only after experiments on human volunteers.