

Isolation of Vi Antigen and a Simple Method for Its Measurement

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Received for publication 8 May 1972

Vi antigen was purified from saline extract of *Citrobacter* strain 5396/38 by enzymatic digestion, concentration with ethanol, and precipitation with hexadecyltrimethylammonium bromide. The Vi antigen prepared by this method was 18 and 230 times more protective in mice than two other preparations isolated by an earlier method utilizing acid hydrolysis. A sensitive and specific method of electroimmunodiffusion was described for measurement of Vi antigen.

The presence of Vi antigen is correlated with virulence and immunogenic properties of *Salmonella typhosa* in mice (6). However, its significance in human immunity remains to be ascertained (2, 7), and its influence in potency assay of typhoid vaccines in mice has been of much concern to biologics control laboratories (22). A number of methods have been developed for isolation and purification of Vi antigen from various species of the *Enterobacteriaceae*. Webster et al. (19) fractionated bacterial extracts with ethanol in the presence of sodium chloride, followed by hydrolysis with acetic acid. Baker et al. (1) used ethanol-saline extraction at various temperatures, and Jarvis et al. (8) employed continuous-flow, paper curtain electrophoresis. The Vi preparations yielded by these methods differ in physicochemical and immunological properties (11).

There have been few studies on measurement of Vi antigen. Landy (9) claimed to have measured the relative amounts of Vi antigen in various enteric organisms by an active immunization mouse protection test. Webster et al. (20) used a turbidimetric method which depends on the tendency of acidic mucopolysaccharides to flocculate with albumin at low pH. Sensitivity of this procedure ranges from 125 to 500 μg of antigen per ml, but the reaction is not specific for the Vi antigen. Cohen and Engel (3) used serological methods, including complement fixation and erythrocyte sensitization tests, for measurement of Vi antigen in acetone-dried typhoid vaccines.

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This paper presents a relatively simple and mild procedure for isolation of Vi antigen and a sensitive and specific electroimmunodiffusion (EID) method for its measurement.

MATERIALS AND METHODS

Culture. *Citrobacter* strain 5396/38 and *Paracolobactrum ballerup* were grown and treated with acetone, as previously described (22).

Isolation of Vi antigen. The procedure for isolation and purification of Vi antigen from *Citrobacter* strain 5396/38 is given in Fig. 1. Tris(hydroxymethyl)aminomethane was purchased from Sigma Chemical Co., St. Louis, Mo. Deoxyribonuclease 1 (Lot DCL, LIS 5613) and ribonuclease A (lot RAF, 9GA) were from Worthington Biochemical Corp., Freehold, N.J. Pronase (lot 801930) was a product of Calbiochem, Los Angeles, Calif., and hexadecyltrimethylammonium bromide (Cetavlon) was from Eastman Organic Chemicals, Rochester, N.Y.

Vi-W₁ was received in lyophilized form from M. Webster, National Heart and Lung Institute, Bethesda, Md., and Vi-W₂ was supplied as a solution (100 $\mu\text{g}/\text{ml}$) by S. Berman, Walter Reed Army Institute of Research, Washington, D.C. Both Vi antigens were prepared from *Citrobacter* strain 5396/38 by the method of Webster et al. (19).

Serology. Antisera were prepared in New Zealand rabbits weighing 2.5 to 3.5 kg. The animals were given six to eight weekly intravenous injections of acetone-killed and -dried cells suspended in saline. The cell content was adjusted to 10 U.S. opacity units (Division of Biologics Standards, NIH) by using the Lumetron Colorimeter model 400 A (Photovolt Corp., New York, N.Y.) at 530 nm. One milliliter of this suspension was given per injection. *P. ballerup* was used as antigen for the preparation of Vi antiserum, and *Citrobacter* strain 5396/38 was used for citrobacter antiserum. The rabbits were exsanguinated by cardiac puncture 1 week after the last injection. Selected Vi-negative colonies of *Citrobacter* strain

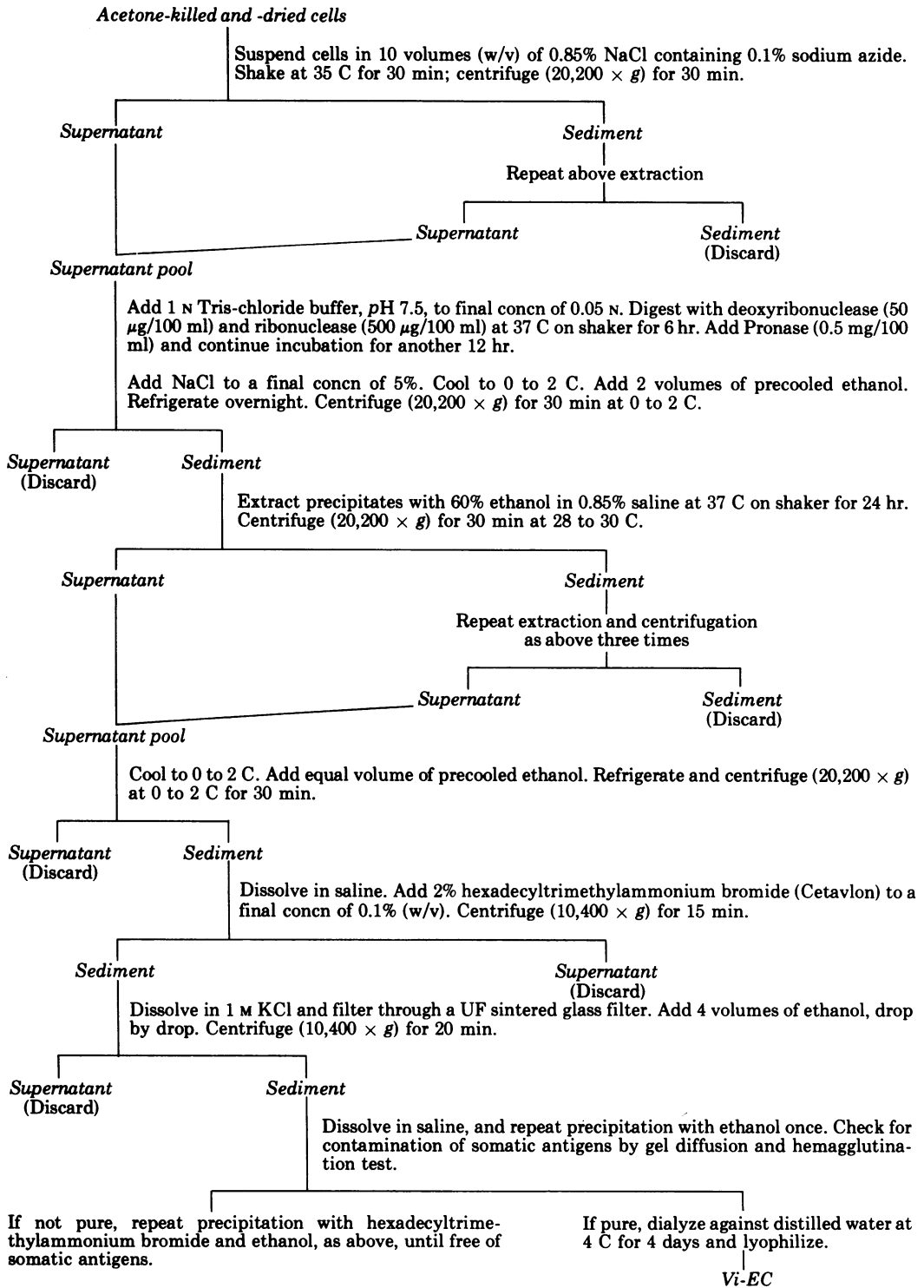


FIG. 1. Isolation of Vi antigen.

5396/38 were used to prepare the somatic antiserum as described by Edwards and Ewing (5).

The method of Landy and Lamb (10) was used for the hemagglutination test, and the gel-diffusion test was done as previously described (21).

Biological assays. All mice were of the NIH strain obtained from the Rodent and Rabbit Production Section, Division of Research Services, NIH.

Toxicity was assayed by injecting into each of five mice (14 to 16 g each) intraperitoneally 1 mg of Vi antigen in 0.5 ml of saline. The control group was given 0.5 ml of saline per mouse. The group weight of the mice was determined immediately prior to injection and at the end of 1, 2, 3, and 7 days after injection (4, 18).

Mice treated with actinomycin D (Lot 2304 M, Merck, Sharp & Dohme, West Point, Pa.) were used to detect trace amounts of endotoxin (14). Groups of five mice (22 to 26 g each) were injected intraperitoneally with 0.5 ml of saline containing 12.5 μ g of actinomycin D and up to 0.25 mg of Vi preparation. For positive control, *Escherichia coli* endotoxin (026:B6, control 547237, Difco), in amounts ranging from 10 to 0.0016 μ g per mouse in serial fivefold dilutions, was used to replace the Vi preparation. Inoculum for the negative control group consisted of 12.5 μ g of actinomycin D in 0.5 ml of saline per mouse. Survival was observed for 3 days, and 50% lethal dose values were calculated by the method of Reed and Muench (16).

Purified Vi preparations were compared by active mouse protection test, the method described in our previous publication (22). Fifty percent effective dose values were calculated by the Wilson-Worcester method (National Institutes of Health Memorandum, revised 1956, unpublished).

Measurement of Vi antigen. Purified Vi antigen was measured by EID (12, 13). Glass slides, 2 inches by 3 inches, were precoated with 1% Ionagar no. 2 (Consolidated Laboratories, Inc., Chicago Heights, Ill.), dried in a 37 C incubator, and stored at 4 C until use. For EID, 1.25 ml of 4% Ionagar, 1.25 ml of Vi antiserum, and 2.5 ml of B-2 buffer, ionic strength 0.15, pH 8.6 (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.) were mixed at 56 C in a water bath. A 4.5-ml amount of this mixture was overlaid on each slide. After the agar solidified, seven circular wells were cut in a line along the short axis of the slide, and each well was filled with 3 μ liters of Vi antigen solution, using a Ziptrol delivery system (Drummond Scientific Co., Broomall, Pa.). The charged slides were subjected to electrophoresis at 10 to 14 ma per slide for various periods of time at room temperature in an Agafor cell (National Instrument Laboratories, Inc., Rockville, Md.) containing B-2 buffer, ionic strength 0.075. After electrophoresis, the slides were soaked in phosphate-buffered saline, pH 7.2, for 4 hr at 4 C, and the length of each precipitate band from the origin was measured.

RESULTS

Isolation of Vi antigen. Treatment of the cell extract with deoxyribonuclease, ribonuclease, and Pronase effectively removed the contaminating nucleic acids and protein. No

nucleic acid or protein absorption peaks were observed in the final product at a concentration of 0.5 mg of Vi antigen per ml, as measured at 260 and 280 nm with a Gilford model 2400 spectrophotometer.

The Vi antigen in the digested cell extract was concentrated by extraction with 60% ethanol in saline at different temperatures (1), after which it was separated from the residual somatic antigens by precipitation with Cetavlon. The Vi-Cetavlon complex was dissolved in 1 M KCl, and the antigen was recovered by precipitation with ethanol. No bromide ion was detected in the final product by either silver nitrate or magenta reagent method (17).

The final yield was 48 mg of Vi antigen from 8 g of acetone-dried cells.

Serological properties. The Vi antigen isolated by this ethanol-Cetavlon precipitation procedure (Vi-EC) was compared serologically with Vi-W₁ prepared by the method of Webster et al. (19), which employed acid hydrolysis for separation of the Vi and O antigens.

Double gel diffusion with antisera against *Citrobacter* strain 5396/38 and *P. ballerup*, respectively, revealed one sharp, narrow band with Vi-EC, and a wide, blurred band with Vi-W₁. These two bands were not identical. Both antigen preparations sensitized human O erythrocytes and gave positive hemagglutination in anti-Vi serum. No reaction of the sensitized red cells was observed with anti-*Citrobacter* O serum, which indicates that somatic antigens were absent from both Vi preparations.

Biological assays. The absence of endotoxin is supported by the toxicity tests. The group of mice injected with 1 mg of Vi-EC per animal showed a weight gain curve not different from that of the normal control group. In assays with mice treated with actinomycin D, no death occurred among those injected with up to 0.25 mg of Vi-EC, the highest dose tested. A 50% lethal dose of 0.2 μ g was obtained for the *E. coli* endotoxin from the positive control group.

The results of active mouse protection tests with three Vi preparations are shown in Tables 1 and 2. Administered by the intraperitoneal route (ip), Vi-EC was 18 and 230 times, respectively, more potent than Vi-W₁ and Vi-W₂. By the subcutaneous route (sc) of immunization, the potency of Vi-EC was 13 times greater than that of Vi-W₁. The mice were better protected by ip than by sc vaccination. The ip to sc ratio for the 50% effective dose of Vi-EC was 3.6, and that for Vi-W₁ was 2.7.

Measurement of Vi antigen. The Vi antigen migrated toward the anode in an electrical

TABLE 1. Comparison of protective activity of Vi-EC and Vi-W₁ in mice

Antigen	ED ₅₀ (μg) Route of immunization			Relative potency (Vi-W ₁ = 1)	
	ip	sc	ip/sc	ip	sc
Vi-EC	0.00361 (0.0023-0.0056) ^b	0.013 (0.01-0.016)	3.6	18.2 (11.8-28.0)	13.4 (9.9-18.1)
Vi-W ₁	0.0657 (0.044-0.099)	0.175 (0.13-0.25)	2.7		

^a Geometric mean of two tests. ED₅₀, Mean effective dose.

^b Range of one standard deviation.

TABLE 2. Comparison of protective activity of Vi-EC and Vi-W₂ in mice

Antigen	ED ₅₀ ^a (μg) ip immunization	Relative potency (Vi-W ₂ = 1)
Vi-EC	0.0023 (0.0015-0.0037) ^b	230 (142.7-369.4)
Vi-W ₂	0.53 (0.33-0.85)	

^a Geometric mean of two tests.

^b Range of one standard deviation.

field, and the length of the precipitate band formed by antigen and antibody reaction in the agar, as measured from the antigen well to the tip of the band, was directly proportional to the antigen concentration. For a given preparation of Vi antigen, factors influencing the length of the precipitate band include: (i) antigen concentration, (ii) antibody concentration, and (iii) duration and voltage of electrophoresis (Fig. 2).

For each determination, three to four appropriate dilutions of the reference Vi preparation were run on the same slide with the test sample. A standard curve was constructed for the reference antigen, and the concentration of Vi antigen in the test sample was read from the standard curve.

Vi antigen preparations isolated by different methods did not give identical reactions in EID at constant concentrations (Table 3). The length of precipitate band and sensitivity limits were influenced by antibody concentration and probably by molecular size of the purified antigen. At 1:20 serum concentration, sensitivity range for Vi-EC was from 10 to 60 μg per ml, whereas that of Vi-W₁ and Vi-W₂, which had been hydrolyzed with hot acetic acid during purification and were known to exist in a somewhat degraded form, was from 3 to 24 μg. As the serum concentration increased, the lower sensitivity limit decreased.

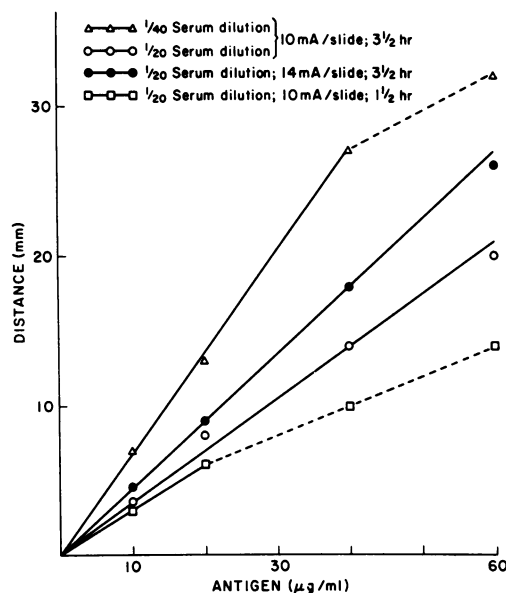


FIG. 2. Measurement of Vi antigen by electroimmunodiffusion. Distance was measured as length of precipitate from origin to end of band.

TABLE 3. Comparison of preparations of Vi antigen by EID^a

Antigen (18 μg/ml)	Length of precipitate band (mm)	Sensitivity range (μg/ml)
Vi-EC	12	10-60
Vi-W ₁	22	3-24
Vi-W ₂	21	2-24

^a Antigens were compared on the same slide after electrophoresis for 3.5 hr at 12 ma. Serum dilution was 1/20.

DISCUSSION

Several attempts have been made to isolate Vi antigen from *S. typhosa* and other enteric organisms. Vi preparations isolated by various methods differ in chemical and immunogenic

properties. The method of Webster et al. (19) yielded highly purified Vi antigen in relatively large quantity, but it required hydrolysis with hot acetic acid which might depolymerize the antigen and affect its immunogenicity. Baker et al. (1) obtained separation of the Vi antigen from somatic antigens in extracts of *P. ballerup* strain 481 and *S. typhosa* strain 61 by fractionation with 60% ethanol in saline at 37 C and precipitation at 0 C. However, when *E. coli* strain 136 and *S. typhosa* Ty 2 were used, separation was not complete (1). Jarvis et al. (8) described a purification method using continuous-flow, paper curtain electrophoresis. The Vi antigen they obtained was found to be more immunogenic in mice than that obtained by the method of Webster et al. No conclusive serological data were obtained in human volunteers for these two antigen preparations because of the limited size of the trial (11).

We describe here a method which does not require drastic chemical treatment or expensive equipment. Vi antigen was separated from somatic antigens in a crude extract of *Citrobacter* strain 5396/38, as indicated by serological and immunological tests. Both ethanol-saline concentration and precipitation with Cetavlon were necessary for complete separation of the Vi antigen from somatic antigens in the cell extract. On preliminary studies, we found that neither was effective alone.

Hornick et al. used Vi-W₂ to immunize human volunteers and found no protection against challenge with *S. typhosa* (7). We demonstrated here that the potency of Vi-W₂ in mice was only 1/230th of the potency of Vi-EC. Work is in progress in our laboratory to determine whether a Vi preparation of high mouse potency isolated from *S. typhosa* is immunogenic in man.

Vi antigen appeared to be more protective in mice by the ip route of immunization than the sc route. This is in agreement with previous work with cellular typhoid vaccines. Pittman and Bohner (15) showed that in a mouse protection assay in which ip vaccination and challenge with *S. typhosa* suspended in mucin were used, acetone-inactivated vaccine K was 3.69 times more potent than the heat-phenol-inactivated vaccine L, whereas with sc immunization, vaccine K was only 0.78 as potent. The ip values reflected the relative efficacy of these two vaccines in field trials. Wong et al. (22) treated the acetone-inactivated vaccine 6A and vaccine L with a Vi-degrading enzyme and showed that these vaccines differed in the amount or the quality of their Vi antigen, which

was a major protective factor in an assay where ip vaccination was used.

Chemically, Vi antigen is a polymer of *N*-acetyl-D-galactosamine-uronic acid. The quality and quantity of this antigen have caused much concern in the control of typhoid vaccines. As compared with other quantitative methods (3, 9, 20), EID, as described here, has the advantage of being sensitive, specific, and relatively simple. EID has been used successfully by other investigators for measuring immunoglobulins and other proteins (12, 13). Depending on the serum concentration, accurate measurement could be made with as little as 10 μ g of Vi antigen per ml in crude cell extract. This method shows promise of being convenient for determining quantitatively extractable Vi antigen from different lots of typhoid vaccine prepared by the same procedure.

ACKNOWLEDGMENTS

We thank M. E. Webster of National Heart and Lung Institute, S. Berman of Walter Reed Army Institute of Research for the reference Vi preparations, and M. Pittman and R. S. Northrup of the Division of Biologics Standards, National Institutes of Health, for their review of this paper.

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