Vibrio fetus Infection in Man: a Serological Test

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Antigen preparations derived from a typical human strain of *Vibrio fetus* were employed in four tests. Of these, the indirect bacterial hemagglutination test proved most sensitive. By this test, antibodies titering 320 to 3,200 were found in five of eight patients with confirmed infections. Two patients without antibodies were on antimetabolites. Antigenic relationship with other compounds, and in particular with *Brucella* organisms, was not observed. No sero-reactors were found among 184 apparently healthy young men; of 401 unselected hospital patients, four had low sero-titers.

The incidence of *Vibrio fetus* infection in man is believed to be considerably higher than indicated by published case reports (5, 7, 18, 20). It was estimated that less than 1% of the cases are recorded (1). Several factors are involved: first, characteristic clinical symptoms are lacking (1); second, accurate timing of collection of blood cultures seems essential; and third, correct identification of *V. fetus* poses problems in laboratories unfamiliar with veterinary bacteriology. Diagnoses of the hitherto confirmed cases were established by the recovery of *V. fetus* from mono-infected tissues, usually blood. The organism has not been isolated from mixed flora of man.

Additional diagnostic tools are desirable. A standardized serological test is a possibility since many patients with generalized vibriosis possess high-titered humoral antibodies to the homologous bacterial strain (3, 5, 20). Apart from the sensitivity and specificity of such a test, its usefulness would be determined mainly by the extent to which the employed antigen represents the antigenic determinants of *V. fetus* infecting man. Working with bovine and ovine strains of *V. fetus*, Morgan (10) allocated 18 strains to a somatic group A and 7 strains to group B. These findings were essentially confirmed by Soderlind (17). King (6) found an even closer antigenic relationship among strains of *V. fetus* from man. Based on somatic antigens, however, Winkler (21) and White and Walsh (19) classified human strains in two distinct serological groups. The somatic antigens were found to be soluble lipopolysaccharide complexes (2, 12, 13). One such compound derived from *V. fetus* var. *intestinalis* consisted of 53% carbohydrates and 28% lipids (23). The lipopolysaccharides were resistant to heat and alkali (2, 12) and readily

adsorbed onto erythrocytes rendering them agglutinable by somatic antiserum (13, 23). Addition of complement to erythrocytes coated with antigen-antibody complexes resulted in lysis (14).

Flagellar antigens of both animal strains (8) and human strains (19) occur in numerous serotypes. K-antigens are irregular components of *V. fetus* (8).

Thus, only the somatic antigen with its limited number of major determinants lends itself readily to the development of a serological test. This paper describes the results obtained with somatic antigen preparations from a typical human strain of *V. fetus*.

MATERIALS AND METHODS

Media. Thioglycolate broth (Baltimore Biological Laboratories) and Todd-Hewitt broth (Difco) were employed according to the manufacturers' instructions. Chocolate-agar was prepared as follows: 43 g of Brucella agar (Pfizer Diagnostic Division) and 5 g of Pfizer agar (Pfizer Diagnostic Division) were dissolved in 1,000 ml of distilled water, autoclaved at 115°C for 15 min, and cooled to approximately 80°C. After the addition of 50 ml of citrated human blood, the mixture was cooled to 50°C and distributed into petri dishes (8.5 cm). Chocolate-agar prepared by the addition of 1% hemoglobin (Difco) to the base medium before autoclaving was equally effective.

Antigens. The following human *V. fetus* strains were used for antigen production: a strain recently isolated here from blood (St. Luke's strain); and for comparison, strains A3208 and A3430 (19) kindly supplied by F. H. White, Department of Veterinary Science, University of Florida.

For maximal antigen production it was necessary to regenerate the strains before use. Accordingly, the strains were passaged at 2-day intervals to fresh thioglycolate broth; adequate growth was obtained after the second or third transfer. During the ex-

ponential growth phase, cultures were diluted in Todd-Hewitt broth to yield 4×10^4 viable bacteria per ml; 0.05 ml of this preparation (approximately 2×10^3 microorganisms) was seeded on chocolate-agar. The plates were incubated at 36 to 37 C for 5 days in an atmosphere of 81% N₂, 12% CO₂, and 7% O₂. The bacterial growth from each petri dish was harvested in 5 ml of saline (pH 7.0), checked for purity, and pooled. The following antigen preparations were made from this dense suspension.

(i) Formalinized bacterial suspension: 10% Formalin was added to the bacterial suspension in the proportion 1:20.

(ii) Heat-killed bacterial suspension: a sample of the bacterial suspension was heated to 100 C for 2 hr, washed twice in buffer, and resuspended in 0.5% Formalin in buffer.

(iii) Antigenic extract: unless otherwise stated, the bacterial suspension was heated to 100 C for 2 hr, cooled to room temperature, and centrifuged at $12,000 \times g$ for 45 min. Two volumes of the supernatant fraction were treated with one volume of 0.25 N NaOH at 56 C for 30 min. The mixture was cooled to room temperature, adjusted to pH 7.2 with 0.25 N HCl, and centrifuged at $20,000 \times g$ for 45 min. This antigenic extract occasionally caused clumping of the erythrocytes. Dialysis in Visking tubes, pore size 0.0024 μ m, against 0.15 M NaCl at 4 C for 48 hr removed the erythrocyte-damaging factor. Antigenically weak solutions were concentrated in the same type of tubes by dialysis against a saturated solution of sucrose at room temperature. The purified product was stored at -12 C.

(iv) Ultrasonic treatment: a Branson sonifier model S125 operating at 20 kc was used for ultrasonic treatment of 10-ml amounts of bacterial suspension.

V. fetus rabbit antiserum. Albino rabbits (approximately 2.0 kg) were injected intravenously with a formalinized suspension of heated and washed *V. fetus* cells adjusted to ca. 10^9 organisms per ml. The antigen was administered four times, at 3- to 4-day intervals, in the following volumes: 1.0 ml, 1.0 ml, 2.0 ml, and 2.0 ml, respectively. The animals were bled 7 days after the last injection. High-titered sera were pooled, mixed with an equal volume of chemically pure glycerin, and stored at 4 C.

V. fetus sera from man. Sera, obtained from bacteriologically confirmed cases described in a previous paper (1), were stored at -12 C.

Other antisera. The specificity of the *V. fetus* hemagglutination test was checked with a variety of antisera. These and their corresponding antigens were partly purchased (Difco Laboratories and Baltimore Biological Laboratories) and partly obtained from colleagues as cited below.

Buffer. Difco phosphate hemagglutination buffer, pH 7.2 to 7.3, was used throughout the experiments for washings, suspensions, and dilutions.

Bacterial agglutination. Serum in serial twofold dilutions (0.25 ml) was mixed with an equal volume of bacterial suspension adjusted to ca. 10^9 organisms per ml. The mixture was incubated at 37 C for 18 hr, centrifuged at $500 \times g$ for 3 min, and read.

Latex agglutination. Latex suspension (0.81 μ m,

Difco) was diluted 1:50 in buffer and added to an equal volume of antigen solution. The mixture was incubated at 37 C for 30 min. To 0.5 ml of this reagent, antiserum in serial dilutions (0.5 ml) was added. The mixtures were incubated at 56 C for 90 min, centrifuged at $800 \times g$ for 3 min, and examined for gross agglutination.

Indirect bacterial hemagglutination. The hemagglutination test (HA test) was carried out as described by Neter et al. (11). Briefly, human type O erythrocytes (2.5% suspension) were washed three times in buffer. The sediment was resuspended to 2.5% in a dilution of antigen that would produce an HA titer of 1,600 to 3,200 against a glycerinated rabbit antiserum. The mixture was incubated at 37 C for 30 min; the modified erythrocytes were washed five times in excess buffer to remove unadsorbed antigen, and resuspended in buffer. The treated erythrocytes (0.2 ml) were mixed with twofold serially diluted antiserum (0.2 ml), and incubated at 37 C for 30 min. The resulting hemagglutination was read grossly after centrifugation at $800 \times g$ for 3 min. Readings obtained with erythrocyte suspensions titrating less than 1,600 or more than 3,200 with a glycerinated rabbit antiserum were discarded. All sera were examined a second time, and reactive sera were tested for agglutinins to uncoated erythrocytes.

RESULTS

Selection of indicator system. All four antigen preparations listed in Table 1 were titrated simultaneously against samples of a single set of serially diluted rabbit antiserum. The HA test was the most sensitive of the reactions examined; it was selected as our indicator system. Heated, washed bacterial cells gave clear-cut readings but were less sensitive. The employed antigenic extract did not lend itself readily to latex agglutination.

Antigen extraction. Regardless of the extraction method employed, NaOH hydrolysis was required to produce an antigenic solution capable of modifying the erythrocyte suspension to an HA titer of 1,600 to 3,200 against the glycerinated rabbit antiserum. Table 2 shows that heat treatment of the bacterial cells (boiling or autoclaving) produced the most suitable extracts for subse-

TABLE 1. Sensitivity of indicator systems for the detection of *Vibrio fetus* antibodies

Indicator system	Titer (reciprocal) ^a
Bacterial suspensions	
Unheated, formalinized	400
Heated, washed	1,600
Hemagglutination ^b	6,400
Latex agglutination ^b	<50

^a Against glycerinated rabbit antiserum.

^b Antigenic extracts diluted 1:2.

TABLE 2. Hemagglutination titers of antigen extracts of *Vibrio fetus* obtained by various treatments

Treatment ^a	Hemagglutination titer (reciprocal) ^b from		
	Whole bacterial suspension	Super-natant fluid ^c	Sediment ^c
None	< 50	< 50	< 50
100 C, 2 hr	6,400	< 50	3,200
121 C, 20 min	6,400	< 50	6,400
Ultrasonic, 10 min	1,600	< 50	Not done

^a Treatment was followed by NaOH hydrolysis of supernatant fluid.

^b Against glycerinated rabbit antiserum.

^c Obtained after centrifugation of samples of bacterial suspension.

TABLE 3. Antigenic relationship to other *Vibrio fetus* strains

Bacterial suspensions	Hemagglutination titers (reciprocal) of <i>V. fetus</i> antisera		
	St. Luke's	A3208	A3430
St. Luke's	3,200	800	< 25
A3208 ^a	3,200	800	< 25
A3430 ^a	< 25	< 25	800

^a Strains from F. H. White's collection (19).

quent hydrolysis. Ultrasonic treatment of the bacterial suspension produced weaker extracts. Little antigenic material was released into the supernatant fraction from a bacterial suspension at room temperature.

Extraction of a heat-killed (80 C, 10 min) suspension of *V. fetus* with 0.02 N NaOH at 100 C for 5 min (22) failed to yield potent antigenic preparations. Regardless of the extraction method employed, centrifugation of the hydrolyzed extract at $20,000 \times g$ for 45 min improved its modifying capacity.

Antigenic relationship to other strains. Glycerinated antisera were prepared to St. Luke's strain, A3208, and A3430. Cross-agglutination (Table 3) and absorption experiments with heated bacterial cells revealed that the somatic antigens of St. Luke's strain and strain A3208 were similar if not identical. There was no evidence of antigenic relationship between these strains and strain A3420.

Antibody levels in confirmed cases. Five of eight patients with confirmed *V. fetus* infections (1) showed moderate to high HA-antibody titers at the time of bacteremia (Table 4). Two of the patients without measurable antibody

TABLE 4. Antibody levels in sera from *Vibrio fetus* patients at time of bacteremia

Underlying disorder	Source of <i>V. fetus</i>	Hemagglutination titer (reciprocal)
Chronic alcoholism	Blood	3,200
Chronic myelogenic leukemia	Blood	6,400
Chronic renal disorder	Blood	1,600
Primary hepatoma	Blood	400
Chronic lymphatic leukemia	Blood	< 20
Pyarthrosis	Knee	320
Liver cirrhosis	Blood	< 20
Lymphosarcoma ^a	Blood	< 20

^a Treated with anti-metabolites.

received antimetabolites for some months before the blood collection; in one of these, the immunoglobulin G level was 320 mg per 100 ml of serum (normal 1,200 to 1,800); it was not measured in the other patient. The third patient without measurable *V. fetus* antibodies suffered from cirrhosis of the liver. The antigenic composition of the infecting strain was unknown, as was the presence or absence of humoral antibodies to that particular strain.

Antibody levels in patients with high titers declined following clinical recovery, and 4 to 6 months later they were in the 40 to 320 range. In two patients with clinical relapse, the titers remained high until approximately a month after the beginning of the curative treatment.

Specificity of *V. fetus* HA test. A series of antisera with homologous titers from 160 to 6,400 failed to agglutinate *V. fetus*-modified erythrocytes over a dilution range of 20 to 200: *Escherichia coli* 026:B6, 055:B5, 0111:B4, and 0127:B8; *Shigella* groups A through D; *Salmonella* groups A through I; *Streptococcus* groups A through G; common enterobacterial antigen (kindly supplied by E. Neter, Children's Hospital, Buffalo, N.Y.); *Mycoplasma hominis* or *arthritidis* (kindly supplied by J. G. Tully, Laboratory of Bacterial Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md.); and *T. pallidum* (kindly supplied by N. J. Richardson, South African Institute for Medical Research, Johannesburg, South Africa). An antigenic relationship between *V. fetus* and *Brucella* organisms (4, 18) was not detected with our preparations (Table 5).

The HA test also failed to detect *V. fetus* antibodies among patients with moderate to high titers of humoral antibodies to: *S. typhi*, (10 patients); *S. paratyphi* A, (4 patients); *S. paratyphi* B, (4 patients); *Proteus* 0 $\times$ 19, (5 patients); streptolysin-O (77 patients); cephalin-

TABLE 5. *Antigenic relationship of Vibrio fetus and Brucella organisms*

Antigen prepn	Titers (reciprocal) of antisera to		
	<i>V. fetus</i>	<i>B. melitensis</i>	<i>B. abortus</i>
<i>V. fetus</i> HA.....	5,120	<20	<20
<i>B. melitensis</i> ^a	<20	160	160
<i>B. abortus</i> ^a	<20	160	320

^a Bacterial suspension.TABLE 6. *Antibody levels in groups of apparently healthy young men and unselected hospital patients*

Groups	Total no.	Reactors		Hemagglutination titers (reciprocal)
		No.	%	
Healthy men, 18-25 years.....	184	0	0	<20
Unselected hospital patients ^a	401	4	1	40-160

^a Sera from hospital patients whose blood was submitted for reagin testing with antigen preparation from Venereal Disease Research Laboratories, Atlanta, Ga.

cholesterol (Hanger's test, 49 patients); sheep erythrocytes (Paul Bunnell, 17 patients); C-poly-saccharide of *Pneumococcus* (38 patients); *Brucella* organisms (48 patients); and gamma globulin (rheumatoid factor, 44 patients).

Rabbit antiserum to *V. fetus* neither reacted with the above-mentioned antigens, nor contained immobilizing antibodies to *T. pallidum* (N. J. Richardson, South African Institute for Medical Research, Johannesburg, South Africa).

Additional evidence of the specificity of the test was derived from the absence of measurable amounts of antibodies in sera from 184 apparently healthy young men (Table 6). Antibodies in low titers were present in 1% of random hospital patients.

Stability of *V. fetus* antibodies. Antibody levels in human and rabbit sera, with titers from 20 to 1,600, were unaffected by repeated freezing and thawing, exposure to 56 C for 30 min, and by storage at room temperature for 1 week in 1:10,000 Merthiolate.

DISCUSSION

A human isolate of *V. fetus* possesses an antigen which modifies erythrocytes for agglutination by homologous, but not heterologous, antisera. Likely, this antigen is analogous to the lipopolysaccharide extracted from animal strains

(2, 12-14, 23). The antigen is firmly lodged in the bacterial cell. It may be extracted by prolonged heat treatment and requires hydrolysis at high pH for activation.

St. Luke's strain of *V. fetus* employed in the experiments was chosen because: (i) it had been isolated recently from a human case; (ii) it shared antigenic determinants with the human strain 1134 (6); (iii) it agglutinated readily in homologous antiserum; and (iv) it yielded a potent antigenic extract. The latter property was the reason for choosing St. Luke's strain over strain 1134, which allegedly had lost some of its potency for antigen production. The somatic antigen of St. Luke's strain is similar, if not identical, to strain A3208, a representative of the larger of White's two groups of serotypes from human hosts (19). Although White's serogroups probably cover most human isolates of *V. fetus*, we have recently observed a strain that did not share antigens with either group. Thus, St. Luke's strain appears to be antigenically representative of most, but not all, serotypes of *V. fetus* infecting man. This is further corroborated by the presence of antibodies, detectable by St. Luke's strain antigen, in the sera of most of the bacteriologically confirmed cases.

The high HA titers observed in most patients at the time of bacteremia declined significantly over a period of 4 to 6 months after clinical recovery. The low titers found in some hospital patients from whom *V. fetus* was not isolated may be attributed to: (i) prior infections; (ii) exposure to similar antigens from sources other than *V. fetus*; (iii) localized intestinal infection with *V. fetus* var. *intestinalis*; or (iv) "natural antibodies" (22). Antigenic relationship between *V. fetus* and *Brucella* organisms was claimed by Spink (18). Kiggins et al. (4) observed that antisera prepared from 3 of 16 strains of *V. fetus* agglutinated *Brucella* organisms to one-fourth or one-eighth of the homologous titer. The remaining 13 strains of *V. fetus* were antigenically unrelated to *Brucella* organisms, as was the St. Luke's strain. Winter (22), working with *V. fetus* var. *intestinalis*, attributed low titers in sera of older cows to the presence of "natural antibodies." He conceded, however, that absorption of antigenically related substances could have engendered the antibody response. This is of particular interest to us since there is accumulating evidence that most human cases are caused by the intestinal rather than by the venereal variant of *V. fetus*. Using Laing's biochemical classification (9), White (19) found that 12 of 12 strains of *V. fetus* recovered from man belonged to the intestinal form. Similarly, in this laboratory we identified 10 of 14 isolates as

V. fetus var. *intestinalis* (unpublished data). All strains were recovered from mono-infected tissues (1). Conceivably the tissues were invaded from a site where the vibrios formed a part of a mixed flora, for example from the intestinal tract. The hypothesis that *V. fetus* could exist temporarily in the human gut offers an attractive explanation for the transmission of the organisms from animals and birds (15, 16) to man, for the presence of low-titered *V. fetus* agglutinins in apparently noninfected individuals, and for the development of clinical infection in debilitated individuals.

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