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Enhancement of Coagglutination Reactions of the Phadebact Gonococcus Test by Ethylenediaminetetraacetate and Ethylene Glycol-bis(β-Aminoethyl Ether)-*N*,*N*-Tetraacetate

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Incubation of gonococci under conditions optimal for autolysis resulted in increased sensitivity and enhancement of the coagglutination reaction of the Phadebact gonococcus test. These conditions included an alkaline pH (pH 8.3) and the presence of divalent cation chelators such as ethylenediaminetetraacetic acid or ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid. Heating cell suspensions at 90°C for 15 min before assay by coagglutination produced a further increase in sensitivity and enhancement of the reaction. Gonococcal lipopolysaccharide was found to be an important antigen in these coagglutination reactions. The detection of lipopolysaccharide was markedly enhanced by the addition of chelating agents.

The Phadebact gonococcus test (Pharmacia Diagnostics, Piscataway, N.J.) is a slide coagglutination procedure which involves an immunoabsorbent of specifically purified immunoglobulin G-class gonococcal antibodies. The antibodies are absorbed so that the cross-reactive antibodies are removed and coupled to the protein A of heat-killed intact staphylococci. The immunoabsorbent reagent reacts specifically with gonococcal cell surface antigens. Fresh clinical isolates and actively growing cells give the best coagglutination reaction. However, this latter factor often necessitates transfer and additional incubation time.

Gonococci which are suspended in buffer at an alkaline pH undergo rapid autolysis (21). Autolysis can be prevented by the addition of divalent cations such as Mg^{2+} and Ca^{2+} (21). These divalent cations are responsible for the integrity of the outer membranes of gram-negative bacteria (2, 13). Chelation of divalent cations with ethylenediaminetetraacetate/acetic acid (EDTA) results in the release of lipopolysaccharide (LPS) and outer membrane proteins (6, 16). These cell surface components are important cell surface antigens and may be involved in the coagglutination reaction. Therefore, autolytic conditions and the presence of divalent cation chelators were evaluated as means of increasing the sensitivity as well as enhancing the coagglutination reaction of the Phadebact gonococcus test.

MATERIALS AND METHODS

Organisms. Twenty confirmed clinical strains of *Neisseria gonorrhoeae*, including 1 pharyngeal, 5 cervical, and 14 urethral isolates, were randomly selected from 500 isolates obtained from the Multnomah County Health Department and stored frozen at −70°C (12) after one subculture on GC agar (Difco Laboratories) plates. Stock cultures of *Neisseria meningitidis* (four strains), nonpathogenic neisseriae (eight strains), and *Branhamella catarrhalis* (eight strains) were also used.

Cell suspensions and counting. Isolates were streaked on GC agar plates and incubated overnight at 37°C with increased CO_2 (4%). Sufficient growth was removed from the agar plates, suspended in 1 ml of 0.85% NaCl containing 0.1% cysteine (pH 6.4) (12), and mixed to break up clumps of bacteria. The total number of cells was estimated by direct counting in a Petroff-Hausser counting chamber. Single cocci and diplococci were counted as one cell. The cell suspensions were serially diluted (twofold dilutions) in 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 8.3) to give a range of cell concentrations (ca. 10^7 to 10^9 cells per ml). The pH of the buffer was varied (see Table 1). Stock solutions (0.1 M) of EDTA or ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA) were prepared in 0.1 M HEPES buffer (same pH as the suspending buffer) and added to give a final concentration of 9 mM. Cell suspensions were incubated at room temperature for 15 min before being assayed by coagglutination. In some experiments, the cell suspensions were heated at 90°C for 15 min before assay by coagglutination.

Coagglutination test. Phadebact test kits were obtained from Gary Britton (Pharmacia Diagnostics). The coagglutination test was performed by mixing 10 μl of the cell suspension and 10 μl of the rehydrated Phadebact gonococcus reagent or the Phadebact control reagent on a clean microscope slide. The slide was rocked for 2 to 3 min in accordance with the directions

TABLE 1. *Effect of pH on coagglutination reactions with 20 strains of N. gonorrhoeae*

Addition	pH	No. of tests	No. of tests with reaction:					
			0	1	2	3	4	5
None	6.3	50	20 (50)[a]	8	2	6	8	6
	7.4	50	14 (50)	6	8	3	6	13
	8.3	50	10 (50)	7	4	6	5	18
EDTA (9 mM)	6.3	59	12 (59)	7	5	7	10	18
	7.4	59	5 (56)	4 (3)	9	7	10	24
	8.3	59	3 (56)	3 (2)	8 (1)	4	10	30
EGTA (9 mM)	6.3	59	17 (59)	6	5	7	7	17
	7.4	59	9 (53)	5 (6)	6	6	5	28
	8.3	59	3 (51)	5 (8)	5	4	1	39

[a] Numbers in parentheses indicate the numbers of tests giving the indicated reactions with the control reagent. In all instances of a positive reaction (1 or 2) with the control reagent, the comparable reactions with the gonococcus reagent were scored as ≥4.

TABLE 2. *Effect of EDTA, EGTA, and heating on the sensitivity of the Phadebact gonococcus test*

Addition	Heating[a]	Negative reaction	
		Mean[b]	SD[c]
None	−	2.4	1.9
	+	0.9	0.7
EDTA (9 mM)	−	1.1	0.9
	+	0.5	0.3
EGTA (9 mM)	−	1.2	0.9
	+	0.5	0.3

[a] Cell suspensions were either heated at 90°C for 15 min (+) or incubated at room temperature for 15 min (−).

[b] Average number of cells (10^6) giving a negative coagglutination reaction.

[c] SD, Standard deviation (10^6).

of the manufacturer and observed with a dissecting microscope. Coagglutination reactions were scored on a scale of 0 to 5 by a single individual (Izakson). A negative reaction exhibited a uniform background and was designated as 0. Positive reactions (1 through 4) exhibited increasing sizes of clumps of agglutinated staphylococci. Reactions scored as 5 had a completely clear background with several large clumps of agglutinated staphylococci.

LPS. LPS was extracted and purified from *N. gonorrhoeae* strain 1361 (type 1) by the procedure of Galanos et al. (1). This preparation contained ca. 5% (wt/wt) protein, as estimated by the method of Lowry et al. (10), with bovine serum albumin as a standard.

RESULTS

Effect of pH. Gonococcal autolysis is markedly affected by pH. The rate of autolysis is maximal at an alkaline pH (pH 8 to 9) and suppressed at an acid pH (pH, <6.5) (21). To examine the effect of pH, 10 gonococcal isolates were suspended and serially diluted in 0.1 M HEPES buffer at pH 6.3, 7.4, or 8.3. After being heated for 15 min at 90°C, the cell suspensions were tested for coagglutination with either the gonococcus or the control reagent. The number of positive reactions as well as the strength of the coagglutination reaction increased with increasing pH (Table 1). No positive coagglutination reactions were observed with the control reagent. The addition of EDTA or EGTA before heating increased the number of positive coagglutination reactions as well as enhanced their strength. These effects increased with increasing pH. Positive coagglutination reactions with the control reagent were observed with cells heated at pH 7.4 and 8.3 in the presence of EDTA and EGTA. However, positive reactions were only observed at the higher cell concentrations. In all of these instances, the coagglutination reactions with the gonococcus reagent were scored as ≥4. Gonococci suspended in buffer under these conditions were heated for 15 min at 90°C, and the result was the release of antigens reactive in the Phadebact gonococcus test. After centrifugation, the supernatant gave a positive coagglutination reaction with the gonococcus reagent (data not shown). These reactions were weakest at pH 6.3 and in the absence of EDTA or EGTA.

Factors affecting sensitivity of the coagglutination reaction. Twenty isolates of *N. gonorrhoeae* were suspended and serially diluted in 0.1 M HEPES buffer (pH 8.3). The suspensions received chelators or were heated as indicated or both received chelators and were heated. The serially diluted cell suspensions were sequentially tested for coagglutination until a negative reaction was obtained. The num-

TABLE 3. *Effect of EDTA, EGTA, and heating on coagglutination reactions with N. gonorrhoeae*

Addition	Heating[a]	Reaction			Positive reaction[b]	
		No. of tests	Positive	Negative	Mean	SD
None	−	95	32	3	2.0	1.2
	+	95	58	0	3.3	1.6
EDTA (9 mM)	−	95	52	0	3.0	1.5
	+	95	75	0	3.7	1.6
EGTA (9 mM)	−	95	51	0	3.1	1.6
	+	95	75	0	4.0	1.6

[a] Cell suspensions were either heated at 90°C for 15 min (+) or incubated at room temperature for 15 min (−).

[b] Means and standard deviations (SD) of positive reactions scored on a scale of 1 to 5.

TABLE 4. *Detection of gonococcal LPS by coagglutination*

Source	LPS (ng)	Reaction with addition:[a]		
		None	EDTA	EGTA
N. gonorrhoeae	175	1	5	5
	87	0	5	5
	44	0	4	4
	22	0	3	3
	11	0	2	3
	6.5	0	1	2
	2.7	0	1	1
	1.3	0	0	0
E. coli	≥10,000	0	0	0
S. typhimurium	≥10,000	0	0	0

[a] The coagglutination reactions are scored on a 0 (negative) to 5 scale.

bers of cells in the suspensions giving negative reactions were used for comparing the effects of EDTA, EGTA, and heating on the sensitivity of the coagglutination test. The addition of EDTA or EGTA produced a twofold increase in sensitivity when the cell suspensions were incubated at room temperature for 15 min before being tested for coagglutination (Table 2). Heating the cell suspensions at 90°C for 15 min resulted in a further 2- to 2.7-fold increase in sensitivity. This increase is statistically significant ($P = 0.005$), as determined by Student's t test.

Factors affecting strength of the coagglutination reaction. Heating and the addition of EDTA or EGTA increased the number of positive coagglutination reactions with serially diluted cell suspensions of *N. gonorrhoeae* (Table 3). Three isolates gave negative coagglutination reactions at all cell concentrations when incubated for 15 min at room temperature in the absence of EDTA or EGTA. These isolates gave a positive coagglutination reaction after the cell suspensions were heated at 90°C for 15 min. The percentage of randomly detected isolates exhibiting this phenomenon (15%) was similar to the percentage reported by Helstad and Bruns (18%) (5). The strengths of the coagglutination reactions, as measured by the means of the scores of all positive reactions, were increased by both heating and the presence of EDTA or EGTA.

Detection of gonococcal LPS by coagglutination. Gonococcal LPS was extracted and purified as previously described (1). The LPS was suspended, serially diluted, and tested for coagglutination with the gonococcus and control reagents. Either EDTA or EGTA was required for the optimal detection of gonococcal LPS by coagglutination (Table 4). As little as 2.7 ng of gonococcal LPS gave a positive coagglutination reaction. Negative coagglutination reactions were obtained with LPS from *Escherichia coli* and *Salmonella typhimurium* at concentrations of ≥10,000 ng. Negative coagglutination reactions were also obtained with the control reagent for all three types of LPS.

Factors affecting specificity of the coagglutination reaction. Twenty stock cultures of *Neisseria* spp. and *B. catarrhalis* (described above) were suspended and serially diluted in 0.1 M HEPES buffer (pH 8.3). The suspensions received chelators or were heated at 90°C for 15 min or both received chelators and were heated. These suspensions were then tested for coagglutination with both the control and gonococcus reagents. Twelve cultures exhibited negative coagglutination reactions with both reagents. The remaining eight cultures gave weak positive reactions (reactions scored as 1 to 2) in both the control and gonococcus reagents. These reactions were only observed at cell concentrations of $>5 \times 10^7$. Since similar reactions were ob-

served with both reagents, these cultures were not misidentified as *N. gonorrhoeae*.

DISCUSSION

Coagglutination tests for laboratory identification of several organisms are presently available (3, 11) and are reported to be accurate, reliable, and easy to perform (4, 7-9, 19). Coagglutination tests have also been used to detect bacterial antigens in body fluids (17, 20). Detecting bacterial antigens in body fluids probably involves fewer organisms than are required for the laboratory identification of whole organisms by coagglutination.

Gonococci are highly autolytic bacteria. Therefore, we took advantage of this phenomenon and examined conditions which, by maximizing autolysis and release of cell surface components, increase the sensitivity and strength of the coagglutination reactions of the Phadebact gonococcus test. A pH of 8.3 gave the greatest number of positive reactions. This pH is within the range of optimal gonococcal autolysis. The addition of the divalent cation chelators EDTA and EGTA further enhanced the sensitivity of the test as well as the strength of the coagglutination reactions.

The chelation of divalent cations (Ca^{2+}, Mg^{2+}) by EDTA results in the release of LPS from gram-negative bacteria (6, 16). LPS appears to be an important antigen in the Phadebact coagglutination test. The preparation of LPS used in this study contained ca. 5% (wt/wt) protein. This level of protein is comparable to those of other preparations of gonococcal LPS (18). We cannot exclude the possibility that these contaminating proteins may contribute to the coagglutination reaction at the higher concentrations of LPS. The detection of gonococcal LPS by coagglutination was markedly enhanced by the presence of either EDTA or EGTA. Ionic interactions play a role in the physical structure of LPS. Olins and Warner (14) found that the dialysis of LPS from *Azotobacter vinelandii* against buffered EDTA results in a reduction in the polymer size. Thus, it is likely that a similar reduction in the polymer size of gonococcal LPS was responsible for the enhancing effect of EDTA and EGTA.

Previous experiments have determined that the dry weight of a single gonococcus is ca. 6×10^{-13} g (data not shown). Gonococcal LPS has been isolated in yields of 1 to 1.7% of the cell dry weight (15). Assuming that these values reflect the cellular content of LPS, each cell would contain 1×10^{-5} to 6×10^{-6} ng of LPS. The minimal amount of LPS detected by coagglutination was 2.7 ng (Table 4). This amount would correspond to ca. 2.7×10^5 to 4.5×10^5 cells and is within the range of the sensitivity of the assay for whole cells treated with EDTA or EGTA. Other cell surface components, such as outer membrane proteins, may also be important antigens in the coagglutination reaction of the Phadebact gonococcus test. In summary, the significance of this study is that conditions which are optimal for cell lysis and release of cell surface antigens increased both the sensitivity and strength of the coagglutination reaction.

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