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# Hemagglutinating Properties of *Shigella dysenteriae* Type 1 and Other *Shigella* Species

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**Strains of *Shigella dysenteriae* type 1 cultured in Casamino Acids-yeast extract broth medium in the presence of 1 mM calcium chloride at 37°C for 22 h induced hemagglutination of erythrocytes that was inhibited by *N*-acetylneuraminic acid, *N*-acetylneuramin-lactose, and $\alpha_1$-glycoprotein. The hemagglutination was heat labile, and the absence of cell-surface appendages suggested a nonfimbrial adhesin(s). Under the same conditions, strains of *Shigella flexneri* (types 1a, 1b, 2a, and 2b) showed *N*-acetylneuraminic acid-resistant hemagglutination of erythrocytes.**

Bacillary dysentery caused by shigellae is a complex disease which involves invasion of the colonic epithelial cells and multiplication of the bacteria therein (4, 20). Relatively little information, however, is available on the mechanisms of attachment of shigellae to cells on the mucosal surface (19), although adherence is recognized as an important initial step in other bacterial infections (3, 6, 8, 11, 14).

Although the binding of strains of *Shigella flexneri* to erythrocytes, causing their hemagglutination (HA), has been reported previously (9, 13, 22), this property has not been demonstrated in strains of *Shigella dysenteriae* type 1 and other *Shigella* species. The aim of this study was to test the ability of *Shigella* species and strains grown under a range of different culture conditions to hemagglutinate erythrocytes.

Bacterial strains used in the study (Table 1) were obtained from patients at the Clinical Research Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka. Nonpathogenic *Escherichia coli* 36000 was obtained from the Centers for Disease Control (Atlanta, Ga.). Strains stored at −70°C were subcultured (25) and grown in Casamino Acids-yeast extract (CYE) broth (5) in screw-cap test tubes containing 5 ml of medium for 22 h at 37°C with shaking (250 rpm). A 2+ HA reaction was recorded for strains of *S. dysenteriae* type 1. HA (12) was carried out on slides by using 20 μl of 2% (vol/vol) erythrocytes and an equal volume of bacterial suspension ($5 \times 10^{10}$ CFU/ml), and the reaction was rated as 4+ (completed within 1 min), 2+ (5 min), or negative. HA was not detected when strains of *S. dysenteriae* type 1 were cultured in CYE static broth or in CYE agar (Table 2). HA was also not detected when bacteria were cultured in any other media tested. HA by *S. dysenteriae* type 1 was increased from a 2+ to a 4+ reaction by the addition of 1 mM calcium chloride to CYE broth. Magnesium chloride and ferrous sulfate were without effect. The addition of the iron chelator 2,2′-dipyridyl (0.2 mM) to CYE broth completely inhibited HA. Strains of *S. dysenteriae* type 1 hemagglutinated erythrocytes obtained from guinea pigs, sheep, monkeys, rabbits, cows, newborn piglets, and humans (blood types O, A, and B). The minimum number of bacteria required for the HA reaction was $5 \times 10^9$ CFU/ml. There was no variation in the HA of erythrocytes obtained from different animals of a given species. HA was not observed with chicken erythrocytes. HA was shown to be dependent on the duration of incubation of the bacteria in the culture medium (8 to 48 h), with a 4+ reaction observed only between 22 and 24 h of bacterial growth. After 24 h of growth, clumping of cells was observed and HA decreased.

HA intensity was strongly dependent on the temperature at which the bacteria were cultured, with 37°C being the optimum. Moderate HA was observed at 35 or 40°C and completely disappeared below 20 or above 45°C. Optimum HA occurred when organisms were grown in CYE medium at pH 7.4, and HA was absent at pHs below 6.5 or above 8.0. Bacterial growth was poor at pHs above 8.0 and below 6.0. Conditions of storage also influenced HA of *S. dysenteriae* type 1. Strains stored on Dorset egg slopes, Trypticase soy agar, or nutrient agar media (9) for 1 year or more showed either reduced or no HA activity, even after repeated subcultures in CYE broth. The same strains stored at −70°C in Trypticase soy broth containing 15% glycerol induced strong HA on culture but showed a much slower or negative reaction when kept at room temperature for about a week. Previous studies (9, 22) failed to demonstrate HA by *S. dysenteriae* type 1 strains, presumably because the strains tested had been stored for long periods on nutrient agar slopes and had also been cultured in the same medium either on agar slopes or in static broth medium to detect HA. The ability to hemagglutinate appeared to be closely dependent on culture conditions, as has been observed for other gram-negative enteric bacteria (2, 16, 17).

A comparison between the nutritionally rich media used in this study, in which *S. dysenteriae* type 1 failed to hemagglutinate (1, 9, 10, 18, 25), and the CYE medium (5) shows that the latter contains over three times the amount of acid-hydrolyzed casein. This suggests that higher amounts of amino acids present in CYE medium may have influenced the HA reaction of *S. dysenteriae* type 1. In addition, supplementation of the CYE medium with calcium ions led to increased HA activity, analogous to their effect in increasing adhesion of *S. flexneri* 2a to cultured cells (19, 24). It is, however, important to remember that in the gut, environmental conditions and nutritional availability are not necessarily those encountered in laboratory culture of the bacteria. Hence, the characteristics determined by growth in vitro may not necessarily correlate with their expression in vivo.

HA by *S. dysenteriae* type 1 was inhibited by 5 mM

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TABLE 1. Hemagglutinating properties of *Shigella* strains[a]

| Species and serotype (no. of strains tested) | No. of strains positive for HA | No. of strains showing a 4+ HA reaction |
|---|---|---|
| *S. dysenteriae* type 1 (20) | 20 | 20 |
| *S. flexneri* type 1a (6) | 1 | 0 |
| *S. flexneri* type 1b (1) | 1 | 1 |
| *S. flexneri* type 2a (6) | 2 | 1 |
| *S. flexneri* type 2b (6) | 5 | 4 |
| *S. flexneri* type 3a (1) | 0 | 0 |
| *S. boydii* type 1-6 (2) | 0 | 0 |
| *S. boydii* type 7-11 (4) | 0 | 0 |
| *S. boydii* type 12-15 (4) | 0 | 0 |
| *S. sonnei* form I (5) | 0 | 0 |
| *S. sonnei* form II (5) | 0 | 0 |
| *E. coli* (enterotoxigenic) (1) | 1 | 1 |
| *E. coli* (nonpathogenic) (1) | 0 | 0 |

[a] Results were confirmed on three separate occasions, using 2% suspensions of guinea pig erythrocytes. Strains of enterotoxigenic *E. coli* and nonpathogenic *E. coli* were used as positive and negative controls, respectively, for the HA assays.

*N*-acetylneuraminic acid (NANA), 5 mM *N*-acetylneuraminlactose (Sigma Chemical Co., St. Louis, Mo.), and 0.67 mM $\alpha_1$-glycoprotein (Scottish Blood-transfusion Association, Edinburgh), which contains 2% NANA. This inhibition of HA was observed with erythrocytes from the different species of animals studied. A wide range of other carbohydrates with concentrations ranging from 5 to 100 mM did not have any effect (21, 23, 29). The pattern of inhibition of HA by *S. dysenteriae* type 1 suggests that the bacteria interact with sialic acid-specific glycoproteins on the surface of erythrocytes, as has been shown for enterotoxigenic *E. coli* and other bacteria (21, 26, 28).

Strains of *S. dysenteriae* type 1 (12588 and 3351) appeared nonfimbriated with a smooth surface when examined by transmission electron microscopy and stained with uranyl acetate (2) or 1% phosphotungstic acid (7). This suggests that HA by *S. dysenteriae* type 1 was caused by a nonfimbrial adhesin, as observed for some pathogenic strains of *E. coli* (7, 15, 29). The HA by *S. dysenteriae* type 1 was sensitive to heat being lost on incubation at 50°C for 10 min. When mixtures of bacteria (200 μl, $5 \times 10^{10}$ CFU/ml) and guinea pig erythrocytes (200 μl of a 2% [vol/vol] suspension) were heated at 50°C for 10 min, the HA property was lost.

TABLE 2. HA of *S. dysenteriae* type 1 grown in different media

| Culture medium[a] | HA reaction with guinea pig erythrocytes |
|---|---|
| CYE broth (5) | 2+ |
| CYE broth + $CaCl_2$ | 4+ |
| CYE broth + $MgCl_2$ | 2+ |
| CYE broth + $FeSO_4$ | 2+ |
| CYE broth + 2,2′-dipyridyl | − |
| Colonization factor antigen broth (10) ± $CaCl_2$ | − |
| AKI broth (18) ± $CaCl_2$ | − |
| Nutrient broth (9) ± $CaCl_2$ | − |
| Minimal broth medium (1) | − |
| Trypticase soy broth (25) ± $CaCl_2$ | − |
| Trypticase soy agar ± $CaCl_2$ | − |
| Colonization factor antigen agar ± $CaCl_2$ | − |
| CYE agar ± $CaCl_2$ | − |

[a] Concentrations of $MgCl_2$, $CaCl_2$, and $FeSO_4$ in the culture media were 1 mM, and that of 2,2′-dipyridyl was 200 μM. Other conditions are described in the text.

Agglutination failed to recur after cooling to 20°C, suggesting that HA by *S. dysenteriae* type 1 can be described as an eluting type (7) which is sensitive to heat.

Of 20 strains of *S. flexneri* (Table 1) belonging to five different serotypes, 9 induced HA of erythrocytes obtained from different animal species. These strains of *S. flexneri* belonged to serotypes 1a, 1b, 2a, and 2b. Not all strains of a particular serotype of *S. flexneri* showed this property. For serotypes 1a and 2a, between 16 and 33% of the strains were positive. For *S. flexneri* 2b, HA appeared to be more common and over 80% of the strains were positive. HA was not sensitive to NANA or other substances tested. The hemagglutinating property was not detected in strains of *Shigella sonnei* and *Shigella boydii*. This is consistent with previous findings (9, 22), but a more systematic and comprehensive study needs to be conducted before a firm conclusion can be reached.

It was observed that both virulent strains and Congo red- (25) and Sereny- (27) negative avirulent variants of *S. dysenteriae* type 1 (10 strains tested) showed similar hemagglutinating activity. HA did not correlate with uptake of Congo red, which is a characteristic of virulent strains. In addition, bacteria grown in other media (listed in Table 2) were Congo red positive and Sereny positive but were negative for HA. Therefore, it is yet to be demonstrated whether HA is indeed an independent and essential virulence factor.

In summary, we demonstrated that the HA property is present in all strains of *S. dysenteriae* type 1 studied. Further work is being carried out to correlate HA with attachment to epithelial cell lines (HeLa and Henle 407) and determine its contribution to virulence and disease. In addition, studies are being carried out to extract and characterize the sialic acid-specific adhesin from *S. dysenteriae* type 1.

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