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Mannose-Resistant Hemagglutination of Human Erythrocytes by Enterotoxigenic *Escherichia coli* with Colonization Factor Antigen II

DOYLE J. EVANS, JR.,[1,2]* DOLORES G. EVANS,[1,2] SHERIDAN R. DIAZ,[2,3] AND DAVID Y. GRAHAM[1,2]

The Bacterial Enteropathogen Laboratory, Digestive Disease Section, Department of Medicine,[1] and Department of Pathology,[3] Veterans Administration Medical Center, Houston, Texas 77211, and Department of Medicine, Baylor College of Medicine, Houston, Texas 77030[2]

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The human erythrocyte receptor which mediates mannose-resistant hemagglutination by enterotoxigenic *Escherichia coli* possessing colonization factor antigen II is not universally distributed among donors. Mannose-resistant hemagglutination-positive erythrocytes are more common among black donors than nonblack donors; tests with erythrocytes of known antigenic makeup confirm this correlation. Colonization factor antigen II receptor activity of mannose-resistant hemagglutination-positive erythrocytes is unstable when whole blood is stored at 4°C. Also, screening of donors is best performed with enterotoxigenic *E. coli* possessing colonization factor antigen II composed of the coli surface antigen 1 (CS1) plus CS3, since these consistently produce stronger hemagglutination reactions than strains with colonization factor antigen II composed of either CS2 plus CS3 or CS3 only.

Fimbrial colonization factor antigens (CFAs) of the enterotoxigenic *Escherichia coli* (ETEC) have a major role in host and tissue specificity of these enteropathogens (1, 5, 9, 15). Although the total number of CFAs is unknown, most human-associated ETEC serotypes produce either CFA/I, CFA/II, or PCF8775 (2, 4, 5, 9, 15). ETEC possessing either CFA/I or PCF8775 hemagglutinate human erythrocytes apparently irrespective of the blood donor (8, 14). Identification of the erythrocyte receptors which interact with these CFAs should help in determining the identity of the corresponding receptors which reside on the target cells in the intestine. Our interest in identifying the CFA receptors led us to reexamine our original (and often confirmed) finding that ETEC with CFA/II hemagglutinate bovine but not human erythrocytes (6). The difference in hemagglutination (HA) pattern between CFA/I and CFA/II has been valuable in interpreting the results of HA tests to screen clinical isolates of ETEC (8). Cravioto et al. (4), however, did observe two CFA/II-positive O6:H16 *E. coli* isolates which gave mannose-resistant HA (MRHA) with both human and calf erythrocytes.

Here we present evidence that there is a phenotype of human erythrocyte which does hemagglutinate with CFA/II-positive ETEC and that this phenotype predominates in the black race. Also, the human erythrocyte-binding activity for CFA/II is remarkably unstable in comparison to binding activity for CFA/I.

MATERIALS AND METHODS

Bacterial strains and culture conditions. CFA/II-positive ETEC test strains included the rhamnose-negative, coli surface antigen 1 (CS1)- and CS3-positive strains E-1392, PB-407, PB-176, and GV-50B (all belonging to serotype O6:H16 except GV-50B, which is O6:H$^-$); other strains were all rhamnose positive and positive for either CS2 plus CS3 or CS3 only, including the serotype O6:H16 strains E-4833 (CS2 plus CS3), TD-219C3, H-22743, and TD-415C2, and one O6:H$^-$ strain, RSJB-10. Strains belonging to serotype O8:H9 included strain E-7463 (CS3 only), H-15862, and H-16160-2. All of these strains produced MRHA with bovine erythrocytes, and HA (bovine)-negative derivatives were freshly isolated from each strain for use as controls. Strains E-1392, E-4833, and E-7463 were provided by Alejandro Cravioto; all strains were derived from patients in studies on acute diarrhea (6, 7, 12). Bacterial cultures were preserved and grown for CFA production by standardized methods described elsewhere (8).

HA tests. Bovine erythrocytes (Bethyl Laboratories, Inc., Montgomery, Tex.) suspended in phosphate-buffered saline (pH 7.2) containing 1.0% mannose were used for quality control of cultures and for isolation of CFA/II-negative derivative strains. Bacteria were harvested from cultures grown for 18 h at 37°C on CFA agar with sterile cotton swabs, suspended in phosphate-buffered saline plus 1% mannose, and adjusted to an optical density of 1.0 at 640 nm. MRHA tests were performed by placing approximately 20 μl of blood (obtained with syringe and needle after mixing tube contents by repeated inversion) upon a large microscope slide, followed by the addition of an equal volume of the bacterial suspension. Slides were placed on ice unless HA occurred within 1 min at room temperature. HA reactions were graded as 4+ (maximum; i.e., rapid, with all erythrocytes agglutinated), 3+ (strong), 2+ (moderate), 1+ (slow, weak HA), or negative.

Human blood samples were obtained as outdated laboratory specimens and used in accordance with approved human studies guidelines. Routinely, these specimens were not refrigerated until at least 12 h postcollection and not assayed until 1 to 3 days postcollection. One group of samples was kept refrigerated for 2 weeks before testing. In a separate study in which blood specimens were collected prospectively, the specimen tubes were immediately placed in ice and kept refrigerated for the duration of the study.

Purchased human erythrocytes (Ortho Diagnostic Systems, Inc., Raritan, N.J.) of known antigenic composition were concentrated by low-speed centrifugation and sus-

* Corresponding author.

pended in phosphate-buffered saline plus 1% mannose at approximately 2% concentration to perform HA tests.

RESULTS

Survey of human blood samples for MRHA reaction with CFA/II-positive ETEC. Initial studies were carried out by screening 1,111 blood specimens for MRHA with CFA/II-positive ETEC test strains over approximately 6 months. Although the majority of samples was from a general patient population, this had no detectable influence on the results since MRHA-positive individuals were also found among 79 free-living healthy donors included in this series and among 53 samples obtained from volunteers in another study reported here.

There was a decline in the percentage of samples testing positive for MRHA with the CFA/II-positive ETEC when the samples were not tested on the day after collection. Samples tested within 24 h showed 169 (31.9%) positive specimens among 529 specimens tested. In general, 2- to 3-day-old specimens which were MRHA-positive showed a distinct shift toward less intense (for example, fewer 4+ and more 2+) HA reactions (48 [10.4%] positive specimens among 461 specimens tested). Only 3.3% (4 of 121) of the 2-week-old samples were MRHA positive, although only well-preserved samples had been used. This result is very different from that obtained with CFA/I-positive ETEC such as strains H-10407 (serotype O78:H11), used here as a control. All donor erythrocytes were MRHA positive with CFA/I-positive ETEC, irrespective of blood type and postcollection age of the blood samples. Also, we consistently observed that the ETEC with CFA/II composed of CS1 plus CS3 produced stronger HA with individual blood samples than the ETEC with the CS2 plus CS3 or CS3 only antigenic types of CFA/II. For example, samples producing a 2+ to 3+ reaction with strain PB-407 (CS1 plus CS3) produced only 1+ or negative tests with all of the CFA/II-positive ETEC lacking the CS1 antigen component. All of the CFA/II-negative derivative strains, selected as colonies negative for MRHA with bovine erythrocytes, were MRHA negative with human erythrocytes, which gave 4+ MRHA with the CFA/II-positive parent cultures.

With respect to the donors in this survey, there was no correlation between a positive or negative MRHA test result with CFA/II-positive ETEC and either ABO or Rh blood type. However, 17 of the MRHA-positive donors were black, whereas donors known to be nonblack were MRHA negative. The significance of this observation could not be determined directly, as the vast majority of the donors were not identified by race. One presumably nonblack group could be identified by surname; that is, donors with distinctively Hispanic surnames. When the data from the largest and most reliable group (HA tests performed within 24 h of collection) was analyzed, only 2.2% of 92 samples bearing Hispanic surnames were MRHA positive, whereas 169 of 437 (38.2%) of the remaining samples were MRHA positive.

Survey of human erythrocytes of known antigenic composition for reactivity with CFA/II-positive ETEC. Human erythrocytes of known antigenic composition but not identified according to ethnic background or race were obtained from a commercial source. Of 26 samples, 8 were obtained specifically to represent the Duffy Fy(a)-negative, Fy(b)-negative phenotype, which is a legitimate marker for the black race (16). The Rh-related V^+ VS^+ phenotype is also a reliable marker indicating black race; to a lesser extent this is true for the S(−)s(+)M(−)N(+) phenotype and strong P1 antigenicity (16). For example, 5 of the 8 FY(a)-negative, Fy(b)-negative test specimens had two of the other phenotypic characteristics noted above, and two had one other selected phenotypic marker (V^+ VS^+); only 1 had none of the other race-related phenotypes.

The results of MRHA assays were as follows. The CFA/II-positive ETEC produced strong MRHA with 5 of the 26 specimens; 3 of these 5 were Fy(a) negative, Fy(b) negative and also had either strong P1 antigenicity or the S(−)s(+)M(−)N(+) phenotype. The other two MRHA-positive specimens had both strong P1 antigenicity and the S(−)s(+)M(−)N(+) phenotype, although not the minus/minus Duffy phenotype. All of the 17 erythrocyte specimens with one or none of the selected phenotypes were MRHA negative. These results indicate that the MRHA-positive erythrocytes were from black donors. On the other hand, these same results also show that none of the four selected phenotypes represents the CFA/II receptor, since none of these showed a 100% correlation with MRHA activity. It is also of interest that the reactivity of these MRHA-positive erythrocyte specimens remained stable over a long period of time (months), indicating that few if any false-negative results were obtained with these specially preserved reagents.

TABLE 1. Assay for MRHA of CFA/II-positive ETEC with prospectively collected blood samples from black and nonblack individuals

Day postcollection	Specimen source	No. with MRHA with strain E-1392[a]/total (%)
0 (3 to 5 h)	Black	29/33 (88)
	Nonblack	6/20 (30)
1	Black	20/33 (61)
	Nonblack	4/20 (20)
4	Black	11/33 (33)
	Nonblack	1/20 (5)
6	Black	8/33 (24)
	Nonblack	0/20 (0)

[a] ETEC strain E-1392 is serotype O6:H16, positive for CFA/II (CSI plus CS3), and rhamnose negative.

Prospective study of blood samples obtained from black and nonblack individuals and instability of the CFA/II receptor. Fifty-three blood samples were obtained from volunteers on a college campus and placed on ice immediately after collection. The first HA tests were performed from 3 to 5 h postcollection. Several aspects of these results (Table 1) are of interest. First, 88% of a group of 33 blood samples from black individuals produced MRHA with CS1 plus CS3 CFA/II-positive ETEC, confirming previous indications that the erythrocyte receptor for CFA/II is common in the black race. Second, 30% of the 20 samples from nonblack individuals were MRHA positive; this was higher than expected but consistent with the fact that these samples had been cooled immediately while those in the original survey had not been refrigerated until 12 to 24 h postcollection.

MRHA tests performed 1, 4, and 6 days postcollection further confirm the instability of the CFA/II receptor activity of the human erythrocyte. By day 6 the only MRHA-positive samples were those from black donors, accounting for 24% of those donors.

DISCUSSION

Although not all ETEC isolates produce CFA/I, CFA/II, or PCF8775, these three CFAs account for the majority of human-associated ETEC serotypes (6, 7, 9, 15). Also, any *E. coli* isolate found to produce one of these CFAs can be assumed, for practical purposes, to be enterotoxigenic because the plasmids which encode for each of these surface antigens also encode for one or both ETEC toxins (5, 10, 12, 13). CFA/I, CFA/II, and PCF8775 are all hemagglutinins that are detectable with bovine erythrocytes; however, CFA/I and PCF8775 are best detected by HA with human erythrocytes (4, 8, 14). In addition to being valuable as a simple and reliable presumptive test (usually confirmed serologically) for ETEC CFAs, HA tests have greatly facilitated studies on the genetics of CFA and enterotoxin production (5, 10–13) and the epidemiology of ETEC diarrhea (1, 2, 4, 9, 15).

We are particularly interested in another aspect of HA of human erythrocytes by the CFAs of human-associated ETEC: the potential for determining the molecular composition and structure of the receptors that play a major role in the pathogenesis of ETEC diarrhea. Since CFA-receptor binding is a very specific molecular interaction, it is reasonable that identification of the erythrocyte receptors which mediate HA might facilitate identification of the corresponding intestinal epithelial cell receptors. For example, the pyelonephritis-associated pili of uropathogenic *E. coli* produce MRHA of human erythrocytes by interaction with a receptor which also occurs on epithelial cells of the urinary tract (3).

Interestingly, the inability to demonstrate MRHA with human erythrocytes with ETEC belonging to serogroups O6 and O8 played an important part in the discovery of CFA/II (6). Also, it has been widely established that CFA/II-positive ETEC produce MRHA with bovine but not human erythrocytes, as we first reported (1, 2, 4, 6, 8). Thus with the exception of one documented comment (4), the results reported here seem contradictory to the experience of many investigators. On the other hand, our results clearly demonstrate several factors which explain the apparent discrepancy. HA tests are usually carried out with erythrocytes that have been stored at refrigeration temperature for various lengths of time and which by chance were donated by individuals not possessing the red cell receptor for CFA/II. Also, in testing our collection of CFA/II-positive ETEC we have observed that strains possessing the CS1 and CS3 antigenic components of CFA/II produce stronger, more rapid HA with bovine erythrocytes than those with CS2 and CS3 or with CS3 only; this difference is even more profound when human erythrocytes are used.

A survey of commercially available, typed erythrocytes did not reveal a correlation between any particular known erythrocyte antigen and reactivity with CFA/II-ETEC; however, although a small number of such erythrocytes were tested, the results are consistent with the idea that expression of the erythrocyte CFA/II receptor is common in the black race. Five of 9 (55%) of samples possessing two or more race-associated phenotypes were HA positive with CFA/II-positive ETEC, in contrast to none of 17 samples with 0 or 1 such phenotype. Also, three of six (50%) of the erythrocytes with V^+ VS^+ antigenicity were HA positive. Thus, a correlation was found between antigen combinations known, statistically, to predominate in the black race and reactivity with CFA/II-positive ETEC. Finally, 88% of a group of 33 black individuals were positive for MRHA with CFA/II (CS1 and CS3)-positive ETEC. It is significant that for blood samples from black individuals 24.2% (8 of 33) were still reactive with the CFA/II-positive ETEC 1 week after collection of blood. These results may reflect quantitative as well as qualitative differences in the CFA/II receptor on erythrocytes of individuals of different genetic backgrounds. Studies are now in progress in an effort to identify the human erythrocyte receptor for CFA/II.

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