those from HeLa cells. Soluble antigen obtained from EE cells reacted strongly with anti-HeLa rabbit serum in the indirect hemagglutination test. These investigations were conducted to see whether indirect hemagglutination offered advantages in sensitivity over direct hemagglutination which might be useful for future approaches to the problem of immunologic identification within species of cells in continuous culture.

Summary. Material obtained from sonically disrupted HeLa cells by extraction of trichloracetic-acid precipitate with buffered saline was used to sensitize tanned sheep erythrocytes. Sensitized erythrocytes were agglutinated to high titer by anti-HeLa rabbit serum, but minimally by normal rabbit serum. Reactivity of the sensitizing extract was considerably reduced by treatment of HeLa cells with tannic acid prior to disruption and extraction.

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Infectious Agents in Infant Diarrhea: I. A Hemagglutination-Inhibition Procedure for Detection of Bacterial Fractions in Infant Sera. (26201)

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Hemagglutination (HA) procedures have been widely utilized since the technic was first described by Keogh *et al.*(1). Modifications of this technic using lipopolysaccharide antigens have been found to be superior to agglutination reactions for detecting antibody titer rises in cases of shigellosis and colienteritis(2,3). Landy and Trapani(4), however, reported that trace amounts of unbound antigen greatly inhibit the hemagglutination test. In some diseases, such as severe pneumococcal pneumonia(5) and typhoid fever(6) circulating antigen has been demonstrated in the blood by means of a precipitin test. The extremely small amounts of antigen required to inhibit the hemagglutination reaction suggested that this phenomenon might provide a basis for an even more sensitive procedure. Indeed Noyes *et al.*(7) were able to detect bacterial endotoxins in the plasma of dogs with experimental peritonitis utilizing a technic based on this suggestion. The present report describes a hemagglutination-inhibition (HI) test developed along similar lines. With this technic it was shown that sera from infants with diarrhea often contain inhibitors that are antigenically related to bacterial strains isolated from their fecal flora.

Method. Preparation of normal human erythrocytes (NHE).* Human O Rh negative blood is diluted with an equal volume of Alsever's solution(8) and allowed to equilibrate for several days before use. The red blood cells can be used until evidence of spontaneous hemolysis is observed, usually a period of 4 to 5 weeks. Just before use the NHE are washed 3 times with 3 volumes of triethanolamine buffered saline (TBS)(9) and packed by centrifuging for 3 minutes at

* This method was developed from information presented by Landy, M., Trapani, R. J., Clark, W. R., Walter Reed Army Inst. of Research Rep., 1955, (WRAIR-56-55).

TABLE I. Standardization of *Shigella flexneri* 2a HA Antigen Lot #30.

Antigen dilution	Final dilution of hyperimmune rabbit serum										
	80	160	320	640	1280	2560	5120	10240	20480	NHE	Titer
1:5	+	+	+	+	+	+	+	+	+	—	20480+
1:10*	+	+	+	+	+	+	+	+	±		10240
1:20	+	+	+	+	+	+	+	±	±		5120
1:40	+	+	+	+	+	+	±	±	—		2560
1:80	+	+	+	+	+	±	±	—	—		1280

* The antigen dilution of choice is 1:10.

+, positive reaction; —, negative reaction; ±, incomplete agglutination, not considered positive reaction.

1800 rpm. A 10% suspension prepared in TBS is used for antigen sensitization.

Production of sensitizing antigen. Smooth colonies, selected on the basis of colony appearance, uniform suspension on growth in broth and lack of autoagglutination on heating in physiological saline at 100°C for 1 hour, are inoculated heavily onto freshly prepared plates of trypticase soy agar containing 5% glycerine. The plates are incubated under 5% CO_2 at 37°C for 18 to 24 hours. The growth is washed off in small amounts of physiological saline and an equal quantity of 0.1N NaOH is added to the harvest. The mixture is heated in a water bath for 60 minutes at 100°C, adjusted to pH 7.0 with HCl and dialyzed in the cold against distilled water for 24 hours using several water changes of approximately 100 volumes each. The dialysant is centrifuged at 1800 rpm for 1 hour and the supernate Seitz filtered. The resulting water clear lipopolysaccharide antigen may be standardized without further treatment or lyophilized and standardized on a dry weight basis.

Standardization of lipopolysaccharide antigen. The highest dilution of lipopolysaccharide giving an HA titer of 2560 to 10240 with hyper-immune rabbit sera prepared according to the method recommended by Edwards & Ewing(10) is determined next. Two-fold dilutions (1:5-1:80) of the liquid lipopolysaccharide antigen are prepared in TBS and each combined with an equal volume of 10% NHE. Each tube is then shaken thoroughly and incubated in a water bath for 2 hours at 37°C with occasional shaking. The resulting antigen-sensitized human erythrocytes (ASHE) are washed 3 times in TBS and resuspended to make a 1% suspension. Serial 2-fold dilutions (1:140-1:10240) of hyperimmune rabbit serum inactivated at 56°C for 30 minutes are prepared in TBS in sufficient quantity for testing each dilution of lipopolysaccharide antigen. For the titration, transfer 0.2 ml of each of the antiserum dilutions to Wasserman tubes to prepare a set of serum dilutions for each of the 5 ASHE preparations. Add 0.2 ml of each ASHE preparation to a row of serially diluted antiserum, mix and incubate in a 37°C water bath for 2 hours. Controls consist of (a) 1% NHE and serum, (b) 1% NHE and TBS, (c) ASHE and TBS. The HA titer is read as the reciprocal of the highest dilution giving complete agglutination of the red blood cells. A sample protocol showing standardization of *Shigella flexneri* 2a antigen is presented in Table I. In this instance the highest HA titer between 2560 and 10240 was given by antigen dilution 1:10 and this dilution would be the one used for all further tests with this particular lot of HA antigen.

Performance of the hemagglutination test. A preliminary HA test is carried out on all sera to be examined for presence of inhibitors, as antibodies would combine with any such material rendering detection of a circulating antigen by this method impossible. One volume of the optimum dilution of sensitizing antigen (see standardization of antigen) is mixed with an equal volume of 10% NHE and incubated in the water bath at 37°C for 2 hours with occasional shaking. The sensitized cells are washed 3 times with TBS and a 1% suspension prepared in TBS. Two-fold dilutions of the patient's inactivated serum (1:4-1:1024) are prepared in 0.2 ml amounts. A hyperimmune rabbit se-

TABLE II. HA Tests with Hyperimmune Rabbit Sera and 1% ASHE (Critical Dilutions To Be Used for HI Test).

E. coli serotypes	Final dilutions of hyperimmune rabbit serum								
	80	160	320	640	1280	2560	5120	10240	20180
01	+	+	C+	C+	C+	C+	C+	C±	C—
02	+	+	+	C+	C+	C+	C+	C+	C—
015	+	+	C+	C+	C+	C+	C+	C—	—

+, positive reaction; —, negative reaction; ±, incomplete agglutination, not considered positive reaction. C = Critical dilutions to be used in HI test.
Note: Lower dilutions may also be used if patient sera is sufficient.

rum control is also diluted as described in the section under antigen standardization. Two-tenths ml of ASHE is then added to each. Other controls are prepared as previously described. Tests are incubated for 2 hours at 37°C in a water bath and read.

Performance of HI test. Sera showing no hemagglutinins in the preliminary HA test may be tested for presence of inhibitors which are antigenically related to bacterial strains isolated from a patient's intestinal tract. Two-fold dilutions of hyperimmune rabbit sera are prepared of such strength that addition of an equal volume of patient's serum will yield critical dilutions. In this case, critical dilutions should include the end point, one 2-fold dilution beyond the end point, and 4 (or more) 2-fold dilutions preceding the end point as shown in Table II. These dilutions of hyperimmune sera are incubated together with a constant amount of patient's serum, undiluted or diluted 1:2 or more if amount of serum available is insufficient for the HI test. Similar dilutions of hyperimmune rabbit sera are incubated together with constant amounts of TBS. They are then placed for 1 hour in a 37°C water bath. ASHE are added and the tubes reincubated for 2 hours at 37°C and read. The hyperimmune rabbit sera are titrated each time the HI test is performed to determine exact titer levels. Controls are carried out as for the HA test. All patient sera yielding inhibition are retested to establish reproducibility of the results.

Discussion. There are several possibilities as to nature of the substance producing the inhibition. "Incomplete" antibodies against somatic (O) antigens of enteric pathogens have been shown by Neter *et al.*(11) to be present in human gamma globulin. The possible interference of such antibodies in the HA and HI tests was investigated as follows: (a) A modified Coombs test (antiglobulin hemagglutination) was performed with patient's sera in which presence of inhibitors had been established. Results did not indicate the presence of incomplete antibodies. (b) In the HI test, antihuman globulin was added to patients' sera prior to incubation with hyperimmune rabbit sera. No change in inhibitory activity could be demonstrated. In further efforts to study the inhibitor an attempt was made to sensitize NHE with the inhibitory substance present in the patients' sera. NHE and inhibitory sera were incubated together, the cells washed and hyperimmune rabbit sera against the bacteria isolated from the patients' fecal sample added. The negative result obtained might be interpreted as due to lack of sensitizing properties. On the other hand, the amount of inhibiting substance might have been insufficient to sensitize cells for the HA test even though it had such properties. In another experiment human antiglobulin was added to patient sera prior to an attempt to sensitize NHE with inhibitory substance. Otherwise the test was carried out as the previous one. Again negative results were obtained.

The possibility exists that blood group substances could be the interfering agents in our HA tests. "A" group substances are known to be widespread in nature. Springer *et al.*(12) have shown that enteropathogenic *Escherichia coli* 086 has a polysaccharide substance related to blood group B antigens. By use of human O Rh negative blood cells, however, it is hoped that cross reactions with these substances are eliminated.

As a further indication of the specificity of the reaction it should be pointed out that

HI tests for 2 or more bacterial strains isolated from an infant with diarrhea usually yield findings at considerable variance from one another. These would be difficult to explain except on the assumption that the HI test is specific. For example, the acute phase serum from a 5-months-old infant had no demonstrable antibodies or inhibitors for enteropathogenic *E. coli* 0127 isolated from his feces, but had a 3 tube inhibition against *E. coli* 015 which he also carried in his intestine. Similarly, the acute phase serum obtained from a 7-months-old child had a hemagglutinin titer of 128 for *E. coli* 018 and a 3 tube inhibition for *E. coli* 015 though both serotypes were isolated from his feces at the same time.

The HI test presented here has been successfully used to provide evidence for the presence in sera of patients with diarrhea of substances which are apparently related antigenically to bacteria present in their intestine. Results of such tests are presented elsewhere(13). The nature of the circulating inhibitor is unknown. It might be a complex substance or a simple sugar(14). Studies are continuing to further its characterization.

Summary. A simple hemagglutination-inhibition (HI) technic is presented which enables detection of inhibitors in patients' sera during diarrhea. These inhibitors thus far appear to be antigenically related to bacteria isolated from the patients' intestine at the same time and may well be substances derived from them.

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Infectious Agents in Infant Diarrhea: II. Serological Reactions with *Escherichia coli* 01 through 025. (26202)

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A number of *Escherichia coli* serotypes have been established as being associated with diarrhea in infants and young children, *i.e., E. coli* 0111:B_4, 086:B_7, 026:B_6, 0.55:B_5, etc.(1). Some of the *E. coli* serotypes 01 through 025 have also been described as possessing pathogenic properties(2,3). Grönroos(4), in an attempt to relate *E. coli* serotypes 01 through 025 to infant diarrhea, studied the feces of healthy and diarrheal infants and was unable to demonstrate significant differences in the two groups. Ørskov (5) carried out a similar study with 130 *E. coli* 0 immune sera and found that strains representing specific serotypes were found in about the same frequency in both groups of