FFA bound with albumin to be reduced with an increase in total serum lipids. A concomitant increase in non-albumin bound free fatty acids along with an increase in serum triglycerides in accordance with the results herein presented would be expected to lead to an increase in REC values. The observations that traces of moisture in the chloroform used for extraction increased REC values in sera with high REC concentrations, but seldom in sera with low REC values suggests that the moisture effect may also be related to the amount of non-albumin bound free fatty acids, although we have no direct evidence that such is the case.

Summary. A study has been conducted on factors which affect the "readily extractable" cholesterol concentrations in serum. The results showed that (a) in general, REC values increased as triglyceride and cholesterol contents of the serum and chylomicra increased, but a notable exception was high REC values in nephrotic subjects in whom chylomicron contents of cholesterol and triglyceride were low, (b) addition of the sodium salts of long chain fatty acids to serum prior to lyophilization markedly increased cholesterol extractability, (c) addition of crystalline human serum albumin to sera with high REC concentrations markedly reduced REC values; the albumin also reduced REC concentration in most normal sera but in no case did it completely disappear, (d) traces of moisture in the chloroform used for the extraction had little or no effect on most sera with low REC concentration but markedly increased it in sera with high REC concentration. It is suggested that the concentration of non-albumin bound free fatty acids may play a major role in regulating REC concentrations in various sera.

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Detection of Anti-HeLa Antibodies in Rabbit Antiserum by Indirect Hemagglutination* (26200)

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Production of species-specific antibodies in animals injected with cells of various mammalian cultures has been described(1-4). These antiserums agglutinated erythrocytes of the same animal species(1,2), or were effectively cytotoxic *in vitro* for cells of homologous lines(3,4). These and other findings(5) substantiate the validity of immunologic identification of the species of mammalian cells in continuous culture.

Extreme sensitivity of the antigen-antibody reaction in indirect hemagglutination suggested application of the technic to detection and measurement of antibodies to cultured mammalian cells. This preliminary report describes methodology of an indirect hemagglutination test used for detection of anti-HeLa antibodies in serum of immunized

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rabbits, and describes preparation of a sensitizing antigen from HeLa cells.

Materials and methods. Antigen suspensions were prepared from 4-day-old monolayer cultures of HeLa cells washed repeatedly with 0.85% NaCl solution and scraped from glass with a rubber policeman. Suspended cells were diluted with 0.85% NaCl solution to a concentration of $3x10^6$ cells per ml, disrupted by treatment at 9 Kc per second for 5 minutes in a Raytheon magnetostrictor, and treated with 5% cold trichloracetic acid (TCA) for 30 minutes at 8°C to precipitate protein. After centrifugation, sedimented material was resuspended to original volume in a solution at pH 7.2-7.4 containing equal parts of phosphate-buffer 0.15 M and 0.85% NaCl. Resuspended material was stored for 1 hour at 8°C to extract soluble protein from the sediment, and recentrifuged. The supernate, containing from 30 to 40 μg/ml of protein (Kjeldahl) nitrogen, was used for sensitization of tanned erythrocytes.

Sheep erythrocytes for tanning and sensitization were obtained from sheep blood collected in modified Alsever's solution(6), kept at 8°C for 3 days before use. Erythrocytes were washed with 0.85% NaCl solution and tanned by addition of 1 ml of packed cells to 19 ml of freshly prepared tannic acid (Merck), 1:20,000 (w/v) in 0.85% NaCl solution. Erythrocyte suspension in the tannic acid was incubated at 37°C for 10 minutes with frequent agitation(7), sedimented by centrifugation, washed with 0.85% NaCl solution and diluted in the same solution to a concentration of 2.5% according to packed cell volume. This tanned erythrocyte suspension was mixed with an equal amount of soluble protein obtained from HeLa cells, incubated at room temperature for 20 minutes with occasional agitation, and centrifuged. The antigen-coated erythrocytes so produced were washed once with 0.85% NaCl solution and diluted to a final 0.75% suspension.

Indirect hemagglutination was tested by mixing 0.25 ml of sensitized sheep-erythrocyte suspension with an equal volume of serially 2-fold diluted serum in 13x100 mm round bottomed tubes. After thorough agitation, tubes were incubated at room temperature until erythrocytes in control tubes settled evenly, usually in 90 to 120 minutes. Results were scored according to the pattern of settling cells(7); titers were read as the reciprocal of the highest reactive serum dilution. Incubation of tests overnight at 8°C produced similar results. Test serums were diluted in a 1% solution of bovine albumin (prepared from bovine albumin 30% solution, Armour Laboratories), which did not reduce sensitivity of the test and served as an excellent stabilizer of the tanned erythrocyte suspensions.

Anti-cellular serums were prepared by injection of rabbits with HeLa cells grown in yeast extract medium(8) supplemented with 20% human serum. Cells were washed repeatedly with 0.85% NaCl solution, scraped from glass and made into a suspension containing approximately $2x10^6$ cells per ml, frozen and thawed repeatedly, freed of coarse sediment by centrifugation at 500 x g and administered intravenously to 4 rabbits by marginal ear vein. Four injections per rabbit of 2 ml of cell material were given at 4-day intervals. After 1 week, 1 rabbit was given a fifth injection of $5x10^6$ sonically disrupted HeLa cells in Freund's adjuvant distributed at various body sites subcutaneously and intramuscularly. None of the animals showed anaphylactic reactions. Blood was taken from the animals 10 days after the last injection, and serum was stored at –20°C. Before use, samples of test serums were thawed and inactivated for 20 minutes at 56°C. Control serums were collected from randomly selected uninjected rabbits.

Results. Sensitizing antigens from HeLa cells were prepared and coated on tanned sheep erythrocytes as described. To study the effect of tannic acid on cellular sensitizing antigens, HeLa monolayers also were washed repeatedly with 0.85% NaCl solution, treated with 1:2,000 tannic acid for 10 minutes at 37°C, rewashed once with saline solution, scraped from glass, disrupted by sonic vibration, precipitated with TCA and extracted with buffered saline. Protein nitrogen of sensitizing suspensions of cellular material so prepared varied from 15 to 20 μg/ml.

TABLE I. Agglutination of Human and Coated Sheep Erythrocytes by Rabbit Antisera to HeLa Cells.

Rabbit serum		Hemagglutination titers with: Untreated human O, Rh− erythrocytes*	Tanned sheep erythrocytes coated with extract of*: Untanned HeLa	Tanned HeLa
Anti-HeLa hyperimmune†		512	8192	64
Anti-HeLa immune rabbit	1	64	512	32
	2	64	1024	32
	3	32	256	16
Normal rabbit	1	<4	<4	<4
	2	<4	<4	<4
	3	<4	8	<4
Anti-human‡		<4	<4	<4

* Titers expressed as reciprocals of highest reactive 2-fold serum dilutions.

† Rabbits immunized with washed frozen-thawed HeLa cells ("immune rabbit"); 1 rabbit ("hyperimmune") also given sonically-disrupted HeLa cells in adjuvant.

‡ Lederle Labs.

Tanned sheep erythrocytes sensitized with these antigen preparations were tested for hemagglutination by a) antisera from 3 rabbits immunized with freeze-thaw disrupted HeLa cells, b) serum from one rabbit so immunized and hyperimmunized with sonically disrupted cells in adjuvant, c) normal rabbit sera, and d) commercially prepared anti-human rabbit serum (Lederle Laboratories). For comparison, these sera were tested also for direct agglutination of human blood-group O, Rh-negative erythrocytes(2). As shown in Table I, serum titers determined by indirect hemagglutination were 8 to 16 times higher than titers determined by direct hemagglutination. In this and other experiments, low titers not greater than 16 occasionally resulted from interaction of sensitized sheep erythrocytes and presumably normal rabbit sera. In all experiments, agglutination was absent in tubes containing sensitized sheep erythrocytes in 1% bovine albumin, or tanned or untanned sheep erythrocytes and normal or immune rabbit serum. Other control tubes containing anti-HeLa rabbit serum and HeLa cell protein-sensitized sheep cells, and anti-human rabbit serum and yeast extract-sensitized sheep cells, likewise showed no agglutination.

Discussion. Agglutination by rabbit antisera to HeLa cells of tanned sheep erythrocytes coated with protein materials extracted from HeLa cells produced satisfactorily high titers ranging from 256 to 8,192, when titers of normal sera did not exceed 16. This indirect hemagglutination test proved considerably more sensitive than direct agglutination of human erythrocytes by rabbit antisera to human cells in continuous culture. Tanned sheep erythrocytes treated with yeast extract medium used for routine cultivation of HeLa cells were not agglutinated by anti-HeLa serums, and tanned erythrocytes sensitized with HeLa-cell material were not agglutinated by anti-human rabbit serum, indicating no contribution by the medium to the hemagglutination reaction.

Early experiments indicated that simple disruption of cells by sonic vibration did not yield an antigen adequate for production of reactive coated tanned erythrocytes. This failure was considered to result from large amounts of lipid material released by cellular disruption, which interfered with adsorption of protein antigen by tanned erythrocytes. No success followed ether-chloroform extraction of lipid succeeded by alcohol-precipitation of protein. A highly reactive antigen for coating tanned erythrocytes was obtained by the procedure described herein. Protein nature of this reactive antigen preparation was indicated by its incapacity to sensitize untreated sheep erythrocytes. The preliminary studies reported here indicate that tannic-acid treatment of mammalian cells may alter cellular antigenic constitution, as reflected by partial loss of reactivity of material extracted from tannic-acid treated HeLa cells and reduction in the concentration of protein nitrogen of such preparations. These findings are of interest in relation to the work of such investigators as Brading (9) who reported reduction in agglutinability of tannic-acid treated erythrocytes.

In experiments not reported here, human esophageal epithelial cells of normal origin in continuous culture (Minn. EE 55-12-1) yielded antigen preparations as reactive as

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).