Erythrocyte Sensitization by Blood Group–Specific Bacterial Antigens

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ABSTRACT Human and chicken erythrocytes are readily coated *in vitro* by blood group active protein-lipopolysaccharides and lipopolysaccharides from *E. coli* O_{86} and *E. coli* O_{128}. Serum albumin, α_2- and β-lipoproteins inhibit this sensitization. Blood group B specific agglutination of erythrocytes with B or B-like antigens was obtained with antibodies purified by adsorption on and elution from B erythrocytes. Anti-blood group B and *E. coli* O_{86}-specific antibodies could be eluted from *E. coli* O_{86}-coated O erythrocytes. Eel anti-H(O) serum agglutinated O erythrocytes and only those A_1B red cells which were coated with blood group H(O) active *E. coli* products. Blood group active substances specifically inhibited agglutination of lipopolysaccharide-coated erythrocytes by anti-B and anti-H(O) agglutinins. Demonstrable amounts of lipopolysaccharide could only be removed from coated erythrocytes by washing them at elevated temperatures (58°C) in physiological solutions. Red cell sensitization with B active *E. coli* O_{86} substances was achieved *in vivo* in a minority of severely diseased infants and in germ-free and ordinary chicks which were in tourniquet shock after treatment with cathartics. Therefore, a possible mode by which erythrocytes of patients with severe intestinal disorders acquire antigens is the fixation of bacterial substances to their surfaces, if there are not enough of the normally interfering plasma factors present.

INTRODUCTION

Numerous Gram-negative bacteria contain immunogenic blood group A, B, and H(O) active polysaccharide structures (*cf.* 1). Most of these bacteria are *Enterobacteriaceae* and humans would be expected to come in contact with them early in life and throughout it.

It was the aim of the present study to determine whether blood group active bacteria bear a relation to the surprising observation (2–5) that patients with severe intestinal or genito-urinary disorders or with extensive gangrene may acquire *de novo* blood group B-like antigens as a temporary characteristic of their erythrocytes, resulting in a transient change of phenotype. Such an

investigation seemed especially indicated since Stratton and Renton (5) thought that the acquired B antigen may be bacterial polysaccharide. Both in severe intestinal disorders and in extensive gangrene the permeability of the damaged body surfaces is increased, and hence macromolecular substances may penetrate these surfaces more easily. The acquisition of extraneous substances by erythrocytes may have general physiological implications pointing to a transport and perhaps detoxifying function of these cells (6). Such studies may also further the understanding of the pathogenesis of some acquired hemolytic anemias (*cf.* 7).

Earlier work (*cf.* 8) has shown that untreated washed red cells may be coated with certain bacterial antigens *in vitro*. Plasma, however, contains powerful inhibitors which prevent coating of red cells (8).

It was attempted in the studies reported here to sensitize *in vitro* and *in vivo* the erythrocytes of healthy and sick humans and those of ordinary and germfree chicks with blood group B and H(O) active polysaccharides from Gram-negative bacteria and other sources. Blood group B active *E. coli* O_{86} is readily established in the intestines of germfree White Leghorn chickens but not in ordinary chickens and healthy humans (9, 10). It was thought that monocontaminated "germfree" chicks harboring a large quantity of blood group active bacteria may more readily show an acquisition of bacterial blood group-like antigens on their erythrocytes than humans or chicks who are continuously exposed to and react with a much larger number of antigenic and noxious agents in their less controlled environment. It was also tried to determine some of the factors which influence the coating reaction. Irreversible coating of erythrocytes with blood group B and H(O) active *E. coli* products is easily achieved *in vitro* provided little or no plasma or the plasma components albumin, α_2- or β-lipoprotein are present. Coating of erythrocytes *in vivo* is difficult to obtain and is demonstrable only under severely pathological conditions of the host or upon intravenous injection of large doses of lipopolysaccharide.

MATERIALS AND METHODS

Experimental Subjects and Their Treatment

Healthy humans of all age groups, as well as individuals suffering from severe diarrhea, cancer of the colon, ulcerative colitis, and leukemia were investigated. Germfree and ordinary White Leghorn chickens were raised and maintained under conditions similar to those previously reported (9). Apart from the immunological procedures described below, tourniquet shock as a form of extreme physical stress was produced in some groups of chickens by application of a heavy rubber band to the distal part of either one or both thighs for 1 to 2 hours. Sterile aqueous suspensions of *Podophyllum* and *Cascara sagrada*, two strong intestinal mucosa irritants, were given to some chickens by mouth with a graduated dropper. A daily dose for 5 days consisted of *Podophyllum* 20 mg and/or *Cascara sagrada* approximately 64 mg. Cortisone

acetate (0.5 mg/chick/day) was given ip for 8 days to another group of birds (Table VI, experiment 4).

Bacterial Antigens

Smooth *E. coli* $O_{86}B$:7 No. 6019 and *E. coli* O_{128} or O antigen preparations obtained from these bacterial strains were used throughout. The bacteria were grown following a uniform procedure as described previously (1). This included a fully defined medium shown to be devoid of blood group A_1, B, and H(O) active substances (1). Vigorously growing cultures were used in the feeding experiments of adult humans or ordinary and germfree White Leghorn chicks. For preparation of large quantities of bacteria, 24 to 48 liter cultures were grown for approximately 48 hours at 37°C and harvested by centrifugation at 30,000 *g*. The bacteria were then washed once with 0.85 per cent NaCl solution, dried *in vacuo* over KOH in the presence of toluene vapor, and subsequently ground in a mortar. The resulting powder was either employed in the feeding experiments or used to prepare protein-lipopolysaccharides and lipopolysaccharides by extraction with pyridine-water or hot phenol-water according to established procedures (11, 12). The nucleic acids were removed in part from the aqueous solution by ethanol precipitation in the presence of 0.1 per cent sodium acetate. Subsequently, the alcohol in the supernatant fluid was evaporated *in vacuo* and the polysaccharide complex sedimented by centrifugation at 105,400 *g* for 3 hours; its nucleic acid content was still between 5 and 9 per cent.

The blood group active substances from higher plants or mammals, used in this study, have been described previously (13, 14). Precautions were taken to exclude bacterial contamination as far as possible. The meconium was collected with swabs or applicators from the infants shortly after defecation, placed in sterile Petri dishes at 4°C, and stored at −20°C until processed by a technique used previously (14).

Microbiological Testing Procedures

These procedures have been described earlier for germfree chickens (19). In man, stool specimens were collected with sterile swabs immediately on defecation and Gram-negative bacteria were isolated by standard procedures of clinical bacteriology. Ten coli-like cultures were picked from each stool specimen at random from blood agar and MacConkey plates and each of these subcultured as described previously under Bacterial Antigens. The bacteria were then harvested by centrifugation, boiled for 2½ hours in distilled water, and tested for A_1, B, and H(O) blood group activity (*cf.* 1, 14).

Sensitization and Immunization

(A) IN MAN Both healthy and sick individuals ranging in age from 4 days (including premature babies) to 52 years were fed killed *E. coli* O_{86}. All the subjects were of blood group A or O. The feedings were scheduled as follows: for the infants 500 mg *E. coli* O_{86} 3 times daily for 7 days, for the adults 2 to 3 gm given within 24 hours in 1 gm lots. This plan could not be strictly adhered to because some individuals regurgitated feedings whereupon the feeding was interrupted for several days. Some adults received approximately 10^7 live *E. coli* O_{86} in 6 ounces of cow's milk.

(B) IN CHICKENS Immunization by feeding live *E. coli* O_{86} was successful only in germ-free chickens. The procedures have been described in an earlier paper (9). Parenteral sensitization and immunization of both ordinary and germfree chicks 6 to 20 weeks old was accomplished by intravenous injection of up to 20 mg/kg of body weight of *E. coli* O_{86} lipopolysaccharide dissolved and autoclaved in buffered saline (see Solutions). Higher doses were lethal.

Erythrocytes

A_1, B, A_1B, and O erythrocytes from humans of all age groups, stored for less than 14 days in one-third volume "anticoagulant acid citrate dextrose" solution (1.47 per cent glucose, 1.32 per cent sodium citrate, 0.44 per cent citric acid) at 1–3°C, were used immediately after 3 washings with 15 to 20 volumes of buffered 0.85 per cent aqueous NaCl solution. Chicken blood was collected by heart puncture into 1 volume of sterile modified Alsever's solution (1 liter contains 8 gm $Na_3\ C_6H_5O_7 \cdot 2\ H_2O$, 0.55 gm $C_3H_4OH\ (COOH)_3 \cdot H_2O$, 4.18 gm NaCl, and 18.66 gm D-glucose, final pH 6.4).

Antisera

Human anti-A and anti-B sera, both "natural" and immune, were used (1). For some adsorption and elution experiments sera from patients who had received incorrect blood transfusions and showed correspondingly high titers were also employed. All sera of a given specificity reacted qualitatively alike. Eel serum containing potent anti-H(O) agglutinins, after absorption with human A_1B erythrocytes, was used in the agglutination of O erythrocytes (1). The sera used in agglutination experiments against coated chicken erythrocytes were first absorbed with ordinary chicken erythrocytes until these were no longer agglutinated. Unabsorbed alligator and snapping turtle (*Chelydra serpentina*) sera were also used in some experiments. Plasma and sera were decomplemented at 56°C for 25 minutes except eel serum which was decomplemented for 50 minutes. The samples were then stored in 3 to 5 ml lots at −20°C until use.

For the direct antiglobulin test on chicken erythrocytes anti-chicken globulin serum (cappel 65/501, lot 4355) was employed. Anti-human globulin sera (pentex lots 41 and 43), in order to investigate sensitization by blocking human antibodies, were also used where indicated.

Antibody Adsorptions and Elutions

Adsorption with an equal volume of washed, packed erythrocytes was performed as previously reported (9). Eluates were obtained by a modified Landsteiner-Miller procedure (15) similar to that described previously (9); the proportion of high titered serum to red cells to eluting solution was usually 3:1:1. Difficulties were encountered in demonstrating the elution of more than a small fraction of the homologous antibodies adsorbed onto erythrocytes coated with bacterial lipopolysaccharides. Here the procedure described by Vos and Kelsall (16) was successfully used[1] with the

[1] Carried out with the help of Dr. Gisela Nass.

following modifications: The serum was preabsorbed with *E. coli* O_{86}-coated O red cells until it did not agglutinate these erythrocytes. Also, the eluate was titrated in 0.85 per cent saline or in serum of A_1B individuals from which anti-*E. coli* O_{86} antibodies had been removed by absorption with bacteria (17). In some instances serum of germ-free chickens was also used because of its lack of detectable blood group B-like substances and anti-human blood group B antibodies.

Solution

The diluent and the erythrocyte suspending solution in all tests was 0.85 per cent saline, containing 0.02 M phosphate buffer, pH 7.2.

Serological Procedures

All tests were done 3 or more times. *In vitro* sensitization of erythrocytes for the "passive" hemagglutination test was based on established methods (8). Unless stated otherwise, a solution containing 250 to 500 μg/ml *E. coli* O_{86} or *E. coli* O_{128} lipopolysaccharide in buffered saline was heated in a boiling water bath for 3 hours, brought to room temperature, and insoluble material removed by centrifugation at 2200 *g*. Ten volumes of this solution were then incubated with 1 volume of thrice washed, packed erythrocytes at 37°C for 2 hours with agitation at approximately 15 minute intervals. Optimal coating resulted from this procedure. The erythrocytes were then thoroughly washed 3 times at room temperature with 25 volumes of buffered saline; no demonstrable difference in agglutinability of the erythrocytes resulted after the third washing even if the washing procedure was repeated 12 times. No blood group active material was detected with the methods employed in concentrates of the supernate after the third washing. As controls, blood group substances of human and animal origin (13, 14), blood group inactive lipopolysaccharides from Gram-negative bacteria,[2] dextran, and starch were included in some experiments.

Inhibition of coating by plasma and some of its fractions was assessed by adding 1 part of these fluids to 1 part washed erythrocytes immediately prior to addition of 10 parts *E. coli* solution.

The mode of titration and interpretation of agglutination of all except the antiglobulin sera have been previously described (9, 14). Agglutination was read with the microscope after 2 to 4 hours' incubation at 22–25°C. Each titration included as a negative control a saline suspension of the coated or uncoated erythrocytes under study. Titers are expressed in terms of twofold geometrical dilution of serum before addition of red cells.

Anti-human globulin sera were used in accordance with the instructions by the manufacturer. The anti-chicken γ-globulin serum was absorbed for 1 hour at 22–25°C and 15 minutes at 37°C with one-quarter volume of washed, packed not sensitized White Leghorn chicken erythrocytes. The red cells were removed by centrifugation and 2 drops of the antiglobulin serum were added to 2 drops of the washed chicken erythrocyte suspensions (2 per cent). The samples were incubated for 30 minutes at 37°C and then centrifuged at 2500 RPM for 1 minute. Negative controls

[2] Some of them given by Professor O. Westphal and Dr. O. Lüderitz.

were included in each experiment, but no appropriate positive control was available. The tests were read microscopically.

Hemagglutination Inhibition

The method described previously (1) for studying inhibition of hemagglutination has been employed except that the steps of dilution of inhibiting substances were fivefold; blood group active substances prepared earlier (14, 18) and 4 minimum hemagglutinating doses of antibody were used.

TABLE I

REACTION OF HUMAN ANTI-B AGGLUTININS WITH B ERYTHROCYTES AND *E. COLI*-SENSITIZED O ERYTHROCYTES

Erythrocytes		Anti-B titer (reciprocal)	
Group	Sensitized with	Whole immune serum	Eluate from B erythrocytes*
B	Nothing	512	32
O	Nothing	<1	<1
O	*E. coli* O_{86}	64	32
O	*E. coli* O_{55}	16–32	<1
O	*E. coli* O_{11}	16	<1

* Landsteiner-Miller procedure.

RESULTS

In Vitro Coating of Saline-Suspended Erythrocytes with Blood Group Active and Inactive Bacterial Polysaccharides

The culture medium after removal of bacteria (60 hours' incubation at 37°C), protein-lipopolysaccharides and lipopolysaccharides had similar erythrocyte coating effects *in vitro*, although the growth medium showed significant quantitative variations from batch to batch. The results reported were obtained with the isolated protein-lipopolysaccharides or lipopolysaccharides which will not be differentiated because of their similar effects.

The susceptibility to "coating" of human erythrocytes from individuals of all ages, blood groups, and states of health was too similar, when tested with a given blood group active lipopolysaccharide preparation and the same serum, to be reliably differentiated by the techniques employed here. Chicken erythrocytes were coated similarly to human erythrocytes. Two alligator sera tested and 1 of 5 snapping turtle sera strongly agglutinated B erythrocytes and O red cells coated with *E. coli* O_{86} while they had little or no effect on ordinary A and O erythrocytes. Sera from individuals of blood group A_1B who do not possess either anti-blood group A or B antibodies, also agglutinated erythrocytes sensitized with products from *E. coli*, regardless of whether the *E. coli*

preparations themselves were blood group active or not. This interference is due to anti-*E. coli* antibodies, which were present in all human sera beyond infancy and which obscured the reactions because of their polyagglutinating effect. Table I gives an example of this situation (column 3). It also shows the results after selective isolation of anti-B agglutinins by adsorption on and elution from erythrocytes of blood group B (column 4).

In contrast to human, rabbit, and chicken sera, eel anti-H(O) serum which had been absorbed with A_1B erythrocytes contained no antibodies reacting

TABLE II

HUMAN ANTI-BLOOD GROUP B AGGLUTININS: ADSORPTION ON AND ELUTION FROM O ERYTHROCYTES COATED WITH *E. COLI* O_{86} LIPOPOLYSACCHARIDES

Anti-B agglutinins from A_1 or O individuals	Reciprocal titer against	
	B erythrocytes	O erythrocytes, coated with *E. coli* O_{86}
Serum, unabsorbed	128–256	32–64
Serum, absorbed	8	<1–2
Eluate	32–64*	1–4‡

* A_1 erythrocytes were also agglutinated to about one-tenth of the anti-B titer if the eluate originated from the serum of a group O individual.
‡ Higher titers were obtained, when eluates were prepared by the Vos-Kelsall procedure.

with blood group inactive lipopolysaccharide, although the same red cells coated with blood group H(O) active *E. coli* O_{128} polysaccharide were strongly agglutinated (see Table III).

Absorption and Elution of Blood Group-Specific Antibodies with Coated and Uncoated Erythrocytes

Two systems were studied and appropriate controls were included: (*a*) B-anti-B including the B cross-reacting *E. coli* O_{86} and (*b*) O(H)-eel anti-H(O) including the H(O) cross-reacting *E. coli* O_{128}.

(*a*) Anti-B antibodies from either blood group A or blood group O individuals were adsorbed onto and eluted from B erythrocytes. In general, all anti-B agglutinins were absorbed. The decrease in anti-*E. coli* O_{86} agglutinin content in the absorbed sera was scarcely significant but amounted regularly to 1 to 2 tubes. The eluate specifically agglutinated B erythrocytes and to a lesser degree (titer of one-eighth to one-quarter) O erythrocytes coated with *E. coli* O_{86} lipopolysaccharide.

If, on the other hand, O erythrocytes coated with *E. coli* O_{86} lipopolysaccharide were employed, one absorption of anti-B sera by such coated erythrocytes resulted in the removal of 75 to 94 per cent of anti-B agglutinins and

over 94 per cent of the agglutinins against *E. coli* O_{86}. All eluates from these cells agglutinated human B erythrocytes to a considerably higher titer than O erythrocytes coated with *E. coli* O_{86} lipopolysaccharides which sometimes were barely agglutinated with Landsteiner-Miller eluates. Eluates obtained by the Vos-Kelsall procedure, however, regularly gave significant titers against sensitized O cells. The most potent eluates were from sera of patients immunized by incorrect blood transfusions. A summary of the results obtained with *E. coli* O_{86}-coated O erythrocytes is depicted in Table II.

TABLE III

ANTI-BLOOD GROUP H(O) EEL AGGLUTININS: ADSORPTION ON AND ELUTION FROM A_1B ERYTHROCYTES COATED WITH *E. COLI* O_{128} LIPOPOLYSACCHARIDES

Anti-H(O) eel serum	Reciprocal titer against erythrocytes			
		A_1B coated with		
	O	Nothing	*E. coli* O_{128}	*E. coli* O_{55}
Serum				
Before absorption with coated erythrocytes	64–128	<1	256–512	<1
After absorption with coated erythrocytes	4	<1	8	
Eluate	16	<1	32–64	<1

Eluates from *E. coli* O_{86}-coated O erythrocytes also agglutinated weakly O erythrocytes sensitized with blood group inactive lipopolysaccharides from *E. coli* and *Salmonella*. Erythrocytes coated with blood group inactive *E. coli* or *Salmonella* lipopolysaccharides, however, did not absorb significant amounts of blood group A or B antibodies.

(*b*) Absorption of eel anti-H(O) serum with *E. coli* O_{128}-coated A_1B red cells removed more than 90 per cent of anti-H(O) and other antibodies reacting with *E. coli* O_{128} (Table III). One absorption of this serum with human O erythrocytes removed only between 50 to 75 per cent of the antibodies reacting with human erythrocytes of blood group O or *E. coli* O_{128}. Anti-blood group M, N, or Rh_0 (D) antibodies were not adsorbed by red cell–bound *E. coli* O_{86} or *E. coli* O_{128} lipopolysaccharides.

Inhibition of Eluted Anti-blood Group and Anti-E. coli Agglutinins by Blood Group Active Substances

Eluted antibodies were used in the hemagglutination inhibition experiments in order to assure specificity. The results are listed in Table IV. The inhibitory action was strictly specific; only B active substances inhibited, regardless of whether the eluates were obtained from ordinary B erythrocytes or erythro-

cytes made B active with *E. coli* O_{86} lipopolysaccharide. Agglutination of B erythrocytes, but not of *E. coli* O_{86} coated O erythrocytes, was inhibited to approximately the same extent by all highly B active polysaccharide preparations.

Strictly comparable results were obtained in the inhibition of eel anti-H(O) agglutinins (eluted either from O erythrocytes or *E. coli* O_{128}-sensitized A_1B red cells) in their action on either O or *E. coli* O_{128}-coated A_1B erythrocytes.

TABLE IV

INHIBITION OF ELUTED ANTI-BLOOD GROUP B AND ANTI-*E. COLI* O_{86} AGGLUTININS FROM HUMAN SERUM (BLOOD GROUP A INDIVIDUAL)

Blood group active material from		Micrograms per milliliter completely inhibiting 4 hemagglutinating doses			
		Eluates from			
		B erythrocytes		*E. coli* O_{86}-coated O erythrocytes	
		Tested against erythrocytes			
Group	Material	B	O, *E. coli* O_{86}-coated	B	O, *E. coli* O_{86}-coated
B	Meconium	10	100	±10	100
	Ovarian cyst	10	500	10	100
	Horse gastric mucin	±10	500	100–500	500
	E. coli O_{86}	<10	10	10	10
O	Ovarian cyst	>5000	>5000	>1000	>5000
A	Ovarian cyst	>5000	>5000	>5000	>5000
Blood group inactive *S. abortus equi* or *E. coli* O_{11}		>5000	>5000		>5000

Attempts to Coat Erythrocytes with Blood Group Active Substances of Other than Bacterial Origin

In all these experiments the conditions were identical to those employed for the bacterial lipopolysaccharides, except that coating was also attempted at polysaccharide concentrations exceeding those of the microbial preparations by a factor of 10. Ordinary unabsorbed sera were used to determine coating.

No coating of erythrocytes was demonstrable by blood group A or B or H(O) mucoid from human ovarian cysts or horse stomach, nor by blood group H(O) active polysaccharides from *Taxus* and *Sassafras* or by blood group inactive dextran. In contrast to these negative findings blood group A and B preparations from human meconium (precipitating from boiled aqueous meconium extracts at 48 to 60 per cent final ethanol concentration) sensitized O erythrocytes irreversibly as measured with human antisera. Sera from AB individuals who do not possess isoagglutinins also weakly agglutinated these

erythrocytes. The coating effect was clearly demonstrable only when the meconium fractions, like those isolated from Gram-negative bacteria, were heated at pH 7.2. Anti-*E. coli* antibodies were removed from the testing sera by absorbing with ½ volume of dried *E. coli* O_{86} (*cf.* 17). While all antibodies against *E. coli* O_{86}-coated O erythrocytes were removed, the decrease in titer against O erythrocytes coated with meconium A and B blood group substances never amounted to more than 1½ tubes as compared to the preabsorption titer. With eel serum as reagent, no coating effect of A_1B erythrocytes was noted with any of the above polysaccharide preparations.

TABLE V
INTERFERENCE OF PLASMA WITH *E. COLI* O_{86}-COATING OF O ERYTHROCYTES

Measured with human anti-B sera		
Source of plasma	Amount of plasma*	Reciprocal titer against erythrocytes
Human, blood group A_1B	None	32
	0.1	8
	0.3	4
	0.5	1-2
Chick	None	32
	0.05	16
	0.15	4
	0.25	1-2
	0.5	<1

* Plasma added to 0.5 ml tightly packed erythrocytes, subsequently mixed with 5 ml buffered saline containing 100 μg lipopolysaccharide per ml; adjusted to 6 ml total volume (see Materials and Methods).

Influence of Plasma on Erythrocyte Coating in Vitro

Plasma inhibits erythrocyte sensitization by bacterial lipopolysaccharides (19, 20). The influence of plasma and of serum on the erythrocyte coating effect of lipopolysaccharides from *E. coli* O_{86} and *E. coli* O_{128} was therefore investigated. No difference was noted between the inhibiting power of ordinary plasma or serum. Neither heating these fluids at 56°C for 30 minutes nor the anticoagulants used in the present investigation influenced their activity. However, the inhibitory effect was much decreased by heating these fluids (diluted 1:10) for 15 minutes in a boiling water bath before testing. The concentration dependence of the inhibitory effect of unheated plasma is depicted in Table V. When plasma from healthy human adults of blood group AB was added in a concentration exceeding 10 per cent (0.5 ml) of the volume of the standard coating solution containing 0.01 per cent lipopolysaccharide (total

amount of lipopolysaccharide = 500 μg) coating was prevented. A final concentration of 0.5 per cent plasma did not significantly inhibit coating. The inhibiting capacity of chicken plasma appeared to be somewhat higher than that of human plasma. Plasma from newborn infants, premature or term, plasma from a number of patients suffering from acute leukemia, ulcerative colitis, hypogammaglobulinemia,[3] or renal failure, and from patients fed gram quantities of dead *E. coli* O_{86} was also investigated for its inhibitory effect. In general, plasma from even severely diseased persons inhibited to the same degree as that of healthy individuals; but plasma from a patient with acute renal failure (subsequent to trauma) had less than 25 per cent of the inhibitory potency found in other adults. Human, equine, and bovine serum albumin inhibited strongly as did the α_2- and β-lipoproteins (0.5 to 1.0 mg/ml) while human β- and γ-globulins (7S and 19S) did not prevent coating to a significant degree (> 7.5 mg/ml under standard conditions). Sera from patients with both congenital and acquired "agammaglobulinemia" inhibited as well as those from healthy individuals. Eel serum and alligator serum inhibited like normal human serum.[4]

Attempts to Remove the Acquired Agglutinogens from the Erythrocytes

Ordinary saline washings at room temperature did not remove demonstrable amounts of fixed lipopolysaccharides. Four volumes ot erythrocytes maximally sensitized with lipopolysaccharide from *E. coli* O_{86} were therefore eluted into 1 volume of physiological saline, plasma, or 4 to 5 per cent human albumin at 37°C. The erythrocytes were resuspended after centrifugation at 37°C in the same solutions and recentrifuged at the above temperatures. No hemagglutination inhibiting substances could be demonstrated in the supernates nor did the coated cells show any decreased titer after elution at 37°C for from 8 to 30 minutes. However, elution and centrifugation at 58–59°C fo 8 minutes gave a different result. The red cells generally lost three-quarters or more of detectable lipopolysaccharide. In agreement with this finding, lipopolysaccharide was demonstrable in the eluates with serological methods.

In Vivo Coating of Erythrocytes with Blood Group B Active Products from E. coli O_{86}

CHICKEN ERYTHROCYTES Previous experiments had shown that bacteria with high blood group B activity established themselves as a pure culture in

[3] Some of the sera from patients with acquired or congenital agammaglobulinemia were donated by Dr. R. A. Good.

[4] Pure human albumin, α_2- and β-lipoprotein and 7S- and 19S-γ-globulin obtained from Professor H. E. Schultze and Dr. G. Schwick, Behringwerke, Marburg, Germany. Human serum albumin, lot RCA 29C, β- and γ-globulin kindly given by Mr. J. Smolens. Alligator serum was donated by Dr. E. Cohen.

TABLE VI

ATTEMPTS TO COAT CHICKEN ERYTHROCYTES *IN VIVO* BY FEEDING LIVE BLOOD GROUP ACTIVE *E. COLI* O_{86}

Chicken	Experiment No.	Treatment	Degree of coating: Age in days‡	+++	++	+	$\oplus$	Age in days‡	+++	++	+	$\oplus$
Ordinary	1	*E. coli* O_{86} 2nd day of life *Cascara* and podophyllin where indicated on 28th and 57th days	9–15			1/9	8/9	29 (58)			3/9 (2/9)	6/9 (7/9)
Germfree	1				1/14	5/14	6/14		1§/13 (1/12)¶	2/13	4‖/13 (1/12)	6/13 (10/12)
Ordinary	2	** *E. coli* O_{86} on 77th day. *Cascara* on 79th, tourniquet on 80th day	80 and 82			1/4	3/4					
Germfree	2		1 hr. post-tourniquet	1/7	1/7	2/7	3/7					
Ordinary	3	*E. coli* O_{86} and *Cascara* on 10th day. Tourniquet on 12th day	13		1/8		7/8					
Germfree	3				1/11	1/11	9/11					
Ordinary	4	Cortisone from 8th to 15th day. *E. coli* O_{86} on 10th day, tourniquet on 15th day	16			1/6	5/6					
Germfree	4				1/8	2/8	5/8					
Ordinary	5	*E. coli* O_{86} on 50th day, *Cascara* 51st day, tourniquet 57th day	57				9/9	58			2/9	7/9
Germfree	5		5 hrs. after tourniquet		1/8	2/8	5/8				2/6	4/6

* +++ agglutination by 6 sera of 6; ++ agglutination by at least 3 of 6 sera; + agglutination by at least 1 of 6 sera; average titer 1:1–1:2; $\oplus$ doubtful or no agglutination.

‡ When bled.

§ Got *Cascara* and podophyllin.

‖ 3 got *Cascara* and podophyllin.

¶ See text.

** These birds (originally 4 ordinary and 10 germfree) were bled on days 10, 28, 56, and 70; their cells showed no coating.

germfree chickens and induced very high titers of anti-human blood group B agglutinins (9). *E. coli* O_{86} was therefore fed to germfree and ordinary chicks and its influence on their erythrocytes determined. The youngest chicks bled were 9 days and the oldest 140 days of age. The erythrocyte coating effect was determined with 6 different sera: 2 from humans of blood group A, 2 from donors of blood group O, and 2 rabbit anti-*E. coli* O_{86} sera (17), one from short and the other from long term immunization.

As can be seen from Table VI, experiment 1, when newly hatched germfree and ordinary chicks were fed live *E. coli* O_{86}, there was faint coating of erythrocytes demonstrable in 6 of 14 of the germfree chicks during the 2nd week of life, but only 1 of 9 ordinary chicks showed such an effect. Therefore, attempts were made to increase intestinal permeability of the chicks by feeding them purgatives and to upset the circulatory systems by producing shock. Experiment 1 in Table VI shows that the agglutinability of red cells of ordinary chicks at the age of 29 days was only slightly increased while 3 of the surviving 13 germfree chicks bled exhibited a significant agglutinability of their cells. The figures in parentheses in the last column of experiment 1 show the situation after the chickens had doubled their age. In a second experiment the birds were fed live B active *E. coli* on the 77th day of life and were given *Cascara* and shock a few days afterwards. The red cells of 2 of 7 germfree chicks were agglutinated by 3 or more of the 6 antisera. Three additional experiments gave similar results. The administration of cortisone over a period of 8 days did not increase the sensitization of chicken erythrocytes by components of *E. coli* O_{86} (experiment 4, Table VI).

The red cells of only 1 ordinary chick of a total of 27 which had received tourniquet shock were agglutinated by one-half of the antisera employed. In contrast the erythrocytes of 5 of 34 germfree chicks with tourniquet shock were so agglutinated.

Of a total of 51 germfree chicks, 31 indicated no coating with bacterial lipopolysaccharide as determined by either saline agglutinins or by the antiglobulin test (see Table VI, including** footnote); 30 of 36 ordinary chicks showed no coating.

In another series of experiments blood group B active *E. coli* polysaccharide was injected into the ventral wing vein of 13 ordinary and 18 germfree chickens and its effect on erythrocytes withdrawn from the other wing measured repeatedly during the 1st hour after injection (Table VII). If possible, additional blood samples were taken 24 hours and 7 days after the injection. Blood was collected from 22 of the 31 birds immediately prior to lipopolysaccharide injection. No erythrocyte coating was observed in any of the preinjection specimens. Seven of 13 ordinary chickens exhibited a coating of their erythrocytes subsequent to lipopolysaccharide injection. The strongest erythrocyte coating was found in those birds which had received tourniquet shock treat-

TABLE VII

ATTEMPTS TO COAT CHICKEN ERYTHROCYTES *IN VIVO* BY INTRAVENOUS INJECTION OF *E. COLI* O_{86} LIPOPOLYSACCHARIDE

	Chicken No.	Amount injected mg/kg chicken	Bleedings: Immediately prior to injection	Post-injection: 3 to 20 min.	60 min.	24 hrs.	7 days
Ordinary	7389	3		⊕*	⊕		
	7390‡	3		+*	+		⊕
	7414	6		+	+		⊕
	7415‡	6	⊕	⊕	⊕	⊕	
	7416‡	6		⊕	⊕		
	7484	6		⊕	⊕		
	7495‡	6		+	+		⊕
	7463§	6		⊕	⊕		
	7464§	6		+	++*		+
	7465§	6		⊕	+		+
	7433	20		⊕		+	
	7434	20		+	+		⊕
	7437	20		⊕	⊕		⊕
Germfree	7391	3		+	+		
	7392‡	3		⊕	+		
	7393‡	3		+	+		
	7401	6		⊕	+		⊕
	7402	6		+	+		
	7403	6		⊕			⊕
	7404	6		⊕	⊕		⊕
	7405	6	⊕	+			
	7407	6		⊕	⊕		⊕
	7408‡	6		⊕	⊕	⊕	⊕
	7496	6		⊕	⊕		
	7497	6		⊕	⊕		
	7426	20		⊕	⊕		⊕
	7427	20		⊕	⊕	⊕	
	7429	20		+	++		+
	7485	20		⊕			
	7499	20		⊕	⊕	⊕	
	7500	20		⊕	⊕	⊕	

(The "Immediately prior to injection" ⊕ is bracketed for chickens 7389–7463 (ordinary) and 7391–7427 (germfree).)

* For interpretation of agglutination see Table VI.

‡ In addition to saline agglutinins from man and rabbit, anti-chicken γ-globulin sera were also employed with similar results. Positive reactions with anti-γ-globulin sera were obtained in ordinary chickens 42 days and older, and in germ-free birds of 140 days, as indicated in table.

§ Injected immediately after 2 hours of tourniquet shock.

ment for 2 hours immediately before injection. This procedure proved fatal to the majority of birds (not listed in Table VII). In only 2 instances was coating still demonstrable 7 days after lipopolysaccharide injection into

ordinary chickens. The results obtained with the germfree birds were similar; 6 of 18 chickens, none treated with tourniquet, showed coating of their erythrocytes. The heaviest coating was observed after injection of 20 mg of lipopolysaccharide into healthy birds. Even with this large dose, only a minority showed uptake of lipopolysaccharide by their erythrocytes. Anti-chicken γ-globulin sera were employed for measurement of sensitization of chicken erythrocytes by the chickens' own antibodies. Some of those chicken erythrocytes, obtained from older chickens, which were clumped by saline agglutinating antibodies from humans and rabbits, also showed erythrocyte sensitization by the chickens' own antibodies during the first 60 minutes following lipopolysaccharide injection (see Table VII).

An effort was also made to find out whether or not "blocking" antibodies were preventing a detection of coating which had actually taken place in chickens which harbored *E. coli* O_{86} in their intestines. Red cells coated *in vitro* with *E. coli* showed that saline agglutination titers obtained with the sera of human origin used differed by not more than 1 tube from those obtained with anti-human globulin sera. It was, therefore, only investigated whether or not blocking chicken antibodies sensitized the chickens' own erythrocytes *in vivo.* For this purpose absorbed anti-chicken γ-globulin antiserum was added to erythrocyte suspensions of 14 ordinary and 14 germfree birds under investigation. A positive anti-γ-globulin reaction was obtained for 2 ordinary and 4 germfree chickens; 3 of these 6 chickens had also indicated erythrocyte coating by bacterial products when measured with saline agglutinating antibodies.

HUMAN ERYTHROCYTES Fifteen sick babies, most of them with diarrhea, and 8 healthy infants aged between 4 days and 8 months were fed between 8 and 12 gm of dried powdered *E. coli* O_{86} in their formula over a period of 5 to 10 days; 5 other healthy infants not fed *E. coli* served as controls. The erythrocytes of 8 of the sick infants were agglutinated by at least one of the antisera described above when examined at the end of the 7 days feeding period. The red cells of 3 of these children, ranging in age from 3½ weeks to 3 months, were agglutinated by over one-half of the sera employed. One of the sick infants of blood group A (G. A.) who had a blood group B active *Proteus mirabilis* in its stool showed microscopic agglutination of its red cells after addition of anti-B serum from A individuals even before it was fed *E. coli* O_{86}. From an additional infant (O. B.) whose red cells indicated coating and in whose stool a B active bacterium was demonstrated, no pretreatment blood could be obtained. Among the healthy infants who were fed *E. coli* O_{86} only 1 of 1 week of age showed weak but distinct coating of its erythrocytes. This coating was still demonstrable 1 week after termination of feeding and this premature infant (B. B.) had live blood group active *E. coli* in its stool. In 2 of the diarrheic infants the coating effect was demonstrable 2 weeks after termination of

TABLE VIII

ATTEMPTS TO COAT HUMAN ERYTHROCYTES *IN VIVO* BY FEEDING 8 TO 12 gm KILLED *E. COLI* O_{86} PER INFANT

Baby's name	Blood group	Age in weeks	Disease	Microbiological assay of stool	Bleedings: *Pre*-treatment	*Post*-treatment: (after last feeding) immediately	7 to 10 days	12 to 14 days	1 to 2 months	2 to 6 months
Sick:										
T. T.	O+	2	Diarrhea (mild)	*Aerobacter cloacae* B active	⊕*	+*	⊕			
A. J.	O+	2	Diarrhea (severe)	*Aerobacter*, *Proteus*, B active	⊕	+		⊕		
J. N.	A+	2	Diarrhea (mild)	*n.a.*‡	⊕	⊕		⊕		
G. A.	A_1+	3½	Diarrhea (severe)	*Proteus mirabilis* B active	++*	++	++	++	⊕	⊕
J. B.	A_1+	4	Diarrhea (mild)	*n.i.*§	⊕	⊕	⊕			
R. S.	O	6½	Diarrhea (severe)	*Klebsiella* and *E. coli*, H(O) active	⊕	⊕			⊕	
J. W.	A_1−	8	Diarrhea (severe)	*E. coli*, weakly H(O) active	n.i.	++		++		
C. J.	A_1+	8½	Diarrhea (severe)	*E. coli*, A active, H(O) active	⊕	+	(blood transfusion) ⊕			
A. N.	O+	11	Premature, anemia, transfusion	*n.i.*	⊕	⊕		⊕		
O. B.	O+	12	Diarrhea (severe)	*Aerobacter*, B active	n.i.	++		±	⊕	
W. C.	O+	13	Diarrhea (mild)	*n.a.*	⊕	+				
V. R.	A_1+	13	Diarrhea	*E. coli*, *Aerobacter*, ±A; +H(O) active	⊕	+	⊕			
D. L.	O+	17½	Diarrhea	*n.i.*	⊕	⊕		⊕		
G. M.	A_1+	17½	?	*n.a.*	⊕	⊕		⊕		
E. D.	O−	35	?	no B active bacteria	⊕	⊕		⊕		
Healthy:										
7 infants	A and O	½ to 10	Healthy	few B active but mostly inactive bacteria	⊕	⊕	⊕	⊕	⊕	
5 infants, fed no *E. coli*	A and O	½ to 15	Healthy	no B active bacteria	⊕	⊕	⊕	⊕	⊕	⊕
B. B.	O+	1	Healthy	*E. coli*, A active	⊕	+	+		⊕	

* For interpretation of agglutination see Table VI.
‡ *n.a.*, not active.
§ *n.i.*, not investigated.

feeding, but it could not be shown in bleedings taken 1 to 2 months after termination of bacteria feeding. The results are summarized in Table VIII.

Living *E. coli* O_{86} were only fed to adults because of their potential pathogenicity for small children (*cf.* 21). Eight healthy human volunteers (22 to 50 years old, 4 of blood group O and 4 of blood group A_1) were fed live smooth *E. coli* O_{86} (see Materials and Methods). These bacteria did not establish themselves in the intestines in a way demonstrable with the methods employed. No *E. coli* O_{86} were found by the bacteriological culturing procedures in the stool of the volunteers 5 and 12 days later, nor were their erythrocytes coated to render them agglutinable by anti-B sera. Five additional healthy volunteers of blood group O were fed 2 gm dried powdered *E. coli* O_{86}. Neither they nor 3 similarly fed patients with severe intestinal disorders, showed any indication of coating of their erythrocytes by B-like antigens. Adults were fed less than one-twentieth the dose of *E. coli* O_{86} per kg body weight which was given to infants.

DISCUSSION

As the results reported in this paper and in an earlier abstract (22) show, blood group active bacterial substances are readily acquired *in vitro* by saline-suspended human and chicken erythrocytes. These findings agree with older experiments which demonstrate that red blood cells of man and animals may become sensitized *in vitro* by a variety of bacterial products (*cf.* 8). The paramount pathophysiological problem of the present study is whether or not blood group active bacterial substances may be acquired by human or chicken erythrocytes *in vivo*. The data presented show that erythrocytes of infants with serious intestinal disorders may become sensitized by blood group B-like antigens from bacteria. The *in vivo* coating of red cells was uniformly weak and never exceeded a titer of 1:4 either with human anti-B agglutinins or with rabbit anti-*E. coli* O_{86} antisera. Also, a considerable number of infants fed *E. coli* O_{86} showed no coating of their red cells. We obtained similar results in experiments with ordinary chicks. In general, erythrocytes from only those birds which had serious intestinal damage and suffered shock exhibited a significant coating.

Intravenous injection of large amounts of *E. coli* O_{86} lipopolysaccharide also led to coating of erythrocytes in a minority of birds. The *in vivo* and *in vitro* experiments indicate that 3 necessary conditions must be fulfilled in order to permit the coating effect by bacterial endotoxic products to occur *in vivo*: (*a*) large amounts of these substances must be present; (*b*) the permeability of the internal (or external) body surfaces must be increased; and (*c*) plasma components such as albumin and some lipoproteins which are found normally and in most pathological conditions must be below a critical level. It is impossible

to decide how much blood group active material may have entered under various conditions the circulation of a germfree chick which harbors a pure culture of *E. coli* O_{86}, nor can it be assessed how much of the killed *E. coli* O_{86} fed to diarrheic infants may have entered their circulation. It is not unlikely that many cell surfaces with which lipopolysaccharides come in contact may adsorb them. A simple, conservative calculation, however, shows the following: According to the literature (23) 2 μg of alkali-activated lipopolysaccharide suffice to render specifically agglutinable (serum dilution 1:50) 10^9 saline suspended human erythrocytes. In the present studies the human blood grouping sera and the homologous rabbit anti-*E. coli* O_{86} sera were undiluted. Therefore, the ability of these sera to detect coating should be enhanced.

A newborn infant has about 300 to 400 ml of blood, and since 1 mm^3 contains approximately 5×10^6 erythrocytes, 300 ml have 1.5×10^{12} and 400 ml 2×10^{12} red cells; it follows that approximately 3 to 4 mg are needed to coat all erythrocytes of a newborn demonstrably *in vitro*. Even under the supposition that only a part of the offered lipopolysaccharide is coating, it will be readily seen that the average amount of dead *E. coli* O_{86} (*ca.* 10 gm) fed to infants exceeds the above optimum by a factor of 1000. It has been observed uniformly by us that blood group active substances are not confined to the lipopolysaccharide fraction of *E. coli* O_{86}, but we found them, to a somewhat lesser extent, in all its macromolecular components. The observed *in vivo* agglutinations were of the spotty type described by others for acquired B in humans (2–5) indicating that only a portion of the erythrocytes were weakly coated. This irregular and weak agglutinability contrasts with that which was described in the present report for *in vitro* experiments (*e.g.* Table I and II).

A calculation, similar to that for humans, showed that the injection of 6 to 20 mg/kg activated lipopolysaccharides into chicks constitutes a 10- to 100-fold excess over that needed to coat *all* the chicks' erythrocytes *in vitro*. Newly hatched chicks have *ca.* 3×10^6 erythrocytes per mm^3 of blood; their hematocrit (28 to 31 per cent) and mean blood volume per cent of body weight (*ca.* 8 to 10 per cent) remain remarkably constant during the first 10 weeks of life (24). It is likely that larger amounts of B-like bacterial substances entered the circulation of those chicks which harbored *E. coli* O_{86} in their intestines, which had suffered damage to the surface of their guts due to purgatives, and which were in tourniquet shock.

It has been reported (25), while our own studies were in progress, that surviving chickens who suffered from severe *Salmonella gallinarum* bacteremia showed erythrocyte sensitization, which was only detectable with the antiglobulin test. Injection of crude *S. gallinarum* polysaccharide led to similar results; the possibility that substances besides polysaccharide had coated the erythrocytes was not excluded. It is remarkable that only antiglobulin sera detected *in vivo* sensitization. The present experiments showed an insig-

nificant increase in chick erythrocyte sensitization only when anti-human antiglobulin serum was employed in addition to saline agglutinating antibodies of human origin. Evidence was obtained in tests with anti-chicken globulin sera from the rabbit, that in some of the older chicks erythrocytes sensitized with *E. coli* O_{86} products had bound some of the chickens' own blocking antibodies suggesting their interference with detection of coating by the human and rabbit anti-B and anti-*E. coli* O_{86} test sera.

In vivo sensitization of erythrocytes by products of Gram-negative bacteria was recently observed in 2 infants (26) suffering from severe intestinal diseases. There was chemical as well as serological indication that the coating agents were not of lipopolysaccharide nature.

The most striking findings in the *in vitro* experiments were polyagglutinability of the red cells following coating by bacterial lipopolysaccharide complexes and the apparent irreversibility of the coating process at body temperature. Polyagglutinability was occasionally observed on erythrocytes coated *in vivo* and appeared to be due to the presence of antibodies against antigenic components of the adsorbed bacterial lipopolysaccharide other than blood group-specific substances. This is not surprising since individuals who are capable of producing blood group agglutinins also possess antibodies against enteric bacteria. Furthermore, sera of individuals of blood group AB also agglutinated lipopolysaccharide-coated erythrocytes. The correctness of this interpretation of polyagglutinability was shown in those experiments where anti-B agglutinins only, obtained by adsorption-elution techniques, were employed. Also, the eel is not known to harbor coliform organisms and eel anti-H(O) serum (after absorption with A_1B erythrocytes) did not agglutinate A_1B erythrocytes coated with blood group inactive *E. coli*, but only those coated with H(O)-specific lipopolysaccharide of *E. coli* O_{128}.

A puzzling *in vitro* observation was the uniform difficulty in eluting by the Landsteiner-Miller procedure homologous anti-*E. coli* antibodies from *E. coli* O_{86}-coated erythrocytes (Table II). While all antibodies reacting with *E. coli* O_{86}-coated erythrocytes are removed from human anti-B sera, the amount of eluted agglutinins which react with *E. coli* O_{86}-coated erythrocytes is quite small. Agglutination of ordinary B erythrocytes was considerably stronger with these cross-reacting anti-B antibodies. This finding recalls observations on the plant anti-N from *Vicia graminea* (27), which could only be eluted from MM and MN but not from NN erythrocytes by the Landsteiner-Miller procedure. It may be that some antibodies or antibody-like substances are bound so tightly to their homologous complementary structure, that they are not eluted by this procedure. Such an interpretation is supported by the here reported observation that the more vigorous elution method of Vos and Kelsall yielded a more potent anti-*E. coli* O_{86} eluate from *E. coli* O_{86}-coated red cells. However, lipopolysaccharides may be eluted together with antibodies at the

elevated temperature of the Landsteiner-Miller procedure and thus prevent eluted antibodies from reacting.

Landsteiner-Miller eluates from B erythrocytes always contained agglutinins which reacted with B erythrocytes and *E. coli* O_{86}-coated O erythrocytes (Table I). These findings were made regularly with 7 different anti-B sera. They are at variance with qualitative observations by Andersen (28).

Of the blood group active water-soluble polysaccharides from mammals or higher plants only some fractions isolated from human meconium coated erythrocytes. Those fractions which did coat also exhibited the highest *in vitro* blood group activity. This coating effect amounted to less than 10 per cent of that observed for *E. coli* O_{86} lipopolysaccharides (weight basis); it may be due to the lipid-rich blood group substances of meconium, but contaminants have not been excluded in spite of the precautions listed under Results.

Finally, other modes of acquisition of blood group-specific antigens by erythrocytes *in vivo* have not been excluded by the experimental results presented here which implicate bacterial substances. Thus, endogenous or extraneous enzymes may transform blood group A-specific structures into those of B specificity. Such a transformation may be possible (4), since there is little chemical difference between A and B blood group-specific antigens. Also, Dr. R. R. Race (F.R.S.) has informed us that of 27 adult patients with acquired B-like antigen, 26 were of blood group A_1 while the 27th possessed blood group A_2. This is in contrast to our experiments in infants in which some of blood group A as well as blood group O showed acquisition of B-like antigen. It may be assumed that anti-B antibodies from adult O persons eliminate B-like bacterial antigens more readily from the circulation than those of individuals from blood group A. An alternate postulate would be the more ready, enzymatic transformation from A into B than from O into B. However, as yet no alteration whatever of blood group A specific structures into those possessing B activity has been shown (*cf.* reference 29) and enzymatic transformation of blood group specific lipopolysaccharide from *E. coli* O_{86} was from B into H (O) only (*cf.* reference 30).

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