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Research Article

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Blood Group Isoantibody Stimulation in Man by Feeding Blood Group—Active Bacteria

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ABSTRACT It was investigated whether or not the human blood group isoantibodies A and B could be induced by immunogenic stimuli via natural routes with a kind of antigenic substance to which all humans are commonly exposed, or if the appearance of these antibodies is independent of antigenic stimuli as has long been believed.

Escherichia coli O₈₆, which possess high human blood group B and faint A activity in vitro, were fed to healthy humans and those with intestinal disorders. 80% of the sick individuals of blood group O and A responded with a significant rise of anti-B antibodies which was frequently *de novo* in infants; significant increase of anti-A isoantibodies among blood group O individuals was less frequent. Over one-third of the healthy individuals also had a significant isoantibody increase. Intestinal lesions favor isoantibody stimulation by intestinal bacteria; this view was supported by the study of control infants. Persons of blood group A responded more frequently with anti-B and anti-*E. coli* O₈₆ antibody production than those of blood group O. Isoantibody increase was accompanied with antibody rise against *E. coli* O₈₆. Inhalation of *E. coli* O₈₆ or blood group AH(O)-specific hog mucin also evoked isoantibodies.

The induced isoantibodies were specifically inhibited by small amounts of human blood group substances. *E. coli* O₈₆-induced anti-blood group antibodies in germ-free chickens and preexisting blood group antibodies in ordinary chickens were neutralized by intravenous injection of *E. coli* O₈₆ lipopolysaccharide.

This study demonstrates that human isoantibodies A and B are readily elicited via physiological routes, by

blood group-active *E. coli*, provided the genetically determined apparatus of the host is responsive. Antibodies against a person's own blood group were not formed. Interpretation of these results permits some careful generalizations as to the origin of so-called natural antibodies.

INTRODUCTION

Two contradictory views on the origin in humans of demonstrable blood group A and B isoantibodies have been advanced. According to the older genetic hypothesis these antibodies are inherited by pairs of linked genes, each representing a blood group antigen and its complementary antibody. The immunogenetic hypothesis, on the other hand, contends that while the ability to manufacture antibodies is inherited, isoantibodies become demonstrable only if the antibody-producing machinery of the host is exposed to exogenous cross-reactive or homologous antigens (*cf.* 1-3). More recent experimental evidence, namely parenteral immunization in man (4, 5) and feeding of fowl with blood group-active substances, including those of microbial origin (6), strongly supports the immunogenetic hypothesis of isoantibody origin. Further circumstantial evidence for the immunogenetic origin of isoagglutinins has been adduced by demonstration of the wide distribution of substances with human blood group A, B, and H(O) specificity among enteric bacteria (7). However, direct proof has so far been lacking of blood group isoagglutinin stimulation in man by microbes or its formation as a result of stimulation via truly physiological routes, namely, the gastrointestinal or respiratory tracts.

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The present paper reports the stimulation (frequently *de novo*) of saline agglutinating and blocking¹ isoantibodies in infants and the increase of isoantibodies in adults by feeding blood group B-specific bacteria. Increased production of isoantibodies occasionally resulted from inhalation of blood group-active bacteria or hog gastric mucin. Some biological properties of the induced antibodies are described. In vivo neutralization of anti-human blood group antibodies of germfree and ordinary chickens has also been achieved.

METHODS

Subjects. Healthy humans of both sexes and all age groups, infants with mild to severe diarrhea, and adults with ulcerative colitis and cancer of the colon were investigated. The majority of the infants were awaiting placement in foster homes and the blood groups of their mothers were not available. None of the individuals had received a recent blood transfusion or vaccination. Germfree and ordinary male and female White Leghorn chickens raised and maintained under conditions closely similar to those previously reported were used (6).

Blood group-specific antigens. Human blood group B-specific smooth *E. coli* O₈₆ B:7 was the most frequent source of antigen. Immunization of chickens and rabbits with *E. coli* O₈₆ indicated that these bacteria also possess blood group A specificity (6, 8) and we have found that the in vitro blood group A activity of smooth *E. coli* O₈₆, as measured with human anti-blood group A sera and A₁ or A₂ erythrocytes, amounts to 0.2–2.5% of its blood group B activity.² The bacteria were grown as previously described on fully defined media devoid of blood group-active substances (9). Cultures in the logarithmic phase of growth were used in the feeding experiments with live bacteria. Killed *E. coli* O₈₆ for feeding or inhalation were obtained from 24- to 48-liter cultures grown for approximately 48 hr at 37°C. The bacteria were harvested by centrifugation at 30,000 *g*, washed with 0.85% aqueous NaCl, dried *in vacuo* over KOH in the presence of toluene vapor, and subsequently ground in a mortar to produce a powder. No bacteria could be cultured from this powder on blood agar plates. For injection, blood group-active lipopolysaccharide (L.P.S.) was extracted from *E. coli* O₈₆ by established procedures and purified as described previously (10). 10 µg/ml of the powder and 1.2 µg/ml of the lipopolysaccharide completely inhibited four hemagglutinating doses of human anti-blood group B serum under standard conditions (7). Commercial hog gastric mucin (Wilson & Co., No. 106 465) which possessed high human A and H(O) blood group activity (0.3 µg/ml inhibited four doses of anti-A serum) was employed in inhalation experiments.

Immunization. (a) *Man.* Immunization was attempted by feeding and inhalation. In the feeding experiments both healthy and sick individuals ranging in age from less than 1 wk to 70 yr were given killed *E. coli* O₈₆. All subjects were of blood group A or O, except two, one of whom had blood group B and the other A₂B. For infants the feedings were scheduled to be 500 mg *E. coli* O₈₆ three times daily

for 7 days and for adults 1–3 g given within 24 hr in 1 g lots. This plan could not be strictly adhered to because some individuals regurgitated the bacterial preparations whereupon the feeding was interrupted for several days. Some adult volunteers received a single dose of 10⁷–10⁸ live, smooth *E. coli* O₈₆ in 1 oz of milk.

Immunization by nasal inhalation was attempted in adults 19–61 yr old with aerosol sprays from plastic 10-ml squeeze bottles. The spray contained either killed *E. coli* O₈₆ bacteria in 0.1% final concentration or hog mucin in 1.0% final concentration in buffered saline (see below) containing 0.05% Zephiran and 1% peppermint spirits. Each bottle delivered about 0.07 ml per squeeze. This volume was applied to each nostril three times daily for 7 days; this schedule amounted to ca. 3 mg of *E. coli* O₈₆ and ca. 30 mg of hog mucin. 12 males inhaled the *E. coli* O₈₆ spray; eight of these were of blood group A and four of blood group O. 12 different males inhaled hog mucin; three of them were of blood group B, the remainder of blood group O. At least six inhalations per individual were supervised. After the assigned period all subjects returned the spray bottles and the amount of material used was determined from the residue by difference. In the case of hog mucin 99.65% of the amount planned was inhaled, with a range from 54 to 143%; for the *E. coli* spray the average was 109% with a range from 68 to 153%.

(b) *Chickens.* The procedure of bacterial "monocontamination" of germfree chicks has been previously described (6). In these peroral immunizations, which were also employed in the ordinary chickens, the birds were given once live *E. coli* O₈₆ in their drinking water. 1–2 months after the feeding of bacteria the chickens were given one intravenous injection of 3 mg/kg of body weight of *E. coli* O₈₆ L.P.S. dissolved and autoclaved in buffered saline.

Microbiological testing procedures. These have been described earlier for germfree chickens (6). In man, stool specimens of those given killed *E. coli* were collected with sterile swabs immediately on defecation within a few hours before the experiments and during the experiments as indicated. Stool specimens from those fed live *E. coli* were taken 3 days before bacterial feeding, a number of hours before this feeding, and one each 24 hr, 5 days, and 12 days after ingestion of the *E. coli*. Bacteria were cultured and Gram-negative organisms isolated by standard procedures of clinical bacteriology. 6–11 coli-like cultures per stool specimen were picked at random from blood agar and MacConkey plates and each of these were subcultured as described above under "Blood group-specific antigens." The bacteria were then harvested by centrifugation, boiled for 2½ hr in distilled water and their final dry weight adjusted to 0.5–1% and tested for blood group activity (*cf.* 7).

Blood and saliva samples. Blood was always collected immediately before the first and after the last immunization as indicated in the "Result" section. Venous blood was obtained from the arm and rarely from the heel in humans; the chickens were bled from the main wing vein. Human blood, 2–20 ml per session, and up to 10 ml of chicken blood were collected into sterile tubes (*cf.* 10). Red cells were separated by centrifugation and the sera frozen in 2-ml lots at –20°C. The scarcity of the sera prevented their physicochemical investigation.

Saliva was collected with cotton swabs and extracted with buffered saline, the eluates were boiled for 10 min and centrifuged. Those supernatants which inhibited hemagglutination (see below) were considered to originate from "secretors."

Erythrocytes. Blood group A₁, A₂, B, and O erythrocytes

¹ As blocking antibodies are defined those which did not agglutinate red cells suspended in saline, but which were demonstrable by the anti-human serum test.

² Springer, G. F., and H. Tegtmeier. Unpublished observations.

from adults, stored for less than 14 days in one-third volume anticoagulant acid citrate dextrose solution (2.45% glucose, 2.20% trisodium citrate, 0.8% citric acid) at 1–3°C were used immediately after three washings with 15–20 volumes of buffered saline. For determination of antibodies directed against *E. coli* O₈₆ human blood group O erythrocytes were sensitized as described earlier (10); the minimal amount of *E. coli* O₈₆ lipopolysaccharide which afforded maximal agglutination was used for sensitization.

Control antisera, absorptions, and elutions. Commercial human hyperimmune anti-A and anti-B sera were used as controls. Absorption of some human sera was done where indicated in the "Results" section sometimes over 1 year after the other experiments. Where possible undiluted sera were used and absorbed with one volume washed, packed erythrocytes as outlined previously (6). All chicken antisera were absorbed with human blood group O erythrocytes before use as in previous experiments (6). Eluates were obtained

by a modified Landsteiner-Miller procedure as described earlier (6).

Anti-human serum (Lot No. 7352-2) produced in rabbits and used to detect those blood group antibodies which do not agglutinate in saline was purchased from Ortho Pharmaceutical Laboratories and rabbit anti-chicken γ -globulin serum (Lot No. R.P. 12-65) from Hyland Laboratories.

Solutions. The diluent for all reagents and the erythrocyte-suspending solution in all tests was 0.125 M aqueous NaCl containing 0.025 M phosphate buffer, pH 7.3 (buffered saline).

Hemagglutination. All tests were done three or more times. Arithmetic averages were reported. Only titer differences which were fourfold or greater were considered to be significant. Titrations of pre- and all postimmunization sera of a given individual were always done in parallel. The method of titration and interpretation of agglutination using saline agglutinating antibodies has been described previously

TABLE I
Blood Group B Isoantibody Stimulation in Infants by Feeding Killed Blood Group B-Specific E. coli O₈₆*

Subject	Blood group	Age at beginning of experiment (wk) and sex	Reciprocal agglutinin titer with human B erythrocytes		
			Immediately before ingestion	After last ingestion	
				7–28 days	1–2 months
Diarrhea					
A. J. [†]	O+ss§	2, f	<1 (<2)¶	<1 (<2)	
T. T. ^a	O+S	2, f	2 (\pm 4)	1 (8)	
J. N. ^b	A ₁ +ss	2, m	<1	16	
R. R. ^d	O+ss	2, m	1	4	8
G. A. ^a	A ₁ +S	3, f	4 (8)	32 (64)	8 (16)
J. B. ^d	O	6, m	1	8	
R. S. ^{a,b}	O+S	6, m	2	4**	
C. J. ^b	A ₁ +ss	9, m	1 (\pm 1)	64 (128)	16 (64)
J. W. ^c	A ₁ neg ss	10, m	4	128	64
O. B. ^a	O+S	13, m	2	32	32
D. T. ^b	A ₁ +S	13, m	<1 (<2)	2 (<4)	
V. R. ^b	A ₁ +	14, m	<1 (4)	64 (256)	
W. C. ^c	O+S	15, m	<1 (<2)	64 (128)	
D. L. ^d	O+	17, f	8 (16)	64 (128)	
G. M. ^c	A ₁ +S	17, m	<1 (<2)	16 (32)	32 (128)
E. D. ^c	O neg	35, m	32 (64)	512 (2048)	
Healthy					
C. O. ^d	O+ss	<1, m	4	8	8
B. B. ^c	O neg	1, f	4 (>128)	4 (>128)	4
L. E. ^d	O+ss	2, f	2	4	2
T. P. ^b	O+S	7, m	<1 (<2)	16	4 (16)
R. B. ^d	A ₁ +	9, m	1 (2)		4–8 (32)
A. N. ^c	O+	11, m	1 (8)	2 (8)	
B. T. ^b	O+S	11, f	1 (32)	4 (16–32)	

* For controls not fed *E. coli* O₈₆ see text.

† a = Blood group B-active bacteria cultured from stool obtained immediately before beginning of bacteria feeding. b = Bacteria of low blood group A activity cultured at beginning of experiment. c = No blood group A- or B-active bacteria cultured from stool at beginning of experiment. d = Stool not investigated.

§ Symbols: + = Rh₀(D) positive; neg = Rh₀(D) negative; S = secretor; ss = nonsecretor.

|| f = female; m = male.

¶ Values in parentheses represent results of anti-human serum test.

** Sample collected 12 hr after termination of feeding.

(6). The procedure was carried out at 23°–25°C. A different pipette was used for each tube in a titration series. A 0.5% suspension of erythrocytes was used in all tests except indirect antiglobulin reactions. Agglutination was read with the microscope after 1½ hr of incubation. The last tube showing agglutination was taken as the end point. The tests were read independently by two individuals. One was frequently an observer to whom the nature of the samples was unknown. Each titration included as control a saline suspension of the erythrocytes under study. All activities are given as reciprocal titers in terms of dilution of serum used before addition of red cells (called "titers" throughout); an increase from <1:1 to 1:1 was considered to be twofold.

The anti-human serum test was carried out with the following variations from the manufacturer's instructions: serial dilutions with 0.1-ml volumes were made throughout. Incubation of red cells with serum was for 1 hr at 23°–25°C. The erythrocyte suspension had a concentration of 1% and one drop of anti-human serum or anti-chicken globulin serum was added after thorough washing. Interpretation of agglutination was by microscope.

Hemagglutination inhibition assays were performed on the sera of individuals fed *E. coli* O₈₆ at the height of their immune response with human blood group A and B glycoproteins isolated from human ovarian cysts and meconium (9, 11) and with *E. coli* O₈₆ L.P.S. (10). These tests were also

performed with commercial antisera and with human saliva. The procedure was similar to that described previously (12) but scaled down to 0.02 ml of all reagents. The first tube showing no inhibition was taken as the end point. Each titration series included controls consisting of a serum standard diluted to four to eight minimum hemagglutinating doses, and then titrated in twofold geometrical dilutions, as well as an erythrocyte suspension in saline.

RESULTS

Stimulation of anti-blood group B isoantibodies in humans by feeding of E. coli O₈₆. It was found that humans of all ages and both sexes may form isoantibodies as response to ingesting or inhaling blood group-active *E. coli* O₈₆. This response occurred more frequently in persons of blood group A than those of blood group O (see "Discussion").

Some of the most striking results of feeding experiments in infants are shown in Table I. More than three-quarters of the diarrheic infants, 13 of 16, responded with a significant increase (fourfold or greater) of anti-B isoagglutinins. 1–4 wk after the last *E. coli* O₈₆ ingestion 11 of these 13 showed an 8- to greater than

TABLE II
Blood Group B Isoantibody Stimulation in Adults by Feeding E. coli O₈₆*

Subject and diagnosis	Blood group	Age (yr) and sex	Reciprocal agglutinin titer with human B erythrocytes		
			Immediately before ingestion	After last ingestion	
				2-7 days	7-28 days
III					
Ulcerative colitis					
E. O. ^{a†}	O+§	35, m§	32 (256)§		128 (4096)
M. L. ^b	A ₂	52, f	64 (128)	512 (4096)	128 (1024)
Carcinoma					
J. B., ^b liver, metast.	A ₂	66, f	2 (16)		16 (512)
W. H., ^b rectum	O	60, m	8 (512)	16 (512)	16 (512)
Healthy					
C. L. ^b	A ₂ +	19, f	64 (64)	256 (1024)	256 (4096)
H. T. ^b	A ₁ +	19, m	64 (64)	128 (128)	256 (512)
B. R. ^{a¶}	A ₁ neg	22, f	256 (1024)	512 (1024)	512 (1024)
J. S. ^{a¶}	O neg	22, m	64 (256)	64 (256)	64 (256)
H. Te. ^b	O+	25, f	64 (64)	64 (128)	32 (128)
I. S. ^{a¶}	O+	25, f	32 (128)	32 (64)	32 (64)
W. P. ^b	O+	28, m	8 (64)	64 (256)	64 (256)
G. S. ^a	O+	37, m	32 (128)	64 (128)	128 (2048)
Y. N. ^{a¶}	A ₁ +	38, m	8 (8)	16 (64)	32 (128)
D. A. ^{b**}	O	69, m	16 (128)		16 (256)

* Killed bacteria, unless indicated otherwise.

† a = No blood group B-active bacteria cultured from stool at beginning of experiment. b = Stool not investigated.

§ See footnotes Table I.

|| Ambulatory patient, no proven intestinal lesions.

¶ Fed live *E. coli* O₈₆ once at beginning of experiment.

** Fully recovered from operation for cancer of colon.

64-fold increase of saline agglutinins. All sick children 9 wk and older had a significant titer increase. Blocking antibodies were measured in 10 of these infants and were found to generally parallel the antibody response determined with saline agglutinins. There was *de novo* isoagglutinin stimulation from <1 to 16 or 64 in six of the seven diarrheic infants. The single infant who possessed no anti-blood group B antibodies at the end of the experiment was A. J., aged 2 wk at the outset. In six of these children agglutinins were also determined more than 1 month after termination of bacteria feeding. In only one instance was there any further significant increase and only of blocking anti-B isoantibodies but in two cases the isoantibodies had decreased significantly. The anti-B isoagglutinin titers of six of these infants were followed up to 13 months. The titer either remained unchanged or decreased as much as 16-fold.

Table I shows, in addition, that healthy infants also may produce blood group anti-B isoantibodies upon being fed *E. coli* O₈₆. They had a somewhat lesser response; four of seven infants had a significant increase of saline agglutinins. Blocking antibodies were determined in five of these healthy infants; R. B. and T. P. showed a 16-fold titer increase.

The average titer increase of those 13 diarrheic infants who responded to *E. coli* O₈₆ with significant anti-blood group B antibody production was 42-fold for saline agglutinins and 55-fold for blocking anti-B antibodies (determined in only eight of the responding children). Comparative figures for the healthy infants fed *E. coli* O₈₆ were a 14-fold increase of saline agglutinins and a 16-fold rise of blocking antibodies.

14 adults were also fed *E. coli* O₈₆. The most pertinent data are depicted in Table II. Both patients with ulcerative colitis (E. O.; M. L.), who suffered from an acute episode of their disorder at the beginning of the experiment, showed a prompt four- and eightfold increase of saline agglutinating anti-blood group B antibodies and 16- and 32-fold increase of blocking antibodies subsequent to the feeding of killed *E. coli* O₈₆. Similarly one of the two carcinomatous patients (J. B.) responded with an eightfold rise of saline agglutinins and a 32-fold increase in blocking anti-B isoantibodies. The fourth patient (W. H.) with carcinoma of the rectum showed no significant rise of either kind of anti-B isoantibodies. The two ulcerative colitis patients (E. O.; M. L.) were observed for an additional 6 months during which the blocking antibodies decreased 8- and 16-fold, and the saline agglutinins fourfold.

6 of the 10 healthy adults listed in Table II were fed killed *E. coli* O₈₆ while four received live *E. coli* O₈₆. One of the latter (Y. N.) responded with a fourfold increase in anti-B saline agglutinins and a 16-fold rise in blocking anti-B antibodies; the three other volunteers

TABLE III
Individuals Responding with Significant Increases of Anti-Blood Group A Antibodies to Feeding of E. coli O₈₆*

Subject†	Age	Erythrocytes tested against	Reciprocal agglutinin titer	
			Immediately before ingestion	7-28 days after last ingestion
W. C.	15 wk	A ₁	1	16
D. L.	17 wk	A ₁	8 (16)§	128 (256)
		A ₂	4 (<8)	32 (128)
D. W.	35 wk	A ₁	2 (4)	8 (8)
		A ₂	2 (2)	8 (8)
E. D.	35 wk	A ₁	4	128
		A ₂	2	128
D. A.	69 yr	A ₁	32 (256)	32 (512)
		A ₂	8 (32-64)	8-16 (256)

* For total population investigated see text.

† D. W. is blood group B, all others are blood group O. For Rh type, secretor status, and bacteria in stool cultures of these subjects see Tables I and II and text.

§ Values in parentheses represent results of anti-human serum test.

did not produce an elevated titer. Blood group B-active bacteria were not found in the preingestion stool specimens of any of these individuals. Subsequent to their ingestion blood group B-specific bacteria were demonstrable in large amounts in specimens collected from Y. N. between the 1st and 12th day and in the stools of 2 additional volunteers. From the remaining volunteer only three weakly B-active cultures were isolated. Four of the healthy adults (C. L.; H. T.; W. P.; and G. S.) who were fed killed *E. coli* O₈₆ showed a four- to eightfold increase of saline agglutinins and a 4- to 64-fold rise in blocking antibodies.

The average titer increase of those ill adults who had a significant response was 5 times for saline agglutinins and 27 times for blocking antibodies, while the corresponding figures for the healthy adults were 5 and 22.

Stimulation of anti-blood group A antibodies in humans by feeding of E. coli O₈₆. The anti-blood group A response of humans to feeding *E. coli* O₈₆ was studied in spite of the low in vitro blood group A activity of this bacterium, since it had been found previously that *E. coli* O₈₆ is able to stimulate anti-A antibodies in animals, some of which cannot be absorbed with blood group B erythrocytes (6, 8). 11 of 22 individuals of blood group O responded with a reproducible increase in anti-A antibodies; however, it was fourfold or greater in only those sera whose titers are depicted in Table III. Comparison of Table III with Table I shows that all three diarrheic infants of blood group O, 15 wk and older, produced a 16- to 64-fold increase of saline agglutinating and blocking anti-A isoantibodies. One infant of blood group B (D. W., 35 wk old, diarrhea), who was fed 2 g of killed *E. coli* O₈₆ had a fourfold increase in

saline agglutinating antibodies against blood group A₁ and A₂ erythrocytes.

One (D. A.) of the eight adults of blood group O listed in Table II had a significant rise in anti-A blocking antibodies (see Table III), and another three, W. H., J. S., and I. S., showed a reproducible two- to fourfold increase in anti-A antibodies.

Stimulation of blood group isoantibodies by inhaling E. coli O₅₆ or hog gastric mucin. 12 adults received nasal sprays of *E. coli* O₅₆ and four had a significant increase in anti-blood group B antibodies. One of these (J. H., A₁, Rh positive) had a four- and eightfold increase in anti-B agglutinins and blocking antibodies respectively in the blood specimens taken 5 and 17 days after termination of the inhalation. The three other responding volunteers, one of blood group A₁, Rh positive (D. O.) and two of blood group O, W. S. (Rh positive) and K. B. (Rh negative) showed a fourfold increase of only blocking anti-blood group B antibodies. These two volunteers of blood group O also had a four- to eightfold increase of blocking anti-A antibodies. Ral. R. (blood group O, Rh positive), who had responded with a twofold anti-B antibody rise, had an eightfold increase of blocking antibodies against A₂ red cells.

Among the 12 males who had inhaled blood group AH(O)-active hog gastric mucin, J. M. (blood group O, Rh negative) showed an eightfold increase of blocking anti-A antibodies and a twofold increase in saline anti-A agglutinins; the other, O. P. (blood group B, Rh positive), had an eightfold increase in blocking

anti-A antibodies when measured with blood group A₂ erythrocytes only. None of the persons who had inhaled hog mucin had a significant increase in blood group B antibodies.

Failure of antibody production against an individual's own blood groups. Four diarrheic infants of blood group A (J. N.; J. W.; D. T.; and G. M., Table I), who had ingested *E. coli* O₅₆ were tested for the formation of anti-A antibodies as were one adult ulcerative colitis patient (M. L.) and one healthy adult (C. L.), also of blood group A. None produced anti-A antibodies although all but D. T. had a significant anti-B response. Similarly, the infant of blood group B (D. W., see Table III) who was fed *E. coli* O₅₆ did not produce anti-B antibodies but showed a rise in anti-A titer only. A sick 70 yr old (G. C., with nontropical sprue, not listed in Table II), blood group A₂B, was fed 2 g of killed *E. coli* O₅₆; he did not produce any kind of anti-B or anti-A antibodies during the 10 day observation following the feeding.

Specific in vitro hemagglutination inhibition. It was attempted to inhibit *E. coli* O₅₆-stimulated anti-A and anti-B blood group isoagglutinins. Table IV shows the results obtained with sera of five individuals of blood group O and three of blood group A. E. O., M. L., and C. L. were from adults, the remainder from children (see Tables I and II). Small amounts of homologous human blood group substances completely neutralized the agglutinating ability of these sera; similarly small quantities of blood group B-specific *E. coli* O₅₆ L.P.S. neutralized

TABLE IV
Specific Inhibition of E. coli O₅₆-Stimulated Human Anti-A and Anti-B Isoagglutinins

Sera obtained from		A ₁ erythrocytes		B erythrocytes		
		Specificity and source of human blood group substance A		Specificity and source of human blood group substance B		<i>E. coli</i> O ₅₆ -L.P.S.
Subject	Blood group	Ovarian cyst	Meconium	Ovarian cyst	Meconium	
W. C.	O	5*	<0.6	0.6	6	20
E. O.	O	1.2-2.5	20	5	1.2	5
D. L.	O	<0.6	2.5	n.d.†	n.d.	n.d.
E. D.	O	1.2	5	n.d.	n.d.	n.d.
O. B.	O	n.d.	n.d.	20	n.d.	20
G. A.	A ₁			n.d.	0.6	160
M. L.	A ₂			10	10	10
C. L.	A ₂			10	10	2.5
Commercial hyperimmune sera§						
Anti-A, No. 8229-3		0.6	1.2			
Anti-B No. 9219-1				20	5	20

* Micrograms per milliliter completely inhibiting four hemagglutinating doses.

† n.d., Not done due to lack of serum.

§ Ortho Pharmaceutical Corporation.

anti-B isoagglutinins; these findings are emphasized by comparing them with the results obtained with commercial anti-A and anti-B sera which were included as controls. Large amounts of the human blood group A substances were without effect in any of the B-anti-B systems as were large quantities of human blood group B substances in the A-anti-A systems.

Absorption of isoantibodies with blood group A₁ and B erythrocytes. The pre- and postimmunization sera of six persons of blood group O who had an isoantibody response to feeding with *E. coli* O₈₆ (see Tables I and II) and those of the single child of blood group B (see above) were absorbed with blood group A₁ and with B erythrocytes. The results are depicted in Table V. One absorption with "homologous" erythrocytes uniformly removed the bacteria-induced isoantibodies by 87 to > 99% from postimmunization sera. The finding that a similar percentage of isoantibodies was removed

from the preimmunization sera by homologous red cells is obvious. On the other hand cross-absorption reduced the isoantibody content of the sera of only two persons and by not more than 50–75%. E. O. and G. S. showed some reduction in postimmunization anti-A after absorption with B erythrocytes and of anti-B antibodies after absorption with A₁ red cells; however, a similar reduction was observed after like absorption of the pre-immunization sera.

Stimulation of anti-E. coli O₈₆ antibodies in humans. Sufficient pre- and postimmunization sera from all 14 adults and of 9 infants who had ingested *E. coli* O₈₆ were available to compare the antibodies reacting with *E. coli* O₈₆ with the isoantibodies. Among these 23 individuals 12 had a 4- to 128-fold increase of antibodies reacting with *E. coli* O₈₆ L.P.S.-coated human blood group O erythrocytes; five of these were infants. The average rise in anti-*E. coli* antibodies of those who had

TABLE V
Effect of Absorbing with Blood Group A₁ and B Erythrocytes on Isoagglutinins Induced by Peroral Immunization with E. coli O₈₆

Subject*	Blood group	Serum absorbed with†	Reciprocal agglutinin titer, erythrocytes			
			Pre-immunization		Postimmunization‡	
			A ₁	B	A ₁	B
W. C.	O	Nothing	1	<1	16–32	32
		A ₁ cells			<2	16–32
		B cells	n.a.§		32	<2
D. L.	O	Nothing	8 (<16)	4 (8)	128 (<256)	32 (64)
		A ₁ cells	n.a.	n.a.	2 (<2)	32 (64)
		B cells	n.a.	n.a.	128 (<256)	2 (<4)
E. D.	O	Nothing	4	4	16	8–16
		A ₁ cells	n.a.	n.a.	<2	8
		B cells	n.a.	n.a.	16	<1
D. W.	B	Nothing	4 (8–16)	<1	16–32 (32)	<1
		A ₁ cells	n.a.		<1	
		B cells	2–4 (<8)		16 (<32)	
E. O.	O	Nothing	16 (32)	16 (64)	16 (64)	64–128 (2048)
		A ₁ cells	<2 (<2)	16 (32)	2 (4)	32 (2048)
		B cells	16 (16–32)	<2 (<2)	16 (16–32)	<2 (<2)
W. P.	O	Nothing	8–16 (32)	8 (32)	16 (32–64)	32 (128)
		A ₁ cells	<1 (2)	8 (32)	<1 (2)	16–32 (128)
		B cells	8 (16–32)	<1 (2)	16 (32–46)	<1 (<1)
G. S.	O	Nothing	32 (64)	32 (32)	32 (64)	64 (256)
		A ₁ cells	<1 (2)	8 (<8)	<1 (2)	32 (64)
		B cells	32 (32)	<1 (2)	32 (64)	<1 (2–4)

* First four are infants, last three adults; for details see Tables I and II and text.

† Performed on sera with maximal isoagglutinin rise within 1 month after immunization, except that sample from E. D. was obtained during rise.

§ n.a., no serum available.

|| Values in parentheses represent results of anti-human serum test.

a significant response was 28-fold for saline agglutinating and 18-fold for blocking antibodies. In addition, a reproducible twofold increase of anti-*E. coli* antibodies was observed in three infants (including D. W., the infant of blood group B) and three adults. Among the remaining five persons four did not respond with either anti-*E. coli* antibodies or isoantibodies. One infant (G. A., 3 wk old) had a significant isoantibody increase only, however, it had a very high *E. coli* titer (64) at the outset of the experiment, apparently transmitted from the mother.

2 of the 12 volunteers who had inhaled *E. coli* O₈₆ showed a significant increase of antibodies reacting with *E. coli* O₈₆ L.P.S.-coated blood group O erythrocytes. J. H. (blood group A₁, Rh positive) had a 16-fold increase of saline agglutinins and a fourfold rise of blocking antibodies 5 days after the experiment; he also had the most powerful rise of anti-blood group B antibodies among the 12 volunteers. J. B. (blood group A₁, Rh positive), showed a fourfold rise of only saline agglutinins against *E. coli* O₈₆ and no significant rise in anti-B antibodies; conversely, three of these volunteers (W. S. and K. B., both blood group O, and D. O., blood group A₁) did not show a significant increase of antibodies reacting with *E. coli* O₈₆ but they had a fourfold rise of blocking anti-B antibodies. Also, volunteer Ral. R., responded with an increase in anti-A and anti-B antibodies, but showed no rise of antibodies reacting with *E. coli* O₈₆. None of the adults who had inhaled hog mucin produced a significantly increased amount of antibodies reacting with *E. coli* O₈₆.

Preexisting blood group-active bacteria in the intestinal flora. Stool specimens of 13 of the 16 diarrheic infants (Table I) were obtained at the beginning of the experiment; blood group B-active bacteria were found in five of these but significant blood group B isoagglutinin stimulation was noted in only two (G. A.; O. B.). In addition, in the stools of four sick infants in whom no blood group B-active bacteria were found, bacteria with faint in vitro blood group A activity were detected. These infants, J. N.; C. J.; D. T.; and V. R., were all blood group A₁, Rh positive. Preingestion stools of four healthy *E. coli* O₈₆-fed infants (Table I) were also investigated. Bacteria with weak blood group A activity only were found in two of them (T. P. and B. T.). No significant change in anti-A titers of any of these infants was observed. The stools of six adults (Table II) were investigated at the outset for the presence of blood group B-active bacteria only and none were demonstrated.

Observation of isoantibodies and antibodies reacting with E. coli O₈₆ in infants not fed E. coli O₈₆. Seven infants 5-7 wk old (average 12 wk) were not fed *E. coli* O₈₆ and served as controls. Their sera were observed for a period of about 2 to about 9 wk (average 6 wk).

Four of the infants, all blood group O, Rh positive, had diarrhea at the beginning of the observation period; three of these (G. U.; B. E.; and J. S.) had no change in either isoantibody titer or titer of antibodies directed against *E. coli* O₈₆, while one, P. A., showed a four- and eightfold increase of agglutinating and blocking anti-A antibodies respectively, a 16-fold rise of blocking anti-B antibodies, and a 2-fold rise of antibodies directed against *E. coli* O₈₆. This latter infant was the only one of the four with diarrhea in whose feces weakly blood group-active bacteria were detected, namely an A-active *Paracolon* and a B-active *E. coli*, in a stool specimen collected during the observation period and 15 days before the serum specimen which showed the antibody increase. Among the three nondiarrheic control infants one, N. D. (O, Rh positive, with B-active *Proteus* in the stool at beginning of observation period), had suffered from erythroblastosis fetalis; another, S. R. (A₁, Rh positive), was healthy; and the last individual, D. P. (O, Rh positive), had an acute upper respiratory infection. Only this latter infant showed an increase in isoantibodies, which was confined to a fourfold rise of anti-A as well as anti-*E. coli* O₈₆ saline agglutinins.

In vivo neutralization of anti-human blood group A and B antibodies in chickens. Anti-human blood group agglutinins can be stimulated in chickens and rabbits with blood group-specific microbes (6, 13). We have now attempted to neutralize in vivo the blood group antibodies of three germfree and two ordinary chickens, 3-4 months old, that had been fed live *E. coli* O₈₆ approximately 2 months after hatching and who had formed optimal anti-blood group B titers. Just before injection the germfree chicken, Nos. 7391, 7392, and 7393, had anti-B agglutinin titers of 512-2048, anti-A titers of 64-256, and blocking antibody titers of 256-512. The agglutinin titers of the two ordinary chickens fed *E. coli* O₈₆ (Nos. 7333 and 7387) were 128-256 for blood group B and 16 for blood group A erythrocytes while the corresponding blocking antibody titers were 32. Also included were two ordinary 6 wk old chickens which had not been fed *E. coli* O₈₆; one, No. 7495, had an anti-B titer of 128, the other, No. 7484, of 32; the anti-A titers of both were < 4. Intravenous injection of *E. coli* O₈₆ L.P.S. reduced the anti-human blood group B agglutinins in two germfree chickens (Nos. 7391 and 7392) within 5 min by about 90% and by > 99% within 1 hr. The anti-A agglutinins were reduced by > 99% within 5 min after injection. Reduction of blocking antibodies ranged from 75% to > 99% after 1 hr. The findings with one ordinary chicken (No. 7387) that was fed *E. coli* O₈₆ paralleled those obtained with the germfree birds. In one of the ordinary birds which had not been given *E. coli* O₈₆ (No. 7495) all anti-B antibodies had disappeared 3 min after injection. After 7 and 15

days the germfree and ordinary chickens had titers equal or twice those at the beginning of the experiment. One germfree (No. 7393) and one ordinary (No. 7333) chicken which had been fed *E. coli* O₈₆ died within 1 hr after injection of L.P.S. as did one (No. 7484) which had not been fed *E. coli* O₈₆. The chickens who died showed less neutralization of blood group antibodies. Neutralization of anti-B agglutinins was from 75 to 92% 20–60 min after injection and that of anti-A agglutinins was 0 and 50% in the two birds who possessed these antibodies at the beginning of the experiment.

DISCUSSION

Human isoantibodies A and B have long been considered to be a prototype of inherited, natural antibodies which arise independent of influences from the environment (14–17), i.e., they are demonstrable without prior immunogenic stimulation. It is not only of theoretical but also of immense practical importance if antibodies indeed could be produced without exogenous stimuli. Based on his theory of the genetic origin of antibodies Hirsfeld hoped “. . . to decipher the epidemiological tale of woe of mankind by the presence of some normal antibodies . . .” as well as for “. . . the possibility of breeding immune animal races with powerful production of normal antibodies . . .” (16, 17). However, while there is agreement that the ability to manufacture antibodies and the way of a particular immune response are inherited, specific antibodies are not generally known to be present without an antigenic stimulus.

Over the years, strong circumstantial evidence has accumulated implicating the environment as the cause of human isoantibodies A and B. This evidence is based first, on the wide distribution of blood group ABH(O)-specific antigens throughout the animal (1, 2, 11) and plant kingdoms (9, 7) and their resulting ubiquitous occurrence in diet (6) as well as dust (18), and secondly, on the failure to differentiate the so-called natural and immune isoantibodies by their biological or chemical properties (1, 4–6). Thirdly, some healthy monozygotic human twins and triplets showed titer differences of > 75% for anti-A, and of > 90% for anti-B isoagglutinins (19, 20). Consequently, a number of investigators believed isoantibodies, like other antibodies, to be the result of exogenous immunogenic stimulation provided largely by cross-reacting antigens (e.g. 21, 1, 2). Investigations into the origin of anti-microbial antibodies in animals also failed to support the view that natural antibodies occur (cf. 22).

The finding of a large number of Gram-negative blood group-active bacteria, including many different genera of *Enterobacteriaceae* (cf. 9, 7, 23), made it realistic to consider their role as important causative agents of isoantibody stimulation. Also, our previous studies on

ordinary and germfree chickens had established that their regularly occurring anti-human blood group B agglutinins are the result of exogenous immunogenic stimuli and that they are not inherited as had been believed (24, 25).

Based on these findings Körner, Maassen, and Pettenkofer investigated possible correlations between intestinal flora and blood group isoagglutinin titers in infants of blood groups A and O (26). In general they found a higher isoagglutinin titer in those infants who had an intestinal flora in which *E. coli* strains predominated, as compared to those whose flora consisted mainly of *Lactobacillus bifidus*. Blood group ABH(O)-active substances have not been found in *L. bifidus* (9, 26) but are common in *E. coli* strains (7–9, 23).

The work reported in this paper was designed to furnish direct evidence either for or against the role of antigenic agents in the origination of blood group antibodies. Antigens were chosen like those to which humans are exposed throughout life and they were applied via the same routes by which they would enter the host under physiological conditions, namely the gastrointestinal tract and the respiratory system. The results clearly show that both saline agglutinins and blocking A and B isoantibodies can be stimulated by blood group-active *E. coli* and in infants, including those less than 1 month old, they may be even evoked *de novo* (Tables I and II). The anti-B response to feeding killed *E. coli* O₈₆ was extraordinary in infants suffering from diarrhea, where over 80% of the patients responded with an average isoantibody increase of over 40 times. Among the healthy infants a smaller percentage responded significantly and with an average increase of both kinds of anti-B antibodies amounting to only about one-third of that of the diarrheic infants.

The foregoing indicates that periods of intestinal disorders with a concomitant increase of antigen absorption may be especially favorable times of isoantibody stimulation in infants. It is likely that damage to any body surface furthers absorption of antigens from this damaged area (cf. reference 10). This view is favored by our observation that of the seven control infants not fed *E. coli* the only two showing an isoantibody response both had damaged body surfaces, one (D. P.) an acute upper respiratory tract infection and the other (P. A.) diarrhea; blood group-active bacteria were found in the feces of the latter infant.

Intestinal damage had a more pronounced effect on the isoantibodies of infants than those of adults. However, the initial titer of the two acutely sick adults (E. O. and M. L.) was as high as the highest ever reached by the majority of the infants. Maximal rise in blood group antibodies after the feeding of *E. coli* O₈₆ occurred in less than 8 days in adults and in less than

3 weeks in infants. Feeding *E. coli* O₈₆ to adults appeared to effect mainly a booster reaction, as is also made likely by the proportionally much larger increase of blocking antibodies. The ratio of increase of blocking to saline agglutinating anti-B antibodies for sick and healthy infants was 1.3:1 and 1.1:1 but 5.4:1 and 4.4:1 for the sick and healthy adults. In most instances there was a response of either both kinds of antibody or neither; exceptions were two infants, A. N. and B. T. (Table I) who showed a saline agglutinin increase only. By the technique employed, an increase in blocking antibodies was demonstrable only if their postimmunization titer was higher than that of the saline agglutinins.

The isoantibody increase was closely similar among the adults of both sexes. A comparison of the response to *E. coli* O₈₆ between male and female infants could not be made since of the seven female infants listed in Table I only two were over 3 wk old at the beginning of the experiment while 13 males were more than 3 wk old.

In spite of the faint in vitro blood group A activity of the *E. coli* that was administered the extent of the blood group A antibody rise of the three blood group O infants was comparable to their increase of anti-B antibodies. The rise of anti-A titers in infants was from low levels throughout, and infants were the only ones, with the exception of D. A. (Table III), to show a fourfold or greater increase of these antibodies. In contrast to the single significant anti-A response among the adults fed *E. coli* O₈₆, three of the four healthy blood group O individuals who inhaled it (K. B.; Ral. R.; and W. S.) responded with a significant blocking anti-blood group A antibody increase; they also showed a rise of anti-B antibodies, but that of Ral. R. was not significant.

Anti-blood group B responses resulting from the inhalation of *E. coli* O₈₆ bacteria or hog gastric mucin were slightly less frequent and the titer increases of those responding were lower than those obtained in healthy adults after ingestion of the antigens. While unknown factors are likely to play a role in these different responses quantitative aspects of the experiments are also likely to be involved. Peroral immunizations with dead bacteria were with 1–10 g (i.e. about 3×10^{10} to 3×10^{11} killed *E. coli*), while the total amount inhaled was never greater than 3 mg (ca. 1×10^{10} bacteria). On the other hand, the weight of the live bacteria consumed by a volunteer was around 10 μ g (ca. 3×10^7 bacteria) but as stool cultures indicated the bacteria persisted up to 12 days.

The specificity of the stimulated isoagglutinins is attested to by their inhibition with small amounts of the corresponding blood group-specific antigens of human origin and by the failure of much larger amounts of blood group antigens of a different specificity to accomplish this (see Table IV).

The isoantibodies reacting with A and B erythrocytes were stimulated by a bacterium which in vitro possesses high blood group B and low A activity. The question arose if the antibodies evoked in blood group O persons (Table II) and which reacted with A₁ and A₂ erythrocytes were strictly anti-A-specific or if they were, at least in part, of the cross-reacting kind frequently observed in individuals of blood group O (27–29). We found that the majority of isoantibodies stimulated by *E. coli* O₈₆ were of the homologous rather than the cross-reacting kind. Previously such cross-reacting antibodies apparently have been studied only in adults. It is, therefore, not possible to state whether or not the lack of stimulation of these antibodies in children is peculiar to *E. coli* O₈₆.

Experiments with chickens had shown that feeding of blood group B- and A-specific *E. coli* O₈₆ does not lead in some mysterious way to unspecific stimulation of anti-blood group antibodies since feeding of blood group-inactive *E. coli* did not evoke antibodies against human blood group antigens (6). It is in accord with these findings that titer increases or their lack against *E. coli* O₈₆ L.P.S.-coated human blood group O erythrocytes went hand in hand with change in blood group antibody concentration. The antibodies reacting with the L.P.S. were also directed against other determinant groups of *E. coli* O₈₆ besides those carrying blood group specificity. Thus, absorption with blood group B erythrocytes led to only a small reduction of the antibody titers against *E. coli* O₈₆ in all 15 postimmunization sera tested. The significance of this antibody reduction, however, was proven in all instances by elution, with the Landsteiner-Miller technique, of specific anti-blood group B antibodies from the B erythrocytes used for the absorptions. All but one person (B. T.) had antibodies reacting with *E. coli* O₈₆ also in their pre-immunization sera and a similar proportion of these could be absorbed and eluted.

While blood group B erythrocytes removed only a fraction of the antibodies directed against *E. coli* O₈₆, >90% removal of anti-B antibodies stimulated by ingestion of *E. coli* O₈₆ was achieved by one absorption with *E. coli* O₈₆ L.P.S.-coated blood group O erythrocytes. These coated cells also removed preexisting anti-B antibodies but to a somewhat lesser extent; this illustrates the complex nature of human blood group B antigens and the heterogeneity of the corresponding antibodies (cf. 27).

A comparison of the anti-B antibody rise of infants and adults, healthy as well as sick, in relation to their blood groups showed no influence of Rh type or secretor status but surprisingly, a remarkable difference was found in response of individuals of blood group A and O. 12 of 14 persons of blood group A responded signifi-

cantly but only 11 of 23 of blood group O; the average increase of saline and blocking antibodies among the responders was similar for individuals of blood group A and O. The findings on antibody increase against *E. coli* O₈₆ were analogous. 7 of 10 individuals of blood group A responded with a significant rise but only 5 of 12 of blood group O showed such an increase; in addition the average titer increase of group A individuals was nearly three times higher. This startling difference in response of isoantibodies and antibodies directed against *E. coli* O₈₆ may possibly be due to a more efficient early elimination of the *E. coli* O₈₆ antigens by antibodies of persons of blood group O. Such a hypothesis finds support in the findings of Dr. R. R. Race, F.R.S., (personal communication and reference 30) that only persons of blood group A with severe lesions of their body surfaces may acquire blood group B-like antigens on their erythrocytes (cf. 10).

Any evaluation of the highly significant responses by the infants receiving *E. coli* O₈₆ must consider preexisting blood group-specific bacteria. The necessity of this consideration is illustrated by the significant, apparently microbe-induced rise, during the observation period, in isoantibodies against blood group A₁, A₂, and B erythrocytes in two of the seven infants who served as controls and were not fed *E. coli* O₈₆. Interestingly, four of the five diarrheic infants in whom blood group A-active bacteria were found were of blood group A, i.e., they did not possess anti-blood group A antibodies.

The failure to demonstrate blood group-active bacteria in a stool does not necessarily prove their absence. However, the examination of the cases investigated was quite thorough (see Methods). Furthermore, while blood group-specific bacteria were found frequently, those with blood group activity amounting to as much as 25-100% of that of the *E. coli* O₈₆ B:7 strain were found only once in this study. Similarly, we noted earlier that among about 300 strains of Gram-negative bacteria which were in part selected, highly active bacteria amounted to no more than 20% of all blood group-active bacteria isolated (7).

The in vivo neutralization of anti-blood group and anti-*E. coli* antibodies in germfree and ordinary chickens was remarkably complete in those birds who survived the injection. That the birds which died had a lesser neutralization of their antibodies than those who survived tempts one to ascribe a protective function to these or concomitantly removed antibodies (cf. 31, 32). However, the presented experiments do not permit a conclusion.

The studies presented here give direct evidence in favor of the necessity of immunogenic stimulation of the genetically determined antibody-producing machinery in order to obtain the human isoantibodies anti-A and anti-B. Yet, even the direct proof of the immunogenic

origin of the blood group anti-A and anti-B antibodies in man furnished in this investigation and earlier in germfree animals (6) does not exclude the possibility that some natural antibodies, including isoantibodies, may be the result of physiological maturation of an organism and possess a fortuitous complementarity to certain antigenic determinants (cf. 16, 17, 33). However, while our results do not vitiate such a possibility, they in no way favor it.

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Dr. R. E. Horton's contribution to this paper was confined to rearing, injecting, and bleeding chickens, as well as evaluating the manuscript. This statement is inserted at the request of Dr. Horton's superiors.