

Antibodies of the IgG, IgM, and IgA Classes in Newborn and Adult Sera Reactive with Gram-Negative Bacteria

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ABSTRACT Umbilical cord serum and adult serum antibodies reactive with heat-stable somatic antigens of Gram-negative bacteria (*Neisseria gonorrhoeae*, *Escherichia coli*, and *Salmonella typhosa*) were assayed by using an indirect fluorescent antibody test. Reactive IgG, IgM, and IgA antibodies were identified by using fluorescein-conjugated antisera specific for these immunoglobulin classes.

IgG antibody titers in cord serum approximated those found in the corresponding maternal sera. IgM and IgA antibodies were present in adult sera but were not demonstrable or were present only in small amounts in cord sera. The presence of IgG and IgM antibodies reactive with Gram-negative bacteria was confirmed by the testing of purified 7S and 19S fractions. In addition, both IgG and IgM reactivities were inhibited by the prior incubation of serum with purified specific lipopolysaccharide preparations.

The ubiquity and magnitude of these natural IgG antibodies in the sera of both adults and neonates have apparently eluded detection in previous studies. The use of bactericidal and agglutination tests, which are apparently more sensitive to the presence of IgM than to IgG antibodies, may account for the failure of previous studies to detect adult and cord IgG antibodies reactive with somatic antigens of Gram-negative bacteria. The presence of these IgG antibodies may be correlated

with the resistance to infection demonstrated by most newborns as they are challenged by the septic extrauterine environment.

INTRODUCTION

Antibodies which are commonly found in the sera of normal individuals and which are not related to obvious immunization or clinical infection may be called natural antibodies (1). A number of investigators employing bactericidal and other tests has concluded that natural antibodies reactive with somatic antigens of Gram-negative bacteria are principally of the IgM (19S, gamma₁-macroglobulin) class (1-3). This finding is of practical importance because it has been shown that the human fetus receives primarily maternal IgG (7S, gamma₂-globulin) antibodies, whereas maternal IgM antibodies do not readily cross the placenta (4). The human newborn is therefore deficient in maternal antibodies of the IgM class.

Studies of natural antibodies reactive with somatic antigens of Gram-negative bacteria in maternal and umbilical cord sera have shown that most cord sera possess little or no bactericidal, agglutinating, or hemagglutinating antibody activity, whereas appreciable activity is demonstrable in the corresponding maternal sera (5-10). It has been suggested (5) that the human newborn is deficient in natural bactericidal antibodies to Gram-negative bacteria because these antibodies are usually present predominantly in the IgM class.

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However, by employing reagents specific for human immunoglobulin classes in an indirect fluorescent antibody (IFA) procedure, we were able to demonstrate that a major portion of natural human antibodies reactive with somatic antigens of Gram-negative bacteria are of the IgG class (11). We also found that cord sera contained these IgG antibodies at titers comparable to those present in the sera of most adults. This finding prompted the more detailed study described in this paper.

IgG, IgM, and IgA (β_2 A-globulin) antibodies reactive with heat-stable somatic antigens of *Neisseria gonorrhoeae*, *Escherichia coli*, and *Salmonella typhosa* were studied by using the IFA method. Individual maternal-cord serum pairs, pooled adult and cord sera, and purified fractions of sera were studied.

METHODS

Sera. 20 maternal-cord serum pairs were obtained through the kindness of Doctors William E. Josey and Frank Smith. These sera were collected from apparently normal mothers and from the umbilical cord bloods of their infants at the time of delivery at Grady Memorial Hospital, Atlanta, Ga. A pool of normal cord sera collected from babies born in hospitals in the Los Angeles area was kindly supplied by Dr. E. Shanbrom and Mr. K. Lou of Hyland Laboratories, Los Angeles, Calif. 36 sera from apparently normal young children were obtained through the kindness of Mr. Charles F. Peters of the Serum Bank of the National Communicable Disease Center at Atlanta, and from Doctors Marshall S. Horwitz and Bayard W. Allmond, Jr., of the Center. All sera were stored at -20°C and were not heated before use. Sera were diluted in phosphate-buffered saline, pH 7.2, (PBS) on the day of testing. Cohn fractions III-1 and II of pooled normal adult sera were obtained from Hyland Laboratories. Cohn fractions III-1 and II derived from human placentas were supplied by Lederle Laboratories, Pearl River, N. Y. The 7S and 19S proteins in these fractions were purified by gel filtration (see below) before use in the IFA test.

Bacteria. The virulent colony type 1 (12) of *Neisseria gonorrhoeae* strain F62 was used. The virulence of this organism was demonstrated by its ability to cause acute gonorrhea in all of 10 human volunteers who were challenged in the course of a concurrent study.¹ The *N. gonorrhoeae* strain was maintained by passage of selected colonies on agar plates made with GC medium

base² (Difco Laboratories, Detroit, Mich.), plus a defined supplement.¹ Cultures were incubated in candle jars at 37°C .

Enteropathogenic strains of *Escherichia coli* 086:B7: N.M. and 0127:B8:H21 (13) and *Salmonella typhosa* 0901 were kindly typed and supplied by Dr. W. H. Ewing of the National Communicable Disease Center. These organisms were grown at 37°C on the surface of plates made with Trypticase soy agar (BBL).

Cells were prepared for testing in the following manner. Colonies were harvested into PBS from the surfaces of agar plates after 15–16 hr of incubation. The bacterial suspensions were adjusted to a standard optical density reading on a Coleman Nephcolorimeter (Coleman Instruments Inc., Maywood, Ill.) that represented a concentration of approximately $2-4 \times 10^9$ bacterial units/ml. The suspensions were heated for 2 hr at 121°C in an autoclave. After heating, the bacteria were sedimented by centrifugation, the supernatant fluid was removed, and the bacteria were resuspended in a volume of fresh PBS equal to that of the bacterial suspension before heating. These suspensions were maintained uncontaminated at 4°C . The *N. gonorrhoeae* cells were diluted 1:10 and the *E. coli* and *S. typhosa* cells 1:5 in PBS just before use. Stock preparations were discarded after periods of 4–5 days. Subsequent fresh preparations were made from subcultures of the original standard cultures which were preserved at -50°C in glycerol broth.

Fluorescent anti-immunoglobulin reagents. Fluorescein-conjugated goat antisera specific for human IgG, IgM, and IgA were kindly supplied by Dr. E. Shanbrom and Mr. K. Lou of Hyland Laboratories. These antisera were stored at -20°C , in small quantities, which were thawed and diluted 1:40 in PBS just before each test.

The specificity of each of these reagents was confirmed in a number of ways. By immunoelectrophoretic analysis in agar gel it was shown that when tested, undiluted, each of the antisera formed only a single precipitin band against normal human serum. Each precipitin band had the conventional location and characteristics expected for antisera specific for IgG, IgM, and IgA. In other gel diffusion studies, each of the fluorescent antisera showed results comparable with those obtained with reference antisera, kindly supplied by Doctors J. Vaughan, J. Fahey, and by Doctors F. Wollheim, and Dr. R. Williams, Jr.

It was also found that the routine 1:40 working dilution of the fluorescent reagents reacted appropriately when used to demonstrate antibody activities within highly purified serum immunoglobulin fractions. For example, the most cathodal fraction of gamma globulin was isolated from normal human serum by the use of continuous-flow paper electrophoresis techniques and this cathodal fraction was then further purified by gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), wherein it yielded a single "7S" peak.

¹ Kellogg, D. S., Jr., I. R. Cohen, L. C. Norins, A. L. Schroeter, and G. Reising. *Neisseria gonorrhoeae* II. Colonial variation and pathogenicity during 35 months *in vitro*. In preparation.

² Trade names are used for identification only and do not represent an endorsement by the Public Health Service or the U. S. Department of Health, Education, and Welfare.

When this purified fraction was overlayed on bacteria in the fluorescent antibody assay, positive results were obtained only with the anti-IgG fluorescent antiserum and not with either the anti-IgM or anti-IgA reagents.

Conversely, a highly purified macroglobulin fraction was obtained by isolating the proteins of beta globulin mobility by continuous-flow paper electrophoresis, and then subjecting these to gel filtration on G-200. The results showed a large "19S" peak and a small "7S" peak. The aliquots which comprised the first portion of the 19S peak were pooled and concentrated, and then subjected to the fluorescent antibody assay. We found that the reactive globulins in this fraction could be "stained" with the anti-IgM fluorescent reagent, but not with the anti-IgG or anti-IgA reagents.

Further, in routine use, the fluorescent antibody reagents revealed antibody activity of a given class only in those serum fractions in which the appropriate immunoglobulins were demonstrable by radial diffusion precipitin techniques.

Fluorescent antibody assay of immunoglobulins. One drop (approximately 0.04 ml) of a heat-treated bacterial suspension (see above) was air-dried at 37°C on a circumscribed area of a glass slide. One drop of the test serum or of the fraction was placed over the bacteria and incubated 30 min at 37°C in a sealed moist chamber. The slide was washed for three 5-min periods in fresh PBS and dried by gently blotting with bibulous paper. The immunoglobulin identity of antibodies binding to the treated bacteria was then determined. One drop of the appropriate fluorescent antiglobulin reagent was placed over the bacterium-antibody complexes on the slide and the slide was incubated, washed, and dried as before. A drop of mounting fluid (1 part glycerol to 9 parts carbonate buffer, pH 9) was placed over the area and a cover slip was added. Negative control slides that consisted of bacteria incubated first with PBS and then with the fluorescent reagents were included in each test.

Slides were coded by number and examined immediately after preparation. An AO microscope with a dark-field condenser and an Osram HB200 W mercury light source was used with Corning 5113 and Schott GG-9 filters. All examinations were performed by the same observer with a 54× oil immersion lens.

Fluorescence that scored 2+ or greater (on a scale of 1+ to 4+) was considered to be a positive reaction indicating the presence of antibodies of the specific immunoglobulin class. Fluorescence that scored 1+ was considered as a trace reaction.

Fractionation of Sera. Sera and Cohn fractions II and III-1 were further fractionated by gel filtration on Sephadex G-200 with a modification of the procedure of Flodin and Killander (14). The gel filtration was carried out in columns (Pharmacia Fine Chemicals, Inc.) 2.5×100 cm, and the eluting buffer was 0.1 M Tris-1.0 M sodium chloride, pH 8.0. We are indebted to Alfredo J. Julian for performing these and other serum protein fractionations.

Quantitation of Immunoglobulins. The concentrations of IgG, IgM, and IgA immunoglobulins in sera and fractions were assayed on Immunoplates (Hyland Labora-

tories). We utilized a radial diffusion precipitin procedure similar to that published by Fahey and McKelvey (15).

RESULTS

IgG, IgM, and IgA antibodies in maternal and cord serum pairs. A comparison of maternal and cord serum IgG, IgM, and IgA antibodies reactive with heat-stable antigens of *N. gonorrhoeae* F62-T1 and *E. coli* 086:B7 is shown in Tables I and II. The titer of the IgG antibodies in each cord serum was approximately equal to the IgG titer of the corresponding maternal serum. The difference in titer in any pair was no greater than one tube dilution (to the log₂). The geometric mean and median values of the cord and maternal serum IgG titers were also approximately equal.

IgM antibody titers, however, appeared to be lower in cord sera than in maternal sera. Most cord sera demonstrated at best a trace (1+)

TABLE I
Maternal and Cord IgG, IgM, and IgA Antibodies Reactive with Heat-Stable Antigens of *N. gonorrhoeae* (T1-F62)*

Serum pairs	Reciprocal of IFA titer					
	IgG		IgM		IgA	
	Mother	Cord	Mother	Cord	Mother	Cord
1	64	64	32	Tr.‡	2	N§
2	32	32	16	Tr.	2	N
3	128	128	16	2	2	Tr.
4	64	32	16	Tr.	2	N
5	32	32	16	Tr.	4	Tr.
6	64	64	16	Tr.	2	N
7	32	32	32	Tr.	4	N
8	32	16	8	Tr.	Tr.	N
9	128	64	32	Tr.	4	N
10	128	128	32	Tr.	4	Tr.
11	32	32	8	Tr.	2	N
12	32	16	16	Tr.	2	N
13	32	64	16	Tr.	2	N
14	32	64	16	N	2	N
15	32	32	8	N	2	N
16	32	32	16	N	Tr.	N
17	16	8	8	N	Tr.	N
18	128	64	8	Tr.	Tr.	N
19	16	32	16	2	2	N
20	64	64	16	Tr.	2	N
Geometric Mean	45	45	15			
Median	32	32	16	Tr.	2	N
Range	16-128	8-128	8-32	N-2	Tr.-4	N-Tr.

* Bacteria were heated 121°C for 2 hr for use as antigens in the IFA procedure (see Methods).

‡ Tr., trace (1+) reaction obtained with undiluted serum or serum diluted 1:2.

§ N, undiluted serum failed to produce a fluorescent reaction of at least 1+.

TABLE II
Maternal and Cord IgG, IgM, and IgA Antibodies Reactive
with Heat-Stable Antigens of *E. coli* (086:B7)*

Serum pairs	Reciprocal of IFA titer					
	IgG		IgM		IgA	
	Mother	Cord	Mother	Cord	Mother	Cord
1	16	16	8	Tr.	Tr.	N
2	32	32	8	Tr.	2	Tr.
3	32	32	16	2	Tr.	Tr.
4	32	16	8	Tr.	2	N
5	32	16	32	Tr.	2	Tr.
6	16	16	8	N	Tr.	N
7	32	64	32	N	4	N
8	16	32	8	Tr.	2	N
9	64	64	32	Tr.	4	N
10	128	64	16	Tr.	Tr.	N
11	16	32	16	Tr.	Tr.	N
12	16	16	16	N	Tr.	N
13	64	32	32	Tr.	Tr.	Tr.
14	64	64	32	Tr.	2	Tr.
15	32	16	8	Tr.	N	N
16	16	16	8	N	Tr.	N
17	16	16	16	Tr.	2	N
18	32	32	16	N	N	N
19	16	32	16	2	2	N
20	16	32	8	Tr.	2	Tr.
Geometric Mean	28	28	14			
Median	32	32	16	Tr.	Tr.-2	N
Range	16-128	16-64	8-32	N-2	N-4	N-Tr.

* See footnotes to Table I.

fluorescent reaction, whereas the reciprocal of the titers demonstrable in the maternal sera ranged from 8 to 32. Two cord sera (Nos. 3 and 19) showed 2+ IgM antibody activity at a titer of 1:2.

IgA antibodies were not detectable or were present only in trace amounts in the cord sera. The majority of maternal sera showed low titer reactions for IgA antibody with both *N. gonorrhoeae* and *E. coli* 086:B7.

Most of the mothers came from an area of Atlanta in which gonorrhea is not an uncommon disease. Although it is likely that some of the mothers were asymptomatic carriers of *N. gonorrhoeae*, the antibody titers found in the maternal sera were very similar to those found in sera from other normal individuals whose medical history tended to exclude the possibility of venereal disease (16).

Antibodies in pooled cord serum. In order to extend our observations beyond the limited number of individual sera, IgG, IgM, and IgA antibodies were assayed in a cord serum pool. This pool was comprised of a large number of normal

cord sera collected in Los Angeles. The results are shown in Table III. The serum titer of IgG antibody activity (1:40 with both *N. gonorrhoeae* and *E. coli* 086:B7) was comparable to the geometric mean titer of the 20 individual cord sera. The undiluted serum gave a trace reaction for IgM antibody, and IgA antibody was not detectable.

Antibody activity in whole sera and in 19S and 7S serum fractions. In order to confirm the class identity of the antibodies demonstrable in whole cord and maternal sera, pooled maternal and cord sera were fractionated into 19S and 7S components (14) by gel filtration on Sephadex G-200.

The purified 19S and 7S fractions of the maternal and cord sera demonstrated antibody activities against the heat-stable somatic antigens of *S. typhosa*, *E. coli*, and *N. gonorrhoeae* which were consistent with the titers found in whole sera.

Fig. 1 illustrates the 19S, 7S, and 3.5S peaks obtained in the fractionation of the maternal and cord serum pools and their relative titers of IgG and IgM antibodies reactive with *N. gonorrhoeae*. The titer of IgM antibody in the maternal 19S fraction reactive with *N. gonorrhoeae* was approximately 10 times higher than that measured in the cord 19S fraction. On the other hand, the IgG antibody activities of the maternal and cord sera were found to be approximately equal.

In concurrent studies of these same 19S and 7S peaks, it was found that the IgG and IgM immunoglobulins were largely separated by the gel-filtration procedure. IgM immunoglobulin eluted with the 19S fraction and very little was detectable in the 7S fraction of either the maternal or the cord sera. The concentration of IgM immunoglobulin was eight times greater in the ma-

TABLE III
IgG, IgM, and IgA Antibodies in Pooled Cord Serum (Los Angeles) Reactive with Heat-Stable Antigens of *N. gonorrhoeae* and *E. coli* (086:B7)*

		Reciprocal of IFA titer		
		IgG	IgM	IgA
Pooled cord serum	<i>N. gonorrhoeae</i>	40	Tr.	N
	<i>E. coli</i> 086:B7	40	Tr.	N

* See footnotes to Table I.

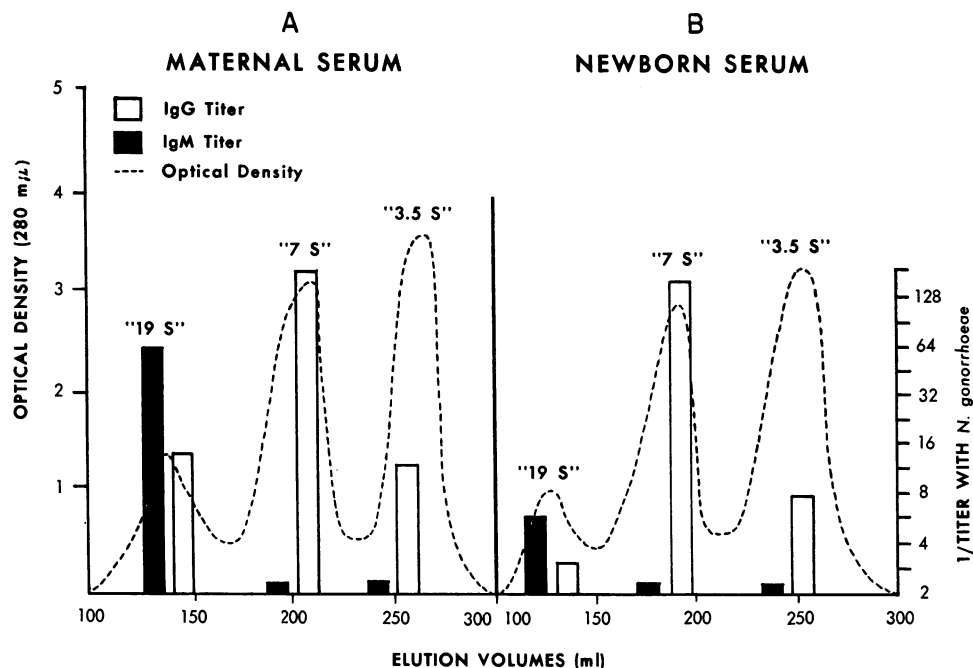


FIGURE 1 IgM and IgG natural antibodies reactive with *N. gonorrhoeae* in the Sephadex G-200 fractions of pooled maternal and cord serum. Note that the IgG antibody titer is approximately equal in the maternal (A) and cord (B) 7S fractions. The 19S fraction of the cord serum (B) appears to contain significantly less IgM antibody than is present in the maternal 19S fraction.

ternal than in the cord 19S fraction. The bulk of the IgG immunoglobulin was found within the 7S fractions of both serum pools and the maternal and cord IgG concentrations were approximately equal.

Results similar to the *N. gonorrhoeae* situation were obtained when the IFA method was used to study antibodies reactive with *E. coli* and *S. typhosa* in purified 7S and 19S fractions.

Table IV shows the titers of IgM and IgG antibodies from the following sources: (a) whole pooled maternal and cord sera, (b) purified 19S and 7S fractions isolated from cord and maternal sera, (c) the 7S fraction isolated from Cohn fraction II, and (d) the 19S fraction isolated from Cohn fraction III-1 of pooled normal human serum. It was found that substantial IgG antibody activity was present in purified 7S fractions of maternal and cord sera and in the 7S fraction of Cohn fraction II, as well as in whole sera. IgM antibody activity was found in the purified 19S fractions of maternal serum and Cohn fraction III-1, but was deficient in cord serum and its 19S fraction.

The finding of antibody activities in the 7S fractions, which were detected by the anti-IgG reagent but not by the anti-IgM reagent, confirmed the presence of IgG antibody in cord and in adult sera, and, in addition, helped to establish the specificity of the anti-immunoglobulin reagents in our assay. The testing of isolated fractions also served to minimize any unknown effects of possible competition for antigenic sites and differences in avidity between IgG and IgM antibodies (17).

Specificity of IFA reactivity. The specificity of the IFA staining of *S. typhosa* 0901 and *E. coli* 0126:B8 was confirmed by cross-absorption studies with specific bacterial lipopolysaccharide preparations (Difco Laboratories, Westphal method) (Table V). The addition to purified 7S and 19S serum fractions of *S. typhosa* 0901 lipopolysaccharide blocked both IgG and IgM reactivity to heat-stable *S. typhosa* antigens. IgG and IgM reactivity to *E. coli* 0127:B8, however, was not significantly altered by this treatment. The addition of *E. coli* 0127:B8 lipopolysaccharide, on the other hand, blocked reactivity to *E. coli* 0127:B8 antigens

TABLE IV
IgG and IgM Antibodies Reactive with *E. coli* and *S. typhosa* in Whole Sera and in 7S and 19S Fractions

Serum	Fraction†	IFA immuno- globulin reagent	Reciprocal of IFA titer with heat-stable antigens*		
			<i>S. typhosa</i> (0901)	<i>E. coli</i> (0127)	<i>E. coli</i> (086)
Pooled maternal§	Whole	IgG	16	32	64
		IgM	16	32	32
	7S	IgG	25	25	50
	19S	IgM	40	40	40
Pooled cord§	Whole	IgG	32	16	32
		IgM	<2	<2	<2
	7S	IgG	40	40	40
	19S	IgM	<5	<5	<5
Cohn II (Hyland)	7S	IgG	50	50	50
	(Lederle)	7S	IgG	200	100
Cohn III-1 (Hyland)	19S	IgM	100	50	50
	(Lederle)	19S	IgM	100	100

* See Methods.

† 7S and 19S protein peaks of whole sera and of Cohn fractions II and III-1 were purified and isolated by gel filtration on Sephadex G-200 (see text). Titers of fractions were adjusted for equal volumes.

§ Aliquots of 10 maternal and 10 corresponding cord sera were pooled and tested in the IFA procedure.

|| Cohn fractions II and III-1 of pooled normal sera were obtained from Hyland and from Lederle Laboratories and dissolved in 0.1 M Tris buffer plus 1 M NaCl before gel filtration.

without appearing to significantly affect antibody reactivity to *S. typhosa*.

Antibody Activities in the Sera of Young Children. IgG, IgM, and IgA antibodies were assayed in the sera of 36 children ranging in age

from 1 month to 2 yr. Fig. 2 shows IgG antibodies reactive with heat-stable antigens of *N. gonorrhoeae* and *E. coli* 086:B7. Fig. 3 shows the IgM titers of the same sera reactive with heat-stable antigens of these bacteria. IgA activity was not detectable in the sera of most of the children younger than 4 months and the titers of older children did not rise above 1:2.

IgM antibody activity appeared to increase with increasing age (Fig. 3). This correlated with the progressive increase in serum IgM immunoglobulin concentration observed during the development of the young child (4). IgG antibody titers, on the other hand, suggested a pattern of development different from that of IgM. IgG antibody titers in both cord sera and in the sera of children older than 4–6 months were found to be similar to adult IgG antibody titers. Children between the ages of 1 and 4 months appeared to have much lower IgG titers (Fig. 2). This pattern of IgG antibody activity seemed to parallel the changes seen in the concentration of serum IgG immunoglobulin during the first years of life (4).

TABLE V
Inhibition of IgG and IgM IFA Reactivity
by Specific Lipopolysaccharides*

Preparation	IFA reagent	Added lipopolysaccharide	IFA reactivity with heat-stable antigens	
			<i>S. typhosa</i>	<i>E. coli</i>
7S fraction of Cohn II	IgG	None	1-2+	2-3+
		<i>S. typhosa</i>	-	2+
		<i>E. coli</i>	1+	±
19S fraction of Cohn III-1	IgM	None	2+	1-2+
		<i>S. typhosa</i>	-	2+
		<i>E. coli</i>	1-2+	-

* The isolated 7S peak from Cohn fraction II (Lederle) and the isolated 19S peak from Cohn fraction III-1 (Lederle) were diluted 1:40 in buffered saline, and 0.2 ml of each was incubated for 30 min at 37°C with 0.5 mg of lipopolysaccharide preparations (Difco Laboratories, Westphal metho) from *S. typhosa* 0901 and *E. coli* 0127:B8. The absorbed fractions were then tested in the IFA procedure with heated (121°C) *S. typhosa* and *E. coli* antigens.

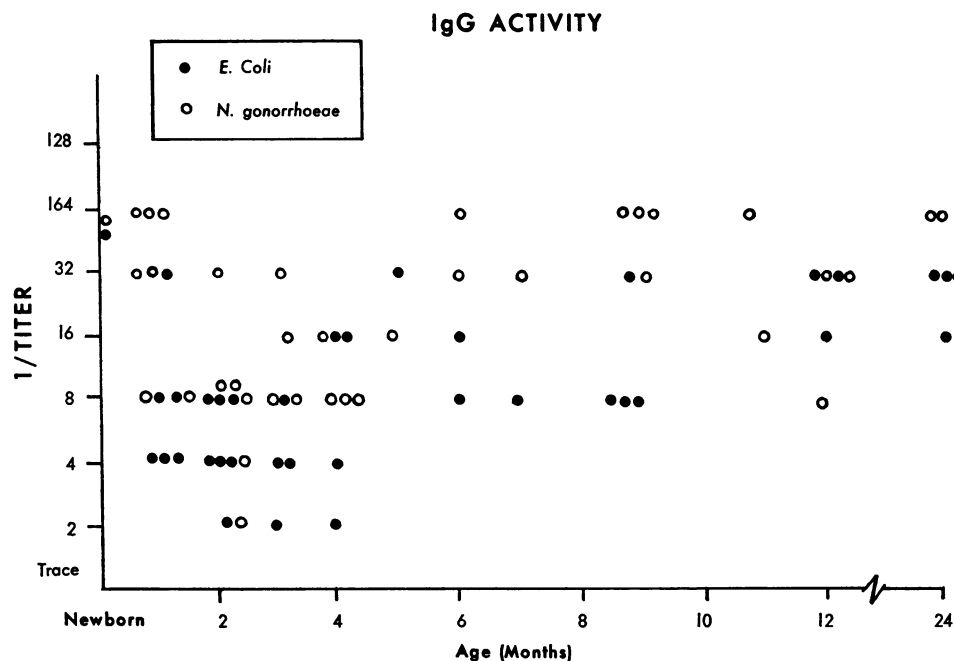


FIGURE 2 IgG antibody titers in the sera of young children reactive with *N. gonorrhoeae* T1-F62 and *E. coli* 086:B7: N.M.

DISCUSSION

It is known that maternal IgM antibodies do not cross the placenta in quantity (4), and that cord sera are relatively deficient in bactericidal, agglutinating, and hemagglutinating antibodies reactive

with somatic antigens of Gram-negative bacteria (5-10).

Although IgG levels are approximately equal in cord and maternal serum, it has recently been pointed out (18-20) that purified IgG antibody

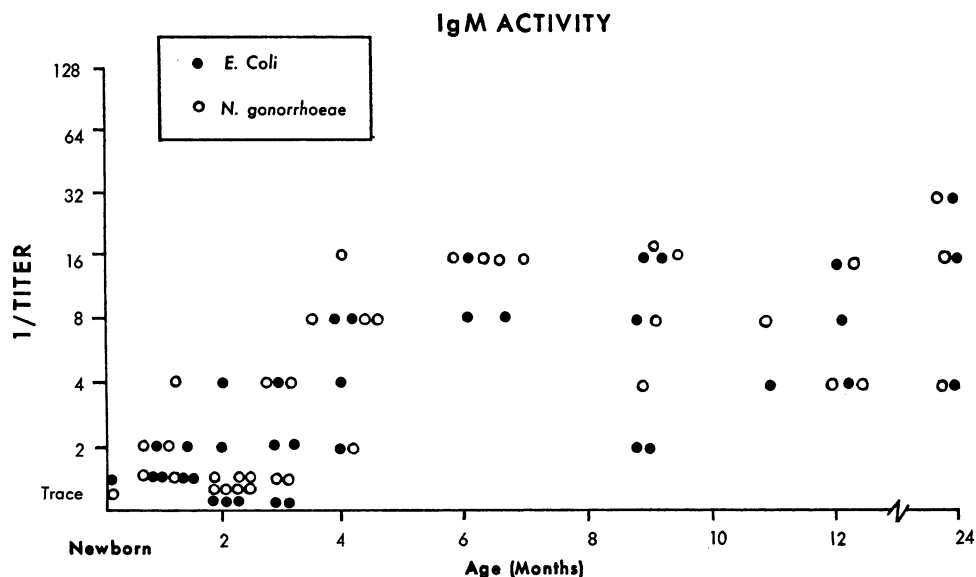


FIGURE 3 IgM antibody titers in the sera of young children reactive with *N. gonorrhoeae* T1-F62 and *E. coli* 086:B7: N.M.

is many times less efficient than purified IgM antibody in producing reactions in bactericidal, agglutination, complement-fixation, and certain other tests. We have recently found (11, 17) that, whereas IFA, bactericidal, and agglutination procedures were all able to detect natural 19S antibodies, the IFA procedure was much more sensitive than bactericidal or agglutination tests in detecting natural 7S antibodies. Thus, substantial antibody activity may be overlooked by bactericidal and agglutination tests.

These findings challenge the generalization that natural antibodies to somatic antigens of Gram-negative bacteria are principally in the IgM class of antibodies (3). They also draw attention to possible limitations in the use of agglutination tests to study the sequence of antibody production to somatic antigens in humans (21, 22) and rabbits (23). The use of agglutination tests to study the immune response of infants to somatic antigens (7, 24) might also be reappraised, since maternal IgG antibody, undetected by the agglutination tests, may have been present in the infants' sera before immunization and may have inhibited or modified the active synthesis of antibody by the infants (25, 26).

It has also been suggested that intensive antigenic stimulation is necessary to elicit IgG antibodies to the somatic antigens of *Salmonella* (27). The applicability of this concept, however, should be limited to bacterial or agglutination test systems, in the light of our demonstration by IFA of appreciable human IgG antibody reactive with *S. typhosa* 0901 in normal adult and cord sera. In short, it appears that tests which are inherently more sensitive to a particular class of antibodies may present a deceptive picture of the character and development of antibodies in the immune response.

We found no evidence that contradicted the specificity of the fluorescent reagents for IgG, IgM, and IgA at the dilution at which they were used in our IFA system. However, the finding of antibodies in purified 7S serum fractions is in itself suggestive of IgG antibody activity. Our conclusions, therefore, do not depend wholly upon the specificity of the antisera for IgG and IgM. It was not practical to determine the relative combining efficiencies of our fluorescent antisera, but in positive control slides each appeared fully able

to recognize antibodies of the appropriate immunoglobulin class.

Neter (28), employing an antiglobulin hemagglutination test, has demonstrated in cord serum "incomplete" antibodies reactive with Gram-negative bacteria. In his antiglobulin test the maternal sera seemed to be reactive at much higher titers than the corresponding cord sera, and it is uncertain which immunoglobulin classes participated in the reaction which he described.

Gitlin, Rosen, and Michael have suggested (5) that the relative deficiency of IgM bactericidal antibody in newborn serum may be responsible, at least in part, for the "unusual" susceptibility of neonates to infection with Gram-negative bacteria. However, although virtually all newborns lack IgM bactericidins to an equal degree, it is apparent that only a very small number succumb to infection with Gram-negative bacteria. The vast majority of neonates accommodate to Gram-negative bacterial contamination without suffering clinical disease, despite a lack of serum bactericidal activity.

There is some evidence that the transplacental transfer of maternal IgG antibody to the fetus may help protect the newborn against infection by Gram-negative bacteria. It was found (29) that mothers who are carriers of enteropathogenic *E. coli* seem to endow their offspring with some protection against diarrhea due to these organisms. Although natural IgG antibody may be poorly reactive in in vitro bactericidal reactions, it may nevertheless perform important in vivo functions.

A small amount of reactive IgM antibody was found to be present in most cord sera. Because it has recently been demonstrated that the normal fetus can synthesize IgM and IgG (30, 31), fetal synthesis may have contributed to at least part of the IgM and IgG cord serum antibodies we observed.

Several facts suggest that the heat-stable antigens reactive in the IFA procedure are comparable to the somatic antigens reactive in other test systems. It has been shown that purified lipopolysaccharide preparations of somatic antigens specifically inhibit the bactericidal action of serum on Gram-negative bacteria (32), and these preparations also inhibited the IFA staining of heat-treated *E. coli* and *S. typhosa*. The finding that both IgG and IgM antibodies were specifically blocked by lipopolysaccharides further suggests

that antibodies of both classes are able to react with lipopolysaccharide antigens. In addition, *E. coli* 086:B7:N.M. and *S. typhosa* 0901 are both nonmotile strains. These bacteria and *N. gonorrhoeae*, therefore, lack the H antigens to which natural human IgG agglutinins have been previously described (7). The heat treatment employed in our study was designed to insure that undetected H antigens and heat-labile capsular antigens would be destroyed. These bacteria thus appear similar to those used in "O" agglutination procedures (33).

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REFERENCES

1. Landy, M., and W. P. Weidanz. 1963. Natural antibodies against gram-negative bacteria. In *Bacterial Endotoxins*. M. Landy and W. Braun, editors. Institute of Microbiology, Rutgers University Press, New Brunswick. 275.
2. Nomenclature for human immunoglobulins. 1964. *Bull. World Health Organ.* 30: 447.
3. Michael, J. G., and F. S. Rosen. 1963. Association of "natural" antibodies to gram-negative bacteria with the γ_1 -macroglobulins. *J. Exptl. Med.* 118: 619.
4. Good, R. A., and B. W. Papermaster. 1964. Ontogeny and phylogeny of adaptive immunity. *Adv. Immunol.* 4: 1.
5. Gitlin, D., F. S. Rosen, and J. G. Michael. 1963. Transient 19S gamma₁-globulin deficiency in the newborn infant, and its significance. *Pediatrics.* 31: 197.
6. Smith, R. T. 1959. Response to active immunization of human infants during the neonatal period. In *Ciba Foundation Symposium on Cellular Aspects of Immunity*. G. E. W. Wolstenholme and M. O'Connor, editors. Little, Brown and Co., Boston. 348.
7. Fink, C. W., W. E. Miller, Jr., B. Dorward, and J. LoSpalluto. 1962. The formation of macroglobulin antibodies. II. Studies on neonatal infants and older children. *J. Clin. Invest.* 41: 1422.
8. Neter, E., O. Westphal, O. Lüderitz, R. M. Gino, and E. A. Gorzynski. 1955. Demonstration of antibodies against enteropathogenic *Escherichia coli* in sera of children of various ages. *Pediatrics.* 16: 801.
9. Yeiven, R., M. Salzberger, and A. L. Olitzki. 1955. Development of antibodies to enteric pathogens: Placental transfer of antibodies and development of immunity in childhood. *Pediatrics.* 18: 19.
10. Sussman, S. 1961. The passive transfer of antibodies to *Escherichia coli* 0111:B4 from mother to offspring. *Pediatrics.* 27: 308.
11. Cohen, I. R., and L. C. Norins. 1966. Natural human antibodies to gram-negative bacteria: Immunoglobulins G, A, and M. *Science.* 152: 1257.
12. Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. I. Pirkle. 1963. *Neisseria gonorrhoeae* I. Virulence genetically linked to clonal variation. *J. Bact.* 85: 1274.
13. Wheeler, W. E. 1964. Infantile diarrhea due to enteropathogenic *Escherichia coli*. In *Communicable and Infectious Diseases*. F. H. Top, editor. Mosby Co., St. Louis. 5th edition. 529.
14. Flodin, P., and J. Killander. 1962. Fractionation of human serum proteins by gel-filtration. *J. Biochim. Biophys. Acta.* 63: 403.
15. Fahey, J. L., and E. M. McKelvey. 1965. Quantitative determination of serum immunoglobulins in antibody-agar plates. *J. Immunol.* 94: 84.
16. Cohen, I. R. 1967. Natural and immune human antibodies reactive with antigens of virulent *Neisseria gonorrhoeae*: Immunoglobulins G, M, and A. *J. Bact.* 94: 141.
17. Cohen, I. R., L. C. Norins, and A. J. Julian. 1966. Competition between, and effectiveness of, IgG and IgM antibodies in indirect fluorescent antibody and other tests. *J. Immunol.* 98: 143.
18. Robbins, J. B., K. Kenny, and E. Suter. 1965. The isolation and biological activities of rabbit γ M- and γ G-anti-*Salmonella typhimurium* antibodies. *J. Exptl. Med.* 122: 385.
19. Onoue, K., N. Tanigaki, Y. Yagi, and D. Pressman. 1965. IgM and IgG anti-hapten antibody: Hemolytic, hemagglutinating and precipitating activity. *Proc. Soc. Exptl. Biol. Med.* 120: 340.
20. Borsos, T., and H. J. Rapp. 1965. Complement fixation on cell surfaces by 19S and 7S antibodies. *Science.* 150: 505.
21. LoSpalluto, J., W. Miller, Jr., B. Dorward, and C. W. Fink. 1962. The formation of macroglobulin antibodies. I. Studies on adult humans. *J. Clin. Invest.* 41: 1415.
22. Turner, M. W., and D. S. Rowe. 1964. Characterization of human antibodies to *Salmonella typhi* by gel-filtration and antigenic analysis. *Immunology.* 7: 639.
23. Bauer, D. C., M. J. Mathies, and A. B. Stavitsky. 1963. Sequences of synthesis of γ_1 macroglobulin and γ_2 globulin antibodies during primary and secondary responses to proteins, *Salmonella* antigens, and phage. *J. Exptl. Med.* 117: 889.
24. Smith, R. T., and D. V. Eitzman. 1964. The development of the immune response. Characterization of the response of the human infant and adult to immunization with *Salmonella* vaccines. *Pediatrics.* 33: 163.
25. Osborn, J. J., J. Dancis, and J. F. Julia. 1952. Studies of the immunology of the newborn infant. II. Interference with active immunization by passive transplacental circulating antibody. *Pediatrics.* 10: 328.
26. Fink, C. W., and J. LoSpalluto. 1964. Effect of prior administration of 7S and 19S antibody on active antibody formation. *J. Pediat.* 65: 1083. (Abstr.)

27. Gotoff, S. P., and W. D. Cochran. 1966. Antibody to the somatic antigen of *Salmonella Newport* in premature infants. *Pediatrics*. 37: 610.
28. Neter, E. 1957. Detection of incomplete enterobacterial antibodies in cord blood by means of anti-globulin hemagglutination test. *Proc. Soc. Exptl. Biol. Med.* 96: 488.
29. Cooper, M. L., H. M. Keller, E. W. Walters, J. C. Partin, and D. E. Boye. 1959. Isolation of enteropathogenic *Escherichia coli* from mothers and newborn infants. *Am. J. Diseases Children*. 97: 255.
30. Martensson, L., and H. H. Fudenberg. 1965. GM genes and γ G-globulin synthesis in the human fetus. *J. Immunol.* 94: 514.
31. Van Furth, R., H. R. E. Schuit, and W. Hijmans. 1965. The immunological development of the human fetus. *J. Exptl. Med.* 122: 1173.
32. Michael, J. G., J. L. Whitby, and M. Landy. 1962. Studies on natural antibodies to gram-negative bacteria. *J. Exptl. Med.* 115: 131.
33. Kauffmann, F. 1954. Enterobacteriaceae. Munksgaard, Copenhagen. 2nd edition.