

## Normally Occurring Human Anti-GM<sub>1</sub> Immunoglobulin M Antibodies and the Immune Response to Bacteria

María E. Alaniz,<sup>1</sup> Ricardo D. Lardone,<sup>1</sup> Silvia L. Yudowski,<sup>2</sup> María I. Farace,<sup>3</sup>  
and Gustavo A. Nore<sup>1\*</sup>

Departamento de Química Biológica "Dr. Ranwel Caputto," CIQUIBIC, CONICET, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba,<sup>1</sup> and Servicio de Bacteriología, Hospital Infantil Municipal,<sup>2</sup> Córdoba, and Servicio Bacteriología Sanitaria, Instituto Nacional de Enfermedades Infecciosas, Administración Nacional de Laboratorios e Institutos de Salud "Carlos G. Malbrán," Buenos Aires,<sup>3</sup> Argentina

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Anti-GM<sub>1</sub> antibodies of the immunoglobulin M (IgM) isotype are normal components of the antibody repertoire of adult human serum. Using a sensitive high-performance thin-layer chromatography (HPTLC) immunostaining assay, we found that these antibodies were absent in the umbilical vein and children <1 month of age but could be detected after 1 month of age. Although most of the children older than 6 months of age were positive, there were still a few negative children. The appearance of anti-GM<sub>1</sub> IgM antibodies showed a perfect concordance with two well-characterized antibacterial antibodies, anti-Forssman and anti-blood group A, which indicates a similar origin. We also studied IgM reactivity with lipopolysaccharides (LPSs) from gram-negative bacteria isolated from stool samples from healthy babies and from *Escherichia coli* HB101 in serum from individuals of different ages. We found a positive reaction with both LPSs in all the children more than 1 month of age analyzed, even in those that were negative for anti-GM<sub>1</sub> antibodies. Anti-GM<sub>1</sub> IgM antibodies were purified from adult serum by affinity chromatography and tested for the ability to bind LPSs from different bacteria. This highly specific preparation showed reactivity only with LPS from a strain of *Campylobacter jejuni* isolated from a patient with diarrhea. We conclude that normally occurring IgM antibodies are generated after birth, probably during the immune defense against specific bacterial strains.

Antibodies reacting with GM<sub>1</sub> are clearly associated with motor neuropathies (11, 14, 18). Although the pathogenic role of the antibodies in the disease is still uncertain, cumulative evidence suggests that they are primarily involved (1, 17, 19, 21, 22, 24). In contrast, very little information is available about how these autoantibodies are originated. Anti-GM<sub>1</sub> immunoglobulin M (IgM) antibodies are part of the antibody repertoire of normal humans (9) and similar antibodies but with higher affinity have been found in patients with neuropathy (6, 10). Studies of anti-GM<sub>1</sub> IgM antibodies (13) cloned from neuropathy patients have shown that they are encoded by somatically mutated diverse V genes. In contrast, although an antibody response can be obtained by immunization of animals with GM<sub>1</sub>, only low-affinity antibodies can be generated (5, 7). Consistent with these findings, all the mouse monoclonal antibodies characterized so far are encoded by genes close to the germ line configuration, with few mutations (25).

Based on comparative studies of experimentally induced versus disease-associated antibodies, high affinity has been postulated as a disease determinant factor in anti-GM<sub>1</sub> antibodies (7), but the origin of the high-affinity antibodies is still hypothetical. Two different hypotheses have been proposed for explaining the appearance of anti-GM<sub>1</sub> antibodies in disease, the antigen mimicry hypothesis (2, 3a, 15, 27) postulates a cross-reactive immune response originally directed to *Campylobacter jejuni* lipopolysaccharides (LPSs), and the binding site drift

hypothesis (6, 8) proposes that the origin of disease-associated (high-affinity) antibodies is spontaneous mutations in the binding site of normally occurring antibodies. An unresolved point of the second hypothesis is the origin of normally occurring antibodies. Since the pioneering work of Springer (20), it had been widely accepted that naturally occurring antibodies recognizing defined glycans such as the Forssman and blood group antigens are produced in response to intestinal or respiratory tract bacteria. In the present paper, we present evidence indicating a similar origin for anti-GM<sub>1</sub> IgM antibodies in healthy humans.

### MATERIALS AND METHODS

**Human serum.** Umbilical vein blood samples were obtained shortly after delivery. Adult blood was obtained from healthy volunteers with negative serology for common infectious diseases. After clot separation (usually less than 3 h after extraction), the serum was frozen at -70°C until use. For infants, we used serum samples extracted for neonatal screening (babies less than 1 week old) or presurgery control (children more than 1 month of age).

**Glycolipids.** GM<sub>1</sub>, GD<sub>1a</sub>, and GD<sub>1b</sub> were obtained from human brains. Asialo-GM<sub>1</sub> (GA<sub>2</sub>) was prepared by acid hydrolysis of cow brain gangliosides (3). Forssman and blood group A glycolipids were obtained from sheep erythrocytes and human A meconium, respectively. Glycolipids were purified by DEAE chromatography (26) and high-pressure liquid chromatography (HPLC) on an latrobeads silica gel column (23).

**Bacterial growth conditions and extraction of LPS.** *Campylobacter jejuni* isolated from a patient with diarrhea was grown on blood agar (Columbia agar base supplemented with 5% human blood and fetal bovine serum) at 42°C for 48 h in a microaerobic atmosphere. Aerobic conditions, 37°C, 48 h, and EMB growth medium were used for *Escherichia coli* HB101, *Salmonella enteritidis*, *Shigella flexneri*, and cultures obtained from stool samples from the babies. Bacterial biomass was harvested and LPSs were extracted with the miniphenol-water method (16). LPS from *Escherichia coli* O127-B8 and *Pseudomonas aeruginosa* serotype 10 were from Sigma (St. Louis, Mo.).

\* Corresponding author. Mailing address: Facultad de Ciencias Químicas (UNC), Ciudad Universitaria, 5000 Córdoba, Argentina. Phone: (54) (351) 4334168. Fax: (54) (351) 4334074. E-mail: gnore@dqbfq.unc.edu.ar.

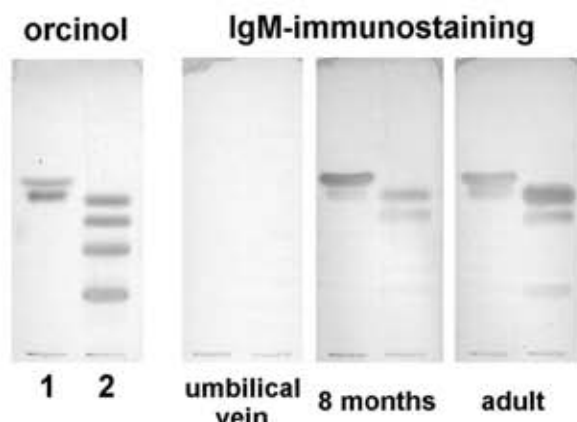


FIG. 1. Antiglycan IgM antibodies in normal human serum. Forssman and blood group A glycolipids (lane 1, upper and lower bands, respectively), and gangliosides GA<sub>1</sub>, GM<sub>1</sub>, GD<sub>1a</sub>, and GD<sub>1b</sub> (lane 2, top to bottom) were separated by HPTLC as described in Materials and Methods. Plates were incubated with the stated human serum, and after being washed, IgM antibody binding was detected with peroxidase-labeled anti-human IgM antibodies. One plate was stained with orcinol reagent for chemical detection of glycolipids.

**HPTLC immunostaining.** Glycolipids (0.3 nmol each) and LPS (50 µg or amounts equivalent to 3 mg of bacteria) were separated on high-performance thin-layer chromatography (HPTLC) plates in running solvent containing chloroform-methanol-aqueous 0.2% CaCl<sub>2</sub> (45:45:10) and 2-propanol-7% NH<sub>4</sub>(OH) (6:4), respectively, with a tank designed to obtain highly reproducible chromatograms (12). After air drying, the plates were coated by being dipped for 2 min in a 0.5% solution of polyisobutylmethacrylate (Plexigum P28; Röhm and Haas, Darmstadt, Germany) in *n*-hexane-chloroform (9:1). The plates were blocked with 1% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20 (BSA-PBS) for 1 h, incubated overnight with BSA-PBS-diluted serum (1:10), and washed thoroughly with PBS. Binding was detected after 2 h of incubation with BSA-PBS-diluted (1:1,000) peroxidase-conjugated anti-human IgM (µ chain) goat antibodies (Sigma). All the incubation steps were performed at 4°C. After washing, color development was achieved in a substrate solution containing 2.8 mM 4-chloro-1-naphthol and 0.01% H<sub>2</sub>O<sub>2</sub> in methanol-20 mM Tris-HCl buffer, pH 7.4 (1:29). The reaction was stopped after 20 min by washing the plates with PBS.

**Preparation of GM<sub>1</sub> affinity column.** The method used, modified from that of Hirabayashi et al. (4), was previously found to be suitable for separation of anti-GM<sub>1</sub> antibody populations (6). Briefly, 1.5 ml of methanol-aqueous 0.2 M KCl (1:1) containing 0.25 µmol of GM<sub>1</sub> was added to 1 ml of octyl-Sepharose (Sigma). The mixture was rotated end over end for 1 h at room temperature. After removal of the solution, the gel was washed three times with 5 volumes of PBS. The remaining steps were performed at 4°C. Small portions of the gel were loaded in a calibrated column and washed with PBS. Serum (1 ml per 0.1 ml of gel) was passed through the column five times. The column was washed with PBS, and retained antibodies were eluted with 1 M KSCN in PBS (10 bed volumes) and desalted in Sephadex G-25 columns. Fractions were supplemented with BSA (1% final concentration), aliquoted, and frozen at -70°C until use.

## RESULTS

Using HPTLC immunostaining, we screened samples of normal human serum, searching for antibodies that reacted with GM<sub>1</sub> and related glycolipids (Fig. 1). As we described previously (9), anti-GM<sub>1</sub> IgM antibodies cross-reacting with GA<sub>1</sub> and GD<sub>1b</sub> were detected in all the adults tested. In contrast, we failed to detect them in umbilical vein serum samples, even at very low serum dilutions (1:2). Antibody binding was observed when an appropriate amount of affinity-purified anti-GM<sub>1</sub> antibodies from adult serum was mixed with umbilical vein serum

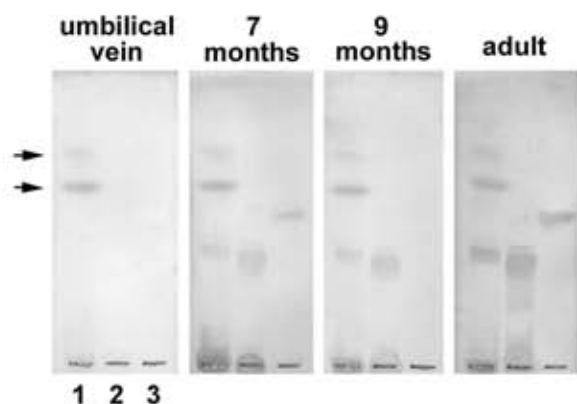


FIG. 2. Normal human serum IgM antibody binding to bacterial LPSs. LPSs from gram-negative bacteria isolated from stool samples from healthy babies (lane 1) and from *Escherichia coli* HB101 (lane 2) were prepared by the miniphenol-water method (16). Both preparations and GM<sub>1</sub> (lane 3) were separated by HPTLC and assayed for IgM antibody binding as described in Materials and Methods. Serum samples from an umbilical vein, an adult, and two children were used. Notice the absence of anti-GM<sub>1</sub> IgM antibody binding in the serum sample from the 9-month-old child. Arrows indicate a nonspecific spot originating from the culture medium.

(results not shown), indicating that if they were present, these antibodies should have been detected by the method used. Anti-GM<sub>1</sub> antibody binding reactivity was also absent in very young babies (5 to 10 days old), but it began to be detectable after approximately 1 month of life. The incidence of these antibodies in serum samples from umbilical veins ( $n = 6$ ), children 5 to 10 days old ( $n = 7$ ), children 1 to 5 months old, 6 to 12 months old, and 13 to 24 months old ( $n = 42$ ), and adults ( $n = 12$ ) was 0, 0, 30, 88, 100, and 100%, respectively. Although most of the children older than 6 months had these antibodies, it is important to highlight that there were still some negative individuals.

In addition to anti-GM<sub>1</sub> antibodies, we also searched for IgM antibodies reacting with two other glycans, the Forssman and blood group A glycolipids (Fig. 1). Comparing anti-GM<sub>1</sub> with anti-Forssman antibodies, a perfect concordance was observed: when one reactivity was absent or present in a serum, the other was also absent or present, respectively. Similar results were observed with anti-A antibodies when only non-A individuals were considered. It has been clearly demonstrated that anti-Forssman and anti-A antibodies are produced in response to bacteria (20). Consequently, these results are strong evidence of a similar origin for normal anti-GM<sub>1</sub> antibodies.

LPSs are the main glycan-carrying molecules in gram-negative bacteria. LPS from gram-negative bacteria isolated from stool samples from healthy babies and LPS from *Escherichia coli* HB101 were prepared and assayed for antibody binding. As shown in Fig. 2, adult serum contained IgM antibodies to both LPSs, but they were not detected in umbilical vein serum samples. Babies less than 1 week old were also devoid of anti-LPS antibodies (results not shown), and as expected, they started to appear later on. Interestingly, antibodies to LPS were detected in serum samples from children that were negative for anti-GM<sub>1</sub> antibodies (Fig. 2). These results suggest



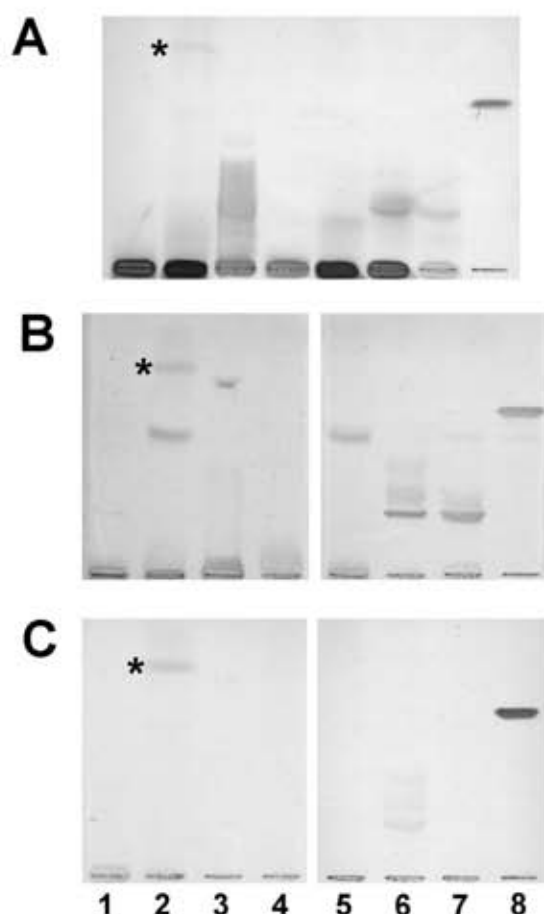


FIG. 3. Cross-reactivity of anti-GM<sub>1</sub> antibodies with LPS from *E. O127-B8* (lane 1), gram-negative bacteria isolated from stool samples from healthy babies (lane 2), *Pseudomonas aeruginosa* serotype 10 (lane 3), *Shigella flexneri* (lane 4), *Salmonella enteritidis* (lane 5), *Campylobacter jejuni* isolate 1 and *C. jejuni* isolate 2 (lane 6), were chromatographed together with GM<sub>1</sub> (lane 8) and assayed for IgM antibody binding as described in Materials and Methods. HPTLC plates were incubated either with total adult serum samples (lane 7) or (B) their anti-GM<sub>1</sub> IgM antibodies purified by affinity chromatography (C). One plate was stained with orcinol reagent (A). The asterisk indicates a nonspecific spot originating from the culture medium.

that anti-GM<sub>1</sub> antibodies are not related to the immune response to symbiotic bacteria.

Adult anti-GM<sub>1</sub> antibodies were purified with a GM<sub>1</sub> column (6). The antibody preparation obtained was highly specific and did not contain anti-Forssman or anti-A antibodies (results not shown). When this preparation was assayed for binding to LPSs obtained from different bacteria, we found that most of the anti-LPS activity present in total serum disappeared (Fig. 3). However, anti-GM<sub>1</sub> antibodies reacted with LPS from a local isolate of *Campylobacter jejuni*. When these LPSs were assayed with serum samples from children, we found results similar to those obtained with LPSs from normal flora bacteria; anti-LPS antibodies were not detected in samples from umbilical veins and babies less than 1 week old but were found in older children, even in those with negative reactivity to GM<sub>1</sub> (results not shown).

## DISCUSSION

Normal adult human serum contains anti-GM<sub>1</sub> antibodies of the IgM isotype (9). They are relatively low-affinity antibodies, cross-reacting with GA<sub>1</sub> and GD<sub>1b</sub>, and probably devoid of GM<sub>1</sub>-mediated biological activity (10). Since early experiments showed that they were not present in umbilical vein serum, we studied infant serum to search for the time of their appearance. As expected, they were also absent in babies less than 1 week old but could be detected after a few months of life. The appearance of anti-GM<sub>1</sub> antibodies seems to be related to the immune response to bacteria, because they showed a perfect concordance (100%) with the presence of two anti-glycan IgM antibodies identified as typical antibacterial antibodies, anti-Forssman and anti-blood group A. Both antibodies are considered to originate in response to bacteria that colonize the intestinal or respiratory tract (20).

Triggering of the production of anti-GM<sub>1</sub> antibody by bacteria can be achieved by at least two mechanisms, by polyclonal lymphocyte activation through a bystander effect of infection, if these antibodies are part of the collection of natural antibodies, and by an antigen-specific immune response. Considering that bacterial colonization of the body starts early after birth, the absence of antibodies in some children more than 1 month of age indicates that symbiotic bacteria are not involved in their generation. This idea is additionally supported by the facts that serum lacking anti-GM<sub>1</sub> antibodies reacted with LPS obtained from gram-negative bacteria isolated from stool samples from babies and with *E. coli* HB101 and that affinity-purified anti-GM<sub>1</sub> antibodies did not react with these LPSs. Although antibody concordance is a clear evidence of the antibacterial origin of the anti-GM<sub>1</sub> antibodies, the rest of the results indicate that bacteria, at least those belonging to normal flora, are not mainly responsible for their generation.

These apparently contradictory results can be explained if we think of an infection with a particular bacterium. In order to search for a bacterial candidate that could act as the immunogen for anti-GM<sub>1</sub> antibodies, LPSs from different bacteria were assayed for reactivity against anti-GM<sub>1</sub> IgM antibodies affinity purified from an adult human. This highly specific antibody preparation only reacted with LPS from a *C. jejuni* strain isolated from the stool sample of a patient with diarrhea but not with other bacterial LPSs, including LPS from a second *C. jejuni* strain isolated from a different patient. Interestingly, children more than 1 month of age, even those negative for anti-GM<sub>1</sub> antibodies, showed immunoreactivity with LPSs from both strains, highlighting the high strain specificity of anti-GM<sub>1</sub> antibodies.

Our results indicate that normally occurring IgM antibodies are generated after birth during the immune defense against specific bacterial strains. In our study, a strain of *C. jejuni* was identified as a candidate for immunogenicity, but other bacteria not related to diarrhea can also be the immunogen responsible. If this bacterium is the immunogen for the antibody production, it appears that an episode of diarrhea is not necessary: in the general population, most children are positive for anti-GM<sub>1</sub> after 1 year of life, but few of them have had the disease associated with *C. jejuni* infection. Subclinical or asymptomatic infection, commonly observed in developing

countries (3a), could be sufficient to generate this immune response.

Finally, Guillain-Barré syndrome with anti-GM<sub>1</sub> antibodies is clearly associated with a preceding infection with GM<sub>1</sub> glycan-carrying strains of *C. jejuni* (2, 15, 27). The relationship between this fact and our results is still an open question, and the answer can shed light on the origin of anti-GM<sub>1</sub> antibodies in disease.

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