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The Repertoire of Human Antiglycan Antibodies and Its Dynamics in the First Year of Life

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Abstract—The repertoire of antiglycan antibodies of peripheral blood was studied using a microarray containing 487 glycan antigens: fragments of mammalian glycans (N- and O-chains of glycoproteins, as well as glycolipids) and also bacterial polysaccharides. The sera samples correspond to the third, sixth, and twelfth months of life. The infants were divided into four groups according to their nutrition type: breast milk, standard formula, and partially or extensively hydrolyzed formula. During the first year of life, the total amount of IgG decreased; presumably, the lifetime of maternal IgG in the newborns' bloodstream is much greater than is generally assumed. At the same time, the IgM content was low during the first six months and increased significantly by the twelfth month. The antiglycan IgM repertoire of one-year-old infants was still different from that of their mothers, as well as from the repertoire of unrelated donors, in particular, by the absence of antibodies against the Galβ1-3GlcNAc (Le^C) disaccharide, which is found in almost all healthy humans. It is noteworthy that the level of IgM of breast-fed infants was significantly lower than that of formula-fed by the twelfth month.

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It is generally accepted that foreign antigens cause the formation of antibodies that are known as adaptive, and their levels and affinities are directly related to the presence of these antigens. At the same time, extensive experimental material has accumulated about antibodies whose level and affinity for the antigen are practically constant throughout life [1, 2]. These immunoglobulins, in contrast to the adaptive ones, are known as natural antibodies (nAbs). nAbs, being part of innate immunity, mediate various functions such as protection from pathogens, clearance of metabolites, surveillance of transformed cells, and regulation [3-7]. All classes of biomolecules can be found among the antigens recognized by nAbs; this is explained by the importance of the corresponding protective processes [3, 4]. In the context of nAbs origin, early age is of particular interest, since the immune system of a child has a special status: despite the very early onset of the development of immunity in ontogenesis, it is functionally immature; the child receives immunoglobulins G (IgG) from the mother's blood system, and there are practically no immunoglobulins M (IgM) in the child's blood [8-12].

It is generally believed that nAbs are primed largely under the influence of the intestinal microflora; therefore, the study of the relationship between the developing immunity, nutrition, and the microbial community is of great interest [13-15]. However, model organisms, in particular mice, with their specific microbiota composition, are not adequate models for studying human nAbs [16, 17]. Here we investigated the dynamics of antibody repertoires in children aged 3, 6, and 12 months, taking into account the effect of their nutrition. Pathogenic microorganisms possess a myriad of antigens, the structure of their glycoconjugates differing dramatically from those of mammals; hence, it is not surprising that nAbs against

Abbreviations: BM, breast milk; EHF, extensively hydrolyzed formula (milk); nAbs, natural antibodies; PHF, partially hydrolyzed formula (milk); SF, standard formula (milk).

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carbohydrate epitopes constitute an essential, if not dominant, part of their repertoire [18-20]. This allows us to make general immunological conclusions based on the study of antiglycan nAbs. At the same time, it is risky to make generalizations based on the study of only a few antibodies. Therefore, we profiled the nAbs using a representative array of 487 antigens.

MATERIALS AND METHODS

Microarrays were prepared from 341 different synthetic amine-functionalized glycans and 146 bacterial O-polysaccharides, using N-hydroxysuccinimide-derivatized glass slides (slide H; Schott-Nexterion, Germany), as described elsewhere [21]. The glycan library included blood group antigens and some of the most frequently occurring terminal oligosaccharides, as well as core motifs of mammalian N- and O-linked glycoproteins and glycolipids, tumor-associated carbohydrate antigens, and bacterial polysaccharides mainly as a carbohydrate part of lipopolysaccharides. Synthetic glycan structures (>95% purity, generally synthetized in the Laboratory of Carbohydrates, Institute of Bioorganic Chemistry, Russian Academy of Sciences) are structurally the same as natural ones. Structures, NMR data of polysaccharides, and related references can be found in http://csdb.glycoscience.ru/bacterial (Zelinsky Institute of Organic Chemistry, Russia). The glycans at concentrations of 50 μM and 10 μg/ml (for oligo- and polysaccharides, respectively) were printed in 6-12 replicates. A complete list of printed glycans can be found in Table S1 of the Supplement to this report on the journal website (http://protein.bio.msu.ru/biokhimiya) and Springer site (Link.springer.com). Two chips from each batch were analyzed using standard Complex Immunoglobulin Preparation (CIP; Immunogem, Russia) at a concentration of 1 mg/ml with biotinylated goat-anti-human Abs (Thermo Fisher Scientific, USA) at concentration of 10 μg/ml followed by streptavidin–Alexa Fluor 555 Conjugate (Thermo Fisher Scientific) at concentration of 1 μg/ml. Batches of printed microarrays with intra- and inter-chip correlation more than 0.9 were used. After printing, glycochips were blocked for 90 min at 25°C with blocking buffer (100 mM boric acid, 25 mM ethanolamine, 0.2% (v/v) Tween 20, pH 8.5) (Sigma-Aldrich, USA), which was then washed out with milli-Q grade water (Mediana-Filtr, Russia) and dried by the air using a Galaxy Mini-Array centrifuge (VWR International, South Korea). Blocked microarrays were stored at –20°C for further analysis. Before serum analysis, microarrays were incubated in an incubation chamber (Simport, Canada) for 15 min at 25°C with PBS (Sigma-Aldrich) plus 0.1% (v/v) Tween 20 (buffer 1), and the buffer was then carefully removed from the microchip surface using Whatman filter paper (Sigma-Aldrich). Human sera were diluted 1 : 15 in PBS plus 1% (w/v) BSA (Sigma-Aldrich) and 0.1% (v/v) Tween 20 (buffer 2). Diluted serum was spread over the slide surface and incubated with agitation (32-36 rpm) at 37°C for 90 min. After the incubation and a round of washing steps with buffer 1 and buffer 3 (PBS with 0.01% v/v Tween 20), the microarrays were incubated for 1 h at 37°C (32-36 rpm) with a mixture of goat-anti-human IgG conjugated to Alexa Fluor 555 and goat-anti-human IgM conjugated to Alexa Fluor 647 (Thermo Fisher Scientific) at a concentration of 8 μg/ml (each) in buffer 2. After another round of washing (buffer 1, buffer 3, and finally milli-Q grade water), the microarrays were dried by airflow using the Galaxy Mini-Array centrifuge. The microarrays were scanned using a ScanArray Gx scanner (PerkinElmer, USA) using excitation wavelength 543 and 635 nm. The resulting data were processed using ScanArray Express 4.0 software and the fixed 70 μm-diameter circle method as well as Microsoft Excel software. From 6 to 12 spot replicates represent each oligosaccharide or polysaccharide on the array, and data are reported as median RFU (relative fluorescent units) of replicates. Median deviation was measured as interquartile range. A signal as RFU exceeding the fluorescence intensity of the background value by a factor of five was considered as significant.

The blood of healthy adult donors, children aged 3, 6, and 12 months, as well as their mothers, was obtained by a standard fence from the ulnar vein. When selecting donors for the study, pathology of gestation, congenital diseases, and infections were excluded.

RESULTS

Antiglycan IgG and IgM during the first year of life. We used a glycan microarray to study the repertoires of children's antibodies at ages of 3, 6, and 12 months and those of their mothers immediately after delivery. The children were divided into groups according to the type of nutrition: breast milk (BM), standard formula milk (SF), as well as two specialized types: extensively hydrolyzed (EHF) and partially hydrolyzed (PHF) formulas.

During the first year of life, IgG diversity decreases. The opposite tendency is characteristic for IgM: at the age of 3 and 6 months, antiglycan IgM is practically undetectable, but at the age of 12 months the repertoire of antibodies of this class is diverse and comparable to adults. Figure 1 shows the time course of antiglycan IgG and IgM diversity (the specificity will be considered below) in the sera of children during the first year of life. Each bar represents the total number of antigens to which serum antibodies are bound, and also for comparison, the corresponding data for mothers and unrelated healthy adult donors are presented. The latter group was necessary because pregnant and lactating women are characterized by physiological immunodeficiency [22, 23].

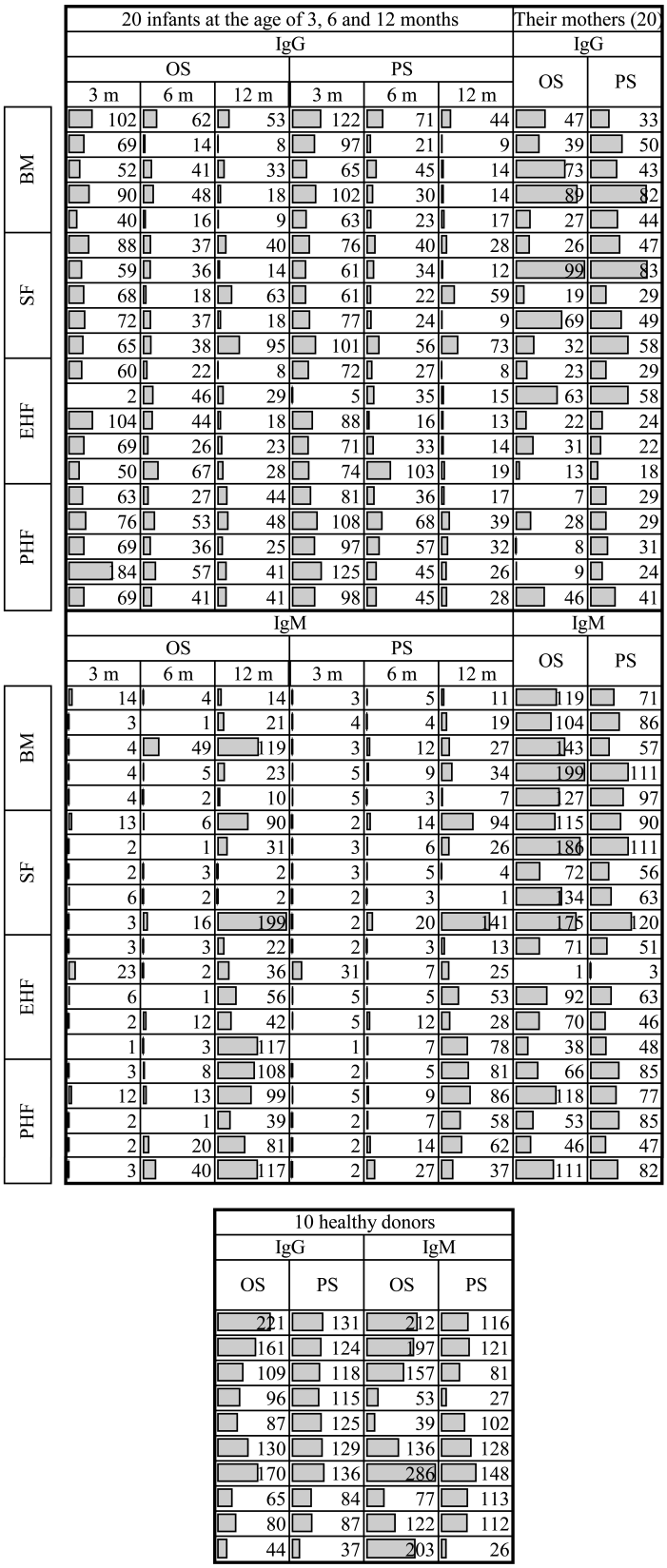


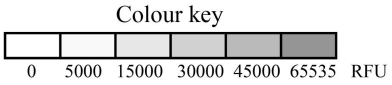
Fig. 1. Dynamics of antiglycan IgG and IgM in the sera of 3-, 6-, and 12-month-old infants with different types of nutrition, and in the sera of their mothers, and in 10 healthy unrelated adult donors. Each number represents the absolute number of glycans (oligo- and polysaccharides) that bind antibodies in the sera samples. OS, oligosaccharides; PS, polysaccharides; 3m, 6m, and 12m, age of children (months) at the time of blood sampling; BM, breast milk; SF, standard formula; PHF and EHF, partially and extensively hydrolyzed formula, respectively.

IgM repertoires in groups of infants who received different types of nutrition. Tendencies towards a decrease in the diversity of antiglycan IgG and the appearance of antiglycan IgM by only 12 months are characteristic of all children whose blood serums were studied in this work, regardless of what type of nutrition they received during the first year of life. At the age of 3 and 6 months, IgM is practically undetectable in children, and at 12 months, the repertoire of antiglycan antibodies of this class is comparable in diversity to an adult (Fig. 1). It should be noted that among bacterial polysaccharides to which there are antibodies, neither structural nor generic similarity is found; apparently, the appearance of antibodies to them occurs individually. In Fig. 2, a list of glycans is presented in the form of a color map demonstrating the maximum IgM binding signals in the blood sera of one-year-old children.

Comparison of the repertoires of IgM of infants receiving different types of nutrition and those of adult donors showed that infants fed with PHF exhibited the greatest variety of glycan-binding immunoglobulins, which is typical of adults. The table shows such a comparison for oligosaccharides and Table S2 (Supplement) for polysaccharides. Supplementary Table S3 contains all fluorescence signals for IgM capable of binding glycans in the serum of infants, their mothers, and unrelated adults.

It should be noted that almost all glycans (highlighted in the table) against which adults, but not 12-month-old children, have antibodies are structurally related to the Le^C disaccharide (Galβ1-3GlcNAcβ). Earlier, we have described nAbs with this specificity as anti-Le^C antibodies [24, 25].

IgG repertoires in the groups of infants who received different types of nutrition. The data are shown in Table S4 (Supplement). There are two reasons why we omit detailed discussion of IgG: (i) the nAbs mainly belong to IgM; (ii) it is impossible to discriminate between infant and maternal IgG, because IgG can cross the placental barrier during intrauterine development [26].



# glycan	Structure	Infants with different type of nutrition																			
		BM					SF					EHF					PHF				
002	Galα																				
003	Galβ																				
100	Galβ1-6Galβ																				
101	GalNAcα1-3GalNAcβ																				
102	GalNAcα1-3Galβ																				
103	GalNAcα1-3GalNAcα																				
111	Glcβ1-4Glcβ																				
113	GlcNAcβ1-3GalNAcα																				
118	GlcNAcβ1-6GalNAcα																				
137	Galβ1-4Glcβ-amino acid																				
142	GlcNAcα1-3GalNAcβ																				
146	Galβ1-4(6-O-Su)Glcβ																				
166	GlcAβ1-6Galβ																				
225	Galα1-4Galβ1-4GlcNAcβ																				
242	GlcNAcα1-3Galβ1-4GlcNAcβ																				
264	Galβ1-4Galβ1-4GlcNAcβ																				
396	$(GlcNAc\beta1)_3$-3,4,6-GalNAcα																				
485	Galβ1-4GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ																				
506	Ara_6																				
1008	S. enterica O67																				
1232	E. coli O14																				
1233	E. coli O19ab																				
1234	E. coli O27																				
1239	E. coli O54																				
1403	P. aerginosa O11ab																				
1603	P. mirabilis 3B-m																				
1605	P. mirabilis O11																				
1606	P. mirabilis O13																				
1610	P. mirabilis O31																				
1614	P. mirabilis O3ab																				

Fig. 2. Glycans showed maximal fluorescence signals of binding with IgM in sera of 12-month-old infants who received different type of nutrition. The data are represented as a thermal map where a darker shade of red color corresponds to a higher fluorescent signal. Designations as in Fig. 1 legend.

Comparison of IgM repertoires in 12-month-old children and unrelated adult donors

Glycan no.	Structure	Groups of children fed by different types of nutrition			
		BM	SF	PHF	EHF
020	Rhaα	+	+ +	+ +	+ + +
080	Galα1-3GlcNAcβ	+ +	+ + +	+	+ + + +
243	GlcNAcα1-3Galβ1-4GlcNAcβ	+ +	+ + +	+ +	+ + + + +
399	Galβ1-3GlcNAcα1-3Galβ1-3GlcNAcβ	+	+	+ + +	+ +
103	GalNAcα1-3GalNAcα	+ +	+ +	+ + + + +	+ + + +
102	GalNAcα1-3Galβ	+	+ +	+ + +	+ + + + +
142	GlcNAcα1-3GalNAcβ	+	+ + +	+ + +	+ + + + +
101	GalNAcα1-3GalNAcβ	+ + +	+ +	+ + + + +	+ + + + +
251	GlcNAcβ1-4Galβ1-4GlcNAcβ	+ +	+ + +	+ + +	+ + +
074	Fucβ1-3GlcNAcβ	+	+	–	–
140	Galα1-3GalNAc(fur)β	–	–	+	–
398	Galβ1-3GlcN(Fm)β1-3Galβ1-3GlcNAcβ	+	+	+	+ +
118	GlcNAcβ1-6GalNAcα	+	+ + +	–	+ + + + +
331	Neu5Gcα2-3Galβ1-3GlcNAcβ	+	+	–	–
307	KDNα2-3Galβ1-3GlcNAcβ	+	–	–	–
055	3-O-Su-GlcNAcβ	+	+	–	+ +
256	GlcNAcβ1-6(GlcNAcβ1-4)GalNAcα	+ +	+ +	+ + +	+ + +
267	GlcNAcβ1-3Galβ1-3GlcNAcβ	+	+ + +	–	–
264	Galβ1-4Galβ1-4GlcNAcβ	+ + + +	+ + +	+ + + + +	+ + + + +
085	Galβ1-3GlcNAcβ	+	–	–	–
246	GlcNAcβ1-2Galβ1-3GalNAcα	–	+ +	–	–
401	Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAcβ	+	–	–	–
378	Galβ1-3GlcNAcα1-3Galβ1-4GlcNAcβ	+	+	+ + +	+ + +
397	Galβ1-3GlcN(Fm)β1-3Galβ1-4GlcNAcβ	+	+	–	–
081	Galα1-4GlcNAcβ	–	+ +	+	+ + +
375	Galα1-4GlcNAcβ1-3Galβ1-4GlcNAcβ	+	+ +	+ + +	+ + + +
082	Galα1-4GlcNAcβ	–	+	+	–
072	Fucα1-3GlcNAcβ	–	+ +	–	–
019	ManNAcβ-Gly	+ +	+ +	+ +	+ + + + +
113	GlcNAcβ1-3GalNAcα	–	+ + +	+ + +	+ + + + +
149	GlcNAcβ1-4(6-O-Su)GlcNAcβ	+ +	+ +	+	+ +
117	GlcNAcβ1-4GlcNAcβ-Gly	+	+ +	–	+ + + +
382	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ	–	–	+ + +	–
164	GlcAβ1-3GlcNAcβ	–	+ +	–	–
380	Galβ1-3GlcNAcα1-6Galβ1-4GlcNAcβ	–	–	–	–
161	6-O-Su-Galβ1-3GlcNAcβ	+	–	–	–
073	Fucα1-4GlcNAcβ	–	+ +	–	–
263	$(GalNAcβ\text{-}PEG_2)_3$-β-Lys-Lys	+ +	+ +	+ +	+ + + +
299	Neu5Acα2-3Galβ1-3GlcNAcβ	+	–	–	–
010	GlcNAcβ	–	+	–	–

Note: The table represents 40 oligosaccharides whose signals for binding to antibodies have the maximum intensity in adult donors (glycans are sorted in descending order of the signal; for 10 donors, the median of the signal values is measured). The right side of the table presents the signals of binding of antibodies to these glycans in the serum of 12-month-old children who received different types of nutrition. The presence of a meaningful binding signal for each child is indicated as "+". The binding of antibodies to glycans, highlighted in gray in the table, is practically absent in children.

DISCUSSION

By the time of birth, the B-cell system of immunity is morphologically formed. In contrast, functional maturity develops for a rather long time after birth, almost until adolescence [9, 27]. However, the most significant events from the point of view of the formation of the immune system occur during the first year of life [13-15]. The origin of nAbs has been studied in model systems, but the results of these studies should be interpreted with caution, since the repertoire of animal (including mouse) nAbs differs significantly from the human one [16, 17]. In addition, other animals and humans substantially differ in the permeability of the intestinal epithelium for immunoglobulins; for example, maternal IgG of rodents, cattle, and cats enter the bloodstream of the offspring through milk. In humans, the process of IgG transfer from mother to child ends at the time of their physical separation, i.e., at the moment of birth [28].

The first months of a child's life are characterized by the so-called physiological (in other words, natural and necessary) immunosuppression, with reduced numbers of neutrophils and proinflammatory cytokines [29, 30]. At first glance, a baby may seem defenseless, but this immunosuppression proves necessary, since it provides the possibility of forming tolerance to antigens of regular microbiota and food; it also ensures synchronous development of the components of the immune system in the course of interaction of the infant with new environmental antigens [31-33]. While humoral immunity is gradually forming, the role of protection is performed by acute phase proteins, which are capable of providing short-term nonspecific protection in the cases of injuries and infections, as well as components of the mother's milk, such as antimicrobial peptides. The recognition of antigens by antigen-presenting cells (APCs) also has age-related characteristics. Specifically, the production of TNFα (tumor necrosis factor-alpha), IFNγ (interferon-gamma), and IL-12 (interleukin-12) is decreased in newborns, while IL-10 (interleukin-10) and IL-6 (interleukin-6) are secreted at the rate typical of normal adults [34, 35].

Glycans of microorganisms are among the antigens that are the most important for the early development of the immune system, because, in contrast to peptide antigens, they can be recognized, first, without the formation of an MHC (main histocompatibility complex) complex and second, directly by B cells, which in this case act as APCs due to the characteristic structure of the B cell receptor (BCR). At the same time, the amount of the antigen itself can influence the immune response of B cells: a small amount of glycan cannot activate the B cell, because the BCR molecule has to simultaneously bind many copies of this antigen to transmit the signal into the cell [36-39].

Antiglycan antibodies are a convenient object for studying the general principles of the formation of nAbs. The repertoire of antiglycan Abs is wide [19, 40], and a considerable part of it (almost all non-allo-antibodies) is the same in all individuals; in addition, there is a convenient tool for their identification, namely, our glycochip [41-43]. We studied the sera of healthy infants as well as their mothers and ten unrelated healthy adults. This third group was necessary for estimating the average repertoire in the population, because of the specifics of the antibody level and composition in pregnant and lactating women [22, 23].

The diversity of antiglycan IgG in the period from 3 to 12 months of age decreased (Fig. 1); this was observed for both antibodies against mammalian glycans and those against bacterial polysaccharides. The maximum diversity of IgG observed at the age of 3 months corresponded to the maternal repertoire; during the next several months, until the age of one year, their diversity was decreasing; this agreed with the literature data [44, 45]. However, there is one important contradiction. It is known [46] that the half-life of the IgG molecule does not exceed 25 days, which means that either infants' antibodies at the age of 3 months are not of maternal origin or their circulation time in children's blood is much longer than it is believed to be. We assume that IgG transferred into the fetus can have an extended circulating time compared with conventional IgG. Indeed, according to some studies, antibodies of pregnant women have a number of specific characteristics, in particular, an altered glycosylation [47, 48], which may increase the circulation time of the immunoglobulin. By the way, the longer circulation provides protection for the period necessary for the appearance of IgM. It is also likely that the late IgM production and prolonged maternal IgG existence both maintain the formation of oral tolerance against self-antigens and commensal bacteria.

The time course of IgM changes in the period from 3 to 12 months of age was completely different than in the case of IgG; at ages of 3 and 6 months, the IgM range was still narrow (Fig. 1) compared with adults. By the age of 12 months their repertoire was extended. Since IgM in normal pregnancy cannot cross the placenta, the production of IgM in infants has not yet reach a sufficient level; therefore, according to the published data, the proportion of antibodies of this class in newborns does not exceed 6-10% of the adult level [44]. It should be noted that, in the group of breastfed (BM) children, the antibody repertoire was different from the three other groups; the repertoire of their antiglycan IgM was the least similar to the "adult" one. At first glance, this finding is unexpected, because the BM infant remains in close contact with the mother; i.e., they are under much more favorable conditions than the children from the other three groups. A possible explanation is that breast milk contains antimicrobial peptides, proteins (including immunoglobulins), and glycans (free glycans and glycoconjugates) [35, 49], which fulfill the function of antibodies, if only partly. In other

words, a wide variety of antibodies under these conditions is not necessary. Then, the accelerated formation of nAbs under the conditions of artificial nutrition can be interpreted as untimely and, hence, less demanded. However, it should be noted that this difference in the repertoire is not extremely pronounced.

The maximum variety of IgM was observed in children fed with PHF, which is a mixture of partially hydrolyzed milk proteins, mostly peptides of higher sizes, which are a reservoir of antigenic determinants. These peptides may serve as mimotopes [50-54] in the process of priming of antiglycan B1 cells.

We should especially note the absence of antibodies against the Le^C epitope in all of the 20 children at the age of 12 months; conversely, in practically all tested adults (~150), anti-Le^C belongs to antibodies of top rank [55]. These antibodies seem to play a role in the surveillance of the appearance of tumor cells [56-58]. Why they are completely absent at early age, when they appear, and what triggers their priming, remains to be seen. At the same time, most other top-rank antiglycan nAbs, such as those against Rha, Galα1-4(3)GlcNAc, and GlcNAcα1-3Galβ1-4GlcNAcβ, are already present in children at 12 months of age (table). Moreover, the titers and the occurrence of other antibodies against tumor-associated glycans, including GalNAcα1-3GalNAcβ (Fs-2, carcinomas [59], no. 101 in Fig. 2) and Galβ1-4Galβ1-4GlcNAcβ (melanoma, no. 264 [60]), in children are higher than those of other antibodies.

In summary, the natural antibodies of children only approach the repertoire for adults by 12 months of age. It is interesting that the appearance of some antibodies, such as anti-Le^C, is delayed to an older age in all studied children. This can be explained by the fact that Le^C is not merely a tumor-associated, but also onco-embryonic antigen; this will need to be explored experimentally. In addition, judging by our results, the lifetime of the maternal IgG in the infant's body seems to be substantially longer than commonly believed.

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Conflict of interest. The authors declare no conflict of interest in financial or in any other area.

Ethical approval. The blood of healthy adult donors, children aged 3, 6, and 12 months, as well as their mothers, was obtained by a standard fence from the ulnar vein with the informed consent of the responsible persons and/or their representatives in accordance with the principles described in the Helsinki Declaration and confirmed by the decision of the Ethical Committee of the University of Frontera (Temuco, Chile).

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