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Polysaccharide Kinase Activity of Human Milk IgG Antibodies

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Abstract—A small fraction of human milk IgG antibodies is shown to possess polysaccharide kinase activity for the first time. Unlike all known kinases, IgG antibodies can use as phosphate donor not only [γ-^{32}P]ATP, but also directly [^{32}P]*ortho*-phosphate. Human milk IgGs therefore possess high affinity to *ortho*-phosphate (K_m = 9-71 μM), which is a more effective substrate than ATP. IgG antibodies possessing polysaccharide kinase activity are yet another example of natural abzymes possessing not hydrolytic, but synthetic enzymatic activity.

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Investigation of antibodies (Ab) possessing enzymatic activities (abzymes) is one of new directions in immunology and biochemistry during recent years [1]. At present, the number of reactions catalyzed by abzymes against chemically stable analogs of intermediates is more than 100, and the substrate specificity of some abzymes exceeds that in enzymes with analogous activities [1-4]. Some abzymes have no analogs among known enzymes [1-5].

The first example of natural abzymes was Ab catalyzing hydrolysis of vasoactive neuropeptide from blood of patients with asthma [4-9], then IgG hydrolyzing DNA [10] and RNA [11] were discovered in patients with systemic lupus erythematosus (SLE). Now IgG and/or IgM Abs hydrolyzing DNA, RNA, proteins, and polysaccharides have been described in blood of patients with various autoimmune diseases (AID) (SLE, autoimmune thyroiditis, polyarthritis, multiple sclerosis, lymphoproliferative diseases, polyneuritis, malignant tumors, as well as virus hepatitis, and AIDS) [4-8]. On the other hand, antibodies from blood of healthy volunteers and patients with influenza, pneumonia, tuberculosis, tonsillitis, duodenal ulcer, and uterus, breast, or intestinal cancers did not display any DNA- or RNA-hydrolyzing activity [12-14].

The first example of abzymes in humans with no detected diseases were human milk sIgA catalyzing phosphorylation of ~15 milk proteins in milk of healthy women in labor [15-17]. The milk of women in labor proved to be a unique source of a number of various abzymes. sIgA and/or IgG discovered later were hydrolyzing DNA and RNA [18-20], ATP and other nucleotides [21, 22], polysaccharides [23-25], and casein [26]. A particular feature of these abzymes is their higher activity than in Abs from blood of patients with various AIDs [4-8, 18-25].

Fractions of unique IgG and sIgA Abs tightly bound to minor lipids of unusual structure and effectively phosphorylating these lipids are found in human milk [27-29]. A small fraction of milk sIgA Abs is shown also to possess polysaccharide kinase activity [30]. Interaction of milk sIgA Abs with DNA and RNA and their influence on protein and lipid kinase activities, as well as interaction of Abs with chemically active ATP analog are studied [31-34].

On the basis of literature data analysis (some of these data are given below) we came to the conclusion that women during pregnancy and beginning of lactation are characterized by specific immune status [4-8, 20]. The blood of pregnant and lactating women, like blood of patients with AIDs, contains DNA at elevated concentrations [35, 36]. Besides, the blood of pregnant women contains fetal cells, and a correlation is found between elevated frequency of their occurrence and autoimmune scleroderma morbidity [37]. Rheumatoid arthritis abates or disappears during pregnancy [36], and the state of patients with SLE and anti-phospholipid syndrome, as well as number of other AIDs becomes apparently worsened [38,

Abbreviations: Ab) antibody; AI) autoimmune; AID) autoimmune diseases; DTT) dithiothreitol; SLE) systemic lupus erythematosus; TLC) thin-layer chromatography.

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39]. Autoimmune stress, i.e. acute exacerbation of autoimmune (AI) reactions, is sometimes observed just after delivery. Independently of whether women in labor have autoimmune stress, some of them develop a number of postnatal AI pathologies, such as thyroiditis, kidney insufficiency, hemolytic-uremic syndrome, idiopathic polymyositis, anti-phospholipid syndrome, and AI-myocarditis [37-39]. Postnatal AIDs can manifest just after delivery or later. Postnatal autoimmune thyroiditis is one of the most common postnatal disorders. The frequency of its occurrence evaluated by various investigators varies from 1.9 to 16.7% [40, 41], while disappearance of AID signs in women in late postnatal period is normal, although in some cases "temporal activation of AI processes" sometimes gradually or drastically (through AI-stress) transits into typical chronic AI processes.

From our point of view, a particular feature of immune system reorganization in pregnant woman is "switch-on" a special immune memory, which accumulates during the pregnancy all the information on dangerous factors of the environment [4-8, 19, 20]. This information is "used" by the immune system particularly during the pregnancy period, but basically just after the beginning of lactation. In mammals, this results in the fact that their immunization with various antigens 1-3 months (but no longer) before the delivery results in very high content of Abs to these antigens in their milk [42, 43]. The route of the antigen entrance into the mammalian body (injection into blood or per oral, as a component of food, bacteria, or viruses) makes no difference.

Data on the dynamics of changes in abzyme activity in blood of pregnant and lactating women are important for understanding the special role of the initial lactation period in "switching of the immune system" of pregnant women. A tendency to elevation of Abs hydrolyzing DNA and ATP in the first and/or third trimester of pregnancy is revealed [44]. Relative activities of abzymes in blood of women are elevated 4-5 times just after the beginning of lactation. However, the activity of milk abzymes in the same women is 5-600-fold higher than that in blood [44]. The level of DNase activity in IgG and IgM Abs from blood of pregnant women with obvious AI-thyroiditis induced by pregnancy is 4-5-fold higher than in healthy women [44]. Note, that the levels of DNA- and RNA-hydrolyzing abzymes in milk of women increased by several orders of magnitude when they suffered from viral or allergic disorders during their pregnancy [19, 20]. It was suggested that some programming of the immune system may happen as a result of specific pre-immunization of a woman's body to produce not only Ab which is capable of binding antigens, but also specific abzymes playing an important role in defense of the newborn child from harmful environmental factors [4-8, 44].

In the initial lactation period, the concentration of oligosaccharides in human milk reaches 20 g/liter with subsequent decrease to 13 g/liter [45, 46]. More than 100 types of milk oligosaccharides have been described, which are divided into two general groups. The first consists of neutral oligosaccharides, the central part of which composes lactose. They contain also fructose, galactose, and N-acetylglucosamine. The second group consists of acidic oligosaccharides, containing, besides the listed carbohydrates, also N-acetyl-neuraminic acid [47, 48]. Enzymes are known that are responsible for the synthesis of oligosaccharides in mammary glands [49].

Oligosaccharides in human milk act as prebiotics [50] as well as inhibit binding of various intestinal and respiratory pathogens, such as *Streptococcus pneumonia*, *Haemophilus influenzae* [51], enteropathogenic strains of *Escherichia coli* [52, 53], and *Campylobacter jejuni* [54], to specific cellular receptors. A protective effect of milk oligosaccharides against *Neisseria meningitis pili* has been described [55]. Protective functions of oligosaccharides are determined by their ability to act as cellular receptor analogs, which bind to pathogens, as well as to take the place of the pathogens by binding with their specific cellular receptors [56].

Human milk also contains acidic polysaccharides—glucosaminoglycans—interacting with membranes of milk fat globules [57]. These polysaccharides containing chondroitin sulfate hinder the binding of glycoprotein gp120 HIV with CD4-receptors on target cells [58].

During the first several months of life, the immune system of newborns is not yet fully formed: the mucous membranes of the respiratory and alimentary tracts are devoid of Abs [59, 60]. It is thought that protective mechanisms in newborns (passive immunity) are mostly provided by Abs from maternal milk [59, 60]. Human milk contains IgG, IgM, IgA, and sIgA. The most part comprise sIgA (80-90%), which are produced by B-lymphocytes of the local immune system of the mammary gland [61]. There is data that the total pool of IgG is formed due to Ab transport from blood as well as a result of their local excretion by B-cells of the mammary gland [61]. It is thought that unlike sIgA, which cover the alimentary mucous membrane, IgG of milk of women in labor penetrate into the blood of newborns through their intestinal epithelium.

As we have shown earlier, the specific activity of sIgA in DNA, RNA, and ATP hydrolysis is substantially higher that that of IgG Abs [3-8]. Moreover, sIgA and IgG abzymes with these activities differ in their substrate specificity and particularly in structure of their active centers [3-8]. Taking into account this data, as well as different origin and different functions of milk sIgA and IgG [20], it is of interest to compare enzymatic functions of these Abs in the reaction of polysaccharide phosphorylation.

In the present work we found that human milk IgG possess the ability to phosphorylate endogenous tightly bound oligo- and polysaccharides. The activity of IgG antibodies is compared with earlier described sIgA with polysaccharide kinase activity [30].

MATERIALS AND METHODS

Purification of antibodies. Milk of 15 women (19-35 years old), clinically healthy in all respects, was collected 1-4 weeks after the beginning of lactation. To remove fats, lipids, and cells the milk (0.1-0.15 liters) was centrifuged for 60 min at $1.2 \cdot 10^4$ rpm with subsequent removal of upper lipid fraction and cellular pellet. Ig preparations were obtained as described elsewhere [19, 28-30], but with some modifications. The supernatant was applied on a Protein G-Sepharose column (2 ml), pre-equilibrated with the buffer A containing 20 mM Tris-HCl, pH 7.5, and 150 mM NaCl. Another column with Protein A-Sepharose (20 ml) was connected with the effluent of this column for specific sorption of sIgA and IgM Abs. Proteins, which do not interact with the sorbents were washed with the same buffer to the complete disappearance of optical absorption at 280 nm. Nonspecifically absorbed proteins were eluted with the buffer A containing 1% Triton X-100, and the columns were washed with 50 mM Tris-HCl, pH 7.5. IgG Abs were eluted from Protein G-Sepharose (after detachment from the column with Protein A-Sepharose) with 0.1 M glycine-HCl, pH 2.6. The fractions were neutralized with Tris-HCl buffer (pH 9.0) just after their elution and dialyzed for 12 h against 20 mM Tris-HCl, pH 7.5.

FPLC gel filtration on the column with Superdex 200 HR 10/30 (Pfizer, USA) using a BioCAD chromatograph (Applied Biosystems, USA) was carried out for additional purification of IgG preparations, as well as to prove that the activity belongs directly to the Abs. With this aim, the IgG preparations (1-3 mg/ml, 0.1 ml) were incubated for 30 min at 30°C in 20 mM glycine-HCl buffer, pH 2.6, containing 0.2 M NaCl. The column was equilibrated with the same buffer. Fractions were collected into tubes each containing 50 μl 1 M Tris-HCl, pH 9.0, for neutralization, and then the samples were additionally neutralized with the same buffer and dialyzed against 20 mM Tris-HCl, pH 7.5, for 16 h at 4°C. Since Abs were subjected to "acid shock", the activity in the fractions collected after chromatography and gel filtration was determined after 1-2 weeks of their incubation in neutral solutions at 4°C.

Electrophoresis of antibodies in polyacrylamide gel in the presence of SDS. Homogeneity of IgG preparations at all stages of the study was analyzed by electrophoresis under non-dissociating conditions in 5-16% polyacrylamide gel containing 0.1% SDS. Electrophoresis under dissociating conditions was carried out in 12% polyacrylamide gel in the presence of 50 mM dithiothreitol (DTT) (0.1% SDS), as described earlier [28-30]. Before the electrophoresis the Ab preparations were boiled for 5 min in buffer containing 1% SDS. After the electrophoretic separation, the proteins were stained with $AgNO_3$ [62]. Intact IgG and their separate units were additionally identified by immunoblotting using monoclonal murine Abs against light and heavy chains of human Abs, as described earlier [28-30].

Analysis of polysaccharide kinase activity of Abs. Polysaccharide kinase activity was measured out under optimum conditions found by varying concentrations of NaCl (1-100 mM), $MgCl_2$ (0.1-3 mM), and buffer (5-50 mM), as well as pH of the buffer (from 6 to 8). These conditions were virtually no different from those described earlier for the study of polysaccharide kinase activity in sIgA Abs [30]. The reaction mixture (10-20 μl) contained 10 mM Tris-HCl, pH 7.5, 70 mM NaCl, 1 mM $MgCl_2$, 0.025-1 μM [^{32}P]*ortho*-phosphate (10-20 μCi) or 0.025-1 μM [γ-^{32}P]ATP (10-20 μCi), and 0.01-0.5 mg/ml IgG. The reaction was initiated by the addition of Ab, and the samples were incubated at 37°C for 0.5-2 h. The reaction was stopped by the addition of 10-20 μl 10% TCA, and the pellet was removed by centrifugation. The supernatant was then brought to the volume of 50 μl, and lipids were extracted with 150 μl of chloroform–methanol mixture (2 : 1 v/v). After vigorous shaking, the mixtures were incubated to complete separation of organic and aqueous phases, the upper aqueous phase was sampled and dried, and the product was dissolved in 10 μl of water. The reaction products of oligosaccharide phosphorylation were analyzed by thin-layer chromatography (TLC) on Kieselgel F_{254} plates (Merck, Germany) in the system dioxane–ammonium–water (5 : 1 : 4 v/v). The plates were dried and subjected to autoradiography.

The stability of the complex between oligosaccharides and Abs was determined via inhibition of the phosphorylation reaction of exogenous oligo- and polysaccharides bound to Abs. To do this, standard reaction mixtures containing 1.3 mg/ml Ab were preincubated for 24 h at 37°C with one of the following polysaccharides (0.01 mg/ml): β-glucan, xylan, inulin, mannan, lichenan, and arabinogalactan. Then the phosphorylation reaction was initiated by the addition of [^{32}P]*ortho*-phosphate. The extent of phosphorylation of oligosaccharides was analyzed by TLC as described above.

Determination of polysaccharide kinase activity of Ab after electrophoresis in polyacrylamide gel. Homogeneous preparations of Abs (7-10 μg) were subjected to electrophoresis in 4-15% polyacrylamide gel containing 0.1% SDS. The Abs were treated for 10 min with buffer containing 1% SDS at 37°C before the electrophoresis. Then the lengthwise gel of the control lane was stained with Coomassie R-250 solution. The gel strips corresponding to sample lanes were washed with water (3 times, 10-15 min each) and dissected to into cross-sections 2-3 mm in length. To elute the proteins from the gel, as well as reconstitute Ab activity, the gel was thoroughly ground and incubated for a week with 50 μl of buffer containing 50 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$ at 4°C with periodic shaking. The gel was removed by centrifugation (10 min at 4000 rpm), and aliquots of the supernatant (15-20 μl) were used for catalytic activity determination.

It was shown in separate experiments using complexes between Abs and ^{32}P-labeled oligo- and polysaccharides that Ab preparations virtually completely loose their bound ^{32}P-labeled ligands after SDS-PAGE. Taking this fact into account, exogenous oligosaccharides were added to the reaction mixtures when polysaccharide kinase activity was determined in preparations of Abs extracted from the gel. For the preparation of concentrated solutions of exogenous oligosaccharides, lipids were extracted from 0.5 ml of Ab solution (6 mg/ml) with 1.5 ml chloroform–methanol mixture. Proteins were precipitated by the addition of 100 μl 0.1 M HCl, and the acid was then removed by evaporation of the solution to dryness on a rotary evaporator. Then the dry residue was solved twice in small volumes of water and subjected to repeated evaporation. The product was dissolved in 100 μl of water and evaporated to 10-20 μl. Aliquots of 3-5 μl were taken and added to the reaction mixture (30 μl) containing 15-20 μl of Abs extracted from gel. For binding of Ab with oligosaccharide, the mixture was incubated for 24 h at 37°C. The phosphorylation reaction was initiated by the addition of 10-20 μCi [^{32}P]*ortho*-phosphate, and the relative activity of fractions was determined by TLC or electrophoresis as described above.

Obtaining and analysis of IgG Fab-fragments. Fab-fragments were generated by the hydrolysis of IgG with papain [63]. The reaction mixture (1 ml) containing 0.5-1.0 mg of Ab, 50 mM Tris-HCl buffer, pH 7.5, 200 mM NaCl, 1 mM EDTA, and 1 mg papain was incubated for 2 h at 37°C. The mixture was dialyzed for 14 h at 8°C against buffer A. Fab-fragments were separated from intact immunoglobulins and Fc-fragments by chromatography on Protein A-Sepharose by analogy with the technique of Ab isolation on Protein G-Sepharose (see above). The fraction eluted during the application containing Fab-fragments and papain was dialyzed for 14 h at 4°C against 20 mM Tris-HCl buffer (pH 7.5), concentrated, and applied on a column with Superdex-75 equilibrated with the same buffer. The protein was eluted with 0.5 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl. The fractions containing Fab-fragments homogeneous according to electrophoresis data were combined and dialyzed for 14 h at 4°C against buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.01% NaN_3. Then the catalytic activity was analyzed in the Fab-fragment preparations.

Hydrolysis of ^{32}P-labeled oligo- and polysaccharides. Preparative Ab-dependent phosphorylation of oligo- and polysaccharides (0.2 mg Ab) was carried out as described above to analyze hydrolysis of oligo- and polysaccharides firmly bound to IgG by various polysaccharide-hydrolyzing enzymes. Then the fraction of ^{32}P-labeled oligo- and polysaccharides was separated from proteins and lipids according to the technique described above (about 60 μl of the solution was obtained) and used for the analysis of their hydrolysis by various enzymes.

The reaction mixture (10-20 μl) contained 10 mM Tris-HCl (pH 7.5), 0.01 mg/ml of one from 11 glycosidases (Table 1), and 5 μl of ^{32}P-labeled oligo- and polysaccharide solution. The hydrolysis reaction was carried out at 37°C for 24 h. The products of ^{32}P-labeled oligosaccharide hydrolysis (3-5 μl reaction mixture) were analyzed by TLC, and ^{32}P-labeled polysaccharides were analyzed by electrophoresis in 20% polyacrylamide gel with SDS in buffer containing 0.1 M Tris-borate, pH 8.6, according to the method described in [30]. Plates for TLC and gels after electrophoresis were scanned, and then the hydrolysis extent of initial oligo- and polysaccharides was evaluated using Image Quant 5.2 software.

Determination of kinetic parameters of the phosphorylation reaction. All the kinetics parameters were measured using the initial sections of linear dependence of accumulation of phosphorylation products of oligo- and polysaccharides on the time and Ab concentration. When K_m values were determined, the concentrations of both *ortho*-phosphate and ATP were varied in broad ranges (0.1-50 μM). The data of autoradiogram scanning and a calibration curve obtained using various amounts of [^{32}P]*ortho*-phosphate or [γ-^{32}P]ATP with known radioactivity were used for calculation of the formed product. The values of K_m and V_{max} (k_{cat}) were obtained by nonlinear regression using Microcal Origin v5.0 software, as well as by linearization of data according to the methods of Lineweaver–Burk, Eadie–Hofstee, and Cornish–Bowden [59]. The error of value determinations did not exceed 20-30%.

Chemicals and materials. The following chemicals were used in the study: acrylamide, N,N′-methylene-bis-acrylamide, glycine, Triton X-100, bromophenol blue, TEMED (Sigma, USA); SDS and Kieselgel F_{254} plates (Merck); dithiothreitol (Serva, Germany); Tris (Helicon, Russia); sorbents Protein G-Sepharose, Protein A-Sepharose, and Superdex 200 HR 10/30 (Pharmacia, Sweden); polysaccharide-hydrolyzing enzymes from Sigma and ICN (USA); radioactively labeled chemicals (3000 Ci/mmol) from Amersham (USA). All other chemicals were of the highest purity grade.

RESULTS AND DISCUSSION

Preparation of IgG antibodies. In the present work, we studied catalytic properties of IgG abzymes phosphorylating oligo- and polysaccharides and compared them with sIgA described in an earlier study [30]. Total fractions of Ig (sIgA + IgG + IgM) were obtained earlier by the affinity chromatography of milk proteins on Protein A-Sepharose with following separation of these Abs into subfractions by FPLC gel filtration [17-26]. In the present study, electrophoretically and immunologically homogeneous IgG Ab preparations were isolated for the first time by affinity chromatography on Protein G-

Table 1. Relative hydrolysis efficacy of ^{32}P-labeled oligo- and polysaccharides isolated from IgG preparations by eleven polysaccharide-hydrolyzing enzymes

No.	Enzyme/source	Enzymes and type of hydrolyzed bond	Band number on electrophoresis*				Band number on TLC*	
			2	3	4	5	I	II
1	β-Glycosidase	β-1,6-glycosidase; EC 3.2.1.21	—	+++	+++	++	+++	+
2	Cellobiohydrolase from *Geotrichum candidum*	1,4-β-D-glucan-cellobiohydrolase (1,4-β-cellobiohydrolase); EC 3.2.1.91	—	+++	++	+	+	+
3	Glucoamylase from *Aspergillus awamori*	1,4-α-D-glucan-glucohydrolase; EC 3.2.1.3	—	—	+	—	—	—
4	α-Galactosidase from *Trichoderma reesei*	α-D-galactoside galactohydrolase (melibiase); EC 3.2.1.22	+**	+++	++	++	+	+++
5	β-1,3-Glucanase from yeast	1,3-β-glucanase; EC 3.2.1.39	—	—	+	+	—	—
6	Lichenase from *Bacillus licheniformis*	1,3-1,4-β-D-glucan-4-glucanohydrolase (lichenase); EC 3.2.1.73	++	+++	+	+	+	+
7	α-Mannosidase from *Oerskovia* sp.	α-D-mannoside mannohydrolase; EC 3.2.1.24	—	+++	+++	+++	++	+++
8	Exo-1,3-β-glucanase from *Trichoderma viride*	1,3-β-D-glucan-glucanohydrolase; EC 3.2.1.58	—	++	+	—	—	+
9	α-Amylase from pancreas	1,4-α-D-glucan-glucanohydrolase; EC 3.2.1.1	—	++	+	+	+	+
10	Endo-F from *Chryseobacterium meningosepticum*	endo-β-N-acetylglucosamidase F; EC 3.2.1.96	++	difficult to determine ***	—	+++	—	—
11	α-L-Fucosidase from *Thermus* sp.	α-L-fucoside-fucosidase; EC 3.2.1.51	—	—	—	—	—	—

* The data corresponds to the mean from three experiments given in Fig. 6.
** Symbol "+++" corresponds to maximum hydrolysis level in the analyzed oligo- or polysaccharide band.
*** In some cases located above oligo- or polysaccharides give hydrolysis products comparable with the initial compounds in their mobility.

Sepharose under conditions destroying nonspecific complexes and selectively eluting Abs (Fig. 1a). Silver staining after SDS-PAGE of intact preparations showed only IgG, any admixtures of other proteins were absent, and only light and heavy chains were detected after the reduction of the Ab with DTT (Fig. 1b). Identity of the protein bands to IgG was confirmed by immunoblotting with monoclonal Abs against light and heavy chains of human IgG.

There are no data in the literature concerning enzymes using *ortho*-phosphate as a phosphate group donor for phosphorylation of any substrates, while our data are evidence that sIgA (but not other human milk proteins) phosphorylates proteins, lipids, and polysaccharides in the presence of [^{32}P]*ortho*-phosphate [27-30]. Milk IgG possessing lipid kinase activity was shown also to use *ortho*-phosphate as a substrate [28, 29]. Taking into account this unique substrate specificity of milk Abs with kinase activities, one may anticipate that only milk IgG can phosphorylate oligo- and polysaccharides in the presence of [^{32}P]*ortho*-phosphate. To test this supposition, it is necessary to remove all Abs from milk. With this aim, a column with Protein A-Sepharose binding IgA, sIgA, and IgM Abs was tandemly attached to the column with

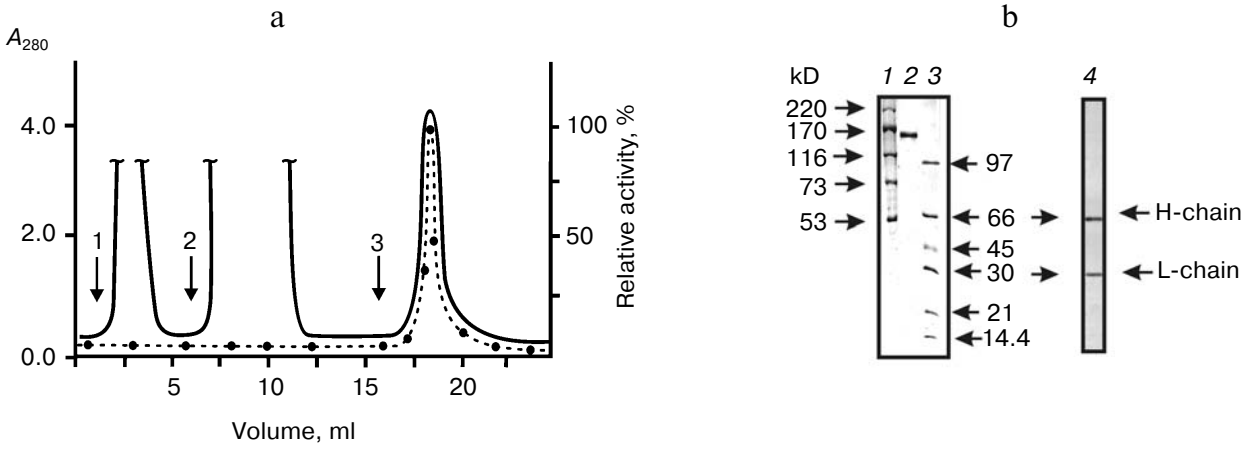

Fig. 1. a) Human milk protein profile from affinity chromatography on a Protein G-Sepharose column tandemly joined with a Protein A-Sepharose column. Solid line, the eluate optical absorbance at 280 nm; points, polysaccharide kinase activity profile (the activity of the fraction with maximum phosphorylation level is taken as 100%). The following fractions are indicated: fractions corresponding to the loading of milk proteins (peak 1), to the elution with buffer containing 1% Triton X-100 and 0.5 M NaCl (peak 2), and to the elution of IgG antibody from Protein G-Sepharose with acidic buffer, pH 2.6 (peak 3). b) Analysis of homogeneity of IgG preparations by SDS-PAGE in a 5-16% gradient polyacrylamide gel under non-dissociating conditions (lane *2*) and in 12% polyacrylamide gel under dissociating conditions (lane *4*). Lanes *1* and *3* correspond to protein markers with known molecular masses.

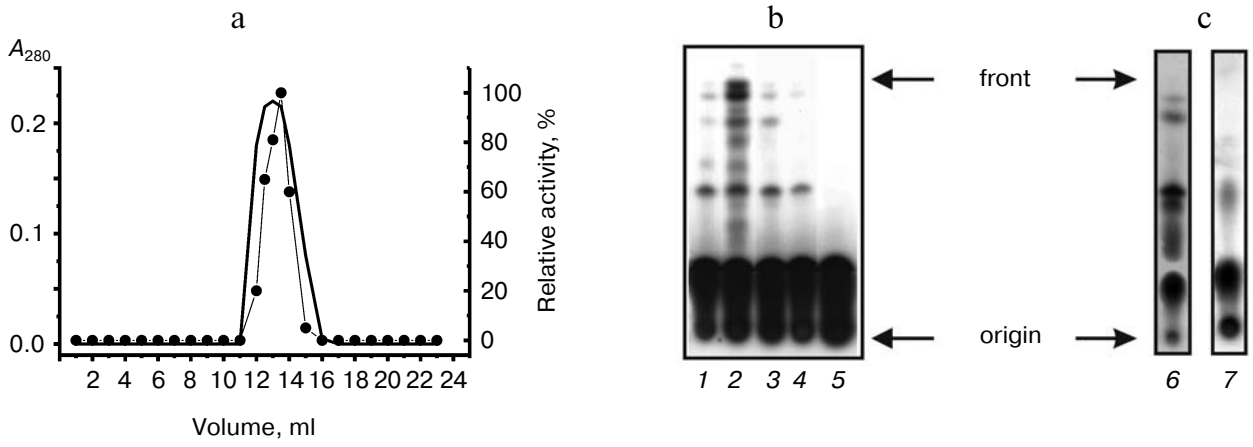

Fig. 2. a) Profile of IgG preparation during gel filtration on Superdex 200 HR 10/30 under "acid shock" conditions. Solid line, protein optical density at 280 nm; points, oligosaccharide kinase activity profile (the activity of the fraction with maximum phosphorylation level is taken as 100%). b) Analysis of oligosaccharide phosphorylation by TLC. Lanes: *1-4*) incubation of IgG isolated from milk of four different individuals, with [^{32}P]*ortho*-phosphate; *5*) incubation of [^{32}P]*ortho*-phosphate in absence of IgG. c) Comparative analysis of oligosaccharide phosphorylation by a single IgG preparation in the presence of [^{32}P]*ortho*-phosphate (lane *6*) and [γ-^{32}P]ATP (lane *7*).

Protein G-Sepharose interacting with IgG Abs. Note, in the case when [^{32}P]*ortho*-phosphate was used as a substrate, the milk proteins not interacting with these sorbents, did not possess oligo- and polysaccharide kinase activity (fractions 1 and 2, Fig. 1a). This is evidence that human milk does not contain any enzymes except Abs capable of phosphorylating oligo- and polysaccharides in the presence of [^{32}P]*ortho*-phosphate.

One of the criteria supporting attribution of the activity directly to Ab is the coincidence of profiles of optical absorption of protein material and their activity under gel filtration in acidic buffer providing dissociation of tight noncovalent complexes of various proteins [4-8]. To test this criterion and exclude possible artifacts of co-purification of Abs with any traces of other proteins, all 15 IgG preparations used in the study were subjected to gel filtration under "acidic shock" (Fig. 2a). This requirement was shown to be fulfilled, and profiles of protein optical density and catalytic activity of Ab virtually coincided. All the experiments described below were carried out with IgG preparations additionally purified by FPLC gel filtration.

Study of polysaccharide kinase activity in IgG. The fraction of polyclonal sIgA was shown earlier to contain Abs tightly bound to minor lipids of unusual structure, as well as oligo- and polysaccharides, which may be phosphorylated by these Abs [27-30]. In connection with this, when protein kinase activity was studied in sIgA and IgG, the incubation of homogeneous Ab preparations with exogenous casein and [γ-^{32}P]ATP resulted in phosphorylation of not only casein, but also lipids, as well as oligo- and polysaccharides [27-30]. When the casein phosphorylation was analyzed by electrophoresis in polyacrylamide gel in the presence of SDS, the decomposition of the complexes of Ab with ^{32}P-labeled lipids and oligo- and polysaccharides occurred. This resulted in distribution of these ^{32}P-labeled species along the gel and the formation of strong radioactive background [28]. A similar pattern of distribution of low and high molecular weight products of non-protein nature was observed in the absence of exogenous casein [28-30]. The lipid and polysaccharide nature of these ^{32}P-labeled products forming the background was demonstrated, and approaches to the separate analysis of efficacy of Ab-dependent phosphorylation of proteins, lipids, and polysaccharides were developed [28-30]. Phosphorylated proteins were shown also to be easily removed by their precipitation with 5% TCA, and ^{32}P-labeled lipids and oligo- and polysaccharides remain in the solution. ^{32}P-Labeled lipids can be quantitatively removed from the solution using methanol–chloroform [29, 30], which is standard for their extraction, and only ^{32}P-labeled oligo- and polysaccharides and radioactive substrate remain in the solution. Special conditions for TLC were developed for the analysis of ^{32}P-labeling efficacy of lipids and oligosaccharides, and for ^{32}P-polysaccharides electrophoresis in 20% polyacrylamide gel with SDS was used [28-30]. Oligomeric and polymeric phosphorylation products remaining in the solution were shown to be not cleaved by proteases, DNases, or RNases, but subject to hydrolysis by polysaccharide hydrolyzing enzymes (see below). The described approach was used earlier for the analysis of sIgA-dependent phosphorylation [30], and in the present work for IgG-dependent phosphorylation of oligo- and polysaccharides.

It is known that virtually all protein-, lipid-, and polysaccharide kinases use ATP as a donor of the phosphate group, and rarely use other macroergic nucleoside-5′-triphosphates. There are no examples known from the literature for kinases using directly *ortho*-phosphate as a donor of the phosphate group. On the other hand, human milk sIgA and IgG with the described earlier kinase activities possess such unusual capacity [17, 28-30]. Human milk sIgA also catalyze *ortho*-phosphate-dependent phosphorylation of oligo- and polysaccharides [30].

We have drawn a comparison between phosphorylation efficiencies of oligo- and polysaccharides tightly bound to milk IgG. We have shown that for 10 studied IgG preparations the total ^{32}P-label incorporation into the bound oligosaccharides (from three to seven residues) and polysaccharides was 2-7 times higher in the presence of *ortho*-phosphate than that after incubation with ATP during the same time range. The data for one IgG preparation are given in Fig. 2c as a typical example. A similar pattern was obtained when polysaccharide kinase activity was studied in milk sIgA Abs [30]. In the case of protein kinase activity of sIgA, GTP was the best substrate (100%), whereas the phosphorylation efficacy by other NTP or dNTP (10-40%) or *ortho*-phosphate (25%) was lower [17]. In contrast, the efficacy of lipid phosphorylation (lipid kinase activity) in sIgA and IgG Abs was comparable for ATP (100%), *ortho*-phosphate (60-80%), and other NTP (30-100%) [29]. Thus, abzymes of human milk possess unique substrate specificity in comparison with known kinases in respect to phosphate group donor. Therefore, further study of polysaccharide kinase activity in human milk IgG was generally done using *ortho*-phosphate.

All 15 IgG preparations possess significantly marked oligo- and polysaccharide kinase activities, but relative substrate phosphorylation level depended substantially on the milk donor. Oligosaccharide phosphorylation data are given for an example in Fig. 2b for four studied IgG preparations. It is seen that these preparations differ in efficacy and number of significantly phosphorylated oligosaccharides. Nevertheless, 2-3 major ^{32}P-labeled oligosaccharides were detected in all the studied IgG preparations (Fig. 2b). The number of major oligosaccharides phosphorylated by sIgA Abs (and efficacy of their relative ^{32}P-labeling), dependent on milk donor, also varies and can reach 5-6 [30]. Note that in the case of distinct IgG and sIgA preparations the mobility of some phosphorylated oligosaccharides is virtually the same, whereas this exact coincidence of mobility for major phosphorylation products in other preparations is absent. However, chromatographic mobility of phosphorylation products cannot be a clear characterization of their structural features. As we shall demonstrate below, several different oligosaccharides with similar chromatographic mobility, but different resistance to glycosidase action can correspond to each band of ^{32}P-labeled product. In general, it was shown that IgG and sIgA can differ in number of phosphorylated oligosaccharides and to some extent in their chromatographic mobility. However, univocal interpretation of the data on the comparison of chromatographic mobility in oligosaccharides bound to IgG and sIgA presents difficulties.

Stability of IgG–oligosaccharide complexes. A small portion of sIgA Abs was shown to be bound with specific oligo- and polysaccharides containing various monosaccharide residues [30]. In the present work we studied stability of milk IgG complexes with oligo- and polysaccharides phosphorylated by these Abs. Oligo- and polysaccharides remained bound to these IgG after affinity chro-

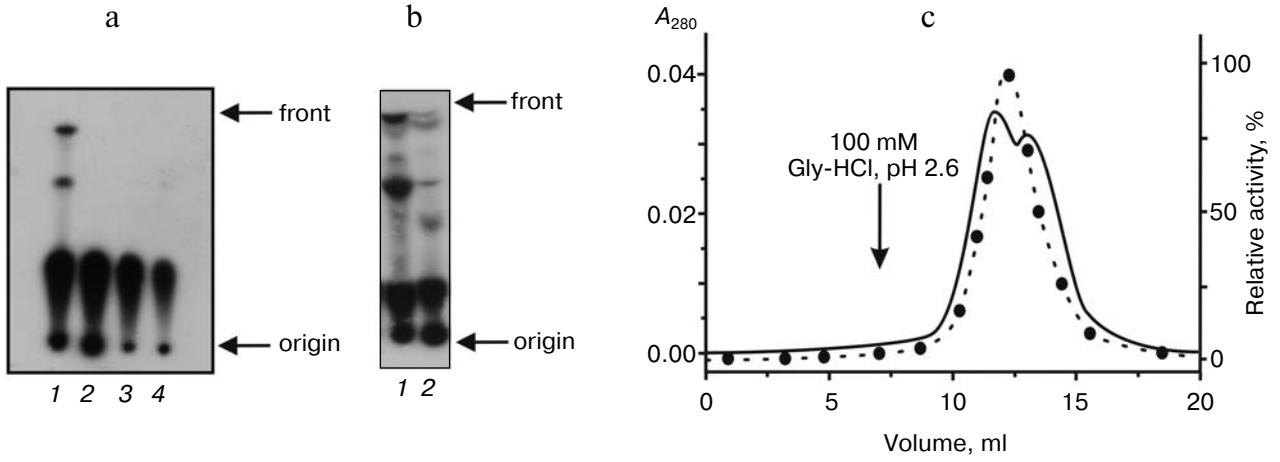

Fig. 3. a) Analysis of oligosaccharide phosphorylation in the presence of [^{32}P]*ortho*-phosphate: *1*) native IgG; *2*) [^{32}P]*ortho*-phosphate without Ab; *3*) Ab after thermal inactivation; *4*) mixture of oligo- and polysaccharides isolated from IgG preparations. b) Comparative analysis of oligosaccharide phosphorylation by intact IgG preparation IgG (lane *1*) and its Fab-fragments (lane *2*). c) Chromatography of IgG on Sepharose with immobilized murine IgG against light chains of human Ab. Solid line, protein optical absorbance at 280 nm; points, relative activity of IgG-dependent oligosaccharide phosphorylation. The activity of fraction with maximum activity level is taken as 100%.

matography on Protein G-Sepharose after washing the column with buffer containing 1% Triton X-100, as well as with acid buffer (Fig. 1a). "Acid shock" (30 min, 37°C) of Ab complexes with oligo- and polysaccharides and their subsequent gel filtration in acid buffer led to a decrease in phosphorylation efficacy by 30-40%, but the major part of the active complex was preserved (Fig. 2a). Polysaccharide kinase activity in IgG did not disappear after the treatment of Abs with 3 M NaCl or 3 M $MgCl_2$, which destroy (with subsequent gel filtration) relatively tight noncovalent complexes. IgG complexes with ^{32}P-labeled oligo- and polysaccharides were virtually totally decomposed after their incubation in buffer containing 1-2 M $MgCl_2$ and 20-30% ethanol or dioxane with subsequent gel filtration on a column equilibrated with the same buffer. According to SDS-PAGE data, 7-15% oligo- and polysaccharides remain in the complex with Ab after precipitation of Abs with 10% TCA. However, these complexes are destroyed during electrophoresis, and ^{32}P-labeled polysaccharides have mobility generally comparable to proteins with molecular mass of 4-15 kD. According to the data obtained by some of the methods described above, the stability of sIgA abzyme complexes with oligo- and polysaccharides [30] is comparable with those for IgG.

Confirmation of assignment of polysaccharide kinase activity to IgG. Several approaches were used to attribute the polysaccharide kinase activity to IgG, and a number of standard strict criteria first introduced by Paul et al. [9] and later expanded by others [4-8] were verified. IgG homogeneity on electrophoresis with subsequent silver staining of protein was demonstrated (Fig. 1b). No ^{32}P-labeled products was detected comparable in their mobility on TLC with the mobility of typical [^{32}P]oligosaccharides after the incubation of standard reaction medium containing only [^{32}P]*ortho*-phosphate or [γ-^{32}P]ATP, as well as oligo- and polysaccharides isolated from IgG preparations in the presence of [^{32}P]*ortho*-phosphate or [γ-^{32}P]ATP. No oligosaccharide phosphorylation products was detected after the incubation of [^{32}P]*ortho*-phosphate with Ab preparations preliminary subjected to 10 min inactivation by heating at 100°C. Oligosaccharide phosphorylation was observed only in the case of native IgG (Fig. 3a). IgG lost their phosphorylating activity after removal of tightly attached lipids and oligo- and polysaccharides from the Ab preparations by FPLC gel filtration in buffer containing 2 M $MgCl_2$ and 30% ethanol or dioxane, but the activity was partially recovered after the addition of exogenous oligosaccharides isolated from Ab preparations.

It was shown that gel filtration of IgG under "acid shock" conditions does not result in loss of activity, and the position of the peak of kinase activity corresponds to the IgG protein peak (Fig. 2). Complete adsorption of catalytic activity was observed during the incubation of IgG with anti-IgG-Sepharose (containing immobilized monoclonal murine Ab against human Ab light chains). Only one protein peak corresponding to IgG and coinciding with the peak of polysaccharide kinase activity was detected (no other peaks of activity was observed) when the proteins were eluted from the sorbent by buffer of low pH value (2.6) (Fig. 3c). IgG Fab-fragments possessed polysaccharide kinase activity (Fig. 3b).

Note that several contemporary criteria were met, unambiguously suggesting that the activity is attributed to Ab but not to admixtures. First, the detection of activity in Ab after protein separation by SDS-PAGE is one such criterion [4-8]. We showed earlier that fulfillment of this

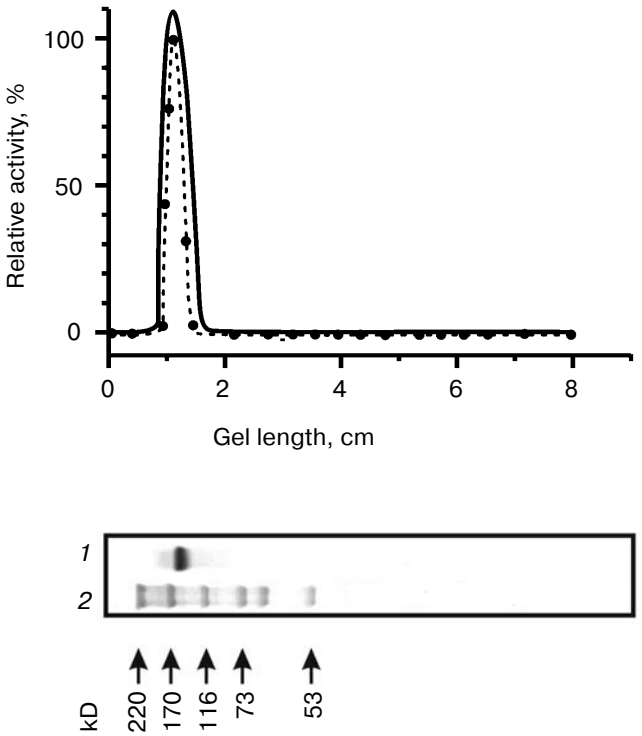

Fig. 4. Analysis of catalytic activity of human milk IgG after SDS-PAGE. A lane of the gel after the electrophoresis was divided onto 2-3-mm slices. The relative activity in phosphorylation of exogenous oligosaccharides was determined after extraction of the protein from the gel slices (points). The activity of the eluate with maximum oligosaccharide phosphorylation level is taken as 100%. Lanes *1* and *2*, positions of IgG and protein markers with known molecular masses after the electrophoresis, respectively; proteins are stained with Coomassie R-250.

strictest requirement is evidence for the fulfillment of other (less strict) criteria [4-8].

As seen from Fig. 4, the protein band possessing oligosaccharide kinase activity corresponds only to the IgG Ab protein band. SDS-PAGE is known as one of the most robust methods of decomposition of various noncovalent complexes. The lack of catalytic activity and other protein bands after SDS electrophoresis is evidence that the catalytic activity is the intrinsic property of IgG and is not due to admixtures of milk enzymes.

All of our experiments described above were done earlier in sIgA abzymes [30], and we have performed all of them for IgG.

As we have shown above, the substrate specificity of IgG, which use *ortho*-phosphate as a phosphate group donor, is unique and has been discovered only in milk abzymes [18, 28-30]. Besides, *ortho*-phosphate has slightly higher affinity to IgG in comparison with that in known enzymes (see below). The bulk of this data is univocal evidence that polysaccharide kinase activity is an intrinsic property of IgG.

Nature of oligomeric and polymeric phosphorylated products. As we have said above, phosphorylated oligomeric and polymeric products according to the data of TLC and electrophoresis cannot be cleaved by various enzymes except glycosidases. Glycosidases are known to possess very narrow specificity and are in use for structural studies of polysaccharides. To study the structure of ^{32}P-labeled oligo- and polysaccharides, we have used 11 various polysaccharide-hydrolyzing enzymes. Figure 5a shows the data of electrophoretic analysis of ^{32}P-labeled polysaccharide hydrolysis by various enzymes. Five

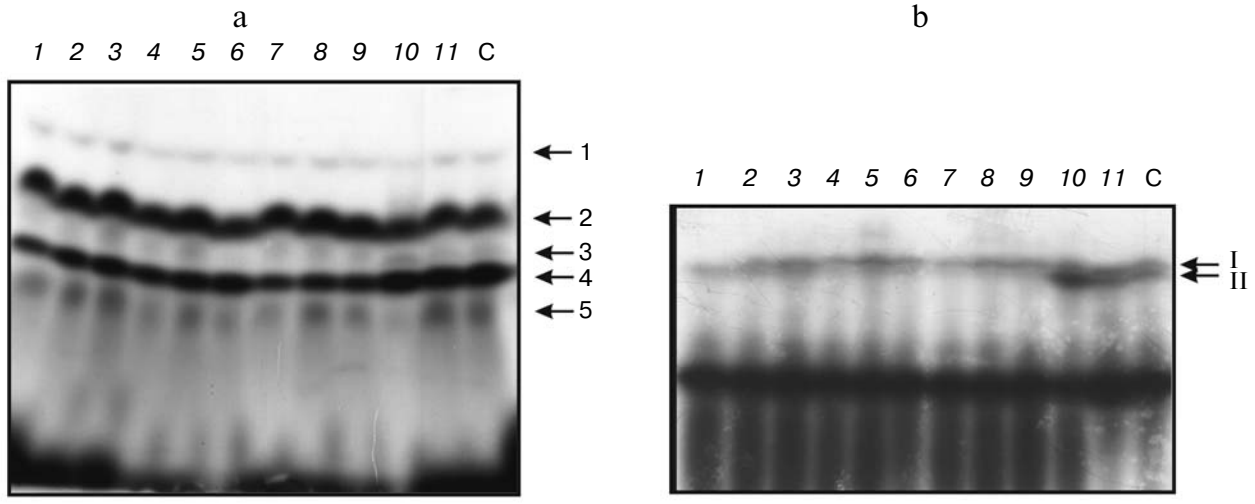

Fig. 5. Analysis of hydrolysis efficacy of ^{32}P-labeled poly- and oligosaccharides (from IgG preparations) by various glycosidases by SDS-PAGE in 20% polyacrylamide gel (a) and TLC (b), respectively. The following enzymes were used for the hydrolysis of poly- and oligosaccharides: β-glycosidase (*1*), cellobiohydrolase (*2*), glucoamylase (*3*), galactosidase (*4*), β-1,3-glucanase (*5*), lichenase (*6*), α-mannosidase (*7*), β-glucanase (*8*), α-amylase (*9*), endo F-enzyme (*10*), α-L-fucosidase (*11*); C, mixture of ^{32}P-labeled oligo- and polysaccharides incubated without the enzymes.

radioactive bands can be distinguished corresponding to the products of polysaccharide phosphorylation; two of them (numbers 2 and 4) are major and three (numbers 1, 3, and 5) are minor. These ^{32}P-labeled polysaccharides are detected in all IgG preparations, but the ratio of the label amounts in these five bands varies significantly depending on the milk donor. Figure 5a demonstrates the data on polysaccharide hydrolysis of one IgG preparation containing measured amounts of all five phosphorylation products. Note that significant hydrolysis of the minor polysaccharide-1 possessing the highest molecular mass was found only in the case of β-glycosidase, α-galactosidase, lichenase, and endo-F enzyme (mean data from three experiments) and to some extent (in sensitivity to various glycosylases) it resembles polysaccharides bound with sIgA [30]. Averages from three experiments on the evaluation of relative hydrolytic efficacy for polysaccharides numbers 2-5, obtained on the basis of scanning of relative label amounts in distinct bands, are given in Table 1.

Interestingly, some glycosidases (glucoamylase, 1,3-glucanase, and α-L-fucosidase) caused virtually no hydrolysis of these four polysaccharides isolated from IgG Ab preparations (Table 1). Working earlier with polysaccharides bound to sIgA, we found the absence of effective hydrolysis by only one of these enzymes (1,3-glucanase), and in three other enzymes—β-glycosidase, cellobiohydrolase, and α-galactosidase [30]. Interestingly, these three last enzymes most effectively cleaved polysaccharides bound to IgG Abs (Table 1). Besides, polysaccharides isolated from sIgA Ab preparations were effectively cleaved by α-L-fucosidase [30], which had virtually no effect on the polysaccharides from IgG Ab preparations (Table 1). α-Mannosidase is the only enzyme, which more effectively cleaves both polysaccharides bound to sIgA [30] and to IgG Abs. Other enzymes (1,3-glucanase and endo-F enzyme) relatively weakly cleave polysaccharides obtained either from sIgA [30] or from IgG Ab preparations. The most effective cleavage occurs in polysaccharides 3 and 4 and to lesser extent in 2 and 5 (Table 1). Similar specificity in selective cleavage of polysaccharides corresponding to the bands with different electrophoretic mobility using these 11 glycosidases was detected earlier in the case of polysaccharides isolated from sIgA preparations as well [30].

To analyze the hydrolysis efficacy of various oligosaccharides bound to IgG by various glycosidases, we used oligosaccharides isolated from several Ab preparations, and similar results were obtained. The general pattern of sensitivity of the preparations to various glycosidases can be satisfactorily described by the example of one preparation, which contained mainly two major bands with similar mobility on TLC (Fig. 5b).

The maximum hydrolysis level of IgG oligosaccharides, like polysaccharides, was observed under the action of α-mannosidase, β-glycosidase, and α-galactosidase, but a difference was observed in the action of these enzymes on oligosaccharides I and II (Fig. 5b and Table 1). As shown earlier, α-mannosidase also more actively hydrolyzes sIgA oligosaccharides, whereas β-glycosidase and α-galactosidase are either not hydrolyzed or very weakly cleaved oligosaccharides bound to sIgA [30]. The eight other glycosidases used in our experiments either very weakly hydrolyzed oligosaccharides corresponding to IgG or did not express activity to these substrates at any significantly detectable level (Fig. 5b and Table 1). On the other hand, some of these eight enzymes (glucoamylase, exo-1,3-β-glucanase, and α-F-fucosidase) effectively cleaved oligosaccharides bound to sIgA Abs [30]. Thus, the data obtained can be evidence that phosphorylated and tightly bound to IgG and sIgA Abs oligo- and polysaccharides, which are similar in their mobility on TLC and electrophoresis, can differ in their structure.

We emphasize that the incubation of polysaccharides corresponding to IgG Abs with any tested enzyme did not result in their complete hydrolysis even on very prolonged incubation. This suggests that several polysaccharides different in structure can correspond to each phosphorylation product with distinct electrophoretic mobility. Besides, all the used glycosidases varied substantially in the hydrolysis efficacy of oligo- and polysaccharides corresponding to the radioactive bands with various electrophoretic and chromatographic mobilities (Table 1). These data are in a good agreement with the results of the analysis of the sIgA-bound ligand hydrolysis and suggest that oligo- and polysaccharides tightly bound to IgG and sIgA Abs are not homopolymers [30].

For better understanding of the difference in the structure of monosaccharides and type of bonds between monomers composing the phosphorylated oligo- and polysaccharides, it is helpful to mention the specificity of enzymes used in our study. In general, oligo- and polysaccharides from sIgA [30] and IgG antibody preparations are virtually not cleaved by β-1,3-glucanase hydrolyzing 1,3-β-D-glycoside bonds. Hence, it seems that oligo- and polysaccharides of sIgA [30] and IgG in human milk do not contain significant amounts of 1,3-β-D-glycans. Very weak hydrolysis of polysaccharides 4 and 5 by β-1,3-glucanase (Table 1) can be due to heterogeneous composition of the polysaccharides corresponding to these bands, wherein only minor part of some specific fragments composing the analyzed polysaccharides and containing 1,3-β-D-glycoside bond can be hydrolyzed by this enzyme. There are no 1,4-α-D-glycan bonds hydrolyzed by glucoamylase and fucoside bonds cleaved by α-L-fucosidase in the analyzed IgG oligo- and polysaccharides, but these two enzymes are active in the case of hydrolysis of sIgA oligo- and polysaccharides [30].

Interestingly, maximum hydrolysis level of IgG oligo- and polysaccharides is observed in the case of β-galactosidase, α-galactosidase, and α-mannosidase. Similar but nevertheless lower activity was displayed by

lichenase (Table 1). α-Amylase displayed a notable activity in the case of one major polysaccharide (number 3) (Table 1). β-Glycosidase hydrolyzes terminal nonreducible β-D-glucose residue from oligo- and polysaccharide molecules and displays broad specificity to the following β-D-glycosides: β-D-galactosides, α-L-arabinosides, β-D-xylosides, and β-D-fucosides. α-Galactosidase cleaves α-(1-3) and α-(1-6) bonds of nonreduced terminal galactose residues from oligo- or polysaccharides. The enzyme α-mannosidase hydrolyzes terminal residue of α-D-mannose from non-reducible terminus of α-D-mannosides. Lichenase hydrolyzes α-(1,4)-glycan bonds. α-Amylase is an endohydrolase cleaving 1-4-α-glycoside bonds. Thus, IgG-associated oligo- and polysaccharides contain various residues of monosaccharides, which are bound together by various glycoside bonds. Moreover, on the basis of the data one can suppose that these polysaccharides can contain complex branched structure and, in spite of appearance of sufficiently discrete bands on electrophoresis, are most probably heterogeneous to some extent with respect to various monosaccharides composing the polysaccharides, corresponding to the bands with distinct electrophoretic mobility. Our data suggest that IgG and sIgA milk antibodies mainly interact with oligo- and polysaccharides of different structure, although their overlapping is not excluded.

We earlier studied the possibility of substitution of oligosaccharides associated with sIgA by various natural polysaccharides [30]. We showed that no remarkable decrease in oligosaccharide phosphorylation is observed after 5-h preincubation of sIgA preparations with six various polysaccharides [30]. However, after incubation for 24 h we observed significant inhibition of phosphorylation of bound oligosaccharides in the case of β-glucan (contains 1,3:1,6-joint glucan residues) and xylan (β-1,4-polymer consisting of β-D-xylopyranosyl chains, decorated with L-arabinose, D-glucuronic acid, and 4-O-methylglucuronic acid residues (simultaneously or separately)). Interestingly, two polymers containing fructose residues—inulin (linear polyfructan in which fructose residues are joined by β-(2→1)-bonds, terminated by a sucrose residue) and arabinogalactan (β-D-(1-3)-galactan, in which the main and side chains contain various numbers of D-galactose and L-arabinose residues), almost completely displaced oligosaccharides from the complexes with sIgA. Other polysaccharides had no strong effect on the sIgA-dependent phosphorylation of oligosaccharides [30].

Analogous experiments were carried out in the case of IgG antibody. Abs were preincubated with six various polysaccharides at high concentrations (0.01 mg/ml), then the reaction was initiated by the addition of [^{32}P]*ortho*-phosphate. Interestingly, none of these polysaccharides inhibited to any remarkable extent the IgG-dependent phosphorylation of oligosaccharides even after 24-h preincubation (Fig. 6). Moreover, remarkable and reproducible activation of phosphorylation of firmly attached oligosaccharides was observed in the case of mannan and especially lichenan (Fig. 6). These data suggest high stability of complexes of IgG antibody with oligo- and polysaccharides.

The observed difference in inhibitory effect of various polysaccharides on sIgA- and IgG-dependent phosphorylation of firmly bound oligosaccharides can be due to various causes. First, IgG probably forms tighter complexes with oligo- and polysaccharides than does sIgA antibody. Second, oligo- and polysaccharides bound to sIgA and IgG antibody differ in their structure and are differently hydrolyzed by 11 polysaccharide hydrolyzing enzymes varying in their substrate specificity. Thus, oligosaccharides from sIgA preparations are easily hydrolyzed by enzymes hydrolyzing polymers containing fructose residues. This fact correlates with almost complete substitution of oligosaccharides from sIgA complex in the case of polyfructan or arabinogalactan containing fructose residues. On the other hand, α-L-fucosidase hydrolyzing polymers containing fructose residues do not hydrolyze oligo- or polysaccharides isolated from IgG antibody preparations (Table 1). Thus, the data on hydrolysis of oligo- and polysaccharides by various glycosidases and on inhibition of the phosphorylation reaction of firmly bound oligosaccharides suggest that sIgA and IgG antibodies can recognize various oligosaccharide

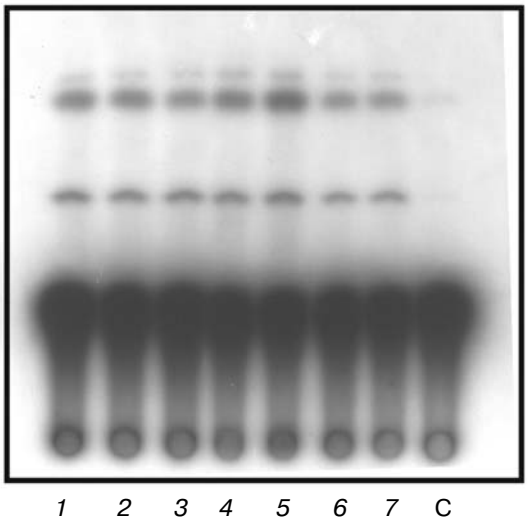

Fig. 6. Effect of various canonic polysaccharides on phosphorylation of oligosaccharides catalyzed by IgG. IgG preparations were preincubated for 24 h with one of the polysaccharides (0.01 mg/ml) or without it, then the phosphorylation reaction was initiated by the addition of [^{32}P]*ortho*-phosphate. The phosphorylation products were analyzed by TLC, the following polysaccharides being used: β-glucan (*1*), xylan (*2*), inulin (*3*), mannan (*4*), lichenan (*5*), arabinogalactan (*6*), IgG without polysaccharide additions (*7*), [^{32}P]*ortho*-phosphate without Ab and polysaccharides (C).

sequences in oligo- and polysaccharides. Taking into account this finding, the existence of oligosaccharide structures effectively interacting with IgG antibody in the structure of six natural polysaccharides used in our study cannot be excluded. As a result, any effective competition between exogenous polysaccharides and endogenous oligosaccharides bound to IgG for binding sites may be absent, but these polysaccharides effectively interact with sIgA [30].

The minor activating effect in the case of mannan and lichenan (Fig. 6) can be due to the polysaccharide binding site of IgG antibody being not completely "overlapped" with relatively short tightly bound oligosaccharides and, as a consequence, being able to additionally interact with exogenous polysaccharide molecules. This can result in the activation of oligosaccharide phosphorylation. Besides, the IgG antibody molecule contains two sites of antigen binding; one of them can be bound to oligo- or polysaccharides and another not. In this case, a cooperative influence of exogenous polymer molecules bound to one of the sites on phosphorylation of oligosaccharide bound to another site of the Ab may be yet another possible explanation of the activation.

Evaluation of affinity of *ortho*-phosphate and ATP to phosphorylating IgG antibody. We evaluated apparent values of K_m and V_{max} for *ortho*-phosphate and ATP substrates in reactions of oligosaccharide phosphorylation catalyzed by IgG antibody. As an example, the data for one IgG preparation are given in Fig. 7. Two of the studied Ab preparations were shown to be characterized by at least two K_m and V_{max} values for each used substrate. The data are summarized in Table 2.

The apparent K_m and k_{cat} values for ATP in the case of IgG1 and IgG2 preparations were comparable (Table 2). K_m values for [^{32}P]*ortho*-phosphate in the case of the same IgG preparations were close to those for ATP, and k_{cat} values for *ortho*-phosphate were remarkably higher than for ATP. Interestingly, 2-3 values of K_m for ATP and *ortho*-phosphate were in the range of 14-27 and 15-77 μM, respectively, in the reaction of oligosaccharide phosphorylation by three sIgA preparations [30]. Thus, ATP and *ortho*-phosphate affinity to IgG- and sIgA-fractions of milk polyclonal Abs catalyzing phosphorylation of oligosaccharides is high and rather similar.

The values of K_d for *ortho*-phosphate comprise 10^{-2}-10^{-3} M for most proteins and enzymes interacting with *ortho*-phosphate, and only for a limited number of enzymes these values are about 10^{-4} M [63]. Hence, the *ortho*-phosphate affinity to the studied milk abzymes (Table 2) is 1-3 orders higher than that in the case of canonical proteins and enzymes interacting with this ligand.

The existence of two K_m values characterizing the interaction of substrates with IgG is in agreement with the observation of 2-3 K_m values for *ortho*-phosphate and ATP in the case of sIgA antibody phosphorylating oligo- and polysaccharides [30] and can be due to several causes. From the theoretical point of view, the immune system is known to produce up to 10^6 different Ab variants in response to the action of a distinct single antigen. As an example, we have shown using abzymes hydrolyzing DNA, RNA, ATP, polysaccharides, and proteins, the polyclonal Abs isolated from blood of patients with various AID and blood or milk of healthy women are very heterogeneous in their composition. They can contain subfractions of abzymes differing in the type of their light chain (λ or κ), total charge, metal ion dependence/independence, thermal stability, pH optimum, substrate specificity, and affinity to substrates [4-8]. There are several additional factors that can result in appearance of

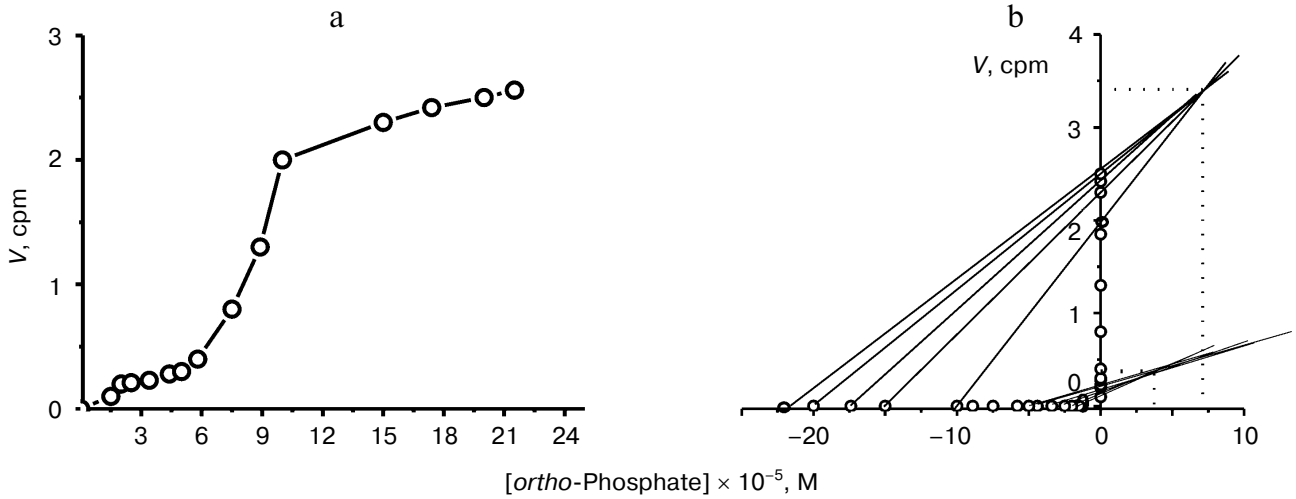

Fig. 7. Dependences of initial reaction rates of oligosaccharide phosphorylation on concentration of [^{32}P]*ortho*-phosphate catalyzed by IgG2, in {*V*– *S*} coordinates (a) and Eisenthal and Cornish–Bowden plots (b).

Table 2. Kinetics constants for *ortho*-phosphate and ATP characterizing IgG-dependent phosphorylation of oligosaccharides tightly bound to these Abs

Ab		$K_m(1)$, μM*	$K_m(2)$, μM	$K_m(2)/K_m(1)$	$k_{cat}(1) \times 10^2$, min^{-1}	$k_{cat}(2) \times 10^2$, min^{-1}	$k_{cat}(2)/k_{cat}(1)$
IgG1	P_i	9.0 ± 3.0	43.0 ± 15.0	4.8	0.17 ± 0.07	1.0 ± 0.3	5.9
	ATP	9.7 ± 2.5	29.0 ± 10.0	3.2	0.19 ± 0.07	0.47 ± 0.015	2.5
IgG2	P_i	34.0 ± 12.0	71.0 ± 25.0	2.1	0.8 ± 0.2	7.5 ± 0.06	9.4
	ATP	21.0 ± 8.0	60.0 ± 10.0	2.9	0.12 ± 0.03	0.62 ± 0.25	5.2

* Means from three experiments are given.

several values of K_m and k_{cat} in the case of IgG antibody phosphorylating oligo- and polysaccharides. These values can change depending on the type of oligo- or polysaccharide bound to the active center. Differences in the behavior of IgG molecules bound to one or two oligo- and polysaccharide molecules as a result of cooperative interactions between the sites of binding cannot be excluded.

Since milk oligo- and polysaccharides are tightly bound to IgG antibody, their saturation of phosphorylation enables evaluation of the portion of Ab that is bound to oligo- and polysaccharides. To saturate phosphorylation the IgG antibody were incubated with [^{32}P]*ortho*-phosphate at high concentration (0.2 mM) up to the plateau on the phosphorylation curve. Saturated incorporation of *ortho*-phosphate was shown to occur with stoichiometry of 0.01-0.03 mol of [^{32}P]P_i per mol IgG depending on the sample. In other words, the amount of IgG bound to oligo- and polysaccharides and capable of phosphorylating these ligands comprises 1-3%. In the case of sIgA, the portion of such Ab capable of phosphorylating polysaccharides was evaluated close to 1-3% as well [30]. As follows from Table 2, k_{cat} values characterizing incorporation of *ortho*-phosphate into oligosaccharides comprise $(0.17\text{-}7.5)\cdot10^{-2}$ min^{-1}. The content of IgG antibody bound directly to oligosaccharides in the case of IgG1 and IgG2 was 0.8-1.2%. Thus, one can presume that k_{cat} values for individual fractions of these Ab bound to oligosaccharides are approximately two orders of magnitude higher (0.1-7.5 min^{-1}). For such Ab, the values of k_{cat} in milk are possibly still higher, because Ab partly become denatured during their purification under strict treatment conditions, and upon reactivation the recovery of Ab activity can be incomplete [4, 5].

The biological significance of abzymes phosphorylating milk oligo- and polysaccharides, as well as abzymes with protein- and lipid kinase activities, remains unclear. Phosphorylations play important roles in regulation of an organism's vital activity—phosphorylation of proteins, lipids, and polysaccharides sometimes resulting in changes in their biological functions. Since during the first 4-6 months of pregnancy the immune system of newborns is not fully formed, maternal IgG and sIgA play an important role in providing the humoral response [20, 59, 60]. IgG can activate complement and possess cytotoxicity [20], and they can cause a shortage of IgA-response in various pathologies and fulfill some specific functions of sIgA [20, 59, 60].

As we have noted above, oligo- and polysaccharides are important for the protection of humans against various pathogens. Taking into account the active participation of IgG together with sIgA in the formation of passive immunity in children, some important, yet unknown, role of their polysaccharide kinase activity in the protective action of these Ig cannot be ruled out.

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