0273-2289/00/83/0115/$14.00

Secretory Immunoglobulin A from Health y Human Mothers' Milk Catalyzes Nucleic Acid Hydrolysis

Georgy A. Nevinsky,* Tat'yana G. Kanyshkova, Dmitry V. Semenov, Alexander V. Vlassov, Anastasiya V. Gal'vita, and Valentina N. Buneva

Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of Russian Academy of Sciences, Novosibirsk 630090, Lavrentieva Ave., 8, Russia, E-mail: Nevinsky@niboch.nsc.ru

Abstract

The human milk secretory immune system is known to be the first line of protection for the newborn infant against various pathogens. Secretory IgA (sIgA), the typical immunoglobulin found in secretions, can fight infections through many mechanisms. Using different methods, we have shown that sIgAfrom the milk of healthy women possesses DNAse and RNAse activities. The catalytic center is localized in the light chain of catalytic sIgA, while the DNA-binding center is predominantly formed by its heavy chain. The enzymic properties and substrate specificity of catalytic sIgA distinguish it from other known DNases and RNases. It is reasonable to assume that the milk DNA- and RNA-hydrolyzing antibodies are capable not only of neutralizing viral and bacterial nucleic acids by binding these antigens as well as by hydrolyzing them. The DNA-hydrolyzing activity of Abs raises the possibility that these catalytic Abs may provide protective functions for the newborn through the hydrolysis of viral and bacterial nucleic acids.

Index Entries: Human milk; sIgA; catalytic antibodies; DNA- and RNA-hydrolyzing activities.

Introduction

Secretory IgA (sIgA) immunoglobulins are the main effectors of the mucosal immune system, and their protective aspects are well-documented *(1–6)*. Milk contains a wide array of antibodies (Abs) to bacterial, viral, and protozoal antigens *(7,8)* which reach the mucosal surfaces of the intestinal

*Author to whom all correspondence and reprint requests should be addressed.

and respiratory tracts and as a result protect infants from infection and disease. However, the mechanism by which sIgA exert their antimicrobial activity is still a matter of debate. Many sIgA protective functions have been demonstrated, such as neutralization and agglutination of pathogens, activation of alternate complement pathways, and inhibition of foreign antigen entry into the mucosa and attachment *(2–4)*. sIgA also appears to opsonize bacteria *(5)*. The mucosal and systemic immune systems of newborn infants are largely undeveloped at birth, so maternal breast milk provides the infant with disease protection for the first months of life *(6)*.

Taking into account the above data, we hypothesized that healthy mothers possess a unique immune status that allows the generation of catalytic Abs in breast milk. We have recently shown that the milk of normal human mothers contains sIgA-possessing protein kinase activity *(9,10)* and IgG Abs hydrolyzing DNA and RNA *(11–13)*, as well as ribo- and deoxyribonucleotides *(14)*.

The sIgA are active at the mucosal surface in opposing the replication and colonization of pathogenic microorganisms, as well as in limiting the access of environmental antigens *(7,8)*. The injection of conventional nucleases into the circulatory system or local treatment of human respiratory mucosal surfaces with DNases and RNases has been proven to provide protection against different viral and bacterial diseases *(15,16)*. Recently, an inverse correlation between mammary-tumor incidence and the amount of RNase activity in human milk was revealed *(17)*. Therefore, we have investigated whether the RNA-hydrolyzing and DNA-hydrolyzing catalytic sIgA Abs could be generated in breast milk. Such immunoglobulins are analogous to conventional nucleases in principle, and may contribute to the protective role of Abs through the hydrolysis of viral and bacterial nucleic acids. In this article, we present evidence that sIgA from the milk of healthy mothers and its isolated light chains are capable of catalyzing nucleic-acid hydrolysis.

Materials and Methods

Materials

Reagents and enzymes used in this work were obtained mainly from Merck (Germany). We also used Protein A-Sepharose, $tRNA^{Lys}$ (Sigma, USA), diethylaminoethyl (DEAE) cellulose DE-52 (Whatman, England), and Toyopearl HW-55 fine (Toyo Soda, Japan). Radioisotopes were purchased from Amersham (3000 Ci/m*M*) (England).

Antibody Purification

Samples of milk were taken from healthy human mothers (19–35-yr-old). The milk was collected within the period from 1 wk until 4 mo of lactation. Electrophoretically and immunologically homogeneous sIgA was obtained by sequential chromatography of human-milk proteins on Pro-

tein A-Sepharose, DEAE-cellulose, antihuman IgA-Sepharose columns, and by gel-filtration on Toyopearl HW-55 (using a variant of acid shock conditions) as described previously *(11–13)*.

The amount of sIgA, IgG, and IgM antibody in the bound and nonbound fractions from different adsorbents including Protein A-Sepharose (and in different peaks under various chromatography conditions) was estimated using the Ouchterlony double-immunodiffusion method with human anti-IgA, anti-IgG, and anti-IgM Abs. At all steps of sIgA purification, different immunoglobulin fractions were analyzed by immunoblotting.

Immunoblotting Analysis of Abs

Identification of IgG, IgA, and IgM Abs, as well as the types of light (L) and heavy (H) chains of Abs was carried out using specific antihuman Abs (Sigma) according to *(18)*. Separation of proteins in SDS-polyacrylamide gels, and their subsequent transfer to a nitrocellulose membrane, were performed as described previously *(18)*. The nitrocellulose membrane containing the blotted proteins was then incubated with alkaline phosphatase conjugated with specific polyclonal Abs to IgA, IgM, IgG, λ light chain, or κ light chain for 2 h at 37°C. The membranes were then washed five times with 50 m*M* sodium phosphate buffer (pH 7.5), containing 0.2 *M* NaCl. 1-Naphthyl phosphate and amido black were then used for the staining of proteins.

DNA-Cellulose Chromatography

A glutaraldehyde-modified DNA-cellulose *(19)* column (5 mL) was equilibrated in 20 m*M* Tris-HCl, pH 7.5, and sIgA (2–3 mg) applied to the adsorbent was eluted with a concentration gradient of NaCl (0–1 *M*) in the same buffer. In order to separate the Ab subunits, sIgA (2 mg/mL) was dissociated by incubation in 20 m*M* Tris-HCl, pH 7.5, containing 0.3 M dithiothreitol (DTT) for 2 h at 25°C, addition of urea to a final concentration of 8 *M*, and incubation for a further 30 min. sIgA subunits were then separated on the DNA-cellulose column using the above buffer (containing 0.3 *M* DTT and 5.0 *M* urea) and an NaCl gradient (0–1 *M*). The protein peaks were analyzed by SDS-PAGE and by immunoblotting, and each fraction was analyzed for nuclease activity. For screening column fractions during all steps of Ab purification, 1–5 μL of each fraction (after dialysis) was incubated in 20 μL standard reaction mixture. The products of 5′-[^{32}P](pU)$_{10}$ or 5′-[^{32}P]d(pT)$_{10}$ hydrolysis were identified by autoradiography.

DNA-Hydrolyzing Activity

Specific activity of sIgA was measured after dialysis of the protein in all cases. The reaction mixture (20 μl) containing 150 ng supercoiled pBR322 DNA, 5.0 m*M* $MgCl_2$, 1.0 m*M* EDTA, 20 m*M* Tris-HCl buffer, pH 7.5, and 1–3 μg of sIgA was incubated for 1–3 h at 37°C. The cleavage products were analyzed by electrophoresis in 0.8% agarose gel. Ethidium bromide-stained

gels were photographed, and the films were scanned. Relative activities of sIgA preparations were determined from the scanning data.

Hydrolysis of Oligonucleotides

For evaluation of the nuclease activity of sIgA, various 5′-[^{32}P]ribo- and deoxyribooligonucleotides (100 pmol) were incubated with 20 pmol of sIgA in a 15 μL mixture containing 20 m*M* Tris-HCl, pH 7.5, 5.0 m*M* $MgCl_2$ (or in the absence of $MgCl_2$), and 1.0 m*M* EDTA, for 2–4 h at 37°C, and the products were analyzed on a 20% PAGE gel containing 8 *M* urea. Experiments with human $tRNA^{Lys}$ was performed as described previously *(12,13)*.

Gel-Filtration of Antibodies After pH Shock

Antibodies (sIgA purified on a antihuman IgA-Sepharose column; 1–2 mg/mL, 0.1 mL) were incubated in 50 m*M* glycine-HCl, pH 2.3, containing 0.2 *M* NaCl for 2 h at 25°C and then chromatographed in this buffer on Toyopearl HW 55 (8 × 180 mm). The fractions were dialyzed against 20 m*M* Tris-HCl buffer, pH 7.5, and the nuclease activities were measured as described above.

Affinity Labeling

Affinity labeling of the sIgA was carried out in a 20-μL reaction mixture containing 5 μg Abs and 100 pmol of the 4-(*N*-2-chloroethyl-*N*-methylamino) 5′-phosphoamide derivative of [^{32}P]$d(pT)_{10}$ (or [^{32}P]$d(pT)_{10}$ as a control) in 50 m*M* imidazole-acetate buffer, pH 7.0, 5 m*M* $MgCl_2$, incubated for 30 min at 20°C and analyzed by SDS-PAGE *(14)*. The alkylating derivative of $d(pT)_{10}$ was synthesized as in *(20)*.

In situ *Gel Assay of DNase Activity*

DNase activity in a 7–15% gradient (in the case of reducing conditions) or in a 7% (nonreducing conditions) SDS-PAGE gel containing 30 mg/mL phage λ DNA was detected as described previously *(11–13)*. The gel was washed five times with water. To allow protein renaturation, the gel was incubated for 16 h at 37°C in 20 m*M* Tris-HCl buffer, pH 7.5, containing 5.0 m*M* $MgCl_2$ and 1.0 m*M* EDTA. To reveal the products of the DNAhydrolysis, the gel was stained with ethidium bromide *(21)*. The positions of the sIgA subunits on the gel were revealed by Coomassie blue staining.

SDS-PAGE Analysis

SDS-PAGE analysis of Ab fractions for homogeneity in a nonreducing condition was done in 7–16% gradient gels (0.1% SDS) or in a reducing 12% gel (in the presence of 0.1% SDS and 1% 2-mercaptoethanol) according to Laemmli *(22)*.

Results and Discussion

Human milk contains various types of Abs (IgG, IgM, IgA, and sIgA), of which sIgA is the major component (> 85-90%) *(23,24)*. As mentioned above, IgG from healthy maternal milk possesses different catalytic activities *(9–14)*. Moreover, such catalytic IgGs were detected in the sera of mothers during the lactation period *(13)*. Today, the source of IgG in milk is still debated. Milk IgG may be partially synthesized locally by specific cells of the mammary gland, and partially transferred from the maternal blood *(24)*. sIgA is produced by B lymphocytes of the local immune system of the mammary gland *(23)*. The sIgA antibodies are present in Payer's patch lymphoid cells, which migrate to mucosal sites and generate local sIgA Ab responses. This suggests that the mechanisms of production of catalytic IgG and sIgA found in milk may be different. It is important to characterize the catalytic activities of sIgA in order to better understand how milk-catalytic Abs arise and what role they may play in the healthy organism.

To determine this, sIgA from human milk was purified by sequential chromatography on Protein A-Sepharose, DEAE-cellulose (separation of IgG and sIgA Abs), antihuman IgA-Sepharose column, and gel-filtration on Toyopearl HW-55 as described previously *(11–13)*. The sIgA possessed DNA- and RNA-hydrolyzing activity after each step of the purification protocol. In contrast to IgGs from normal human sera, the sIgA from 45 of 50 milk samples (90%) had detectable levels of nuclease activity. The nuclease activity of sIgA was comparable to or lower than that of milk-catalytic IgG described previously *(11–13)*.

According to immunoanalytical data, the protein peak eluted from antihuman IgA-Sepharose column by the acidic buffer (pH 2.6) was composed of Abs with a positive reaction with antihuman IgA Abs. There were no detectable IgG or IgM Abs as evaluated using the Ouchterlony double-immunodiffusion method with anti-IgA, anti-IgG, and anti-IgM Abs.

SDS-electrophoresis in nonreducing conditions following by silver staining (*see* Fig. 1A) of the Ab fraction obtained at the final step of purification revealed two protein bands (300 and 380 kDa), both of which had a positive reaction with anti-IgA Abs by immunoblotting (*see* Fig. 1A). After complete reduction of sIgA and SDS-electrophoresis in the presence of 2-mercaptoethanol, only three bands (72, 62, and 23 kDa) corresponding to the secretory component, the heavy chain, and the light chain of sIgA were revealed (*see* Fig. 1B). The mobility of the L- and J-components is similar in the Laemmli system used in our studies *(25)*. All four components of sIgA were identified as previously described *(26)*, using electrophoresis having two coordinates—molecular size and isoelectric point (not shown). The 380 kDa-purified Ab band corresponds in size to the $(L_2H_2)_2$JS-form of sIgA (*23,25,26*; 1 S-subunit, 72 kDa; 4 heavy chains, 62 kDa each; 4 light chains, 23 kDa each, and 1 J-chain, 23–26 kDa). These findings (*see* Fig. 1) demonstrate that sIgA preparations do not contain detectable contaminating proteins.

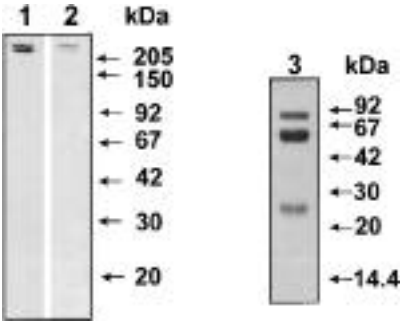

Fig. 1. Silver-stained SDS-PAGE of sIgA (3–5 μg per sample) purified by gel filtration on Toyopearl HW 55 in a nonreducing 7%–15% gradient gel (lane 1) and in a reducing 12% gel (lane 3) (for assay conditions, *see* Methods). Lane 2 corresponds to an immunoblot of lane 1 using Abs against human IgA. Arrows indicate the positions of molecular mass markers.

It seems reasonable to suggest that the 300 kDa form of the Ab lacks one of the subunits of the complete $(L_2H_2)_2$JS-oligomeric form of sIgA. Since human milk contains sIgA (IgA2 or sIgA2 types) in which not all light chains are linked covalently to the oligomer by disulfide bonds *(27)*, it is probable that such molecules of sIgA have lost the light subunits under the drastic conditions used for Ab purification.

Further control experiments were performed to rule out possible artifacts caused by copurifying nucleases. Incubation of sIgA at each stage of purification with immobilized anti-sIgA Abs led to essentially complete binding of Abs to the adsorbent and the disappearance of RNase and DNase activities from the solution. During protein elution from the anti-sIgA column with an acidic buffer (pH 2.6), the nuclease activities coincided exactly with the sIgA peak, and there were no other peaks of activity (data not shown).

A further approach provided direct evidence that sIgA possesses DNA- and RNA-hydrolyzing activities. After SDS-PAGE (using nonreducing conditions and a gel containing DNA) of a preparation of native sIgA, an in-gel assay showed DNase activity only in a single protein band corresponding to the complete 380 kDa $(H_2L_2)_2$JS-form of sIgA (*see* Fig. 2A). A photograph of the gel after development of nuclease activity with ethidium bromide revealed a sharp dark band on a fluorescent background of DNA-bound ethidium bromide. After partial reduction of sIgA using 2-mercaptoethanol, the in-gel assay showed DNA-hydrolyzing activity in the bands corresponding to the original $(H_2L_2)_2$JS-form of sIgA, its partially reduced form (H_2L_2), and the separated L chain (*see* Fig. 2B).

Further purification of sIgA by chromatography on DNA-cellulose was performed to separate Abs capable of binding nucleic acids from the total Ab fraction (*see* Fig. 3A, curve 1). The chromatography showed the sIgA (from one donor) to be composed of Ab fractions with different affinities for DNA. The same experiment was performed using sIgA from

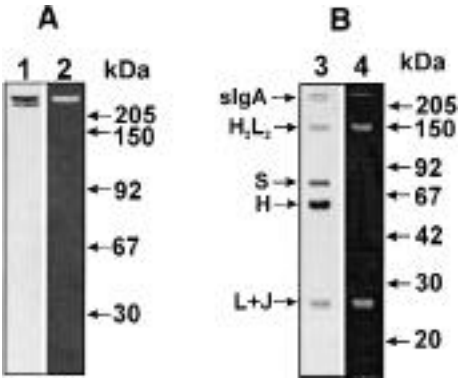

Fig. 2. *In situ* gel assay of DNase activity of catalytic sIgA (5–7 μg per sample) separated by SDS-PAGE in a gel containing DNA. Before electrophoresis the samples were incubated in nonreducing (lanes 1, 2) or reducing conditions (lanes 3, 4) (*see* Methods). DNase activity was revealed as a sharp dark band on the fluorescent background by ethidium bromide staining (lanes 2 and 4) (the negatives of the films are shown). Gels stained with Coomassie blue show sIgA (lane 1) and sIgA and its different fragments (lane 3) after incubation and electrophoresis of Abs in a nonreducing condition. Arrows indicate the positions of molecular mass markers.

20 different donors. The amount of sIgA in peaks 1–2 in Fig. 3A varied depending on the milk donors. About 30–70% of the total sIgA from 20 different donors was bound to the column. Both the bound and nonbound fractions of sIgA (having lower affinity and eluting by buffer without salt) possessed the DNase and RNase activities.

The milk IgG possessed a heterogeneous affinity for DNA—40–70% of homogeneous IgG from 30 different donors was bound to the column, and this fraction possessed nuclease activities, while the nonbound fraction of antibodies was catalytically inactive *(11–13)*.

In an earlier experiments with IgG, we developed a method to dissociate the Ab subunits using urea and DTT *(11–13)* and to separate the L- and H-subunits of the catalytic IgG having different affinities for DNA-cellulose. In the case of the catalytic IgG, DNA-cellulose adsorbed only the light chain, which was then observed to hydrolyze DNA and RNA. In contrast to catalytic IgG, the light chain of sIgA had low affinity for DNA, as shown by separation of the subunits on DNA-cellulose (*see* Fig. 3A, curve 2; Fig. 3B). As in the case of *in situ* experiments, the fraction of heavy chains (*see* Fig. 3B) that was bound by DNA-cellulose did not hydrolyze DNA or RNA (*see* Fig. 3A). Thus, it is reasonable to conclude that both the light and heavy chain subunits are important for organization of the sIgA catalytic center. The same conclusion may be drawn from the data on sIgA affinity modification by an alkylating derivative of an oligonucleotide. While affinity modification of IgG by a chemically reactive derivative of $d(pT)_{10}$ led to preferential modification of the L-chain *(12,13)*, both the L and H chains were labeled after incubation of sIgA

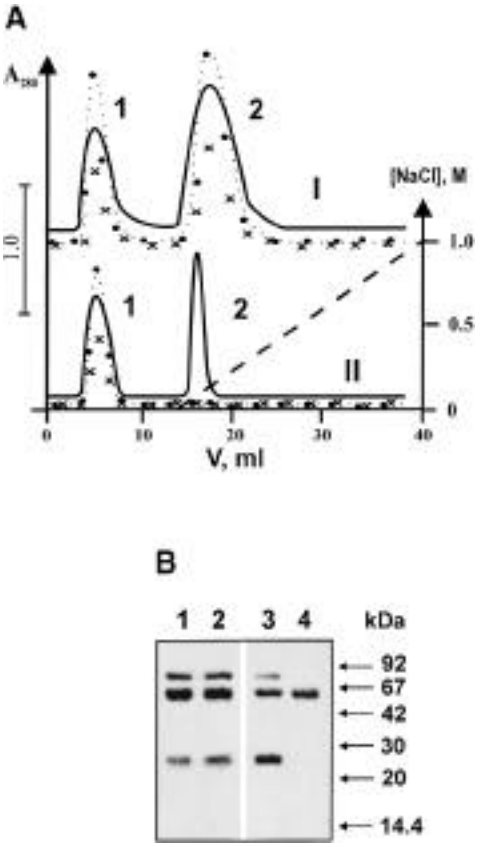

Fig. 3. **(A)** Chromatography of sIgA purified by gel filtration on DNA-cellulose before (curve I) and after dissociation into subunits (curve II) using DTT and urea (*see* Methods): (—), absorption at 280 nm; (•) and (×), activity of Abs in 5′-[^{32}P](pU)$_{10}$ and 5′-[^{32}P]d(pT)$_{10}$ hydrolysis, respectively (*see* Methods), relative to the activity of the fraction having maximal (pU)$_{10}$ hydrolyzing activity (100%). **(B)** Silver-stained SDS-PAGE of peak fractions from panel A in a reducing 12% gel. Lanes 1 and 2 correspond to peaks 1 and 2 of curve I and lanes 3 and 4 to peaks 1 and 2 of curve II. Arrows indicate the positions of molecular mass markers.

with an affinity probe for DNA-binding sites [4-(*N*-2-chloroethyl-*N*-methylamino) amide of [5′-^{32}P]d(pT)$_{10}$] (*see* Fig. 4).

From the crystal structure of a catalytic Ab with esterase-like activity, it was concluded that the ligand *p*-nitrophenyl ester interacts with amino-acid residues of both light and heavy chains of abzymes, and that both types of subunit are required for catalysis *(28)*. The same situation probably occurs in the case of DNA-hydrolyzing sIgA from human milk.

Approximately 90% of catalytic sIgA was adsorbed on immobilized MAbs against κ-light chain of human Abs. No detectable catalytic sIgA adsorption was observed when an anti-λ-light chain Sepharose column was used (*see* Fig. 5). The only explanation for all of these data is that the

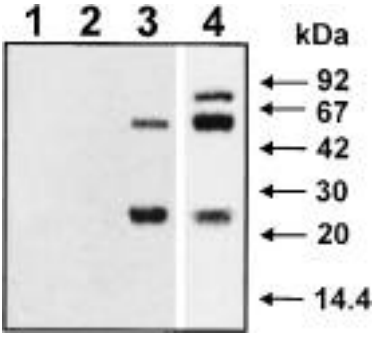

Fig. 4. Affinity labeling of the L and H chains by incubation of sIgA with the 4-(*N*-2-chloroethyl-*N*-methylamino) 5′-phosphoamide derivative of 5′-[^{32}P]d(pT)$_{10}$. Autoradiographs of a SDS-PAGE gel (reducing conditions were used, *see* Methods) of sIgA after incubation with [^{32}P]d(pT)$_{10}$. Lane 1: control experiment with chemically reactive d(pT)$_{10}$; sIgA before incubation (zero incubation time) and after incubation of sIgA for 30 min at 20°C with the alkylating agent 4-(*N*-2-chloroethyl-*N*-methylamino) 5′-phosphoamide [^{32}P]probe (lanes 2 and 3, respectively); lane 4, gel stained with silver.

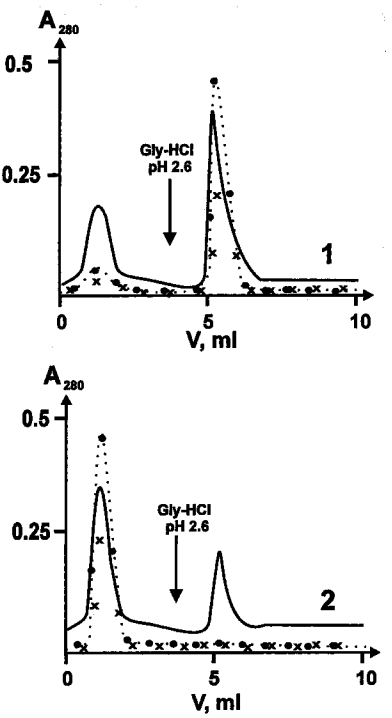

Fig. 5. Chromatography of sIgA on Sepharose containing immobilized MAbs against κ-light chain (1) or against λ-chain of human IgA (2): (—), absorption at 280 nm; (•) and (×), relative activity of Abs in 5′-[^{32}P](pU)$_{10}$ and 5′-[^{32}P]d(pT)$_{10}$ hydrolysis, respectively, assayed using 3 μL of each fraction in 20 μL of reaction mixture (*see* Methods) relative to the activity of the fraction having maximal (pU)$_{10}$ hydrolyzing activity (100%).

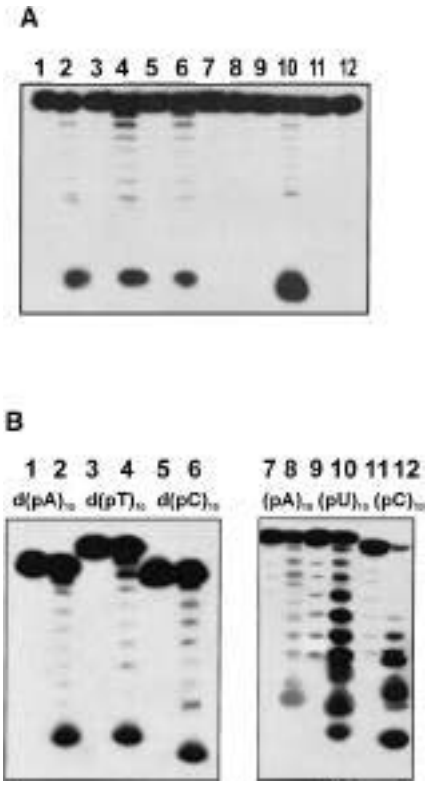

Fig. 6. **(A)** Influence of different metal ions (5 m*M*) on the relative activity of catalytic sIgA in the hydrolysis of 5′-[^{32}P]d(pT)$_{10}$: without metal ions *(1,2)*; Mg^{2+} *(3,4)*; Mn^{2+} *(5,6)*; Ca^{2+} *(7,8)*; Zn^{2+} *(9,10)*; and Cu^{2+} *(11,12)*. **(B)** Cleavage of 5′-[^{32}P]oligonucleotides by milk sIgA (after gel filtration): d(pA)$_{10}$ *(1,2)*; d(pT)$_{10}$ *(3,4)*; d(pC)$_{10}$ *(5,6)*; (pA)$_{10}$ *(7,8)*; (pU)$_{10}$ *(9,10)*; (pC)$_{10}$ *(11,12)*. In panels (A) and (B) the odd-numbered and even-numbered lanes show the products after incubation without or with antibodies, respectively. The products were analyzed in a 20% PAGE gel containing 8 *M* urea. For assay conditions, *see* Methods.

DNA- and RNA-hydrolyzing activities are intrinsic properties of different oligomeric forms of L- and H-subunits of sIgA and its separated light chains.

The properties of the DNase of milk sIgA distinguished it from other known DNases. The sIgA hydrolyzed DNA within the entire pH range tested (5.5–9.0), with an optimum at pH 7.0–7.5 (data not shown), a value markedly higher than that of human blood DNase II [pH optimum 5.2 *(29)*]. Whereas DNase I is metal-dependent *(29)*, the DNase activity of milk sIgA was only slightly activated by Mg^{2+}, Mn^{2+}, or Zn^{2+}, and cleavage of DNA substrates was inhibited by Ca^{2+} and Cu^{2+} (*see* Fig. 6A). Importantly, the minimal products of hydrolysis of deoxyribonucleotides by known DNases are dinucleotides *(29)*, while degradation of this particular deoxyribonucleotide by sIgA led to the appearance of mononucleotides as the final products of the reaction (*see* Fig. 6). Figure 6B demonstrates

Table 1
Kinetic Parameters for Hydrolysis of Different Ribooligonucleotides *(r)* and Deoxyribooligonucleotides *(d)* by Catalytic sIgA from Milk of One Donor

Substrate	K_m (μM*)	$k_{cat} \times 10^3$ (min^{-1})	k_{cat}/K_m ($M^{-1} \times min^{-1}$)
dA_{10}	7.6	11.3	1.5×10^3
dC_{10}	6.5	7.0	1.1×10^3
dT_{10}	4.6	8.3	1.8×10^3
rA_{10}	2.7	2.7	1.0×10^3
rC_{10}	6.9	90.6	13.1×10^3
rU_{10}	1.4	18.1	12.9×10^3

*Errors in K_m and k_{cat} were within 10–30%.

that catalytic sIgA are capable of hydrolyzing different ribo- and deoxyribooligonucleotides. The data on the specificity of sIgA-dependent hydrolysis of ribo- and deoxyribonucleotides in the case of Abs from one donor are summarized in Table 1.

As was shown previously *(13)*, the specific activities of the DNase and RNase of milk-catalytic IgG with polymer substrates were 0.5–10% (means of 20 milk samples) of those of DNase II and RNase A, and varied for different donors. The apparent K_m and k_{cat} values for different ribo- and deoxyribonucleotides were estimated to be in the range 0.1–1.5 μ*M* and 2–14 × 10^{-2} min^{-1}, respectively. sIgA also hydrolyzed various ribo- and deoxyribonucleotides, but the affinities of the sIgA for the substrate were about one order of magnitude lower (1.4–7.6 μ*M*; *see* Table 1) than of IgG. This probably explains why a large fraction of catalytic sIgA cannot be effectively adsorbed by the DNA-cellulose column.

The 80 kDa and 14 kDa RNases so far reported in human milk *(17)*, like the known five human blood RNases *(29)* and pancreatic RNase A *(29,30)*, cannot hydrolyze oligo(A). Unlike all known human RNases, milk sIgA efficiently hydrolyzed oligo(A) (*see* Table 1), and was not capable of hydrolyzing cCMP (data not shown).

The IgG from serum of patients with systemic lupus erythematosus hydrolyzes homooligoribonucleotides 20–50 times faster than oligodeoxyribonucleotides. The best substrates are $d(pA)_n$ and $(pA)_n$, and hydrolysis of $d(pT)_n$ and $(pU)_n$ is 10–15 times slower *(31)*. In addition to these differences, the RNase activity of milk IgG and sIgA can be distinguished from RNase A and from other human RNases by its pattern of cleavage of $tRNA^{Lys}$. The cleavage of this substrate was more pronounced in the region between nucleotides 52 and 56 of $tRNA^{Lys}$, although the major cleavage positions were similar *(13)*. Cleavage by IgG and by sIgA between nucleotides 52-53 and 55-56 was stimulated by NaCl and $MgCl_2$, whereas cleavage by RNase A was essentially completely inhibited *(13,32)*.

These data, taken together, show that the DNase and RNase activities of human milk sIgA are intrinsic properties of its L chain, and are not caused by copurifying nucleases.

We studied the RNA- and DNA-hydrolyzing activities of sIgA using the same preparations of catalytic Abs. It is possible that the two catalytic activities reside in the same protein, since we have recently shown *(33)* that monoclonal lupus IgG—which recognizes specific DNA sequences—shows both DNase and RNase activities (the RNase activity is 50–100 times greater than the DNase activity). Yet a mixture of different catalytic Abs that hydrolyze either DNA or RNA may be present in the catalytic sIgAs, since these are polyclonal preparations. We have recently developed methods to separate IgG fractions *(34)* that hydrolyze only RNA, only DNA, or both substrates simultaneously. Therefore, it seems possible that polyclonal sIgA may also contain a mixture of such Abs hydrolyzing only RNA, only DNA, or both substrates.

It should be noted that the specific activities for RNA and DNA hydrolysis by the sIgA were 0.02–2% (50 milk samples) of those of DNase II and RNase A. In principle, such levels of activities are comparable with those of certain known sequence-specific RNases and DNases *(29)*, and are comparable to or higher than those of certain catalytic Abs, such as antithyroglobulin Abs *(35)*, DNA-hydrolyzing IgG *(19)*, and artificial abzymes against transition-state analogs *(36)*. Several reasons allow us to suggest that the true specific activities of RNA- and DNA-hydrolyzing sIgA fractions of certain mothers may be significantly higher than those observed for the sIgA preparations we analyzed. First, the relative specific activities of the 50 sIgA preparations—which probably also contain noncatalytic antibodies—were estimated using total concentrations of protein. The purification procedure does not provide complete separation of RNA-hydrolyzing sIgA from catalytically inactive sIgA interacting with nucleic acids. *In situ* investigation of catalytic activity of native sIgA (*see* Fig. 2) also demonstrated that the 300 kDa fraction of the Abs are not catalytically active. Thus, the specific activities are probably underestimated. Second, acidic treatments during Ab purification can lead to partial inactivation of catalytic activity *(34)*. Finally, catalytic sIgA hydrolysis of polymer substrates, such as plasmid DNA, is about 5–10 times faster than for short oligonucleotides.

A number of catalytically active Abs have been detected recently in the sera of patients with autoimmune pathologies (*11–14,19,31–37*, and references therein), whose sera contain nucleic acids and certain proteins at higher concentrations than those in the sera of normal humans. These components of sera could cause autoimmunization. It is commonly believed that the presence of catalytic Abs is associated with autoimmunization, and that autoantibodies in such patients may be of antiidiotypic nature. The question concerning the genesis of the catalytic Abs in human milk remains unanswered. We have also recently shown that the milk contains sIgA with protein kinase activity *(9,10,26)* and IgG with DNAse, RNAse *(11–13)*, and nucleotide-hydrolyzing activities *(14)*. It is important to note that we did not find any detectable nuclease activity in IgG from the sera of 50 normal humans (men and women) *(12,13,34)*. In contrast to IgG from

the sera of normal humans, IgG from the milk and serum samples of seven mothers had detectable levels of DNAse and RNase activities *(13)*. Based on these observations, we propose that an as-yet unidentified process of immunization of healthy mothers leads to the production of Abs with different catalytic activities found both in serum and in milk.

From an analysis of published data, a possible process by which pregnant women are directly immunized may be a result of a specific response of their immune system to certain components of viruses, bacteria, or foods. Thus, immunization of animals by injection of protein antigens or by oral administration no more than 1–3 mo before birth of neonates leads to the production of antiprotein Abs, which can then be detected in the milk at high concentrations *(38–40)*. This suggests that in contrast to other humans, pregnant women may be more effectively immunized by contact with components of various viruses and bacteria. Moreover, production of milk Abs 1–3 mo after immunization speaks in favor of the existence of a specific "immunomemory" in pregnant women. In this connection, we observed a significant increase of the level of RNA- and DNA-hydrolyzing Abs in the milk and sera of healthy mothers who suffered from viral or allergic diseases during pregnancy *(12,13)*. Since we did not detect catalytic Abs in sera of nonpregnant patients with influenza, pneumonia, tuberculosis, tonsilitis, duodenal ulcer, or some types of cancer *(41)*, it is quite possible that the immune system of healthy humans (men and women) cannot be stimulated by proteins and nucleic acids in the same way as that of pregnant women. Perhaps the autoimmunization of pregnant mothers is similar to that in autoimmune patients—an increased level of nucleic acids in sera of healthy women during the first three months of pregnancy, like that in cases of autoimmune diseases, was recently revealed *(42)*. Recently, the increased apoptosis of cells during the last three months of pregnancy was demonstrated *(43)*. A very low concentration of embryo cells is present in the blood of pregnant women *(44)*. Taken together, we believe that the catalytic antibody response during pregnancy may be related to immunization with various components of viruses and bacteria, including those that stimulate an antiidiotypic response as in autoimmune diseases.

As yet, the important question of the biological role of catalytic Abs in the blood of patients with various autoimmune diseases remains unanswered. It is unknown whether catalysis is a normal function or a dysfunction of autoimmune Abs. We believe that catalytic Abs of maternal milk may have a positive function during breastfeeding of the newborn. As noted above, therapy of patients with nucleases leads to protection from different viral and bacterial diseases (ref. *45*) and references therein). It is reasonable to conclude that the milk DNA- and RNA-hydrolyzing Abs are capable not only of neutralizing viral and bacterial nucleic acids through binding, but also through hydrolyzing them and thus serving a protective function. The specific production of various Abs—including catalytic Abs—by the immune system of mothers as a result of autoimmunization and/or viral and bacterial infection may be a way of strengthening the protective

function of breast milk, and may play an important role in providing passive immunity to the neonate.

Acknowledgments

The research was made possible in part by a grant (98-04-49719) from the Russian Fund of Basic Research and a grant from the Siberian Division of Russian Academy of Sciences.

References

1. Hanson, L. A., Hahn-Zoric, M., Berndes, M., Ashraf, R., Herias, V., Jalil, F., Bhutta, T. I., Laeeq, A., and Mattsby-Baltzer, I. (1994), *Acta Paediatr. Jpn.* **36,** 557–561.
2. Mestecky, J., Russell, M. W., Jackson, S., and Brown, T. A. (1986), *Clin. Immunol. Immunopathol.* **40(1),** 105–114.
3. Gregory, R. L. (1994), *Lab. Med.* **25,** 724–728.
4. Gregory, R. L., Kindle, J. C., Hobbs, L. C., Filler, S. J., and Malmstrom, H. S. (1990), *Oral Microbiol. Immunol.* **5(4),** 181–188.
5. Eggert, F. M. and Gurner, B. W. (1984), *Infect. Immun.* **44(3),** 660–664.
6. Newman, J. (1995), *Sci. Am.*, **273(6),** 76–79.
7. Redhead, K., Hill, T., and Mullroy, B. (1990), *FEMS Microbiol. Lett.* **58,** 269–273.
8. Gillin, F. D., Reiner, D. S., and Wang, C. S. (1983), *Science* **221,** 1290–1292.
9. Kit, Yu.Ya., Semenov, D. V., and Nevinsky, G. A. (1995), *Mol. Biol. (Moscow)* **29,** 893–906.
10. Kit, Yu.Ya., Semenov, D. V., and Nevinsky, G. A. (1996), *Biochem. Mol. Biol. Int.* **39,** 521–527.
11. Kanyshkova, T. G., Semenov, D. V., Khlimankov, D. Yu., Buneva, V. N., and Nevinsky, G. A. (1997), *FEBS Lett.* **416,** 23–27.
12. Kanyshkova, T. G., Semenov, D. V., Vlassov, A. V., Khlimankov D. Yu., Baranovskii, A. G., Shipitzin, M. V., Yamkovoi, V. I., Buneva, V. N., and Nevinsky, G. A. (1997), *Mol. Biol. (Moscow)* **31(6),** 927–934.
13. Buneva, V. N., Kanyshkova, T. G., Vlassov, A. V., Semenov, D. V., Khlimankov, D. Yu., Breusova, L. R., and Nevinsky, G. A. (1998), *Appl. Biochem. Biotechnol.* **75,** 63–76.
14. Semenov, D. V., Kanyshkova, T. G., Kit, Yu.Ya., Khlimankov D. Yu., Akimzhanov, A. M., Gorbunov, D. A., Buneva, V. N., and Nevinsky, G. A. (1998), *Biochemistry (Moscow)* **63(8),** 935–943.
15. Glukhov, B. M., Ierusalimskii, R. P., and Salganik, R. I. (1968), *J. Neurol. Psychiatr. (Russian)* **10,** 361–368.
16. Mashkovsky, M. D. (1984), in *Medical Preparations*, Medicine Press, Moscow, Russia, **2,** pp. 53–54.
17. Ramaswamy, H., Swamy, Ch.V. B., and Das, M. R. (1993), *J. Biol. Chem.* **268,** 4181–4187.
18. Towbin, H., Staehein, T., and Gordon, J. (1979), Proc. Natl. Acad. Sci. USA *76, 4350–4354.*
19. Gololobov, G. V., Chernova, E. A., Schourov, D. V., Smirnov, I. V., Kudelina, I. A., and Gabibov, A. G. (1995), *Proc. Natl. Acad. Sci. USA* **92,** 254–257.
20. Mishenina, G. F., Samukov, V. V., Shubina, T. N. (1979), *Bioorganic. Chem. (Moscow)* **5,** 886–894.
21. Rosenthal, A. L. and Lacks, S. A. (1977), *Anal. Biochem.* **80,** 76–82.
22. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
23. Hanson, L. A., Carlsson, B., and Cruz, J. R. (1979), in *Immunology of Breast Milk*, Ogra, P. L. and Dayton, D. H., eds., Raven Press, NY, pp. 145–157.
24. Kim, K., Keller, M. A., and Heiner, D. C. (1992), *Acta Paediatr.* **81,** 113–118.
25. Koshland, M. E. (1975), *Adv. Immunol.* **20,** 41–67.
26. Nevinsky G. A., Kit Yu. Ya., Semenov D. V., and Buneva V. N. (1998), *Appl. Biochem. Biotechnol.* to be published.
27. Gray, H. M., Abel, C. A., Yount, W. J., and Kunkel, H. G. (1968), *J. Exp. Med.* **128,** 1223–1236.

28. Golinelly-Pimpaneu, B., Gigant, B., Bizebard, T., Nazava, J., Saludjian, P., Zemel, R., Tawfik, D. S., Eshhar, Z., Green, B. S., and Knossow, M. (1994), *Current Biology Structure* **2,** 175–218.
29. Shapot, V. S. (1968), in *Nucleases*, Medicine Press, Moscow, Russia, pp. 1–162.
30. delCardayre, S. B. and Raines, R. T. (1995), *J. Mol. Biol.* **252(3),** 328–336.
31. Andrievskaya, O. A., Buneva, V. N., Zabara, V. G., Naumov, V. A., Yamkovoi, V. I., and Nevinsky, G. A. (1998), *Molekul. Biol. (Moscow)* **32(5),** 908–915.
32. Vlassov, A. V., Andrievskaya, O. A., Kanyshkova, T. G., Baranovsky, A. G., Naumov, V. A., Breusov, A. A., Giege, R., Buneva, V. N., and Nevinsky, G. A. (1997), *Biochemistry (Moscow)* **62(5),** 474–479.
33. Andrievskaya, O. A., Kanyshkova, T. G., Yamkovoi, V. I., Buneva, V. N., and Nevinskii, G. A. (1997), *Dokl. Russian Akad. Nauk (Moscow)* **355,** 401–403.
34. Baranovskii, A. G., Kanyshkova, T. G., Mogilnitskii, A. S., Naumov, V. A., Buneva, V. N., Gusev, E. I., Boiko, A. N., Zargarova, T. A., Favorova, O. O., and Nevinsky, G. A. (1998), *Biochemistry (Moscow)* **63(11),** 1459–1469.
35. Li, L., Paul, S., Tyutyulkova, S., Kazatchkine, M. D., and Kaveri, S. (1995), *J. Immunol.* **154,** 3328–3332.
36. Suzuki, H. (1994), *J. Biochem.* **115,** 623–628.
37. Generalov, I. I. and Novikov, D. K. (1998), *Adv. Modern. Biology (Russian)* **118,** 178–193.
38. Fey, H. R., Burtler, R., and Marti, F. (1973), *Vox Sang.* **25,** 245–253.
39. Gregory R. L. and Filler S. J. (1987), *Infect. Immun.* **55(10),** 2409–2415.
40. Englund J., Glezen W. P., Piedra P. A. (1998), *Vaccine* **16(14–15),** 1456–1463.
41. Baranovskii, A. G., Matyushin, V. G., Vlassov, A. V., Naumov, V. A., Giege, R., Buneva, V. N., and Nevinsky, G. A. (1997), *Biochemistry (Moscow)* **62,** 1358–1366.
42. Kazakov, V. I., Bozhkov, V. M., Linde, V. A., Repina, M. A., and Mikhailov V. M. (1995), *Citology (Moscow)* **37,** 232–235.
43. Mikhailov, V. M., Linde, V. A., Rozanov, Yu. M., Tatarova, N. A., Susloparov, L. A., and Konycheva, E. A. (1992), *Citology (Moscow)* **34,** 67–73.
44. Lo, D. Y. M., Tein, M. S. C., Lau, T. K., Haines, C. J., Leung, T. N., Poon, P. M. K., Wainscoat, J. S., Johnson, P. J., Chang, A. M. Z., and Hjelm, N. M. (1998), *Am. J. Hum. Genet.* **62,** 768–775.
45. Glukhov, B. M., Ierusalimskii, R. P., and Salganik, R. I. (1968), *Zh. Nevropatol. Psikhiatr. (Moscow)* **68,** 361–368.

Discussion

Sela: The pure immunoglobulins you isolated from milk are polyclonals containing all the various specificities and properties due to different molecules. Did you try to affinity-fractionate so that you separate the RNase activity from DNase activity? If not, I can't understand the meaning of the dissociation constants, and so on.

Nevinsky: First we purified IgG and then separated IgG and IgA. Then by affinity chromatography on different matrices we separated protein-kinase activity, ATP-hydrolyzing activity, and so on. We cannot separate RNA- and DNA-hydrolyzing activity because the antibodies have affinity for both nucleic acids. Sometimes we can obtain small amounts of antibodies after separation on DNA-affinity columns with only DNA-hydrolyzing activity. But usually the major fraction has both activities, and I do not know whether the same antibodies can have both activities. We do have other results concerning the RNA-/DNA-hydrolyzing activity of monoclonal antibodies to different DNA sequences. We have found that RNA-hydrolyzing activity of monoclonal antibodies against DNA is about 100 times higher than its

DNA-hydrolyzing activity. It is possible that the same antibodies can have RNA- and DNA-hydrolyzing activity at varying levels.

Sela: Your answer leads to my other comment, which is not only for your work, but also for some of the others. It would be very useful to get the gene and produce the antibody by genetic engineering. Then, once and for all, you eliminate worries about this being a contamination. You have such interesting properties. Nobody believed this 40 or 50 years ago because of contamination. Now this work is so superbly done that I believe it completely. It is not so surprising that antibodies have proteolytic activity, DNase activity, and so on. The moment you can get heavy and light chains by genetic engineering, nobody can doubt the conclusions.

Paul: Having studied the contamination issue myself, I will respond with a somewhat less optimistic position. Even after you engineer the molecule genetically, the bacteria have proteases which must be removed. I think the reason why we start believing or disbelieving in certain activities is partly when they make mechanistic sense, and then worries about contaminations slowly disappear. Site-directed mutagenesis is really a good way to prove that the activity is resident in the molecules. One question to you—I know of no good way to separate catalytic from noncatalytic antibodies. I'm speaking of contamination of one antibody with another antibody. Did you imply that you were able to separate the catalytic fraction completely from the noncatalytic fraction?

Nevinsky: No. I said that we cannot separate DNA binding and DNA-/RNA-hydrolyzing antibodies. We can separate only an ATP-hydrolyzing fraction from DNA- and RNA-hydrolyzing fractions. We can only separate different activities.

Paul: It is then appropriate to refer to the antibodies as enriched catalytic antibodies. The kinetic parameters are only nominal K_m and k_{cat} values.

Nevinsky: We tried to separate different catalytic antibodies according to their affinities. For example, in the case of the kinase activity, we saw many peaks when we changed elution buffers and a small fraction was eluted with magnesium chloride. These peaks have different affinity and different K_m value for ATP. So these antibodies are polyclonal. For example, if you use three elution steps at three different magnesium chloride concentrations, you will have further separation of catalytic species.

Marchalonis: I agree with Dr. Sela, and I'm not as pessimistic as Dr. Paul. I think the recombinant molecules must be made, and the proof is really in the site-directed mutagenesis—that you can modify the catalytic function by changing particular residues. It's hard to explain that by contamination. Another point I found very interesting is that there seem to be different mechanisms of catalysis. Some of your activities required divalent metal ions, and others did not. It may well be that there is a whole spectrum of catalytic mechanisms involved.