Human autoantibodies with amylolytic activity

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We have investigated the activity of IgG and IgM fractions from patients suffering from multiple sclerosis, as well as that of IgG and sIgA fractions isolated from human milk of healthy women, in the hydrolysis of artificial substrates and maltooligosaccharides with different degrees of polymerisation. All electrophoretically-homogeneous preparations of IgG and its Fab fragments, as well as sIgA and IgM antibodies, possessed amylolytic activity. The specific activities of catalytic antibodies from human milk varied in the range from 0.11 up to 0.2 U/mg, i.e. about three orders higher than those for IgGs from the sera of multiple sclerosis patients and one order higher than those for cancer patients. Milk IgG and sIgA fractions revealed Michaelis constants for hydrolysis of p-nitrophenyl α-D-maltooligosides in the range of 10^{-4} M. Fractions of autoantibodies from various donors revealed different modes of action in hydrolysis of maltooligosaccharides, p-nitrophenyl maltooligosaccharides and p-nitrophenyl α-D-glucopyranoside.

Key words: autoantibodies from human, α-amylase, autoimmunity disease.

Introduction

The field of artificial and natural catalytically-active antibodies (Abs) or abzymes has been amply reviewed recently (Lerner & Tramontano, 1981; Tramontano et al., 1986; Lerner et al., 1991; Benkovic, 1992; Hilvert, 1992; Suzuki, 1994; Nevinsky et al., 2000a). In autoimmune diseases, there can be spontaneous induction of anti-idiotypic Abs which are elicited by a primary antigen. These anti-idiotypic Abs may have characteristics of the primary antigen, including catalytic activity (Earnshaw & Rothfield, 1985; Reimer et al., 1987). Catalytic IgGs

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hydrolysing peptides (PAUL et al., 1989), proteins (KALAGA et al., 1995), DNA (SHUSTER et al., 1992; GOLOLOBOV et al., 1995), DNA and RNA (BUNEVA et al., 1994; VLASSOV et al., 1998a, 1998b; ANDRIEVSXAIA et al., 2000; BARANOVSKII et al., 2001) were detected in the sera of patients with several autoimmune pathologies: bronchial asthma (PAUL et al., 1989), SLE (systemic lupus erythematosus) (SHUSTER et al., 1992, 1995; GOLOLOBOV et al., 1998a, 1998b; ANDRIEVSXAIA et al., 2000), Hashimoto’s thyroiditis, polyarthritis (KANDRNA) and RNA (BETNE), proteins (KALAGA et al., 1995; VLASSOV et al., 1998a, 1998b; ANDRIEVSXAIA et al., 2000), multiple sclerosis (BARANOVSKII et al., 2001). In spite of the lack of obvious immunising factors that are found in normal humans, we have recently shown that, in the case of pregnant women, there is a previously-unidentified process of autoimmunization that leads to production of Abs with different catalytic activities, found both in serum and in milk (BUNEVA et al., 1998; NEVINSKY et al., 1998, 2000a, 2000b). As was shown, the milk of normal human mothers contains slgA possessing protein kinase (NEVINSKY et al., 1998) and DNase or RNase activities (KANYSHKOVA et al., 1997; BUNEVA et al., 1998; NEVINSKY et al., 2000b), as well as IgG and slgA that can hydrolyse ATP (SENOV et al., 1998). Recently, we have described the amylolytic activity of IgG fractions and its Fab fragments from the sera of oncological patients and pregnant women (SAVEL’EV et al., 1999).

Here we have compared the amylolytic activity of IgG and IgM fractions of patients suffering from multiple sclerosis and the amylolytic activity of slgA and IgG fractions from milk of healthy women. We also present evidence that IgG and slgA from human milk are able to catalyse the hydrolysis of artificial and natural substrates of amylase. Data on their substrate specificity and kinetic parameters as well as possible origin of these catalytic Abs are discussed.

**Material and methods**

**Chemicals**

p-Nitrophenyl-α-D-glucopyranoside (PNPGlc), methyl umbelliferyl glucoside and maltooligosaccharides were purchased from Sigma; p-nitrophenyl 4,6-O-ethylidene-α-D-maltoheptaoside (EPS) was from Boehringer Mannheim (Germany); other chemicals were purchased from Sigma (USA) or Merck (Germany). Anti-IgA, anti-IgM and anti-IgG Sepharoses 4B were obtained by coupling anti-human IgA (clone no. GA-112; Sigma), goat anti-human IgM and IgG (Sigma), respectively, with BrCN-activated Sepharose 4B according to the Pharmacia manual.

**Enzymes**

α-Glucosidase from yeast was purchased from Charlock, Int. (UK); cyclodextrin glucosyltransferase (CGT-ase; E.C. 2.4.1.19) was isolated from Bacillus sp. after growth at 37°C in a medium containing 0.5% soluble starch; 0.2% (NH4)2SO4; 0.1% KH2PO4; 0.05% MgSO4·7H2O; 0.03% CaCl2·2H2O. The enzyme was purified according to the following procedure. Cells were separated by centrifugation, the resulting supernatant was concentrated with hollow fibres, transferred to 20 mM Tris-HCl buffer, pH 7.4, with 20 mM CaCl2, and applied to a DEAE Sephadex A-50 column equilibrated with the same buffer. Fractions with CGT-ase activity were eluted with 0.5 M NaCl in the above buffer, pooled and concentrated using an Amicon PM-30 membrane, and dialysed against 20 mM PIPES, pH 6.0, 10 mM CaCl2 (buffer A). The CGT-ase fraction was loaded on a column coupled with raw starch equilibrated with buffer A, and desorbed with 20 mM β-cyclodextrin (in buffer A). The enzyme was separated from β-cyclodextrin on a Sephadex G-50 column and used for enzymatic synthesis of substrates. The unit of α-CGT-ase activity was defined according to the method described by SCHMID (1989). MUF α-glucoside (0.35 mM), β-cyclodextrin (0.25 mM) and 10 units of CGT-ase were mixed in 5 mL of 20 mM PIPES buffer, pH 6.0, supplemented with 20 mM CaCl2 and 5 mL of methanol. The reaction mixture was incubated at 37°C, overnight. A set of MUF-(Glc)_n (n = 1–7) was applied on the PREP-ODS column (Gasukuro Kogio Inc., Japan) and fractions of individual oligosaccharides were separated using a linear gradient of acetone (0–90%) in water. Enzymatic synthesis of p-nitrophenyl maltooligosaccharides was carried out in the same manner, using PNPGlc as acceptor.

Modification of pNP and MUF maltooligosaccharides was performed as follows. Benzylidene derivatives of pNP-(Glc)_n and MUF-(Glc)_n (n = 2–5) were prepared as described by SATOMURA et al. (1988). The reaction mixture contained 1 mM of chromogenic or fluorescent substrate, 3 mM of benzaldehyde dimethylacetal and 1 mg of p-toluene sulfonic acid monohydrate in 5 mL of dry acetonitrile (DMFA was used only with MUF-substrates). The mixture was stirred at 40°C for 60 min, then evaporated and purified by HPLC techniques as described above.

**Donors and patients**

Samples of milk were taken from healthy human mothers (19–35 yr old). The milk was collected within the period from 1 wk to 4 mo of lactation. Autoimmune patients were diagnosed at the Novosibirsk Army Hospital and at the Gatchina Hospital. The diagnosis was confirmed and its reliability was checked according to the criteria developed by the American Rheumatoid Association (SLE) and according to the POSSER criteria (multiple sclerosis) (POSSER, 1984). Sera from cancer patients were collected from 26th Hospital of St. Petersburg.
Antibody purification

Electrophoretically and immunologically homogeneous IgG and secreted IgA (sIgA) were obtained from human milk after removing the lipid phase as described by Buneva et al. (1998) and Nevinisky et al. (2000b). Milk was loaded on a Protein-A Sepharose column equilibrated with buffer: 150 mM NaCl, 20 mM Tris-HCl, pH 7.4. The column was washed with buffer containing 1% Triton X-100 and 0.3 M NaCl followed by 0.1 M sodium citrate, pH 4.6. Abs were eluted with 50 mM glycine-HCl, pH 2.6. Column fractions were immediately neutralised and dialysed against 10 mM Tris-HCl, pH 7.5. The Abs fraction was loaded on a DEAE-Toyopearl column equilibrated with 20 mM Tris-HCl buffer, pH 7.3 where sIgA was removed from the IgG fraction by elution in a linear gradient (from 0 to 0.5 M NaCl). Abs were additionally purified on Sepharoses bearing anti-IgG or anti-IgA Abs, respectively, according to the procedure described above for protein A-Sepharose. Fractions of Abs were dialysed against 10 mM Tris-HCl, pH 7.5, 1 mM NaN$_3$, and used in the following experiments. Fractions of IgM from blood of patients and healthy human donors were isolated as described by Savel’ev et al. (1999), with an additional stage of purification on anti-IgG Sepharose 4B.

IgM from human blood were isolated according to the following procedure. Antibodies were precipitated from human serum using ammonium sulphate. The precipitate was dialysed against 20 mM Tris-HCl buffer, pH 7.5. To reveal substrate hydrolysis, the gel after SDS-PAGE, SDS was removed by incubating the gel for 1 h at 37°C with 2 M urea. The gel was washed 5 times with H$_2$O, and to allow protein to renature, it was incubated for 16 h at 37°C in 20 mM Tris-HCl buffer, pH 7.5. To reveal substrate hydrolysis, longitudinal 2 mm slices of the gel were cut, extracted in 20 mM Tris-HCl buffer, pH 7.5, and incubated with 1 mM maltoligosaccharides or 0.16 mM EPS as described above, while parallel longitudinal lanes were used for detecting the position of IgG and sIgA on the gel by silver or Coomassie R250 staining.

Immunoblotting analysis of Abs

Identification of IgG, IgM, and sIgA Abs was carried out by Western blotting using specific antihuman Abs (Sigma). The nitrocellulose membrane was incubated with conjugates of alkaline phosphatase with specific polyclonal anti-IgA and anti-IgG antibodies for 2 h at 37°C. The membranes were then washed 5 times with 50 mM sodium phosphate buffer (pH 7.5), containing 0.2 M NaCl. 1-Naphthyl phosphate and amido black were then used for the detection of proteins and bound antibodies.

Activity assays

Measurements of the IgG, Fab, IgM and sIgA amylolytic activities were carried out in 30 mM Tris-HCl buffer, pH 7.5, 1 mM NaN$_3$, at 30°C, at Abs concentrations of 0.05 to 0.1 mg per mL. Amylolytic activity toward maltoligosaccharides and starch was measured by the Somogyi-Nelson method. Activity toward EPS was measured according to Savel’ev et al. (1999). One unit of the amylolytic activity was defined as the amount of enzyme that released 1 μmole of reducing sugar from an appropriate substrate per min at 30°C, pH 7.5. One unit of α-glucosidase activity of Abs was defined as the enzyme amount liberating 1 μmole of p-nitrophenol from p-nitrophenyl-α-D-glycopyranoside per min at 37°C in 20 mM Tris-HCl, pH 7.5. The $K_M$ and $V_{max}$ values for the hydrolysis of several substrates were determined by the standard method of initial rates; linearization was carried out by the Lineweaver-Burk method.

To investigate the mode of action of Abs, maltoligosaccharides with different degree of polymerisation were used. Products of hydrolysis were identified by TLC on Kieselgel 60 (Merck) with a mobile phase of 1-butanol-acetic acid-H$_2$O (2:2:1), and maltoligosaccharides (Sigma) were used as standards. Plates were developed at 25°C until the solvent front was ca 5 mm from the upper plate border, air dried, sprayed for charring with a 5% H$_2$SO$_4$ solution in 1-propanol, and incubated at 110°C for 8 min. Alternatively, HPLC was employed for determination of hydrolysis products using a Dextra-Fak$^\text{TM}$ cartridge column (Waters Co.), 8 × 100 mm. Maltoligosaccharides (Sigma) were used as standards. When pNP- and MUF-maltoligosaccharides and their derivatives were tested as substrates in these experiments, TLC on Kieselgel 60 (Merck) plates was used in a mobile system consisting of 1-butanol-acetic acid-water (4:2:1) and detection of products was carried out as described above. Alternatively, the products of the hydrolysis were identified by HPLC on a Lichrosorb C18 column, Pharmacia LKB, using a gradient of acetonitrile in water from 0 to 100%. Quantitative interpretation of the results was performed by integration of the respective chromatographic peaks.

Other methods

Experiments with immunoadsorption and gel-filtration of Abs on Sephacryl S-200 and Superose 12 (Pharmacia) under drastic conditions (6 M guanidine chloride or acidic buffer, pH 2.6) were performed as described previously (Savel’ev et al., 1999). Ab concentrations were estimated spectrophotometrically ($A_{280}$) assuming 0.8 mg IgG per mL corresponds to 1 optical density unit. Moreover, the amount of sIgA and IgG antibody
in bound and non-bound fractions from different adsorbents including Protein A-Sepharose (and in different peaks under various chromatography conditions) was evaluated using the Ouchterlony double immunodiffusion method against human anti-IgA, anti-IgG Abs. At all steps of sIgA purification different immunoglobulin fractions were analysed using immunoblotting (TOWN et al., 1979). To prepare Fab fragments, antibodies were digested with papain as described (LEE et al., 1982). The Fab fragments obtained were purified by gel-permeation chromatography on a Sephacryl S-200 (1.6 × 100 cm) column in 50 mM sodium phosphate, 100 mM NaCl, Tween 20 (0.25 mL per L of the buffer), pH 7.0, 1 mM NaN₃, followed by chromatography on an immobilised Protein A column (KALAGA et al., 1995). The Fab fraction was dialysed against 20 mM Tris HCl, 1 mM NaN₃, pH 7.0, and used for activity assays. The Fab fragments were composed of a single 50-kDa band when analysed by nonreducing SDS-PAGE. Concentrations of IgG and Fab fractions were estimated spectrophotometrically (A₂₈₀) assuming that 0.8 mg IgG per mL corresponds to 1 OD unit (PAUL et al., 1991).

Results and discussion

Preparations of total human Abs are polyclonal and consist of many Igs having different affinities for various antigens including proteins, enzymes, nucleic acid etc., which may be tightly combined with these Abs. Thus, in abzyme analysis it is of the utmost importance to ensure that the method of IgG, IgM and sIgA purification does separate the Abs from any minor contaminants such as highly active enzymes. Human milk contains various types of Abs (IgG, IgM, IgA, and sIgA), of which sIgA is the major component (>85–90%) (HANSON et al., 1979, 1994; KIM et al., 1992). A method of Abs purification leading to electrophoretically and immunoanalytically homogeneous preparations of IgG and sIgA was used. After SDS-PAGE of native sIgA or IgG preparations, Abs had a single protein band corresponding to the complete 380 kDa (H₂L₂)₂JS-form of sIgA or the complete 150 kDa H₂L₂-form of IgG (staining by silver). Recently, a number of strict criteria were developed in studies of catalytic human autoantibodies to test experimentally for evidence that the observed catalytic activity is an intrinsic property of human abzymes and is not due to co-purifying enzymes (PAUL et al., 1989; KALAGA et al., 1995). Here we used some of these criteria for isolated IgG, IgM, and sIgA including: a) immunoprecipitation of abzymes by anti-IgG, anti-IgM or anti-IgA Abs and disappearance of the α-amylase activity from the solution; b) complete adsorption of these activities by anti-IgG, anti-IgM or anti-IgA Sepharose and their elution from the adsorbents with buffer of low pH; c) gel-filtration of IgG, IgM or sIgA under conditions of "acidic shock" did not lead to disappearance of the activities of the Abs and the peak of activities tracked exactly with 150 kDa IgG or 380 kDa sIgA Abs; d) coincidence of the α-amylase activity with the Abs peak after the Superose 12 chromatography in 50 mM sodium phosphate buffer, pH 6.5, containing 6 M guanidine chloride, followed by renaturation by dialysis against 20 mM Tris-HCl, pH 7.4, until guanidine chloride concentration decreased to 1 mM (Fig. 1); e) Fab fragments obtained from IgG and purified as described above possessed amylolytic activity (Fig. 1). The data obtained from the above-listed experiments indicated that the amylolytic activity did belong to the considered Abs.

Specific amylolytic activities of IgG and sIgA

![Fig. 1. Analytical gel-filtration of the amylolytic activity of IgG (A) and the amylolytic activity of Fab fraction (B) on a Sephacryl S-200 column (1.6 × 100 mm) in a 6 M guanidine chloride: , activity; ---, absorbance at 280 nm. Carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), lactate dehydrogenase (158 kDa), and β-amylase (200 kDa) were used as standards.](image-url)
Table 1. Kinetic parameters of the hydrolysis of PNP-\(\text{Glc}\)\(_5\) catalyzed by several different samples of milk IgG and sIgA.

<table>
<thead>
<tr>
<th>Fraction of immunoglobulin</th>
<th>(K_M) [mM]</th>
<th>(V_{max}) [mole/(min·mg)]</th>
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<tbody>
<tr>
<td>sIgA3</td>
<td>0.54 ± 0.01</td>
<td>0.019 ± 0.001</td>
</tr>
<tr>
<td>sIgA8</td>
<td>0.40 ± 0.01</td>
<td>0.020 ± 0.001</td>
</tr>
<tr>
<td>sIgA10</td>
<td>0.41 ± 0.01</td>
<td>0.016 ± 0.001</td>
</tr>
<tr>
<td>IgG10</td>
<td>0.32 ± 0.01</td>
<td>0.018 ± 0.001</td>
</tr>
<tr>
<td>IgG5</td>
<td>0.30 ± 0.01</td>
<td>0.014 ± 0.001</td>
</tr>
<tr>
<td>sIgA7</td>
<td>0.22 ± 0.01</td>
<td>0.013 ± 0.001</td>
</tr>
</tbody>
</table>

Fig. 3. pH-Dependence of the amylolytic activity of IgG and sIgA antibodies from milk of different donors: – sIgA3; – sIgA7; † sIgA8; □ IgG10.
Fig. 4. Analysis of the hydrolysis of PNP(Glc)$_5$ as a function of its concentration in the case of IgG and sIgA antibodies from milk of different donors by the method of Lineweaver-Burk: •, sIgA3; ○, sIgA7; □, sIgA8; ▲, IgG10.

Fig. 5. Specific amylolytic (PNP-maltohexaose) and α-glucosidase (PNPGlc) activities of IgM from patients with multiple sclerosis.

(KALAGA et al., 1995). $K_M$ values of the same order were determined for monoclonal antibodies with glycosidase activity (SUGA et al., 1994). It should be noted that $K_M$ values for autoantibody fractions purified from different humans differed only slightly; similar small differences of $K_M$ values (not more than 1.4 fold) were described for IgG with proteolytic activity (KALAGA et al., 1995).

As shown in Figure 5, there are significant differences in activities of the IgM fractions from patients suffering from multiple sclerosis toward PNPGlc. This mode of action is characteristic for putative α-glucosidases and has been found only for IgM fractions. Clear differences in the mode of action of IgMs with amylolytic activity exhibiting the presence or absence of exo-glucosidase activity provide additional evidence that amylolytic activity is due to Abs. Hypothetical contaminant α-amylases, which might be assumed to present in IgM samples, should not differ dramatically in this property.

We have examined amylolytic activities of IgM and IgG fractions from the sera of pregnant women (12 donors), healthy humans, and patients with autoimmune diseases: multiple sclerosis and SLE (10–15 samples) (Fig. 6). It should be mentioned, that in contrast to milk Abs, IgG fractions from the above donors and patients exhibited much lower levels of amylolytic activities, although all the preparations showed the presence of some amylolytic activity. These findings speak in favour of the generation of such enzymes only in the milk of lactating mothers.

Catalytic Abs with glycosidase activity were generated for the first time using induction of a catalytic Ab to a half-chair transition-state analogue. Synthesis of such an analogue (hapten) and monoclonal Ab production were based on an idea that glycosidases stabilise the developing half-chair conformers along the hydrolysis pathway (SUGA et al., 1994). The significance of such activity of sIgA fractions from human milk is still unclear. However, we have shown recently significant differences between the substrate specificity, and specific activities of catalytic Abs from the sera of autoimmune patients (VLASSOV et al., 1998a,
and RNA-hydrolysing activities (A
SLE monoclonal Abs against DNA possess DNA
to production of anti-idiotypic abzymes. Since
enzymes could cause autoimmunization leading
than the sera of normal humans. These serum
enzymes and proteins at higher concentrations
healthy mothers (B

A number of catalytically active Abs have
been detected recently in the sera of patients with autoimmune pathologies (P
1994; Li et al.,
1995; PAUL et al., 1997; for a review see NEVINSKY
et al., 2000a), whose sera contain certain
enzymes and proteins at higher concentrations
than the sera of normal humans. These serum
enzymes could cause autoimmunization leading
to production of anti-idiotypic abzymes. Since
SLE monoclonal Abs against DNA possess DNA
and RNA-hydrolysing activities (ANDRIEVSKAIA
et al., 1997), one can believe that in some au-
toimmune patients with high concentration of nu-
cleic acids (first of all in SLE patients) there
may be production of abzymes as Abs to nucleic
acids or to DNA or RNA in complexes with cer-
tain proteins. Interestingly, VIP (vasoactive in-
testinal peptide)- and thyroglobulin-hydrolysing
Abs detected in sera of patients with asthma and
Hashimoto’s thyroiditis, respectively, are Abs di-
rectly against these proteins (PAUL, 1994; Li et al.,
1995). Thus, it is obvious that, in autoim-
mune patients, production of abzymes may oc-
cur via different mechanisms. Such Abs may be
both anti-idiotypic abzymes and Abs against spe-
sic antigens. One cannot exclude the possibility
that some potential antigens can change their con-
formation due to interaction with certain proteins
or enzymes and, as a consequence, the structure of the antigens in such complexes can mimic the
structure of the transition state of the reaction in-
volving this antigen conversion.

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