DNA-hydrolysing activity of IgG antibodies from the sera of patients with diseases caused by different bacterial infections

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Abstract

DNase autoantibodies (Abs) can be found in the blood of patients with several autoimmune diseases, while the blood of healthy donors or patients with diseases with insignificant disturbances of the immune status does not contain the DNase Abs. Here we have analysed for the first time the DNase activity in the patients with diseases caused by several bacterial infections. Several rigid criteria have been applied to show that the DNase activity is an intrinsic property of IgGs from the sera of patients with bacterial diseases but not from healthy donors. The relative activity of IgGs has been shown to vary extensively between the diseases analysed and from patient to patient, but most of the preparations had detectable levels of the DNase activity. On average, the catalytic activities were significantly lower than in patients with autoimmune pathologies and increased in the following order: streptococcal infection (erysipelas) < urogenital chlamydiosis associated with arthritis (Reiter’s disease) < meningococcal meningitis < shigellosis < supplicative surgical infections caused by Staphylococcus aureus < supplicative surgical infections caused by epidermal staphylococci < urogenital ureaplasmosis associated with reactive arthritis. While intact IgGs possessed this catalytic activity, separated light chains of polyclonal Abs appeared to be even more active in the hydrolysis of DNA.

Keywords: bacterial infections • abzymes • DNA hydrolysis

Introduction

Artificial (antibodies, or Abs, against transition state analogues of reactions) and natural abzymes (Abzs) are novel biological catalysts that have attracted much interest in the last years (reviewed in [1–5]). Natural catalytic Abzs hydrolysing DNA, RNA, polysaccharides, oligopeptides, and proteins exist in the sera of patients with many autoimmune (AI) and viral diseases [2–4]. Healthy human beings cannot develop Abzs with detectable DNase and RNase activities, their levels being usually on the borderline of sensitivity of the detection methods [6–8]. In addition, there was no confirmed nuclease Abs in the sera of patients with many different diseases (influenza, tonsillitis, duodenal ulcer, several types of cancer and other diseases) with insignificant AI reactions [7, 8]. We have shown that appearance of Abzs with nuclease activities is among the earliest and clear signs of AI reactions in a number of AI and viral diseases: systemic lupus erythematosus (SLE), Hashimoto’s thyroiditis, polyarthritis, multiple sclerosis, hepatitis ([2–5] and refs. therein).

Auto-Abs are characteristic not only of AI patients but also of healthy human beings; different auto-Abs including anti-DNA IgGs can be detected in the sera of healthy volunteers [5, 9]. Concentration of anti-DNA Abs is shown to be higher in patients with SLE (36% of SLE patients), multiple sclerosis (17–18%) [9, 10], primary Sjogren’s syndrome (18%), Hashimoto thyroiditis (23%), myasthenia gravis (6%) and rheumatoid arthritis (7%) than in healthy donors [10]. The auto-Abs of healthy human beings can, in principle, contain Abs with catalytic activities, but the concentration of Abzs in healthy volunteers may be significantly lower than in AI diseases. However, some catalytic Abs have been detected in the sera of healthy human beings. For example, Abzs with low catalytic activity hydrolysing vasoactive
intestinal peptide (VIP) were found in healthy human beings [11]. Abzs hydrolysing thyroglobulin were found in patients with rheumatoid arthritis, but IgGs from some healthy controls also demonstrated detectable levels of the thyroglobulin-hydrolysing activity [12]. Ab amylase activity in healthy donors was ~40–100-fold lower than in AI patients [13]. Interestingly, anti-DNA Abs in healthy volunteers and AI patients are quite different, since only Abs from the sera of AI patients hydrolyse DNA [2–5]. According to our data, the catalytic activity of nuclease Abzs is usually very easily detectable even at the beginning of AI diseases when the concentrations of Abs to DNA or other auto-antigens have not yet increased significantly and correspond to their ranges for healthy donors [2–5].

Because of their ability to bind a variety of exogenous antigens, including those on bacteria and viruses, natural Abs can play a major role in the primary line of defence against infections. Sepsis is the leading cause of death in intensive care units and results from a deleterious systemic host response to an infection. The presence of IgGs with serine protease-like activity was shown in patients with sepsis [14]. Although initially perceived as potentially deleterious, Abzs have also been proposed to participate in the control of disseminated microvascular thrombosis. It was shown that the presence of IgG with serine protease-like activity in the serum strongly correlates with survival after sepsis [14].

VIP-hydrolysing Abs of patients with asthma can have an important effect in the pathogenesis by decreasing the concentrations of VIP, which plays an important role in asthma pathophysiology [15]. Serine protease-like and metal-dependent proteolytic IgGs, IgMs and IgAs from patients with multiple sclerosis hydrolyse myelin basic protein of the myelin-proteolipid sheath of axons and therefore can play an important role in pathogenesis of this AI pathology [16, 17].

Abs against DNA and Abzs hydrolysing DNA or RNA are characteristic of SLE patients but could at a first glance be considered non-specific side-products of the AI processes in the sera of patients with many other AI and viral diseases (Hashimoto’s thyroiditis, polyarthritis, multiple sclerosis, AIDS, hepatitis). However, DNase Abzs from SLE, lymphoproliferative diseases [18], multiple sclerosis patients [4] and DNA-hydrolysing Bence-Jones proteins from multiple myeloma patients [19] are cytotoxic, cause nuclear DNA fragmentation and induce cell death by apoptosis. Therefore, it cannot be excluded that DNase Abzs play an important role in the pathogenesis of different AI diseases [2–5] and refs therein.

Abzs with different activities can be obtained by immunization of healthy animals with protein, DNA, RNA, etc. [20]. During many diseases caused by infections, the human organism is subjected to action of different bacterial components including protein, DNA, RNA and polysaccharides. Sera of mice infected with Salmonella typhimurium [21, 22], malaria [23], Plasmodium chabaudi [24] and human beings infected with Trypanosoma cruzi [25] and other bacterial pathogens [26, 27] contain a variety of Abs to microbial antigens and to human lipids, proteins and nuclear components, including anti-DNA Abs. The origin of anti-DNA Abs at infections remains speculative; some of them may arise inadvertently in normal immune response due to induction by Abs that bear structures (mimotopes) mimicking DNA [27]. The immunoregulatory effect of the infection seems to be related, at least partially, to the increase in a particular population of Abs, the polyreactive antibodies [22]. Some results suggest that the synthesis of antibodies directed against bacterial antigens and natural antibodies follow, at least partially, distinct pathways, but with the existing experimental data it is impossible to determine unambiguously whether these two antibody populations are produced by the same or distinct B-cell subpopulations [21].

Taking this into account, one cannot exclude a possibility of production of catalytic Abs not only with proteolytic [14] but also with other activities in human beings infected with some bacterial pathogens.

Here we have analysed for the first time a possible correlation of the relative DNA-hydrolysing activities of IgGs with some infections. DNase activity in bacterial infections was compared with that in AI and viral diseases.

**Materials and methods**

Reagents and chromatographic sorbents used in this work were obtained mainly from Sigma or Pharmacia.

**Description of patients and methods of diagnosis**

The blood sampling protocol conformed to the local hospital human ethics committee guidelines. Sera of healthy donors and patients with different diseases caused by various pathogenic bacteria were used to search for DNase Abzs. Patient’s clinical diagnoses were validated by a full set of physical and laboratory data (see below). The patients were treated according to standard protocols, including antibiotics therapy. The blood was collected within 7–10 days of the admission to the hospital. The sera of seven patients was collected before treatment with antibiotics. The characteristics of the patients are given in the Table 1.

To isolate streptococcal species, 5% blood Columbia agar was used. Staphylococci were grown on highly selective yolk-salt agar with sodium azide. Enterobacteria were primarily cultured using lactose-containing Endo, McConkey or similar media. Shigella identification was done according to the established protocol. Briefly, patient’s faeces were plated on Endo or McConkey agar with a parallel inoculation into the selenite selective broth for enrichment. After the primary growth, the microbial cells were re-inoculated into Kligler or a similar medium. The final identification of the cultures was made by type-specific serum agglutination and biochemical tests. The laboratory diagnosis of patients with suppurative surgical infections was confirmed by isolation of the causative agents from the primary site of infection with further identification of cultures with the ATB Expression system (Biomerieux, France). Patients with meningococc meningitis were diagnosed by microscopic detection of N. meningitidis (Gram-negative bean-shaped diplococci) in the cerebrospinal fluid followed by a positive latex agglutination test for detection of meningococcal antigens. Verification of urogenital chlamydiosis associated with arthritis (Reiter’s disease) was made by PCR combined with an immunofluorescent test or ELISA.
ELISA of anti-DNA Abs

The titres of anti-DNA Abs was determined using standard assay plates with immobilized double-stranded DNA as described in [9]. After consecutive treatment of the wells with the blood sera and horseradish peroxidase-conjugated rabbit Abs against mouse IgG, the reaction mixtures were incubated with tetraethyl benzidine and hydrogen peroxide. The reaction was stopped with sulphuric acid and optical density (A450) of the solutions was determined using a Labsystems Uniskan II plate reader. The relative concentrations of anti-DNA Abs in the samples was expressed as a difference in the relative absorbance at 450 nm (average of three measurements) between the experimental and control samples; controls using DNA without Abs and with Abs not interacting with DNA produced the same results.

IgG purification

Electrophoretically and immunologically homogeneous IgGs were obtained by sequential affinity chromatography of the serum proteins on protein A-Sepharose and FPLC gel filtration according to [16, 28]. Blood (3 ml) of healthy donors and patients with different diseases was incubated for 3–5 hrs at 4°C to achieve complete coagulation, carefully separated from the clot and centrifuged. Proteins were precipitated with ammonium sulphate (50% saturation). The pellet was dissolved in 1 ml of 20 mM Tris-HCl buffer (pH 7.5), dialysed for 12 hrs at 4°C against 200 ml of buffer A (150 mM NaCl, 50 mM Tris-HCl, pH 7.5), and loaded on an 1-ml protein A-Sepharose column equilibrated in buffer A. The column was washed with 15 ml of buffer A. Proteins adsorbed non-specifically were eluted with this buffer (15 ml) containing 1% Triton X-100 and 0.3 M NaCl, followed by 0.1 M sodium citrate (pH 4.6) and the column was washed with buffer A to zero optical density. The total Ab (IgG + IgA + IgM) fraction was eluted in 0.1 M glycine-HCl (pH 2.6), the column fractions were collected to cooled tubes containing 50 μl of 0.5 M Tris-HCl (pH 9.0) and finally each fraction was additionally neutralized with this buffer and dialysed against 10 mM Tris-HCl (pH 7.5), containing 0.3 KCl, and concentrated for additional purification.

FPLC gel filtration of this fraction was performed on a Superdex 200 HR 10/30 column (Pfizer, New York, NY, USA), using the BioCAD workstation (Applied Biosystems, Foster City, CA, USA) under ‘acid shock’ conditions analogous to human IgG as described previously [25]. Abs (2–3 mg/ml, 0.3 ml) were incubated for 20 min. at 25°C in 0.1 M sodium citrate (pH 2.6), containing 0.3 M NaCl and then subjected to the gel filtration on the column equilibrated with buffer A. The fractions collected were

Table 1 Characteristics of healthy human beings and patients with bacterial infections and the relative DNA-hydrolysing activities of polyclonal IgGs from patients with different diseases

| Disease                                           | Number of patients (men/women) | Average years old | Range of activities, % | Average activity, % * | Additional information, relative activity, % |
|---------------------------------------------------|--------------------------------|-------------------|------------------------|-----------------------|-----------------------------------------------|
| Healthy human beings                              | 10 (5/5)                       | 29.0 ± 15.0       | 0                      | 0                     | -                                             |
| Streptococcal infection (erysipelas)              | 19 (4/15)                      | 48.3 ± 7.8        | 0–36.1                 | 17.2 ± 7.0            |                                               |
| Urogenital chlamydiosis associated with arthritis (Reiter’s disease) | 17 (11/6)                     | 32.1 ± 7.3        | 2.0–58.4               | 17.8 ± 10.1           |                                               |
| Meningococcal meningitis                          | 5 (3/2)                        | 37.0 ± 11.5       | 2.1–46.8               | 19.0 ± 13.7           |                                               |
| Bacterial dysentery (shigellosis)                 | 11 (6/5)                       | 3.8–54.8          | 21.3 ± 13.7            |                      |                                               |
| Suppurative surgical infection caused by *Staphylococcus aureus* | 7 (3/4)                       | 39.2 ± 8.1        | 21.4–44.7              | 32.1 ± 7.4            | phlegmonas (n = 4), osteomyelitis (n = 3)    |
| Suppurative surgical infection caused by epidermal staphylococci | 4 (2/2)                       | 43.6 ± 13.6       | 16.2–50.1              | 34.5 ± 9.6            | phlegmonas (n = 3), peritonitis (n = 1)      |
| Urogenital ureaplasmosis associated with reactive arthritis | 3 (1/2)                       | 29.0 ± 10.0       | 95–97                  | 96.3 ± 1.1            | -                                             |

*The RAs were calculated from activities of IgGs corresponding to patients treated and untreated with antibiotics using the same fixed concentration of DNA (10 μg/ml), different concentrations of IgGs and 1–16 hrs of incubation, with the RAs normalized to 0.1 mg/ml IgGs and 2 hrs of incubation; for each patient, the mean of three repeats is used; the results are reported as the mean and the standard deviation.
dialysed against 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl. The type of Abs (IgA, IgG or IgM) in the fractions during the purification was determined by Western blotting as in [21–25]. In order to protect Ab preparations from bacterial and viral contaminations, they were filtered through a Millipore syringe-driven filter units (0.2 μm) and kept in sterilized tubes. Sterility control testing of purified IgG samples did not reveal any bacterial or fungal contamination. After 1–4 weeks of storage at 4°C for refolding the Abs were used in activity assays as described below. To exclude possible artifacts due to hypothetical traces of contaminating enzymes, the rigid criteria for assigning the DNase activity to IgGs were checked (see below).

In some cases, electrophoretically homogeneous IgGs were chromatographed on Sepharose bearing immobilized polyclonal mouse IgG against human IgG. The protein was applied to the column (1 ml) equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.1 M KCl and the column was washed with this buffer containing 0.3 M NaCl. Abs were eluted in 0.1 M glycine-HCl (pH 2.6), neutralized and dialysed as described above.

**DNase activity assay**

DNA-hydrolysing activity was analysed similarly to [7, 8, 28, 29]. The reaction mixture (20 μl) contained 10–20 μg/ml supercoiled DNA pBluescript, 2.5 mM MgCl₂, 1 mM EDTA, 20 mM Tris-HCl (pH 7.5) and 0.001–0.2 mg/ml Abs, and was incubated for 1–24 hrs (standard time 2 hrs) at 30°C. The cleavage products were analysed by electrophoresis in 1% agarose. The ethidium bromide-stained gels were photographed and the films were scanned.

All quantitative measurements of the relative DNase activity of Abs were performed according to general methods of enzyme-specific activity determination [30, 31] using scDNA as described below. The activities of IgG preparations were determined from the scanning data (Gel-Pro Analyzer v9.11, Media Cybernetics, Bethesda, MD, USA) as a percentage of DNA converted to the relaxed form from the initial supercoiled form, corrected for the distribution of DNA between these bands in the control (incubation of pBluescript in the absence of Abs). All measurements (initial rates) were taken within the linear regions of the time courses (15–40% of DNA hydrolysis) and a complete transition of supercoiled to nicked DNA (10 μg/ml = 3.35 nM) within 2 hrs was taken for the 100% activity. If the activity was low (<5–10% of scDNA disappearance), the incubation was prolonged to 3–24 hrs, depending on the sample. If the degradation of supercoiled DNA during 2 hrs of incubation exceeded 50%, the concentration of Abs was lowered 2–100-fold, depending on the sample analysed. All measurements (initial rates) were taken within the linear regions of the time courses and Ab concentration curves. This allowed to normalize the relative activities (RAs), like in the case of determination of the specific activity of enzymes [27, 28], to any standard condition, for example, 0.1 mg/ml (6.7 × 10⁻⁷ M) IgGs and a 2-hr incubation. In this case, 100% DNase activity corresponds to the specific activity of Abs (16.7 nM hydrolysed DNA/1 hr)/1 mg of Abs, and the apparent kcat value of the reaction at the fixed concentration of IgGs and DNA is -4.2 × 10⁻⁴ min⁻¹.

**SDS-PAGE assay of DNase activity**

SDS-PAGE analysis of Abs for the homogeneity under non-reducing conditions was performed in 5–16% gradient gels containing 0.1% SDS, and for the polypeptide spectrum, in reducing 12% gels containing 0.1% SDS and 50 mM DTT (Laemmli system) as described in [16, 28, 29]. The polypeptides were visualized by silver staining or by Western blotting on a nitrocellulose membrane [28, 29].

The IgG DNase activity after SDS-PAGE was analysed in gels containing calf thymus DNA (5 μg/ml) under non-reducing or reducing conditions as in [29]. Before the electrophoresis, the IgG samples were incubated at 22°C for 20–40 min. in 20 mM Tris-HCl (pH 7.5) containing 0.1% SDS with or without 50 mM DTT. To restore the enzymatic activity after SDS-PAGE, SDS was removed by incubating the gel for 1 hr at 22°C in 20 mM Tris-HCl (pH 7.5) and washing the gel five times with the same buffer. To allow protein refolding and to assay for DNase activity, longitudinal slices of the gel were incubated at 25°C for 15–48 hrs in the reaction buffer containing 20 mM Tris-HCl (pH 7.5), 4 mM MgCl₂, 0.2 mM CaCl₂. To visualize the products of the DNA hydrolysis, the gel was stained with ethidium bromide. The same ethidium bromide-stained or parallel longitudinal slices were used to detect the position of IgG in the gel by Coomasie Blue staining.

**Gel filtration of Abs after acid shock**

IgGs were incubated in 50 mM glycine-HCl (pH 2.6) containing 0.2 M NaCl for 30 min. at 25°C. Separation of the IgGs under ‘acid shock’ conditions was done by FPLC gel-filtration on Superdex 200 HR 10/30 (Pharmacia) equilibrated with 50 mM glycine-HCl (pH 2.6) as described previously [28, 29]. Fractions collected after the gel filtration were dialysed against 20 mM Tris-HCl (pH 7.5) containing 50 mM KCl and used in activity assays.

**Kinetic parameters**

The initial rates of the Ab-dependent hydrolysis of plasmid DNA were measured in kinetic experiments from the slopes of the time courses at the zero time. The reaction mixtures contained the standard components and 4–200 nM supercoiled pBluescript. The Kₘ and Vmax (kcat) values were calculated from the kinetic data by least-squares non-linear regression fitting using Microcal Origin v5.0 software and presented as linear transformations using a Lineweaver-Burk plot [30, 31]. Errors in the values were within 10–30%.

**Statistical analysis**

The results are reported as mean ± standard deviation of at least three different experiments for each sample of IgGs. The differences between IgG samples were analysed by Student’s t-test, P = 0.05 was considered statistically significant.

**Results**

To search for DNase Abzs in the cases of bacterial infection, the sera of patients with the following diseases were used: shigellosis, streptococcal infection (erysipelas), urogenital chlamydiosis associated with arthritis (Reiter’s disease), suppurrative surgical infections caused by *Staphylococcus aureus* and *Staphylococcus epidermidis*, urogenital ureaplasmosis associated with reactive arthritis and meningococcal meningitis. The characteristics of the healthy donors and different patients are given in the Table 1.
We have measured the titres of anti-DNA Abs in the sera of all patients and 10 healthy volunteers. The titres of anti-DNA Abs for healthy donors were not zero and varied from 0.01 to 0.29 A450 units. For all diseased patients, the anti-DNA titres were comparable (0.05–0.35 A450 units) with those for healthy volunteers, and only ~3–4% of all infected patients demonstrated significantly higher titres. This result agrees with the literature data that the sera of healthy donors usually contain different auto-Abs including Abs interacting with DNA [32, 33]. The titres of anti-DNA Abs in healthy human beings determined in this work may be an overestimate, since immobilized DNA can interact not only with Abs against DNA but also with Abs against phospholipids, polysaccharides, cell surface protein, etc. that demonstrate cross-reactivity with DNA [5, 32–34]. At the same time, despite anti-double-stranded DNA Abs are considered a serologic hallmark for SLE, the concentration of anti-DNA Abs was shown to be higher in comparison with healthy donors only in 36% of patients with SLE, and this number is significantly lower for other diseases (7–17%) [10] (see above). In untreated patients, the titres of anti-DNA Abs, in principle, may be higher after disease progression later than 7–10 days after the admission to the hospital. For example, the levels of mouse IgM and IgG auto-Abs against all antigens started to increase remarkably from 15 days and lasted until 6 weeks after the infection [35]. Our data, however, suggest that during 7–10 days after the admission of infected patients to the hospital the change in the titres of anti-DNA Abs is not statistically significant.

Interestingly, due to catalytic turnover, detection of abzymes is much more sensitive than determination of concentration of anti-DNA Abs by ELISA. We have shown that the appearance of Abzs specifically hydrolysing DNA is among the earliest and clear signs of AI reactions in a number of AI diseases when titres of Abs to DNA or other auto-antigens are not yet increased significantly and correspond to their ranges for healthy donors [2–5]. This result was confirmed recently using MRL-lpr/lpr mice where the increase in anti-DNA Abs and DNase Abzs was measured in dynamic of SLE development [36]. It was shown that during pre-disease and the beginning of disease, when specific and lineage-specific changes occur in differentiation and proliferation of bone marrow haematopoietic stem cells and in lymphocyte proliferation in different organs, the change in concentration of anti-DNA IgGs is not statistically significant, while the changes in RAs of Ab DNase and ATPase activities are statistically significant.

Purification and characterization of antibodies

IgG fractions were purified individually from the sera of 10 healthy donors and 66 patients with different diseases by chromatography on Protein A-Sepharose using a special protocol to remove non-specifically bound proteins. Strong non-covalent protein complexes usually dissociate under acidic conditions. As was shown previously, the purification conditions used excluded possible artefacts due to hypothetical traces of contaminating enzymes ([2–5] and refs. therein).

In order to obtain IgGs, the purified total IgGs (IgG + IgA + IgM) were subjected to gel filtration. The homogeneity of the typical 150-kD IgG was confirmed by SDS-PAGE with silver staining, which showed a single band under nonreducing conditions (for example, Fig. 1A) and two bands corresponding to the H and L chains after reduction (for example, Fig. 1B).

In principle, Abzs can be produced not only in the organisms of AI patients, but also in healthy human beings (see above). However, even nicking of plasmid scDNA, the most sensitive DNase assay, did not detect DNase activity of Abs from healthy human beings [2–5, 6]. Similar results were obtained for healthy mice, in which detectable activity of IgGs was revealed only after immunization with DNA or spontaneous development of SLE [36]. Interestingly, separated light chains of IgGs interacting with DNA are usually more active that intact IgGs [2–5] and sometimes, using scDNA, it is possible to reveal a very low activity of a small fraction of healthy human IgGs after their reduction with 2-mercaptoethanol [37].

We first used 10 IgG preparations from healthy donors and confirmed the previously published data ([2–6] and refs therein) that IgGs from healthy human beings do not possess detectable DNase activity (Fig. 2A). Although the sera from healthy human beings contained auto-Ab-interacting with DNA, they were inactive even after 24 hrs incubation of scDNA in the presence of 1 mg/ml IgGs (Fig. 2B). Thus, even if auto-Abs from healthy mammals interacting with DNA could possess DNase activity, this activity of intact IgGs is extremely low.

The RAs of IgGs from the sera of patients with different infectious diseases significantly varied from patient to patient but most of the Abs had a detectable DNase activity (Fig. 2A). We have confirmed that the DNase activity is an intrinsic property of IgGs of patients with diseases caused by the infections analysed...
(see below) and that the purified Abs can be used to evaluate their RA without additional purification of the Ab preparations.

As was shown previously, a 2-hrs incubation of scDNA with Abzs (0.03 mg/ml) from some patients with different AI diseases can cause only single breaks in one strand of the scDNA producing relaxed DNA, whereas others Abzs cause multiple breaks leading to formation of linear DNA [2–5]. The most active Abzs fragment DNA into short- and medium-length oligonucleotides. The percentage of patients with low (formation of rDNA only), intermediate (formation of partially degraded DNA) and high DNase activity (formation of short oligonucleotides) usually significantly changes from one pathology to another ([2–5] and refs therein).

Figure 2A illustrates cleavage of plasmid DNA after 2-hrs incubation with Abs from different patients with various infectious diseases. Interestingly, no IgGs from these patients were found to convert scDNA to fragmented DNA under these conditions, and only relaxed plasmid DNA was formed.

First, we have analysed the Ab-dependent hydrolysis of plasmid DNA in kinetic experiments to estimate initial rates from the slopes of the time course in the linear parts of these dependencies (Fig. 3A). To estimate quantitatively the DNase activity, we have found the concentration for each IgG preparation corresponding to the linear part of the rate dependencies upon Ab concentration (the conditions of the reaction of the pseudo-first order) and time of incubation where scDNA is converted into the relaxed (nicked) form without formation of linear or fragmented DNA after 0.2–12 hrs of incubation and the DNA hydrolysis corresponds to the linear regions of the time courses (15–40% of DNA cleaved, for example, Fig. 3B).

The efficiency of DNA cleavage was calculated from the percentage of DNA in the bands of scDNA and relaxed DNA taking into account the relative amounts of DNA in these two bands for control reactions incubated in the absence of IgGs or with Abs from healthy volunteers. Three independent experiments were used to estimate the average RA values for each Ab preparation (for example, Fig. 3B). Since all measurements (initial rates) were taken within the linear regions of the time courses and Ab concentration curves, the measured RAs for IgGs were normalized to standard conditions (0.1 mg/ml Abs, 2 hrs) and a complete transition of scDNA to its relaxed form was taken as 100% of DNase activity. The relative DNase activity corresponding to the complete transition of 10 μg/ml of scDNA into its relaxed form (3.35 nM, 100%) in the presence of 0.1 mg/ml Abs is equal to 16.7 nM nicked DNA / 1 hr / 1 mg of Abs. For each IgG preparation, the RA

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**Figure 2**
DNase activities of catalytic IgGs (0.1 mg/ml, 2 hrs) from patients with diseases caused by various bacterial infections (A) in the cleavage of supercoiled (sc) pBluescript plasmid DNA: lane 4, erysipelas; lane 5, suppurative surgical infection caused by *Staphylococcus aureus*; lane 6, suppurative surgical infection caused by epidermal staphylococci; lane 7, Reiter's disease; lanes 8 and 9, shigellosis. Lanes 2 and 3, DNA incubated with Abs from the sera of two healthy donors; lane 1, DNA incubated alone. DNA was incubated for 24 hrs with 1 mg/ml IgGs from the sera of three healthy donors (lanes 3–5) or DNA alone (lanes 1–2) (B). The hydrolysis of scDNA was analysed by agarose electrophoresis and ethidium bromide staining.

**Figure 3**
(A) Time dependence of scDNA hydrolysis in the presence of 0.15 mg/ml IgG of one patient with shigellosis; the percentage of hydrolysis estimated as 39%, normalization to standard condition (0.1 mg/ml Abs, 2 hrs) gives a relative activity ~26%. Electrophoretic analysis data of scDNA hydrolysis are given in the inset; lanes 1–7 correspond to 0, 0.26, 0.53, 1.2, 2, 3.4 and 4.6 hrs of the incubation. (B) Typical electrophoretic data used to estimate the DNase RAs of IgGs from different patients. The data corresponding to two different patients with urogenital chlamydiosis are given; lanes 1 and 5, scDNA incubated alone; lanes 2–4 and 6–8 correspond to independent repeats of DNase activity measurements. scDNA was incubated in the presence of 0.194 mg/ml IgG from the first patient (lanes 2–4) for 5 hrs (25.2 ± 0.5% of scDNA hydrolysis; RA = 5.2 ± 0.1%) and in the presence of 0.05 mg/ml IgG (lanes 6–8) of the second patient for 1 hr (14.6 ± 0.6% of scDNA hydrolysis, RA = 58.4%).
was determined from three independent experiments, the standard error of estimate was within 5–7%. The average values of the RAs of Abs corresponding to all groups of patients with various diseases (see above) were calculated. The data are summarized in the Table 1.

IgGs from seven patients with erysipelas, Reiter’s disease, meningitis and shigellosis, which were not treated with antibiotics before blood collection, demonstrated detectable or high DNase activity (~8–48%; 25.1 ± 12.9%), while 45 treated patients with these four and 59 patients with all seven diseases (Table 1) were characterized by average RAs 18.8 ± 13.9% and 25.4 ± 21.8%, respectively. There was no statistically significant difference in the RAs of IgGs from untreated and treated patients corresponding to four (P = 0.095) and all seven diseases (P = 0.31).

On average, the RAs of IgGs corresponding to patients treated and not treated with antibiotics increased in the order: streptococcal infection (17.2 ± 7.0%) ≤ urogenital chlamydiosis associated with arthritis (17.8 ± 10.1) ≤ meningococcal meningitis (19.0 ± 13.7%) ≤ shigellosis (21.3 ± 13.7%) ≤ suppurative surgical infections caused by S. aureus (32.1 ± 7.4%) ≤ suppurative surgical infections caused by epidermal staphyloccoci (34.5 ± 9.6%) < urogenital ureaplasmosis associated with reactive arthritis (96.3 ± 1.1%).

The coefficients of correlation between the numerical values of the anti-DNA Abs titres and RAs of Abs on the whole were estimated for all patients combined (0.75), patients with erysipelas (0.71), Reiter’s disease (0.78) and shigellosis (0.79) (in all cases P = 0.05).

It should be mentioned that the DNase RAs of IgGs in patients with different AI and viral diseases also depend very much on the patient and the disease analysed, the RAs usually increasing approximately in the order: diabetes < viral hepatitis < polyarthritis ≤ Hashimoto’s thyroiditis < AID ≈ multiple sclerosis < systemic lupus erythematosus ([2–5] and refs therein). The IgG RAs of patients with diseases caused by bacterial infections are statistically significantly higher than those for diabetes but there is no statistically significant difference with those for viral hepatitis and polyarthritis, for which RAs ≈100% are very rare. In other AI and
viral pathologies, 15–35% of IgG samples, depending on the disease, hydrolysed DNA more efficiently and, normalized to standard conditions, yielded RAs from 80% to 500%, with a small number of Ab samples showing RAs >1000%; these Abs demonstrate higher activities than those from patients with bacterial infections. Thus, the DNase Abzs from patients with bacterial infections were generally much less active than those from some AI diseases, especially SLE and multiple sclerosis.

Application of the strict criteria

To prove that the DNase activity of IgGs from the sera of patients with infections belongs to the Abs and is not due to co-purifying enzymes, we have applied several previously developed strict criteria [2–5, 11]. They may be summarized as follows: (i) the IgGs were electrophoretically homogeneous (Fig. 1); (ii) gel filtration of IgGs under conditions dissociating strong noncovalent complexes in an acidic buffer (pH 2.6, Fig. 4) did not lead to a disappearance of DNase activity, and the peaks of the activity tracked exactly with the intact IgGs; (iii) immobilized mouse polyclonal IgGs against human IgGs completely absorbed the DNase activity, and this activity corresponded only to the peak of IgGs eluted with an acidic buffer (Fig. 5).

To exclude possible artifacts due to hypothetical traces of contaminating enzymes, the IgGs were subjected to SDS-PAGE using a gel co-polymerized with calf thymus DNA, and their DNase activity was detected by incubating the gel in the standard reaction buffer, human urine DNase I and IgGs from healthy human beings were used as controls (Fig. 6). It should be mentioned that the incubation of IgGs with SDS and the following SDS-PAGE usually denatures the Abs to a significant extent, and the incubation of the gels in a refolding buffer restores the catalytic activities only of preparations very active initially [2–5]. Ethidium bromide staining of the gels after the electrophoresis and refolding of IgGs with initial RA ~45–100% (Table 1) revealed a sharp dark band on a fluorescent background of DNA. Without treatment of the Abs with DTT, the activity bands were revealed only at the positions of intact IgGs (Fig. 6B, lanes 1–2) and DNase I (lane 4), while after the DTT reduction, the separated light chains were also active (Fig. 6C). Since SDS dissociates all protein complexes, the detection of the activity in the gel region corresponding only to IgGs or their light chains, together with the absence of any other band of the activity or protein (Fig. 6), provides a direct evidence that the IgGs possess the DNA-hydrolysing activity.

Interestingly, the catalytic centres of IgGs of AI patients that hydrolyse proteins, DNA and RNA are most often located in the variable part of light chains of Abs ([2–5] and refs therein). In the crystal structure of a catalytic Ab with an esterase-like activity, p-nitrophenyl ester interacts with amino acid residues in both light and heavy chains of the IgG, and both subunits are required for catalysis [38]. The DNase centre of human milk sigAs is located in the light chain, while the DNA-binding centre is mainly formed by the heavy chain [39]. The active site of milk ATPase IgGs is formed by both light and heavy chains of the Abzs at their interface, and the separated subunits are not catalytically active [40].

As mentioned above, the DNase activity of intact IgG after SDS-PAGE was observed only in the case of Ab preparations with the highest, ~45–100% initial RAs (for example, Fig. 6B). After the reduction of disulfide bonds with DTT or 2-mercaptoethanol, the DNase activity of light chains was easily revealed by the in-gel approach even in the case of IgGs with moderate and sometimes low RA, 20–40% (Fig. 6C). During prolonged storage (3–5 months) of the Ab preparations in a neutral buffer (pH 7.5)
containing NaN3, some IgGs partially disintegrated forming small amounts of free L- and H-chains. In these cases, it was possible to detect the catalytic activity of separated light chains even for the IgG preparations with low RAs, the intact molecules of which are not active after a typical incubation with DTT and reducing SDS-PAGE in a gel containing DNA (data not shown). Separated light chains of Abs with different catalytic activities are very often more active than the intact Abs [2–5, 43]. For example, the light chains hydrolyse VIP with specific activity 32-fold greater than that of Fab fragments [43].

The DNase activity of the light chain is presumably due to a presence of an enzyme-like active site, in which activation of functional groups of certain amino acids may occur via intramolecular interactions similar to those found in proteolytic abzymes and natural enzymes [44]. It may be hypothesized that the DNase activity of the light chain arises from its ability to recognize the transition state of the reaction of DNA hydrolysis.

The heavy chain rather than the light chain is generally considered to play an important role in binding and recognizing antigens ([45, 46] and refs therein) including DNA [47]. Using DNase Abs from the sera of patients with SLE and multiple sclerosis [5], we have shown previously that separated light chains possess $10^2$–$10^3$-fold lower affinity for scDNA than intact IgGs interacting with DNA through both heavy and light chains and catalyse hydrolysis of DNA 50–500-fold faster than intact Abs. According to [48], the reduced $k_{cat}$ implies that stabilizing interactions of the ground state with the heavy chain are evidently lost in the transition state, resulting in $k_{cat}$ reduction for intact Abs. Similar reasons for the increase in the RA of separated light chains as compared with intact IgGs may be true for Abs from AI and infected patients.

**Characteristics of IgG-dependent hydrolysis of DNA**

As mentioned above, the RAs of IgGs varied widely between individuals with different infectious diseases. It was shown previously that polyclonal Abzs from patients with different AI diseases can contain one to several subfractions of Abzs characterized with different $K_M$ and $k_{cat}$ values, as well as Abs interacting with DNA but having no catalytic activity [2–5]. Unfortunately, there are presently no ways to separate catalytically active and inactive Ab subfractions. Therefore, RA of polyclonal Abzs from different patients and their affinity for substrates (in terms of $K_M$) are usually characterized by average values of apparent $K_M$ and $k_{cat}$ calculated using the total concentration of Abs [2–5].

The $K_M$ and $k_{cat}$ values were measured using the total concentration of polyclonal IgGs and the conditions of the reaction of pseudo first order. In this case, catalytically inactive IgG subfractions, either interacting with DNA or not, do not affect the apparent $K_M$ and $k_{cat}$ values. The dependencies of $1/V$ upon $1/[S]$ provide an estimate of average values characterizing the Abz subfractions with comparable $K_M$ values; in the case of significant differences in $K_M$ or $k_{cat}$ values ($>5$-fold) between different catalytic IgG subfractions, several pairs of these values can be found [2–5]. The measured $k_{cat}$ values usually characterize the relative content of
Typical estimates of the apparent $K_M$ and $V_{max}$ ($k_{cat}$) values for plasmid DNA hydrolysis catalysed by IgGs from patients with bacterial dysentery (shigellosis) (A) urogenital ureaplasmosis associated with reactive arthritis (B): 1.2 μM and 0.12 μM IgGs, respectively, were used in these experiments.

Fig. 7 Lineweaver-Burk plot of $K_M$ and $V_{max}$ determined for plasmid DNA hydrolysis catalysed by IgGs from patients with bacterial dysentery (shigellosis) (A) urogenital ureaplasmosis associated with reactive arthritis (B). One IgG from one patient with urogenital ureaplasmosis showed $K_M$ values and $V_{max}$ that correspond to hyperbolic curves of Abs saturation with DNA substrate (for example, Fig. 7B). One IgG from one patient with shigellosis showed $K_M$ and $k_{cat}$ values corresponding to two values of these parameters were also revealed for IgG with the highest activity from one patients with streptococcal infection (erysipelas): $K_M$ (I) = 41 ± 8 nM ($k_{cat} = (1.3 ± 0.4 \times 10^{-3})$ min$^{-1}$) and $K_M$ (2) = 360 ± 45 nM ($k_{cat} = (7.0 ± 1.0) \times 10^{-3}$ min$^{-1}$).

In principle, one cannot exclude that the biphasic character of these dependencies can reflect allosteric interaction between two DNA binding centres of IgG molecules or activation of IgGs by an excess of substrate. We have shown previously that polyclonal IgGs from the sera of patients with viral and AI diseases can contain one to several Abz subfractions with different affinities for DNA, which can be separated by chromatography on DNA-cellulose [5]. All separated fractions usually demonstrate monophasic curves, while initial non-separated IgG preparations can show from 2 to 4 or even more sets of $K_M$ and $k_{cat}$ values corresponding to these values of the enzyme subfractions with different affinities for DNA-cellulose [5]. The number of Abz subfractions with different affinities for DNA is usually increased with the increase in RAs and the progress of pathology. Therefore, the observed $K_M$ and $k_{cat}$ for IgGs from the sera of infected patients most probably also characterize the average $K_M$ and $k_{cat}$ values corresponding to two major DNase IgG subfractions in the polyclonal Ab preparations analysed.

Interestingly, two $K_M$ values for one IgG preparation are in agreement with the general situation of the very high diversity of polyclonal Abzs with various catalytic activities in the sera of AI patients [3–5]. For example, polyclonal nuclease and polysaccharide-hydrolysing Abzs contain κ or λ light chains, demonstrate the highest activity at various optimal pHs, are activated or not by metal ions and can be characterized by many different net charges as well as $k_{cat}$ and $K_M$ values and by different substrate specificities [3–5]. It was shown that at the beginning of AI diseases it is usually possible to reveal only one $K_M$ value for DNA or RNA as substrates of nuclease Abs, while after a long time of pathology development or at its exacerbation, the repertoire of monoclonal DNase Abs is usually much wider, and the polyclonal Abzs may be characterized by 2–4 or even more distinguishable $K_M$ values for some samples analysed, producing a constant increase in the reaction rate with an increase in the substrate concentrations [5].

The catalysis mediated by artificial Abzs is usually characterized by $10^2$–$10^5$-fold lower $k_{cat}$ values than for the canonical enzymes ([1–5] and refs therein). The known $k_{cat}$ values for natural Abzs from AI patients vary in the range 0.01–15.6 min$^{-1}$ [5, 11, 49–51]. The data on the measured $k_{cat}$ values allow us to estimate the range of these values for IgGs from different patients with various infections approximately from $(1 ± 0.25) \times 10^{-5}$ to 0.07 min$^{-1}$. The highest $k_{cat}$ value determined in our experiments is about 200 times lower than the $k_{cat}$ value for hydrolysis of scDNA by SLE IgGs (14 min$^{-1}$) and significantly lower than for DNase I $(1.0 – 3.2 \times 10^2$ min$^{-1}$) [51]. The specific DNase activities of polyclonal (non-separated on DNA-cellulose) IgGs from patients with bacterial infections are comparable with those of known natural Abzs including DNase Abs from patients with other AI and viral diseases (see above).
The first $K_m$ for plasmid DNA for all analysed IgGs varied in the 38–104 nM range, and the second one, in the range of 204–360 nM. Both values correspond to typical $K_d$ values for Ab interaction with different antigens, and they are comparable with the $K_d$ value for plasmid DNA (43 nM) reported previously for IgG from SLE patients [51], but higher than for DNase IgGs (0.34 nM) from the sera of patients with multiple sclerosis [52]. These data suggest that the immune systems of patients with SLE, MS and bacterial infections can generate DNase IgGs of different types.

Different anti-DNA Abs in AI and bacterial infection diseases may form in different ways. It was shown that bacterial DNA is more immunogenic for healthy mammals than mammalian DNA and can induce formation of anti-DNA Abs, since it is rich in GC dinucleotides and methylated adenosines and possesses unique immunogenic epitopes [53]. Some data [22, 54] demonstrate that bacterial DNA can induce AI reactions against DNA mostly in organisms lacking correct mechanisms of tolerance to autoantigens, while in healthy mammals the formation of mainly specific Abs against bacterial DNAs is observed.

It was shown that the specific reorganization of immune system during the spontaneous development of a profound SLE-like pathology in MRL-lpr/lpr mice is associated with changes in the differentiation profile and the level of proliferation of bone marrow haematopoietic stem cells and with production of DNase, ATPase, and amylase Abs [36]. Immunization of healthy mice with DNA also leads to production of these Abs; however, it is associated only with increased lymphocyte proliferation and suppression of apoptosis of lymphocytes in different organs (especially spleen), but not with a change in differentiation of the bone marrow cells [36]. Immune processes after immunization of mammals with bacterial DNA during many infectious diseases may be considered similar to those after immunization of healthy mice with DNA. According to theoretical analysis, the adaptive improvement of the catalytic turnover is limited by the rate of B cell receptor signal transduction, as rapid release of antigen fragments from catalytic B cell receptors aborts clonal selection, but production of catalysts can occur at increased levels under conditions of rapid B cell signalling in AI disease [55]. In addition, the RA's of DNase Abs increased with the progress of the AI pathology [5], while the time course of immunization associated with infections is usually not so long as compared with AI diseases, which can have chronic character.

It was shown that the presence of IgG endowed with serine protease-like hydrolytic activity in the plasma of patients with sepsis strongly correlates with their survival [14]. In contrast to DNase abzymes, the polysaccharide-hydrolysing Abs are usually present even in the sera of healthy human beings and their activity remarkably increases in the sera of patients and animals with different AI diseases [13, 42] and especially with pathologies caused by infections (V.N. Buneva, unpublished). Formation of specific Abs against bacterial DNA during bacterial infections of healthy mammals suggests that the specific catalytic Abs can mostly hydrolyse bacterial DNA. One cannot exclude that co-action of Abs with proteolytic and polysaccharide-hydrolysing activities can at least partially degrade the bacterial cell wall and facilitate the entry of DNase Abs into bacterial cells and the hydrolysis of bacterial DNA. This cooperative action of abzymes with different catalytic activities may have a protective effect against diseases caused by infections.

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