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Natural Hidden Autoantibodies React with Negatively Charged Carbohydrates and Xenoantigen Bdi

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Abstract—Immunoglobulin preparations from sera of healthy donors contain polyspecific autoantibodies interacting with DNA and other charged antigens. These antibodies belong to the IgG class and can exist in the free or hidden state. The hidden antibody activity can be revealed after ion-exchange chromatography on QAE-Sephadex A-50. Immunoenzyme assay was used to assess the interactions of both free and hidden antibodies with different carbohydrates. The hidden antibodies were only able to interact with different polyanionic carbohydrates and neutral xenoantigen Bdi.

Key words: natural antibodies, hidden antibodies, polyspecificity of antibodies, antibodies against DNA, antibodies against carbohydrates

Natural antibodies are common components of the immune system. They are present abundantly (to the extent of 20%) in blood sera from healthy humans and other animals; they are encoded by fetal genes and produced without specific antigen stimulation [1]. A characteristic feature of natural antibodies is their polyspecificity and ability to interact with multiple antigens, such as DNA, phospholipids, cytoskeletal and nuclear proteins, or membrane components [2].

The functions of natural antibodies are still not clearly understood. These antibodies are supposed to be involved in immunoregulatory processes, defense against outer pathogens, binding and excretion of the products of cell decay, and in the pathogenesis of some pathologies such as dysfunctions of blood coagulation, autoimmune processes, or cytotoxicity.

We pioneered in the discovery of two coexisting natural antibody subpopulations, free and hidden, in blood sera of healthy humans (or in immunoglobulin preparations), which interact with DNA [3]. The hidden antibody activity can be revealed after either ion-exchange chromatography or gel filtration at pH 4.5 [4], which result in the decomposition of immune complexes. We previously showed that the free and hidden antibodies differ significantly in their properties. Thus, the hidden antibodies only influence blood coagulation processes and interact with a series of human or animal cells resulting in their death; in so doing, these antibodies interact preferably with antigens carrying a well-expressed negative charge [5, 6].

Considering these facts, we investigated the comparative interactions of both free and hidden natural antibodies with different antigens, primarily with negatively charged carbohydrates that are characteristic of membrane glycoproteins.

MATERIALS AND METHODS

Reagents. Human immunoglobulins for intravenous injections (Sandoglobulin, Switzerland; Intraglobin, Germany; Gabrioglobin, Russia), QAE-Sephadex A-50 and DNA from calf thymus (Sigma, USA), and anti-human IgG antibodies peroxidase conjugate (Sevac, Czech Republic) were used. Polyacrylamide conjugates of mono- and oligosaccharides (molecular mass 35 kD) were purchased from Syntesome (Munich, Germany).

Isolation of free and hidden DNA-binding antibodies. Antibodies were isolated from commercial immunoglobulin preparations containing 98-99% IgG according to the manufacturer.

DNA-cellulose was prepared by the method of Litman [7]. IgG solutions (10-12 mg/ml) in buffer containing 0.15 M NaCl, 0.05 M Tris-HCl, pH 8.0, and

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0.005 M EDTA were chromatographed on DNA-cellulose. Free DNA-specific IgGs were eluted in 2 M NaCl, dialyzed against buffer containing 0.14 M NaCl, 50 mM sodium phosphate, pH 7.4, and concentrated. QAE-Sephadex for chromatography was prepared by incubation for one day in 0.01 M potassium phosphate buffer and then by washings several times in the same buffer, then in 0.5 M NaCl, and then again in the chromatography buffer. The IgG fraction, which was not bound to the affinity matrix, was dialyzed against 0.01 M potassium phosphate buffer and subjected to ion-exchange chromatography on QAE-Sephadex A-50 in the same buffer. Two fractions were obtained: 1) that not bound to the matrix; 2) that adsorbed by the QAE-Sephadex and then eluted with 0.5 M NaCl. Both fractions were subjected to affinity chromatography on DNA-cellulose as described above, and hidden antibodies were collected separately from the first and the second fraction. Protein concentration was measured by spectrophotometry at $\lambda = 280$ nm. The antibodies were stored at –25°C.

Immunoenzyme assay. DNA from calf thymus and mono- and oligosaccharide conjugates with polyacrylamide [8] were used as antigens.

DNA solution (100 μl, 10 μg/ml) in phosphate-buffered saline (0.14 M NaCl, 50 mM sodium phosphate, pH 7.4) was placed into each well of 96-well immunological plate (Labstar) and incubated for 18 h at room temperature. Carbohydrates (10 μg/ml) or bacterial lipopolysaccharides (20 μg/ml) in 0.05 M carbonate buffer, pH 9.2, were coated on immunological plates (Nunc) for 18 h at 37°C. Gelatin (0.5%) in phosphate-buffered saline containing 0.05% Tween 20 was used as blocking solution. The same solution was used for incubation with both antibodies (different dilutions) and peroxidase anti-IgG conjugate (1 h at 37°C). *o*-Phenylenediamine was used as the substrate. The reaction was terminated with 1 M H_2SO_4. Optical density was determined using a Multiscan reader at $\lambda = 492$ nm.

The inhibition of antibody-carbohydrate interactions was tested by immunoenzyme assay as follows. DNA, the inhibitor, at concentrations from 1 μg to 1 ng per sample, was added to 1 μg of hidden antibodies in blocking solution and incubated for 1 h at 37°C and then for 18 h at room temperature. The immunoenzyme assay was then performed as described above. The inhibition of hidden antibody–DNA interactions by carbohydrate conjugates was studied in the same way. Results are expressed as percentage of inhibition.

RESULTS AND DISCUSSION

Earlier, we showed that human immunoglobulin preparations contain two subpopulations of polyspecific antibodies, free and hidden, that demonstrate cross-reactivity to DNA and many other antigens [3]. The contents of free antibodies in different IgG preparations used in this study varied from 0.22 to 0.48%; hidden antibodies comprised from 0.9 to 1.23% in the IgG1 fraction after QAE-Sephadex and from 1.32 to 1.8% in the IgG2 fraction. Figure 1 demonstrates some typical results from experiments on the interaction of both free and hidden antibodies (in the IgG2 fraction after QAE-Sephadex) with DNA in comparison with the initial IgG preparation. This preparation interacted slightly with DNA, possibly because of the presence of free antibodies. However, both free and especially hidden affinity antibodies interacted much more effectively with DNA. Antibodies from the IgG1 fraction demonstrated the same results. The threshold level of optical density in the absence of antigen did not exceeded 0.14 for free antibodies and 0.22 for hidden antibodies (taken at 10 μg per sample) and decreased regularly when antibody concentration decreased.

However, these subpopulations differ in their ability to interact with carbohydrates. Both intact IgG preparations and free antibodies slightly interact with carbohydrate antigens, as shown in the table. Hidden antibodies demonstrated a higher level of binding with all antigens studied that carried negative charge and also with neutral Bdi antigen.

To elucidate whether the same or closely positioned molecular sites are involved in the interaction of hidden antibodies with DNA and carbohydrates, we performed experiments on inhibition in immunoenzyme assay. The results presented in Fig. 2 show that DNA at concentra-

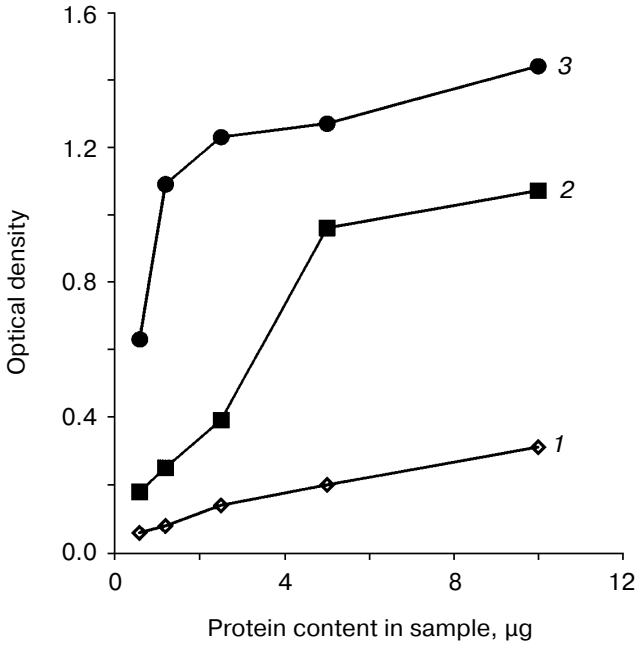

Fig. 1. Interactions of IgG, free, and hidden antibody preparations with DNA in immunoenzyme assay: *1*) initial IgG preparation; *2*) free antibodies; *3*) hidden antibodies.

Interaction of IgG preparations and free and hidden antibodies with carbohydrates in immunoenzyme assay (optical density units)

Antibodies	Protein in sample, μg	3-HSO_3-Gal-PAA	6-HSO_3-GlcNAc-PAA	Man-6-P-PAA	3'-HSO_3-Le^a-PAA	$SiaLe^x$-PAA	Sia_{5-6}-PAA	Glcβ-PAA	Bdi-PAA
IgG	10	0.45 ± 0.05	0.43 ± 0.09	0.40 ± 0.05	0.39 ± 0.07	0.38 ± 0.10	0.48 ± 0.09	0.27 ± 0.07	0.52 ± 0.07
	5	0.30 ± 0.06	0.38 ± 0.07	0.34 ± 0.02	0.33 ± 0.05	0.25 ± 0.07	0.41 ± 0.10	0.22 ± 0.07	0.41 ± 0.07
	2.5	0.21 ± 0.04	0.29 ± 0.03	0.25 ± 0.04	0.28 ± 0.05	0.20 ± 0.06	0.35 ± 0.05	0.17 ± 0.04	0.28 ± 0.05
	1.25	0.17 ± 0.05	0.20 ± 0.06	0.21 ± 0.04	0.20 ± 0.02	0.16 ± 0.06	0.28 ± 0.05	0.14 ± 0.04	0.23 ± 0.03
	0.6	0.15 ± 0.03	0.18 ± 0.03	0.19 ± 0.03	0.15 ± 0.04	0.14 ± 0.03	0.21 ± 0.04	0.13 ± 0.03	0.21 ± 0.04
Free antibodies	10	0.47 ± 0.09	0.42 ± 0.09	0.43 ± 0.03	0.43 ± 0.03	0.41 ± 0.12	0.40 ± 0.02	0.24 ± 0.08	0.46 ± 0.05
	5	0.24 ± 0.05	0.37 ± 0.10	0.33 ± 0.01	0.35 ± 0.12	0.29 ± 0.08	0.35 ± 0.04	0.22 ± 0.07	0.32 ± 0.06
	2.5	0.21 ± 0.05	0.27 ± 0.02	0.22 ± 0.06	0.38 ± 0.11	0.23 ± 0.07	0.31 ± 0.03	0.16 ± 0.09	0.21 ± 0.02
	1.25	0.20 ± 0.01	0.21 ± 0.02	0.25 ± 0.02	0.23 ± 0.02	0.17 ± 0.05	0.27 ± 0.04	0.12 ± 0.08	0.18 ± 0.04
	0.6	0.16 ± 0.03	0.20 ± 0.02	0.20 ± 0.03	0.19 ± 0.02	0.16 ± 0.05	0.21 ± 0.03	0.12 ± 0.04	0.15 ± 0.03
Hidden antibodies	10	1.58 ± 0.05	1.35 ± 0.22	1.32 ± 0.12	1.57 ± 0.05	1.31 ± 0.13	1.55 ± 0.05	0.63 ± 0.12	1.52 ± 0.08
	5	1.42 ± 0.08	1.19 ± 0.09	1.16 ± 0.12	1.35 ± 0.13	1.20 ± 0.09	1.31 ± 0.06	0.49 ± 0.03	1.35 ± 0.02
	2.5	1.25 ± 0.08	1.03 ± 0.09	1.05 ± 0.11	1.12 ± 0.08	1.13 ± 0.11	1.18 ± 0.09	0.38 ± 0.08	1.12 ± 0.11
	1.25	1.02 ± 0.11	0.92 ± 0.07	0.90 ± 0.08	0.88 ± 0.10	0.82 ± 0.13	1.02 ± 0.08	0.35 ± 0.05	1.02 ± 0.07
	0.6	0.78 ± 0.07	0.66 ± 0.05	0.79 ± 0.08	0.72 ± 0.07	0.64 ± 0.08	0.81 ± 0.08	0.31 ± 0.06	0.93 ± 0.07

tions from 10 ng to 1 μg strongly inhibits the interaction of hidden antibodies with three different antigens. Reciprocal experiments on the competition of hidden antibodies from the IgG2 fraction for DNA binding (Fig. 3) showed that both sialic acids and Bdi antigen inhibit the interaction of the antibodies with DNA.

We performed additional experiments to determine whether the ability of hidden antibodies to interact with carbohydrates is their intrinsic and unique property or whether it results from ion-exchange chromatography on QAE-Sephadex. Free antibodies were subjected to an additional step of ion-exchange chromatography on QAE-Sephadex followed by testing of their interaction with carbohydrates in immunoenzyme assay. Because the binding level was unchanged (data not shown), we concluded that the carbohydrate-binding activity is a characteristic feature of hidden antibodies. Also, the results on the interaction of both free and hidden antibodies to bacterial lipopolysaccharides (Fig. 4) indicate that in this case the higher level of antigen binding is characteristic of the hidden antibodies.

Cross-reactivity of hidden antibodies to DNA and carbohydrates is additional proof of the fact that the polyspecificity is characteristic of the natural antibodies. Multiple antigen-binding sites on the antibody molecule

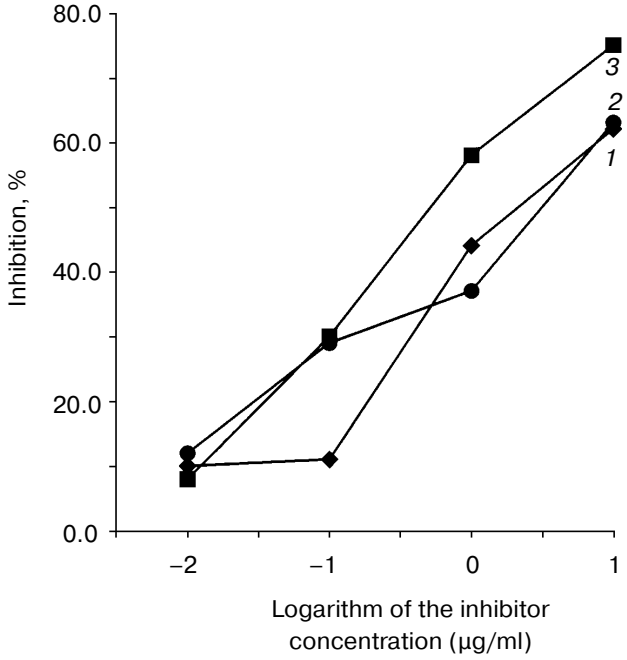

Fig. 2. Inhibition of hidden antibody (IgG2 fraction) binding with carbohydrates by thymus DNA in immunoenzyme assay: *1*) 3'-HSO_3-Le^a-PAA; *2*) 6-HSO_3-GlcNAc-PAA; *3*) Sia_{5-6}-PAA.

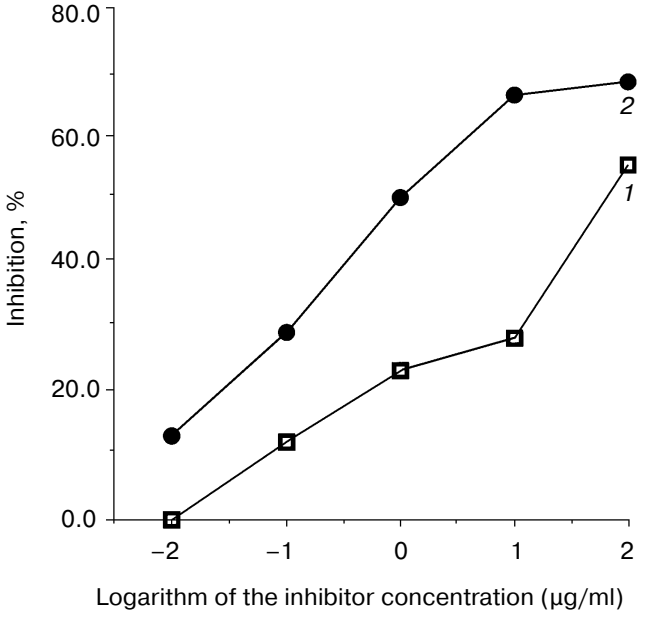

Fig. 3. Inhibition of hidden antibody (IgG2 fraction) binding with DNA by carbohydrate conjugates in immunoenzyme assay: *1*) $Sia_{5\text{-}6}$-PAA; *2*) Bdi-PAA.

as well as the presence of sites possessing an extraordinarily high electric charge or hydrophobicity may be the reason for the polyspecificity. We suggested earlier that the interaction of hidden antibodies with antigens is mainly due to electrostatic interactions. Indeed, as shown previously, the hidden antibodies, unlike free ones, interact preferably with molecules carrying multiple negative charges, such as DNA, phosphatidylserine, cardiolipin [6], and dextran sulfate. On the other hand, according to our previous estimates, the maximal p*I* values for the hidden antibodies are 8.0-9.5, and for the free antibodies they are from 5.5 to 6.5. It is very likely that the hidden antibody molecules contain higher levels of such amino acids as arginine and lysine, this determining their high p*I* values. This feature is characteristic of many antibodies that are able to interact with DNA and other anionic molecules [9], and they are the most pathogenic with systemic lupus erythematosus (SLE) [10].

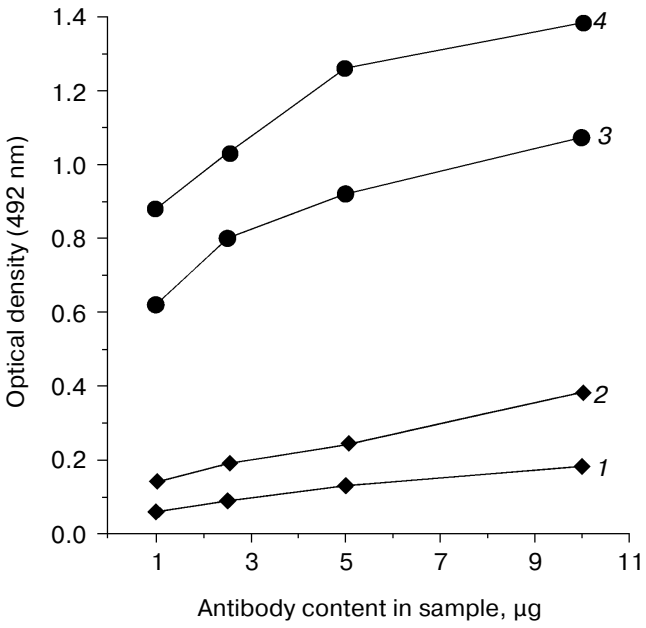

Fig. 4. Interaction of free and hidden antibodies with lipopolysaccharides of *E. coli* (*1*, *3*) and *Salmonella typhimurium* (*2*, *4*) in immunoenzyme assay: *1*, *2*) free antibodies; *3*, *4*) hidden antibodies from the IgG2 fraction.

Natural autoantibodies reacting with DNA are commonly the products of normal embryonic genes [11] and are present in blood sera of healthy humans and animals. The polyspecificity of the natural antibodies in this case may indicate that the lipopolysaccharides from Gram-negative bacteria instead of DNA are the initial antigens. In such a situation, cross-interactions may come from epitope conformation similarity as well as charge interactions. It is interesting to note that many of the natural antibodies, such as antibodies specific to carbohydrates [12] or DNA [13], are synthesized by $CD5^+$ B-cells and possibly represent the same antibody population.

The fact that hidden antibodies are very similar to those characteristic of autoimmune diseases in a number of properties attracts attention. The hidden antibodies belong to the IgG class, react with closely related antigens, and possess high affinity constants. Such antibodies, because of their cross-reactivity with DNA, phospholipids, and carbohydrates, may form the immune complexes both in blood serum and on cell surfaces, this resulting in a progression of autoimmune processes specific for SLE [14], phospholipid syndrome [15], or neuropathies [16].

It should be mentioned that the carbohydrates we studied are typical components of membrane glycolipids and glycoproteins and play an important role in the processes of cell response, proliferation, reception, and endocytosis. Antibodies interacting with such carbohydrates can certainly influence these processes. Actually, we have demonstrated that, unlike free antibodies, the hidden ones interact actively with surfaces of different human and other mammalian cells: thymocytes, thymoma cells, lymphocytes, platelets, and endotheliocytes [17, 18], resulting in massive (30-40%) cell death. It is very likely that some membrane carbohydrates common for cells of with different tissue and species specificity could, in this instance, appear as antigen determinants. Moreover, it has been shown in a number of studies that normal human and animal blood sera contain natural antibodies against carbohydrates; these antibodies display a cytotoxic effect [19] inhibitable by such carbohydrates as sialic acids, D-galactose, N-acetylglucosamine, and complex oligosaccharides.

It should be noted that we have tested, in addition to six negatively charged carbohydrates, two neutral ones: Glc as a possibly negative control and Bdi as a xenoantigen (see below). The data presented indicate that all antibodies tested bind Glc; however, the binding level is markedly lower than that for charged antibodies with Bdi. The level of binding with singly charged antigens is virtually independent of the nature of the carbohydrate (HSO_3Gal, 6-$HSO_3GlcNAc$, Man-6-P, HSO_3Le^a, $SiaLe^x$) or charged group (sulfate, carboxyl); however, interaction with Sia_{5-6}-PAA (mixture of pentamer and hexamer of sialic acids) is significantly stronger, possibly reflecting the higher negative charge of this antigen.

The strong interaction with the neutral Bdi-PAA (Bdi is Galα1-3Gal) is evidence that not only electrostatic forces determine hidden antibody binding.

Specific interest arises from the ability of hidden polyspecific antibodies to interact with carbohydrate xenoantigen Bdi. It is known that antibodies against Bdi do arise in humans and Old World primates but do not in the other monkeys and other animals [20]. The content of natural anti-Bdi antibodies in serum may run as high as 1-2% of the circulating IgGs. However, our data indicates that such antibodies may be in the hidden state, this resulting in additional complications with xenotransplantation.

When discussing possible functions of natural hidden antibodies, it should be kept in mind that in the blood sera of healthy individuals these antibodies are present as complexes, so they do not expose their reactivity.

As we have previously shown, the activity of hidden antibodies develops, when the immune complexes dissociate under chromatography of IgGs on anion-exchange Sephadexes, gel filtration at pH 4.5, or affinity chromatography of blood sera on Protein A-Sepharose. These facts suggest that the hidden antibodies are able to form two types of complexes: 1) with negatively charged antigens, and 2) with anti-idiotypic antibodies or other proteins in serum. It has been demonstrated in a number of studies [21, 22] that various natural antibodies do constitute such immune complexes. Hence, either overproduction of natural hidden antibodies or deficiency of blocking components (antigens) may lead to autoimmune processes and other pathologies. Moreover, we found previously that the release of bound hidden antibodies may result from some injuries, such as traumatic or heat shock [23, 24].

Blood plasma components blocking hidden antibodies as well as factors inducing the disruption of complexes *in vivo* will be the subject for further detailed investigations.

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