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Four Free Cysteine Residues Found in Human IgG1 of Healthy Donors

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Abstract—Modifications with different thiol reagents demonstrated that 28 of 32 cysteine residues of human IgG1 are involved in the formation of disulfide bonds, and four cysteines remain free. So IgG1 is a protein possessing both free SH-groups and disulfide bonds. Only one of the four SH-groups is accessible for silver or mercury ions and hydrophobic reagents, whereas the remaining three SH-groups are masked and can be revealed only after deep denaturation of the protein. Detection of the masked cysteine residues was shown to depend on the kinetics of intramolecular changes occurring during denaturation of the protein and on the method of the assay of the SH-groups.

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Progress in investigation of the structure of IgG is connected with the discovery of the domain organization of the macromolecule and determination of the functional role of each domain. Numerous studies including physicochemical, protein, and immunochemical analysis have demonstrated that the IgG molecule contains two heavy and two light chains that include 12 domains. The domains have similar packing of the polypeptide chain, and each domain possesses one disulfide bond in its hydrophobic nucleus [1-3]. Four polypeptide chains form three large substructures, two identical Fab fragments containing the antigen-binding site and Fc fragment that exerts the effector functions. The Fab fragments are connected with the Fc fragment through constant sites of the heavy chains named the hinge region [3, 4]. The characteristic feature of this region is a high content of cysteine residues involved in the formation of the interchain disulfide bonds, providing rigidity of the molecule.

The IgG1 molecule is considered to have 16 disulfide bonds and no free cysteine residues [4, 5]. However, there are a number of works demonstrating that IgG of humans [6-10] and animals [11, 12] contain free SH-groups (0.3-2 mol/mol protein), and their content is reduced in pathology [12-14]. IgG molecules can bind covalently to albumin [15] and can interact with each other forming intermolecular covalent bonds [16]. The SH-containing fragment was shown to be exposed into the reaction medium on the interaction of the antibody with the antigen [17, 18]. In spite of the fact that the data on the content of the SH-groups are fragmentary and ambiguous, and the results have not been discussed or interpreted as the consequence of breaking of the labile interchain disulfide bonds [8, 9], these works suggest the possible existence of free cysteine residues potentially capable due to their reactivity, which may participate in the functioning of the antibodies. Free SH-groups were not found in monoclonal and myeloma antibodies (0.02-0.3 SH) [5] used for investigation, but they were detected in serum antibodies. Considering these facts, investigation of the content of free cysteine residues in native IgG of healthy donors seems to be of interest.

The goal of the present work was to determine the content of free cysteine residues and disulfide bonds in human IgG1 of healthy donors.

MATERIALS AND METHODS

Chemicals. In this work we used the following chemicals: $AgNO_3$, DAZ (N-dansyl aziridine), iodoacetic

Abbreviations: ABD-F) 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole; DAZ) N-dansyl aziridine; DMF) dimethylformamide; DTNB) 5,5′-dithiobis-(2-nitrobenzoic acid); PMSF) phenylmethylsulfonyl fluoride.

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acid, DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)), trypsin, EDTA, ammonium bicarbonate, and sodium chloride (Sigma, USA); 4-vinylpyridine (Ferak, Germany); $HgCl_2$ and ABD-F (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole) (Fluka, Germany); DEAE-Sepharose FF (Amersham Biosciences, Sweden); Sephadex G-200 (Pharmacia, Sweden); Biogel P-6 (Bio-Rad, USA); Calbiosorb (Calbiochem, USA); urea and sodium dodecylsulfate (SDS) (Serva, Germany); antisera against human immunoglobulins and subclass of IgG were obtained at the G. N. Gabrichevsky Center for Production of Bacterial Preparations (Russia). Urea and SDS were recrystallized twice; urea solution was deionized. Buffer solutions were prepared using doubly distilled water purified on a Milli-Q unit (Millipore, USA).

Materials. Immunoglobulins IgG1 were isolated from blood sera of healthy donors ($n = 20$) with normal characteristics of the immunogram. Immunoglobulins were precipitated from the serum (10 ml) by the addition of a saturated ammonium sulfate solution (pH 7.5) to 35% saturation. The precipitate was removed by centrifugation (15 min, 20,000*g*, $r_{av} = 6.6$ cm, 4°C), dissolved, and saturated with ammonium sulfate to 27%. The precipitate was centrifuged and dialyzed against 0.02 M sodium phosphate buffer, pH 7.8, containing 2 mM EDTA. The fraction containing IgG was applied to a DEAE-Sepharose column (1 × 6.5 cm) equilibrated with the same buffer. The sample was then chromatographed further as described in [19]. The IgG1 fraction was eluted within the dead volume of the column. Then the IgG1 fraction was purified by gel filtration on a Sephadex G-200 column equilibrated with 0.1 M ammonium bicarbonate buffer, pH 7.5, containing 2 mM EDTA (buffer I).

The homogeneity of the preparation was tested using SDS-PAGE following Laemmli [20] and by immunoelectrophoresis [21]. The class and subclass of immunoglobulins were confirmed by the Ouchterlony double radial immunodiffusion method using the corresponding antisera [22], and also by analysis of the N-terminal amino acid sequence of the Fc fragment by the Edman method [23].

To obtain the Fc fragment, a solution of IgG1 (2 mg/ml) was subjected to limited trypsinolysis (enzyme to substrate ratio, 1 : 50) in buffer I at 37°C for 4 h with constant stirring under nitrogen. The reaction was stopped by the addition of PMSF (phenylmethylsulfonyl fluoride). IgG1 was removed by gel filtration on a Sephadex G-200 column in buffer I. Fab and Fc fragments were separated by liquid chromatography on a DEAE-Sepharose column (1 × 6.5 cm) equilibrated with 20 mM ammonium bicarbonate buffer, pH 8.0, containing 2 mM EDTA (with Pharmacia-LKB chromatograph, Sweden). The Fab fragment was eluted with the same buffer, and the Fc fragment was eluted with a linear gradient (0.02-1 M) of ammonium bicarbonate, pH 8.0, as described in [24].

Assaying of SH-groups and S–S bonds. To reveal the readily accessible SH-groups, a solution of IgG1 (1-1.5 mg/ml) was incubated for 5 min in buffer I without detergent in the dark under a nitrogen atmosphere at 37°C (conditions A).

To determine the content of the masked SH-group and disulfide bonds, IgG1 were subjected to different extent of denaturation as described in [25] in modification. The conditions used for protein denaturation were: 8 M urea in buffer I (conditions B); 8 M urea, 2% SDS in buffer I (conditions C); 8 M urea, 4% SDS in buffer I (conditions D). The conditions of protein denaturation also differed in the way urea was introduced. In case B, urea was added singly, in case C and D urea was added every 30 min, each time to 2 M. In all cases, the protein was incubated for 120 min at the room temperature in the dark under nitrogen.

Amperometric titration of SH-groups with silver and mercury ions. Solutions of IgG1 preincubated under conditions A, B, C, and D, were titrated with 0.5 mM $AgNO_3$ or 0.5 mM $HgCl_2$. The protein (5-10 nmol) was added into 20 ml of 0.25 M ammonium nitrate buffer, pH 7.5, or 0.1 M Tris-HCl, pH 7.5, respectively. SH-groups were determined in the presence or in the absence of the denaturing agent at concentrations corresponding to the conditions of incubation. A Hg/HgI_2 element was used as the reference electrode. The sample was titrated until the emergence of linear dependence between the current and the amount of added reagent. The disulfide bonds in IgG1 (preincubated under conditions C or D) were detected in the same sample after the complete mercaptidation of free cysteine residues and subsequent addition of a saturated solution of sodium sulfite (0.1-0.5 ml) according to [25]. The total content of SH-groups (mol/mol protein) was determined as the sum of all free SH-groups (readily accessible and masked) and those revealed by sulfitolysis. Also, to determine the total content of SH-groups, sodium sulfite was added to the protein preincubated under conditions C or D directly before the titration, as described in [26].

Determination of SH-groups with DTNB. A solution of IgG1 (1.5 mg/ml) preincubated under conditions A or B was supplemented with an 80-fold molar excess of DTNB. The reaction was performed at pH 8.0 for 30 min in the dark at room temperature under nitrogen according to [9]. The absorption of the resulting solution was measured at 412 nm, and the content of the SH-groups reacted with DTNB was estimated using the molar absorption coefficient 14,140 $M^{-1} \cdot cm^{-1}$ [27].

Determination of SH-groups with alkylating reagents. In all cases, a solution of IgG1 (1.5 mg/ml) preincubated under conditions A, B, or C was modified at pH 7.5 at room temperature in the dark under nitrogen and with constant monitoring of the pH value of the medium. After the alkylation, the excess of the reagents was removed by gel filtration on a Bio-Gel P-6 column

equilibrated with buffer I. The excess of SDS was removed by incubation of the protein solution in the presence of the Calbiosorb sorbent for 15 min at room temperature. The sorbent was removed by centrifugation. The resulting solution was concentrated by ultrafiltration using a PM-10 membrane (Amicon, USA). The content of the modified cysteine residues was determined by measuring the content of the alkylated cysteines or by measuring the content of the unmodified cysteines. The latter were determined by the amperometric titration after preincubation of the alkylated IgG1 under conditions C.

Alkylation with iodoacetic acid. The carboxymethylation reaction was performed according to [28] with some modifications [29]. A sample of IgG1 was incubated with an 80-fold molar excess of recrystallized iodoacetic acid for 30 min. The amount of the protein and the S-carboxymethylcysteine was determined by amino acid analysis.

Alkylation with DAZ. A solution of DAZ in dimethylformamide (DMF) (40-fold molar excess of the alkylating reagent) was added to a sample of IgG1 twice with an interval of 1 h [30]. The mixture was incubated for 2 h in the presence of 10% DMF. The incorporation of the label was detected spectrophotometrically using the molar absorption coefficient for the dansyl chromophore 4400 $M^{-1}\cdot cm^{-1}$ at 335 nm [31].

Alkylation with ABD-F. A sample of IgG1 was supplemented twice with 1 h interval with equal portions of ABD-F to the final concentration of 4 mM according to [32]. The reaction mixture was incubated for 2 h in the presence of 10% DMF. The incorporation of the label was detected spectrophotometrically using the molar absorption coefficient for ABD 7800 $M^{-1}\cdot cm^{-1}$ at 384 nm [33].

Alkylation with 4-vinylpyridine. The protein was alkylated according to [34]. A sample of IgG1 was supplemented twice (with an interval of 1 h) with a 40-fold molar excess of distilled 4-vinylpyridine. Half of the protein was taken for analysis 2 h after the beginning of the reaction, and the rest portion was supplemented with another initial portion of 4-vinylpyridine and urea to the final concentration of 10 M. The total reaction time was 6 h. The amount of the protein and S-β-(4-pyridylethyl)cysteine was determined by amino acid analysis. Protein concentration was determined by the Lowry method [35] using BSA solution as the standard.

Amino acid analysis was performed using a Biotronic LC 6000 amino acid analyzer (Germany) after hydrolysis of the protein sample in 5.7 M HCl at 110°C under vacuum for 24 h.

N-Terminal amino acid sequence was determined by Edman's procedure [23] using a 491 Procise Protein Sequencing System (Applied Biosystems, USA). Phenylthiohydantoin derivatives of the amino acids were identified using a 785A PTH-analyzer (Applied Biosystems).

The results were statistically analyzed using the parametric criteria of Biostat software (1998). Analysis of variance with determination of the arithmetic mean (M) and the standard deviation (m) was used. The significance of the differences between the mean values of the number of cysteine residues in IgG1 modified under conditions A, B, and C was estimated using the paired Student's t-test (p). The difference was considered to be significant at $p < 0.05$.

RESULTS

When isolating IgG1, the main goal was to obtain a homogeneous preparation with native quaternary structure of the protein. From the wide spectrum of methods suggested for isolation of immunoglobulins G, we chose the precipitation of the antibodies with saturated ammonium sulfate solution, pH 7.5. This method allows a rather high purification [36], does not result in irreversible denaturation [37], and also stabilizes the conformation of the molecule [38]. In contrast to the known conditions of the ammonium sulfate precipitation [39], we elaborated an approach including two-step precipitation of the immunoglobulins with the decrease in ammonium sulfate concentration used on the second step of precipitation from 35 to 27%, this allowing isolation of the fraction enriched with IgG.

The resulting fraction was purified by ion-exchange chromatography on DEAE-Sepharose. Considering the difference in the values of the isoelectric points of different subclasses of immunoglobulins, we used the conditions under which IgG1 did not bind to the sorbent [19, 40]. To remove a high-molecular-weight admixture, the protein was purified on Sephadex G-200. As a result, an electrophoretically homogeneous fraction of IgG1 was obtained, this being confirmed by Ouchterlony radial immunodiffusion analysis [22]. The belonging of the preparation of antibodies to the G1 subclass was confirmed by the determination of the N-terminal sequence of the Fc fragment (^{223}THTC/$_2$PPC/$_2$PAPEL^{234}) isolated after separation of the products of limited trypsinolysis of IgG1, which is in agreement with the literature [41].

Determination of SH-groups and S–S bonds in IgG1. The absence of unambiguous information concerning SH-groups in the IgG1 molecule, and also considering that IgG1 is a complex protein, we determined the content of the SH-groups both under native and denaturing conditions, this allowing differentiation of all SH-groups into free cysteines (readily accessible and masked) and those involved in the formation of disulfide bonds. To obtain complete characteristic of the SH-groups and to determine the optimal way for their detection, different types of known reactions were used: mercaptidation (with $AgNO_3$ and $HgCl_2$), thiol-disulfide exchange (with DTNB), and alkylation with hydrophilic and hydropho-

Accessibility of SH-groups of IgG1 for thiol reagents depending on the conditions of denaturation and method of assay

Thiol reagents used for the detection of SH-groups	Conditions of IgG1 preincubation*	SH-groups, mol/mol protein ($M \pm m$)	
		readily accessible (modified)	masked (non-modified)**
$AgNO_3$ or $HgCl_2$ ($n = 20$)	A	1.1 ± 0.03	—
	B	2.2 ± 0.04	—
	C	4.0 ± 0.03	no
4-Vinylpyridine ($n = 10$)	A	0.7 ± 0.04	3.1 ± 0.03
	B	1.3 ± 0.06	2.5 ± 0.06
	C	2.9 ± 0.05	1.0 ± 0.03
	C_6***	3.78 ± 0.02	0.2 ± 0.02
ABD-F ($n = 5$)	A	0.6 ± 0.03	3.2 ± 0.03
	B	1.1 ± 0.06	2.8 ± 0.06
	C	2.5 ± 0.04	1.6 ± 0.03
DAZ ($n = 12$)	A	0.8 ± 0.03	3.10 ± 0.03
	B	1.5 ± 0.05	2.4 ± 0.04
	C	3.1 ± 0.04	1.1 ± 0.03
DTNB ($n = 5$)	A	0.2 ± 0.02	—
	B	0.6 ± 0.04	—
	C	—	—
Iodoacetic acid ($n = 5$)	A	0.1 ± 0.03	3.5 ± 0.03
	B	0.3 ± 0.04	3.3 ± 0.05
	C	0.9 ± 0.05	2.9 ± 0.05

Notes: "–" means no data; n is number of preparations of IgG1. The significance of the differences in the content of SH-groups under conditions A, B, and C: $p < 0.05$.

* Conditions A, B, and C are described in "Materials and Methods".

** Non-modified SH-groups, determined by amperometric titration after preincubation of the alkylated IgG1 under conditions C.

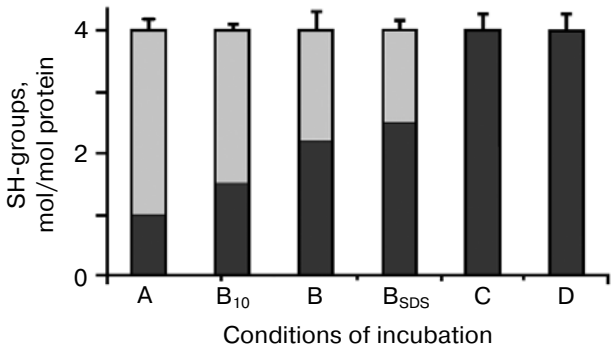
*** After 6 h of alkylation of IgG1 preincubated under conditions C.

bic reagents (iodoacetic acid, 4-vinylpyridine, DAZ, and ABD-F).

Readily accessible SH-groups. Using amperometric titration, one SH-group (1.1 ± 0.03 mol/mol protein) in native IgG1 preparations ($n = 20$) was shown to react easily with silver and mercury ions in the absence of detergents. To obtain more detailed characteristic on the reactivity of the free cysteine residue, preparations ($n = 5$) were compared in terms of their reactivity towards different thiol reagents. It appeared that, depending on the reagent employed, different content of SH-groups (0.1-0.8 mol/mol protein) was determined in the same preparations of IgG1 (table, conditions A). The highest values were obtained when the protein was modified with the hydrophobic reagent DAZ, and the lowest content of SH-groups was obtained in the presence of iodoacetic acid. In terms of the affinity to the cysteine residue, the reagents can be arranged in the following order: Ag^+/Hg^{2+} (1.0 ± 0.06) > DAZ (0.8 ± 0.06) > 4-vinylpyridine (0.7 ± 0.04) > ABD-F (0.6 ± 0.03) > DTNB (0.2 ± 0.02) > iodoacetic acid (0.1 ± 0.03). These data indicate that in native IgG1 the free SH-group is in a hydrophobic surrounding, being readily accessible for heavy metals ions and hydrophobic reagents but masked for hydrophilic reagents.

Masked SH-groups and S–S bonds. Since it is known that the IgG1 molecule has 32 cysteine residues involved in the formation of disulfide bonds [2], the detection of one free SH-group implies the existence of at least one masked cysteine residue inaccessible for thiol reagents under native conditions.

To reveal the masked cysteine residues, the same IgG1 preparations ($n = 5$) that were investigated under the native conditions were subjected to different extent of denaturation with subsequent investigation of the accessibility of the SH-groups to thiol reagents. Determination of the SH-groups by amperometric titration showed that the immediate introduction of a high concentration of the denaturing agent caused a limited destruction of the IgG1 molecule that was insufficient to reveal the deeply masked cysteine residues even after a long time of incubation. For example, 10 min of incubation of the IgG1 in buffer I containing 8 M urea (figure, conditions B_{10}) revealed 1.5 ± 0.03 SH-groups. After 2 h of incubation (figure, conditions B), no more than 2.2 ± 0.1 SH-groups were revealed. The addition of 2% SDS to the protein incubated under conditions B, little affected the results: after 2 h of incubation, 2.5 ± 0.04 SH-groups were determined (figure, conditions B_{SDS}). A gradual increase in urea concentration (figure, conditions C) resulted in more profound denaturation of the protein with liberation of 4.0 ± 0.08 SH-groups. To test the possibility of the existence of SH-groups remaining masked under condi-



Readily accessible (black columns) and masked SH-groups (gray columns) of IgG1 ($M \pm m$, $n = 5$) determined by amperometric titration depending on the conditions of protein denaturation. Conditions A, B, C, and D are described in "Materials and Methods". B_{10}, 10 min of incubation of IgG1 under conditions B; B_{SDS}, conditions B in the presence of 2% SDS. The significance of the differences in the content of SH-groups was determined with respect to the results obtained under conditions A ($p < 0.05$)

tions C, the IgG1 preparation was denatured under severe conditions, i.e., gradual increase in urea concentration to 10 M in the presence of 4% SDS, which leads to the destruction of the intramolecular interactions except for the S–S bonds [26]. However, these conditions allowed determination of no more than four cysteine residues (figure, conditions D).

It may seem that under conditions C all free cysteine residues must be accessible for each of the thiol reagents. Actually, no more than 3.1 cysteines were modified. In terms of the affinity to the SH-groups, reagents can be arranged in the following order: Ag^+/Hg^{2+} (4.0 ± 0.03) > DAZ (3.1 ± 0.04) > 4-vinylpyridine (2.9 ± 0.05) > ABD-F (2.5 ± 0.04) > iodoacetic acid (0.9 ± 0.05). A higher extent of modification of the cysteine residues (3.78 ± 0.02) was achieved after 6 h of incubation of the protein with 4-vinylpyridine in the case of gradual addition of the reagent and urea to 10 M (table, conditions C_6). These conditions were elaborated previously for the modification of the deeply masked cysteine residues of Na,K-ATPase from pig kidneys [25]. Thus, using alkylating agents, four free cysteines were determined.

To determine the number of disulfide bonds, we used both the classical [26] and modified [25] methods of amperometric titration. After mercaptidation of the free cysteine residues and the addition of sodium sulfite, 28 SH-groups were found to be liberated after the destruction of the S–S bonds. In the same sample treated with sodium sulfite before the amperometric titration, 32 SH-groups were determined, this corresponding to the data on the primary structure of the molecule [2]. Thus, in IgG1 of healthy donors, 14 S–S bonds were determined, rather than 16 (as it was considered before), and four free cysteines residue, three of which were detected only after severe denaturation of the protein.

DISCUSSION

Among all functional groups of proteins, SH-groups are strong nucleophiles and can participate in various chemical reactions. Their reactivity varies over wide ranges and depends on many factors: effect of functional groups of the surrounding amino acids, packing of the polypeptide chains in the molecule, and spatial situation in the protein globule [42, 43]. The detection of reactive SH-groups on the protein surface is not a difficult task, while detection of the cysteine residues in hydrophobic clusters, especially in multidomain proteins, is a complicated problem that is difficult to solve with the use of the widely employed standard approaches. Underestimation of the masked cysteine residues, which has occurred on investigation of IgG, results in an erroneous detection of the disulfide bonds, since the latter is based on the determination of the modified cysteine residues before and after the compete reduction of a protein.

Thus, the crucial moment in determination of the S–S bonds is the quantitative modification of all free SH-groups of the protein, which can be incorrect in the case of the presence of deeply masked cysteine residues. Previously, we demonstrated that amperometric titration with $AgNO_3$ or $HgCl_2$ is the most adequate method allowing the quantitative determination of the free cysteines (especially masked) [25, 34]. The method has a number of advantages: it allows determination of all cysteine residues (readily accessible, masked, and involved in disulfide bonds) in one sample, investigation of turbid and colored solutions in the presence of high concentrations of detergents and even in suspensions, and monitoring of the efficiency of the alkylation. An important advantage of this method is the fact that heavy metal ions penetrate into the sites of the molecule that are sterically inaccessible to other SH-reagents. Exhibiting a high affinity to sulfur atoms, they displace the cysteines from intramolecular noncovalent interactions [42]. While investigation IgG1, amperometric titration was more informative than other methods. Finding of four free cysteine residues using this method was a reason for the search for conditions for their modification, which is of great importance for elucidation of the localization of free cysteine residues in the protein molecule.

In spite of the fact that protein chemistry often deals with the problem of masking of free cysteine residues, there is no single viewpoint concerning their functional significance and mechanisms of their screening. To explain the mechanism of the masking, two hypotheses have been suggested: (i) steric screening of the SH-groups by neighboring amino acid residues, and (ii) chemical screening, i.e., participation of the SH-groups in intramolecular noncovalent bonds [42]. In the case of IgG1, both mechanisms occur. The impossibility of detection of free cysteine residues inserted into the C_H1 domain of IgG by directed mutagenesis with the use of 4,4-dithiopyridine was demonstrated by Lyons [7]. These data illustrated the steric inaccessibility of the SH-groups. Our investigations demonstrate that detection of the SH-groups in IgG1 significantly depends on both the kinetics of the intermolecular changes occurring during denaturation of the protein and on the method of the assay, i.e., on the nature of the thiol reagents, this suggesting the existence of both mechanisms.

Taken together, our results have elucidated the causes that prevented detection of the masked SH-groups by many authors [2, 6, 7, 9], this resulting in the erroneous determination of the number of the disulfide bonds and in the denial of the existence of free cysteine residues in IgG. Negative results can be explained by the insufficient denaturation of the molecule that is necessary for demasking of all SH-groups [6], by inability of the used thiol reagents to penetrate into the hydrophobic clusters containing SH-groups [5-7, 44-48], and by an increased stability of myeloma and monoclonal antibodies on

which basic researches were conducted to the denaturing agents compared to native IgG [49].

The characteristic feature of the present work is the fact that the SH-groups were investigated in the native IgG1 isolated from the serum of healthy donors using different thiol reagents under conditions providing severe denaturation of the protein. As a result of this work, four free cysteine residues and 14 S–S bonds were found in the IgG1 molecule. Thus, it is demonstrated that IgG1 contains simultaneously free SH-groups and S–S bonds, which alters significantly the viewpoint on the role of cysteine residues in the structure of IgG and raises a question concerning their participation in the functioning of the antibodies.

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