DIFFERENCES IN SENSITIVITY TO REDUCTION OF RAT IMMUNOGLOBULIN IgG SUBCLASSES

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1. Introduction

Immunoglobulin IgG can be separated into distinct subclasses that differ by some biological properties such as activation of complement, cytophilic activity, interaction with staphyloccocal protein A [1] and by structural characteristics. In particular, human IgG subclasses have been found differentially affected by reduction [2], IgG4 being reduced through the HL subunit intermediate, although in IgG1, IgG2 and IgG3, the intermediates are H_2L, H_2 and HL.

In the rat, four isotypes of IgG: IgG1, IgG2a, IgG2b and IgG2c, have been identified [3]. In an attempt to define structural differences between rat IgG subclasses and to relate them to the IgG of other species, we have investigated the sensitivity to reduction of different types of monoclonal IgG proteins produced by rat LOU immunocytomas. Our results point out clear differences between some of the subclasses, and some relationship with human IgG subclasses.

2. Material and methods

2.1. *Purification of IgG proteins*

Immunocytomas were transplanted in rats LOU/M and in (LOU/Wistar) or (LOU/OKA) F_1 hybrids as in [4]. Monoclonal IgG were isolated from ascites or sera of rats bearing tumors by ammonium sulphate precipitation followed by chromatography on DEAE- or CM-cellulose and filtration on Ultrogel AcA 34 (LKB, Sweden) [3]. Their heavy chain subclasses were typed by immunodiffusion against specific antisera [3]. Normal IgG2a was isolated from serum of randomly-bred Wistar rats by DEAE-cellulose chromatography [5].

2.2. *Reduction experiments*

2.2.1. Dithioerythritol (DTE) reduction

Proteins (2 mg/ml) in 0.2 M Tris–HCl buffer (pH 8.2) were reduced at room temperature with 0.05–20 mM dithioerythritol. After 1 h incubation under nitrogen, alkylation was performed at 0°C with iodoacetamide (50% molar excess over total thiol).

2.2.2. Dithiothreitol (DTT) reduction

Proteins (10 mg/ml) in 0.2 M Tris–HCl buffer (pH 8.6) were reduced at room temperature with 0.2–20 mM dithiothreitol. After 1 h incubation, the pH was lowered to 8.0 by addition of 0.2 M Tris–HCl (pH 7.2) and alkylation was carried out at 0°C with iodoacetic acid (10% molar excess over total thiol).

2.3. *Analysis of the reduction products*

Reduction products were studied by SDS–polyacrylamide gel electrophoresis as in [6] with the following modifications: a 5–20% acrylamide gradient was used for the separation and the stacking gel (5% acrylamide) was buffered by 0.1 M Tris–H_2SO_4, (pH 6.8). Samples to be electrophoresed were diluted to 1 mg/ml in 2% (w/w) SDS, 0.075 M iodoacetamide, heated 5 min at 95°C and a further 30 min at 45°C. The following molecular weight markers were run together with the reduction products: rat IgE

(mol. wt 189 000 [7]; rat IgG2a (mol. wt 150 000); phosphorylase *b* (mol. wt 96 800); human serum albumin (mol. wt 66 300); egg albumin (mol. wt 43 000); cytochrome *c* (mol. wt 11 700). The molecular weight of the reduction products was calculated from a calibration curve of the logarithm of molecular weight of the markers against the logarithm of the concentration of acrylamide reached by the marker [8]. Quantitation of the reduction intermediates and of unreduced protein was performed by scanning the gels with an Helena Autoscanner Quick Quant II.

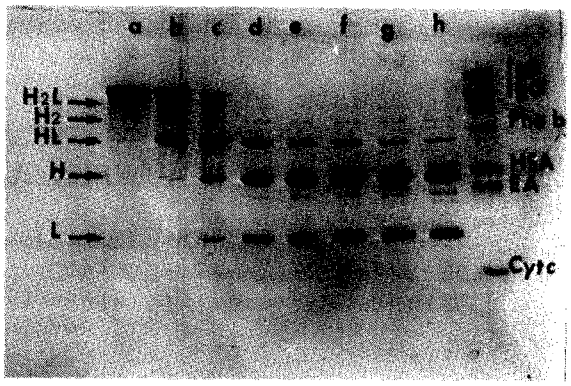

Fig.1. SDS–polyacrylamide gel electrophoresis of protein IR 530 (IgG2a subclass) after reduction by dithioerythritol (DTE) and alkylation: (a) unreduced protein; (b) DTE 0.05 mM; (c) 0.1 mM; (d) 0.5 mM; (e) 1 mM; (f) 5 mM; (g) 10 mM; (h) 20 mM. IgE: rat IgE (mol. wt 189 000); IgG (rat IgG2a, mol. wt 150 000) Pho-b: phosphorylase *b* (mol. wt 96 800) HSA: human serum albumin (mol. wt. 66 300) EA: egg albumin (mol. wt 43 000) cyt *c*: cytochrome *c* (mol. wt 11 700).

3. Results

Two monoclonal proteins of subclasses IgG1 and IgG2a, three proteins of subclass IgG2c and one of subclass IgG2b were studied together with IgG2a isolated from normal rat serum.

Tables 1 and 2 show the relative percentage of

Table 1
Relative proportions of reduction intermediates and of unreduced protein (H_2L_2) in proteins of different heavy chain subclasses, after dithioerythritol (DTE) reduction

Subclass	Protein	DTE	H_2L_2	H_2L	H_2	HL
IgG1	IR 595	0.1 mM	31.6	28.2	12.6	27.6
		1.0 mM	5.8	15.2	20.5	58.5
	IR 656	0.1 mM	42.9	21.0	5.6	30.5
		1.0 mM	3.5	20.0	19.2	57.3
IgG2a	IR 530	0.1 mM	16.5	4.1	2.4	77.0
		1.0 mM	0	0	5.4	94.6
	IR 418	0.1 mM	16.1	4.5	2.6	76.8
		1.0 mM	0	0	4.6	95.4
	Normal	0.1 mM	20.5	21.1	2.6	55.8
		1.0 mM	0	0	0	100
IgG2b	IR 863	0.1 mM	10.9	21.2	23.2	44.7
		1.0 mM	2.7	0	56.3	41.0
IgG2c	IR 304	0.1 mM	8.1	34.7	29.3	27.9
		1.0 mM	0	4.2	78.3	17.5
	IR 759	0.1 mM	5.9	12.6	40.9	40.6
		1.0 mM	0	5.4	66.5	28.1
	IR 558	0.1 mM	0	32.3	23.9	43.8
		1.0 mM	0	56.4	18.9	24.7

Table 2
Relative proportions of reduction intermediates and of unreduced protein in proteins of different heavy chain subclasses, after dithiothreitol (DTT) reduction

Subclass	Protein	DTT	H_2L_2	H_2L	H_2	HL
IgG1	IR 595	0.2 mM	60.4	26.7	7.8	5.1
		1.0 mM	12.0	24.6	10.0	53.4
	IR 656	0.2 mM	42.7	35.9	6.2	15.2
		1.0 mM	10.6	23.7	11.4	54.3
IgG 2a	IR 530	0.2 mM	23.0	6.2	4.3	66.5
		1.0 mM	0	0	5.1	94.9
	IR 418	0.2 mM	25.6	3.4	6.2	64.8
		1.0 mM	0	0	3.8	96.2
	Normal	0.2 mM	36.0	19.8	1.8	42.4
		1.0 mM	0	4.0	4.0	92.0
IgG2b	IR 863	0.2 mM	31.4	15.1	21.8	31.7
		1.0 mM	7.5	17.3	35.6	39.6
IgG 2c	IR 304	0.2 mM	5.8	20.1	35.3	38.8
		1.0 mM	0	0	58.8	41.2
	IR 759	0.2 mM	23.5	34.0	17.6	24.9
		1.0 mM	1.2	16.2	60.5	22.1
	IR 558	0.2 mM	2.2	30.7	15.8	51.3
		1.0 mM	0	24.1	5.4	70.5

unreduced protein (H_2L_2) and of reduction intermediates (H_2L, H_2 and HL) with dithioerythritol (0.1 mM and 1 mM) and dithiothreitol (0.2 mM and 1 mM). The two procedures give almost the same results (except for monoclonal IgG2c IR 558). IgG2a appears clearly different from the other subclasses: in effect, the two monoclonal IgG2a proteins and normal IgG2a are almost entirely reduced via the HL subunit (fig.1). For the other IgG subclasses H_2L and H_2 reduction intermediates are also present in significant amounts. However in the IgG1 proteins studied, the HL subunit is the major product (fig.2),

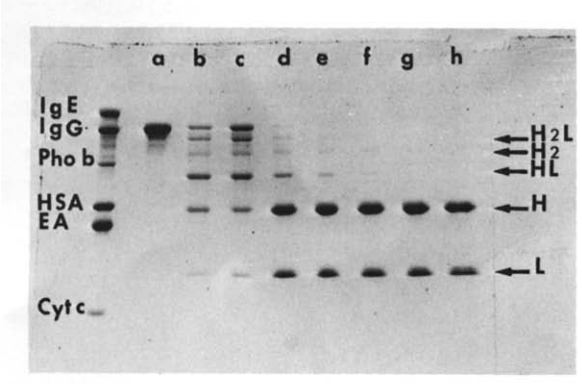

Fig.2. SDS–polyacrylamide gel electrophoresis of protein IR 656 (IgG1 subclass) after reduction by DTE. For legend, see fig.1.

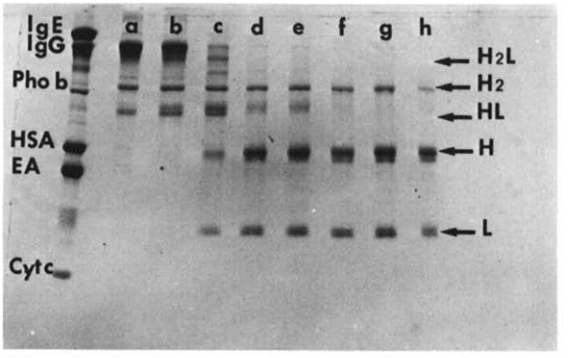

Fig.3. SDS–polyacrylamide gel electrophoresis of protein IR 863 of IgG2b subclass after reduction by DTE. For legend, see fig.1.

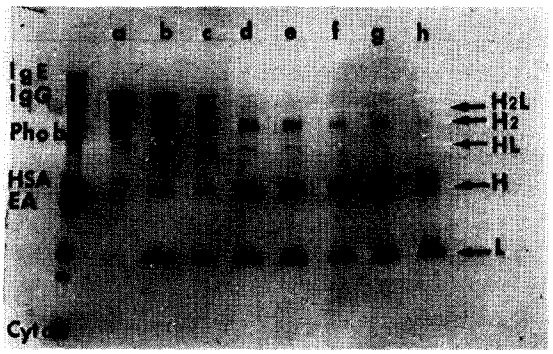

Fig.4. SDS–polyacrylamide gel electrophoresis of protein IR 304 of IgG2c subclass after reduction by DTE. For legend, see fig.1.

whereas in the monoclonal IgG2b, and two of the three IgG2c proteins studied, the H_2 intermediate is the predominant product, and remains present even with high concentrations of reducing agent (fig.3,4). A discrepancy between the results of DTE and DTT reduction is observed for the monoclonal IgG2c IR 558 immunoglobulin, the major intermediate being the H_2L with DTE and the HL with DTT.

4. Discussion

Reduction experiments have clearly shown differences between the IgG proteins studied and part of these differences can be related to the subclass. In IgG2a (monoclonal or normal) proteins, the almost exclusive product of partial reduction is the HL subunit with only traces of other intermediates. This implies that in IgG2a the inter heavy-chain disulphide bridges are very sensitive to reduction and suggest that the inter heavy-chain non-covalent interactions in the hinge region are somewhat weak. We have noticed that the molecular weight of the heavy chain of rat IgG2a proteins is smaller than the one of the other IgG subclasses, in particular IgG2b and IgG2c. This could be due to a smaller size of the hinge region. Experiments are undertaken to study the structure of the hinge of the different subclasses, and answer this hypothesis. In IgG1 proteins, although H_2L and H_2 intermediates are present, the major product is the HL subunit, suggesting that the inter heavy-chain bonds are more sensitive to reduction than the inter heavy–light chains bridges. For IgG2b and IgG2c proteins, the results cannot be considered as characteristic of the subclass. In effect, the IgG2c proteins behave differently, and only one IgG2b monoclonal immunoglobulin is available. This protein is remarkable for the resistance of the inter heavy-chain bridge to reduction. It will be interesting in the future to study whether this property is found in IgG2b from normal serum.

Rat IgG2a is reduced in a similar manner to human IgG4 [2]. This is probably linked to common structural features of the hinge region, but cannot be related to common biological properties, as human IgG4 does not bind complement, interacts poorly with macrophages and binds staphyloccocal protein A, whereas the reverse is true for rat IgG2a [9]. Rat IgG2a behaves differently from mouse IgG2a, where H_2L and H_2 intermediates are present [10]. These results together with differences in the products obtained by proteolytic cleavage [11] suggest that the hinge regions of rat and mouse IgG2a do not have the same conformation.

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