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Neutralization capacity and antibody dependent cell-mediated cytotoxicity of separated IgG subclasses 1, 3 and 4 against herpes simplex virus

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SUMMARY

IgG subclasses 1, 3 and 4 in sera from herpes simplex virus type 1 (HSV-1) seropositive donors were separated and their functions assayed. The main neutralizing activity to HSV-1 was found in the IgG1 fractions. Both IgG3 and IgG4 possessed higher neutralizing titres than IgG1 in relation to the respective HSV IgG subclass enzyme-linked immunosorbent assay (ELISA) titre. Addition of complement resulted in a strong enhancement of IgG3 neutralizing activity. HSV neutralizations by IgG1 and, surprisingly, IgG4 were also somewhat enhanced by complement. With the addition of complement, the contribution to neutralizing activity of IgG3 was calculated to increase from 31 to 40% of total IgG in HSV neutralization in native sera. The avidities of the IgG fractions to HSV glycoprotein C (gC) were estimated in a few sera but could not be correlated to neutralization results. Antibody dependent cell-mediated cytotoxicity (ADCC) was detectable mainly in IgG1 and 3 fractions of sera with high anti-HSV antibody titres.

Keywords IgG subclasses virus neutralization antibody-dependent cell-mediated cytotoxicity herpes simplex virus

INTRODUCTION

Human IgG has been separated into four antigenically and functionally different subclasses, IgG1–4 (Dray, 1960; Grey & Kunkel, 1979; Terry & Fahey, 1964; for a review see Shakib, 1986). HSV neutralization *in vitro* is mainly dependent on IgG from HSV seropositive patients (Ratner, Sanford & Smith, 1979). Previous reports state that IgG3 and IgG1 bind to peripheral blood mononuclear cells (Woof *et al.*, 1984). Some functional antiviral properties of the subclasses have been studied in sera separated by protein A-sepharose column (Beck, 1981) and in sera treated by enzymatic digestion (Ehrnst, 1978). IgG3 was reported to neutralize virus more actively in relation to the IgG concentration than IgG1, 2 or 4 (Beck, 1981). Results of complement binding are equivocal (Augener, 1971; Schumaker, 1976; Ehrnst, 1978).

Still, little is known about antiviral effector mechanisms of the various subclasses. HSV-IgG1 is almost always present in HSV-1 seropositive healthy persons; HSV-IgG3 is present in 50%, while HSV IgG4 is less common and probably correlates with frequent reactivations of HSV infection. HSV-IgG2 is found in just a few sera (Sundqvist, Linde & Wahren, 1984; Gilljam *et al.*, 1985). IgG1 and 3 were therefore the main candidates for functional activities in seropositive donors.

Antibody avidity has been reported to play a major role in neutralization (Blank, Leslie & Clem, 1972). Among HSV-1 glycoproteins, gC has been shown to be one target for neutralizing antibodies (Norrild, Shore & Nahmias, 1979; for a review see Norrild *et al.*, 1984). In an attempt to elucidate further the influence of the different IgG subclasses regarding their functional antiviral properties, polyclonal preparations of single IgG subclasses were obtained from immune sera by using subclass-specific affinity chromatography. HSV type 1 neutralization, complement-mediated cytotoxicity and ADCC activity (Kohl *et al.*, 1977; Shore *et al.*, 1976; Subramaniam & Rawls 1977; Ballew, 1986) were then measured. The neutralizing titre of an antibody fraction was compared with its avidity to gC, since a higher avidity could be expected to render better neutralization.

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MATERIALS AND METHODS

IgG subclass-specific affinity chromatography

Sera were obtained from seven HSV seropositive healthy donors. The IgG subclasses were separated according to Persson (1987). Briefly, sera were desalted and run through

combinations of affinity columns with antibodies to IgA, IgM and all but one of the IgG subclasses. Only non-bound proteins were collected, concentrated and used for subsequent experiments. The following antibodies were coupled to agarose matrix (Affigel 10, BioRad, Richmond, CA, USA): rabbit anti-human IgA and anti-human IgG antisera were purchased from Dako Immunoglobulins, Copenhagen, Denmark (product code A092 and A091, respectively). Subclass-specific monoclonal antibodies were obtained from Unipath, Bedford, UK: clone JL512 (anti-IgG1), GOM1 (anti-IgG2), ZG4 (anti-IgG3, GB7B (anti-IgG4) and lal (anti-IgG1, 2, 3, i.e. anti-non-IgG4). Their characteristics have been published previously (Lowe *et al.*, 1982). Monoclonals reacting with all subclasses except one were generously donated by Dr Roy Jefferis, Birmingham, UK: clones TM-10 (anti-IgG2, 3, 4) and VC9 (anti-IgG1, 2, 4) (Bird *et al.*, 1984). Clones HP 6030 (anti-IgG2, 3, 4) and HP6019 (anti-IgG1, 3, 4) were gifts from Dr Charles B. Reimer, Atlanta, GA, as were clones HP6014, HP6002 (both anti-IgG2); characteristics of those four monoclonals have been reported (Reimer *et al.*, 1984).

The amounts of remaining IgG subclass proteins and accordingly the purity of the IgG subclass preparations were determined by single radial immunodiffusion (SRID) with monoclonal anti-IgG subclass antibodies (clones JL512, GOM1, ZG4, GB7B and RJ4) according to the manufacturers instructions (Unipath, Bedford, UK) and for specific antibodies by a sensitive HSV IgG subclass ELISA (Sundqvist *et al.*, 1984). Internal laboratory standard sera were used as positive and negative controls.

Complement fixation

This was carried out according to the method of Wellings & Lewis (1986). Herpes simplex type 1 prepared in Vero cells (National Bacteriological Laboratory) was used as antigen.

HSV neutralization assay

All sera were tested in duplicate. Sera and serum containing media were heat inactivated (56°C for 30 min). Sera (100 μl) diluted 1:5 in Eagle's medium (Gibco, Paisley, Scotland) were added to sterile Linbro microplates (Flow Laboratories, Irvine, Scotland). One hundred units of the 50% tissue culture infecting dose ($TCID_{50}$) HSV type 1 was added. For testing complement-enhanced neutralization, guinea-pig complement was added to a concentration of 15%; for controls, heat-inactivated complement was used. After 60 min, 30,000 baby hamster kidney (BHK) cells were added. All incubations were in 6·7% CO_2 at 37°C. After 82 h the cells were washed with phosphate-buffered saline (PBS according to Dulbecco, National Bacteriological Laboratory) and fixed in the plates with 1:1 methanol:acetone for 1 min at −20°C. HSV antigen was quantified by ELISA. The reagents were 100 μl of rabbit anti HSV-1 antiserum (182:6, National Bacteriological Laboratory) diluted 1:120 and horseradish peroxidase-labelled mouse anti-rabbit IgG (Dakopatts, Copenhagen, Denmark) diluted 1:2000; the substrate was ortho-phenylenediamine (OPD, Dakopatts) activated by H_2O_2. Sulphuric acid terminated the reaction; the optical density (OD) at 490 nm A_{490} was read in a Dynatech MR 600 (Arlington, VA, USA). The neutralization titre for each separated serum was expressed as the reciprocal value of the highest serum dilution at which the antigen ELISA OD was below cut-off (0·200 equals mean A 490 nm + 2s.d. for negative sera). No cytopathic effect (CPE) was detected in the wells negative for antigen ELISA.

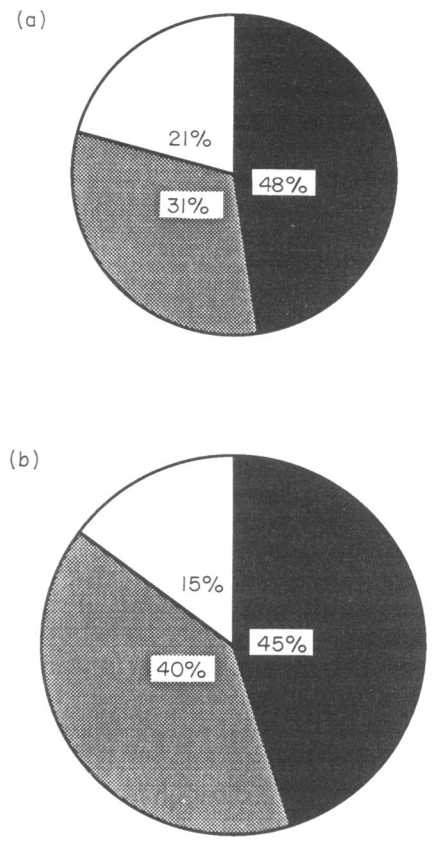

Fig. 1. Estimated original HSV neutralizing activities of IgG1, 3 and 4. Values were calculated from yields and titres obtained after separation and tested in neutralizing assays (a) without or (b) with complement as described in Materials and Methods. IgG1 is the dominant HSV neutralizing antibody. The relative importance of IgG3 increases with addition of complement. (■)IgG1; (■)IgG3; (□)IgG4.

For data presented in Fig. 1 the neutralization titre of each separated subclass fraction was multiplied by the inverted yield. The resulting values, taken to represent neutralization of the respective subclasses in native sera, were used to calculate percentages of HSV neutralization for every subclass in each patient. The validity of the estimations was controlled by comparing theoretical total neutralization with experimental data available for patients. It was found to correlate well (data not shown).

ADCC

BHK cells were labelled with ^{51}Cr (Amersham, UK; 1 mCi sodium chromate/10^6 cells; incubated at 37°C for 90 min). They were infected with 10^3 $TCID_{50}$ HSV type 1 for 45 min. After washing, 10,000 cells were added to each well of sterile 96-well microplates (Nunc, Aarhus, Denmark). After 18 h a CPE was seen in more than 90% of the cells and surface antigens could be detected by ELISA withour 182:6 antiserum. The sera were tested in 2-fold dilutions and added in 50 μl fractions to the wells. Serum from HSV seronegative donors were used as controls. Peripheral blood mononuclear cells (PBMC) from an HSV seronegative donor were purified by density gradient centrifugation (Lymphoprep, Nyegaard, Oslo, Norway). Fifty

Table 1. ELISA HSV IgG subclass titres and percentage of IgG recovered after separation of the respective serum fractions analysed

Fraction no.	HSV subclass titre IgG1	IgG3	IgG4	% recovered
85·1	2000	—	—	90%
86·1	5000	—	—	61%
82·1	9000	—	—	52%
83·1	20000	—	—	41%
81·2	10000	—	—	36%
50·3	3000	—	—	48%
81·3	—	1000	—	19%
82·2	—	2500	—	37%
85·3	—	5000	—	58%
83·2	—	3000	—	33%
50·4	—	300	—	61%
81·1	—	—	600	70%
83·3	—	—	1500	18%
86·2	—	—	200	30%
84·2	—	—	600	67%
50·5	—	—	200	27%

— not detectable.
Anti-HSV-IgG2 was not detected in any serum fraction.

thousand cells were added to each well for 4 h. Cells were harvested in a Skatron semiautomatic cell harvester (Lier, Norway). The radiation was measured with a Packard 5110 gamma counter (Packard, Downers Grove, IL). Lysis was calculated by dividing radioactivity in the supernatant by radioactivity remaining in the cells. Values above the mean lysis + 2S.D. for controls were considered positive. The ADCC titre was recorded as the reciprocal dilution of the highest sample dilution resulting in a positive ADCC.

Avidities of separated IgG antibodies to gC

These were estimated by using an inhibition ELISA. Samples were titrated to give similar optical densities (OD) in ELISA with gC-coated microplates (Linbro). The conjugate was alkaline phosphatase-conjugated rabbit anti-human IgG (Dakopatts). Free gC was added in increasing amounts to the wells. The gC concentration leading to a 50% decrease of initial OD was recorded as the IC_{50}. The relative avidity of the separated IgGs to gC was taken as to $1/IC_{50}$. Purified gC was a gift from Dr Stig Jeansson, Gothenburg.

RESULTS

Anti-HSV-IgG1, 3 and 4 in separated serum fractions

Sixteen anti-HSV subclass fractions were obtained from six seropositive human sera (Table 1). None of the serum samples contained sufficient amunts of HSV-IgG2 for purification. As demonstrated by immunodiffusion the contents of each separated fraction were IgG but not IgM or IgA. Yields of antiviral IgG, measured by HSV-IgG subclass ELISA and calculated as a percentage of the initial amount, varied between 18 and 90% (Table 1). HSV-IgG1 ELISA titres were higher than IgG3 and 4 titres. All fractions contained one IgG subclass only as demonstrated by subclass-specific SRID and further supported by the

Table 2. Neutralization of HSV using separated IgG subclasses with and without complement

Fraction	IgG subclass	NT Without C	NT With C	NT/ELISA titre ×1000 Without C	NT/ELISA titre ×1000 With C
85·1	1	20	40	10	20
86·1	1	40	80	8	16
82·1	1	160	320	18	36
83·1	1	320	640	16	32
81·2	1	160	320	16	32
50·3	1	40	80	13	27
81·3	3	40	60	40	60
82·2	3	40	120	16	48
85·3	3	160	640	32	128
83·2	3	20	160	7	53
50·4	3	10	20	33	67
81·1	4	80	120	133	200
83·3	4	80	160	53	107
86·2	4	20	40	100	200
84·2	4	10	40	17	67
50·5	4	10	10	50	50

The actual neutralization titres (NT) from the experiments are shown together with values calculated by dividing neutralization titres (without complement, C) with the respective HSV IgG subclass titre. This was done to enable comparison of neutralization per unit of anti-HSV IgG.

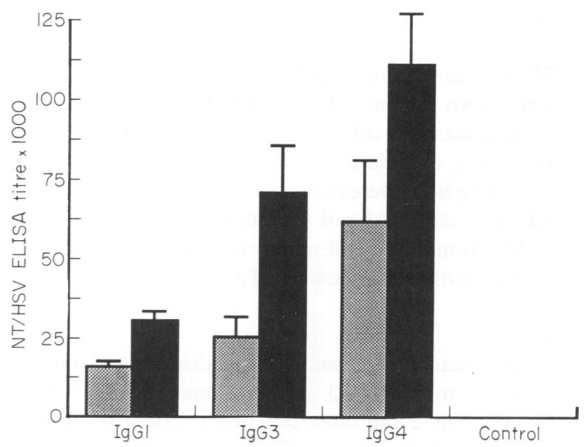

Fig. 2. HSV neutralization titres increase with addition of complement. An increase of the mean neutralization titre was present in IgG1, 3 and 4. The largest difference with addition of complement occurs in IgG3 fractions. Controls with complement did not show detectable neutralization in absence of HSV antiserum. (■) No complement; (■) with complement.

Table 3. ADCC against HSV type 1 infected cells of separated IgG1, 3 and 4

Fraction no.	Control* titre	ADCC titre	HSV IgG subclass ELISA titre		
			IgG1	IgG3	IgG4
85·1	< 10	20	2000	—	—
83·1	< 10	80	20000	—	—
50·3	< 10	20	10000	—	—
82·2	< 5	< 5	—	2500	—
85·3	< 10	20	—	5000	—
83·2	< 5	10	—	3000	—
50·4	< 5	< 5	—	300	—
83·3	< 5	< 5	—	—	1500
84·2	< 5	< 5	—	—	600
50·5	10	10	—	—	200

*Controls consisted of sera from an HSV seronegative donor instead of subclass preparations.

Table 4. IC_{50} and its reciprocal value for five anti-gC positive serum fractions

Fraction No.	IC_{50}	$1/IC_{50}$	IgG subclass	and titre	Neutralization titre
81·2	0·053	19·0	IgG1	10000	160
82·1	0·019	53·5	IgG1	9000	160
83·1	0·031	32·4	IgG1	20000	320
82·2	0·060	16·7	IgG3	2500	40
83·3	0·062	16·2	IgG4	1500	80

fact that the absorbance values for the respective discarded IgG subclasses were always lower than the negative control. The recovered subclasses were in accordance with subclasses present before separation (not shown).

Anti HSV-1 neutralization titres

These are shown for the different preparations in Table 2. The highest neutralization titres were found in IgG1 followed by IgG3 and 4 (Fig. 1). Relative to HSV IgG subclass ELISA titres, the neutralizing titres were highest with IgG4, followed by IgG3 and IgG1. The same order of activity was obtained by dividing the neutralization titre by the amount of total IgG present in the respective fractions (not shown in Tables).

Complement

Adding complement increased neutralization in 6/6 IgG1 preparations, in 5/5 IgG3 preparations and in 4/5 IgG4 preparations (Fig. 2 and table 2). The increase was 2-fold to 6-fold with IgG3, 2-fold with IgG1 and 1·5-fold to 4-fold with IgG4. In the complement-fixation assay, which is less sensitive than ELISA (Wellings & Lewis, 1986), HSV antibodies were detectable in eight of the 16 serum fractions. Four of these belonged to the IgG1 subclass, two to the IgG3 subclass and two to the IgG4 subclass.

ADCC

Results are shown in Table 3. The HSV infection resulted in CPE in the target cells and a mean lysis of 19% in control cells. HSV-specific ADCC activity was demonstrated in three IgG1 fractions, two of which had high anti- HSV-IgG1 ELISA titres. The activity was also seen in two fractions with high anti-HSV-IgG3 ELISA titres. Owing to the restricted amounts of separated sera available, only 10 serum fractions could be evaluated in these tests.

IgG reactive with gC

This was found in three IgG1, one IgG3 and one IgG4 fraction. Table 4 shows that the avidities to gC were higher in all three IgG1 fractions than in the IgG3 and 4 fractions. A high IgG-gC avidity was not correlated to either neutralization or complement-activated neutralization.

DISCUSSION

We have demonstrated HSV type 1 neutralizing activity in all three separated IgG subclasses. Both HSV ELISA titres and neutralization titres were highest in IgG1 preparations. However, in relation to HSV subclass ELISA titres, the neutralizing capacity was highest in the IgG4 subclass, followed by IgG3 and IgG1. These findings partially agree with Beck's results (Beck, 1981) although we have not been able to confirm either a 10-fold difference between neutralization titres/unit of immunoglobulin of IgG3 and the other subclasses together or an antiviral neutralization exclusively in the IgG3 separations as Beck claimed. It is possible that the IgG subclasses that had to be eluted from protein A columns in Beck's experiment, i.e. IgG1, 2 and 4, were affected by the elution procedures while the IgG3 proteins remained unaffected as this subclass does not bind to protein A and hence does not need to be eluted by low pH or high ion concentration.

A high anti-HSV IgG titre was required for detection of ADCC. Our test, utilizing target cells with lytic infection, is probably less sensitive than that used by Norrild *et al.* (1979). ADCC is mediated by macrophages, granulocytes and moncytes (Herberman, 1986). The Fc-receptors on these cells bind IgG1 and IgG3 with high affinity compared to IgG2 and IgG4 (Woof *et al.*, 1984; Hay, Torrigiani & Roitt, 1972; Naegel, Young & Reynolds, (1984), which is also true of lymphocytes comprising the majority of PBMC. Thys the finding of ADCC in IgG1 and 3 fractions was not unexpected.

Previous findings of IgG subclass complement binding have been contradictory (Augener, 1971; Schumaker, 1976). The present experiments showed that the IgG1, 3 and 4 subclasses can bind and activate complement to some degree, although IgG3 neutralization was by far the most affected by complement addition.

The now classical complement fixation test was also used to determine HSV antibody titres. This test is less sensitive than ELISA, which explains why only half the serum fractions were HSV positive by complement fixation. The subclass distribution of the positive samples does however, strengthen the finding of IgG4 complement activation.

The relationship between antibody activity to defined viral antigens, and viral neutralization was addressed in our estimates of IgG subclass avidities to gC. The results from the few gC positive preparations seemed to relate high avidity to gC to

IgG1, but this was not conclusive. In fact, for these five subclass preparations for titre correlates well with the neutralization results ($r=0{\cdot}9818$, $P<0{\cdot}01$, linear regression analysis). However, the corresponding values for the whole sample does not support such a relation, in particular not for the IgG3 and IgG4 subclasses (for IgG1 $r=0{\cdot}9890$, $P<0{\cdot}01$; for IgG3 $r=0{\cdot}8208$, $P>0{\cdot}20$; for IgG4 $r=0{\cdot}7175$, $P>0{\cdot}20$) which seem to have a better neutralizing capacity than their titres would predict. Accordingly, further studies on anti-viral antibodies of these subclasses in particular may be warranted, to correctly assess their importance in the humoral response to viral antigens.

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