Immunology 1985 **54** 565

Binding site and subclass specificity of the herpes simplex virus type 1-induced Fc receptor*

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Accepted for publication 20 November 1984

Summary. Immunoglobulin Fc-binding activity was detected by indirect immunofluorescence employing fluorochrome conjugated $F(ab')_2$ antibody fragments on acetone-fixed cell cultures infected with herpes simplex virus type 1 (HSV-1). Using this method the Fc receptor-like activity seemed to be restricted to the IgG class of human immunoglobulins. While IgG1, IgG2, and IgG4 myeloma proteins bind to this putative Fcγ receptor at a concentration of 0·002 mg/ml, IgG3 myeloma proteins were without activity at 0·1 mg/ml. The binding activity was associated with the Fc fragments of IgG, while the pFc' fragments of IgG appeared to be unable to bind in this assay system. The reactivity and specificity of the HSV-1 Fc receptor was independent of both the type of tissue culture cells used and the strain of HSV-1 inducing the Fc receptor-like activity. The HSV-1-induced Fc receptor has a similar specificity for human immunoglobulin class and subclasses as staphylococcal Protein A. However, these two Fc receptors exhibit at least one striking difference. The IgG3 G3m(st) protein which binds to Protein A does not bind to HSV-1-induced Fc receptor. A possible reaction site for the HSV-1 Fc receptor on IgG could be at or near Asp 276.

*Part of the results described here was presented at the 8th Scandinavian Virus Symposium, Aarhus, Denmark, May 1983.

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INTRODUCTION

Receptor-like activity for the Fc region of immunoglobulins from many different species has been demonstrated on a variety of cell types (Unkeless, Fleit & Mellman, 1981). The IgG Fc-receptor activity on monocytes and lymphocytes has been especially well characterized (Frøland, Wisløff & Michaelsen, 1974; Dickler 1976). Fc-receptor activity can also be induced in cells that normally lack or display very little of this activity, by infecting the cells with human herpes viruses such as cytomegalovirus (CMV), herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV) and Epstein-Barr virus (EBV) (Watkins, 1964; Furukawa *et al.*, 1975; Costa *et al.*, 1978; Ogata & Shigeta, 1979; Yee *et al.*, 1982). This Fc-binding property is well known because of the problems observed when acetone-fixed virus infected cells are used as a substrate to measure serum antibody titre by indirect immunofluorescence (Feorino, Shore & Reimer, 1977). The HSV-1 Fc receptor has been particularly well characterized biochemically and is thought to be a glycoprotein (gE) that is not only on the infected cell but also on the intact viron (Baucke & Spear, 1979; Para, Bauke & Spear, 1980).

The HSV-1 receptor does not bind human or rabbit IgM nor human IgA, while polyclonal rabbit and human IgG lacking antibody to HSV bind quite strongly to the virus-infected cells (Yasuda & Milgrom, 1968; Feorino *et al.*, 1977). Similar specificity

has been shown for the HSV-2 Fc receptor-binding activity which also has been shown to be inhibited by monoclonal proteins of IgG1 and IgG2 subclasses (Nakamura *et al.*, 1978). The binding has been shown to be with the Fc region of IgG, as isolated $F(ab')_2$ fragments from rabbit or human IgG are without binding activity (Westmoreland & Watkins, 1974; Feorino *et al.*, 1977). We have studied the specificity of the HSV-1-induced Fc receptor with respect to binding capacity for human immunoglobulin classes, IgG subclasses, and IgG fragments.

MATERIALS AND METHODS

Cells and virus

Herpes simplex virus type 1 BB strain was used for most of this work. In certain experiments, the LMM strain of HSV-1 was also used. These low passage, clinical isolates of HSV-1 were typed by D. M. Lonsdale using restriction enzymes (Lonsdale, 1979). The type specificity of these isolates was confirmed using an enzyme-linked immunosorbent assay (ELISA) with reagents from Daco, Copenhagen, Denmark (Vestergaard & Jensen, 1981). Virus was grown in roller bottles of low passage, semicontinuous human fibroblast cells produced in our laboratory or in flasks of BHK-21 clone 13 cells (Flow Laboratories, Rockville, MD) using Eagle's minimal essential medium (MEM) supplemented with 2% fetal calf serum. When extensive cytopathic effect was evident, the cultures were frozen and thawed and then stored at $-70°$. Titrations of viral infectivity were made in the cell types used to prepare the stock virus.

Induction of Fc receptor-like activity by HSV 1

Roller bottles of human fibroblasts or flasks of BHK-21 clone 13 cells were inoculated with HSV-1 BB strain at a multiplicity of infection of 10 plaque-forming units/cell. The virus was absorbed to the monolayer at room temperature for 1·5 hr before the inoculum was removed, and replaced with MEM without serum. After incubation at 36° for 2, 3, 4, 6 or 24 hr, the medium was removed and the cells were loosened from the flasks by treatment for approximately 30 sec with 0·25% Bacto trypsin (Difco). The cells were washed from the bottle with approximately 20 ml of phosphate-buffered saline (PBS). After an additional wash in PBS, the cells were resuspended to a concentration of $1·5 \times 10^6$ cells/ml. The virus-infected cells were mixed with a control cell preparation which had been treated in the same manner, at a virus/control cell ratio of 1:5, in order to provide a negative contrast and to act as an internal control in the stained slides. Approximately 25 μl of this suspension was applied to each of 10 wells on PTFE (Teflon)-coated slides, air-dried, fixed in acetone at room temperature for 5 min, and frozen at $-20°$ until use. In certain experiments, slide preparations made from human fibroblast cells infected with the LMM strain of HSV-1 were used. Since earlier reports have indicated that the Fc-binding activity on HSV types 1 and 2 infected cells is trypsin sensitive, parallel experiments were carried out using slides made from virus-infected cells that had been scraped from the culture bottles (McTaggart *et al.*, 1978; Costa *et al.*, 1978). The Fc-binding activity of these preparations was essentially the same as when trypsin was used. However, large aggregates of cells, especially in the 2, 3, 4 and 6 hr post-infection preparations made the interpretation of immunofluorescence difficult. All batches of slides were tested by titrating a standard preparation of serum, IgG, and $F(ab')_2$ from a HSV antibody-negative individual.

Fluorochrome-conjugated antibodies

Species-specific antibodies were produced by immunizing rabbits or sheep with 0·04–0·4 mg of IgG from the appropriate animal emulsified in Freund's complete adjuvant. Anti-human Ig and IgG were made in a similar manner by immunizing with $F(ab')_2$ or Fc fragments from pooled normal IgG, respectively. Anti-human IgG3 antiserum was obtained by immunizing with $F(ab')_2$ of a IgG3 myeloma protein followed by absorption of the antisera on immunosorbent columns containing Fc and Fab fragments of the myeloma protein used for immunization (Michaelsen & Natvig, 1974). The species, class and subclass specificity were tested in ELISA where the antibodies were shown to react only with the immunogen and homologous antigens. These antibodies were digested with pepsin at pH 4·0 and filtered through a Sephadex G-200 column to isolate the $F(ab')_2$ fragment. The $F(ab')_2$ preparations were then labelled with fluorescein isothiocyanate (FITC), Isomer I, or tetramethylrhodamine isothiocyanate (MRITC) (Becton-Dickinson, Cockeyville, MD) essentially as described by Brandtzaeg (1973). The optimally labelled $F(ab')_2$ was isolated by ion-exchange chromatography on DEAE cellulose (Whatman DE-52). The FITC conjugates had F/P ratios of 2–3 and for the optimally labelled MRITC conjugates the OD ratio 280/495 nm was 1·5–2.

Indirect immunofluorescence test

Dilutions of serum and purified protein preparations in PBS with 0·5% bovine serum albumin (BSA) were applied to the wells of the slides and incubated in a moisture chamber at 37° for 30 min. The slides were washed three times for 5 min in PBS and air-dried before the appropriate conjugate, diluted in PBS-BSA, was applied to the wells. After incubation and washing in PBS as before, the slides were counterstained in 0·01% Evans blue, air-dried and mounted with a cover glass using buffered glycerine, pH 7·5. Counterstaining was not done when MRITC was used. The slides were coded and read using a Zeiss standard IFD fluorescence microscope. Controls were always included to verify that the FITC- or MRITC-conjugated immunoglobulins did not bind directly to the HSV-infected cells.

Human immunoglobulin preparations

IgG from the sera of two individuals lacking antibodies to HSV was isolated by DEAE-ion exchange chromatography as previously described (Michaelsen & Natvig, 1973). One of the two sera also lacked antibodies to varicella zoster virus (VZV) while the other serum contained antibodies to VZV as determined by the fluorescent antibody to membrane antigen (FAMA) test (Williams, Gershon & Brunell, 1974). Monoclonal IgG from the different subclasses and the IgA, IgM, IgD and IgE proteins were isolated from the sera of patients suffering from multiple myelomas or macroglobulinemias using DEAE-ion exchange chromatography combined with Sephadex G-200 gel filtration as previously described (Wisløff, Michaelson & Frøland, 1974). The purity of the different preparations was more than 95% as judged by agarose gel electrophoresis, immunoelectrophoresis, single radial immunodiffusion, and double immunodiffusion employing class-specific antisera, as well as anti-κ and anti-λ specific antisera as previously described (Wisløff *et al.*, 1974). Some of the IgM, IgA and IgD preparations which were slightly contaminated with IgG were further purified by passage through a column containing Protein A (Sepharose 4B CL Prot. A, Pharmacia, Uppsala, Sweden).

Fragments of IgG

Fab and Fc fragments of IgG were prepared by papain digestion with or without cysteine depending on the IgG subclass, and the Fab and Fc were separated from undigested IgG by gel filtration through Sephadex G-200, as previously described (Michaelsen & Natvig, 1972). Fab was separated from Fc by DEAE-cellulose chromatography where Fab was usually eluted by 0·015 M phosphate buffer pH 7·6 and the Fc was eluted by 0·3 M phosphate buffer pH 7·6 (Michaelsen & Natvig, 1971). Alternatively, the Fc and Fab were separated by passage through a Protein A column where Fab was eluted by PBS pH 7·3 and Fc was subsequently eluted by 0·1 M glycine buffer pH 3·5. This was not done with the IgG3 proteins since, with the exception of the G3m(st) variant, they do not bind to Protein A (Kronwall & Williams, 1966; Recht *et al.*, 1981). $F(ab)_2$ and pFc′ fragments were prepared by pepsin digestion at pH 4·5 followed by gel filtration through a Sephadex G-200 column (Michaelsen & Natvig, 1974; Michaelsen, Fragione & Franklin, 1977). The purity of the different fragments was tested by double immunodiffusion analysis employing Fab- and Fc-specific antisera as well as anti-κ and anti-λ antisera. Some of the preparations were also tested in a sensitive ELISA system as previously described (Michaelsen & Kolberg, 1984).

RESULTS

Time-dependent appearance of Fc receptors

When human fibroblast cell cultures were infected with HSV-1 at high multiplicity, the Fc receptor-like activity could be detected within 3 hr after infection (Table 1). At this time the immunofluorescence due to the Fc receptor, was located in a perinuclear area in most cells (Fig. 1a). By 6 hr post-infection, when maximum binding with IgG was observed, the immunofluorescence was distributed throughout the cytoplasm often as large granular deposits, and as uneven granular membrane fluorescence (Fig. 1b).

At the early time of 2 hr post-infection, HSV antigens were present on the virus-infected cells since sera taken from patients after primary or recurrent infections with HSV-1 or HSV-2 gave high titres when slides made from these cells were used to assay for antibody to HSV in indirect immunofluorescence. However, these cells lacked or had very little Fc-binding activity since sera, IgG or Fc fragments from HSV antibody-negative individuals did not bind to the cells. Consequently, these slides were used as a control to test all protein preparations for the presence of specific antibody to HSV, without interference from the Fc receptor.

It is possible that the development and immunoglobulin specificity of the Fc receptor detected by this technique could depend on the cell type used and/or

Table 1. Development of IgG-binding activity on human fibroblast cells infected with HSV type 1

Hours post-infection	Lowest concentration of protein or highest dilution of serum showing binding			
	Polyclonal IgG* (mg/ml)	Monoclonal IgG† (mg/ml)	Serum A‡	Serum B§
2	>0·5	>1·0	>1:5	>1:5
3	0·025–0·006	NT¶	1:400	1:100
4	0·025–0·006	NT	1:800	1:400
6	0·006–0·002	0·002	NT	1:1600
24	0·002	0·002	1:1600	1:1600

* IgG purified from serum A.
† IgG2 monoclonal protein (Fj).
‡ Serum A: antibody-negative to HSV, antibody-positive to VZV.
§ Serum B: antibody-negative to both HSV and VZV.
¶ NT, not tested.

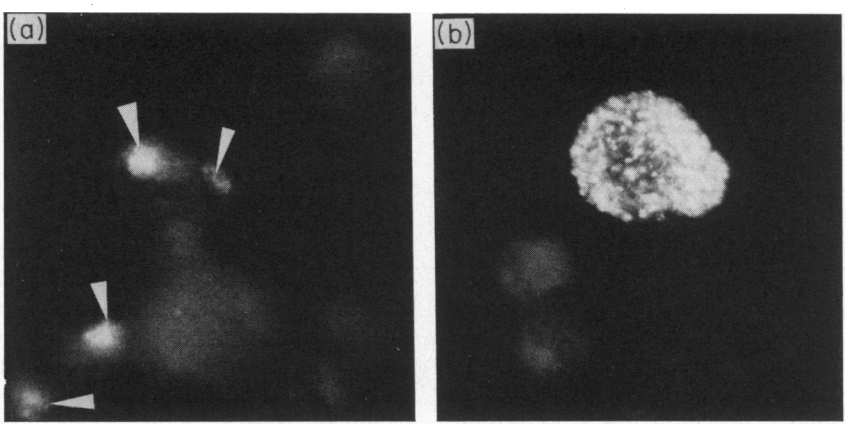

Figure 1. Location of HSV-1-induced Fc-receptor activity in human fibroblast cells infected with the BB strain of virus. Indirect immunofluorescence using FITC-conjugated $F(ab')_2$ of sheep anti-human IgG antibodies with a 1:20 dilution of HSV antibody-negative human serum on acetone fixed cells: (a) 3 hr post-infection, location of early Fc-receptor activity indicated by arrows; (b) 6 hr post-infection, showing a granular distribution of the Fc activity. (Magnification × 320.)

the strain of HSV-1 infecting the cells. HSV-1 BB strain was passed three times in the BHK 21 clone 13 cell line before a similar time study on the development of the Fc receptor. The Fc-binding activity seemed to develop earlier in this cell line than with the human fibroblast cells with activity detected initially at 2–2·5 hr post-infection and maximum binding occurring 2 hr later. However, the specificity for immunoglobulin class and IgG subclass was the same as that exhibited on the human fibroblast-infected cells. This was also true when the LMM strain of HSV-1 was used (data not shown). Thus, it seems that the specificity of the HSV-1 Fc receptor is independent of both the strain of HSV-1 and the cell type used.

Human immunoglobulin class specificity of HSV Fc receptor

The binding of human sera to HSV-induced Fc receptor appeared to be due to the IgG content of the sera. The other classes of immunoglobulins exhibit very little, if any, binding to the HSV-infected cells (Table 2). Since very low concentrations of IgG, 2–6 μg/ml, bind to the HSV-infected cells, it was necessary to purify some of the myeloma proteins on Protein A columns. Any binding activity present in the IgM, IgA, IgE or IgD myeloma preparations was always retained in the small amounts of protein binding to the Protein A column and the PBS eluate, which was not

Table 2. Binding of human immunoglobulin classes to HSV-infected human fibroblasts 24 hr post-infection

Immunoglobulin class	Number of proteins tested	Minimum concentration showing binding (mg/ml)
IgG polyclonal*	2	0·006–0·002
IgA monoclonal	2	>0·1
IgM monoclonal	3	>0·1
IgE monoclonal	1	>0·1
IgD monoclonal	2	>0·1

* From HSV antibody-negative individuals.

retained on the column and contained the myeloma protein, always lacked Fc receptor-binding activity. Polyclonal IgG from the HSV antibody-negative, VZV antibody-positive individual, and the HSV, VZV, antibody-negative individual showed a similar degree of binding with the Fc-positive HSV-1 infected cells. However, both preparations were negative with the HSV-1-infected cells obtained at 2 hr post-infection. In addition, high concentrations of $F(ab')_2$ from the VZV+ HSV− and the VZV− HSV− individuals did not react with slides prepared from 24 hr post-infection cells that contained large amounts of both Fc receptor and HSV-specific antigens. This seemed to indicate that antibodies to VZV did not cross react with the HSV in the assay system and that neither of these sera contained antibody activity to HSV.

IgG subclass specificity of the HSV Fc receptor

Different IgG myeloma proteins were tested with respect to their ability to bind the HSV-1-induced Fc receptor (Table 3). The IgG1, IgG2 and IgG4 proteins bound to the same extent as polyclonal IgG. However, none of the five IgG3 proteins which were tested exhibited significant binding with the HSV-infected cells. Included among the IgG3 proteins was the G3m(st) genetic variant which binds to staphylococcal Protein A. This Goe protein was also without binding activity to the HSV-1 Fc receptor.

Binding of IgG fragments to the HSV-induced Fc receptor

Fragments from several of the IgG myeloma proteins were tested in order to determine if the binding activity was located on the Fc fragment of these proteins (Table 4). The Fab and $F(ab')_2$ fragments of IgG1 and IgG2 proteins were without activity while the Fc fragments exhibited binding at approximately the same concentration as the intact myeloma proteins from which they were derived. However, the Fc

Table 3. Binding of human IgG monoclonal proteins to HSV-1-infected cells 24 hr post-infection

IgG subclass	Protein	Gm and light chain type	Minimum concentration binding (mg/ml)
IgG1	Rø	λG1m(f)	0·002
	Ar	λG1m(f)	0·002
IgG2	Fj	κG2m(n−)	0·002
	The	κG2m(n−)	0·002
	Ed	λG2m(n+)	0·002
IgG3	Br	κG3m(b^0)	>0·1
	Jo	λG3m(g)	>0·1
	Hus	κG3m(g)	>0·1
	Her	λG3m(g)	>0·1
	Goe	κG3m(st)	>0·1
IgG4	Ger	κ	0·002
	Joh	κ	0·002

fragment of an IgG3 myeloma protein was without binding as shown for the intact IgG3 molecule. The pFc′ fragment, which roughly corresponds to the Cγ3 homology region of the IgG heavy chain, was also without activity. The binding pattern with the different fragments derived from the IgG of an HSV-negative individual was essentially the same as with the myeloma protein fragments (Table 5). Partial reduction and alkylation of the Fc fragment only slightly reduced the ability to bind to the Fc receptor. As with the myeloma proteins, isolated pFc′ did not seem able to bind to the HSV-1-induced Fc receptor.

DISCUSSION

Semicontinuous lines of human fibroblast cells express Fc receptor-like activity after infection with herpes simplex type 1 as reported here and by others (Yasuda & Milgrom, 1968; Westmoreland & Watkins, 1974). We used an indirect immunofluorescence technique on trypsin-treated acetone-fixed HSV-1-infected cells to detect the Fc-receptor activity. In our work the short exposure to trypsin did not seem to affect the Fc-binding activity probably because intracellular Fc receptor is also detected due to acetone fixation.

The immunofluorescent conjugates were employed as $F(ab')_2$ fragments of the antibodies otherwise direct binding of these conjugates was observed with the HSV-infected cells. These results appear to be in contrast to others who have indicated that fluorochrome labelling of rabbit IgG interfered with the ability to bind to the Fc receptor (Thrasher *et al.*, 1975). However, as has been shown with the CMV-induced Fc receptor, which is more in agreement with our observations, fluorochrome labelling only reduces the ability of IgG to bind to the Fc receptor but does not eliminate this binding (Keller, 1976).

Table 4. Binding of fragments from monoclonal IgG to HSV-infected cells 24 hr/post-infection

	Minimum concentration (mg/ml) of fragments binding				
Protein	$F(ab')_2$	Fab	Fc	pFc′	pr/a IgG*
IgG Rø	ND†	ND	0·006	>0·1	ND
IgG1 Ar	ND	>0·5	0·025	ND	ND
IgG2 Fj	>0·1	>0·5	0·006	ND	0·025
IgG3 Her	ND	ND	>0·5	ND	ND

* Partially reduced and alkylated.
† ND, not determined.

Table 5. Binding of polyclonal IgG and fragments to HSV-infected cells 24 hr post-infection

Preparations tested	Minimum concentration or highest dilution binding to HSV-infected cells
Serum A*	1:1600*
IgG from serum A†	0·006 mg/ml
Δ agg. IgG from serum A‡	0·006 mg/ml
$F(ab')_2$ from serum A	>0·5
Fab from serum A	>0·5
Fc from serum A	0·002
pr/a Fc§	0·006
pFc′	>0·1

* Serum A is HSV-negative VZV-positive.
† The IgG concentration of serum A is approximately 12 mg/ml, therefore, A 1:1600 dilution contains about 0·007 mg IgG.
‡ Heat aggregated at 63° for 10 min.
§ Partially reduced and alkylated.

The IgG Fc specificity of the receptor was assayed by using polyclonal IgG and IgG fragments from two sera lacking antibodies to HSV. The $F(ab')_2$ and Fab fragments from these sera did not bind to the 24-hr post-infection preparations even though they contained Fc receptor- and herpes simplex-specific antigens, while the Fc fragments bound to about the same extent as the intact IgG. One of these individuals also lacked antibodies to VZV thus avoiding possible problems with crossreacting antibodies to HSV. Furthermore, we have tested fragments from IgG myeloma proteins and binding was only observed with the Fc fragments and not with the $F(ab')_2$ or Fab fragments of the myeloma protein.

The IgG Fc specificity was verified by testing individual myeloma proteins of other immunoglobulin classes. As others have shown with rabbit IgM (Yasuda & Milgrom, 1968) and pools of human IgM and IgA myeloma proteins Feorino *et al.*, 1977), neither of these immunoglobulin classes were able to bind in our Fc receptor test system. In addition, we found that IgD and IgE myeloma proteins were not able to bind. For all classes except IgE, several myeloma proteins were tested and all gave negative results. We did not observe any individual differences among these proteins but the possibility cannot be excluded that certain rare myelomas belonging to these classes might bind to HSV-1 Fc receptor as has been reported for Protein A (Harboe & Følling, 1974).

When the IgG subclass specificity of the HSV-1 Fc receptor was studied, it was found that IgG1, IgG2 and IgG4 subclasses reacted equally well irrespective of allotype. However, the proteins of the IgG3 subclass were completely negative, also in the form of isolated Fc fragments. Attempts to determine the reactive site on the IgG molecule were performed using fragments of IgG from HSV-negative individuals as well as fragments of IgG from the IgG1 and IgG2 myeloma proteins. The Fc fragments were positive in binding while the pFc′ fragments, corresponding to the C-terminal, CH3 domain, were negative. Such non-pFc′ specificity of the HSV-1 Fc receptor is shared with the FcR II receptor on the human K-cells (Wisløff *et al.*, 1974; Gergely *et al.*, 1982). On the other hand, the HSV-1 Fc receptor seems to be different from the FcR II receptor on the K-cells with respect to specificity for the IgG subclasses. Thus, the FcR II receptor has high reactivity for IgG1 and IgG3 while IgG2 only reacts after heat aggregation and IgG4 is without activity (Wisløff *et al.*, 1974) but this is quite different from the HSV-1 Fc receptor specificity presented here.

The HSV-1-induced Fc receptor has a close resemblance to its well characterized bacterial counterpart, Protein A from *Staphylococcus aureus*, since Protein A also has a specificity for IgG1, IgG2 and IgG4, while IgG3 does not bind (Kronwall & Williams, 1966). The interaction between Protein A and IgG1 has also been analysed by X-ray diffraction and two interaction sites, one on $C\gamma2$ and the other on $C\gamma3$, have been demonstrated (Deisenhofer, Jones & Huber, 1978). In the $C\gamma3$ region, it is the His 435 which binds strongly to Protein A but in IgG3 this His 435 is substituted with Arg and thus explains why IgG3 is not reactive (Michaelsen *et al.*, 1977). Recently two IgG3 proteins have been described which bind to Protein A, and both of these have His in position 435 (Recht *et al.*, 1981, Matsumoto *et al.*, 1983). We have tested one of these, the Goe protein in our HSV-1 Fc-receptor system, and it did not bind. Thus, the binding site of HSV-1 Fc receptor and Protein A must be different. One strong candidate for the HSV-1 Fc receptor-binding site could be Asp 276 which is substituted with Lys in IgG3, as it is in the Goe protein. Further experiments are in progress in order to verify this proposition.

During the preparation of this manuscript, we became aware of an article by Johansson *et al.* (1984) reporting that the HSV-1 Fc receptor has specificity for IgG1, IgG2 and IgG4. However, they observed a quantitative difference among these subclasses which we have not observed using our method of detecting Fc-binding activity.

ACKNOWLEDGMENTS

The authors appreciate the constructive criticism of Dr R. Wiger and Professor M. Degré. The excellent technical assistance of Ms I. Modahl and Ms M. F. Løfsgaard is gratefully acknowledged.

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