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Induction of Immunoglobulin G Fc Receptors by Recombinant Vaccinia Viruses Expressing Glycoproteins E and I of Herpes Simplex Virus Type 1

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Glycoprotein E (gE) of herpes simplex virus type 1 (HSV-1) will bind immunoglobulin G (IgG) (Fc) affinity columns (R. B. Bauke and P. G. Spear, J. Virol. 32:779–789, 1979), but recent evidence suggests that the HSV-1 Fc receptor is composed of a complex of gE and glycoprotein I (gI) and that both gI and gE are required for Fc receptor activity (D. C. Johnson and V. Feenstra, J. Virol. 61:2208–2216, 1987; D. C. Johnson, M. C. Frame, M. W. Ligas, A. M. Cross, and N. D. Stow, J. Virol. 62:1347–1354, 1988). We have expressed gE and gI, either alone or in combination, on the surface of HeLa cells by using recombinant vaccinia viruses and have measured Fc receptor activity by Fc-rosetting or IgG-binding assays. Expression of gE alone resulted in the induction of Fc receptor activity, while expression of gI alone gave no detectable Fc binding. Coexpression of gE and gI resulted in higher levels of IgG binding than did expression of gE alone, despite the fact that under conditions of coexpression, the levels of surface gE were reduced. We propose that gE and gI together form a receptor of higher affinity than gE alone and that HSV-1 therefore has the potential to induce two Fc receptors of different affinities.

It has been recognized for many years that herpes simplex virus (HSV) induces the expression of an immunoglobulin G (IgG) Fc receptor on the surfaces of infected cells and enveloped virions (16, 23, 24). The significance of this receptor is uncertain, but it is assumed that it provides the virus with a mechanism of immune evasion by blocking effector functions requiring Fc recognition, a view supported by in vitro studies of immune cytolysis of infected cells (1) and by the demonstration that bipolar binding of antibody to HSV type 1 (HSV-1) particles greatly reduces the efficiency of complement-mediated neutralization (7). Bauke and Spear (2) identified a virus-specific glycoprotein, subsequently designated glycoprotein E (gE), that would bind to IgG affinity columns, and mapping experiments showed that gE was the product of the HSV-1 Us8 gene (11, 15, 18, 19). More recently, Johnson and Feenstra (8) identified a second component, glycoprotein I (gI), in a complex with gE that is precipitated by Fc binding. gI is encoded by the Us7 gene of HSV-1 (12, 15), and a role for gI in the formation of the virus-induced Fc receptor was demonstrated by showing that virus mutants in which either Us7 or Us8 was inactivated failed to induce Fc receptors (9). Unlike the many different Fc receptors found in mammalian cells, the HSV Fc receptor therefore appears to be a heteropolymer. However, the assertion that both gE and gI are directly involved in Fc binding is dependent on negative evidence obtained by using deletion mutants, and the available evidence does not formally exclude the involvement of other virus-specific proteins in Fc binding. Indeed, Johnson et al. (9) reported difficulty in obtaining consistent Fc receptor activity on transfected cells expressing both gE and gI. In this report, we describe the induction of Fc receptors on cells infected with recombinant vaccinia viruses expressing HSV-1 gE and gI. We find that cells expressing gE alone bear Fc receptors but that Fc binding is enhanced by the simultaneous expression of gI.

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MATERIALS AND METHODS

Cells and viruses. HeLa, CV-1, and 143-TK$^-$ cells were grown in Glasgow modified Eagle medium supplemented with 10% fetal calf serum (FCS). BHK-21 cells were grown in Glasgow modified Eagle medium supplemented with 10% tryptose phosphate broth and 10% newborn calf serum. HSV-1 strain SC16 was grown and assayed in BHK-21 cells. Vaccinia virus strain WR and recombinants derived from it were grown in BHK-21 cells and assayed in CV-1 cells.

Recombinant vaccinia viruses. A recombinant vaccinia virus expressing HSV-1 gI was described by Sullivan and Smith (22) and was a gift from the authors. For convenience, this virus will be called Vac-gI. Vaccinia virus recombinants expressing gE were constructed according to the methods outlined by Mackett and Smith (13) as follows. A *Dde*I fragment of HSV-1 strain 17 (Us base pairs 8398 to 10737 [15]) was partially digested with *Sst*II to yield a *Sst*II-*Dde*I fragment of approximately 2.2 kilobases (Us base pairs 8526 to 10737) in which the first ATG triplet was at base pair 8639, the start of the gE coding sequence. The *Sst*II-*Dde*I fragment was end repaired with T4 DNA polymerase and ligated into two insertion vectors: (i) the *Sma*I site of pSC11 such that the gE coding sequence was downstream of a vaccinia virus promoter taken from a gene encoding a 7.5K protein and was flanked by the vaccinia virus thymidine kinase gene (4) and (ii) the *Sma*I site of pRK19 such that the gE coding sequence was downstream of a promoter for a vaccinia virus late gene (the 4b gene) and was flanked by the vaccinia virus thymidine kinase gene (R. Kent and G. Smith, unpublished results). The resulting plasmids were examined by restriction endonuclease digestion to determine the insert orientations, and plasmids with the desired orientation were trans-

fected into vaccinia virus-infected CV-1 cells. Progeny virus was used to form plaques on 143-TK$^-$ cells in the presence of 25 μg of bromodeoxyuridine per ml, and recombinant plaques among the thymidine kinase-negative progeny were identified by β-galactosidase activity (pSC11-derived recombinants) or by dot blot hybridization (pRK19-derived recombinants). A positive recombinant from each transfection was identified and plaque was purified, and stocks were prepared as described by Mackett et al. (14). The recombinant in which gE was expressed under the control of the 7.5K promoter was named Vac 7.5-gE, and the recombinant in which gE was expressed under the control of the 4b promoter was named Vac 4b-gE.

Antibodies. Monoclonal antibodies 3114, specific for HSV-1 gE, and 3104, specific for HSV-1 gI, were gifts from Anne Cross, Institute of Virology, Glasgow, United Kingdom, and were provided as ascites fluids. These antibodies were prepared as described by Cross et al. (6). Fluorescein-labeled rabbit anti-mouse immunoglobulin was obtained from DAKO (Copenhagen, Denmark) and was stored at 4°C. Fluorescein-labeled normal rabbit IgG was obtained from DAKO. The preparation had been stored at −20°C for several years and had been subjected to thawing and refreezing. Normal rabbit serum used for blocking Fc receptors had been similarly stored. These preparations were assumed to contain IgG aggregates. An IgG fraction prepared from a rabbit anti-ox erythrocyte (RBC) serum was a gift from Anne Wilson, Department of Pathology, University of Cambridge.

Surface fluorescence. All manipulations were at room temperature. HeLa cells (5×10^6) infected with HSV-1 or with vaccinia virus recombinants were harvested 14 h after infection by scraping and pipetting in phosphate-buffered saline containing 1% FCS–1% bovine serum albumin (PBS-FCS-BSA) to achieve a suspension of single cells. Monoclonal antibody was added to a final dilution of 1 in 300 to a suspension of 10^6 cells in 2 ml of PBS-FCS-BSA and allowed to react for 30 min with occasional inversion. The cells were sedimented at 500 × *g* for 3 min and suspended in 5 ml of PBS-FCS-BSA containing 2% normal rabbit serum. This washing procedure was repeated three times. The cells were then resuspended in 2 ml of PBS-FCS-BSA–2% normal rabbit serum, and fluorescein-labeled rabbit anti-mouse immunoglobulin was added to a dilution of 1 in 80 and allowed to react for 30 min. The previous washing procedure was repeated, and the cells were suspended in 1 ml of PBS containing 1% BSA and fixed by addition of an equal volume of PBS containing 2% formaldehyde–1% BSA. The cells were stored at 4°C in the dark before analysis. The normal rabbit serum was effective in blocking Fc receptors because when monoclonal antibodies were omitted from the first stage of the assay, addition of fluorescein-labeled rabbit anti-mouse immunoglobulin gave low background staining and the background on HSV-1-infected cells (which bore Fc receptors) was indistinguishable from the background on wild-type vaccinia virus-infected cells (which did not bear Fc receptors).

Fc-binding assay. A sample of 10^6 infected HeLa cells prepared as a single-cell suspension as described in the previous section was suspended in 2 ml of PBS-FCS-BSA containing 250 μg of fluorescein-labeled rabbit IgG per ml and incubated for 30 min at room temperature. The cells were then washed three times and fixed in 1% formaldehyde as described in the previous section.

Flow cytometric analysis. An Epics-Profile flow cytometer (Coulter Electronics, Inc., Hialeah, Fla.) was used to analyze cell surface fluorescence in antibody-binding or Fc-binding assays. The single-cell population was identified by forward and 90° scattering, and each analysis was performed on at least 10^4 cells.

RBC antibody rosettes. Assays were performed as follows. RBCs were prepared from fresh ox blood by being washed in PBS and sedimented at 400 × *g* six times to remove buffy coat leukocytes. A 2% suspension of RBCs in PBS was treated with a subagglutinating dose of an IgG fraction of rabbit anti-ox RBC serum (approximately 100 μg/ml) for 30 min. The sensitized cells were washed three times in PBS and stored at 4°C as a 1% suspension in PBS. A 50-μl sample of a suspension of infected HeLa cells at 10^6 cells per ml in PBS–5% FCS was mixed with an equal volume of sensitized RBCs. The mixture was centrifuged at 400 × *g* for 2 min, and the pellet was left undisturbed for 30 min at room temperature. The mixture was gently resuspended and examined microscopically. Unsensitized RBCs were used as negative controls. Rosettes were never observed with unsensitized RBCs.

Immunoprecipitation. Monolayers of 5×10^6 HeLa cells were infected with HSV-1, vaccinia virus, or recombinant vaccinia viruses at a multiplicity of infection (MOI) of 10. After 10 h, the medium was replaced with methionine-free medium containing 50 μCi of ^{35}S-methionine (Amersham International, Amersham, United Kingdom) per ml and incubated for a further 4 h. The subsequent steps of cell lysis, immune precipitation, collection of precipitates with protein A-Sepharose, electrophoresis, and autoradiography were as described previously (20).

RESULTS

Vaccinia virus recombinants expressing HSV-1 gE. The objective of preparing two vaccinia virus gE recombinants utilizing different promoters driving the same coding sequence was to achieve different levels of gE in infected cells. The 7.5K promoter utilized in pSC11 (4) and in Vac 7.5-gE is active both before and after viral DNA synthesis (5, 14). The 4b promoter utilized in pRK19 (Kent and Smith, unpublished results) and the recombinant virus Vac 4b-gE is active only after viral DNA synthesis but drives the expression of a major virion core component (17, 21) and at late times in infection is stronger than the 7.5K promoter (R. Kent and G. Smith, personal communication). Figure 1 shows gE precipitated from infected cells pulsed with ^{35}S-methionine from 10 to 14 h postinfection. The main point of the experiment was to show that gE synthesized by the recombinant vaccinia viruses is qualitatively indistinguishable from gE synthesized by HSV-1. However, it is also notable that cells infected with Vac 4b-gE incorporated more isotope into gE during the labeling period than cells infected with Vac 7.5-gE did. This is consistent with the results of experiments designed to measure more directly the relative amount of gE present on the surface of recombinant-infected cells, described later in this paper.

Fc rosetting. HeLa cells were infected with HSV-1, wild-type vaccinia virus, or recombinant vaccinia viruses either singly or in combination. All infections were at an overall MOI of 10. When cells were doubly infected with recombinant viruses, each virus was used at an MOI of 5. When cells were infected with a single recombinant, an MOI of 5 was used and the cells were simultaneously infected with wild-type vaccinia virus at an MOI of 5. Each vaccinia virus infection was therefore equalized for the overall MOI and recombinant gene dose. After 14 h, the cells were harvested and rosetting assays were performed. This is a very sensitive

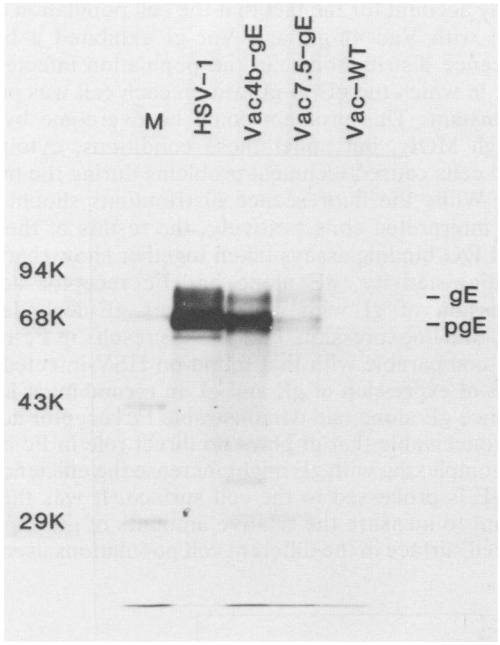

FIG. 1. Expression of gE by recombinant vaccinia viruses. HeLa cells were infected at an MOI of 10 with HSV-1, vaccinia virus, Vac 7.5-gE, or Vac 4b-gE and labeled with ^{35}S-methionine from 10 to 14 h postinfection. Immune precipitates were prepared from cell lysates by using a monoclonal antibody against HSV-1 gE, and the products were electrophoresed through a 7.5% polyacrylamide gel. Lane M contains marker proteins whose molecular weights are given on the left. WT, Wild type; pgE, high-mannose precursor of gE.

assay because of the large number of binding sites per RBC, but it is inevitably somewhat subjective, and the assay is only semiquantitative. Representative rosettes are shown in Fig. 2, and the results can be summarized as follows. Rosettes were never observed with cells infected with wild-type vaccinia virus or with Vac-gI. Rosettes were observed with cells infected with vaccinia virus recombinants expressing gE, but the rosettes were more impressive when gI and gE were expressed in the same cells. In particular, the rosettes observed with cells infected with Vac 7.5-gE were weak, consisting of a halo of RBCs around the infected HeLa cell. In cells infected with Vac 7.5-gE plus Vac-gI, the RBCs often formed a continuous mosaic over the surface of the infected HeLa cell, similar to that seen with HSV-1-infected HeLa cells. We also noted that rosettes formed by cells infected with Vac 4b-gE were stronger than those formed by cells infected with Vac 7.5-gE, implying a relationship between the levels of gE expressed and the numbers of Fc receptors.

IgG-binding assays. HeLa cells were infected as described for the previous experiment and assayed for their ability to bind fluorescein-labeled normal rabbit IgG as described in Materials and Methods. The relative geometric mean fluorescence values for each infected-cell population are given in Table 1, and comparisons of the fluorescence distribution of selected populations are shown as smoothed histograms in Fig. 3. The results are consistent with those of the rosetting assays. Although we were unable to detect IgG binding to cells infected with Vac 7.5-gE, coinfection with Vac-gI resulted in significant Fc binding. When gE was expressed under the control of a strong promoter (in cells infected with Vac 4b-gE), IgG binding was observed, but coinfection with

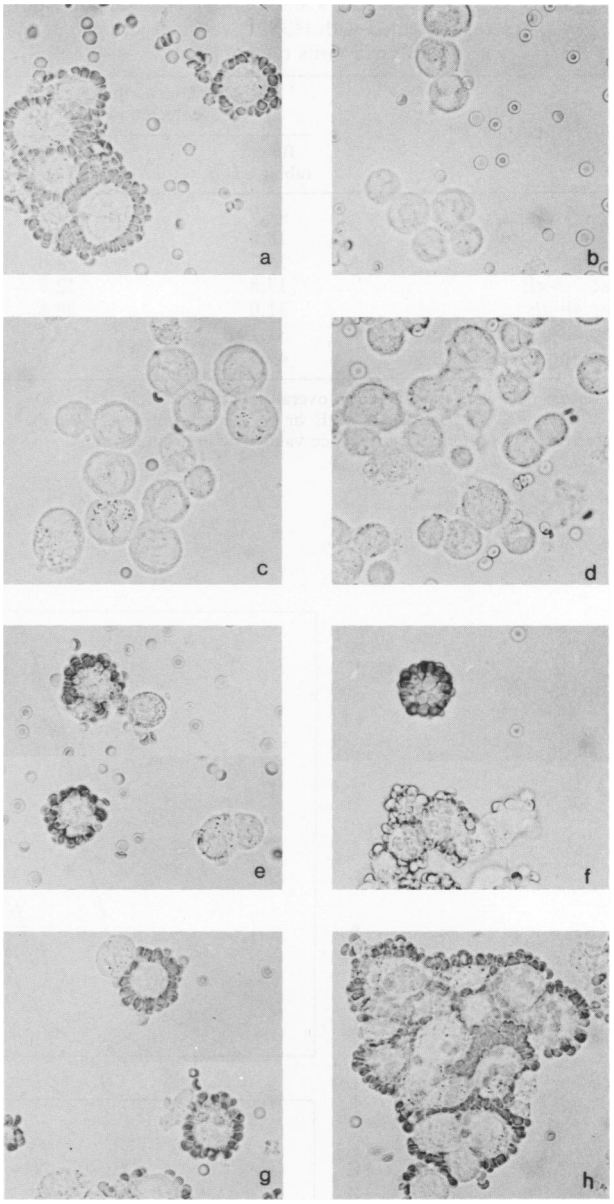

FIG. 2. Fc rosettes formed around HeLa cells infected with HSV-1, vaccinia virus, or recombinant vaccinia viruses. Cells were infected at an overall MOI of 10, harvested 14 h after infection, and mixed with IgG-sensitized ox RBCs as described in Materials and Methods, with the exception of the results shown in panel b, in which unsensitized RBCs were used. The figure shows selected but representative cells. Virus infections were with HSV-1 (a and b), vaccinia virus (c), Vac-gI (d), Vac 7.5-gE (e), Vac 4b-gE (f), Vac 7.5-gE plus Vac-gI (g), and Vac 4b-gE plus Vac-gI (h).

Vac-gI resulted in increased binding at mean levels slightly higher than those observed with HSV-1-infected cells. Expression of gI alone gave no increase in binding compared with the level observed in vaccinia virus-infected cells. It should be noted that these cell populations were far from homogeneous because of the Poissonian distribution of viruses among cells. In particular, when cells are doubly infected with recombinants expressing gE and gI, different cells receive different doses of each virus and are likely therefore to express different levels of each glycoprotein.

TABLE 1. Surface gE levels and IgG-binding characteristics of cells infected with HSV-1, vaccinia virus, or vaccinia virus recombinants[a]

Infection	Geometric mean fluorescence detected by:	
	Bound rabbit IgG	Bound anti-gE antibody
HSV-1	44.9	97.3
Vaccinia virus	18.0	3.6
Vac-gI	18.2	4.8
Vac 7.5-gE	17.8	12.7
Vac 4b-gE	31.0	38.8
Vac 7.5-gE + Vac-gI	31.1	7.1
Vac 4b-gE + Vac-gI	49.5	19.0

[a] HeLa cells were infected at an overall multiplicity of 10, harvested 14 h after infection, and assayed for gE or for Fc receptors as described in Materials and Methods. Fluorescence values are in arbitrary units.

This may account for the fact that the cell population doubly infected with Vac 4b-gE and Vac-gI exhibited a broader fluorescence distribution than the population infected with HSV-1, in which the gE-to-gI ratio in each cell was presumably constant. This problem could be overcome by using very high MOIs, but under these conditions, cytolysis of infected cells caused technical problems during the manipulations. While the fluorescence distributions should therefore be interpreted conservatively, the results of the rosetting and IgG-binding assays taken together show that gI has no binding activity, gE alone has Fc receptor activity, coexpression of gI with gE enhances gE-dependent Fc binding, and coexpression of gI and gE results in Fc-binding activity comparable with that found on HSV-infected cells.

Levels of expression of gE and gI on recombinant-infected cells. Since gE alone had demonstrable Fc receptor activity, it was conceivable that gI plays no direct role in Fc binding but by complexing with gE might increase the efficiency with which gE is processed to the cell surface. It was therefore important to measure the relative amounts of gE expressed on the cell surface in the different cell populations used in the

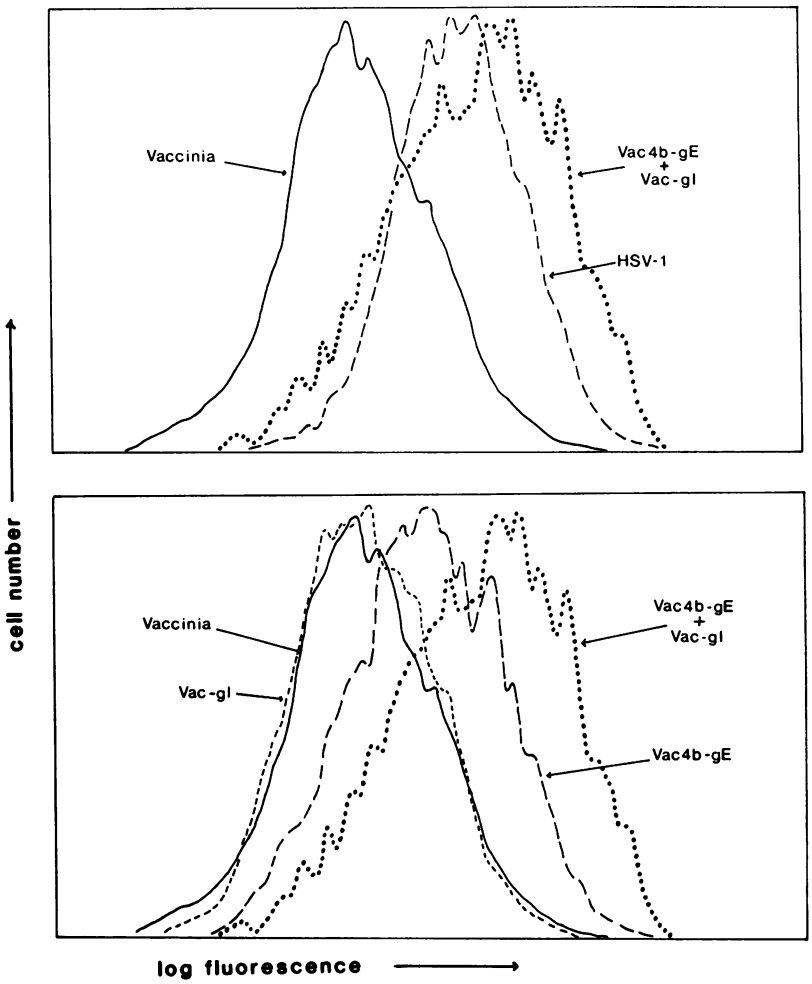

FIG. 3. Binding of IgG to infected HeLa cells. Cells were infected with HSV-1, vaccinia virus, or recombinant vaccinia viruses as described for Fig. 2 and reacted with fluorescein-labeled rabbit IgG as described in Materials and Methods. A minimum of 10^4 cells was analyzed by flow cytometry, and populations of single cells, selected by scatter characteristics, were analyzed for fluorescence signals. The data are presented as smoothed frequency histograms in which the histograms are normalized to identical peak values to allow convenient comparison of the different distributions. The geometric mean fluorescence values of each of these populations are given in Table 1 and are calculated from the raw data.

TABLE 2. Surface gE and gI levels in cells infected with HSV-1, vaccinia virus, and recombinant vaccinia viruses[a]

Infection	Geometric mean fluorescence detected by:	
	Anti-gE antibody	Anti-gI antibody
HSV-1	43.8	29.7
Vaccinia virus	2.9	3.0
Vac 7.5-gE	16.0	3.9
Vac-gI	3.5	43.0
Vac 7.5-gE + Vac-gI	12.5	10.7

[a] HeLa cells were infected at an overall MOI of 10 with HSV-1, vaccinia virus, or recombinant vaccinia viruses and harvested 14 h after infection. Samples of each cell population were reacted with monoclonal antibodies to gE or gI, and bound antibody was detected with fluorescein-labeled rabbit anti-mouse immunoglobulin. Surface fluorescence was determined by flow cytometry. Units are arbitrary.

Fc-binding assays. Infected cells were prepared as described in the previous two sections and were reacted with monoclonal antibodies to gE or gI. After the Fc receptors were blocked with normal rabbit serum, the bound monoclonal antibody was detected with fluorescein-labeled anti-mouse immunoglobulin and cells were analyzed by cytofluorometry. The geometric mean fluorescence values are given in Table 1. As predicted from the data in Fig. 1 and from our knowledge of the vaccinia virus promoters used, cells infected with Vac 4b-gE expressed more surface gE than cells infected with Vac 7.5-gE. However, neither recombinant produced levels of gE as high as HSV-1-infected cells. The effect of coinfection with Vac-gI was to decrease surface expression of gE as detected by antibody 3114. The simplest explanation is that the gE-gI complex is processed and transported to the cell surface more slowly than gE alone is, but it is also possible that the conformation of gE is altered in the complex such that antibody 3114 reacts with lower affinity. Table 2 shows the results of a further experiment in which both gE and gI were measured. It is notable that cells infected with Vac-gI alone expressed more gI than HSV-1-infected cells, despite the fact that in this recombinant, gI is expressed under the control of the relatively weak 7.5K promoter (22). Coexpression of gE and gI decreased surface expression of both glycoproteins compared with expression of either alone, and although it is apparent from comparison of Tables 1 and 2 that the relative levels in different cell populations are not highly reproducible, it appears that the complex is processed more slowly than either glycoprotein alone. What is certainly clear from these results is that we cannot account for the effect of gI in enhancing Fc-binding activity simply in terms of increased gE expression. gI must therefore play some direct role in Fc binding.

DISCUSSION

We have used recombinant vaccinia viruses to express HSV-1 gI and gE either alone or in combination on the surface of HeLa cells and to investigate the nature of the HSV-1 Fc receptor. The strength of this approach is that it provides positive rather than negative evidence for function. The weakness is that it is impossible to mimic the glycoprotein levels, both relative and absolute, found on HSV-1-infected cells. Thus, we were never able to achieve levels of gE that corresponded to HSV-1 infection, despite using different promoters to drive the gE gene in vaccinia virus recombinants, and it is therefore dangerous to draw parallels too closely between HSV-1-infected cells and cells infected with combinations of recombinant vaccinia viruses.

Johnson and Feenstra (8) and Johnson et al. (9) reported that gI and gE formed a complex in HSV-1-infected cells, that both glycoproteins were precipitated by IgG, and that both glycoproteins were required for Fc receptor formation. Our results support the view that both gI and gE contribute to Fc binding, and since coexpression of gE and gI resulted in Fc binding comparable to that exhibited by HSV-1-infected cells, it is most unlikely that any other HSV-specific protein is involved. However, we found that gE expressed alone acted as an Fc receptor but that gI enhanced this activity. Since coexpression of gI did not increase the level of surface gE (indeed, it decreased it) the gE-gI complex must form a receptor of higher affinity than gE alone. It is impossible to say whether this increased affinity was due to the interaction of both gE and gI with IgG (Fc). We were unable to detect Fc binding by gI alone, but this does not exclude a very low affinity interaction. The alternative explanation is that interaction of gI with gE modified the conformation of the latter to increase receptor affinity, and this might be addressed by examining the behavior of a collection of gE-specific monoclonal antibodies against gE expressed in the presence or absence of gI.

Most Fc receptors of both high and low affinity identified in mammalian cells are composed of single polypeptide chains (10). The high-affinity receptor for IgE (FcεR1) is an exception, being composed of a tetrameric complex of three subunits (3), but only one of the subunits is responsible for Fc binding. Why does HSV employ two proteins to achieve a function that is adequately performed by a single polypeptide in mammalian cells? Johnson et al. (9) noted that while most of the gI in HSV-1-infected cells could be precipitated as a complex with gE, a large fraction of the gE was not complexed. The obvious conclusion is that HSV-1-infected cells bear two types of Fc receptor, perhaps with different functions: one composed of gE alone and a second, of higher affinity, composed of a complex of gE and gI.

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