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Immunoelectron Microscopic Localization and Analysis of *Herpes simplex* Virus Type 1 Antigens•

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Abstract Antigens of *Herpes simplex* virus type 1 (HSV-1) strain F were immunoblotted to identify the most immunodominant one, and the localization of this antigen was then studied using immunoelectron microscopy. The 67.8 kDa antigen appeared to be the most immunodominant one in a mouse model, and it showed randomly scattered and partially clustered distribution on the surface of the virion. The localization study was performed using immunogold with polyclonal anti-HSV-1 sera produced from BALB/c mice, and immunofluorescence demonstrated that the viral products in the HSV-1 infected Vero cells were distributed throughout the infected host cell, however, mainly on the surface of the host membrane.

Key words: *Herpes simplex* virus type 1, immunodominant antigen, localization, immunogold labeling, immunofluorescence

The family Herpesviridae *is* divided into three subfamilies based on biological properties: *Alpha-*, *Beta-*, and *Gammaherpesviridae* [30]. *Herpes simplex* virus type 1 (HSV-1) is a member of *Alphaherpesviridae*, whose viruses typically have a broad host range and a short replicative cycle, are highly cytotoxic to cultured cells, and can establish latent infections in the cells of the nervous system of the natural host [31].

Early studies on purified HSV-1 virions suggested that they contain more than 30 proteins, designated as virion polypeptides (VP), and were given serial numbers [13, 33]. Eleven glycoproteins were designated as gB (VP7 and VP8.5), gC (VP8), gD (VP17 and VP18), gE (VP12.3 and VP12.6), gG, gH, gI, gJ, gK, gL, and gM [13, 33]. The biologic properties of some of these glycoproteins have already been identified. In most cases, five viral envelope glycoproteins (gB, gC, gD, gH, and gL) mediate the virus binding to cells and the entry [21, 40]. The initial interaction of the virus with cells is the binding of gC, and also gB in some cases, to cell surface glycosaminoglycans (GAGs), preferentially heparan sulfate [40]. gB is required for infectivity and elicits a potent neutralizing antibody in HSV infection [6]. The gC is not essential for virus production in a cell culture [14], yet serves multiple accessory functions including a role in the virus attachment to cells upon infection via heparan sulphate moieties on the cell surface membrane and the ability to bind the C3b component of a complement [9]. Since gD can bind to any one of several specific cell-surface receptors [18, 40], gD is likely related to viral infectivity and is the most potent inducer of neutralizing antibodies [11, 25]. In addition, the expression of gD in transfected cells is sufficient to render the cells resistant to HSV infection, which has led to a proposal that the presence of gD in membranes of infected cells may prevent the super-infection of the cell by escaping progeny virions [32]. Although gC is dispensable for the infection of many cultured cells, gB, gD, gH, and gL are required for mediating the fusion between the virion envelope and the cell membrane that allows the viral penetration [3, 4, 22]. gE is an HSV-induced receptor for the Fc component of immunoglobulin G [1]. gG provides antigenic specificity to HSV and, therefore, results in an antibody response that allows for the distinction between HSV-1 (gG-1) and HSV-2 (gG-2) [38]. gI has biologic properties that are thought to be involved with gE at the Fc receptor [23].

HSV glycoproteins have also been implicated as the major target antigens on the surfaces of infected cells for both humoral and cellular HSV-specific immune responses [2, 10, 26]. A requirement for the immune response to be effective in limiting HSV multiplication and dissemination during primary infection is the ability to recognize and eliminate HSV-infected cells before the production of significant levels of infectious viral progeny [16]. For this reason, the kinetics of the appearance of glycoproteins on

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the surfaces of infected cells has been investigated; by sensitive flow cytometric techniques [16], the oligomerization of HSV glycoproteins by chemical cross-linkers [12], and HSV surface glycoproteins by an electron microscope to reveal their morphological properties [24, 34, 35].

In this study, attention was focused mainly on the localization and analysis of putative immunodominant HSV-1 surface antigens to ascertain the most immunodominant antigens of HSV-1 in a mouse model and the distribution of these antigens in a virus particle. The results obtained in this study will be used in designing neutralizing antibodies and constructing vaccines for HSV-1.

MATERIALS AND METHODS

Cells and Cell Culture

African green monkey kidney cells (Vero, ATCC CCl 81) were obtained from the Korean Type Culture Collection (KTCC). The cells were grown at 37°C in an Eagle's minimum essential medium (EMEM) supplemented with 5% fetal bovine serum (FBS), 100 units of penicillin G sodium per ml, 100 μg of streptomycin sulfate per ml, 2.5 μg of amphotericin B per ml, and 0.2% sodium bicarbonate.

Viruses and Virus Assay

The *Herpes simplex* virus type 1 (HSV-1) strain F (ATCC VR-733, Rockville, MD, U.S.A.) [8] was obtained from the Korean National Institute of Health (KNIH). Virus stocks were prepared in Vero cells in EMEM supplemented with 2% FBS [34] and tittered in Vero cells by a $TCID_{50}$ [28]. All virus stocks were stored at -70°C until use.

Preparation of Antisera

Six-week-old female BALB/c inbred mice were immunized intraperitoneally with a mixture of 250 μl of the virus suspension and the same amount of complete Freund's adjuvant (GIBCO/BRL, U.S.A.). Two booster immunizations using incomplete Freund's adjuvant (GIBCO/BRL, U.S.A.) were performed at two week intervals with the same dosages. Two weeks after the final boosting, the mice were sacrificed and approximately 700 μl of blood was directly collected from the heart and incubated at 37°C for 1 h. The tube was flicked several times to dislodge the blood clot, transferred to 4°C for overnight, and then spun at 9,000 ×g for 20 min at 4°C. The supernatant was recentrifuged at 9,000 ×g for 10 min to remove the remaining cell pellet, and then sodium azide was added to 0.02%. The prepared polyclonal antisera were stored at -20°C until use.

Immunofluorescence Microscopy

Immunofluorescence microscopy for the detection of the expressed HSV-1 proteins in the Vero cells was carried out as described previously [20]. The Vero cells were diluted to 5×10^5 cells per ml in a complete tissue culture medium of 2% FBS, and then mixed with 200 μl of the virus suspension to give a multiplicity of infection (M.O.I.) of 10 PFU/cell. Twenty μl of the mixture was placed in each well of a sterile 12-well multiwell slide (Flow Laboratory Inc., U.S.A.). The slide was placed in a sterile Petri dish to avoid drying and incubated at 37°C for 24 h in a humidified CO_2 incubator. After incubation, the cells were briefly rinsed with PBS, and then fixed by immersing the slide in acetone in a glass Petri dish for 10 min at -20°C. The slide was gently washed with PBS three times, then an incubation buffer (0.5% bovine serum albumin, 0.1% gelatin, 20 mM sodium azide in PBS) was applied to each well and the slide was incubated for 15 min at room temperature. The Primary antisera were diluted 1:20 (v/v) in the incubation buffer and allowed to react for 30 min with fixed cells at room temperature. The cells were washed with the incubation buffer three times for 15 min each to wash off any unbound antibodies, and then a dilution of 1:100 (v/v) fluorescein isothiocyanate-conjugated goat IgG+IgA+IgM anti-mouse immunoglobulins (CAPPEL, U.S.A.) in the incubation buffer was allowed to react for 20 min at room temperature. Finally, the samples were washed three times for 15 min with PBS and an adequate amount of a mounting buffer (glycerol:PBS=1:5) was placed throughout the slide which was then covered with a coverslip while trying not to trap any air bubbles between the coverslip and the slide. The samples were examined under an Axioplan-2 Universal Microscope (Carl Zeiss, Germany).

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 12% polyacrylamide gel under denaturing conditions at constant 20 mA, according to the previously described method [19]. The Vero cells were infected with HSV-1 as mentioned above, and once the cytopathic effect (CPE) stopped progressing, the cells were freeze-thawed three times and harvested by centrifuging at 1,400 ×g for 20 min. The pellet was resuspended in 16 μl of sterile water and then mixed with 4 μl of 5× electrophoresis sample buffer [final concentration=60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% (SDS), 0.1% bromophenol blue, and 14.4 mM β-mercaptoethanol]. The samples prepared in this way were co-electrophoresed with intact Vero cell extracts to distinguish the HSV-1 proteins from those of the Vero cells. For the molecular weight determinations, broad-range standard molecular markers (Bio-Rad, U.S.A.) were used: rabbit skeletal myosin (200 kDa), β-galactosidase of *E. coli* (116 kDa), phosphorylase B of rabbit muscle (97.4 kDa), bovine serum albumin (66 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), hen egg white lysozyme (14.5 kDa), and bovine pancreas aprotinin (6.5 kDa). After

electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Co., U.S.A.), and then the apparent molecular weights of the HSV-1 proteins were calculated from their electrophoretic mobility relative to those of the standard proteins with known molecular weights.

Western Blotting

Western blotting was carried out by a previously described technique [36]. After SDS-PAGE, the proteins were electrophoretically transferred to a nitrocellulose membrane, after which nonspecific binding was blocked using 1% (w/v) skimmed milk in Tris-Buffered Saline (TBS; final concentration of 2 M Tris-HCl, pH 7.5, was diluted to 10 mM and 4 M NaCl to 150 mM, respectively). The primary antibody incubations were carried out in the same solution at a dilution of 1:200 for 2 h at 37°C. After washing in TBS three times, a nitrocellulose membrane was incubated with HRP (horseradish peroxidase)-conjugated secondary antiserum (Sigma Chemical Co., U.S.A.) at a dilution of 1:2,000 for 2 h at 37°C, and then the blots were developed using 4-chloro-1-naphtol (Sigma Chemical Co., USA) after washing in TBS a further three times.

Preparation of Virus Samples for Electron Microscopy

For the negative staining of unlabeled HSVs, the viruses were concentrated by precipitation with polyethylene glycol 8000 (PEG 8000; Yakuri Pure Chemical Co. Ltd., Japan). Briefly, 50% PEG (w/v) in a TNE buffer (0.15 M NaCl, 0.05 M Tris-HCl, 0.001 M EDTA) was first prepared and then added while stirring to the virus-containing cell culture supernatant to a final concentration of 10%. The mixture was stirred slowly for 90 min at 4°C, centrifuged at 1,400 ×g for 15 min, and then the supernatant was discarded. The pellet was resuspended in a TNE buffer at a fiftieth of the original volume of the supernatant, and then stored either at -70°C until use or used immediately [37]. For immunogold labeling, the viruses were concentrated simply by ultracentrifuging at 20,000 rpm for 2 h at 4°C in a Beckman SW28.1 rotor.

Negative Staining and Electron Microscopy of Unlabeled HSV-1

For the negative staining of the specimen for transmission electron microscopy, the virus preparations were adsorbed onto the support film using the drop-to-drop method [27]. A drop of the virus sample concentrated through precipitation with PEG 8000 was placed on a Parafilm surface, and a Formvar-carbon coated 300-mesh nickle grid (Electron Microscopy Sciences, U.S.A.) was floated with the film facing side down on the drop for 15 min to allow the virus to adsorb. The excess sample was removed by touching the edge of the grid with torn filter paper. The grid was then transferred to a drop of 2% uranyl acetate for 1 min. The excess stain was removed by touching the edge of the grid with torn filter paper. After air drying, the grid was examined under a JEOL JEM-1200EX II transmission electron microscope (JEOL, Japan).

Immunogold Labeling

The virus sample was concentrated to about 10^4 to 10^6 particles per ml by centrifuging at 20,000 rpm for 2 h at 4°C in a Beckman SW28.1 rotor. The primary antiserum was pretreated by heating at about 55°C for 30 min, and then centrifuged at 12,000 rpm at 4°C for 1 h to avoid any possible nonspecific reactions due to the presence of a complement or from the presence of proteases that could have damaged the particles [5]. The immunogold labeling was performed using goat anti-mouse IgG+IgM complexed with 10 nm gold particles (Electron Microscopy Sciences, U.S.A.). This gold-conjugated secondary antibody was diluted 20 to 100-fold with an incubation buffer (0.5% bovine serum albumin, 0.1% gelatin, 20 mM sodium azide in PBS). The virus sample was adsorbed to a grid surface for 15 min and the excess sample was removed. Then, the grid was floated on a drop of the primary antiserum diluted 10 to 80-fold with the incubation buffer for 1 h at room temperature in a sterile Petri dish to avoid drying. After incubation, the grid was washed by floating on 6 consecutive drops of the incubation buffer for 5 min. The grid was then floated on a drop of the 20 to 100-fold diluted gold-conjugated secondary antibody, and washed by floating on 6 consecutive drops of the incubation buffer, and 2 drops of distilled water for 5 min [5, 17]. The decorated grid was negatively stained with 2% uranyl acetate as described above, air dried, and finally examined under a JEOL JEM-1200EX II transmission electron microscope (JEOL, Japan).

RESULTS AND DISCUSSION

SDS-PAGE Analysis of HSV-1 Antigens

The HSV-1 antigens as distinguished from the Vero cell antigens appeared as 149, 115.8, 85.9, 67.8, 56.8, 47, 39.3, 31.6, and 24 kDa bands (Fig. 1). According to SDS-PAGE, the apparent molecular weights of fully matured glycoproteins and of their partially glycosylated precursors have been previously reported [1]. In summary, the apparent molecular weights of the mature forms of gB, gC, gD, and gE are 120, 115-130, 59-65, and 80 kDa, and their precursors are 110, 86-105, 51, and 66 kDa, respectively. Based on the reference previously reported, the 115.8 kDa antigen would appear to be a mature form of gB or gC and the 85.9 kDa antigen a precursor of gC. The 67.8 kDa and 56.8 kDa antigens would appear to be the mature form of gD, yet the 67.8 kDa antigen would also appear to be a precursor of gE.

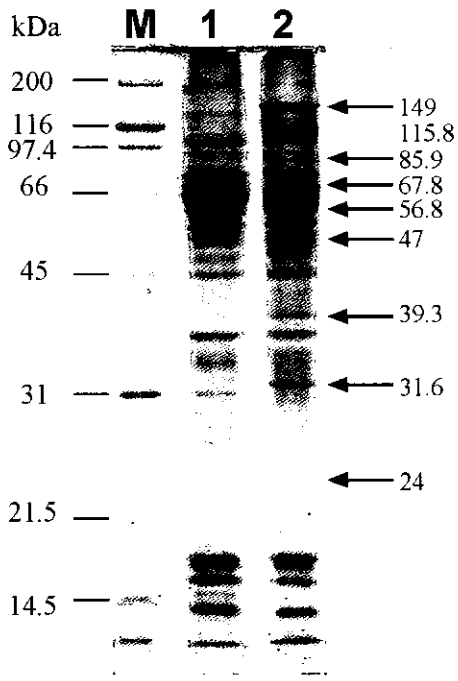

Fig. 1. SDS-PAGE analysis of proteins produced by Vero cells infected with HSV-1.
Coomassie Brilliant Blue R-250 stained 12% SDS-acrylamide gel slab showing the electrophoretically separated HSV-1 infected Vero cell antigens and intact Vero cell antigens. Lane M, standard molecular markers; Lane 1, intact Vero cell antigens; Lane 2, HSV-1 infected Vero cell antigens of 78 h post-infection. Black arrows indicate major bands of HSV-1 antigens.

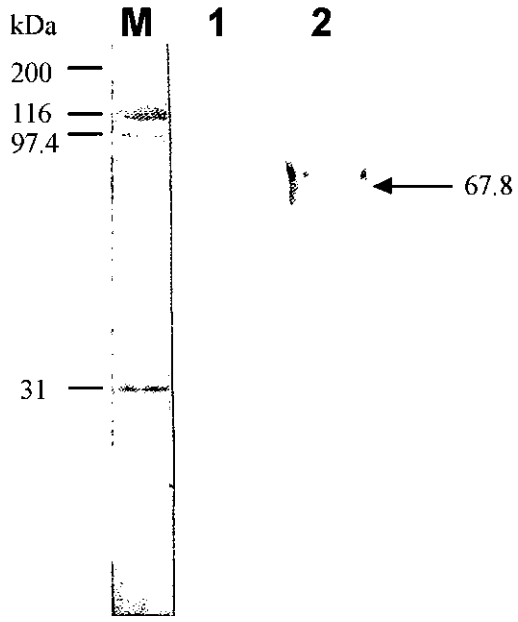

Fig. 2. Western blotting analysis of HSV-1 infected Vero cell antigens.
After SDS-PAGE, antigens were electrophoretically transferred to nitrocellulose membrane, reacted with polyclonal HSV-1 antiserum, and then reacted with goat anti-mouse IgG conjugated with horse-radish peroxidase. Lane M, standard molecular markers; Lane 1, intact Vero cell antigens; Lane 2, HSV-1 infected Vero cell antigens. The black arrow indicates the most immunodominant antigen of HSV-1.

Western Blot Analysis of HSV-1 Antigens Using Mouse Derived Antisera

The result in Fig. 2 demonstrates that the 67.8 kDa band appears to be the most immunodominant antigen of HSV-1 in a mouse model. Isolated gD-1 and gD-2 also induce the production of neutralizing antibodies [7]. A comparison of three different HSV-1 glycoproteins as immunogens (gB-1, gC-1, and gD-1) revealed that gD-1 induced the highest titer of neutralizing antibodies [39]. In contrast, monoclonal antibodies specific for gB, gC, and gD have been reported to exhibit a neutralizing activity, and in the absence of a complement, the highest activities have generally been associated with the anti-gD monoclonal antibodies, where quantitative comparisons of neutralizing activity have been presented [15, 29]. Accordingly, in agreement with these studies, it was concluded that the immunodominant 67.8 kDa band was a gD band.

Immunofluorescence Microscopy of HSV-1 Infected Vero Cells

The constructed mouse anti-HSV-1 antisera were examined by indirect membrane immunofluorescence to determine their ability to recognize and react with antigens located on the plasma membrane of the Vero cells infected with HSV-1. As shown in Fig. 3, the periphery of clumped HSV-1 infected Vero cells was labeled well with fluorescein. However, it was not possible to determine whether the labeled viral antigens were located only on the membrane or in the cytoplasm also.

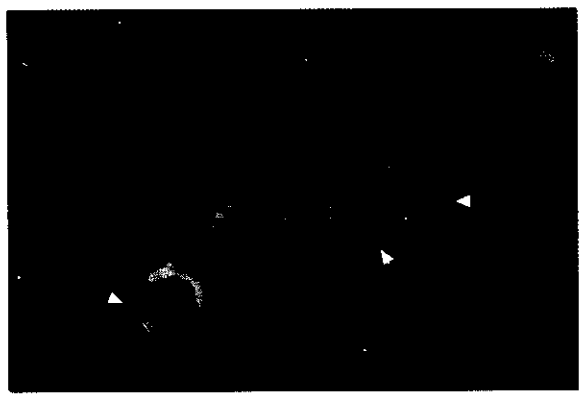

Fig. 3. Detection of HSV-1 virion-associated antigens on Vero cells with immunofluorescence staining.
5×10^5 cells/ml of Vero cells were mixed with HSV-1 at m.o.i. of 10 pfu/cell, and then placed on a 12-well multiwell slide and incubated for 24 h. Infected cells were fixed with cold acetone, and then immunostained with mouse polyclonal HSV-1 antiserum and fluorescein isothiocyanate-conjugated goat IgG+IgA+IgM anti-mouse immunoglobulins. White arrowheads indicate the strong immunofluorescence on the periphery of the Vero cell membrane (×400).

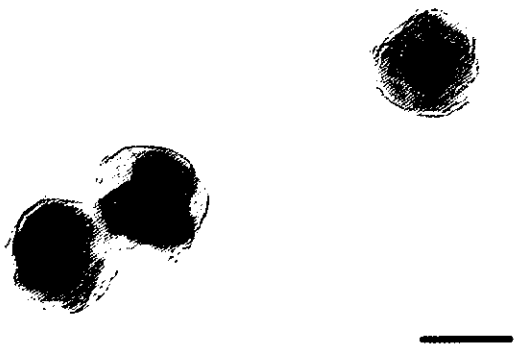

Fig. 4. Transmission Electron Microscopy (TEM) of negatively stained HSV-1 virions.
Double membranes enclose amorphous tegument and capsid. Fused virions shared their envelopes and teguments. Samples were prepared by PEG precipitation, mounted on a Formvar-carbon-coated nickel grid through the drop-to-drop method, and then negatively stained with 2% uranyl acetate. The bar represents 100 nm.

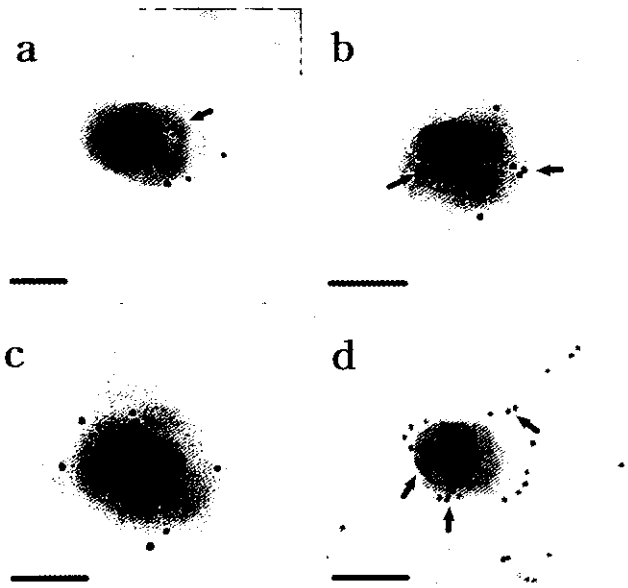

Fig. 5. Detection of immunodominant antigens on HSV-1 virions by immunogold labeling.
Immunodominant antigens on HSV-1 virions were labeled with mouse derived anti-HSV-1 polyclonal antibodies which were subsequently labeled with anti-mouse IgG+IgM complexed with 10 nm gold particles. Primary antibodies were diluted 80-fold and secondary antibodies were diluted 100-fold with incubation buffer (panels a to c). For Fig. 5d, primary antibodies were diluted 20-fold and secondary antibodies were diluted 50-fold. Gold particles appear to bind to the surface, ascertaining that the immunodominant antigens are localized on the surface of HSV-1. Black arrows indicate the clustered antigens of HSV-1. Bar markers represent 100 nm.

Negative Staining and Electron Microscopy of Unlabeled HSV-1

The virus particles recovered from the culture fluid of the Vero cells which had been infected with HSV-1 were shown by electron microscopy to possess the well-known morphology of herpes viruses (Fig. 4). In the samples prepared by the PEG precipitation technique, virions were clearly observed with little background and notably fused particles. Double layered envelopes were distinguishable from some protrusions, yet spikes were hard to distinguish. The teguments, the structure between the capsid and the envelope, had no distinctive feature and varied in amount, and ruptured envelopes made it possible to observe the enclosed nucleocapsid.

Immunogold Labeling of HSV-1 Surface Antigens

The incubation of HSV-1 with mouse derived polyclonal anti-HSV-1 antisera and subsequent incubation with anti-mouse IgG+IgM complexed with 10 nm gold particles showed a distribution pattern of putative immunodominant antigens on viral particles. The primary antisera were diluted from 20 to 80-fold to find out how the immunodominant antigens were specifically distributed on the virions. Generally, the gold particles were randomly distributed, however, some gold particles were clustered on protruding portions (Figs. 5a, 5b) and some showed sparse distribution (Fig. 5c). The perimeters of the envelopes and presence of spikes were distinguishable with the array of gold particles (Fig. 5d). Elegant electron microscopic studies to characterize the features of major HSV-1 surface glycoproteins have been previously described [34, 35]. Some gB spikes are invariably clustered in virion envelope protrusions or "buds", whereas the structures containing gC and gD are randomly distributed, and the gD envelope spikes are short and not clustered [34, 35]. Although it was impossible to confirm that all immunogolds were labeled on one glycoprotein specimen, when polyclonal antisera were applied, according to the result of the Western blot analysis (Fig. 2), most of the labeled immunogolds appeared to be gD. As such, it would also appear that gD is distributed in a clustered form, just as in the distribution pattern of gB.

Based on SDS-PAGE and Western blotting analyses, this study demonstrated that gD elicits a strong humoral immune response in an HSV-1 infected mouse and gD would appear to be distributed in a clustered form. The importance of this study is the confirmation of gD as an immunodominant antigen of HSV-1 in a mouse model and the finding of localization using the immunoelectron microscopic technique. Thus, gD would appear to be a great tool for designing a neutralizing antibody and developing vaccines.

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