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Kinetics of Expression of Herpes Simplex Virus Type 1-Specific Glycoprotein Species on the Surfaces of Infected Murine, Simian, and Human Cells: Flow Cytometric Analysis

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The kinetics of expression of the herpes simplex virus type 1-encoded major glycoprotein species gB, gC, gD, and gE on the surfaces of cells of murine, simian, and human origins were studied. Viable cells were stained with monoclonal antibodies specific for each species, and the levels expressed were determined by fluorescence flow cytometry. Differences were observed in both the kinetics and the levels of expression of individual glycoprotein species, depending upon the origin of the host cells. Glycoprotein gC was expressed early and at high levels in cells of murine and human origins, but late and at relatively low levels in simian cells. In contrast, gE was expressed at high levels in simian cells, but was not detectable until late in the infectious cycle in murine and human cells. The kinetics and levels of expression of gB were similar for all cells investigated, whereas gD, with high levels of expression in all cells late in infection, appeared on the surfaces of murine cells very early postinfection. This approach has allowed a simple quantitative method for comparing levels of glycoprotein expression.

There are two serologically and genotypically distinct types of herpes simplex virus (HSV), designated HSV types 1 and 2 (HSV-1 and HSV-2) (44). Each specifies four major, well-characterized glycoprotein species, designated gB, gC, gD, and gE (42). The genes of these species are colinear in the two genomes, with the coding regions for gB and gC found within the long unique (U_L) component of the prototype HSV genome, while the coding regions for gD and gE are found within the short unique (U_S) component (45). In addition, HSV-2 encodes a glycoprotein species designated gG-2 (25, 38), also encoded within U_S, which appeared initially to have no counterpart within the HSV-1 genome; however, a minor species whose coding region is colinear to gG-2 has recently been identified in HSV-1, and has tentatively been designated as gG-1 (2, 37). Another species, designated gH-1, encoded within U_L (5, 41), has no identified counterpart in HSV-2 at this time. Recently, the entire U_S component of HSV-1 has been sequenced (26), and was shown to contain five open reading frames in addition to the identified and mapped glycoprotein genes, with the characteristics typical of genes encoding glycoprotein species. However, it is not clear whether these open reading frames are transcribed and translated. Nevertheless, it is likely that both HSV-1 and HSV-2 have the potential to encode many more glycoprotein species, albeit only minor species, than those already identified.

The genes of HSV are transcribed and translated in a tightly regulated and orderly manner. The genes are characterized as immediate early (α), early (β), and late (γ) (16, 17). The genes for the major glycoprotein species are generally characterized as γ genes, although gB, gD, and gE appear to be more intermediate between early and late gene translation, designated γ_1 (reviewed in reference 1) or $\beta\gamma$ genes (45). The gene for gC is the only true late (γ_2) gene among the major glycoprotein species. Studies of the maturation of the glycoprotein molecules following translation have identified a series of complex steps, involving multistep glycosylation, acylation of some glycoprotein species occurring predominantly within the Golgi apparatus (18, 19), and insertion of the fully glycosylated species into the cell membranes of infected cells (12, 15). Glycoproteins are also an integral component of the envelope of the mature, infectious virion (43). In addition, certain virus-associated functions are related to the individual glycoprotein species. Glycoprotein gB is essential for virion infectivity (22, 39). Also, gB (14, 24) and gD (27) are associated with cell fusion, gC is an HSV-induced receptor for activated complement component 3b (9), and gE is an HSV-induced receptor for the Fc component of immunoglobulin G (4, 31).

The 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 (6, 8, 10, 13, 21, 23, 28–30, 40). A requirement for the immune response to be effective in limiting HSV multiplication and dissemination during the primary infection is to be able to recognize and eliminate HSV-infected cells before the production of significant levels of infectious viral progeny. An important consideration, therefore, is the kinetics of the appearance of glycoproteins on the surfaces of infected cells.

In this study, we investigated in detail the kinetics of the appearance of gB, gC, gD, and gE on the surfaces of HSV-infected cells of murine origin by sensitive flow cytometric techniques. These studies were performed by using four well-characterized, glycoprotein-specific monoclonal antibodies of murine origin. We have also compared the appearance and levels of expression of individual glycoprotein species in cells of murine, simian, and human origin.

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These data therefore provide a comparative analysis of the appearance of the fully glycosylated, surface-associated forms of the HSV glycoproteins throughout the HSV infectious cycle in a variety of susceptible host cells.

MATERIALS AND METHODS

Cells and cell culture. B6/WT-3, a simian virus 40-transformed mouse embryo fibroblast cell line generated and cloned in this laboratory, has been described previously (36). The cell line was maintained by passage in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum (FCS), 100 μg of streptomycin sulfate per ml, 100 U of penicillin per ml, 0.03% glutamine, 0.075% $NaHCO_3$, and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer. Human epidermoid carcinoma no. 2 (HEp-2) and continuous African green monkey kidney (Vero) cells were maintained by passage in Dulbecco modified Eagle medium as described above but with 10% FCS.

Virus and cell infection. HSV-1 strain KOS was originally obtained from P. A. Schaffer, the Dana-Farber Cancer Institute, Boston, Mass. Stocks were grown in HEp-2 cells, stored at −70°C, and titrated for infectivity on Vero cell monolayers. Cell lines were infected by removing the media from confluent 75-cm^2 flasks, and adding 2 ml of virus suspension diluted in Tris-buffered saline to give a final multiplicity of infection of 2.5 to 10. Mock-infected cells were treated with Tris-buffered saline alone. Cultures were incubated at 37°C for 1 h, and the inoculum was removed and replaced with the appropriate growth medium.

Monoclonal antibodies. Monoclonal antibodies specific for the individual glycoprotein species were as follows: gB (I-144-2B), gD (II-436-1) (18), gC (HC-1) (35), and gE (H600) (32). All antibodies were titrated extensively to ensure that they were present in the reaction mixture in saturating amounts.

Immunofluorescence. At various times postinfection (p.i.), target cells were removed from the flasks by mild trypsin treatment (0.5 mg/ml for 1 min) and washed with FACS buffer, consisting of phosphate-buffered saline with 2% (vol/vol) FCS and 0.1% (wt/vol) sodium azide (NaN_3). The cells were washed twice in FACS buffer, resuspended at 2×10^6 cells per ml, and dispensed in round-bottomed 96-well microtiter plates (Costar, Cambridge, Mass.) at 10^6 cells per well. The cells were pelleted by centrifugation, and the supernatant was removed by suction. The pellet was resuspended in 50 μl of the appropriate antibody dilution, and incubated at 4°C for 1 h. Unbound antibody was removed by adding 150 μl of FACS buffer, followed by 40 μl of FCS to each well, followed by centrifugation. The pellet was resuspended in 50 μl of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Becton Dickinson Monoclonal Center, Mountain View, Calif.) for an additional 1 h at 4°C. The cells were washed twice as described above and analyzed immediately, or fixed in 2% (wt/vol) paraformaldehyde (pH 7.4).

Flow cytometry analysis. Samples were analyzed by using an Epics V flow cytometer/sorter (Coulter Electronics, Inc., Hialeah, Fla.) with excitation of fluorescence of 500 mW at 488 nm. The instrument calibration was optically aligned with 10-μm Fluorospheres (grade II) (Coulter) and calibrated with Fluorotrols (Ortho Diagnostic Systems, Inc., Westwood, Mass.). The coefficients of variation were always less than 2.5. Efforts were made to calibrate the instrument to ensure that individual sample runs were performed under identical conditions. Ninety-degree light scatter was collected by using a 488-nm dichroic mirror and a neutral density filter. Green fluorescence was collected by using a 515-nm interference filter, a 560-nm short-pass, and a 515-nm long-pass glass filter. For each sample, 10,000 cells were analyzed at 500 cells per s, collecting the logarithm of integrated green fluorescence gated on forward-angle light scatter and 90° light scatter. Dead cells, usually less than 5%, were effectively removed by using forward-angle light scatter gates, or by excluding red fluorescing cells after adding propidium iodide in samples analyzed immediately. Histograms were analyzed by simple integration and the immuno program for the Easy 88 computer (Coulter). Results were presented as fluorescence profile histograms, with the number of cells on the *y* axis, and the fluorescence intensity as arbitrary units on a 3-decade logarithmic scale on the *x* axis.

RESULTS

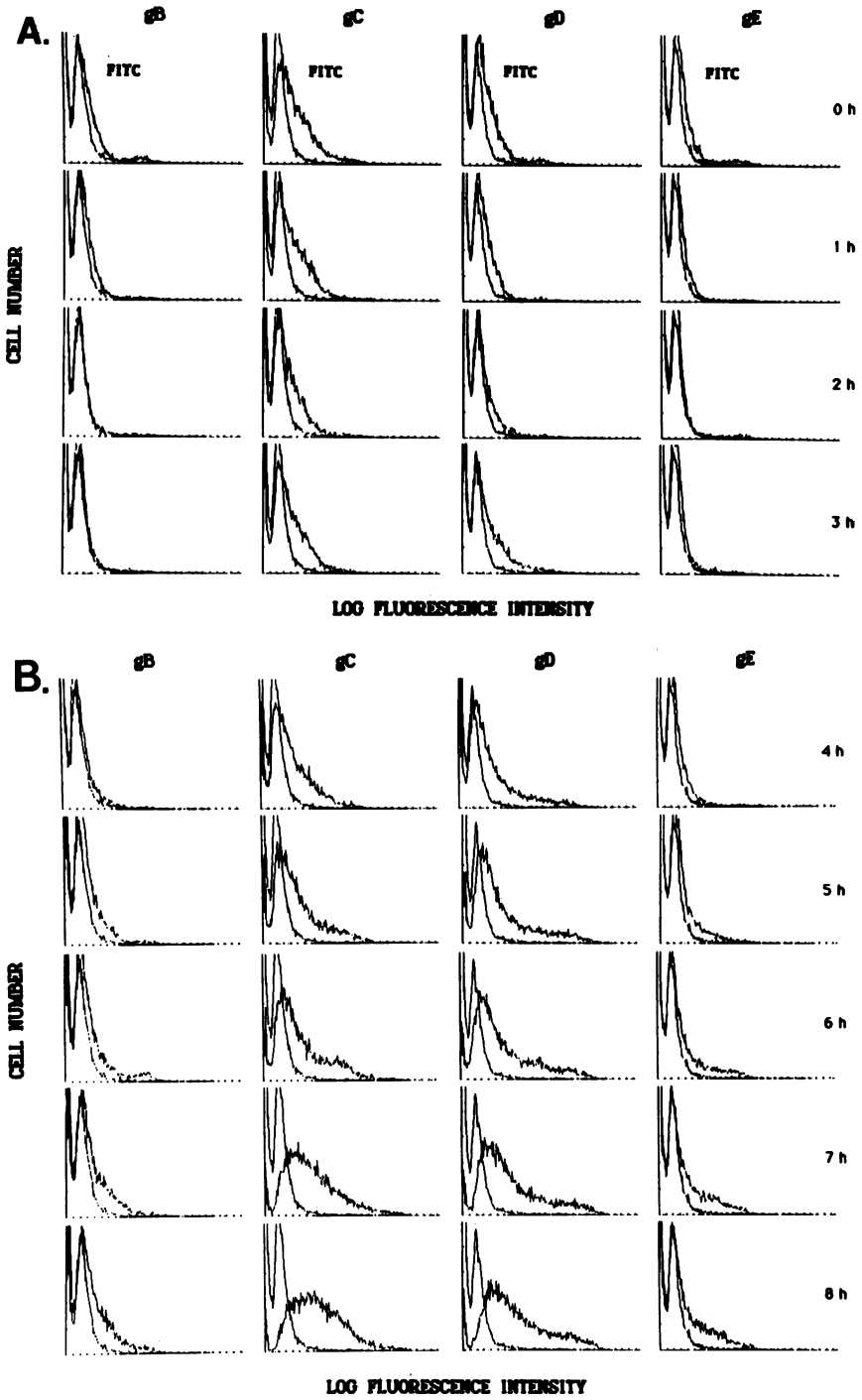
Surface glycoprotein expression on HSV-infected murine cells. The appearance of the major glycoprotein species (gB, gC, gD, and gE) on the surfaces of HSV-1-infected murine (B6/WT-3) cells was monitored (Fig. 1). Fluorescence from infected cells represented only surface staining, as dead cells, which may incorporate fluorescence nonspecifically, were excluded from the analysis. In addition, the staining pattern of live cells was confirmed as surface staining by UV microscopy.

Immediately after the virus absorption period (0 h), low levels of glycoprotein expression, especially gC and gD, were observed (Fig. 1A). This low level was undoubtedly due to glycoproteins derived from input infectious virions adhering to the cell surface. Over the next 3 h, these levels diminished, probably due to viral penetration and the disappearance of input antigen. Differences were evident in the initial expression and disappearance of individual glycoprotein species, as both gB and gE were present at very low levels immediately following absorption, and had decreased to undetectable levels by 2 h p.i., while the expression of gC and gD, while low, was always detectable.

During the period from 4 to 8 h p.i., surface expression of each glycoprotein species increased in the mouse, due to de novo synthesis and transport to the cell membrane (Fig. 1B). Again, the most dramatic increases were observed in the expression of gC and gD, while both gB and gE expression appeared to lag behind. The most interesting feature of this period was the observation that the rate at which serologically detectable gC appeared on the cell surface was apparently identical to that of gD, apparently contradicting the observation that the peak rate of synthesis of the precursor of gD precedes that of gC (3, 7, 20).

Late in infection, glycoprotein expression continued to increase in mouse cells, so that by 12 h p.i., 90 to 95% of infected cells were positive for the expression of each of the individual species (Fig. 1C). However, consistent with earlier time points, the levels of gC and gD were greater than those for gB and gE. Moreover, it was apparent that HSV infection resulted in a marked heterogeneity in surface expression, as indicated by the broad profiles obtained.

Surface glycoprotein expression on HSV-infected simian cells. The characteristics of glycoprotein expression on the surfaces of infected cells of murine origin, in particular the relatively low levels of gB and gE, and the surprisingly early appearance of gC, prompted similar studies in cells of either simian or human origin. The kinetics of appearance of the individual glycoproteins on the surface of simian (Vero) cells are shown in Fig. 2A. As was observed with infected



B6/WT-3 cells, low levels of each of the glycoprotein species were present immediately following virus absorption (0 h), and disappeared over the subsequent 2-h period (Fig. 2A). By 3 h p.i., gB, gD, and gE were again detectable, while gC remained at undetectable levels (Fig. 2B). Between 6 and 12 h p.i., the levels of each species continued to increase. However, gC expression never achieved the levels of other glycoproteins. As observed with virus-infected B6/WT-3 cells, the expression of the individual glycoproteins at the population level was broadly heterogeneous.

Surface glycoprotein expression on HSV-infected human cells. The kinetics of appearance of the glycoproteins on the surface of the human cell line (HEp-2) followed essentially the same pattern as that observed for the other cell lines. Low levels of expression of gB, gC, and gD were observed immediately after infection and decreased slightly over the subsequent 2 h after virus infection (Fig. 3A). However, no expression of gE was detected over this period. In the period from 3 to 12 h p.i., the expression of gB, gC, and gD increased dramatically, reaching peak levels of expression by 6 to 9 h p.i. (Fig. 3B). In contrast, the expression of gE remained low, and did not reach a peak until 12 h p.i. The pattern of its expression suggested that greater levels would be detectable at even later stages in infection. This anoma-

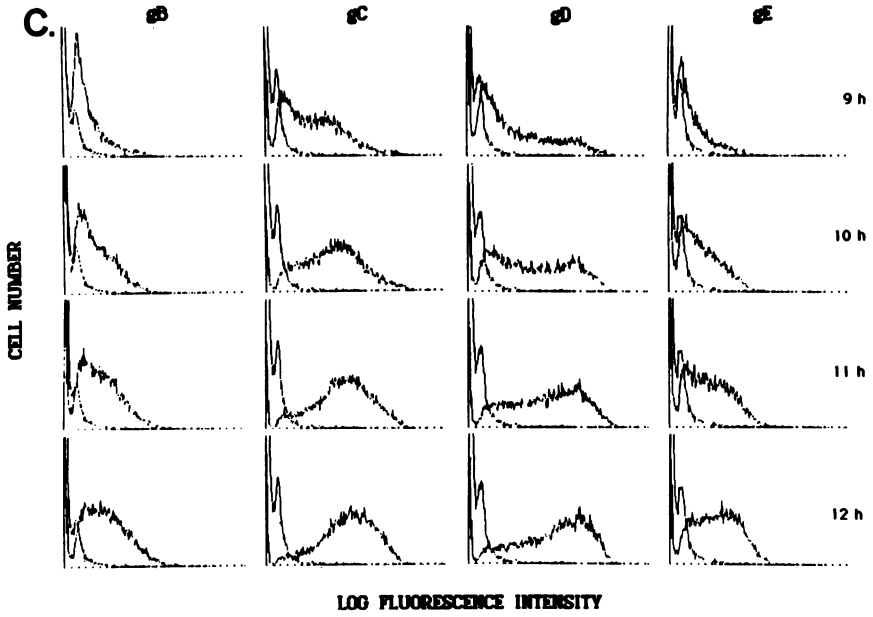


FIG. 1. Surface expression of major HSV-1 glycoprotein species on infected B6/WT-3 (murine) cells: (A) 0 (immediately postabsorption) to 3 h p.i.; (B) 4 to 8 h p.i.; (C) 9 to 12 h p.i. Cells were infected in monolayer cultures, and removed at the indicated time p.i. by mild trypsin treatment. Cells were stained for the surface expression of gB, gC, gD, and gE by using monoclonal antibodies specific for each species (see the text), followed by a second-step, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin antibody for indirect immunofluorescence. Expression was quantitated by fluorescence flow cytometry. Background binding and autofluorescence were determined by staining the cells with the second-step antibody only.

lous low-level expression of gE was not due to a lack of expression of this glycoprotein species by the virus used for infection, as the same lot of HSV-1 was used in these experiments. Further, this observation was made consistently in repeated experiments. This suggested that low levels of gE expression were a characteristic of the interaction of HSV-1 strain KOS and HEp-2 cells under the conditions of these experiments.

Figure 4 graphically summarizes the temporal changes in surface glycoprotein expression for each cell line. The *y* axis represents the change in the mean fluorescence intensity of the HSV-infected cell population relative to background fluorescence intensity of uninfected cells stained with the appropriate glycoprotein-specific monoclonal antibody. The mean fluorescence intensity is a relatively crude measure of surface staining, because it does not take into account cell populations which have a highly heterogeneous staining profile. Although it may not be possible to directly compare different cell lines to each other by using this parameter, the general patterns described above hold, and there are demonstrable differences in the hierarchy of staining patterns for the glycoproteins characteristic for each cell line (Fig. 4).

DISCUSSION

The purpose of this study was to evaluate in detail the kinetics of surface expression of the major HSV-encoded glycoprotein species, specifically gB, gC, gD, and gE, on the surfaces of infected cells of murine, simian, and human origin. Surface expression was determined by the binding of glycoprotein-specific monoclonal antibodies of murine origin to the surfaces of live, HSV-infected cells, and the extent of binding was evaluated by indirect immunofluorescence with a sensitive fluorescence flow cytometric approach.

The sensitivity of this approach was clearly evident when HSV-1 infected cells were evaluated immediately after the absorption period. In all cell lines tested, a small but significant shift of the fluorescence profiles toward the right, indicative of a higher percentage of positive cells, was observed. This indicated that the virion-associated glycoproteins, which presumably become transiently associated with the host cell membrane as the virion attaches and begins penetration, were detectable, albeit at a low level. This transient expression decreased to undetectable levels over the subsequent 2-h period of the infectious cycle. It is unlikely that this low level of detectable expression was due to increased nonspecific attachment of the glycoprotein-specific monoclonal antibodies or the fluorescein isothiocyanate-labeled anti-mouse immunoglobulin antibody to the infected cell membrane, as experiments with an irrelevant first-step antibody failed to produce similar results. Therefore, we would conclude that the early expression was indicative of the low levels of input, virion-associated glycoproteins. This interpretation would agree with that of Shore et al. (40), who showed that input viral antigen expressed on the surfaces of infected cells early in infection provided target structures for early target cell damage by antibody-dependent cellular cytotoxicity effector mechanisms.

A number of points can be made from this study. First, for a particular cell line infected with HSV, there were clear differences in the kinetics of appearance and the levels of expression of the different glycoprotein species. Second, clear differences in the kinetics and levels of expression of individual glycoprotein species were observed when different cell lines were compared. However, despite these differences, the rate at which the glycoproteins appeared on the surfaces of the cell lines of different species origin was essentially the same. This suggested that the mechanisms of HSV glycoprotein gene regulation and the processing events

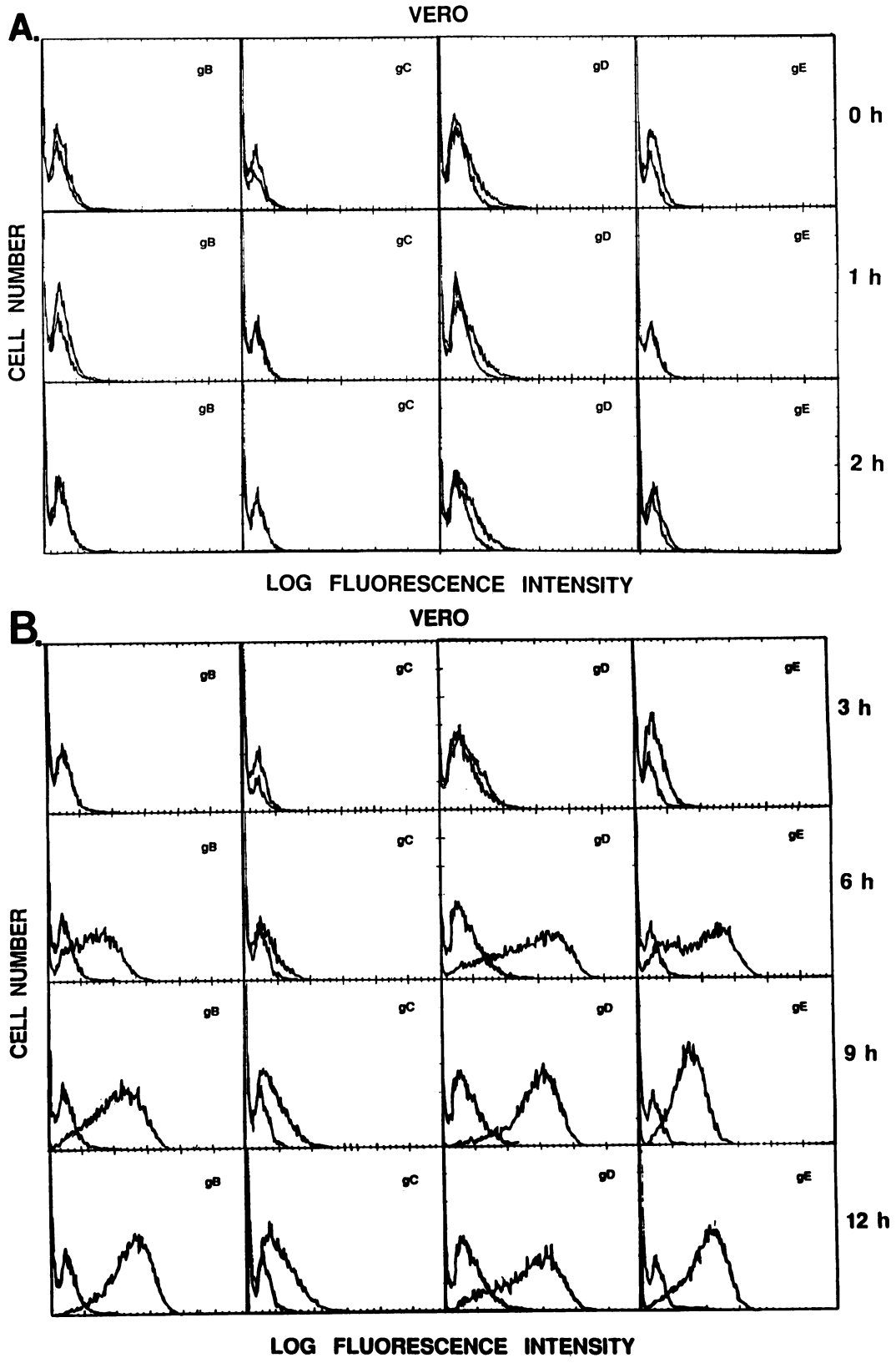


FIG. 2. Surface expression of major HSV-1 glycoprotein species on infected Vero (simian) cells: (A) 0 to 2 h p.i.; (B) 3 to 12 h p.i. See the legend to Fig. 1.

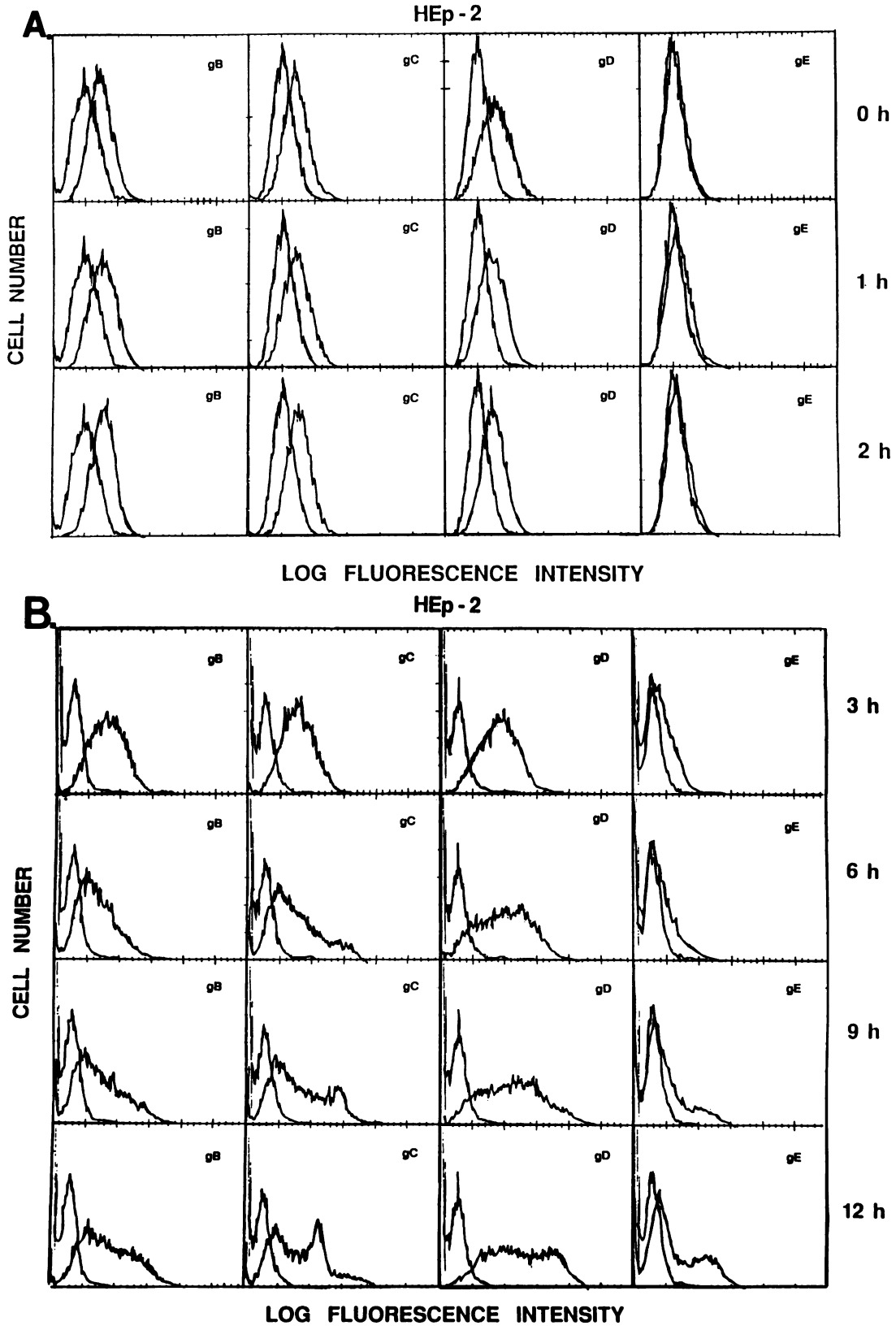


FIG. 3. Surface expression of major HSV-1 glycoprotein species on infected HEp-2 (human) cells: (A) 0 to 2 h p.i.; (B) 3 to 12 h p.i. See the legend to Fig. 1.

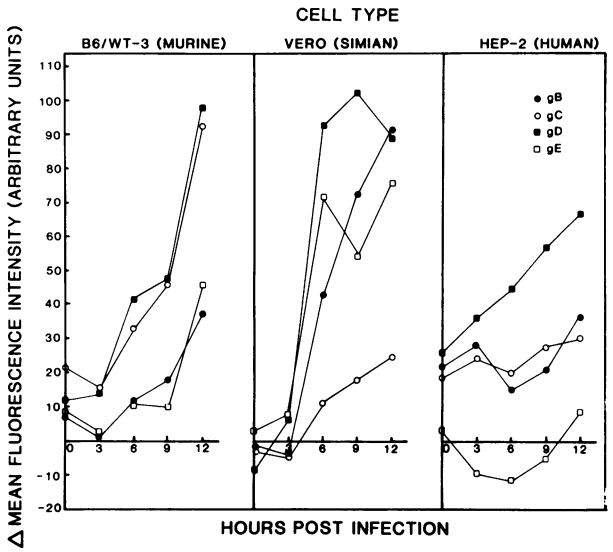


FIG. 4. Summary of temporal changes in surface glycoprotein expression on murine, simian, and human cell lines. The scale on the *y* axis represents changes in the mean fluorescence intensity (in arbitrary units) of the fluorescence profiles (Fig. 1 to 3) relative to the mean fluorescence intensity of uninfected cells stained with the appropriate monoclonal antibody.

involved in glycoprotein maturation and expression were the same in the different host cells.

The most surprising observation made in this study was the finding that the appearance of gC on the surfaces of HSV-infected B6/WT-3 cells of murine origin showed the same kinetics and levels of expression as gD. Based upon the observations of others that the onset and peak synthesis of the precursor form of gC occurred later than for the other species (3, 7, 20), it was expected that the surface expression of gC would also occur later. One explanation of the apparent early appearance of gC on the cell surfaces may be that the processing events leading to the fully mature, surface membrane-associated species proceed more rapidly for gC than other glycoprotein species. An alternative explanation is that gC may be present on the cell surfaces in different forms at various stages of glycosylation, as Glorioso et al. (11) have observed that underglycosylated forms of gC can become cell membrane associated. These alternatives may be differentiated through the use of antibody preparations which can distinguish between different maturational forms of the molecule.

The kinetics of glycoprotein expression on the surfaces of Vero cells followed the pattern predicted by the known regulation of the genes encoding the glycoprotein species. The appearance of newly synthesized gB, gD, and gE occurred simultaneously, with the expression of gD reaching the highest levels. Glycoprotein gC rose later, and never reached high levels. The initial interpretation of this observation would be that gC is translated and modified either later than the other glycoprotein species or is modified more slowly than the other species. However, Pereira and colleagues (33) have shown in a study utilizing a gB-specific monoclonal antibody that gB of HSV-2 was synthesized differentially in HEp-2 cells and Vero cells. Subsequent studies indicated that the differences in processing were dependent upon a proteolytic enzyme present within Vero cells which were absent from HEp-2 cells (34). Similarly, Balachandran and colleagues (3) observed differences in the processing of gB and gD of HSV-2 in Vero and BHK cells. In our study, the low level of gC expression on the surfaces of Vero cells may reflect differential processing, rather than a real difference in gC expression at the cell surface. Indeed, when monoclonal antibodies specific for other epitopes of gC were used, higher levels of gC were detected at the cell surface, although the later appearance of gC was a consistent observation (S. R. Jennings, personal communication). Therefore, quantitation of surface expression by immunofluorescence must account not only for saturating levels of the specific antibody but must also account for its specificity. Ideally, monoclonal antibodies should recognize epitope(s) present on the polypeptide backbone, rather than epitopes generated by posttranslational glycosylation and other modification events.

The results of this study indicated that the levels of expression of the individual species had similar kinetics to each other in HEp-2 cells. However, Norrild et al. (29), using polyclonal, monospecific antisera, found that gB was expressed by 5 h p.i., while the other species rose by 9 h p.i. The differences between these two studies may merely reflect the sensitivities of the methods utilized.

In all the cell lines tested, it was observed that surface expression of the glycoprotein species continued to increase over the period from 9 to 12 h p.i. This period was after the peak levels of precursor glycoproteins were synthesized, and may actually be during a period when their synthesis is shut down (41). This suggested that there may be a limiting factor involved in glycoprotein processing, such that an intracellular pool of precursors may be available for further processing even though synthesis was no longer occurring. Alternatively, the surface glycoproteins measured late in infection may be virion-associated, as Glorioso and Smith (12) have shown that maximal glycoprotein expression corresponds with maximal levels of infectious virus isolated from cells. The methods used to quantify glycoprotein expression in this study cannot distinguish between membrane-associated species and glycoproteins present within the envelope of infectious virions that may remain associated with the cell surface before passing into the extracellular environment (41).

In summary, we have described the quantitation of cell surface-associated glycoprotein species following infection with HSV, using a sensitive fluorescence flow cytometric method. This approach should provide a powerful adjunct to other standard techniques in the complex study of glycoprotein processing and expression, and of the particular processing events leading to the surface expression of the individual glycoprotein species. In addition, the use of monoclonal antibodies with well-defined specificities for different epitopes of the individual species has the potential for providing important information about the three-dimensional structures of surface glycoproteins in HSV-infected cells.

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