HUMAN CYTOMEGALOVIRUS INDUCES STAGE-SPECIFIC EMBRYONIC ANTIGEN 1 IN DIFFERENTIATING HUMAN TERATOCARCINOMA CELLS AND FIBROBLASTS

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Each year in the United States, several thousand children are born suffering from the consequences of congenital infection with human cytomegalovirus (HCMV)1 (1). Apart from fatal cases, the most severe outcome of intrauterine infection is damage to the central nervous system, resulting in psychomotor retardation and neuromuscular disorder, hearing loss, chorioretinitis, or optic atrophy. The mechanism by which HCMV causes these effects is unknown. One possibility is that cytolytic infection of certain critical cells in the embryo or fetus by HCMV leads to the observed abnormalities. On the other hand, HCMV can infect cells without cytolysis and so may act indirectly to effect fetal damage, for example, by altering the expression of cellular genes. To investigate how HCMV might affect the developing human embryo, we have studied the replication of this virus, and its effects on the expression of embryonic cell surface antigens, in cultures of human embryonal carcinoma (EC) cells and human diploid fibroblasts.

EC cells, the stem cells of teratocarcinomas, resemble early embryonic stem cells, and their differentiation in vitro provides a model of embryonic cellular differentiation (2). The TERA-2-derived human EC cell line, NTERA-2 cl.D1 (NT2/D1) (3, 4), is not permissive for HCMV replication until induced to differentiate with retinoic acid (RA) (5–9), when shifts in glycolipid synthesis accompany the appearance of distinct subsets of cells marked by differential expression of specific glycolipid antigens (10). We have suggested that the observed changes in glycolipid synthesis result from regulation of key glycosyl transferases that control the synthesis of different oligosaccharide core structures (11). Such switching of cell surface glycolipids may

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1 Abbreviations used in this paper: EC, embryonal carcinoma; HCMV, human cytomegalovirus; HPTLC, high-performance thin-layer chromatography; moi, multiplicity of infection; PFU, plaque-forming unit; RA, retinoic acid; SSEA-1, stage-specific embryonic antigen 1.
provide important cues for regulating specific cell interactions during embryonic development. We now report that infection of permissive differentiated NT2/D1 cells and diploid human fibroblasts with HCMV induces expression of the lactoseries glycolipid, stage-specific embryonic antigen 1 (SSEA-1), and we hypothesize that this induced expression of SSEA-1 may have relevance for the mechanism of HCMV pathogenesis in the developing fetus.

Materials and Methods

Cells. NTERA-2 cl.D1 (abbreviated NT2/D1) is a cloned EC subline of the human teratocarcinoma cell line TERA-2 (3). These cells were maintained with an EC phenotype by culturing at high cell density as previously described. Differentiation of NT2/D1 cells was induced by seeding 10⁶ cells per 75-cm² flask in medium containing 10⁻⁴ M all-trans-RA (Eastman Kodak Chemical Co., Rochester, NY) as previously described (4). MRC-5 (12) and WI38 (13) diploid human fibroblasts were obtained from the Medical Research Council (United Kingdom) and from Dr. Paul Phillips, the Wistar Institute, respectively.

Virus. The Towne strain of HCMV was plaque purified and propagated on MRC-5 cells as previously described (14). The infectivity of the stock virus, assayed by the virus plaque method (15), was 1 x 10⁶ to 3 x 10⁶ PFU/ml. In additional experiments, we also used the established laboratory HCMV strain Ad-169 (16), obtained from the American Type Culture Collection (Rockville, MD), the low-passage strain Toledo (17), and a fresh isolate provided by the Clinical Virology Laboratory, Children's Hospital of Philadelphia.

Immunoassays of Cell Surface and Viral Antigens. mAbs MC480 (anti-SSEA-1) (18) and A2B5 (19) were used to detect expression of their corresponding cell surface glycolipid antigens, using either immunofluorescence or radioimmunoassay as previously described (3, 20), or immuno-TLC (thin-layer chromatography) analysis (see below). SSEA-1 is a lactoseries glycolipid antigen and its epitope has been identified with the Lex structure (21, 22); Lex⁺ expression was also assayed using mAbs PM 81 (23) and D, 56-22 (24). A2B5 recognizes a ganglioside, GT₁, in NT2/D1 cells (10). mAb A5 (25), recognizing unsubstituted lactoseries core glycolipids, was also used in immuno-TLC analysis.

Viral antigens were detected using a pool of four human convalescent sera (Clinical Virology Laboratory, Children's Hospital of Philadelphia) at a dilution of 1:15. In separate tests, we confirmed that this serum pool detected immediate early (IE), early (E), and late (L) HCMV antigens, but did not react with uninfected, SSEA-1⁺ cells.

The coexpression of cell surface and viral antigens was assayed by a two-color immunofluorescence technique (10), modified as follows. As previously described for flow cytofluorometric analysis (3, 20), cells were reacted in suspension with a murine mAb to the surface antigen followed by rhodamine-conjugated goat anti-mouse Ig antibodies that had been preabsorbed to remove crossreactivity to human Ig (Cappel Laboratories, Malvern, PA). The cells were then cytocentrifuged onto slides, air dried, and fixed with acetone, after which they were sequentially reacted with anti-HCMV sera and fluorescein-conjugated goat anti-human Ig that had been preabsorbed to remove crossreactivity to mouse Ig. In each experiment, controls were included to confirm the specificity of the reagents and their lack of crossreactivity.

Glycolipids. Glycolipids were extracted from pellets of HCMV and cultured cells using isopropyl alcohol/hexane/water (55:25:20). For cultured cells, total lipid extracts were partitioned into an upper and a lower phase according to the method of Folch-Pi (27). The Folch-Pi upper phase was chromatographed on DEAE-Sephadex in order to separate neutral and acidic fractions (28). Neutral glycolipids were eluted with methanol, while acidic glycolipids (gangliosides) were eluted with chloroform/methanol/water (30:60:8) containing 0.8 M sodium acetate. TLC immunostaining was performed as described previously (10), except that TLC plates were dipped in 0.05% polyisobutyl methacrylate (Aldrich Chemical Co., Milwaukee, WI) in diethyl-ether prior to antibody staining.

1 Glycolipids are designated according to the recommendations of the IUPAC Nomenclature Committee, but the suffix OseCer is omitted (26).
Enzyme Assays. Tissue culture cells were homogenized in 2 vol of a buffer composed of 50 mM Hepes buffer, pH 7.2, 0.5 M sucrose, 1 mM EDTA by two strokes of Potter-Elvehjem homogenizer and used as enzyme source in the following assays.

β1→3N-acetylglucosaminyltransferase was assayed as previously described (29) in reaction mixtures containing 2.5 μmol of Hepes buffer, pH 7.2, 40 μg of lactosylceramide, 150 μg of Triton CF-54, 0.5 μmol of MnCl₂, 0.5 μmol of CDP-choline, 50 nmol of UDP[¹⁴C]N-acetylglucosamine (9,000 cpm/nmol), and 375–425 μg of protein in a total volume of 0.05 ml. The reaction mixture was incubated for 2 h at 37°C and terminated by addition of 50 μl of 0.25M EDTA and 0.6 ml chloroform/methanol (2:1). The entire reaction mixture was streaked onto a 4-cm wide strip of Whatman 3 paper and chromatographed with water overnight. The glycolipid remaining at the origin was extracted with 2–5-ml washes of chloroform/methanol/water (10:5:1). The solvent was removed with a nitrogen stream and dissolved in 20 μl chloroform/methanol (2:1). A 10-μl aliquot was removed and spotted onto a high-performance TLC (HPTLC) plate (Merck, Darmstadt, FRG) and developed in a solvent of chloroform/methanol/water (60:40:9) containing 0.02% CaCl₂·2H₂O as a final concentration. Standard glycolipids were visualized by orcinol spray. Radioactive glycolipid bands were located by autoradiography, scraped from the plate, and counted by a liquid scintillation counter. 1 U of activity is defined as transfer of 1 pmol of N-acetylglucosamine per hour under the conditions of the assay.

This methodology was also followed for assays of β1→4galactosyltransferase and α1→3fucosyltransferase activities using the conditions described by Holmes et al. (30). In addition, α1→4galactosyltransferase activity with lactosylceramide as the acceptor was determined under conditions defined by Wiel et al. (31). α-Fucosidase activities were determined as described by Barlow et al. (32).

Protein Determination. Protein concentrations of cell fractions were determined by the method of Lowry et al. (33) using BSA as the standard.

Results

Surface Antigen Expression in HCMV-infected Teratocarcinoma Cells and Fibroblasts. Since HCMV replication occurs in only a subset of differentiated NT2/D1 cells, we used two-color immunofluorescence to determine whether the HCMV-permissive cells are characterized by a particular pattern of surface antigen expression (Table I). Cultures of NT2/D1 cells that had been exposed to 10⁻⁵ M RA for 7 d were reseeded in the absence of RA and infected with HCMV; the expression of viral antigens and cell surface differentiation antigens was assayed 6 d later. Of the cells replicating HCMV and expressing HCMV-specific antigens, 69% were also SSEA-1⁺; conversely, 72% of the SSEA-1⁺ cells were HCMV⁺. Very few SSEA-1⁺ cells were evident in the uninfected control cultures, suggesting that infection with HCMV induces expression of this antigen. By contrast, very few HCMV⁺ cells expressed the ganglioseries antigen A2B5, which is commonly expressed by many differentiated NT2/D1 cells (10).

Notwithstanding the present results, in other experiments SSEA-1 is commonly expressed by some uninfected, differentiated NT2/D1 cells; typically, a peak of expression by ~20% of the cells after 7 d of RA exposure is followed by disappearance of the antigen, so that very few SSEA-1⁺ cells are ordinarily found after a further 7 d (10). Thus, rather than inducing SSEA-1, HCMV might preferentially infect SSEA-1⁺ cells and stabilize expression of this antigen. However, after isolating SSEA-1⁺ and SSEA-1⁻ subsets of differentiated NT2/D1 cells by FACS and testing their relative susceptibilities to HCMV infection, we did not detect any differences that would support this notion (data not shown). Unfortunately, the variability in SSEA-1 expression by uninfected NT2/D1 cells, as well as the heterogeneity of these
| Exp.      | HCMV* cells* | SSEA-1* cells* | A2B5* cells* |
|-----------|--------------|----------------|--------------|
|           | % of Total   | % of SSEA-1*   | % of Total   | % of HCMV*   | % of Total   | % of HCMV*   |
| Noninfected | 0 (0/1,000) | 0 (0/43)       | 1 (3/283)    | 31 (53/173)  |             |              |
| Infected  |              |                |              |              |              |              |
| low moi   | 36 (76/209)  | NC1            | 1 (1/155)    | 32 (90/286)  | 78 (60/77)   | 16 (39/239)  | <1 (0/106)   |
| high moi  | 51 (170/336) | 72 (68/95)     | 4 (4/93)     | 40 (205/507) | 69 (50/72)   | 6 (40/612)   | <1 (0/113)   |

Differentiated NT2/D1 human teratocarcinoma cells, obtained by growth in 10^{-5} M RA for 7 d, were reseeded at 10^6 cells per 25-cm^2 flask and infected with HCMV at 1-2 (low) and 2-4 (high) multiplicities of infection (moi). After culturing for 6 d, the coexpression of viral antigens and the cell surface differentiation antigens SSEA-1 and A2B5 was determined using two-color immunofluorescence. In each experiment, controls were included to confirm the specificity of the reagents and their lack of crossreactivity.

* The numbers of cells counted are shown in parentheses.

1 NC, not counted.
cultures and absence of HCMV permissiveness in all of the cells, posed difficulties for a detailed investigation of the relationship between HCMV infection and SSEA-1 expression in the teratocarcinoma system. We therefore investigated the expression of SSEA-1 in diploid human fibroblasts, which are fully permissive for HCMV replication.

Infection of MRC-5 or WI38 fibroblasts with HCMV also led to a marked induction of SSEA-1 expression, whereas SSEA-1 was not expressed by the uninfected cells at any stage of their growth cycle (Fig. 1). SSEA-1 was first detectable \( \sim 3 \) d after infection, when cytopathic effects were already evident in some cells, and its expression increased up to 6 d after infection, by which time almost all the cells expressed a cytopathic effect. The SSEA-1 epitope has been identified as the fucose \( \alpha 1 \rightarrow 3 \)GlcNAc structure carried on the type 2 polyactosamine chains of lactoseries glycolipids, a carbohydrate structure also known as \( \text{Le}^+ \) (III Fuc\( \text{Le}^- \)) (21, 22). Accordingly, similar results were obtained with monoclonal anti-SSEA-1 and two other monoclonal anti-\( \text{Le}^+ \) antibodies, PM81 and D1-56-22 (23, 24) (Fig. 1). As with the differentiated NT2/D1 cells, only one-third to one-half of the HCMV-infected fibroblasts expressed SSEA-1 (Table II). In addition to the Towne strain of HCMV, this induction of SSEA-1 was also observed (data not shown) upon infection with other strains, including another established laboratory strain (Ad-169), a low-passage strain (Toledo), and a strain freshly isolated in our laboratory. On the other hand, no induction was observed after infection with HSV-1 (data not shown).

**HCMV-induced Changes in Membrane Glycolipids.** In parallel with the cell surface antigen changes, glycolipid changes were also detectable in HCMV-infected MRC-5 fibroblasts: a series of extended lactoseries glycolipids bearing the terminal SSEA-1 (\( \text{Le}^- \)) epitope were identified in the neutral glycolipid fraction of HCMV-infected but not uninfected cells (Fig. 2). These glycolipids had the same TLC mobility as monofucosylated hepta-
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TABLE II

Coexpression of SSEA-1 and Viral Antigens in MRC-5 Fibroblasts Infected with HCMV

| Days after infection | HCMV+ cells* | SSEA-1* cells* |
|---------------------|--------------|----------------|
|                     | % of Total   | % of Total     |
|                     | % of SSEA-1* | % of HCMV+     |
| 3 d                 |              |                |
| Noninfected         | 0 (0/1,000) | 2 (14/521)     |
| Infected            | 29 (57/194) | 90 (90/100)    | 8 (14/175) | 41 (36/88) |
| 6 d                 |              |                |
| Noninfected         | 0 (0/1,000) | 0 (0/1,000)    |
| Infected            | 95 (54/237) | 100 (54/237)   | NC*         |

Confluent cultures of MRC-5 fibroblasts were infected with HCMV (moi = 0.1) on day 0; cells were harvested on day 3 and 6 after infection and assayed by two-color immunofluorescence for the coexpression of SSEA-1 and viral antigens.

* The numbers of cells counted are shown in parentheses.

saccharide ($V^2$FucnLc6, Y2), difucosylated heptasaccharide ($V^2III^2$FucnLc6, diY2), monofucosylated nonasaccharide ($VII^2$FucnLc8, Zt), and difucosylated nonasaccharide ($VII^2$FucnLc8, Z2), respectively (22). A series of extended lactoseries glycolipids bearing the terminal $Le^y$ epitope (Fucα1→2Galβ1→4[Fucα1→3]-GlcNAc) were also detected, and parallel immunofluorescence experiments confirmed cell surface binding of anti-$Le^y$ mAb (data not shown). Glycolipids with $Le^e$ and $Le^y$ structure were not detected in extracts of HCMV itself, confirming that their appearance in MRC-5 cells was not due to passive transfer during viral infection.

An increase in unsubstituted lactoseries core glycolipids nLc6 and nLc8 was also observed in the upper-phase neutral glycolipid fraction of HCMV-infected MRC-5 cells using mAb A5 (25). However, lactoseries glycolipids $Le^e$, $Le^y$, nLc6, and nLc8 were not detected by orcinol spray, suggesting that they are relatively minor mem-
brane components. By contrast to the general increase in lactoseries glycolipids after HCMV infection, the expression of globo series glycolipids was not significantly changed after infection (not shown). Thus, HCMV infection appears to be accompanied by a specific increase in lactoseries glycolipid biosynthesis.

Glycosyltransferase Activities in Infected and Noninfected Fibroblasts. The appearance of SSEA-1 in HCMV-infected cells suggests that viral infection may trigger an increase in the activity of key glycosyltransferases. Indeed, after HCMV infection of MRC-5 cells, the specific activities of \( \beta 1 \rightarrow 4 \)galactosyltransferase and \( \alpha 1 \rightarrow 3 \)fucosyltransferase increased two- and threefold, respectively, while \( \beta 1 \rightarrow 3 \)N-acetylglucosaminyltransferase, associated with synthesis of \( Lc_3 \) from lactosylceramide, the first step in lactoseries core chain synthesis, was undetectable in the uninfected cells but detectable at a low activity (\( \sim 5 \) pmol/h/mg protein) in the HCMV-infected cells (Table III, Fig. 3). The relative specific activities of these enzymes suggest that formation of \( Lc_3 \) is the rate-limiting step in synthesis of SSEA-1 active carbohydrate structures, and that the appearance of a low activity of \( \beta 1 \rightarrow 3 \)N-acetylglucosaminyltransferase is most probably chiefly responsible for the marked effect of HCMV infection on the cell surface expression of lactoseries carbohydrate epitopes. An identical mechanism has been found to be responsible for the accumulation of large quantities of lactoseries antigens during oncogenesis in human colon (30).

A generalized activation of enzymes associated with oligosaccharide biosynthesis and degradation occurring as a consequence of HCMV infection was suggested since

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### Table III

| Enzymes                        | HCMV infection | Specific activity pmol/h/mg protein |
|-------------------------------|----------------|-----------------------------------|
| Lactoseries enzymes           |                |                                   |
| \( \alpha 1 \rightarrow 3 \)fucosyltransferase | -   | 101 ± 8                           |
|                                | +   | 286 ± 18                           |
| \( \beta 1 \rightarrow 4 \)galactosyltransferase | -   | 2,340 ± 28                         |
|                                | +   | 4,340 ± 34                         |
| \( \beta 1 \rightarrow 3 \)N-acetylglucosaminyl transferase | -   | ND                                 |
|                                | +   | 5 (see Fig. 3)                     |
| Other enzymes                 |                |                                   |
| \( \alpha 1 \rightarrow 4 \)galactosyltransferase | -   | 34.3                               |
|                                | +   | 55.9                               |
| \( \alpha \)-fucosidase        | -   | 0.32 (mmol/h/mg protein)           |
|                                | +   | 0.66 (mmol/h/mg protein)           |

Confluent cultures of MRC-5 fibroblasts were infected, or mock infected, with HCMV (moi = 0.1) and harvested after 6 d, when cell lysates were prepared and enzyme activities determined. The fucosyltransferase activity was \( \alpha 1 \rightarrow 3 \) specific, as no transfer into \( Lc_4 \) was detected. In the assay for \( \beta 1 \rightarrow 4 \) galactosyltransferase no synthesis of an \( Lc_4 \), type 1 chain paragloboside product was detected, indicating no type 1 chain lactoseries synthesis in either infected or uninfected cells. The \( \beta (1 \rightarrow 3) \)N-acetyl-glucosaminyl transferase yielded a product as shown in Fig. 3 based on autoradiography or reaction products after separation by TLC. A detectable band was found for the infected cells (lane 4), whereas no significant spot was found for the uninfected cells (lane 3).
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Discussion

In cultures of differentiating teratocarcinoma cells, and of diploid human fibroblasts, many cells infected with HCMV expressed the cell surface antigen SSEA-1, an epitope identified with the Le" hapten, fucose(α1→3)GalNAc (21, 22). TLC analysis of neutral glycolipids from infected and noninfected fibroblasts confirmed a marked increased in the SSEA-1 (Le")-reactive glycolipids of the infected cells. This change in cell surface glycolipid antigen expression resulting from infection appears to be due to subtle alterations in the relative competition for common precursor structures at key points in the biosynthetic pathways, rather than activation of a single glycosyltransferase. In particular, the results suggested that alterations in β1-3N-acetylglucosaminyltransferase activity after infection cause slightly increased synthesis of Lc3 which is rapidly converted to SSEA-1 (Le")-reactive products. However, the increased activities of other enzymes, such as α1-3fucosyltransferase, may also contribute to the final result. It should be noted that, 6 d after infection, only about one-third of the fibroblasts expressed surface-detectable SSEA-1, whereas all the cells were replicating virus; we do not currently understand the basis for such SSEA-1+ and SSEA-1- subsets of infected cells and do not know whether greater increases in the activities of the glycosyltransferases occur in the SSEA-1+ cells.

Changes in carbohydrate antigen expression have also been reported after infection of cells with other viruses. For example, the lactoseries glycolipid Lev was recently found to be induced in human T cell lines after HIV infection (34). Also, transfection of rat cells with the transforming gene of human adenovirus type 12 induced the synthesis of the disialoganglioside GD3, which was subsequently shown
to result from activation of a highly specific sialyltransferase, GD3 synthetase (35, 36). However, in the present case, infection seems to have pleiotropic effects on carbohydrate synthetic enzymes. Indeed, HCMV has been reported to influence expression of a large number of other host enzymes. Examples of the enzymes and other proteins induced after HCMV infection include poly-ADP ribose synthetase (37), RNA polymerase (38), thymidine-kinase (39, 40), ornithine decarboxylase (41), plasminogen activator (42), DNA-poly-merase α (43), bovine growth hormone (44), an inhibitor of IL-1 (45), and a factor that is released into the culture medium and stimulates cellular DNA synthesis (46).

Generally, stimulation of host cell enzymes and production of DNA-binding proteins in HCMV-infected cells precedes viral DNA synthesis; recently the product(s) of the IE2 region has been described as a potent and nonspecific transactivator of heterologous virus and cellular target promoters (47-49). However, the time of SSEA-1 appearance after HCMV infection suggests that its induction may be a late viral function, although we cannot yet exclude the possibility that it is a delayed consequence of IE gene activity.

During embryogenesis in the mouse, SSEA-1 first appears during late cleavage stages; it is subsequently expressed on the primitive ectoderm and, during later stages of development, on various tissues including parts of the nervous system (18, 50-52). Several studies have indicated that carbohydrates bearing the SSEA-1 epitope may play a role in mediating or directing some critical cell–cell interactions during development. For example, carbohydrates bearing multivalent Le\(^{a}\) structures have been reported to block compaction of the morula during early mouse development (53, 54). Other studies have suggested that lactoseries carbohydrates and complementary lectins act as recognition signals that guide the formation of correct neuronal interconnections in parts of the developing rat peripheral nervous system (55-57). Consequently, the observations reported here raise the question of what effects the induction of the lactoseries carbohydrate antigens, such as SSEA-1, may have on the developing fetus if that induction were to occur at an inappropriate time or place. Although the deleterious effects of HCMV infection on the developing fetus might be due to the infection and subsequent death of specific critical cell types, there is no direct evidence for or against such a hypothesis. In view of the ability of HCMV to cause multiple physiological changes in infected cells, as well as its propensity for latent infection, alternative possibilities should also be entertained. It is notable that damage to the central nervous system is a prominent consequence of HCMV infection in utero, while lactoseries carbohydrates, among others, have been proposed to have a role in guiding some neuronal interactions.

**Summary**

Cell surface expression of stage specific embryonic antigen 1 (SSEA-1), or Le\(^{x}\) (IIIF FucnLC\(_4\)), was induced in differentiated human teratocarcinoma cells and in human diploid fibroblasts 3–6 d after infection with human cytomegalovirus (HCMV). In parallel, fucosylated lactoseries glycolipids bearing the SSEA-1/Le\(^{x}\) epitope were readily detected in the infected cells but not in the uninfected cells. HCMV infection also results in altered expression of several glycosyltransferases. SSEA-1/Le\(^{x}\) induction is probably a consequence of both increased expression of β1→3N-acetylglucosaminyltransferase, which catalyzes the rate-limiting step in lactoseries...
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core chain synthesis, and subtle alterations in the relative competition for common precursor structures at key points in the biosynthetic pathway. Since SSEA-1 has been suggested to play a role in some morphogenetic cell–cell interactions during embryonic development, the induction of this antigen at inappropriate times might provide one mechanism whereby intrauterine infection with HCMV can damage the developing fetal nervous system.

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