Antibodies to Surface Antigens of Herpesvirus Type 1- and Type 2-Infected Cells Among Women with Cervical Cancer and Control Women

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Cells infected with herpesvirus type 1 or type 2 develop surface antigens which can be detected by immunofluorescence. Using the indirect immunofluorescence technique, we determined antibody titers to the surface antigens in 20 sera from persons with known type 1 virus infections, 20 sera from persons with known type 2 virus infections, 30 sera from women with cervical cancer, and 30 sera from matched control women. There was a good correlation between antibody activity to the surface antigens and neutralizing antibody activity for both herpesvirus type 1 and herpesvirus type 2 in all groups of sera examined. Women with cervical cancer did not have unusually high or low titers of antibody activity to the surface antigens. In the selected sera examined, analysis of antibodies to the surface antigens was not superior to the microneutralization test in distinguishing women with cervical cancer from control women.

Herpesvirus type 2 has been implicated as a possible etiological agent in carcinoma of the cervix. Neutralizing antibodies to herpesvirus type 2 have been found more frequently among women with carcinoma of the cervix than among control women in certain, but not all, populations (16, 19, 20, 25). In addition, it has been reported that cervical anaplasia occurs more frequently among women with genital herpes than among control women and that cells from cervical neo-plasms contain herpesvirus-specific antigens (17, 26).

By means of the immunofluorescence test using viable cells, several oncogenic and cytolytic viruses have been shown to induce formation of specific antigens on the surface of infected cells (2, 4, 7, 9, 10, 12, 13, 27, 28). These include, among the members of the herpesvirus group, Epstein-Barr virus (EBV) and herpesvirus of Marek’s disease (2, 4, 12), which are associated with lymphoproliferative diseases. Patients with Burkitt’s lymphoma or nasopharyngeal carcinoma may possess high levels of antibodies to surface antigens of cells infected with EBV, and the titers of these antibodies have been reported to fluctuate during regressions and recurrences of the tumors (11).

Infection of cells with herpesvirus type 1 has been reported to induce formation of specific surface antigens (22). Because of the importance of herpesviruses in human disease, the present study was undertaken to examine surface antigens induced by herpesvirus type 1 or 2, which can be demonstrated by means of the immunofluorescence reaction with various human sera.

MATERIALS AND METHODS

Cell cultures. Primary rabbit kidney (PRK), chick embryo (CE), and human embryonic kidney (HEK) cell cultures were prepared and maintained as previously described (5, 15).

Virus and virus assays. Herpesvirus type 1 strains KOS and a mutant C2b, which replicates in CE cells, have been described elsewhere (15, 18). Type 2 herpesvirus, strain SAV, isolated from a vulvar lesion, was used throughout the study.

Stocks of herpesvirus type 1 were prepared in passaged HEK cell cultures (13). Preparation of type 2 virus stocks differed from preparation of type 1 virus stocks in that type 2 preparations were subjected to only one cycle (instead of three) of freezing and thawing, followed by ultrasonic treatment for 30 sec. Virus stocks were placed in ampoules and stored at −70°C. Strain KOS was quantitated by the plaque count method in PRK cell cultures, and strain C2b was assayed in CE cell cultures (15). Strain SAV was quantitated in both PRK and CE cell cultures. Infectious virus concentrations were expressed as plaque-forming units (PFU) per milliliter.

Measurement of neutralizing antibody to herpesvirus. The procedure for determining the titer of neutralizing antibody to herpesvirus types 1 and 2 by the microneutralization test has previously been reported. The relationship of antibody titers to the two herpesviruses
was expressed as the II/I index (18):

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\text{titer log}_{10} \text{to herpesvirus type II} \times 100 = \text{II/I index}
\]

\[
\text{titer log}_{10} \text{to herpesvirus type I}
\]

Demonstration of antibody to surface antigens. Confluent monolayers of CE cell cultures in plastic plates (60 by 15 mm; Falcon Plastics, Los Angeles, Calif.) were inoculated with 0.2 ml of either strain C2b or strain SAV herpesvirus at a multiplicity of 1 to 2 PFU/cell. After a 2-hr adsorption period at 36 C, cell monolayers were refed with 4 ml of Eagle's medium supplemented with 2% fetal calf serum (FCS), and were incubated at 36 C for 20 hr in a 5% CO\textsubscript{2} humidified incubator. For demonstration of surface antigens, a modification of the indirect immunofluorescence reaction was employed, in which viable cells were used (24). Briefly, 20 hr after infection, cells were dispersed with 0.25\% trypsin, suspended in tris-(hydroxymethyl)aminomethane-buffered saline (TBS) containing 10\% FCS, and centrifuged at 1,000 rev/min for 5 min. Cell pellets were resuspended in TBS and counted. Suspensions containing 10\textsuperscript{4} cells were dispensed in plastic test tubes (100 by 13 mm; Falcon Plastics) and washed two additional times in TBS. Cell pellets were resuspended in 0.1 ml of test serum, mixed by gentle pipetting, and incubated for 45 min at 36 C. Sera were tested at twofold dilutions, starting at 1:8. Cells were washed three times in TBS. After the third wash, pellets were gently resuspended by adding 0.1 ml of goat anti-human gamma globulin conjugated with fluorescein isothiocyanate (Hyland Laboratories, Los Angeles, Calif.). After an incubation period of 45 min at 36 C, cells were washed three times with TBS, resuspended with 0.1 ml of TBS, and spread on glass slides. By means of the technique described by Cerottini (1), cell suspensions were air-dried, fixed in methanol for 5 min, and mounted under Elvanol (21); stained cells were observed under a Zeiss microscope.

Study groups. Sera from selected groups were tested for neutralizing antibodies to types 1 and 2 herpesvirus and antibodies to surface antigens of herpesvirus-infected cells. Sera were obtained from 20 patients from whom type 1 herpesvirus had been isolated, and 20 sera were obtained from patients with genital herpes from whom type 2 herpesvirus had been isolated. There were sera from 30 women with histologically proven invasive carcinoma of the cervix and 30 control women. These sera were collected in three geographic areas. In two areas, the patients were Caucasian, and in these areas the occurrence of antibodies to herpesvirus type 2 among cancer patients had been found to be between 37 and 50\%. In the other area, the patients were Negro, and 83\% had antibodies to the virus.

RESULTS

Demonstration of surface immunofluorescence in cells infected with type 1 or type 2 herpesvirus. By use of a 1:4 dilution of serum obtained from a patient with a known past infection with herpesvirus type 1, surface antigens could be demonstrated on CE cells infected with type 1 herpesvirus. Specific fluorescence was not observed until 6 hr after infection of the cultures with 1 to 2 PFU of the C2b strain per cell. The fluorescence pattern at 6 hr was characterized by many bright granules spread evenly over the surface of the infected cells. By 10 hr after infection, the granularity was replaced by a more diffuse pattern of fluorescence in which the periphery of the cells displayed bright rings. An example of this pattern of fluorescence is shown in Fig. 1A.

Infection of CE cell cultures with the SAV strain of herpesvirus type 2 resulted in surface fluorescence when serum collected from a patient with a known type 2 herpesvirus infection was used. The reaction was again first noted at about 6 hr after infection but, unlike type 1 virus, the fluorescence pattern remained granular throughout the 24 hr of observation (Fig. 1B).

Infection of CE cell cultures with two other strains of herpesvirus type 1 which were capable of replicating in CE cells and three other strains of type 2 virus yielded the same results. Surface fluorescence was also observed when HEp-2, PRK, or human embryonic lung cells were used instead of CE cells.

The surface immunofluorescence reaction was found to be specific, since uninfected cells and cells infected with Sindbis, vaccinia, or vesicular stomatitis viruses failed to react when incubated with sera containing antibodies to herpesvirus. In addition, cells infected with herpesvirus which had been inactivated either by heating at 56 C for 30 min or by exposure to ultraviolet light prior to infection failed to react with positive sera. Incorporation of 20 \mu g of cycloheximide/ml into the culture medium also inhibited the formation of surface antigens in both type 1- and type 2-infected cells.

Titration of antibodies against surface antigens.

FIG. 1. Immunofluorescence on the surface of chick embryo cells infected with type 1 or type 2 herpesvirus. Cells were harvested 20 hr after infection and stained before fixation. (A) Ring-type fluorescence characteristic of cells infected with type 1 herpesvirus. (B) Granular-type fluorescence characteristic of cells infected with type 2 herpesvirus.
cells were obtained for sera from the other groups studied.

When sera from 20 patients with virologically proven herpesvirus type 2 infections were examined for neutralizing antibodies against type 2 and for antibodies to surface antigens of cells infected with herpesvirus type 2, a correlation (Fig. 3) similar to that observed for type 1 virus was observed; high titers by neutralization test correlated with high titers of antibodies to surface antigens. This correlation was observed in all groups of sera examined for neutralizing antibodies to herpesvirus type 2 and antibodies to surface antigens of herpesvirus type 2-infected cells.

Previously, it was found that the relative antibody titers, as determined by neutralization test, to the two viruses distinguished persons with past infections with herpesvirus type 2 from persons infected only with type 1 virus. A II/I index of 85 or greater was associated with a past infection with herpesvirus type 2 (18). A similar relationship was found to exist for antibodies to the surface antigens of infected cells (Table 1). The results in Table 1 also show that the mean antibody titers from patients with known type 1 herpesvirus infection, as determined both by neutralization test and by surface reactions, were greater

Upon dilution of reference serum, the intensity of the fluorescence decreased and the percentage of cells showing fluorescence decreased. Determination of antibody titer end points based on the intensity of fluorescence proved unreliable, but consistent titers were obtained when end points based on the percentage of cells showing fluorescence were used. A titer end point in the surface fluorescence test was designated as that dilution of human serum at which approximately 50% of the infected cells showed fluorescence.

CE cells infected with herpesvirus type 1 or type 2 were incubated for 20 hr before testing. The titer of antibody reacting with the surface antigens of CE cells infected with herpesvirus type 1 or type 2 was determined for sera from 20 patients who had known herpesvirus type 1 infections and who gave no history of genital herpes. The antibody titers to the surface antigens of the cells infected with type 1 virus correlated with the neutralizing antibody titers to herpesvirus type 1 (Fig. 2). Similar correlations between the values obtained by the microneutralization test and the surface fluorescence test with type 1- and type 2-infected

**Fig. 2.** Relationship between antibody titers obtained by the microneutralization and the surface fluorescence tests, using type 1 herpesvirus and cells infected with type 1 herpesvirus. Scatter diagram represents results obtained from the examination of sera from 20 patients from whom type 1 herpesvirus had been isolated. The regression line was calculated by the method of least squares; correlation coefficient = 0.82; standard error = 0.11.

**Fig. 3.** Correlation obtained by reacting sera from 20 patients from whom type 2 herpesvirus had been isolated with type 2 herpesvirus and cells infected with type 2 herpesvirus. Correlation coefficient = 0.90; standard error = 0.12.
TABLE 1. Antibody titers obtained by microneutralization and surface immunofluorescence tests among patients with clinical herpesvirus infection, women with carcinoma of the cervix, and control women

| Diagnosis                  | Infection with herpesvirus | Cervical cancer | Control
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<tbody>
<tr>
<td></td>
<td>Type 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Type 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Neutralization</td>
<td>1.98 ± 0.33</td>
<td>1.68 ± 0.42</td>
<td>2.18 ± 0.37</td>
</tr>
<tr>
<td>Herpesvirus type 1</td>
<td>1.37 ± 0.26</td>
<td>1.79 ± 0.40</td>
<td>1.91 ± 0.46</td>
</tr>
<tr>
<td>Herpesvirus type 2</td>
<td>1.89 ± 0.19</td>
<td>1.73 ± 0.30</td>
<td>1.93 ± 0.24</td>
</tr>
<tr>
<td>Surface immunofluorescence</td>
<td>1.50 ± 0.16</td>
<td>1.71 ± 0.27</td>
<td>1.75 ± 0.28</td>
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<tr>
<td>II/I index</td>
<td></td>
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<tr>
<td>Neutralization</td>
<td>70 ± 9</td>
<td>108 ± 16</td>
<td>87 ± 14</td>
</tr>
<tr>
<td>Surface immunofluorescence</td>
<td>79 ± 7</td>
<td>100 ± 10</td>
<td>91 ± 9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean and standard deviation (log<sub>10</sub>) of 20 sera.

<sup>b</sup> Mean and standard deviation (log<sub>10</sub>) of 30 sera.

against type 1 herpesvirus than against type 2 herpesvirus. The mean titers to the two herpesviruses by the two methods were also comparable for sera obtained from patients with known herpesvirus type 2 infection.

**Antibodies to herpesvirus type 1 and type 2 in sera from women with cervical cancer and matched control women.** Sera from 30 women with cervical cancer and from 30 matched control women were titrated for neutralizing antibodies to herpesvirus type 1 and type 2 and for antibodies reacting to the surface antigens of cells infected with the two viruses. As observed with sera from patients with herpesvirus infections proven by virus isolation, there was a good correlation between neutralizing antibody titers and antibody titers to the surface antigens among women with cervical cancer and among control women (Table 1). The correlation between results obtained by the two methods is further illustrated by the similarity of the mean II/I index values obtained for the groups studied (Table 1). When the II/I indices obtained by microneutralization test and by the surface fluorescence test of women with cervical cancer and of control women were compared, no differences were found. As can be seen in Fig. 4, regression lines obtained from the examination of the sera from 30 women with cervical cancers, represented by the solid line, and 30 control women, represented by the broken line, were similar. Thus, women with carcinoma of the cervix could not be distinguished from control women by these antibody titrations.

**DISCUSSION**

In confirmation and extension of previous studies (6, 14), the data presented indicate that cells infected with herpesvirus type 1 or type 2 develop specific surface antigens which can be demonstrated by the immunofluorescence reaction. Specific fluorescence was first observed about 6 hr after infection, at which time the pattern...
displayed was particularly granular in nature. In type 1-infected CE cell cultures, the fluorescence pattern changed by 10 hr after infection to give bright fluorescence rings, but in the type 2-infected cells the pattern remained granular. These patterns of fluorescence were the same for all virus strains examined and were not specific for the source of the antibody; i.e., granular fluorescence of the cell surface was evident when cells infected with type 2 virus were tested against sera from persons with known type 1 infections and ring fluorescence was observed when cells infected with type 1 virus were tested with sera from persons with type 2 infections.

The antigens appearing at the cell surface required infectious virus and the synthesis of new proteins. The reaction did not appear to be due to virus particles, since the surface antigens appeared before the release of infectious virus, and electron microscopy of CE cells 24 hr after infection with either C2b or SAV strains did not reveal significant numbers of virus particles adhering to the cell surface (unpublished data). In addition, alteration of the membrane of cells infected with herpesvirus has been demonstrated by a number of techniques (8, 22, 23). Using a complement-dependent cytolytic technique, Roizman (22) has presented evidence that the surface membrane changes are virus-specific, and that the same antigens are found in the envelope of the virion. The level of cytolytic antibody in sera obtained from herpesvirus-infected rabbits correlated with the titer of neutralizing antibody (24). The data presented in our study of 100 human sera confirm the correlation between antibody activity to cell surface antigens and neutralizing antibody activity. This correlation supports the hypothesis that the antigens demonstrable at the surface of infected cells are similar to those incorporated into the herpesvirus envelope.

The primary objective of this study was to determine whether the surface fluorescence test could be used as a tool to distinguish women with cervical cancer from control women. This approach was taken because of reports showing that surface antigens of cells infected with certain oncogenic viruses could be demonstrated by using the serum of animals immune to transplantation of tumor cells induced by the virus (3). In addition, patients with Burkitt's lymphoma and nasopharyngeal carcinoma have been found to have higher antibody titers to EBV cell surface antigens than patients without these neoplasms (11).

Previously, a greater occurrence of antibodies to herpesvirus type 2, as defined by the relative antibody titers to herpesvirus type 1 and type 2, was found among Negro women than among control women matched for race, age, and social level (16, 20, 25). Among Caucasian women, antibodies to the virus were found in less than half of the women with cervical cancer (19). When sera selected predominantly from Caucasian women were used, antibodies to surface antigens of herpesvirus type 1- and type 2-infected cells were present in the sera of women with carcinoma of the cervix; however, the titers of antibodies detected by immunofluorescence, as with antibodies detected by neutralization, were similar to those found in sera from the selected control women. The surface fluorescence test thus provided no advantages over the micro-neutralization test in distinguishing women with cervical cancer from control women.

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