Serological and Epidemiological Considerations of the Role of Herpes Simplex Virus Type 2 in Cervical Cancer

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Summary

To assess the possible biological significance of the observations that women with cervical cancer tend to be younger at first intercourse than control women, data from 1823 women were analyzed for the relationship between age at 1st intercourse and number of sex partners. Women who were younger at first intercourse had more sex partners than did women who were older at first intercourse. The interdependence of age at first intercourse and number of sex partners does not exclude the possibility that intercourse at an early age represents a biologically significant event in which the neoplastic process is initiated. However, it is equally possible that younger women at first intercourse may have multiple sex partners and be at greater risk of coming in contact with a putative oncogenic agent later in life.

In addition, sera from patients with herpesvirus infections were assayed for cross-reacting and type-specific antibodies. Approximately 80% of the total antibody activity was to the cross-reacting antigen and only 20% was to the type-specific antigens in the sera of patients infected with either type 1 or type 2 virus. Among patients infected with both types of virus, less antibody activity to the type-specific antigens and more antibody activity to the cross-reacting antigens were found. These observations are discussed with respect to case-control seroepidemiological studies.

Introduction

The seroepidemiological evidence associating herpes simplex virus type 2 with squamous cell carcinoma of the cervix has been previously reviewed (2, 8, 12, 14). In most studies, significant differences in patterns of neutralizing antibodies to herpes simplex virus types 1 and 2 have been observed between women with cervical cancer and control women. The meaning of the various antibody patterns observed is not well understood, and interpretation of the case-control differences is difficult (13). This is especially true in terms of attempting to construct a possible pathogenesis of a putative oncogenetic interaction between herpes simplex virus type 2 and cervical epithelial cells.

Studies of the environmental factors involved in the genesis of cervical cancer have provided abundant evidence of a relationship between the neoplasm and patterns of sexual behavior. The 2 variables that best discriminate between women with cervical cancer and control women are the onset of coitus at an early age and exposure to multiple sexual partners (15). These observations and the observed metaplastic changes that normally occur in the cervical epithelium during pubertal development and during the 1st pregnancy (6) suggest that the genesis of cervical cancer may arise from an oncogenic insult to these metaplastic cells. Assuming that herpes simplex virus type 2 is oncogenic, infection of metaplastic cells of the cervical epithelium by the virus during the 2nd decade of life may be responsible for the events leading to the development of cancer.

In examining the above hypothesis, the implications that infection by the virus during the period in life when cervical metaplasia is normally present could be more firmly accepted if the age at 1st intercourse was independent of the number of sex partners. The hypothesis also implies that women with cervical cancer should develop serological evidence of infection by herpes simplex virus type 2 at an earlier age than women who do not develop the disease. These 2 points will be considered in this paper.

Materials and Methods

Study Groups. A total of 1823 women were studied. Of these, 1236 were drawn from the patients attending municipal hospitals serving the city of Houston and the rest of Harris County, Texas. A detailed account of the characteristics of the population and sampling method is described elsewhere (1). The remaining 587 women were drawn from the practices of private physicians and public health clinics in the counties surrounding Charleston, W. Va. A portion of these women were selected because of neoplastic cervical disease, while the remainder were women from small rural communities. The communities were sampled without known bias, and all women within certain age limits were sampled.

A standard questionnaire was administered by 1 of 3 trained nurses. It included a wide range of items including age, race, religion, occupation, place of birth, occupation of husband and father, family income, education, and marital status. Information was also obtained regarding the num-

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2 Presenter.
number of marriages, age at 1st menses, age at 1st pregnancy, number of pregnancies, number of live births, age at 1st sexual intercourse, and number of sexual partners.

Quantitation of Antibodies to Cross-reacting and Specific Antigens. Antibodies were quantitated by a modification of the $^{51}$Cr-release test described by Smith et al. (18). Briefly, BHK21 cells were infected at a multiplicity of 3 to 5 plaque-forming unit per cell and incubated in the presence of $^{51}$Cr ($30 \mu$Ci/ml as sodium chromate) for 18 to 20 hr at 37°C. The cells were then monodispersed. Test sera were heat inactivated and serially diluted in Tris-buffered saline containing 10% heat-inactivated fetal bovine serum. To 0.1 ml of the dilutions of the serum, $5 \times 10^4$ cells in 0.1 ml were added, and the mixtures were incubated for 1 hr at 37°C. Guinea pig complement was then added, and the mixtures were incubated an additional 2 hr, after which 2 ml of cold Tris-buffered saline with fetal calf serum were added and the cells were sedimented by centrifugation. Aliquots of the supernatant fluid were assessed for radioactivity, and the percentage specific $^{51}$Cr release was calculated as previously described (18).

The results of a typical assay are shown in Chart 1. The antibody titer was taken as that dilution which produced 50% specific $^{51}$Cr release; this dilution was also considered to contain 1 biological unit of antibody. To quantitate specific antibody, the serum was diluted to contain 10 units of antibody in 1 ml and adsorbed for 1 hr at 37°C and then overnight at 4°C with $1 \times 10^2$ packed cells, which were infected with one or the other types of virus. The cells were removed by centrifugation and the adsorbed serum was assayed in the $^{51}$Cr release assay, with labeled cells. The titer of specific antibody was taken as the dilution of the adsorbed serum that produced 50% specific $^{51}$Cr release. As exemplified in Chart 1, the total antibody activity of the serum was 1:40, while that of adsorbed serum was 1:8, thus, 20% of the antibody activity of the serum was to the specific antigen and 80% to common antigen.

Results

Age at 1st Intercourse and Number of Sex Partners. The relationship between age at 1st intercourse and the number of sex partners was examined by analyzing data from 1823 women. Since some of the women were selected because they had cervical neoplasia, an initial analysis comparing women with cervical disease and women without cervical disease was performed. No differences with respect to the relationship between age at 1st intercourse and number of sex partners were found; therefore, women with and without cervical disease were pooled in subsequent analysis.

A summary of the attributes examined is presented in Table 1. A greater percentage of the groups of women with the younger mean ages at 1st intercourse had multiple sex partners than the groups of women with the older ages at 1st intercourse. The relationships between age at 1st intercourse and number of sex partners for Negro and Caucasian women of Houston, Texas, are shown in Charts 2 and 3, respectively. A mean of about 6 sex partners was recorded in both groups for women whose age at 1st intercourse was 13 or 14 years. The mean number of sex partners decreased as the age at 1st intercourse became older. The distribution of women with multiple sex partners in relation to age at 1st intercourse is presented in Tables 2 and 3. The mean ± S.D. of age at 1st intercourse was computed for each group. Those whose ages at 1st intercourse were younger than the mean $-1$ S.D. were placed in 1 category. Those whose ages at 1st intercourse were older than the mean $+1$ S.D. were placed in another category, while the remainder were placed in a 3rd category. The attribute of number of sex partners was divided as low for those with less than the mean number and high for the remainder. The data in the tables indicate that the proportion of women with more sex partners was significantly greater for women younger at 1st intercourse than for women older at 1st intercourse.

Women with younger ages at 1st intercourse are, in theory, at risk of having more sex partners than women of comparable age, who were older at 1st intercourse, because the total years of sexual experience would be greater. In order to compensate for this difference in time for exposure to higher numbers of sex partners, the groups were compared diagonally. For example, those women presently 20 to 24 years of age whose ages at 1st intercourse were 14

![Chart 1. Semilogarithmic plot of the percentage specific $^{51}$Cr release from cells infected with type 1 virus versus serum dilution. The antibody titer (dilution producing 50% release) is 1:40 for unadsorbed serum and 1:8 for serum after adsorption with cells infected with type 2 virus.](chart1.png)
Table 1

Summary of study attributes

<table>
<thead>
<tr>
<th>Group</th>
<th>Race</th>
<th>Social Level</th>
<th>No. studied</th>
<th>% of women indicating more than 1 sex partner</th>
<th>Age at 1st intercourse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Houston</td>
<td>Negro</td>
<td>Low</td>
<td>935</td>
<td>88</td>
<td>16.5 ± 2.2*</td>
</tr>
<tr>
<td>Houston</td>
<td>Caucasian</td>
<td>Low</td>
<td>301</td>
<td>66</td>
<td>17.0 ± 3.1</td>
</tr>
<tr>
<td>W. Va.</td>
<td>Caucasian</td>
<td>Low</td>
<td>465</td>
<td>35</td>
<td>17.5 ± 2.5</td>
</tr>
<tr>
<td>W. Va.</td>
<td>Caucasian</td>
<td>Upper</td>
<td>122</td>
<td>11</td>
<td>19.4 ± 2.4</td>
</tr>
</tbody>
</table>

*Mean ± S.D.

Chart 2. Relation of mean number of sex partners to age at 1st intercourse among Negro women of Houston, Texas. The numbers above bars indicate the number of women in each group.

Chart 3. Relation of mean number of sex partners to age at 1st intercourse among Caucasian women of Houston, Texas. The numbers above bars indicate the number of women in each group.
Table 2

<table>
<thead>
<tr>
<th>Race</th>
<th>Social level</th>
<th>Age at 1st intercourse</th>
<th>No. of sex partners</th>
<th>Significance of difference between indicated groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-3</td>
<td>&gt;4</td>
</tr>
<tr>
<td>Negro</td>
<td>Low</td>
<td>&lt;14</td>
<td>73</td>
<td>87 (54)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15-18</td>
<td>330</td>
<td>306 (48)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;19</td>
<td>96</td>
<td>43 (31)</td>
</tr>
<tr>
<td>Caucasian</td>
<td>Low</td>
<td>&lt;14</td>
<td>27</td>
<td>18 (40)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15-19</td>
<td>153</td>
<td>50 (23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;20</td>
<td>37</td>
<td>6 (14)</td>
</tr>
</tbody>
</table>

a Women within 5-year age groups who were younger at 1st intercourse were compared with women in the next-older 5-year age group who had begun heterosexual activity later in life.

Table 3

<table>
<thead>
<tr>
<th>Race</th>
<th>Social level</th>
<th>Age at 1st intercourse</th>
<th>No. of sex partners</th>
<th>Significance of differences between indicated groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>&gt;2</td>
</tr>
<tr>
<td>Caucasian</td>
<td>Low</td>
<td>&lt;15</td>
<td>36</td>
<td>30 (45)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16-19</td>
<td>188</td>
<td>120 (39)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;20</td>
<td>79</td>
<td>12 (13)</td>
</tr>
<tr>
<td>Caucasian</td>
<td>Upper</td>
<td>&lt;17</td>
<td>17</td>
<td>4 (19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18-21</td>
<td>74</td>
<td>7 (9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;22</td>
<td>18</td>
<td>2 (10)</td>
</tr>
</tbody>
</table>

a Women in 5-year age groups who were younger at 1st intercourse were compared with women in the next-older 5-year age group who had begun heterosexual activity later in life.

An appreciation of the influence of present age on the relationship of age at 1st intercourse and number of sex partners can be obtained from Charts 4 and 5 which are derived from data obtained from women in Houston. Among all age groups of Negroes, means of 6 to 7 sex partners were observed for women whose ages at 1st intercourse were 14 years or younger. A significant age-dependent difference in the number of sex partners was also not observed among those women who started heterosexual activity later in life (Chart 4). A somewhat different pattern was found for Caucasian women (Chart 5). An age-associated increase in the mean number of sex partners was not observed among those women whose ages at 1st intercourse were 20 years or older. However, women whose present ages were 39 years or less were compared with those women 25 to 29 years of age whose ages at 1st intercourse were 19 years or older. As can be seen in Tables 2 and 3, the relationship between early age at 1st intercourse and more sex partners remained after adjustment for possible years of sexual exposure. The drop of statistical significance in the groups of Caucasians is explainable by the relatively small numbers of people studied in each age group.

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The data demonstrate that age at 1st intercourse and number of sex partners are not independent variables.

Cross-reacting and Specific Antibodies. Interpretation of seroepidemiological studies is hampered because of the cross-reacting antibodies induced by either type of herpesvirus. In fact, the data in Table 4 suggest that most of the antibody present is cross-reacting, and relatively little antibody is to the specific antigens. Of the total antibody activity to herpesvirus-infected cells, a mean of 17.1% was directed to specific type 2 antigen among patients deemed to have been infected with only type 2 virus. A mean of 19.9% of the total activity of sera from patients infected only with type 1 virus was specific for type 1. The bulk of antibody activity could be adsorbed by cells infected with heterologous virus; means of 82.3 and 79.4% for the 2 groups, respectively. Among patients apparently infected with both types of virus, there was less antibody to specific antigens (7.9%) and more cross-reacting antibody (90.1%).

Estimates of the percentage of cross-reacting antibody by
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X Ml

Age at first intercourse ≤ 14.
Age at first intercourse ≥ 19.

12
11
10
9
8
7
6
5
4
3
2

20 - 29
30 - 39
40 - 49
≥ 50

Present age (years)

Mean number of sex partners

Chart 4. Mean numbers of sex partners in relation to present age and age at 1st intercourse among Negro women of Houston, Texas.

Chart 5. Mean number of sex partners in relation to present age and age at 1st intercourse among Caucasian women of Houston, Texas.

Table 4

Antibodies to cross-reacting and specific antigens

<table>
<thead>
<tr>
<th>Study group</th>
<th>No. of patients</th>
<th>End point titration* (%)</th>
<th>Adsorption with cells infected with heterologous virus* (%)</th>
<th>Specific antibody remaining after adsorption* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 2 infections only</td>
<td>17</td>
<td>67.0 ± 18.4</td>
<td>82.3 ± 10.2</td>
<td>17.1 ± 10.0</td>
</tr>
<tr>
<td>Type 1 infections only</td>
<td>15</td>
<td>25.3 ± 10.8</td>
<td>79.4 ± 8.2</td>
<td>19.9 ± 8.1</td>
</tr>
<tr>
<td>Type 1 and type 2 infections</td>
<td>15</td>
<td>36.7 ± 15.1</td>
<td>90.1 ± 10.1</td>
<td>7.9 ± 10.3</td>
</tr>
</tbody>
</table>

* Percentage cross-reacting antibody = (antibody titer to cells infected by virus yielding lowest titer)/(antibody titer to cells infected by virus yielding highest titer) × 100.

The excess of antibody activity to herpes simplex virus type 2 among women with cervical cancer, compared with control women, cannot be accounted for simply by differences in patterns of sexual behavior of the 2 groups (1). Control women pair-matched to women with cancer on the basis of multiple attributes, including age at 1st intercourse and number of sex partners, had significantly less antibody activity to the virus than the cancer cases. On analysis of cases and the pool of control women, it was the women with cancer who began heterosexual activity at or after 16 years of age and/or who had 1 to 3 sex partners that differed substantially in the occurrence of antibodies from control women with similar attributes. Women with cancer and...
control women who began heterosexual activity at or before 15 years of age and/or who had 4 or more sex partners were similar with regard to occurrence of antibodies to the virus. This would be anticipated with respect to sex partners. Since multiple sex partners increases the risk of being infected by the venereally transmitted herpes simplex virus type 2. The data presented in this paper indicate that women who began heterosexual activity early in life have more sex partners than women who began heterosexual activity later in life. The excess of antibodies to herpes simplex virus type 2 in relation to early age at 1st intercourse may thus represent an interdependence of the age at 1st intercourse and the number of sex partners in a lifetime.

Analysis of neutralizing antibody titers to herpes simplex virus type 2 by age has revealed differences between cases and controls. The mean antibody titers to the virus were found to be age dependent, and increased until age 40 years among control women, while the titers were not age dependent among women with cancer (1, 2). A similar but not statistically significant trend was observed among women of religions other than Moslem, but not among Moslem women, in Yugoslavia (9). This suggests that women with cervical cancer were infected earlier in life by the virus than control women. However, an age-dependent increase in antibody activity was observed among women with abnormal cervical cytology in Birmingham, England, while the activity of control women was constant with age (17). In addition, fluctuations in antibody activity in relation to the presence or absence of cervical lesions have been reported (5, 17, 19, 20). Thus, from case-control retrospective studies, it is not possible to conclude at what age infection with herpes simplex virus type 2 occurred. Data from prospective analysis of selected groups of women indicate that the viral infection precedes the appearance of cervical neoplasia (5, 12); however, the determination of whether or not infection during the period when squamous metaplasia of the cervix normally occurs is associated with an increased risk of developing a neoplastic lesion remains to be established.

Herpesvirus virion antigens appear at the surface of infected cell. Quantitation of antibodies to surface antigens of infected cells has yielded results similar to those obtained by neutralization assays (13). We have found this to be true also for antibody analysis by 31Cr-release test (H. McClung, P. Seth, and W. E. Rawls, unpublished results). With the 31Cr-release test, data have been obtained that suggest that, in human sera, only about 20% of the total antibody activity is to type-specific antigens, while about 80% is to the cross-reacting antigen.

A major structural antigen of herpesvirus has been identified which contains both cross-reacting and type-specific antigenic determinants. In sera from immune rabbits, antibodies to the cross-reacting antigen accounts for all cross-neutralizing activity (16). The data presented in Table 4 suggest that antibodies to the cross-reacting antigens are of varying quality in human sera. Antibodies that appeared following an initial herpes simplex virus type 2 infection reacted with the antigen of cells infected by type 1 virus in approximately equal amounts at 1 hr at 37° and overnight at 4°. Antibodies induced by an initial infection with type 1 virus reacted poorly with the cross-reacting antigen of type 2-infected cells after incubation for 1 hr at 37° compared with overnight at 4°. Patients infected with both types of virus tended to have less antibody activity to the specific antigens and more cross-reacting antibody. Antibody assays that have been used in seroepidemiologic case-control studies thus appear to assess primarily the quantity and quality of antibodies to the cross-reacting antigens.

Infection and, possibly, reinfection with both types of herpesvirus are dependent upon cultural conditions. With reinfection, it is possible that there may be an increase or broadening in cross-reacting antibody. Infection by herpes simplex virus type 1 was found to occur early in life among isolated Indian tribes. With increasing age prior to puberty, there was an increasing percentage of subjects with substantial cross-neutralizing activity to type 2 virus. This apparent broadening of the type 1 response was not observed in subjects from New Haven, Conn. (4). The experience of a population to type 1 virus may influence the relative quality or quantity of antibody to the cross-reacting antigen of type 2 virus. The log10 antibody titer to type 2 virus divided by the log10 antibody titer to type 1 virus and multiplied by 100 (II/I index) is an expression of cross-reaction. The II/I index, which maximally distinguishes women with cervical cancer from control women, was found to be 93 for Negro women and 87 for Caucasian women in a study conducted in Houston, 89 for women in Uganda, and 82 for Israeli women of European origin (2). In general, the percentage of women with cancer who were considered positive at a II/I index of 83 decreased as the socioeconomic conditions of the population from which they were sampled increased (11, 14). While speculative, this could reflect differences in exposure of the populations to type 1 virus, rather than absolute differences in past infections by type 2 virus, among the study subjects from different geographic areas.

While there is an association between neutralizing activity to herpes simplex virus type 2 and carcinoma of the cervix, it has not been established that the association represents one in which the virus is etiologically related to the neoplasm. The presence of antibodies to nonstructural viral antigens among patients with the disease (3, 7) and other data support the hypothesis that the virus is oncogenic. If it is assumed that the virus is oncogenic, further studies will be required to determine the effect of the age at infection upon the development of the disease. Such information would be of value in consideration of possible prevention through vaccination.

Acknowledgments

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