In Vivo and in Vitro Measurements of the Relationship of Human Squamous Carcinomas to Herpes Simplex Virus Tumor-associated Antigens

Ariel C. Hollinshead, Paul B. Chretien, O'Bong Lee, John L. Tarpley, Suzanne E. Kerney, Norman A. Silverman, and John C. Alexander


Summary

An additional 244 unfiltered sera have now been studied in a series of controlled, coded tests to determine the relationship of squamous carcinomas of the head and neck and cervix to the presence of complement-fixing antibodies to herpesvirus-tumor-associated antigens (HSV-TAA) in both tumor-bearing and cured patients. Ninety % of sera from patients with squamous carcinomas had antibodies to HSV-TAA, in contrast to 11% of sera from patients with nonsquamous cancers and 4% of sera from normal individuals. The temporal relationship to Stage 1 laryngeal carcinomas suggests that HSV-TAA appearance precedes the immune defects.

An in vitro correlate of the previously demonstrated specific delayed hypersensitivity reactions in controlled skin tests of squamous carcinoma patients with HSV-TAA is reported. In leukocyte migration inhibition tests, the migration indices after incubation with HSV-TAA of peripheral blood leukocytes from patients with squamous carcinoma (k = 0.847) were in definite contrast to migration indices seen for normal leukocytes (k = 1.037) and patients with nonsquamous solid cancers (k = 1.03). Thus, these polypeptides elicit both humoral antibody response and cell-mediated reactivity.

Introduction

HSV-TAA1 can be separated from human squamous carcinoma cells and can also be induced in several different types of fetal and adult human cell cultures by superinfection with any one of several strains of herpesviruses types 1 and 2. HSV-TAA consists of 2 polypeptide chains that can be gently separated for preservation of immunogenicity. Antibodies to HSV-TAA can be detected in sera from a high proportion of patients with squamous carcinomas. In addition, HSV-TAA also produces delayed hypersensitive skin reactions specific for patients with squamous carcinomas. This in vivo response to HSV-TAA can be correlated with an in vitro measurement of the cellular immune response using the leukocyte migration inhibition test. All of these observations have been reported elsewhere (1, 2, 4, 5-10) and are summarized in this report, with additional reporting of further serological studies.

As shown in Chart 1, separation of HSV-TAA is best accomplished by very gentle sonic disruption of the soluble components, followed by further separations, finally using polyacrylamide gel electrophoresis. The appropriate polyacrylamide gel electrophoresis regions (Fig. 1) are sliced and eluted: the 2 protein bands (Chart 2) can be purified and concentrated for test procedures. Some of the characteristics of HSV-TAA are listed in Table 1.

Materials and Methods

Coded sera were obtained by the Department of Surgery of the National Cancer Institute, Bethesda, Md., both filtered and unfiltered; in this report we show results using only sera that were unfiltered, obtained within 5 months prior to CF testing. CF reactivity to HSV-TAA of 3+ or 4+ was scored as positive; and 0, trace, 1+, or 2+ reactions were considered negative. The diagnosis of squamous carcinoma was histologically confirmed in each patient. Patients were staged by the guidelines established by the American Joint Committee for Cancer Staging and End Results Reporting.

Preparation of HSV-TAA. As previously described (5, 6), thin-layer cultures of HEK cells were grown in 32-oz glass bottles, washed, and incubated with serum-free minimal essential Eagle's media at pH 7.1. The HEK cells were infected with HSV type 1 (KOS strain) or HSV type 2 (SAV strain) at a multiplicity of 35 plaque-forming units/cell. Cells were harvested at 24 hr and 10% cell suspensions in 0.9% NaCl solution were frozen at -70° overnight. The quickly thawed material was subjected to stepwise sequential low-frequency sonic disruption (Raytheon Inc. unit, 9 kc/sec) for 3 time intervals (3, 1.5, and 6 min) with centrifugations without brake at 100,000 g for 1 hr after each interval. The supernatants from each sonic disruption interval were combined and a determination made of protein content.

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1 Presented at the symposium "Immunological Control of Virus-associated Tumors in Man: Prospects and Problems," April 7 to 9, 1975, Bethesda, Md.
2 Presenter.
3 The abbreviations used are: HSV-TAA, herpesvirus-tumor-associated antigens; CF, complement fixing; HEK, human embryonic kidney; HSV, herpes simplex virus; SDS, sodium dodecyl sulfate; RPMI, Roswell Park Memorial Institute; FCS, fetal calf serum.
The method of gradient polyacrylamide gel electrophoresis used for further separation has been described previously (11). For this procedure, sterile Pyrex tubes measuring 6 mm internal diameter x 12 cm were immersed in Eastman Kodak Photo Flo, which has been diluted with 200 parts of sterile, triple-distilled water. This solution is removed so that no Photo Flo remains, and the gradient gels are formed on the wet tubes. Eastman Kodak polyacrylamide stock gel solutions were prepared by adding 2 parts of the stock gel solution to 1 part distilled water mixed with 1 part of stock gel buffer (Tris-buffered to pH 8.9 with 1 N HCl and containing 0.23% N,N,N',N'-tetramethylethylenediamine). This mixture is added to an equal volume of 0.145% ammonium persulfate (Fisher Scientific Company, Pittsburgh, Pa.). The gel solutions are layered as follows: 10% solution to the 6-cm mark, 7% solution to the 7.5-cm mark, 4.75% solution to the 8.5-cm mark, and finally the 3.5% solution to the 10-cm mark. The columns are layered with distilled water about 4 mm above the 3.5% gel solution and allowed to polymerize for 40 min. After polymerization, the water is removed and each tube inserted into the upper buffer chambers of the electrophoretic system. The upper and lower buffer chambers are filled with Tris-glycine buffer, pH 8.3. Tubes with air bubbles are not used. Two drops of bromphenol blue are added to the upper buffer chamber.

HSV-TAA-containing cell sonic extracts are mixed with about 14 µg of sucrose to be sure that the solution is heavier than water. The solution is layered onto the columns with a Hamilton syringe; the material is allowed to flow slowly down the inner edge of the glass. After electrophoresis is done at room temperature at 4 mA/column, the gels are removed by placing them in cool, sterile, distilled water and rimming the glass with a finely tapered sterile needle. Sometimes a small amount of water is injected to help remove the gel. Representative gels from each electrophoresis are stained with Coomassie Brilliant Blue for 10 min, the stain is removed, and the test tube is filled with 12% trichloroacetic acid. These stained gels were kept in the dark and read at Days 1, 2, and 7. On Day 2, the trichloroacetic acid is poured off and the tube is refilled with 5% acetic acid. Analysis of bands at Day 2 permits separation of the remaining material kept at 4°C until the bands are located in the stained gel both by measurement and by densitometry, using a Gelman Inc. Model ACD-15 densitometer. The remaining gels are then sliced for precise regions. Comparable regions are pooled, eluted with 0.9% NaCl solution at 4°C for 50 hr, concentrated by ultrafiltration, rediluted 100-fold, and reconcentrated to help eliminate any toxic dialyzable substances. A standard preparation is established for use in several tests.

An attempt is made to control the conditions for the production of HSV-TAA. However, a great deal depends upon the use of thin, even-cell monolayers; upon the source, age, and passage number of the human embryonic kidney cell cultures; upon penetration by the virus; and upon the many variations that can occur during the complex process necessary for separation and purification of the 2 polypeptide bands used for complement-fixation tests. The crude soluble pool contains many particulates, so that the total protein yield after further separation by polyacrylamide gel electrophoresis is between 20 and 40% of the protein concentration in the crude sonic extract prior to separation (Table 2). If an intermediate step, using Sephadex G-200, is used, the recovery from input protein in the final polyacrylamide gel electrophoresis step is vastly improved. However, we discovered that this intermediate step results in a total overall loss of protein. The recovery of HSV-TAA (Table 2) apparently does not depend upon whether or not the operation is large scale (200 bottles) or small scale (52 bottles), and the total yield of this antigen is between 7 and 11% of the total crude soluble protein. Tests using polyacrylamide gel electrophoresis Region 3 antigens are almost as specific and this region contains 25 to 27% of total protein.

In the 1st experiment the soluble pool was separated by polyacrylamide gel electrophoresis using 159 µg/gel. Two hundred four gels were run. The Region 3 (Chart 2) containing the 2 polypeptide bands was sliced, eluted, and concentrated, for a protein yield of 9.5 mg. This material was appropriately diluted for use in complement fixation tests by standard macrotechnique, using 2 units of complement and 144 µg antigen/test. In the 2nd, 3rd, and 4th experiments the 2 polypeptide bands were sliced, eluted, and concentrated for testing (Chart 2, HSV-TAA). A 2nd polyacrylamide gel electrophoresis separation of 100 µg of each preparation was stained and analyzed to ensure the purity of HSV-TAA. In the 2nd experiment, 240 µg protein per gel was used for separations from 1430 gels, with a yield of 24 mg of HSV-TAA. In the 3rd experiment, 120 µg protein were separated on each of 1300 gels with a yield of 15 mg of HSV-TAA. In the 4th experiment, 99.6 µg protein were separated on each of 230 gels with a yield of 1.67 mg of HSV-TAA. HSV-TAA protein in the amount of 44 µg was used per test.

Detailed analyses of polyacrylamide gel electrophoresis
Herpesvirus Tumor-associated Antigens

Fig. 1. Polyacrylamide gel electrophoresis Numbers 1 and 2 show the lower-molecular-weight protein bands associated with mature herpesviruses types 2 (SAV strain) and 1 (KOS strain), respectively. Gels 3 and 4 show the protein bands associated with cell harvests of the same herpesviruses types 1 and 2 superinfected cells containing low-molecular-weight cell, virion, and nonstructural antigens, including HSV-TAA (arrows).

patterns of HSV-TAA of both infected cells and cancer cells have been published (9). The special method of polyacrylamide gel electrophoresis had to be used since we could not use SDS as it destroys the antigenic activity of one of the HSV-TAA bands. However, HSV-TAA could be separated on SDS gels and did show the same 2 bands. Table 2 contains a summary of the protein yields obtained at each step in the separation procedure. Chart 1 delineates the steps taken in purification and separation of the HSV-TAA polypeptides. Fig. 1 illustrates the difference in polyacrylamide gel electrophoresis patterns between lower-molecular-weight mature HSV types 1 and 2 virus components and shows the presence of the lower bands associated with HSV-TAA in the polyacrylamide gel electrophoresis separations of the early harvests of superinfected HEK cell cultures.

Antigenic Specificity. Although HSV-TAA was used for the last 3 tests, we have found, as with the 1st test in this new series, that polyacrylamide gel electrophoresis Region 3 antigens are pure enough to permit specific CF testing with groups of patient sera. To monitor for specificity, CF titrations of the antigens used in those tests are routinely performed using HSV-TAA specific antisera (our Pools 3 and 6) and are also controlled using specified human squamous cancer sera of known titer. HSV-TAA-specific antisera was prepared by injection of 5 preparations of the 2 polypeptide bands that were specifically sliced and eluted from many gels and reseparated by polyacrylamide gel electrophoresis for purified antigen. Six rabbits were used for antisera preparation. Two each received footpad injections and s.c. injections of 10, 50, and 100 μg HSV-TAA mixed with Freund’s complete adjuvant, respectively, for the 1st injections. This was followed in 1 month with fresh preparations of HSV-TAA mixed with incomplete Freund’s complete adjuvant, respectively, for the 1st injections. This was followed in 1 month with fresh preparations of HSV-TAA mixed with Freund’s complete adjuvant, followed by injections ofHSV-TAA without adjuvant, and in the 3rd month a booster with HSV-TAA plus incomplete Freund’s adjuvant, and in the 4th month another booster with HSV-TAA alone. Immunodiffusion tests indicated specific precipitins in 2 of...
Soluble components PAGE region 3 HSV-TAA

Chart 2. A special method of polyacrylamide gel electrophoresis (PAGE) is used for separation on 10, 7, 4.75, and 3.5~ layered gel solutions of soluble components of HSV-TAA-containing early harvests of herpesvirus-infected HEK cells. Tracking dye (TD) is bromphenol blue. Protein bands were stained with Coomassie Brilliant Blue. Further separation of the more anodic components (Region 3) also contain HSV-TAA. The 2 polypeptide bands (HSV-TAA) can be separated either directly from the soluble components or in a 2-step procedure, from the Region 3 components. HSV-TAA comprises about 7 to 11% of the lower-molecular-weight soluble components present in these polyacrylamide gel electrophoresis separations.

these sera. In CF tests, antisera from Rabbits 3 and 6 were highly specific for the purified HSV-TAA, negative to control preparations of HEK cells and purified herpesviruses as well as homogenates of nonsquamous cancer cells and to purified adenoviruses, cytomegaloviruses, the new SMON herpesviruses. The CF titration of the 2 purified polypeptides is consistent with the CF titration of the polyacrylamide gel electrophoresis Region 3 material adjusted for an equivalent quantity of HSV-TAA and, therefore, the routine use of Region 3 material is satisfactory.

If the same type of cell culture and the same strain of herpesvirus is used throughout a given series of tests and if the freshly prepared antigen is used immediately, the HSV-TAA titer per a given protein concentration has proven to be fairly consistent. In the present tests, for Experiment 1, 144 μg polyacrylamide gel electrophoresis Region 3 were used; and for the last 3 experiments, 44 μg of purified HSV-TAA were used. Each of the 4 preparations were of a consistent titer with specific HSV-TAA antisera (Rabbit Sera 3 and 6) and with control squamous cancer sera of known titer.

**Lymphocyte Migration Inhibition Tests.** Lymphocyte migration inhibition tests were conducted with precision in a continuous, rapid sequence as follows. Heparinized venous blood was separated into 10-ml aliquots, placed in the 15-ml blue-top test tubes, and gravity sedimented at a 45° angle for 1 hr at 37°. With plastic pipets, the leukocyte-rich plasma was aspirated and centrifuged at 1,000 rpm for 5 min at 4°. The plasma was aspirated and the cells were resuspended with 20 ml of RPMI 1640 tissue culture media containing penicillin, streptomycin, and 10% FCS. This suspension was centrifuged and resuspended in RPMI 1640 at 37°C. The cells were then resuspended in RPMI to 1.0 × 10⁸ WBC/ml. After thorough but gentle resuspension, the cells were aliquoted in 200-μl portions into Falcon 3040 Microtest II plate wells. To each well, 50 μl RPMI 1640 + 10% FCS or 50 μl freshly prepared HSV-TAA were added to give a final cell

<table>
<thead>
<tr>
<th>HEK bottles (32 oz)</th>
<th>Protein recovered after sonic disruption, soluble pool (mg)</th>
<th>Amount protein separated on each gel (μg)</th>
<th>Gels used for separation</th>
<th>Protein yield of polyacrylamide gel electrophoresis Regions 1 and 2 (mg)</th>
<th>Protein yield of Region 3, HSV-TAA (mg)</th>
<th>Protein total yield after polyacrylamide gel electrophoresis (mg)</th>
<th>Polyacrylamide gel electrophoresis total protein yield/soluble pool (%)</th>
<th>Region 3 of HSV-TAA protein yield/soluble pool (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>34.36</td>
<td>159</td>
<td>204</td>
<td>4.37</td>
<td>9.5³</td>
<td>13.87</td>
<td>40.37</td>
<td>27.65</td>
</tr>
<tr>
<td>200</td>
<td>342.2</td>
<td>240</td>
<td>1430</td>
<td>47.75</td>
<td>24³</td>
<td>71.75</td>
<td>20.97</td>
<td>7.01</td>
</tr>
<tr>
<td>100</td>
<td>136.32</td>
<td>120</td>
<td>1300</td>
<td>17.25</td>
<td>15³</td>
<td>32.25</td>
<td>23.66</td>
<td>11¹</td>
</tr>
<tr>
<td>52</td>
<td>23.4</td>
<td>99.6</td>
<td>230</td>
<td>5.6</td>
<td>1.67³</td>
<td>7.27</td>
<td>31.07</td>
<td>7.14</td>
</tr>
</tbody>
</table>

³ Lowry method.
³ Polyacrylamide gel electrophoresis Region 3.
³ HSV-TAA.
concentration of $7.5 \times 10^7$ cells/ml. The numbers of assays were limited since the production of small amounts of purified, labile HSV-TAA requires considerable time and expense. The suspensions were incubated for 1 hr at 37° with 5% CO$_2$ and humidity. The cells were then very thoroughly but gently resuspended and drawn into sterilized, siliconized, 25-μl capillary tubes. The tubes were flamed sealed, then cooled and stored in ice. The tubes were placed in the 15-ml conical centrifuge tubes with gauze packed in the bottoms, and they were centrifuged at 3,000 rpm for 10 min at 4°. The tubes were nicked and broken below the WBC platelet layer and placed in the sterilized leukocyte migration plate chamber (LMT Plate; Microbiological Associates, Bethesda, Md.), which had been dotted with 2 dabs of silicone grease to hold the tubes in place. The chambers were sealed with coverslips that were secured with silicone grease. The chambers were incubated at 37°. After 18 hr the migration areas were projected, traced, and measured by planimetry.

A minimum of 7 to 9 replicate tubes for control and 7 to 9 tubes preincubated with HSV-TAA were prepared from each blood sample. The results given for each patient in Chart 3 represent the mean of the individual migration areas of cells in cultures containing antigen divided by the mean migration area of cells in cultures containing only media and FCS. The migration index for each sample was then determined by the formula, $MI = migration in antigen/migration in media$.

Results

The neutralizing and CF antibody titers of sera from patients with squamous carcinoma, normal individuals, and nonsquamous carcinoma patients to mature herpesviruses is evenly distributed, in a series of sera selected for a study of antibody to HSV-TAA. Although the same levels of antibody to the mature virus are measured in the sera, the distribution of positive antibody levels to HSV-TAA is quite different. In an ongoing series of studies of the CF activity of unfiltered human sera to HSV-TAA (Table 3), over 90% of patients with squamous carcinomas of the head and neck and of the cervix reacted with a HSV-TAA, in comparison with only 4% reactivity by sera from normal individuals and only 11% of sera from nonsquamous patients. No significant differences were detected in a study of the distribution of positive reactivity to HSV-TAA according to age, grouping patients less than 50 years of age, 50 to 60 years of age, and greater than 60 years of age. Likewise, no significant differences could be detected between races for degree of positive reactivity with the antigen. Interestingly, the average titer of sera from patients with Stage 1 laryngeal cancer (1:2.6) was lower not only than that of patients with Stage 2 laryngeal tumors (1:3.1) and oral tumors (1:3.5), but less than that of Stage 1 oral tumors (1:4) as well. A relationship was seen to the general immunological status of patients with squamous carcinomas. As shown in Table 4, the general immune reactivity of patients with various types of cancers revealed that there was direct immunosuppression induced in patients with squamous carcinomas and that these patients remain immunosuppressed throughout life.

This is in striking contrast to patients with nonsquamous cancers, where the immune reactivity returns to normal in the cured patients. There is 1 exception to this general observation. Stage 1 laryngeal carcinomas are, by definition, confined to a vocal cord that is not functionally altered by the tumor, and such tumors cannot exceed 3 to 4 mm in diameter, while elsewhere in the head and neck region carcinomas of 2 cm or less are classified as Stage 1. Thus, the State 1 laryngeal carcinomas are the smallest cancers in the head and neck region. In this group of Stage 1 laryngeal...
squamous carcinomas (Table 4), no suppression was seen in the preoperative and cured groups. However, 63% of the patients' sera from these groups were positive for HSV-TAA antibodies. This suggests that the HSV-TAA expression precedes the impaired cellular immunity associated with these cancers.

Cervical and breast cancer patients were age and stage matched and were tested for positive delayed hypersensitive skin reactivity to mumps and to streptokinase-streptodornase. Patients positive to one or both recall antigens were then further tested using HSV-TAA and a 2nd tumor-associated antigen, unrelated to herpesviruses, that were carefully prepared from cervical tumor cells that showed a high concentration of HSV-TAA antigen as tested by highly specific HSV-TAA rabbit antisera. Control materials from similar separations of normal vaginal cells were included in the test. As shown in Table 5, a high percentage of cervical cancer patients reacted to both of the tumor cell antigens but were not responsive to antigens prepared from the normal vaginal cells. The lack of reactivity by breast cancer patients must be studied further, since the number tested would not be statistically valid. In separate studies of tumor-associated antigens from breast cancer cells, we have seen that patients with other forms of gynecological cancers occasionally respond to some of the tissue-associated antigens isolated from the breast cancer cells, although there is also present a fairly specific breast cancer tumor-associated antigen (3). After incubation with HSV-TAA of peripheral blood leukocytes (Chart 3) a significant (p < 0.0005) inhibition of migration of leukocytes from patients with squamous carcinomas of the head, neck, and cervix occurred as compared to normal sera.  

Table 3  
Complement-fixing activity of human sera to HSV-TAA  

<table>
<thead>
<tr>
<th>Squamous carcinoma patients</th>
<th>Positive/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head and neck</td>
<td>85/93 (91)</td>
</tr>
<tr>
<td>Cervix</td>
<td>42/48 (88)</td>
</tr>
<tr>
<td>Total</td>
<td>127/141 (90)</td>
</tr>
<tr>
<td>Normal individuals</td>
<td>3/67 (4)</td>
</tr>
<tr>
<td>Nonsquamous carcinoma patients</td>
<td>4/36 (11)</td>
</tr>
<tr>
<td>Total</td>
<td>7/103 (6)</td>
</tr>
</tbody>
</table>

\* Number in parentheses, percentage of positive patients.

Table 4  
The immune reactivity includes: (a) PHA-induced in vitro lymphocyte reactivity; (b) serum effect on normal lymphocyte reactivity; (c) dinitrochlorobenzene contact sensitivity.  

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Preoperative</th>
<th>Cured</th>
<th>HSV-TAA antibodies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous carcinomas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 1 laryngeal*</td>
<td>No suppression</td>
<td>No suppression</td>
<td>63</td>
</tr>
<tr>
<td>All other head and neck, including Stage 1 oropharyngeal*</td>
<td>Immunosuppression</td>
<td>Immunosuppression</td>
<td>91</td>
</tr>
<tr>
<td>Pelvic squamous carcinomas</td>
<td>Immunosuppression</td>
<td>Immunosuppression</td>
<td>88</td>
</tr>
<tr>
<td>Nonsquamous cancers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcomas</td>
<td>Immunosuppression</td>
<td>No suppression</td>
<td>11</td>
</tr>
<tr>
<td>Melanomas</td>
<td>Immunosuppression</td>
<td>No suppression</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinomas</td>
<td>Immunosuppression</td>
<td>No suppression</td>
<td></td>
</tr>
<tr>
<td>Normal individuals</td>
<td>No suppression</td>
<td>No suppression</td>
<td>4</td>
</tr>
</tbody>
</table>

\* <3 to 4 mm tumor diameter.  
\* ≥2 cm tumor diameter.

Table 5  
Cutaneous hypersensitivity reactions to autologous and allogeneic cervical squamous cancer cell and control vaginal cell polyacrylamide gel electrophoresis regions in cancer patients  

| Delayed hypersensitivity reactions* (no. positive/no. tested) |
|-----------------------------------------------|----------------|---------------|
| Cervical cancer patients                      | Breast cancer patients |
| Cervical tumor cells                          | Polyacrylamide gel electrophoresis Region 1 | 0/16 | 0/9 |
| Polyacrylamide gel electrophoresis Region 2 (TAA) | 15/16 | 0/9 |
| Polyacrylamide gel electrophoresis Region 3 (HSV-TAA+) | 16/16 | 0/9 |
| Vaginal cells, normal                         | Polyacrylamide gel electrophoresis Region 1 | 0/16 | 0/9 |
| Polyacrylamide gel electrophoresis Region 2 | 0/16 | 0/9 |
| Polyacrylamide gel electrophoresis Region 3 (HSV-TAA-) | 0/16 | 0/9 |
| Recall antigens                               | Mumps          | 11/16 | 7/9 |
| Streptokinase-streptodornase                  | 14/16 | 9/9 |

* >5 mm induration at 48 hr is considered positive.
pared with leukocytes from normal individuals and leukocytes from patients with nonsquamous solid cancers. These findings correlate with the observations of delayed hypersensitive skin reactions to HSV-TAA specific for patients with squamous carcinoma and demonstrate that an in vitro correlate of the in vivo cell-mediated immune response may permit measurements of immunological reactivity to purified HSV-TAA.

Discussion

Honess et al. (12) have analyzed type-specific and type-common structural and nonstructural HSV antigens. Using SDS-polyacrylamide gel electrophoresis, they found 15 polypeptides with molecular weights of 25,000 to 100,000, some of which were nonstructural (13). A nonstructural thymidine kinase polypeptide with a molecular weight of 44,000 was associated with a structural glycopolypeptide with a molecular weight of 47,000. The role of HSV-TAA in cell-mediated immunity has been reported and is yet another example of a function for nonstructural antigens (1). Other workers have shown that thymidine kinase genes in HSV-transformed cells are possibly not under the control of cellular mechanisms (14). It stands to reason either that a small fragment of HSV below the level of detection of present annealing experiments, or that a defective herpesvirus that produces nonstructural thymidine kinase antigens and induces virus-specific DNA polymerase could be involved in cell transformation with or without new enzymatic conditions either at the genetic or epigenetic level. This process in different types of human tissue must be further studied.

We have shown previously (9) HSV-TAA is truly virus specific, that it is present in many squamous carcinomas of the cervix and head and neck, and that several herpesvirus strains are capable of inducing the antigens in a number of different cell types. It is important to consider various factors that may influence the expression of HSV-TAA. We have found that HSV-TAA expressions vary not only in the quantities that can be directly isolated from squamous carcinoma cells, but also in the quality of production when different wild-type and domestic HSV strains or different cell cultures are used. Some HSV-TAA expressions may also be shared with other herpesviruses, may vary in cancers from different species, and may meet with interference due to the presence of defective viral particles or competitive antigens in certain cell types. For example, we have found that certain freshly isolated strains of cytomegalovirus also produce similar but not identical antigens, whereas certain HSV-induced hamster tumors contain only 1 of the HSV-TAA antigens, and that certain antigens of human embryonic lung cells are closely associated with and interfere with the expression of 1 component of HSV-TAA when these cultures are used (A. Hollinshead and B. Eddy, unpublished data).

It is important to think not only of genetic evolution but also of genetic revolution. The expression of the highly sophisticated herpesviruses are different in human tumors than in animal models; it would be important to study those models which reflect changes similar to man. The cooperation with other viral forms must also be studied; for example, C-type RNA viruses may be involved in transformation of some tissue types but not in others. We are less concerned with viral structural glycoproteins (9) because these vary in association with tumors and are concerned with the smaller molecular weight HSV-TAA proteins; examination of approximately 100 tumor specimens has convinced us of the constant association with HSV-TAA.

We are concerned with careful identification of the 2 HSV-related antigens associated with human squamous carcinomas and with thorough testing of the relationship of these antigens to the malignant process. The relationship of HSV-TAA to squamous carcinomas is accompanied by what appears to be a lack of relationship to sex, age, race, and tumor status (with 1 exception) of the patients. The findings in patients with Stage 1 carcinoma of the oropharynx may provide insight into the temporal relation between tumor development and suppressed cellular immunity associated with squamous carcinomas (9, 15, 16). Since Stage 1 laryngeal carcinomas are, by definition, confined to a vocal cord that is not functionally altered by the tumor, such tumors cannot exceed 3 to 4 mm in diameter, while elsewhere in the head and neck region carcinomas 2 cm or less are classified as Stage 1. Thus Stage 1 laryngeal carcinomas are the smallest cancers encountered in the head and neck region. That the general immune reactivity of patients with these tumors did not differ from that of the normal population, despite a 63% positive CF reactivity, suggests that HSV-TAA expression precedes the impaired cellular immunity associated with these cancers. The suppressed immune responses in patients with larger tumors support this hypothesis and the corollary that a prolonged period of HSV-TAA expression may be an important antecedent of suppressed host immunity. The normal immune responses of patients with Stage 1 laryngeal tumors who also had positive CF reactivity may reflect the relation between duration of HSV-TAA expression to impaired immunity, since the time since onset of Stage 1 vocal cord tumors, as a generalization, can be presumed to be relatively short. The relatively low CF reactivity of Stage 1 laryngeal cancer patients (63%) provokes reasonable questions concerning the relation between HSV-TAA expression and tumor inductions, particularly in view of the epidemiological correlation between a putative carcinogen, tobacco products, and head and neck squamous carcinoma. It is possible that the 6% incidence of CF reactivity in normals and nonsquamous cancer controls may define a cohort of the population with a relatively greater potential for the development of head and neck cancers when exposed to carcinogens, especially tobacco products, due to increased HSV-TAA expression through the action of tobacco products or other carcinogens. This hypothesis implies high incidences of HSV-TAA expression in patients with even small cancers. The insensitivity of the CF assay may account for our disparate finding. Another correlation between the presence of squamous carcinomas and immune reactivity to HSV-TAA is demonstrated by the skin test assay of a specific delayed hypersensitivity response as well as by the lymphocyte migration inhibition test assay of the specific effect of HSV-TAA on in vitro leukocyte migration. These studies have encouraged us to undertake the development of a radioimmunoassay for...
HSV-TAA in view of the unparalleled sensitivity of this technique.

Thus, these polypeptides elicit both humoral antibody response and cell-mediated reactivity. These findings in this study parallel the results obtained from other assays of cellular reactions to various human cancers using tumor cell extracts. These similarities provoke speculation concerning the relation of the humoral and cellular responses to HSV-TAA in patients with squamous carcinomas to the immunological responses to other antigens associated with or unique to these tumors (1, 9). Studies that define this relationship have relevance to immunotherapy regimens that utilize tumor cells or cell components. The association of squamous cell carcinoma to HSV-TAA as shown by in vitro and in vivo studies provides a rationale for the comparison of the effectiveness of HSV-TAA and other tumor-associated antigens in immunotherapy regimens.

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References

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