Frequency of Antibody to a Virus-induced Tumor-associated Antigen (AG-4) in Japanese Sera from Patients with Cervical Cancer and Controls

Takashi Kawana, J. D. Cornish, Marie F. Smith, and Laure Aurelian

Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo, Japan; and Departments of Laboratory Animal Medicine, Biological and Biophysical Sciences and Microbiology, National Cancer Institute, Baltimore, Maryland

SUMMARY

Microquantitative complement fixation was used to detect antibody to AG-4, a tumor-associated herpesvirus type 2-induced antigen, in sera from Japanese patients with cervical neoplasia. The prevalence of antibody to AG-4 in cervical cancer patients is 47% as compared to 7% in control women. That AG-4 antibody is less prevalent in Japanese than in American cancer patients reflects the frequency of genital herpesvirus type 2 infections in Japan. These findings confirm the association of AG-4 with cervical cancer in a proportion of Japanese patients with neoplastic disease; they implicate factors other than herpesvirus type 2 in another proportion of cervical cancer patients. The possibility that herpesvirus type 1 is one of these factors is considered.

INTRODUCTION

The original observation by Naib et al. (16) associating HSV-2 with squamous cancer of the uterine cervix was followed by numerous seroepidemiological studies inquiring into the presence of HSV-2-neutralizing antibody in both women with cervical cancer and controls. A significantly higher prevalence of HSV-2 antibody in the cancer groups was reported by most of these studies (13), but this prevalence was not confirmed in Japan (12). Thus, using the kinetics of neutralization assay, we found that the frequency of HSV-2 antibody was uniformly high (70%) in Japanese cancer and control subjects. On the other hand, the prevalence of HSV-1 antibody in both Japanese study groups was similar to that reported by other investigators (13), and ranged between 85 and 98%. In view of these results, and since one-half of the genital isolates in this country are biologically and immunologically identical to HSV-1 (11), we suggested that in Japan, HSV-2 was not associated with cervical cancer.

More recently, an "early" virus-induced antigen designated AG-4 was described by Aurelian et al. (2, 4). AG-4 antigenic activity correlates with a high-molecular-weight viral structural polypeptide (7) and appears to be type-specific (L. Aurelian, unpublished observations). Significantly, it is present in biopsies from cervical cancer but not from normal cervical tissue (2), and antibody to AG-4 reflects the progression and/or regression of the cervical tumor (4). Thus, in a recent study (1) of 155 women, the prevalence of AG-4 antibody was significantly higher (85%) in the cancer than in the matched control group (10%), and it correlated with the gradation (14) expected of cervical cancer (atypia, 35%; in situ, 72%). Furthermore, antibody to AG-4 was not observed in 26 women successfully treated for the neoplasia but was present in 6 of 7 women with recurrent cancer. The association of AG-4 antibody with carcinoma of the cervix has recently been confirmed by Notter and Docherty (17) in a study of 50 women with cancer and their matched controls.

The study described in this communication was designed to inquire into the presence of AG-4 antibody in sera from Japanese patients with cervical cancer and matched controls. The data indicate that, in Japan, the frequency of AG-4 antibody in patients with cervical cancer is lower than the one previously reported in the United States, and it parallels the frequency of HSV-2 isolations from genital lesions (11). Nevertheless, the prevalence of AG-4 antibody in Japanese control women is lower than in the cancer group.

MATERIALS AND METHODS

Study Groups. Two series of sera, obtained from women seen in the Department of Obstetrics and Gynecology, Tokyo Hospital, and the Department of Gynecology, National Cancer Hospital, Tokyo, Japan, were analyzed. The 1st series was obtained prior to therapy from 29 patients with histologically diagnosed carcinoma of the uterine cervix. The distribution of the patients according to clinical staging of the disease was: Stage I, 4; Stage II, 7; Stage III, 15; and Stage IV, 3. The sera were stored at 4°C for 1 year prior to use in this study.

The 2nd series of sera was obtained from 14 patients with histologically diagnosed epidermoid carcinoma of the uterine cervix, and from 14 control women without pathological abnormalities after repeated Papanicolaou smears. With the exception of 3 women with myomata, pregnancy, and recurrent genital herpes, respectively, all control subjects were negative for HSV-1 and HSV-2 antibodies.

Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo, Japan; and Departments of Laboratory Animal Medicine, Biological and Biophysical Sciences and Microbiology, National Cancer Institute, Baltimore, Maryland, 21205

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2 The abbreviations used are: HSV-2, herpesvirus type 2; HSV-1, herpesvirus type 1; AG-4, early HSV-2-induced tumor-associated antigen; HEp-2, human epidermoid carcinoma; VB, barbital-buffered saline, pH 7.4, containing 0.46 g barbituric acid, 0.3 g sodium barbital, and 8.38 g sodium chloride per liter of distilled water; gel VB +, barbital-buffered saline with ions and gelatin, contains 1.5 × 10^-4 M CaCl2, 1 × 10^-1 M MgCl2, and 0.01% gelatin; AG-H, control antigen prepared from mock-infected cells.

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were diagnosed as cervical erosion. The controls were matched to the cancer patients for sex and age. The distribution of cancer patients according to clinical staging of the neoplasm was: microinvasive, 2; Stage I, 3; Stage II, 6; and Stage III, 3. Sera were collected prior to therapy and were stored at -20°.

Antigens. Two batches of antigen used in this study were supplied by Dr. L. Aurelian, Johns Hopkins University, Baltimore, Md. They were routinely used in the assay of United States sera and their preparation and properties were previously described (2, 4). Briefly, HEP-2 cells were infected with 0.2 to 0.4 plaque-forming unit of HSV-2 (G strain) per cell. After adsorption at 37° for 1 hr, the cells were overlaid with medium 199 supplemented with 1% calf serum (Microbiological Associates, Bethesda, Md.) and reincubated at 37° for 4 hr. At this time, the cells were collected by scraping, washed with VB, and disrupted by freezing and thawing. Cell debris was removed by centrifugation at 1550 × g for 30 min, and the supernatant, designated AG-4, was used in complement-fixation tests. A crude extract prepared in the same way from HEP-2 cells that were mock infected with maintenance medium and designated AG-H was used as control antigen. The properties and specificity of the AG-4 antigen were studied independently (6, 7).

Complement Fixation. The complement-fixation test used to assay for AG-4 antibody was the modification of the quantitative microassay of Wasserman and Levine (19) adapted to a reaction volume of 0.350 ml, with the use of polyethylene micropipets and tubes as described by Aurelian et al. (2, 4). Also, 1.5 × 10⁻⁴ M CaCl₂, 1 × 10⁻³ M MgCl₂, and gelatin (0.01%) were added to the barbital-buffered saline (gel VB₂⁻). The assay was performed in duplicate. Briefly, a mixture of antigen or gel VB₂⁻ (for antibody or complement controls), antibody (adjusted so that antibody control gave less than 10% complement fixation), and complement (reconstituted lyophilized guinea pig complement; Bioquest, Baltimore, Md.) was incubated at 4° for 18 hr and again at 37° for 20 min. At this time, sensitized sheep erythrocytes (5 × 10⁸ cells/ml) were added and the mixture was again incubated at 37° for 60 min. After addition of gel VB₂⁻ and centrifugation for 20 min at 1550 × g, the adsorbance (A) at 416 nm of the supernatant was read. The number of adsorbance units fixed (ΔA) was determined by subtracting the adsorbance of the reaction mixture from that of the antibody control. The percentage of complement fixation was computed by dividing ΔA by the adsorbance of the antibody control. The reaction was considered positive if more than 10% of the complement was fixed. Sera fixing more than 35% of the complement in the absence of antigen were considered anticomplementary. Sera-fixing complement with both AG-4 and AG-H were considered negative for AG-4 (2, 4).

Neutralization Assays. The multiplicity analysis neutralization assay using an artificial mixture of HSV-2 and a laboratory variant of HSV-1 designated HSV-MP was previously described (3). Briefly, the artificial mixtures containing 1 × 10⁸ plaque-forming units of HSV-2 and HSV-MP, respectively, were exposed to increasing dilutions of immunoglobulins for 1 hr at 37°. At this time, the virus-antibody mixtures were diluted and assayed for surviving virus. The constants of neutralization (K) were calculated for each virus according to the formula log n/v₀ = -0.43 k.c where v₀/v is the proportion of virus surviving neutralization and c equals antibody concentration expressed in terms of the amount of original serum per unit volume of the reaction mixture. Under the conditions of these experiments, keeping virus concentration constant, K may serve as a measure of antibody potency of the serum. A single virus preparation was used throughout this series and tests were performed simultaneously, in order to allow comparison of K values. The ratio of K for HSV-2 to K for HSV-1 was used to determine the presence of antibody to HSV-2, HSV-1, or both (3). A stock of rabbit anti-HSV-1 serum previously used in standardizing the assay (3) was used as control for the K ratio.

Sera Fractionation. The 29 sera in the 1st series were fractionated according to the method of Edelman et al. (10). Briefly, sera were diluted one-half in 0.85% NaCl solution and 0.3-ml samples were layered on preformed 10 to 40% (w/v) linear sucrose gradients and centrifuged at 35,000 rpm for 18 hr in a Spinco SW 50 rotor. Fractions (0.02 ml each) were collected from the bottom of the tube and monitored for absorbance measurements at 280 nm in an ISCO Model UA-4 adsorbance monitor. When 2 peaks of adsorbance were observed, peak fractions were independently pooled, dialyzed against VB, and labeled IgG and IgM, respectively. They were quantitated and assayed for cross-contamination by immunodiffusion using Hyland immunoplates (Hyland Div., Costa Mesa, Calif.). In case good separation was not obtained, the material in the IgM region of the gradient was exposed to a 2nd cycle of rate zonal centrifugation. Macroglobulin quantities recovered after 2 cycles of centrifugation were less than 10 mg/100 ml and accordingly were concentrated 5- to 8-fold by vacuum dialysis prior to assay in complement-fixation tests.

RESULTS

The 1st series of cervical cancer sera was analyzed in order to standardize the assay for Japanese samples. Accordingly, standard United States sera, AG-4 positive and negative (2, 4), were assayed simultaneously with the test Japanese sera, and 2 batches of antigen were tested. Identical results were obtained with both antigen preparations, and the standard United States sera behaved as previously described (2, 4). Of the 29 Japanese sera in this series, 17 (59%) had to be excluded from consideration due to their ability to fix complement in the absence of antigen, and 6 of the remaining 12 sera (50%) fixed complement with AG-4 but not with AG-H (Table 1).

Because a similarly high proportion of anticomplementary sera was not observed by Aurelian et al. (2, 4), we considered the possibility that our sera, stored at 4° for prolonged intervals, contain cryoprecipitates (8, 20) that either mask or bind the antibody to AG-4. To inquire into this possibility and since antibody to AG-4 is a 19 S immunoglobulin (5), all sera were fractionated by rate zonal centrifugation in 10 to 40% sucrose gradients, and pure 19 S immunoglobulins were isolated. These were tested for their ability to fix complement with AG-4 and the control AG-H antigen. Successful macroglobulin purification from sera without anticomplementary activity was obtained following 1 cycle of rate zonal centrifugation whereas 2 cycles...
of centrifugation were necessary to purify 19 S immunoglobulins from the anticomplementary sera. Purity of these fractions and their identification as IgG were established by immunodiffusion (5, 15) following 5- to 8-fold concentration by vacuum dialysis. Eight (47%) of the 17 macroglobulins from the anticomplementary sera and the same 6 (50%) of the 12 macroglobulins from sera without anticomplementary activity fixed complement with AG-4. Cryoprecipitates obtained from the anticomplementary sera by centrifugation at 5000 rpm for 15 min at 4° contained both IgG and IgM as determined by immunodiffusion of the samples dissolved in warm 37° phosphate-buffered saline (8 g sodium chloride per liter distilled water with $268 \times 10^{-3}$ M KCl, $8 \times 10^{-3}$ M Na$_2$HPO$_4$, $1.47 \times 10^{-3}$ M KH$_2$PO$_4$, $4.9 \times 10^{-4}$ M MgCl$_2$$\cdot$$6$H$_2$O, and $9.1 \times 10^{-4}$ M anhydrous CoCl$_2$$)$ (15).

Presence of virus-neutralizing antibody in a serum is an established criterion of previous subject exposure to this infection. Accordingly, the 29 sera studied in this series were assayed for neutralizing antibody to HSV-2 by the multiplicity analysis assay described by Aurelian et al. (3). Of these, only 15 sera were positive for HSV-2. Significantly, all but one were also positive for antibody to AG-4. On the other hand, high titers of antibody to HSV-1 were observed in all the studied sera (Table 2).

Having standardized the sera and established the variables, a 2nd series of 28 freshly drawn sera stored at —20° was assayed under code. These sera consisted of 14 cancer sera were positive for AG-4 antibody (Table 3), whereas only one (7%) of the controls was similarly reactive ($x^2, p < 0.06$). None of the sera fixed complement with AG-H and 57% of the cancer sera and 43% of the controls contained neutralizing antibody to HSV-2. All sera except one (Control Serum 18) had neutralizing antibody to HSV-1. Mean HSV-1 titers expressed as $K$ values were 0.75, titers essentially similar to those observed in United States population groups (Ref. 3; L. Aurelian and I. Royston, unpublished). In this series of sera, as well as in the first, all AG-4-positive sera had neutralizing antibody to HSV-2 (Chart 1). The AG-4-negative cancer sera did not neutralize HSV-2; the AG-4-negative control sera were either positive or negative for neutralizing antibody to HSV-2.

**DISCUSSION**

Confirming previous studies done on United States population groups, the results described in this communication indicate that the frequency of antibody to AG-4, in sera from Japanese patients with epidermoid carcinoma of the uterine cervix, is higher (43%) than that in control women (7%). This difference is of borderline significance ($p < 0.06$). On the other hand, when comparing our results to those from United States study groups (1, 2, 4, 17), it becomes evident that the prevalence of AG-4 antibody in Japanese cancer patients is substantially lower than that observed in the United States. Thus, of a total of 43 sera from cancer patients studied in these series, only 20 (47%) contained AG-4 antibody whereas 78 to 91% of cervical cancer patients from the United States were described as having antibody to AG-4 (1, 2, 4, 17).

The results reported in this communication are not artefacts due to laboratory manipulation, as evidenced by the data pertaining to the specificity and reactivity of the antigen and the sera and the methodological approaches used in these studies. Antigen pools used in these series contain AG-4 since: (a) 2 batches were assayed with identical results, (b) both batches were routinely used in the study of United States sera resulting in an 85% prevalence of antibody to AG-4 in the cancer groups (1, 2, 4), and (c) standard United States sera used as positive controls reacted with the antigen pools as previously described. The antigenicity cannot be attributed to a nonspecific cellular antigen as sera do not react with control antigen (AG-H) prepared from mock-infected HEp-2 cells. Furthermore, AG-4 differs from other viral antigens as evidenced by the results of the neutralization experiments (Table 3) as well as the properties of

<table>
<thead>
<tr>
<th>Patients</th>
<th>No. tested</th>
<th>Mean age</th>
<th>Anticomplementary</th>
<th>Positive for antibody to AG-4</th>
<th>Positive for antibody to AG-H</th>
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<tr>
<td><strong>Japanese sera</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Invasive CaCx</td>
<td>4</td>
<td>54</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Stage I</td>
<td>7</td>
<td>48</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Stage III</td>
<td>15</td>
<td>57</td>
<td>7</td>
<td>4</td>
<td>0</td>
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<tr>
<td>Stage IV</td>
<td>3</td>
<td>57</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>54</td>
<td>17</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td><strong>United States standard sera</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AG-4 Positive</td>
<td>3</td>
<td>49</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>AG-4 Negative</td>
<td>5</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</table>

**Table 2**

<table>
<thead>
<tr>
<th>Sera</th>
<th>No. tested</th>
<th>AG-4</th>
<th>AG-H</th>
<th>HSV-2</th>
<th>HSV-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonanticomplementary</td>
<td>12</td>
<td>6</td>
<td>50</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>IgM from anticomplementary</td>
<td>17</td>
<td>8</td>
<td>47</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td>29</td>
<td>14</td>
<td>48</td>
<td>1</td>
<td>3.5</td>
</tr>
</tbody>
</table>
anticomplementarity and accordingly did not require macroglobulin isolation. In this series, as well, only 43% of the macroglobulins from sera without anticomplementary activity, only those obtained from AG-4-positive sera fixed complement with AG-4.

Finally, the results we report in this communication are not artefacts due to an abnormal reactivity of the sera used in these studies, as their reactivity with both HSV-1 and HSV-2 in neutralization tests is similar to that reported for all other study groups (13). In this respect, it should be pointed out that the prevalence of HSV-2 antibody we find in this series of experiments is slightly lower (43 to 57%) than that (70%) we reported previously (12). On the other hand, the prevalence of HSV-1 antibody is equally high (12). Considering the well-known difficulty in assessing the presence of antibody to HSV-2 in the presence of high levels of cross-reactive HSV-1 antibody (3, 12, 13), it seems reasonable to suggest that this variability results from the use of different neutralization assays. Thus, whereas in our first report we used the kinetics of neutralization calculating the amount of specific anti-HSV-2 antibody (12), in this series of investigations we use the multiplicity analysis plaque reduction assay in which HSV-2 seropositivity is calculated from the ratio of the reactivities to the 2 viruses (3). Despite these difficulties, it is highly significant that, independent of the assay, the prevalence of antibody to HSV-2 in Japanese women was similar in both cancer and control groups (12), and that the results obtained using the multiplicity analysis assay reveal an almost complete correlation with the frequencies of HSV-2 isolations from the genitalia (11).

The 2nd series of cancer and control sera, having been stored at -20°C and for shorter intervals, did not display anticomplementarity and accordingly did not require macroglobulin isolation. In this series, as well, only 43% of the cancer sera fixed complement with AG-4 (Table 3).

The observation that only a total of 47% of sera from Japanese patients with cervical cancer fix complement with AG-4 is amenable to 2 interpretations. First, it is possible that in the AG-4-negative sera, IgM and therefore AG-4 antibody levels are too low for detection by our assay procedures. Alternatively, it is possible that the AG-4-negative sera were obtained from patients that had not been previously exposed to HSV-2 even if they were diagnosed as having cervical cancer. Our data support this interpretation.

### Table 3

<table>
<thead>
<tr>
<th>Patients</th>
<th>No. tested</th>
<th>Mean age</th>
<th>AG-4</th>
<th>%</th>
<th>AG-H</th>
<th>%</th>
<th>HSV-1</th>
<th>%</th>
<th>HSV-2</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive cancer</td>
<td>14</td>
<td>43</td>
<td>6</td>
<td>43</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>100</td>
<td>8</td>
<td>57</td>
</tr>
<tr>
<td>Controls</td>
<td>14</td>
<td>35</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>93</td>
<td>6</td>
<td>43</td>
</tr>
</tbody>
</table>

The AG-4 antigen. Thus, of the 14 sera from control women, 6 are HSV-2 positive whereas only one of them fixes complement with AG-4, suggesting that the antigens reacting in these 2 assays are different. AG-4 synthesis in HSV-2-infected HeP-2 cells is maximal between 4 and 8 hr after infection which is before the time of maximal virus production, and AG-4 does not block the neutralizing potential of hyperimmune rabbit and human anti-HSV-2 sera (6). Finally, antibody to AG-4 is not adsorbed with intact virions precipitated by centrifugation at 25,000 rpm for 1.5 hr in a Spinco SW 27 rotor. On the other hand, adsorption of AG-4-positive sera with lysates of the HSV-2 virions removes their ability to fix complement with AG-4 (L. Aurelian, M. F. Smith, and B. Strnad, in preparation). These observations indicate that AG-4 differs from other viral antigens involved in neutralization. They also suggest that AG-4 is an inner component of the HSV-2 virion, a conclusion independently reached from biochemical studies indicating that the antigenic activity correlates with a high-molecular-weight (161,000 daltons)-infected cell polypeptide designated ICP 10 (7). ICP 10 has been identified as a virion structural protein since it comigrates on polyacrylamide gel electrophoresis with a polypeptide obtained from virus particles purified 250-fold (18).

In view of the method of serum storage (4°C) and the association of cryoglobulins with chronic diseases (8, 20), the presence in a large proportion of these sera of cryoprecipitates that bind or mask the AG-4 antibody and cause anticomplementarity even after heating at 56°C for 30 min (20) is not totally unexpected. In this respect, it is particularly significant that anticytomegalovirus antibodies are present in cryoglobulins (8). The observation that precipitates are removed by low-speed centrifugation suggests that not all of the macroglobulin is in a bound state. The presence of cryoprecipitates did not correlate with the stage of the disease or the patient’s age (Table 1). Reactivity of 8 of the 17 macroglobulins from anticomplementary sera with AG-4 antigen is not an artefact due to serum fractionation since, of the 12 macroglobulins from sera without anticomplementary activity, only those obtained from AG-4-positive sera fixed complement with AG-4.
Thus, AG-4-positive sera from cancer patients have neutralizing antibody to HSV-2, whereas AG-4-negative cancer sera are also negative for HSV-2 antibody (Chart 1). Conversely, in the control group, HSV-2 seropositivity does not correlate with the presence of AG-4 antibody. Of the 6 control sera positive for HSV-2, only 1 fixes complement with AG-4. The data suggest that previous exposure to HSV-2 as well as a diagnosis of cancer are necessary for AG-4 seropositivity. On the other hand, virtually all subjects are HSV-1 seropositive.

The data suggest that AG-4 is a type-specific antigen, in agreement with our previous observations indicating that cells infected with HSV-1 for 4 hr do not contain an antigen similar to AG-4, but rather an antigen that is recognized equally well by both cancer and control sera and that appears to be involved in neutralization (L. Aurelian and M. F. Smith, in preparation). The presence of HSV-1 antibody in all the cancer patients, the AG-4 seropositivity of one-half of these women, and the identification of one-half of the genital herpesvirus isolates as type 1 herpesvirus, on the basis of both immunological and biological properties (11), may be interpreted as suggesting that in Japan, HSV-1 is also associated with cervical cancer. This is not improbable, as the oncogenic potential of HSV-1 for mammalian cells in vitro has been established (9). Studies designed to inquire into this possibility are now in progress in our laboratory.

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