Detection of human serum antibodies that neutralize infectious human papillomavirus type 11 virions

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A selection of human sera were tested for the presence of antibodies that neutralized infectious human papillomavirus (HPV) type 11. Neutralizing antibodies were detected by prevention of HPV-11-induced condylomatous transformation of human foreskin chips transplanted subrenally into athymic mice. Test sera were obtained from 21 female patients with genital condylomas and eight patients with laryngeal papillomas. Control patients consisted of 57 adult random blood donors and five asymptomatic children. ELISAs demonstrated that all sera from patients with genital papillomas were strongly reactive to disrupted papillomavirus (PV) antigens of HPV-11, bovine PV type 1 and cottontail rabbit PV, but only two were weakly reactive to intact HPV-11. None of the eight sera from the laryngeal papilloma bearers reacted significantly to disrupted PV antigens, but four of the eight showed strong specific responses to intact HPV-11 only. The majority of the sera that were reactive to intact HPV-11 by ELISA neutralized HPV-11 infectivity in the athymic mouse xenograft system. The data indicated that ELISA reactivity to intact HPV-11 virions was a good predictor for the presence of HPV-11 neutralizing antibodies.

Human papillomaviruses (HPVs) that infect laryngeal and anogenital regions have been implicated as causative agents in the development of laryngeal papillomas and cervical cancers (Dürst et al., 1983; Hollinger et al., 1968; Pfister, 1984). Antibodies reactive to these HPV types have been detected in the sera of patients with papillomavirus (PV) lesions as well as in patients with cervical cancers. Antibody detection however was not assayed using genital PV antigens from intact viruses because most human lesions contain very few PV particles (Almeida et al., 1969), and because these viruses have not been propagated in tissue culture. Instead, bacterially expressed proteins of the open reading frames (ORFs) determined from sequenced PV DNA (Firzlaff et al., 1988; Jenison et al., 1988; Jochmus-Kudielka et al., 1989; Li et al., 1987; Strike et al., 1989; Dillner et al., 1980), synthetic peptides (Cason et al., 1989; Dillner, 1990; J. Dillner et al., 1989, 1990; Jenison et al., 1989; Müller et al., 1990) and denatured, natural viral proteins from bovine PV (BPV) or HPV-1 virions (Baird, 1986; Beiss et al., 1987; Cubie & Norval, 1988; L. Dillner et al., 1989; Portolani et al., 1987) were used to measure anti-HPV reactivity.

The above antigen sources provide only a subset of the potential antigenic structures that are present on intact genital HPVs because external conformational epitopes on virions are not easily mimicked by bacterially derived fusion proteins and synthetic peptides. The presence of such extra antigenic structures, and their detection by human serum antibodies, was confirmed by studies using intact virions of HPV-1 and -2 (Steele & Gallimore, 1990; Anisimová et al., 1990) and HPV-11 (Bonnez et al., 1991). We have observed that some of these external and predominantly conformational structures on virions define strong neutralization-inducing epitopes (Christensen & Kreider, 1990; Christensen et al., 1990b).

The purpose of this study was to examine sera from selected human patients for the presence of antibodies that neutralize HPV-11. To date, there have been no reports on the detection of serum antibodies that neutralize any HPV type because of the difficulties in propagation of infectious virions. Sera were tested for neutralizing activity against HPV-11 in the athymic mouse xenograft system (Christensen & Kreider, 1990; Kreider et al., 1985).

The study groups were 21 adult female patients with anogenital warts (Philadelphia metropolitan area, Pennsylvania, U.S.A.); five young children with laryngeal papillomatosis (Baltimore metropolitan area, Maryland, U.S.A.) and three patients (two adults and one young child) with laryngeal papillomatosis (Hershey, Pennsylvania, U.S.A.). Control groups consisted of five young
children (Baltimore), and 57 adult random blood donors (Hershey). Thirteen of 21 patients with genital warts were HPV typed by Southern blot hybridization as previously described (Rando et al., 1986). Four of the 13 were HPV-6/11-positive, six were HPV-16/18-positive, one was HPV-31-positive and two were HPV-11-negative. Three patients (Hershey group) with laryngeal papillomas were as typed HPV-6/11-positive by in situ hybridization using the ViraType in situ kit from Life Technologies. The remaining five laryngeal papilloma bearers were typed by Southern blot hybridization (Mounts et al., 1982), but typing data were available for two patients only. Both were positive for HPV-11 DNA.

All patients' sera were initially screened by ELISA in order to detect anti-PV antibody reactivity. HPV-11, BPV-1 and cottontail rabbit PV (CRPV) were attached to ELISA plate wells either as whole virion antigens at neutral pH (Cowsert et al., 1987), or as alkali-disrupted and reduced antigens at pH 10.6 (Favre et al., 1975) as previously described (Christensen et al., 1990a). Human patient sera (heat-inactivated at 56 °C for 30 min) were tested in duplicate at dilutions of 1:100 in blocking buffer consisting of 5% milk protein in PBS, then the reaction was developed with 1:1000 dilution of alkaline phosphatase-conjugated rabbit anti-human (anti-IgG, -IgM and -IgA) antibody (Dako) and substrate.

Means and S.D.s of ELISA absorbance values for the 57 random blood donors for each of the six different PV antigens tested (inact and disrupted HPV-11, BPV-1 and CRPV) were used statistically to define positive and negative human patient sera for each target antigen. Individual values exceeding the mean plus 3 s.d. were excluded, then the remaining readings re-averaged until no further values exceeded the recalculated mean plus 3 s.d. These final means and associated S.D.s were used as the experimental values for assessing whether patients' sera were defined as positive or negative for reactivity to PV antigens. Samples greater than the recalculated means plus 3 s.d. were designated as positive for reactivity to the appropriate PV antigen. Individual ELISA absorbance readings for each patient serum were plotted for each group, and the results are shown in Fig. 1.

Sera from patients with genital lesions did not react strongly to intact virion antigen (two of 21 reacted weakly to intact HPV-11, five of 21 reacted weakly to intact BPV-1, and none were reactive to intact CRPV). Four of the eight sera from laryngeal papillomatosis patients (50%) showed strong antibody reactivity to intact HPV-11 only. One serum from the 57 patients in the random blood donor group (2%) reacted strongly to intact HPV-11 only, and one other serum was weakly reactive to intact BPV-1 only (neither serum responded to disrupted virion antigen).

![Fig. 1. ELISA of patients' sera reactivity to (a) disrupted and (b) intact HPV-11, (c) disrupted and (d) intact BPV-1 and (e) disrupted and (f) intact CRPV. Patients' sera were tested in duplicate at 1:100 dilution against all six PV antigen preparations. Patient groups were A, 57 adult random blood donors; B, 10 sera from children (five with laryngeal papillomas and five asymptomatic); C, 21 adult females with current genital papillomas; D, three patients with laryngeal papillomas; E, various rabbit polyclonal antisera to (●) group-specific antigen, (▲) HPV-11, (●) BPV-1 and (●) CRPV. Horizontal lines represent the mean plus three S.D. of ELISA readings from the 57 random blood donors with the appropriate high readings subtracted as described in the text. Means ± S.E.M. of duplicate absorbance readings have been plotted for several of the polyclonal antisera reactive to various papillomavirus antigens, but S.E.M.s were not plotted for any of the mean absorbance values obtained for human patients' sera.](image-url)
Table 1. ELISA of patient serum samples used in the HPV-11 neutralization assay

<table>
<thead>
<tr>
<th>Serum sample*</th>
<th>HPV-11</th>
<th>BPV-1</th>
<th>CRPV</th>
<th>HPV-11 (intact)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact</td>
<td>Disrupted</td>
<td>Intact</td>
<td>Disrupted</td>
</tr>
<tr>
<td>KS-1</td>
<td>0.371†</td>
<td>ND</td>
<td>0.055</td>
<td>ND</td>
</tr>
<tr>
<td>RR-5</td>
<td>0.098</td>
<td>ND</td>
<td>0.228</td>
<td>ND</td>
</tr>
<tr>
<td>RR-18</td>
<td>0.086</td>
<td>ND</td>
<td>0.228</td>
<td>ND</td>
</tr>
<tr>
<td>RR-3</td>
<td>0.079</td>
<td>ND</td>
<td>0.474</td>
<td>ND</td>
</tr>
<tr>
<td>RR-13</td>
<td>0.074</td>
<td>ND</td>
<td>0.470</td>
<td>ND</td>
</tr>
<tr>
<td>HMC-1</td>
<td>0.255</td>
<td>ND</td>
<td>0.055</td>
<td>ND</td>
</tr>
<tr>
<td>HMC-2</td>
<td>0.161</td>
<td>ND</td>
<td>0.084</td>
<td>ND</td>
</tr>
<tr>
<td>HMC-3</td>
<td>0.287</td>
<td>ND</td>
<td>0.060</td>
<td>ND</td>
</tr>
<tr>
<td>BD-18</td>
<td>0.212</td>
<td>ND</td>
<td>0.071</td>
<td>ND</td>
</tr>
<tr>
<td>BD-44</td>
<td>0.053</td>
<td>ND</td>
<td>0.395</td>
<td>ND</td>
</tr>
<tr>
<td>BD-6</td>
<td>0.023</td>
<td>ND</td>
<td>0.024</td>
<td>ND</td>
</tr>
<tr>
<td>BD-15</td>
<td>0.011</td>
<td>ND</td>
<td>0.024</td>
<td>ND</td>
</tr>
<tr>
<td>BD-19</td>
<td>0.015</td>
<td>ND</td>
<td>0.026</td>
<td>ND</td>
</tr>
<tr>
<td>639§</td>
<td>0.291</td>
<td>ND</td>
<td>0.154</td>
<td>ND</td>
</tr>
<tr>
<td>Dako†</td>
<td>0.055</td>
<td>ND</td>
<td>0.510</td>
<td>ND</td>
</tr>
<tr>
<td>7341‡</td>
<td>0.004</td>
<td>ND</td>
<td>0.000</td>
<td>ND</td>
</tr>
<tr>
<td>3866**</td>
<td>ND</td>
<td>ND</td>
<td>0.963</td>
<td>ND</td>
</tr>
<tr>
<td>7380††</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.708</td>
</tr>
</tbody>
</table>

* Samples 1 and 6 to 8 were from laryngeal papilloma bearers, 2 to 5 from genital papilloma bearers, and 9 to 13 from the random blood donor group.
† Underlined ELISA values represent absorbances defined as antibody-positive.
‡ ND, Not determined.
§ Rabbit polyclonal antiserum against intact HPV-11.
† Rabbit polyclonal antiserum to group-specific antigen from Dako.
¶ Non-immune rabbit serum.
** Rabbit polyclonal antiserum against intact BPV-1.
†† Rabbit polyclonal antiserum against intact CRPV.

Sera from female patients with current genital warts showed strong reactivity to disrupted HPV-11, CRPV and BPV-1 antigen. All 21 patients' sera were positive against disrupted HPV-11, whereas 19 of 21 (90%) and 20 of 21 (95%) respectively were positive for reactivity to disrupted BPV-1 and CRPV. None of the eight sera from laryngeal papillomatosis patients' sera showed any significant reactivity to disrupted HPV-11, BPV-1 or CRPV antigen. Of the 57 random blood donor sera, nine (16%), two (4%) and five (9%) sera reacted to disrupted HPV-11, BPV-1 and CRPV antigen respectively.

Neutralization of HPV-11 by human serum antibodies was assayed in the athymic mouse xenograft system as previously described (Christensen & Kreider, 1990). Dilutions of selected human sera (1:10) were mixed with an equal volume of infectious HPV-11 in PBS, and incubated for 1 h at 37°C. Human foreskin chips (2 x 2 mm) were added to the virus/serum mixture and incubated a further 1 h at 37°C. The chips were grafted under the renal capsule of athymic mice, and cysts were examined for morphological transformation and for the presence of nuclear HPV-11 DNA by in situ hybridization 90 days later (Christensen & Kreider, 1990; Kreider et al., 1987). Morphological transformation was defined as the presence of typical koilocytic nuclei (Koss & Durfee, 1956). A polyclonal rabbit antiserum (no. 639 specific for intact HPV-11 was used as a positive control serum for HPV-11 neutralization. Comparisons between the frequency of HPV-11-induced morphological transformation of foreskin chips after various human serum pretreatments of HPV-11 prior to infection were determined by Fisher's exact probability test.

A total of 13 patients' sera were tested for antibody-mediated neutralization of HPV-11 infectivity in two separate experiments. Sera tested were seven samples that were positive for reactivity to intact HPV-11 by ELISA (four laryngeal papilloma bearers, two patients with genital warts, and one patient from the random blood donor group), three sera that were strongly reactive to disrupted but not to intact PV particles by ELISA (two patients with genital warts, and one patient from the random blood donor group) and three sera that were negative for reactivity to PV antigens by ELISA (all from the random blood donor group). ELISA reactivity for these sera are shown in Table 1.

Two separate neutralization assays using the above 13 human sera were examined using different dilutions of crude extract containing infectious HPV-11. In experiment 1 (Table 2), patients' sera at a final dilution of 1:20 were incubated with a 5 x 10^{-2} dilution of HPV-11...
were positive to intact HPV-11 by ELISA (samples 1, 3 respectively.)

less infectious HPV-11, six of the seven sera (86%) that sera for experiment 2 were 0.00008, 0.00006 and 0.63

(0.20) was observed between the latter two groups of sera. No significant difference (P, 0.054) with all the sera that were positive only to disrupted HPV-11 antigen and a non-significant difference (P, 0.054) with all the negative by ELISA to HPV-11 (samples 11, 12 and 13, in which 13 of 15 were positive for HPV-11 DNA; Table 2),

There was a significant difference (P, 0.004, Fisher’s exact probability test) between the number of cysts that sera reactive with intact HPV-11 only by ELISA (serum samples 1, 6, 7, 8 and 9, in which five of 15 were positive for HPV-11 DNA; Table 2),

In the first experiment, only partial neutralization of HPV-11-induced morphological transformation of human xenografts by several human sera was obtained. Previous experiments in our laboratory have shown that such pretreatment increased the survival of xenografts (Kreider et al., 1990). In the above experiments, graft survival was 46 of 58 (79%) following cyclophosphamide pretreatment and 44 of 69 (64%) without pretreatment (P, 0.025).

In the present work, we have examined sera from patients with genital and laryngeal papillomavirus infections for the presence of antibodies that reacted to laboratory-produced HPV-11 virions by ELISA, and for neutralization of HPV-11 infectivity. The presence of HPV-neutralizing antibodies in the serum of human patients with natural HPV infections has not been verified experimentally prior to this study.

ELISAs revealed that six out of a total of 29 sera from patients with genital or laryngeal papillomas, and one out of 62 sera from control patients, reacted with intact HPV-11 virion antigen. Five of these seven sera did not contain antibodies that were significantly reactive to disrupted HPV-11, BPV-1 or CRPV (a test for the presence of cross-reactive or type-common antibodies), and therefore these five sera would probably not have been identified as containing HPV antibody reactivity either by ELISA or Western blots that utilized bacterially derived fusion proteins and synthetic peptides of PV capsid proteins, as well as disrupted BPV-1, HPV-1 and HPV-11 antigen. All five sera that were reactive by ELISA exclusively to intact HPV-11 antigen contained HPV-11 neutralizing antibodies. These data demonstrated that there was a good correlation between positive responses to intact virions in ELISA and the presence of neutralizing antibodies, and also that serum from some patients who were naturally infected with PV’s contained antibodies that recognized a non-linear,

<table>
<thead>
<tr>
<th>Serum sample*</th>
<th>HPV in wart†</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In situ DNA§</td>
<td>Morphology‡</td>
<td>In situ DNA§</td>
</tr>
<tr>
<td>1KS-1</td>
<td>11+</td>
<td>0/2/5</td>
<td>0/4/5</td>
</tr>
<tr>
<td>2 RR-5</td>
<td>6/11+</td>
<td>2/2/4</td>
<td>1/4/4</td>
</tr>
<tr>
<td>3 RR-18</td>
<td>11−</td>
<td>0/3/4</td>
<td>1/4/4</td>
</tr>
<tr>
<td>4 RR-3</td>
<td>16/18+</td>
<td>1/4/5</td>
<td>2/2/4</td>
</tr>
<tr>
<td>5 RR-13</td>
<td>16/18+</td>
<td>1/3/6</td>
<td>5/6/6</td>
</tr>
<tr>
<td>6 HMC-1</td>
<td>6/11+</td>
<td>0/1/4</td>
<td>0/3/5</td>
</tr>
<tr>
<td>7 HMC-2</td>
<td>6/11+</td>
<td>1/1/2</td>
<td>0/3/6</td>
</tr>
<tr>
<td>8 HMC-3</td>
<td>6/11+</td>
<td>0/5/5</td>
<td>0/5/6</td>
</tr>
<tr>
<td>9 BD-18</td>
<td>NT</td>
<td>1/4/6</td>
<td>0/2/2</td>
</tr>
<tr>
<td>10 BD-44</td>
<td>NT</td>
<td>2/5/6</td>
<td>3/5/5</td>
</tr>
<tr>
<td>11 BD-6</td>
<td>NT</td>
<td>2/4/6</td>
<td>6/6/6</td>
</tr>
<tr>
<td>12 BD-15</td>
<td>NT</td>
<td>3/4/6</td>
<td>1/1/1</td>
</tr>
<tr>
<td>13 BD-19</td>
<td>NT</td>
<td>2/3/6</td>
<td>NS</td>
</tr>
<tr>
<td>14 639</td>
<td>–</td>
<td>0/3/4</td>
<td>0/4/6</td>
</tr>
</tbody>
</table>

Table 2. Morphological and in situ DNA analysis of grafts developed from HPV-11 incubated with various human patients' sera

* Serum samples from patients as described in Table 1.
† HPV type present in wart at time of serum collection.
‡ Number of grafts morphologically transformed/number surviving/number attempted.
§ Number of grafts in situ DNA-positive/number surviving/number attempted.
¶ NS, No survivors.
NT, Not typed.
conformational neutralizing epitope on HPV-11 virus particles.

A preliminary ELISA was conducted to determine the isotype of the antibodies in four of the sera that contained HPV-11 neutralizing antibodies (Table 1). One of the four sera contained only IgM antibodies (HMC-2, 2 year old with laryngeal papillomas), three of the four had strong IgG reactivities, and of these three, one patient had concurrent IgA and the other had concurrent IgM responses to intact HPV-11. Although the ELISA cannot determine which isotype is responsible for neutralization when concurrent reactivities exist, the data suggest, at least, that both IgM and IgG antibodies can neutralize HPV-11. These data are of interest because infections triggered by the subset of genital HPVs are often restricted to mucosal surfaces such that IgA responses may be expected. The ability of serum IgM and IgG neutralizing antibodies to protect against HPV infections of the genital HPV types is unknown, and the isotype of neutralizing antibodies may provide prognostic indicators for effective protection and/or resolution of the lesions.

Although we detected serum antibodies that reacted to HPV-11 (Hershey isolate) by ELISA using intact virions, by neutralization of HPV-11 infectivity, and by immunohistochemical staining of koilocytic nuclei in frozen sections of experimental condyloma (data not shown), not all sera from patients with HPV-6/11 DNA-positive lesions contained HPV-11 neutralizing antibodies. Five of the eight laryngeal papilloma bearers and only four of 13 patients with genital warts (HPV typing data were available for five of the eight laryngeal papilloma bearers, and for 13 of the 21 patients with genital warts) had lesions that were typed as HPV-6/11 DNA-positive. Of these nine patients with confirmed HPV-6/11 DNA-containing lesions, four (44%) contained antibodies that reacted to intact virions of HPV-11 (Hershey) by ELISA. This percentage is in close agreement to data obtained for serum antibody reactivity to HPV-11 (Hershey) from genital condyloma bearers with HPV-6/11-positive lesions (30%), and laryngeal papilloma bearers with HPV-6/11-positive lesions (47%) (Bonnez et al., 1991; W. Bonnez et al., personal communication).

In direct contrast to the predominantly type-specific antibody response to intact HPV-11 virions, serum antibody reactivity to disrupted PV virions was PV-cross-reactive. This PV-non-type-specific cross-reactivity was especially prevalent for sera obtained from patients with genital warts (Fig. 1). These results clearly demonstrated that PV cross-reactive antibodies were triggered in such patients irrespective of the HPV type present in the lesions. The high prevalence and non-type-specificity of serum antibody response from patients with genital warts that was demonstrated in this study is in agreement with other published data using disrupted BPV (Baird, 1986; Beiss et al., 1987; L. Dillner et al., 1989; Portolani et al., 1987) and HPV-1 (Cubie & Norval, 1988). Most of these studies, however, used only one PV as antigen such that cross-reactivity of the responses could only be implied empirically. The results from Fig. 1 indicated that any and all PVs would detect high frequencies of positive ELISA signals when serum from patients with genital warts are examined.

The non-type-specific response to disrupted PV virion antigens was not equivalent to antibody reactivity to the group-specific antigen (GSA) site that occurs upon immunization with disrupted PV virions. We (data not shown) and others (Beiss et al., 1987; Jenison et al., 1988; Steele & Gallimore, 1990; Strike et al., 1989) were not able to detect such high frequencies of positive antibody signals, by Western blotting, to the major capsid protein (L1), which is the target protein for the GSA site(s) (Cowsert et al., 1987; Jenison et al., 1989; Strike et al., 1989). More sensitive assays utilizing bacterially derived fusion proteins demonstrated that some sera from patients with genital warts do contain antibody reactivity to linear epitopes on capsid proteins of genital HPV types (Jenison et al., 1988; L. Dillner et al., 1989). However, in contrast to the ELISA data described in this (Fig. 1) and other studies (Baird, 1986; Beiss et al., 1987; Portolani et al., 1987), the majority of the sera were not positive, and the responses were predominantly type-specific (Jenison et al., 1989), not cross-reactive. The set of virion antigens that is recognized, in ELISA, by serum antibodies from the patients with genital warts therefore still remains to be characterized.

It is intriguing to note that the ELISA using disrupted PV virion antigen was able to identify as positive almost all patients with genital lesions, and to identify lower numbers of positive sera from random adult blood donors and asymptomatic children. For example, 90% of the serum samples from patients with genital warts were positive to disrupted HPV-11, BPV-1 and CRPV, and nine of 62 (15%), two of 57 (4%) and five of 57 (9%) of the control sera were reactive to disrupted HPV-11, BPV-1 and CRPV respectively. These results suggested that the ELISA provides a reasonable correlation with disease symptoms based on DNA analyses of infected and healthy control adult populations (de Villiers et al., 1987; Lorincz et al., 1986). The frequency of patients with genital warts that were typed as HPV DNA-positive was 90% whereas asymptomatic females contained HPV DNA at frequencies between 9 and 11% (de Villiers et al., 1987; Lorincz et al., 1986).

We did not detect any significant responses by ELISA to disrupted PV antigens for the sera from the eight patients with laryngeal papillomas. This result appears to contrast directly with the data for serum antibody
reactivities to disrupted PV antigens from patients with genital warts. However, the laryngeal papilloma bearers tested in this study were predominantly children (two of eight were young adults), and it is possible that cross-reactive antibodies to disrupted PV virion antigens had not yet been triggered. A previous study detected four of nine patients with laryngeal papillomas that were positive to disrupted PV antigens from patients with laryngeal papilloma, but the positive responses obtained were not identified by the patients’ ages (Beiss et al., 1987).

In conclusion, several human serum samples from patients with genital and laryngeal papillomas contained antibodies which neutralized laboratory-produced infectious HPV-11. Most of the patients’ sera that contained neutralizing antibodies reacted only to intact HPV-11 virions by ELISA, stained koliocytotic nuclei in sections of freshly frozen but not formalin-fixed, paraffin-embedded sections of experimental condyloma, and were negative for reactivity to disrupted HPV-11, BPV-1 and CRPV by ELISA. This set of responses was directly comparable to the reactivity obtained by HPV-11 neutralizing monoclonal antibodies (Christensen et al., 1990). These observations indicate that human serum neutralizing antibodies recognized similar conformational determinants on HPV-11 virions which were the predominant target epitopes identified by the set of neutralizing monoclonal antibodies.

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