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Detection of Human Antibody against the Human Papillomavirus Type 16 E7 Protein

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We examined 500 human sera for the presence of antibody against the human papillomavirus type 16 E7 protein by enzyme-linked immunosorbent assay with bacterially expressed fusion protein *lac*-E7, and by radioimmunoprecipitation and immunofluorescence assays with the E7 protein expressed in monkey COS-1 cells. The anti-E7 antibody was detectable in 6 out of 54 cases of cervical carcinoma, but in none of the others, including patients with other gynecologic cancers, those with sexually transmitted diseases, and healthy adults. The data indicate that expression of the E7 protein is closely related to cervical carcinoma.

Key words: HPV 16 E7 — Human anti-E7 antibody — Cervical cancer

Human papillomavirus type 16 (HPV16)[1] is one of the agents probably causing cervical cancer.[2] Its DNA is frequently found in genital cancer biopsies and in cell lines derived from cervical cancers,[2] and is capable of transforming rodent[3–6] and human cells.[7,8] HPV 16 open reading frame E7 encodes a nuclear[9] oncoprotein,[10–18] which is the most abundant HPV protein found in some of the cancer cell lines.[19,20] The E7 protein, therefore, appears to be involved in carcinogenesis in humans.

Investigation of anti-E7 antibody in human sera is an important step to define the role of HPV 16 in carcinogenesis. Whereas the detection of HPV DNA in cancer cells has been facilitated by the nucleic acid hybridization methods, detection of human anti-HPV antibodies has been hampered by the lack of cell cultures providing us with viral antigens, which are essential for serologic studies. Recently, human anti-E7 antibody has been shown by the Western method using fusion proteins expressed in *Escherichia coli* to be detectable in human sera more frequently in patients with cervical cancer than in the normal population.[21] In this study we examined 500 serum samples from various populations for the presence of anti-E7 antibody, using fusion protein *lac*-E7 expressed in bacteria[9] and the E7 protein expressed in monkey cells.[22] We found the antibody against HPV 16 E7 in sera from 6 out of 54 patients with cervical cancer.

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MATERIALS AND METHODS

Sera Five hundred human sera, collected from patients with gynecologic cancers [54 cervical cancers including 8 cases of carcinoma *in situ* (CIS), 23 ovarian cancers, 6 endometrial cancers and 3 choriocarcinomas] or with sexually transmitted diseases (29 condyloma acuminata and 150 genital herpes), from 30 prostitutes and 33 homosexual men, and from 172 healthy adults including pregnant women, were examined for the presence of anti-HPV 16 E7 antibody. The diseases were diagnosed as gynecologic cancers from histopathological findings, as condyloma acuminata from typical vulvar lesions and histopathological findings, and as genital herpes from clinical and virological findings. Sera were obtained from cancer patients before treatment.

Antigens A bacterial subcellular fraction enriched with *lac*-E7 (P10-*lac*-E7) was used as the antigen for an enzyme-linked immunosorbent assay. Fusion protein *lac*-E7 was expressed in *E. coli* strain YA21 from the *lac* promoter in pUC18 plasmid as described previously.[9] The bacteria capable of producing *lac*-E7 were grown overnight, washed once with TN buffer (10 m*M* Tris-HCl, pH 7.5–50 m*M* NaCl), and disintegrated by sonication in TN-1 m*M* phenylmethylsulfonyl fluoride (PMSF). The lysate was centrifuged at 10,000 rpm for 30 min. The pellet was suspended in TN-1 m*M* PMSF. The P10-*lac*-E7 preparation contained *lac*-E7 at approximately 20%, as revealed by gel electrophoresis. For

comparison a bacterial subcellular fraction without *lac*-E7 (P10-*coli*) was similarly prepared from YA21 containing pUC18, a backbone expression plasmid.

Enzyme-linked immunosorbent assay (ELISA) The ELISA procedure was described previously.[23] Human serum samples diluted at 1:10 with phosphate-buffered saline (PBS) were preabsorbed with *E. coli* strain YA21 containing pUC18 plasmid for 2 h at room temperature. After centrifugation at 10,000 rpm for 3 min, the supernatants were used for ELISA. The antigen preparations, P10-*lac*-E7 and P10-*coli*, were diluted in 0.05 *M* sodium carbonate buffer (pH 9.6) to appropriate concentrations (approximately 2 μg/ml) and were used to coat separately 96-well polystyrene microplates (Maxisorp, Nunc, Denmark) at 4°C, overnight. The protein concentrations were chosen so that the sera from 4 healthy adults (anti-HPV 16 E7 antibody-negative as tested by the radioimmunoprecipitation assay described below) gave comparable ELISA absorbances (A) for the two antigen preparations. The human serum samples were tested at a 1:100 dilution, and IgG, IgM and IgA antibodies were detected by using peroxidase-conjugated goat anti-human IgG, IgM, and IgA antisera (Tago, USA) at a 1:2000 dilution. Each well was measured with an automatic plate reader (Titertek Multiskan, Flow Laboratories, USA). The specific A values were calculated by subtracting the A values of P10-*coli*-containing wells from those of corresponding P10-*lac*-E7-containing wells, and were subjected to statistical analyses. Anti-HPV 16 E7 murine monoclonal antibodies (MAbs) (#704 and #730),[22] anti-HPV 16 E7 rabbit serum,[9] anti-HPV 16 E6 murine MAb (#618) (manuscript in preparation), anti-lymphotropic papovavirus murine MAb, and normal rabbit serum were used to check the specificity of ELISA. MAbs and rabbit sera were serially diluted 2-fold from 1:50 to 1:51200, and antibodies were detected with peroxidase-conjugated goat anti-mouse immunoglobulin antiserum and goat anti-rabbit IgG antiserum (Zymed, USA), respectively, at a 1:2000 dilution. The antibody titers, which were expressed as the reciprocal of the highest dilution giving specific A greater than 0.4, of the 3 anti-E7 sera were 6400–25600, whereas the background level was less than 50.

Radioimmunoprecipitation assay (RIPA) Monkey COS-1 cells[24] in a 90 mm dish were transfected by the calcium phosphate method[25] with 10 μg of pSRα-E7P DNA[22] which allows the efficient expression of HPV 16 E7 protein under the control of SV40 transcriptional regulatory elements and R-U5 sequence from LTR of human T-cell leukemia virus type 1.[26] Forty-eight hours later, mock-transfected or transfected cells were labeled with [^{14}C]leucine at 0.2 mCi/ml (CFB. 67, Amersham, UK) for 3 h. The labeled cells were scraped off a culture dish, suspended in 800 μl of modified RIPA buffer [20 m*M* HEPES (pH 7.0)-150 m*M* NaCl-1 m*M* EDTA-1% NP-40-1% sodium deoxycholate-0.1% sodium dodecyl sulfate (SDS)-0.5 m*M* dithiothreitol-0.2 m*M* PMSF], kept on ice for 20 min, and centrifuged at 10,000*g* for 20 min. Aliquots (200 μl) of supernatant were immunoprecipitated with 30 μl of serum. Immunocomplexes were collected with protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology, Sweden) electrophoresed on 15% SDS-polyacrylamide gels, and autoradiographed as described previously.[9]

Immunofluorescence assay (IF) COS-1 cells on cover slips were transfected with pSRα-E7P (HPV 16) or pSRα-H58 E7 (HPV58[27]) plasmid DNA at 1 μg of DNA per cover slip by the DEAE-dextran method.[28] At 48 h after transfection, cells were fixed for 5 min with acetone prechilled at −20°C and air-dried, or fixed for 20 min with 5% formalin in PBS at room temperature and rinsed in PBS. Formalin-fixed cells were incubated in 1% NP-40 in PBS for 20 min at room temperature before staining. The fixed cells were reacted with human sera at a 1:5 dilution, ascites of MAb #730,[22] or anti-*lac*-E7 serum[9] for 40 min at 37°C and then incubated with fluorescein-conjugated anti-human IgG, or anti-mouse IgG, or anti-rabbit IgG (Cappel-Organin Teknika Corp., USA) for 30 min at 37°C. Stained cultures were examined under a Nikon EFD2 UV microscope.

Statistical analyses Differences between proportions of positive sera in different groups were evaluated with the use of the chi-square test. A *P*-value below 0.05 was considered to indicate a significant difference.

RESULTS

Five hundred human sera were examined by ELISA for class-specific anti-E7 against bacterially expressed *lac*-E7 fusion protein and the results are summarized in the histograms shown in Fig. 1. The frequency distributions of the great majority of the samples appeared to be normal and the peaks of distribution were around specific A zero, which is expected for a sero-negative group. In determination of IgG antibody, we found several samples apparently not belonging to the great majority (Fig. 1A). When the mean plus 3×standard deviation (SD) was arbitrarily chosen as a cut-off value, 6 samples could be considered to be positive for IgG antibody. By the same criterion, one sample was apparently positive for IgM antibody (Fig. 1B), but this was considered to be false-positive because it contained rheumatoid factor. No samples were found to be positive for IgA antibody (Fig. 1C).

Selected serum samples were tested for their ability to immunoprecipitate the E7 protein transiently expressed in monkey COS-1 cells. The samples that showed specific A values over the mean plus 1.5×SD and 8 samples

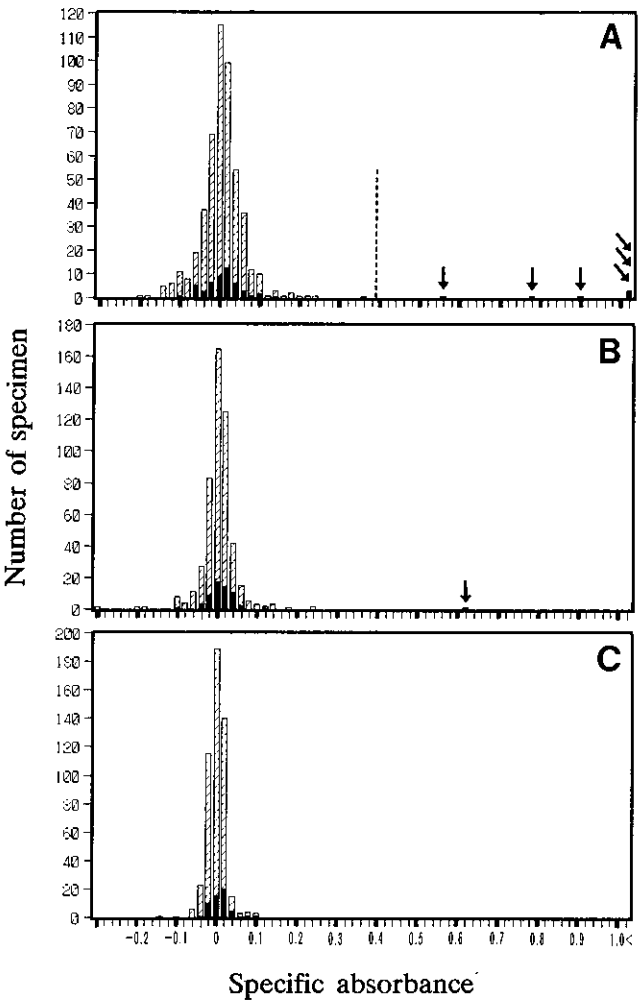

Fig. 1. Detection of class-specific antibody against HPV 16 E7 protein by ELISA. Number of samples is plotted against intervals of specific A. (A) IgG antibody. The broken line indicates an arbitrary cut-off value (the mean + 3 SD of total samples). Arrows indicate samples with A values higher than the mean + 3 SD. (B) IgM antibody. (C) IgA antibody. Filled bars show samples from cervical cancer patients. Shadowed bars show samples other than cervical cancer.

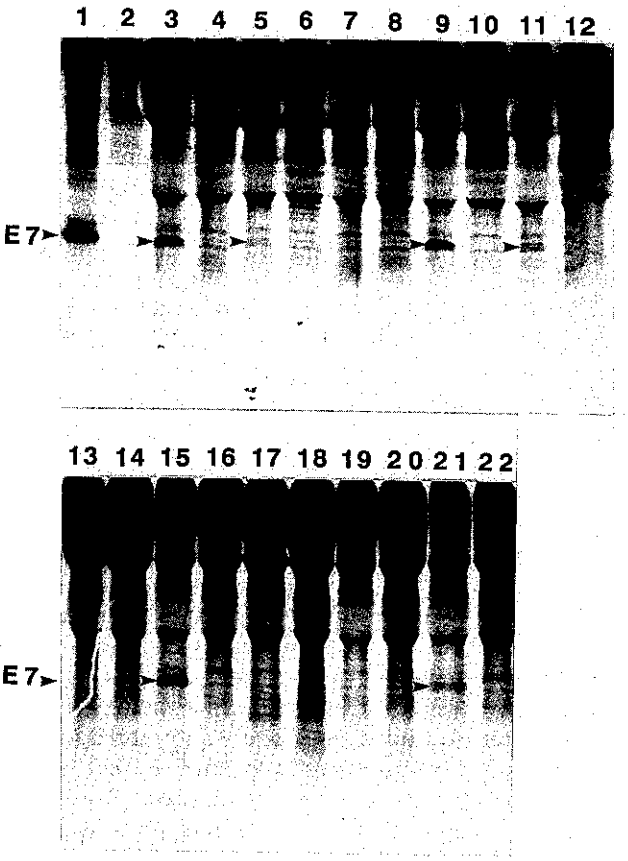

Fig. 2. Detection of anti-E7 antibody by RIPA. Untransfected COS-1 cells (lanes with even numbers) and COS-1 cells transfected with pSRα-E7P (lanes with odd numbers) were labeled with [^{14}C]leucine 48 h after transfection. Extracts from these cells were immunoprecipitated with anti-E7 MAb mixture #704 and #730 (lanes 1 and 2), and human sera, HC1519 (lanes 3 and 4), HC1520 (lanes 5 and 6), HC1530 (lanes 7 and 8), HC1537 (lanes 9 and 10), HC1546 (lanes 11 and 12), H3297 (lanes 13 and 14), G32 (lanes 15 and 16), #249 (lanes 17 and 18), #285 (lanes 19 and 20), and #398 (lanes 21 and 22). Immunocomplexes were collected with protein A-Sepharose, subjected to SDS-15% polyacrylamide gels, and autoradiographed. Arrowheads on the left indicate the position of E7 protein, which forms double bands when expressed under the control of the SRα-promoter in COS-1 cells. The E7 bands immunoprecipitated with human sera are also indicated by arrowheads. These bands were determined to be E7 as described previously[9,22] from the specific association of 20k bands with COS-1 cells transfected with pSRα-E7P, their absence in normal COS-1 cells, and the inability of normal rabbit and human sera to immunoprecipitate the 20k bands.

randomly selected from the apparent sero-negative group were subjected to RIPA and representative data are shown in Fig. 2. The E7 specific bands were detectable with the 6 samples that showed specific A values over the tentative cut-off value, but with none of the other samples (Table I).

The 6 samples positive for anti-E7 (in ELISA and RIPA) were tested, together with rabbit anti-*lac*-E7 and murine MAb #730, by indirect IF for their ability to detect the E7 protein expressed in COS-1 cells. The polyclonal rabbit antiserum can recognize both nuclear and cytoplasmic E7 expressed in COS-1 cells fixed either with acetone or formalin, whereas murine MAb #730 recognizes only cytoplasmic E7 in the culture fixed with acetone and both nuclear and cytoplasmic E7 in the

culture fixed with formalin (Fig. 3).[9, 22] Like the rabbit serum and unlike MAb #730, 4 out of the 6 human samples tested were able to reveal the presence of nuclear E7 protein in the transfected COS-1 cultures fixed with acetone or with formalin (Table I and Fig. 3). None of these 6 human positives stained the cells transfected with the plasmids capable of expressing HPV 58 E7.

The 6 samples positive for anti-E7 in ELISA and RIPA were from patients with cervical carcinoma, which constitute 54 out of the 500 cases examined in this study (Table II). Table III shows the clinical stage and histologic type of the anti-E7 positive cases. All of the positive samples were from patients with squamous cell carcinoma. The age of these patients ranged from 32 to 78 years.

Table I. Cases Positive for Anti-E7

Sample ID	ELISA specific A	RIPA	IF
HC1537	1.308	+	+
#398	1.217	+	+
G32	1.167	+	+
HC1520	0.905	+	−
HC1546	0.791	+	−
HC1519	0.571	+	+
HC1530	0.370	−	−
H3297	0.258	−	−
#249	0.223	−	−
#285	0.217	−	−

Table II. HPV 16 E7 Antibody in Various Populations

Populations	Cases	Anti-E7 IgG positives (%)
Patients with		
cervical cancer	54[a]	6 (11)
ovarian cancer	23	0
endometrial cancer	6	0
choriocarcinoma	3	0
condyloma acuminata	29	0[b]
genital herpes	150	0[c]
Prostitutes	30	0[b]
Homosexual men	33	0[b]
Pregnant women	51	0[d]
Healthy adult men	41	0[b]
Healthy adult women	80	0[d]

a) Including 8 cases of CIS.
b) $P<0.05$, *c*) $P<0.001$, *d*) $P<0.01$, as compared with the positive rate of cervical cancer group.

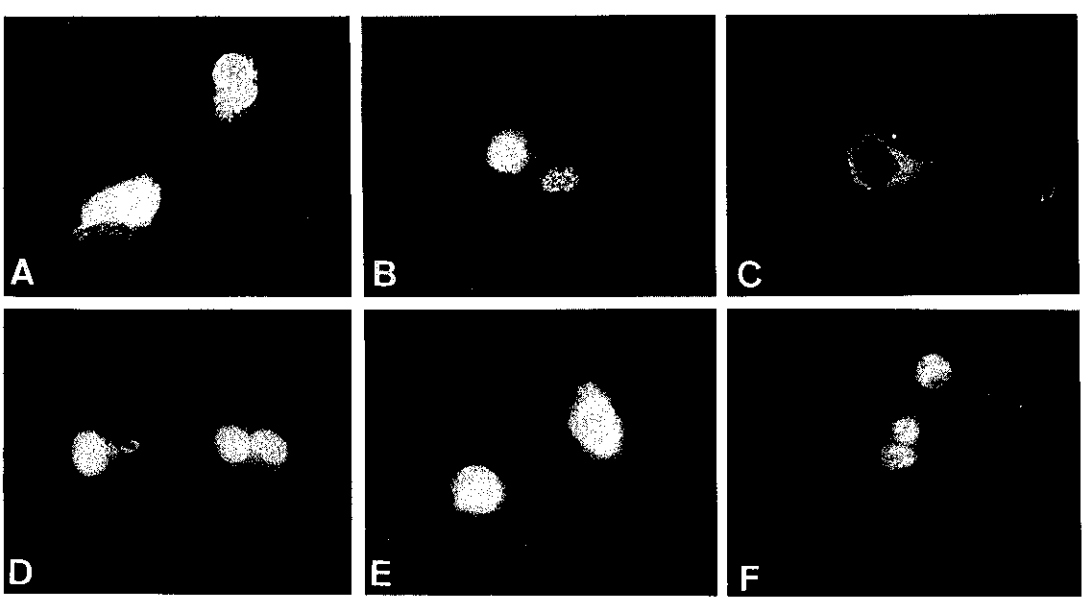

Fig. 3. IF staining of monkey COS-1 cells expressing HPV 16 E7 protein. Cells transfected with pSRα-E7P were fixed with cold acetone (A, B and C) or 5% formalin (D, E and F), and stained with anti-*lac*-E7 (A and D), a human serum sample (B and E), and MAb #730 (C and F). ELISA titers of applied antibodies were comparable.

Table III. Clinical Stage and Histologic Type of Cervical Cancers from Anti-E7 Positive Patients

Clinical stage of cervical cancer	Anti-E7 positives/cases with	
	squamous cell carcinoma	adenocarcinoma
0[a]	0/8	0/0
I	2/18 (#398, HC1520)[c]	0/1
II	2/12 (HC1537, HC1519)	0/3
III	1/6 (HC1546)	0/2
IV, Recur[b]	1/4 (G32)	0/0

a) Equivalent to CIS.
b) Recurrent cervical cancer.
c) Sample IDs of positive cases.

DISCUSSION

We attempted to detect the presence of anti-E7 antibody in human sera, using two types of HPV 16 E7 antigen; bacterially expressed *lac*-E7 and eucaryotic E7 proteins, because we expected that use of the two antigens differing in origin would enhance the specificity of detection. Fusion protein *lac*-E7 was used for ELISA, which is highly sensitive and is suitable for examination of a large number of samples. The antibodies against bacteria in human sera turned out to be problematic in the ELISA. To circumvent this problem, we used *E. coli* to preabsorb anti-bacterial antibodies in the sera and compared the specific A values to detect anti-E7 positives, as described in "Materials and Methods." Some sera were selected from among those tested by ELISA and were subjected to RIPA using COS-1 cells expressing E7. The results obtained from the two assays indicate that the ELISA can detect specific human antibody against the HPV 16 E7 protein and that 6 out of 500 samples were positive for anti-E7 antibody.

The data obtained by IF further confirm the specificity of detection of anti-E7 and give us some information on the epitope that the human antibody recognizes. Since the E7 proteins of HPV 16 and HPV 58 or 33 have the highest amino acid (AA) sequence homology of 60% matching among HPVs 1, 6, 8, 11, 16, 18, 33 and 58, lack of cross-staining of HPV 58 E7 protein with the human sera strongly suggests that the antibody detected in this study is specific for HPV 16 E7. Two regions of HPV 16 E7 protein consisting of 98 AAs, AAs 8 to 22 and AAs 39 to 54, are known to be masked in nuclei of COS-1 cells for reaction with murine MAbs unless the protein is denatured by formalin.[22] Thus, human anti-E7 appears to be different from murine MAbs in that it can recognize intranuclear E7. Recently, it was reported that the epitopes for human antibody against HPV 16 E7 are in the region of AAs 21 to 34 of the protein.[29]

We could detect the anti-E7 antibody in 11% of the 54 patients with cervical carcinoma, but in none of the 446 persons without cervical carcinoma. The results are considered to be essentially consistent with those obtained by the Western method by Jochmus-Kudielka *et al.*,[21] who found anti-E7 positives much more frequently in the patients with cervical carcinoma than in the normal population, because the positives in their control group showed weaker intensity of the reaction. Although it remains to be further studied by various methods whether or not normal persons carry anti-E7 antibody, our and their[21] data demonstrate that expression of the HPV 16 E7 protein is closely associated with cervical carcinomas.

Despite the well-established association of HPV 16 DNA with cervical carcinoma,[1,2] the role of HPV 16 E7 oncoprotein in carcinogenesis is virtually unknown and, thus, the correlation of HPV 16 DNA positivity and the presence of anti-E7 antibody is one of the important questions that remain to be investigated. Our data and those reported earlier[21] do not provide an answer, because materials for DNA studies were unavailable. The anti-E7 positivity (11%) of cervical carcinoma in this study, however, is significantly lower than the HPV 16 DNA positivity for cervical carcinoma (34 or 40%) from Japanese cases.[30,31] A similar difference has been recorded with German cases (21% for antibody[21] vs. 41 to 67% for DNA[31]). In a preliminary study, we tested 5 serum samples from patients with HPV 16 DNA-positive carcinoma, as determined by the Southern blot method, and could detect the anti-E7 antibody in none of them (unpublished data). One possible explanation for the discrepancy between the DNA and antibody data is that the sensitivity is different between the two assays. Indeed, the recent application of the polymerase chain reaction method is expected to widen the gap. Another possible explanation is that not all the patients with cervical carcinoma harboring HPV 16 DNA have anti-E7 antibody. These two hypotheses are not mutually exclusive. The second hypothesis is supported by the reports showing that the integrated HPV 16 DNA in cervical carcinomas is not always transcriptionally active[32] and that in some carcinomas only the antisense HPV RNA transcripts are detectable.[33] Further systematic studies are needed to correlate epidemiologic data on DNA and antibody.

In summary, we have shown that expression of the HPV 16 E7 protein is specifically associated with cervical carcinoma. The results support the hypothesis that the E7 protein is involved in carcinogenesis. In future serological studies, it will be important to examine simultaneously the presence of HPV DNA and mRNA in carcinoma and the presence in patients' sera of antibodies against HPV proteins, preferably including one that can be used as a marker for productive infection of HPV.

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