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Identification of Antibodies against Human Papillomavirus Type 16 E6 and E7 Proteins in Sera of Patients with Cervical Neoplasias

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We have developed a sensitive and specific ELISA method using the s10-fusion proteins of human papillomavirus (HPV) 16 E6 and E7, expressed in *E. coli*. Sera from 97 women (30 patients with invasive cervical cancers, 26 patients with cervical intraepithelial neoplasia III (CIN III) and 38 healthy women) were tested for the presence of antibodies to E6 and E7 proteins. Eight (27%) of the 30 cervical cancer sera, five (19%) of the 26 CIN sera and none of the 38 normal sera were reactive with E6 proteins (cut-off point: absorbance (A) = 0.59, x + 3SD). Ten (33%) of the 30 cervical cancer sera, two (8%) of the 26 CIN III sera and none of the 38 normal sera were reactive with E7 proteins (cut-off point: A = 0.40, x + 3SD). The mean absorbance for anti-E7 antibody in positive cases was higher in cancer patients than in CIN III patients, while that for E6 did not differ between these two groups. Interestingly, six (50%) of 12 cancer sera which reacted with either E6 or E7 proteins were reactive for both proteins, whereas none of the sera from the CIN III patients reacted with both proteins. The high prevalence rates and high absorbance values for HPV 16 E6 and E7 antibodies in association with malignant transformation suggest that detection of these antibodies may be a useful diagnostic aid for cervical cancer-associated HPV 16.

Key words: Human papillomavirus — Antibody — Early gene product — Fusion protein — Tumor marker

Up to the present time, more than 60 distinct types of human papillomavirus (HPVs) have been identified, and some of them have been found to be closely associated with anogenital carcinomas.[1,2] Especially, HPV types 16[3,4] and 18[5] are most commonly found in cervical carcinomas, their precursors (cervical intraepithelial neoplasia) and cervical carcinoma-derived cell lines. In addition, recent molecular biology research has shown that the E7 open reading frame (ORF) of HPV 16 and HPV 18 encodes a transforming gene for rodent cells[6–9] as well as human keratinocytes.[10] The E6 gene of HPV 16 and HPV 18 enhances the transforming ability of ORF E7, especially for human primary cells.[11] Moreover, the E7 protein of HPV 16 and the E6 proteins of HPV 16 and HPV 18 were identified in nuclear and/or cytoplasmic protein fractions from uterine cervical carcinomas.[12,13] Thus, the presence and expression of HPV 16 and HPV 18 genes appear to be a major risk factor for the development of cervical carcinomas. Therefore, identification of these HPV DNAs in routine clinical samples is very important for detecting HPV infection and understanding its role in carcinogenesis.

Although multiple methods, including filter hybridization, *in situ* hybridization and the polymerase chain reaction (PCR) method, have been developed as sensitive and specific methods for the identification of HPV DNAs, such nucleic acid hybridization methods are cumbersome and expensive in practical medicine. One possible avenue for exploration with regard to screening for HPV infection is the detection of circulating HPV-related antigens and/or antibodies in human sera. Exposure to a virus will generally induce antibodies against the virus in the sera of the host, and practical assay systems for detection of antibodies to viruses other than HPVs are already available. However, techniques for HPVs are still under development. Unfortunately, HPV-related antigens for serological assay cannot be obtained from tissue culture systems and so have become available only recently through the application of recombinant DNA technology.

These circumstances prompted us to investigate the antibodies circulating in human sera against E6 and E7 proteins of HPV 16, produced by use of a T7-polymerase-dependent expression vector[14] in *Escherichia coli*.

MATERIALS AND METHODS

Human sera Human sera were obtained from 30 patients with invasive squamous cell carcinoma of the cervix and 26 patients with cervical intraepithelial neoplasia (CIN), grade III. These patients ranged in age from 22 to 76 years and were treated between March 1988 and June

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1991 at the Department of Obstetrics and Gynecology, Osaka University Medical School. As normal controls, 38 serum samples were obtained from five patients with myoma, nine patients with ovarian cyst and 24 normal volunteers; these subjects showed normal cervical cytology and ranged in age from 21 to 65 years. All samples were stored at −20°C until the time of assay.

Cloned HPV DNA, bacterial cells, plasmids and antibodies Cloned HPV 16 DNA was kindly supplied by Dr. H. zur Hausen (German Cancer Virus Research Center, Heidelberg, Germany). The expression plasmids, pET and pARA, which express the protein in the control of T7 promoter and *E. coli* strain BL21 (DE 3) Lys S, were generous gifts of Drs. Kiyono and Ishibashi (Aichi Cancer Center, Nagoya). *E. coli* XL-1-Blue was purchased from Stratagene Inc. (Fukui). Anti-E7 polyclonal antibody was a gift from Dr. Yoshiike (National Institute of Health, Tokyo) and monoclonal antibodies specific for E6 of HPV 16 and HPV 18 were purchased from Chemicon International Inc. (Temecula, CA).

Construction of expression plasmids of HPV 16 E6 and E7 genes HPV 16 DNA was digested at *Rsa* I-*Dde* I site for E6 (nucleotide number: 110–560) and *Sau*3AI-*Nco* I site for E7 (nucleotide number: 580–860). Each E6 and E7 DNA fragment was ligated using DNA ligation kits (Takara Co., Ltd., Osaka) in frame with expression vectors pET and pARA. The pET vector was used for generation of the fusion proteins, i.e., s10-E6 and s10-E7 (s10: 12 amino acid leader sequences of T7 gene 10). The pARA vector was used for generation of s10-protein A-E6 or E7 fusion proteins.

E. coli strain XL-1-Blue was transformed with the recombinant plasmids according to a modification of the method of Cohen *et al.*[15] Ampicillin-resistant colonies were analyzed by colony *in situ* hybridization and restriction analysis to confirm the presence and the proper orientation of the plasmids. Recombinant plasmids pET-E6, pET-E7, pARA-E6 and pARA-E7 were introduced into *E. coli* strain BL21 (DE 3) Lys S, and selection was achieved with 20 μg/ml ampicillin and 25 μg/ml chloramphenicol (Fig. 1).

Purification of fusion proteins BL21 (DF3) Lys S cells containing the expression plasmid were maintained in L-broth (or M9ZB) medium containing 20 μg/ml ampicillin and 25 μg/ml chloramphenicol. Cells were grown at 30°C until the mid log phase of bacterial growth. Subsequently, isopropylthiogalactoside (IPTG) and rifampicin were added to final concentrations of 0.4 m*M* and 200 μg/ml, respectively, and incubation was continued for an additional 2–3 h at 37°C. Lysis of the bacteria and purification of the s10-E6 and s10-E7 fusion proteins were carried out by a modification of the method of Rosenberg *et al.*[14] Briefly, cell pellets were lysed with 0.2 mg/ml lysozyme in hypotonic buffer (50 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0) and sonicated for a few seconds. The lysate was centrifuged at 10,000 rpm for 10 min, and s10-E7 fusion protein was obtained in the supernatant. On the other hand, s10-E6 fusion protein was eluted from the lysate in 50 m*M* Tris-HCl buffer containing 7 *M* urea after removing bacterial proteins soluble in 3 *M* urea in 50 m*M* Tris-HCl buffer. The s10-protein A-E7 and s10-protein A-E6 were purified on IgG Sepharose columns (Pharmacia, Freiburg, Germany) according to the supplier's recommendations. s10-E7 and s10-E6 were used as the antigens for enzyme-linked immunosorbent assay (ELISA). s10-protein A-E7 and s10-protein A-E6 were used as antigens for the immunization of rabbits, whose sera were used as the positive controls for Western blot analysis and ELISA.

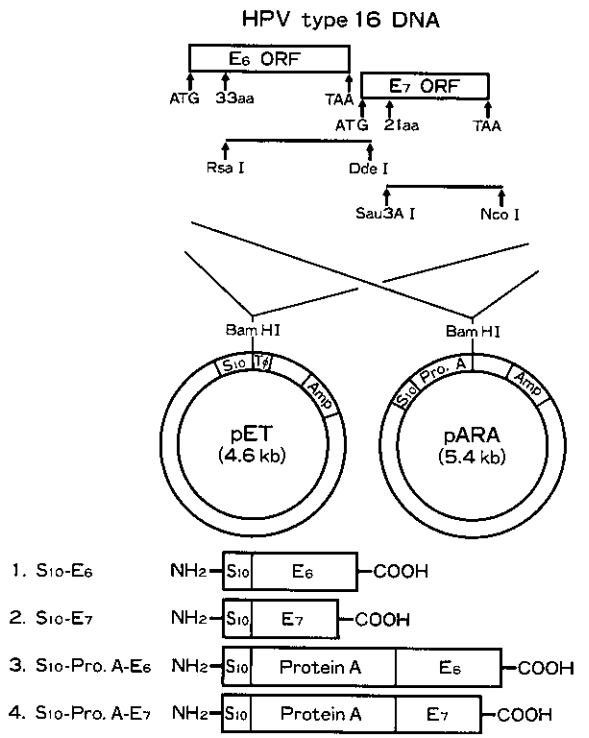


Fig. 1. Schematic representation of the plasmids used for expression of HPV16 E6 and E7 gene products.

Immunization of rabbits Approximately 1 mg of purified fusion protein (s10-protein A-E6 or s10-protein A-E7) was mixed 1:1 with RIBI adjuvant (RIBI Immunochem. Res. Inc., Montana, MT). Rabbits were immunized by intramuscular injection of this mixture into both hind legs. Booster injections of each fusion protein in incomplete Freund's adjuvant were administered 2 and 4 weeks later.

Immunoprecipitation The *E. coli* cells producing a fusion protein were labeled with [^{35}S]cysteine in M9 minimal essential medium. The crude cell lysates were incubated with monoclonal or polyclonal antibodies for 1 h at 4°C, and then protein A Sepharose (Pharmacia, Uppsala, Sweden) was added to the antigen-antibody mixture at 1/100 (v/v). After incubation at 4°C for 30 min, the immunocomplexes were dissociated in 25 m*M* Tris buffer (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, 10% glycerol, and 0.1 *M* dithiothreitol by heating at 100°C for 5 min. The eluted samples were loaded onto a 5% stacking/16% SDS-polyacrylamide gel at 20 mA/gel. After drying, the gel was exposed to Fuji RX film.

Western blotting About 10 μg of lysate of *E. coli* expressing s10 fusion proteins was subjected to gel electrophoresis on 5% stacking/16% separating SDS-polyacrylamide gels and run at 20 mA/gel. The proteins were then electro-blotted onto a nitrocellulose filter (BA Schleicher & Schuell, Dassel, Germany) at 30 mA/gel using a blotting apparatus. The filter was immersed in 0.1 *M* Tris (pH 7.5), 0.15 *M* NaCl and 10% dry milk (M-TBS) for 30 min at room temperature to block nonspecific binding. After washing with TBS-0.05% Tween 20 (T-TBS), the filter was incubated for 3 h at room temperature with human serum which had been preabsorbed overnight with acetone powder of *E. coli* lysate and diluted 1:20 with M-TBS. For the positive control, rabbit anti-E6 and anti-E7 sera diluted 1:100 with M-TBS were applied to the filter. After several washings with T-TBS, the filter was reacted with sheep anti-human or anti-rabbit immunoglobulin labeled with biotin (Amersham Japan, Tokyo) for 30 min at room temperature, followed by incubation with strepto-avidin-alkaline phosphatase GIBCO, BRL, Tokyo). Subsequently, antibody complexes were visualized with NBT and BCIP (GIBCO, BRL) in 100 m*M* Tris-HCl, 100 m*M* NaCl and 50 m*M* MgCl (pH 9.6).

ELISA The fusion proteins (s10-E6 and s10-E7) were diluted to 10 μg/ml in 10 m*M* carbonate buffer, pH 9.6, and held overnight in 100 μl/well microtiter plates (ELISA S-plate, Sumirom Co., Tokyo). Wells coated with *E. coli* lysate containing pET vector without ORF E6 or E7 served as the negative controls. After washing with T-TBS, the plates were blocked with M-TBS for 60 min at 37°C. Human sera were preincubated with an acetone powder of the *E. coli* lysate overnight at 4°C. The preabsorbed human sera were diluted 1:20 with M-TBS and added to the plates, which were then incubated for 180 min at room temperature. After washing with T-TBS, the plates were incubated for 30 min at room temperature with biotin-labeled sheep anti-human immunoglobulin. After washing with T-TBS, strepto-avidin-alkaline phosphatase was added. The plates were washed with TBS-T and incubated with *p*-nitrophenyl phosphate (Kirkeguard & Perry Labs, Inc., Gaithersburg, MD) for 60 min at room temperature. The reaction was stopped by addition of 20 m*M* TE buffer, and the absorbance of each well was measured at 415 nm with a microplate photometer (Titertek Multiskan MCC/340, Japan Flow Labs. Inc., Tokyo). The final absorbance value of each sample was calculated as the absorbance of the fusion protein minus that of the *E. coli* lysate. As the internal standard, the HPV16-positive serum sample (No. 5) was reacted with the respective antigens in each test. The final ELISA absorbances were adjusted in relation to the internal standard to compensate for interassay variation. To assign a cut-off point between reactivity and non-reactivity in our fusion protein-based ELISA, we tested the sera of 38 women showing no abnormalities in their cervico-vaginal smears. The cut-off value was selected as the mean final absorbance value (A) of those sera plus three standard deviations (SD).

Seventeen serum samples were also subjected to Western blot and immunoprecipitation analysis for the purpose of confirming the specificity of the present ELISA assays.

PCR for HPV The PCR was performed to generate amplified fragments of the E6 genes of HPV 16 and 18.[16] The primers used were 5′ATTAGTGAGTATAGACATTA3′ (H1), 5′GGCTTTTGACAGTTAATACA3′ (H2) and 5′GGTTTCTGGCACCGCAGGCA3′ (H3). H1 and H2 correspond to the nucleotide positions (np) 320–339 and 410–429 of HPV 16, respectively. H1 and H3 correspond to np 328–347 and 418–437 of HPV 18, respectively. After 25 cycles of PCR, the amplified DNAs were electrophoresed on 1.5% agarose gel, transferred onto a nylon filter and hybridized with a ^{32}P-labeled oligomer probe specific for E6 of HPV 16 or HPV 18 by the Southern blot method. Caski cells were used as the positive control for HPV 16 and the negative control for HPV 18, respectively. Similarly, HeLa cells served as the negative control for HPV 16 and the positive control for HPV 18. The sensitivity of the present assay is such that 20 viral copies per sample can be detected.

RESULTS

Specificity of fusion proteins The expression products of *E. coli* carrying vectors pET-E6 or -E7, or pARA-E7 are demonstrated on Coomassie blue-stained gels in Fig. 2. The s10-E6 and s10-E7 fusion proteins are represented as strong bands at 17.5 kd and 16 kd in lanes pET-E6(IPTG+) and pET-E7(IPTG+), respectively (Fig. 2A). The s10-protein A-E6 and s10-protein A-E7 fusion proteins are seen at 48.5 kd (Fig. 3A) and 47 kd (Fig. 2B), respectively. In order to confirm that the ex-

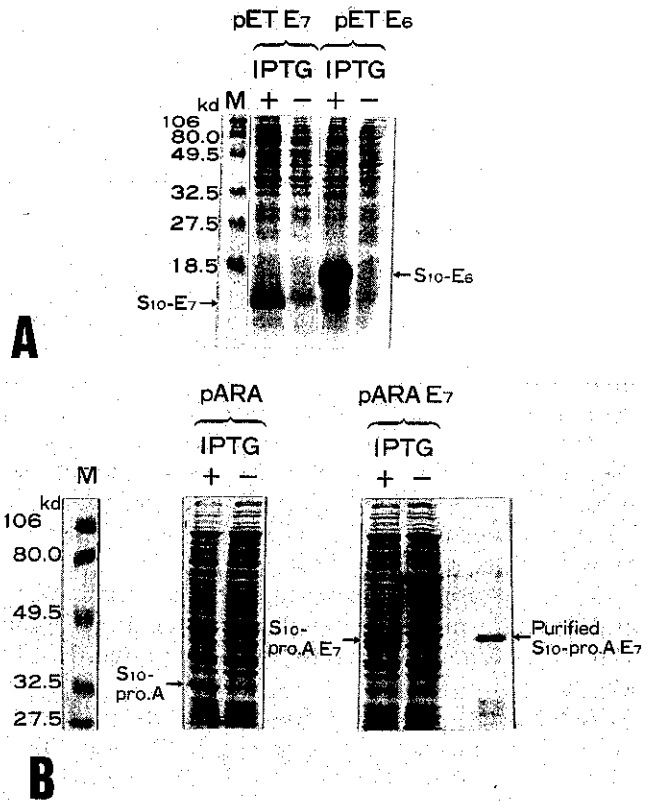


Fig. 2. Demonstration of s10-HPV16 and s10-protein A-HPV16 fusion proteins by Coomassie blue staining. The fusion proteins of s10-HPV16 E6 and s10-HPV16 E7 are depicted at 17.5 kd in lane pET E6/IPTG(+), and 16 kd in lane pET E7/IPTG(+), respectively (A). The fusion proteins of s10-protein A and s10-protein A-HPV16 E7 are depicted at 32 kd in lane pARA/IPTG(+) and at 47 kd in lane pARA E7/IPTG(+), respectively (B). The s10-protein A-E7 purified on an IgG Sepharose column is also depicted in the lane at the right end. M: molecular weight marker.

pressed fusion protein is related to HPV 16 E6 or E7 protein, immunoprecipitation analysis was carried out using monoclonal and polyclonal antibodies specific for HPV 16 E6 or E7 protein (Fig. 3). Monoclonal antibody against HPV 16 E6 protein showed strong reactivity with s10-E6 and s10-protein A-E6 fusion protein, showing single bands at 17.5 kd in the lane pET-E6 and 48.5 kd in the lane pARA-E6, respectively. No reaction was observed in the lane of the pET lysate derived from *E. coli* containing only pET vector or the pET-E6 lysate incubated with another antibody unrelated to HPV protein (anti-X-antibody) (Fig. 3A). Polyclonal antibody against HPV 16 E7 protein immunoprecipitated the specific proteins at 16 kd and 47 kd, as shown in lane 1 of pET-E7 and pARA-E7 (Fig. 3B), but no specific precipitate was detected in the lanes of the pET lysate (Fig. 3A). Normal rabbit serum also did not react with this 16-kd protein. On the other hand, normal rabbit serum reacted with s10-protein A, as shown in lanes 1 and 2 of pARA. However, this band may have been due to the formation of IgG-protein A complexes (Fig. 3B).

Production of polyclonal antibodies against HPV 16 E6 or E7 protein Rabbit antisera prepared against s10-protein A-E6 and s10-protein A-E7 fusion proteins reacted specifically with s10-E6 (17.5 kd) and s10-E7 (16 kd) fusion proteins in Western blotting (Fig. 4). These two antisera were used as positive controls for Western blotting and immunoprecipitation analysis of human sera.

Detection of antibodies against HPV16 E6 or E7 proteins in human sera Serum samples from 12 cervical cancer patients and five healthy controls whose cervical cells were investigated for HPV 16 or 18 DNA by the PCR method were tested by ELISA, Western blotting and immunoprecipitation for reactivity with HPV16 E6 or E7 fusion protein (Table I, Fig. 4). Of six sera from HPV 16-positive cancer patients, four sera and five sera reacted with s10-E6 (>0.59 *A*) and s10-E7 (>0.40 *A*) proteins, respectively, in the ELISA analysis. Western blot and/or immunoprecipitation analysis showed similar results with the exception of Case 4, in which E6 antibodies could not be detected by ELISA and Western blotting but were detected by immunoprecipitation assay. On the other hand, four sera from HPV 18-positive cancer patients and seven sera from women negative for HPV 16 and 18 did not react with these proteins in ELISA, Western blotting and immunoprecipitation.

Serum samples from the 30 patients with invasive cervical cancers and the 26 patients with CIN III were tested by ELISA for reactivity with HPV 16 E6 or E7 fusion proteins (Fig. 5). The cut-off points (mean+3SD) were 0.59 for E6 protein and 0.40 for E7 protein. Sera from 8 (27%) of the 30 cancer patients and five (19%) of the 26 CIN III patients reacted with E6 protein. Similarly, sera from 10 (33%) of the 30 cancer patients and 2 (8%) of the 26 CIN III patients reacted with E7 protein. The frequency of anti-E6-positive or anti-E7-positive sera was thus higher in patients with cervical cancer than CIN III. The mean absorbance of E7 antibody-positive sera of cancer patients was significantly higher than that of CIN III patients (Student's *t* test; $P<0.05$), while the absorbance for E6 protein did not differ between these two patient groups. Interestingly, 6 (50%) of 12 cancer patients whose sera reacted with either E6 or E7 protein were reactive for both proteins, whereas none of the sera from the CIN III patients reacted with both proteins. Moreover, 12 (40%) of the 30 patients with invasive cervical cancers and 7 (27%) of the 26 patients with CIN III reacted with either E6 or E7 protein. It is

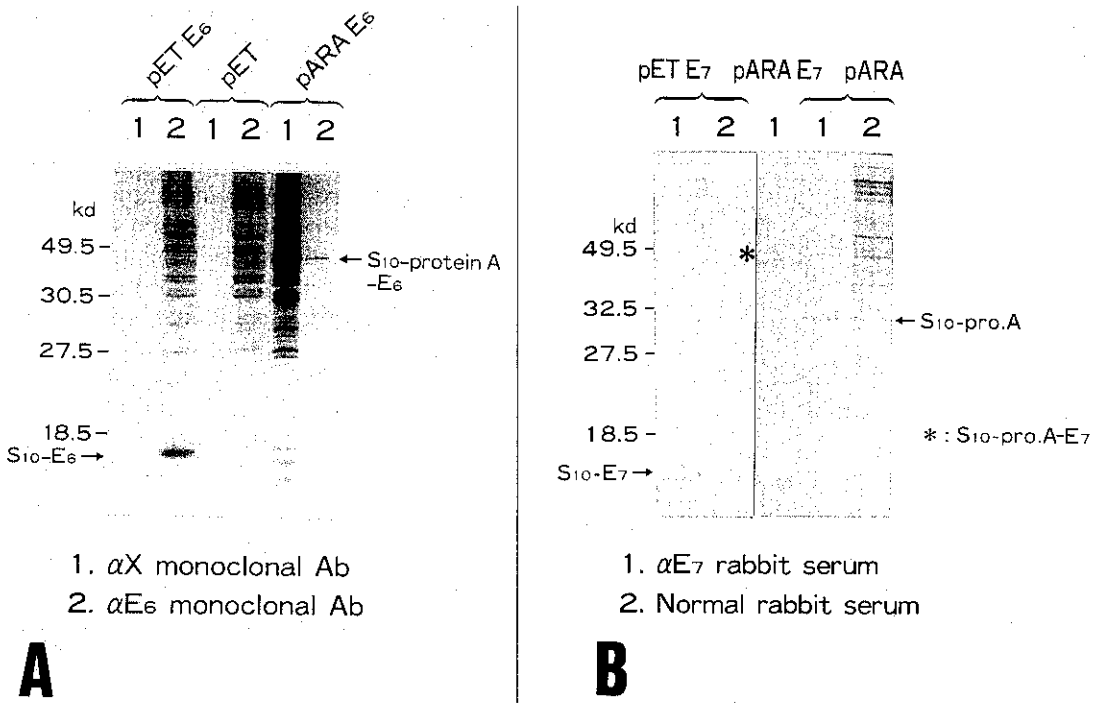


Fig. 3. Immunoprecipitation of s10-HPV16 and s10-protein A-HPV16 fusion proteins. The fusion proteins of s10-E6 and s10-protein A-E6 are depicted at 17.5 kd in lane 2 of pET 6 and at 48.5 kd in lane 2 of pARA E6, respectively. No specific bands are detected in the negative control lysates (lanes 1 and 2 of pET) or in the lysate immunoprecipitated with HPV-unrelated antibody (lane 1 of pET E6 and lane 1 of pARA E6) (A). The fusion proteins of s10-E7 and s10-protein A-E7 are depicted at 16 kd in lane 1 of pET E7 and at 47 kd in lane 1 of pARA E7, respectively, while these proteins are not immunoprecipitated by normal rabbit serum (B). The negative control lysate (pARA) gives an immunoprecipitate at 32 kd with both anti-E7 rabbit serum and normal rabbit serum, but this reaction is derived from nonspecific binding between protein A and IgG.

also notable that six (67%) of nine patients infected with HPV 16 had antibody to either E6 or E7 protein in their sera.

DISCUSSION

Viral infection in humans induces an immune response against the viral antigens, and this phenomenon has permitted the development of practical and specific assay systems for several viral infections. They are now commercially available. However, little is known about the immune response to infection by HPV, despite a great deal of knowledge about molecular analysis. Quite recently, several investigators have focused on the antibodies circulating in human sera against HPV 16 gene products and have devised assay systems for detecting those antibodies as serodiagnostic markers of HPV infection. Some investigators have expressed HPV-derived proteins fused with β-galactosidase[17, 18] or MS-2 polymerase[19] in bacteria and used them as antigens in Western blotting to detect antibodies in human sera. However, such fusion proteins consist of a large amount of protein with a high molecular weight derived from bacteria, and cross-reactivity with bacterial antigen thus can not be excluded. Other investigators have used synthetic peptides,[20, 21] which are easier to prepare than fusion proteins obtained by genetic engineering. However, it is questionable whether such synthetic peptides really respond to antibodies specific for HPV 16-derived proteins because their three-dimensional structures differ from those of native proteins derived from HPV genes. Therefore, We introduced an improved system for obtaining a highly purified fusion protein using a T7 polymerase-dependent expression vector, i.e., pET vector. s10 protein derived from T7 bacteriophage gene 10 is a tiny peptide composed of 12 amino acids. The s10-E6 and s10-E7 fusion proteins used in the present study are proteins in which s10 is connected with the N-terminal of E6 and E7 gene-derived proteins; their molecular weights are 17.5 kd and 16 kd, respectively. Since s10 protein is a

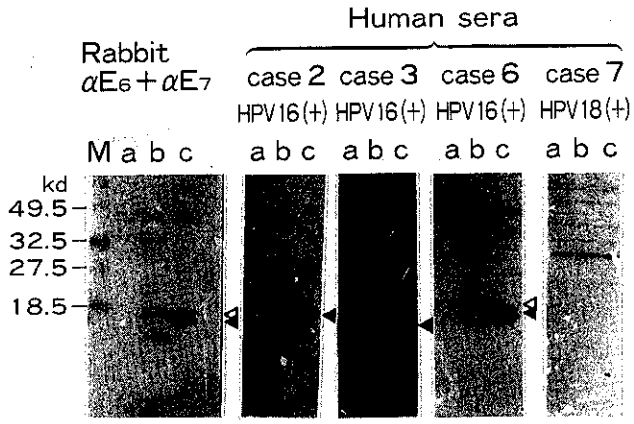


Fig. 4. Western blot analysis of human sera from women whose uterine cervical cells were investigated for HPV16 and 18 DNA. Negative control lysate (*E. coli*; lane a) and fusion protein extracts (lane b: s10-E6; lane c: s10-E7) were transferred onto nitrocellulose filters after gel electrophoresis. The filters were incubated with immunized rabbit serum and human serum. Rabbit serum reacted with the E6 and E7 fusion proteins. The sera obtained from Cases 2, 3 and 6 reacted with E7 protein of HPV16. Case 6 also reacted with E6 protein of HPV16. Case 7 did not react with either protein. The case numbers correspond to those shown in Table I. ◁ s10-E6, ◀ s10-E7.

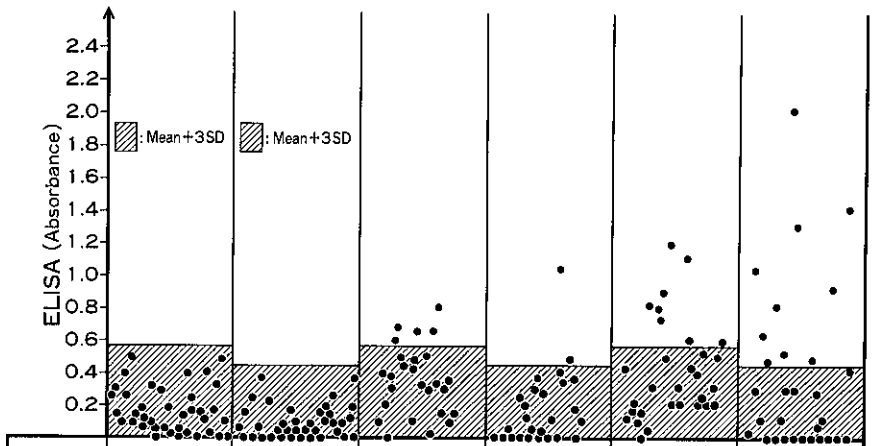
much smaller peptide compared to MS-2 polymerase or β-galactosidase, non-specific reactions in the present ELISA method might be reduced. Moreover, the pET vector has another special characteristic in that expression of its proteins is not reduced by rifampicin, which blocks the function of the host bacterium's RNA polymerase. Therefore, by co-incubation with IPTG and rifampicin, we were able to obtain more of the desired fusion proteins and less bacterial protein. In addition, serum samples were preabsorbed with *E. coli* lysate before assay in order to eliminate the background reaction.

The aforementioned improvements might make the present ELISA method more specific and sensitive. Actually, our results with ELISA mostly agreed with the results of Western blotting and immunoprecipitation assay, as shown in Table I and Fig. 4. However, a few sera with titers around the cut-off point of ELISA showed a discrepancy among the three assay systems; ELISA, Western blot and immunoprecipitation. Such a difference may be due to inevitable interassay variation. All six patients with cervical cancer harboring HPV 16 DNA were shown to have antibody specific for either HPV 16 E6 or E7 by the present ELISA method. In contrast, all the sera from patients harboring HPV 18 and females without HPV infection were negative. Anti-

Table I. Correlations between Infecting HPV Types, ELISA and Western Blotting Analysis in the Human Sera

Case	Diagnosis	HPV type	Antigens					
			s10+E6 (HPV16)			s10+E7 (HPV16)		
			ELISA (*A*)	WB	IP	ELISA (*A*)	WB	IP
1	C.C.	16	0.62	+	+	0.02	−	−
2	C.C.	16	0.63	−	+	1.03	++	+
3	C.C.	16	0.42	−	−	0.62	+	+
4	C.C.	16	0.56	−	+	0.47	−	−
5	C.C.	16	0.69	+	+	2.0	++	++
6	C.C.	16	1.18	+	+	1.21	++	+
7	C.C.	18	0.45	−	−	0.30	−	−
8	C.C.	18	0.35	−	−	0.10	−	−
9	C.C.	18	0.20	−	−	0.0	−	−
10	C.C.	18	0.31	−	−	0.0	−	−
11	C.C.	negative	0.21	−	−	0.0	−	−
12	C.C.	negative	0.25	−	−	0.26	−	−
13	normal	negative	0.12	−	−	0.0	−	−
14	normal	negative	0.39	−	−	0.0	−	−
15	normal	negative	0.25	−	−	0.34	−	−
16	normal	negative	0.13	−	−	0.0	−	−
17	normal	negative	0.13	−	−	0.04	−	−

C.C.: Invasive cervical cancer.
−, Negative; +, weakly positive; ++, strongly positive in Western blotting (WB) and immunoprecipitation (IP).



Antibody	Anti-E6	Anti-E7	Anti-E6	Anti-E7	Anti-E6	Anti-E7
Positivity	0/38 (0%)	0/38 (0%)	5/26 (19%)	2/26 (8%)	8/30 (27%)	10/30 (33%)
Diagnosis (n)	Healthy women (38)		CIN III (26)		Cervical cancer (30)	

Fig. 5. Reactivity of human sera with E6 and E7 fusion proteins.

bodies to HPV 16 E6 were not found in any of the sera from healthy controls, but were detected in 19% of CIN-patients' sera and 27% of cancer-patients' sera. The positive incidence was thus higher in precancer and cancer patients than in the normal controls. Similar results have been reported by Dillner,[20] who synthesized the predicted amino acid sequence of E6 ORF of HPV 16 and detected reactivity with the peptide in 48% of cancer patients' sera and 28% of control sera by the ELISA method. The reason for the discrepancy in the percentage of positive cases (28% in his study vs. 0% in ours) in the normal controls is not clear. However, one possibility is the difference in setting cut-off points between the two studies. In his study, the cut-off point was a background reaction level corresponding to the uncoated well of the ELISA plates, while our cut-off level was mean absorbance value plus three standard deviations of control sera. The second possibility is that Dillner's synthesized peptide may induce non-specific reactions, because it might make an artificial conformation. The third possibility is the difference in race and geography between the two studies. The frequency of HPV infections in the general population in Japan has been estimated to be lower than that in Europe and the United States.[22, 23]

Antibodies to E7 protein have been extensively studied by several investigators using Western blotting[18, 19] and ELISA.[20, 21] According to those reports, antibodies to E7 were detected in 2–4% of normal control sera and 20–30% of cancer patients' sera. These findings are strikingly similar to our results, i.e., positive incidences of 0% in healthy controls, 8% in CIN patients and 33% in cervical cancer patients. The positive incidence and the mean absorbance for E7 protein in the sera of the patients with invasive cervical cancer were higher than in those with precancerous lesions. It is also noteworthy that 12 of 30 cancer patients (40%) were positive for either E7 or E6 antibody, and that 6 of those 12 patients had antibodies to both proteins. In contrast, none of the CIN patients had antibodies to both proteins. These data may support the concept that interaction between E6 and E7 proteins occurs during the process of transformation from precancer to invasive cancer.

HPV infects the squamous epithelium, gains access to the germinal or replicative cells in the basal epithelium via defects in the surface mucosa, and then produces some morphological changes. The interval from exposure to the development of a lesion may be considered to range from a few weeks to several years. During such a long process, multiple events, including a host immune response, may take place. Antibodies to late proteins, such as L1 and L2,[18] are very likely to be produced in consequence of a primary HPV infection. Anti-E4 antibody seems to appear during the stage of viral replication,[24, 25] because the highest proportion of anti-E4 antibody-positive individuals (40.7%) was observed in the age group between 10 and 20 years. A considerable number of individuals under 10 years old were also positive, and the frequency of anti-E4 positive sera was three-fold higher in patients with HPV-associated genital lesions.[19] Although we did not test for antibodies to late gene products or early gene products other than E6 and E7 in the present study, 40% of the cancer patients

harbored antibody to either E6 or E7. Such findings correspond well with the incidence of HPV 16 infection among cervical cancer patients in Japan.[26-28]

The present findings may support the hypothesis that antibody to either E6 or E7 may be produced in the early stage of carcinogenesis, followed by the immunogenic response against both proteins during the establishment of invasiveness and production of antibodies to both proteins as a final event in the host. However, further studies are necessary to clarify the immune response in the event of HPV infection and subsequent malignant transformation. The natural history of human genital papillomavirus infection may be elucidated by serological studies using the sensitive and specific ELISA assay described here.

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