Monoclonal Antibody-Mediated Neutralization of Infectious Human Papillomavirus Type 11

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Monoclonal antibodies recognizing human papillomavirus type 11 (HPV-11) were prepared from BALB/c mice immunized with intact HPV-11 virions obtained from morphologically transformed human foreskin xenografts grown subrenally in athymic mice. Four of five monoclonal antibodies that were reactive by enzyme-linked immunosorbent assay only to intact virions neutralized HPV-11 infectivity in the athymic mouse xenograft system.

Human papillomaviruses (HPVs) infect a variety of epithelial tissues in a species- and tissue-specific manner (14). Assays for testing antibody-mediated neutralization of HPV infectivity have not been available because of ethical considerations and because of the lack of a suitable animal or tissue culture model for measuring HPV infectivity. Neutralizing epitopes have not been defined for any of the more than 60 HPV types described.

We have developed an animal model that allows HPV type 11 (HPV-11) infectivity, viral replication, and morphological transformation of human xenografts transplanted beneath the renal capsule of athymic mice (13, 15). The xenograft model has also been used to detect polyclonal antibody-mediated neutralization of HPV-11-induced morphological transformation of human foreskin tissue, as well as neutralization of bovine papillomavirus type 1 (BPV-1) infection of bovine skin tissue and cottontail rabbit papillomavirus (CRPV) infection of domestic rabbit skin tissue (2). Neutralization of infectivity and prevention of morphological transformation now provide a model for the identification and analysis of neutralizing epitopes on HPV-11 by monoclonal antibodies.

Two other animal models for antibody-mediated neutralization of papillomaviruses have been described. These models include neutralization of BPV-1-induced focus formation of mouse C127 cells in tissue culture (5, 22), protection in cattle following BPV-1 challenge (21), and neutralization of CRPV-induced formation of papillomas on domestic rabbit skin (12).

The purpose of this study was to develop monoclonal antibodies to HPV-11 starting from immunizations with infectious viral stocks which have been shown to induce neutralizing polyclonal antibodies (2). These monoclonal antibodies were screened in an *in vivo* assay to determine whether they could neutralize HPV-11-induced morphological transformation of human foreskin in the xenograft system.

Production, screening, and cloning of monoclonal antibodies. BALB/c mice were immunized subcutaneously with 20 μ g of intact HPV-11 in phosphate-buffered saline (PBS) emulsified in complete Freund adjuvant. The mice were boosted in the hind footpads 1 and 2 months later with 20 μ g

of HPV-11 in incomplete Freund adjuvant and 20 μ g of HPV-11 in PBS, respectively. Three days after the final boost, the spleen and popliteal lymph nodes were removed and a single-cell suspension of lymphocytes was prepared. Separate suspensions of spleen and lymph node cells were fused by polyethylene glycol 1500 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) with P3X63-Ag8.653 (American Type Culture Collection, Rockville, Md.) nonsecreting mouse myeloma fusion partner cells at a 1:1 ratio (8). The fusions were plated into 96-well, flat-bottom microtiter plates in Iscoves modified Dulbecco medium containing 10% fetal bovine serum, hypoxanthine (10^{-4} M), aminopterin (4×10^{-7} M), and thymidine (10^{-5} M). Three to four days later, culture wells were supplemented with 5% conditioned medium obtained from mixed cultures of human foreskin chips and irradiated NIH 3T3 cells. Approximately 1,500 hybridomas developed from fusions containing 6.4×10^7 lymph node cells and 6.7×10^7 spleen cells from a single mouse which tested positive for serum antibody to HPV-11 by immunohistochemical staining of fresh-frozen sections of experimental condyloma.

Supernatants from individual culture wells containing growing hybridomas were assayed for antibody reactivity to HPV-11 by enzyme-linked immunosorbent assay (ELISA) and by immunohistochemical staining of koilocytotic nuclei in fresh-frozen sections of experimental condyloma (3). Cultures identified as positive were cloned at one cell per well in 96-well, flat-bottom microtiter plates containing Iscoves medium supplemented with 5% conditioned medium. After 2 weeks of growth, individual wells were examined microscopically and supernatants from wells containing a single colony of hybridomas were screened for anti-HPV-11 antibody. Monoclonal antibodies were classified with an antibody-isotyping kit (Amersham Corp., Arlington Heights, Ill.).

ELISA. ELISAs of monoclonal antibody reactivity against both intact and disrupted HPV-11, BPV-1, and CRPV were performed as previously described (3, 4). Intact virions (300 ng per well of protein) were attached to round-bottom Corning ELISA plate wells (VWR Scientific, Bridgeport, N.J.) by incubation of virions in PBS for 1 h at 37°C (4). Virus particles were disrupted in 0.2 M Na_2CO_3 buffer (pH 10.6) containing 0.01 M dithiothreitol (7) and dried onto ELISA plate wells by a 3-h incubation at 37°C. Plates were

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TABLE 1. ELISA of monoclonal antibody reactivity to papillomaviruses

Papillomavirus	OD with:											
	H11.A3	H11.B2	H11.E1	H11.F1	H11.G5	H11.H3	BSA-1 ^a	639 ^b	7380 ^c	3866 ^d	DAKO ^e	7341 ^f
HPV-11												
Intact	0.481	0.980	0.062	0.640	0.484	0.430	0.015	0.131	0.000	0.000	0.033	0.000
Disrupted	0.017	0.030	0.251	0.020	0.000	0.008	0.024	0.123	0.020	0.042	0.546	0.000
BPV-1												
Intact	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.326	0.110	0.000
Disrupted	0.003	0.003	0.023	0.002	0.000	0.009	0.041	0.000	0.000	0.584	2.000	0.000
CRPV												
Intact	0.000	0.000	0.024	0.002	0.000	0.000	0.037	0.000	2.000	0.000	0.000	0.000
Disrupted	0.011	0.012	0.247	0.032	0.020	0.014	0.014	0.000	0.009	0.026	0.553	0.000
Control (BSA) ^g	0.005	0.006	0.005	0.004	0.010	0.012	2.000	0.009	0.010	0.014	0.047	0.011

^a Monoclonal antibody to bovine serum albumin.^b Polyclonal antiserum generated against CsCl-banded intact HPV-11.^c Polyclonal antiserum generated against CsCl-banded intact CRPV.^d Polyclonal antiserum generated against CsCl-banded intact BPV-1.^e DAKO GSA antiserum.^f Normal rabbit serum.^g BSA, Bovine serum albumin at 10 µg per well.

washed three times with PBS, and unattached sites were blocked with a blocking buffer consisting of 5% nonfat milk protein (Carnation) in PBS. Monoclonal antibody supernatants were diluted 1:10 in blocking buffer and added to duplicate wells containing antigen for 1 h of incubation. Plates were washed, a 1:1,000 dilution of alkaline phosphatase-conjugated rabbit anti-mouse antibody (DAKO Corp., Via Real, Calif.) was added, and the plates were incubated for 1 h. The plates were washed five times in PBS, substrate was added, and optical densities (OD) were determined in an ELISA plate reader (3). Monoclonal and polyclonal antibody supernatants were tested in duplicate, and mean OD readings at 410 nm were determined on a Dynatech automated microplate reader.

ELISA reactivity demonstrated that five of the six selected monoclonal antibodies (H11.A3, H11.B2, H11.F1, H11.G5, and H11.H3) strongly and specifically bound to intact but not disrupted HPV-11 (Table 1). One monoclonal antibody (H11.E1) reacted with disrupted HPV-11 and CRPV but not to intact papillomavirus or to disrupted BPV-1. The five monoclonal antibodies that were reactive to intact HPV-11 alone (H11.A3, H11.B2, H11.F1, H11.G5, and H11.H3) were examined in a competitive ELISA to determine whether the monoclonal antibodies identified nonoverlapping epitopes located on the intact virus particle. These assays were initiated because the epitope(s) that was identified by the monoclonal antibodies was nonlinear, destroyed by particle disruption, and therefore not amenable to mapping by synthetic peptide analysis. Individual monoclonal antibody supernatants were added to duplicate ELISA plate wells containing bound intact HPV-11 for 30 min and washed three times with PBS, and then each of the five monoclonal antibodies, including BSA-1 (a negative-control monoclonal antibody reactive to bovine serum albumin), was added and incubated for a further 30 min. Monoclonal antibodies detecting nonoverlapping epitopes were identified by ELISA readings in excess of those obtained for the primary monoclonal antibody when added alone (Table 2). These data indicated that H11.A3 bound to a different epitope than that recognized by the other four monoclonal antibodies (H11.B2, H11.F1, H11.G5, and H11.H3), provided that H11.A3 was added first. Some minor differences

were detected between H11.G5 and H11.B2, between H11.G5 and H11.F1, and between H11.H3 and H11.B2.

Immunohistochemical staining of koilocytotic nuclei. The five monoclonal antibodies that detected intact HPV-11 by ELISA (H11.A3, H11.B2, H11.F1, H11.G5, and H11.H3) strongly stained koilocytotic nuclei in fresh-frozen sections but not nuclei in Formalin-fixed, paraffin-embedded sections of experimental condyloma. The monoclonal antibody that reacted to both disrupted HPV-11 and CRPV antigen by ELISA (H11.E1) also stained koilocytotic nuclei in frozen sections, but the staining pattern was weaker and confined to nuclei located in the keratin layer. This monoclonal antibody strongly stained nuclear material in all viable cells (human, rabbit, bovine, and mouse) in Formalin-fixed, paraffin-embedded sections of experimental condylomas and virus-infected cysts and may have identified a common cross-reactive epitope on nuclear protein(s) that was present also on papillomavirus proteins. No viral protein, however, was identified on Western immunoblots by this monoclonal antibody. None of the remaining five monoclonal antibodies bound to viral proteins by Western blot analysis of polyacrylamide gel electrophoresis separated viral proteins either under denatured and reduced conditions or under nondenatured and reduced conditions.

TABLE 2. Competition ELISA of monoclonal antibodies that recognize intact HPV-11 virus

Second monoclonal antibody	OD obtained with first monoclonal antibody:					
	H11.A3	H11.B2	H11.F1	H11.G5	H11.H3	BSA-1
None	0.262	0.932	0.714	0.561	0.546	0.025
H11.A3	0.316	0.868	0.621	0.481	0.611 ^a	NT ^b
H11.B2	1.363 ^c	0.914	0.684	0.704 ^a	0.625 ^a	NT
H11.F1	0.957 ^c	0.823	0.603	0.697 ^a	0.480	NT
H11.G5	0.848 ^c	0.823	0.657	0.560	0.549	NT
H11.H3	0.671 ^a	0.797	0.569	0.530	0.529	NT
BSA-1	0.268	0.752	0.528	0.551	0.579	NT

^a Value is greater than the individual OD obtained for the first monoclonal antibody.^b NT, Not tested.^c Value is approximately the sum of the individual monoclonal antibody values.

TABLE 3. Summary of monoclonal antibody reactivity to HPV-11 as determined by various assays

Monoclonal antibody	Antibody subclass ^a	Lymphocyte origin	Staining ^b in:		Cyst size ^c (mean $\pm$ SEM mm ³)	No. of grafts	
			Fresh-frozen sections	Formalin-fixed, paraffin-embedded sections		By morphology ^d	In situ ^e
H11.A3	IgG2a(κ)	Lymph node	++++	—	2.4 $\pm$ 1.2	2/3/5	2/3/5
H11.B2	IgG2b(κ)	Lymph node	++++	—	0.8 $\pm$ 0.5 ^f	0/5/6	0/4/5
H11.E1	IgM(κ)	Spleen	++	++++ ^g	2.5 $\pm$ 0.8 ^h	4/6/6	5/6/6
H11.F1	IgG2a(κ)	Lymph node	++++	—	0.5 $\pm$ 0.5 ^f	0/2/4	0/2/4
H11.G5	IgG2a(κ)	Spleen	++++	—	0.7 $\pm$ 0.4 ^f	0/4/5	0/4/5
H11.H3	IgG2b(κ)	Lymph node	++++	—	1.5 $\pm$ 0.5	0/3/4	0/3/4
BSA-1	IgG2a(κ)	Lymph node	—	—	3.2 $\pm$ 0.7 ^h	4/6/6	5/6/6
639	N/A ⁱ	N/A	++++	+	0.7 $\pm$ 0.4 ^f	0/3/6	0/3/6
DAKO GSA	N/A	N/A	++	++++	NT ^j	NT	NT

^a IgG2a, Immunoglobulin G2a.^b Immunohistochemical staining of koilocytotic nuclei in sections of experimental condylomas. Staining intensity scale from ++++ (very strong) to + (weak); —, negative.^c Of HPV-11 morphologically transformed human xenografts after 100 days of growth beneath the nude mouse renal capsule.^d Number of grafts transformed/number surviving/number attempted.^e Number of in situ HPV-11 DNA-positive grafts/number surviving/number attempted.^f $P < 0.05$ when compared with BSA-1 (nonneutralizing control group) by Student's t test.^g Nuclear staining of all viable cells.^h $P < 0.05$ when compared with 639 (neutralizing control group) by Student's t test.ⁱ N/A, Not applicable.^j NT, Not tested.

In situ hybridization. The biotinylated probe of HPV-11 was made by incorporation of biotin-11-dUTP (Sigma Chemical Company, St. Louis, Mo.) by random primer extension by using the multiprimer DNA-labeling system (Amersham) and was purified with G-50 Sephadex Quick Spin columns (Boehringer Mannheim Biochemicals). Tissue sections were cut 5 μ m thick and mounted onto acid-cleaned slides coated with 3-aminopropyltriethoxy silane. In situ hybridization was carried out with the Vira Type In Situ kit (GIBCO BRL Life Technologies, Inc., Gaithersburg, Md.), according to the instructions of the manufacturer. Following hybridization and detection, the slides were counterstained with Nuclear Fast Red and inspected microscopically.

Monoclonal antibody-mediated neutralization of HPV-11 infectivity. Neutralization of infectious HPV-11 by monoclonal antibodies was measured by using the xenograft system (2). Supernatants containing monoclonal antibody were prepared by incubating hybridomas for 5 days in Clonetics medium (Clonetics Corp., Boulder, Colo.) supplemented with 5% conditioned medium and 0.5% fetal bovine serum. Antibody titers were tested by spotting fivefold dilutions of supernatants onto nitrocellulose strips followed by development of the strips with alkaline phosphatase-labeled rabbit anti-mouse antibody and substrate. Monoclonal antibodies were detected at dilutions between 1:625 and 1:3,125. Dilutions of hybridoma supernatants (1:5 in PBS) were incubated at 37°C for 1 h with samples of an equal volume of PBS containing infectious HPV-11, and then human foreskin chips were added and incubated for a further 1 h at 37°C. The chips were transplanted beneath the renal capsule of athymic mice, and the grafts were examined 100 days later for condylomatous transformation (15). An HPV-11-specific rabbit polyclonal antiserum (639) was included as a positive control for neutralization (2). Cyst size was determined by measuring the lengths, widths, and heights of the cysts in millimeters, and geometric mean diameters were calculated by taking the cubic roots of the product of length times width times height of the cysts in millimeters. Means of the geometric mean diameters of cysts developing after pretreatment of HPV-11 with monoclonal antibodies and of control cysts that developed after pretreatment of HPV-11 with an

irrelevant monoclonal antibody and also with neutralizing polyclonal rabbit serum were compared by Student's t test.

There was a significant difference ($P < 0.05$) between mean cyst sizes when the polyserum 639 treatment group (positive control for neutralization) was compared with the H11.E1 and BSA-1 monoclonal antibody treatment groups (Table 3). Significant differences ($P < 0.05$) were also obtained when the mean cyst size in the BSA-1 monoclonal antibody treatment group (negative control for neutralization) was compared with the mean cyst sizes in the H11.B2, H11.F1, and H11.G5 monoclonal antibody treatment groups. (Table 3). Sections of individual cysts were examined also for regions of koilocytotic nuclei and for nuclear HPV-11 DNA by in situ hybridization as an indicator of morphological transformation and therefore of a lack of monoclonal antibody-mediated virus neutralization (Table 3). Normal untransformed cysts were obtained from foreskin chips incubated with HPV-11 pretreated with rabbit polyclonal anti-HPV-11 serum (positive control) and monoclonal antibodies H11.B2, H11.F1, H11.G5, and H11.H3. Condylomatous cysts developed from foreskin chips incubated with HPV-11 pretreated with monoclonal antibodies BSA-1 (negative control), H11.E1, and H11.A3. In situ hybridization analysis demonstrated that two cysts that had been scored as morphologically untransformed or normal (one each from the pretreatment groups H11.E1 and BSA-1, which were both nonneutralizing monoclonal antibodies) contained several nuclei that were HPV-11 DNA positive. All remaining normal epithelial cysts were negative for HPV-11 DNA by in situ hybridization.

Four of the six anti-HPV-11-reactive monoclonal antibodies neutralized HPV-11-induced morphological transformation of human foreskin tissue in the xenograft system. All four neutralizing monoclonal antibodies detected only intact virion antigen epitopes, as measured by ELISA. The fifth monoclonal antibody which reacted to intact virion antigen (H11.A3) was nonneutralizing. This later monoclonal antibody appeared to identify a different viral surface epitope than the four neutralizing monoclonal antibodies when analyzed in a competitive-binding ELISA. The four neutralizing monoclonal antibodies did not appear to bind to distantly

related epitopes, as judged by the competitive-binding ELISA. However, both steric hindrance and epitope modification by the binding of the primary monoclonal antibody may have decreased the sequential binding of the second monoclonal antibody if two epitopes were different but adjacent on the capsid surface.

Only nonlinear (conformational) neutralizing epitopes on HPV-11 were defined by the monoclonal antibodies that were developed in this study. The presence of nonlinear (conformational) neutralizing epitopes have been described also for other virus neutralization systems (1, 6, 18, 20). Linear neutralizing epitopes may exist on HPV-11 particles but, if present, may not be immunodominant when presented in the form of an immunogen consisting of whole-virus particles.

The monoclonal antibodies may have neutralized HPV-11 infectivity by preventing HPV-11 from binding to a cell surface receptor molecule(s), as described for many other viral systems (9, 10, 16, 17, 19, 23). In these systems, neutralization was achieved by the binding of the monoclonal antibody to a virus surface structure, which blocked the recognition and binding of the virus to a cell surface receptor. This hypothesis was supported also in this study by the observation that a monoclonal antibody which bound to intact HPV-11 virion (H11.A3) was nonneutralizing. An alternative explanation for virus neutralization was that the monoclonal antibodies blocked a stage in the virus life cycle between uncoating of the virion and transcription, after the papillomavirus had penetrated into the host epithelial cell, as has been found for an RNA virus (11). However, no analogous mechanism for neutralization of DNA viruses has been described.

In conclusion, neutralizing epitopes have been identified on HPV-11 infectious virus particles by monoclonal antibodies. The epitope(s) represented external nonlinear determinants so that it was not possible to localize the region on the viral capsid protein that defined the neutralization site. The athymic mouse xenograft system made possible the production of infectious virions, assay of antibody-mediated neutralization of HPV-11, and identification of neutralizing epitopes.

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