Reactivity between Herpesvirus Type 2-Related Soluble Cervical Tumor Cell Membrane Antigens and Matched Cancer and Control Sera (36853)

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In cells of animal tumors induced by DNA oncogenic viruses, transplantation antigens specific for the inducing virus have been demonstrated (1, 2). These antigens appear to be associated with the cell membrane (3). Specific soluble antigens were separated from membranes isolated from DNA virus-induced tumor cells and were capable of producing immunity in animals against tumor formation when inoculated with viable cells (4, 5).

Herpesvirus type 2, a venerally transmitted virus, has been examined for a possible etiologic role in carcinoma of the cervix. Antibodies to the virus have been found to occur more frequently among women with cervical cancer than among control women (6–8). Cervical anaplastic changes occur at a greater frequency among women with genital herpes followed prospectively than among control women (9) and herpesvirus antigens have been detected by immunofluorescence techniques in cells exfoliated from cervical lesions (10).

Experiments were undertaken to examine sera from women with cervical cancer for antibodies to soluble membrane fractions obtained from cells of invasive lower genital cancers. It was also of interest to see whether such tumor-related antigens might be shared with herpesvirus type 2. Comparable antigens from normal cells as well as sera from matched control women were included in the tests.

Material and Methods. Virus preparation and assay. Herpesvirus type 1 (KOS strain) and herpesvirus type 2 (SAV strain) were grown in human embryonic kidney cells as previously described (11). The stocks were assayed by the terminal dilution method using at least 3 tubes/10-fold dilution. The viruses were semipurified using the two-phase partition method (12).

Preparation of antisera. The antisera against the total virus was prepared by emulsifying 0.5 ml of semipurified virus with 0.5 ml of incomplete Freund's adjuvant, and injecting the footpads of six guinea pigs. Bleedings were performed at 3 and 6 wk. High titer antisera was selected and mixed in a proportion of 0.4 ml antisera to 0.1 ml of whole HEK cells, and homogenized together by hand. This material was incubated in the refrigerator for 24 hr, then centrifuged for 20 min at 1500 rpm at 4°, and the antisera was removed from the top and used for complement-fixation testing. Assay for antigen or antibody was by complement-fixation testing, with appropriate controls.

Preparation of soluble fractions from tumor cell membranes (4, 5, 13). Normal vaginal tissue, skin of the groin area, fibroid tissue and invasive carcinomas of the cervix, vulva, and vagina were surgically excised, debrided of fat and necrotic tissue and washed three times with basal medium. The tissue was finely minced and the cells were disaggregated using a stainless steel sieve. Viable cells were counted in the presence of trypan blue for each tissue and between 10^8 and 10^9 separated cells in 10 ml of saline were placed in suspension and stored at −60°. The material was then thawed and the membranes produced by hypotonic lysis, using isotonic saline, 1/2 isotonic saline and sterile water suspensions, each followed by centrifugation at 2500 rpm for 10 min. The combined supernatants were centrifuged at 100,000*g* for 1 hr, and the membrane pellet was resuspended and washed twice in saline. Human em-

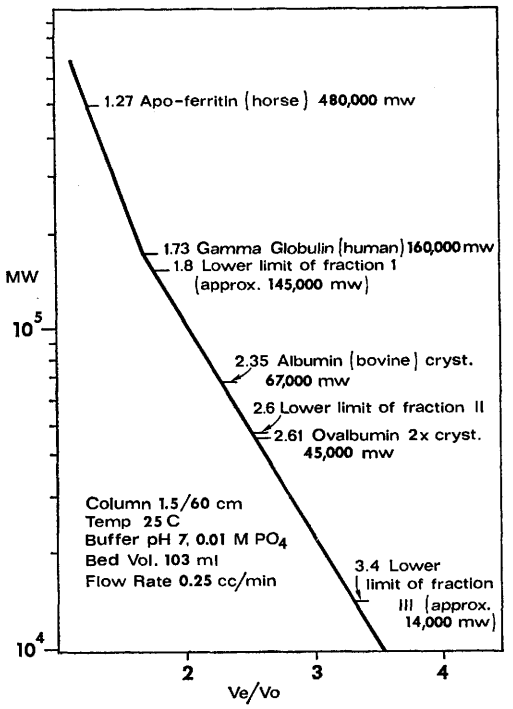

FIG. 1. Relationship between ratio of elution volume to void volume and molecular weight of standard proteins on Sephadex G-200. Superimposed on standard curve are shown the lower limits of fractions I, II and III of separated sonicates of soluble cell membrane preparations. Fraction IV is without the limits of Sephadex G-200 and included final eluates with protein content.

bryonic kidney cells in fourth tissue culture passage were similarly treated. The membranes were then sonic-treated in Raytheon Model S102A oscillator at 9 kHz, 3 min, at 0.3 Å at $< 10°$. The treated material was then centrifuged in a Spinco Model L ultracentrifuge at 100,000*g* for 1 hr at 4°. The membrane pellet was again sonic-treated in 0.5 to 1 ml saline for 1.5 min and centrifuged and this pellet again sonic-treated for 1.5 min and centrifuged. Concentrated supernatants resulting from the above 3, 4.5 and 6 min sequential sonications were fractionated at room temperature on freshly prepared Sephadex G-200 columns (1.5 × 65 cm), equilibrated with 0.01 *M* phosphate buffer, pH 7.0. The void volume was approximately 26 ml. Four fractions of 20 ml each were collected in an ice bath and concentrated to 1 ml by Diaflo ultrafiltration, passed through a Millipore filter and tested for pyrogenicity in rabbits and for sterility by inoculation in thioglycollate and blood agar plates. Lowry protein determinations were made and the soluble fractions were diluted or concentrated for testing. Further separation was by gradient polyacrylamide gel electrophoresis (14, 16).

Patient and control sera. Blood was obtained by venipuncture from women with invasive cervical cancer and from women chosen as controls. The criteria for matching the controls with the cases are described elsewhere (17). The blood from the women was allowed to clot and the serum was removed. Sera were stored at —20° until tested. Complement-fixation testing of soluble membrane antigens and controls for reactivity with coded control and cancer sera was by the standard macrotechnique.

Results. As described in Materials and Methods, soluble components were obtained by low frequency sequential sonication of cell membranes prepared from control tissue and from cervical, vulva and vaginal carcinomas. The soluble components were separated into four fractions after Sephadex gel filtration as shown in Fig. 1, and the protein content of the fractions was adjusted. Herpesvirus type 2 was semipurified and injected into guinea pigs (see Material and Methods). An antiserum was selected which reacted at 1:80 with herpes type 2 and at $<$ 1:5 with both HEK cell membrane antigen and herpes type 1.

The antiserum prepared against herpesvirus type 2 after adsorption with HEK cells, was reacted with antigens from the tumor tissue. The results are shown in Table I. Complement-fixation reactions were observed in all the fraction II soluble antigens from the genital carcinomas. Reactions were not observed with similar fractions from control tissues.

In the first test, serum samples were collected from 10 Caucasian women with invasive cervical cancer and from 10 control women. The mean age for the case group was 45.4 yr and for the control group 46.1 yr. Both in the cancer group and in the control group the mean number of pregnancies per

TABLE I. Complement-Fixation Testing of Soluble Membrane Fractions from Cervical, Vulva and Vaginal Cancer and Control Cells with Hyperimmune Herpesvirus Type 2 Antisera.

Patient no.	Reactive antigens[a]	Complement-fixation titer as measured with herpesvirus type 2 hyperimmune guinea pig sera (1:20)					
		1:2	1:4	1:8	1:16	1:32	1:64
	Cervical tumors						
(1)	Soluble fraction II	4+	4+	4+	4+	4+	3+
(2)	Soluble fraction II	4	4	4	3		
	Vulva tumors						
(3)	Soluble fraction II	4	4	4	3		
(4)	Soluble fraction II	4	4	3			
	Vaginal tumor						
(5)	Soluble fraction II	4	4	4	4	3	
Herpesvirus type 2		4	4	4	4	4	4

[a] All soluble antigens from control tissues of these five patients and soluble fractions I and IV of the tumor cell membranes were <1:2. Two of the five soluble fractions III of tumor cell membranes gave 3+ reactions at 1:4. Complement and cell antigen controls were negative.

woman was 7.

Sera from the women were tested under code with freshly prepared soluble membrane antigens from cancer, fibroid and normal tissues and with soluble membrane antigens prepared from human embryonic kidney cells as well as with purified herpesvirus types 1 and 2 and adenovirus 12. The results are shown in Table II. Reactivity was observed only in the second of the four eluate fractions collected from the Sephadex G-200 column, The sera from 4 cases and 1 control proved to be anti-complementary. Sera from 5 out of 6 cancer cases reacted positively with all four fraction II soluble membrane antigens from the cancer cells of four patients with cervical, vulvar, and vaginal cancers. As shown 22 of 24 tests between specific cancer antigens and cancer sera were positive.

Seven of 9 matched control sera did not react to cancer or control antigens. However, control serum No. 22 reacted positively with vulvar and vaginal cancer antigens but did not react with the two antigen preparations derived from cervical cancers. Further, this serum reacted with herpesvirus type 2. Control serum No. 20 had a similar pattern, but was positive only for the antigens associated with vulvar cancer. Of the total number of tests between cancer antigens and matched control sera, only 3 of 36 reacted. None of sera from the cases and from the control women reacted with membrane antigens prepared from healthy tissue.

The cervical cancer cell soluble membrane fraction II was further separated using gradient polyacrylamide gel electrophoresis (16). Six bands were found in the gel of a sonicated herpesvirus type 2 preparation and 4 bands were found in the gel of cervical cancer antigen. One band of the two preparations was in a similar position on the gels. Ten unstained fraction II preparations were run simultaneously with two preparations stained for presence of bands and the regions were sliced, eluted with sterile saline and concentrated by Diaflo ultrafiltration (15). The materials eluted from regions 1, 2 and 3 of the gels were reacted under code, with matched sera including dilutions of sera from a woman with cancer and from a control woman. Region 2 material was not reactive and region 1 material was either nonspecific or anti-complementary. Region 3 material (which contained the common band) was specific for the cancer sera. The results with the diluted sera showed reactivity of the serum from the case only and the reaction occurred to a dilution of 1:16.

Satisfactory tests were performed on a total of 21 sera from women with cancer and 21 matched control women. The results with the Sephadex fractions (Table II) and region 3 materials are shown in Table III.

TABLE II. Complement-Fixation Tests of Soluble Membrane Antigens from Cancer and Control Cells with Sera from Cancer and Matched Control Patients.

	CF titers[a] (reciprocals); antigens (1:2–1:128)												
	Patient with Cancer cervix			Cancer vulva		Cancer vagina		Cancer cervix			HEK	Herpes	Herpes
Patient sera 1:4	Cancer FxII	Control FxII	Fibroid FxII	Cancer FxII	Control FxII	Cancer FxII	Control FxII	Cancer FxII	Control FxII	Fibroid FxII	FxII 1:5 only	I 1:32 only	2 1:32 to 1:128
Ca[b] 207	4	0	0	0	0	0	0	4	0	0	0	0	0
M[c] 30	0	0	0	0	0	0	0	0	0	0	0	0	0
Ca 125	8	0	0	4	0	16	0	8	0	2	0	0	32
M 69	0	0	0	0	0	0	0	0	0	0	0	2	0
Ca 225[d]													
M 22	0	0	0	4	0	2	0	0	0	0	0	4	32
Ca 3	16	0	0	2	0	2	0	8	0	0	0	0	64
M 183[d]													
Ca 126	32	0	2	8	0	16	0	32	0	0	0	0	128
M 67	0	0	0	0	0	0	0	0	0	0	0	0	0
Ca 247[d]													
M 129	0	0	0	0	0	0	0	0	0	0	0	0	0
Ca 278	64	0	2	8	0	16	0	128	0	0	0	2	128
M 20	0	0	0	4	0	0	0	0	0	0	0	16	128
Ca 177[d]													
M 1	0	0	0	0	0	0	0	0	0	0	0	0	0
Ca 157	8	0	0	8	0	8	0	16	0	0	0	0	64
M 107	0	0	0	0	0	0	0	0	0	0	0	0	0
Ca 179[d]													
M 6	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] 0 indicates $<2^+$ at 1:2.

[b] Ca = case.

[c] M = matched.

[d] Each of these sera were $AC4^+$ at 1:4.

TABLE III. Summary of Reactions of Sera from Cases and Controls, Tested Under Code, with Partially Purified Soluble Membrane Antigens.

Group	No. tested	No. reactive[a]	Percentage reactive
Cervical cancer	21	20[b]	95
Control women	21	5	24

[a] Sera reactive when tested against antigen from one or more cancer specimens.

[b] Case–control difference in reactivity: $X^2 = 19.4$, $p < .001$.

Reaction to one or more antigen preparations was obtained in 20 of 21 (95%) cancer cases and 5 of 21 (24%) control women.

Discussion. An antigen designated "carcinoembryonic antigen (CEA)" has been demonstrated in human colon cancers and was shown to be similar to antigens which appear in fetal gut cells during the first (and second) trimesters of pregnancy (18–21). Using a bis-diazotized benzidine hemagglutination technique, a specific humoral anti-CEA antibody response was detected in sera of 70% of patients with nonmetastatic colon carcinomas (19). Another antigen which produces delayed hypersensitive skin reactions in colon cancer patients has also been separated from soluble membrane preparations from adult colon cancer and fetal gut cells and the structural components differ from CEA, which is present in the same crude extract and can be separated from the skin-reactive antigen (13, 14). It has been shown in animal studies that most tumors which share antigens are caused by viruses. It might be implied, therefore, that any group of human cancers which are found to share a common antigen might also be caused by viruses. However, we could not identify a virus association with human gastrointestinal cancer by testing both fetal antigens and human colon cancer antigens against a battery of enteroviral antisera (Hollinshead, A. and Melnick, J., unpublished data). In other studies, we found no relationship between the soluble tumor-specific transplantation antigen of adenovirus-induced tumors and the adenovirus itself (4).

In the present paper, evidence is presented which suggests that carcinomas of the lower genital tract of women may also possess soluble membrane antigens specific for the tumors. Furthermore, antibodies to these antigens were found in 95% of patients with cervical cancers and in 24% of matched controls. The antigens associated with tumors of the gastrointestinal tract are thought to be expressions of fetal antigens during malignant transformation. Antigens in the genital carcinoma found in our study may also be expressions of fetal antigens. However, serum prepared in a guinea pig with semipurified herpesvirus type 2 reacted with the tumor antigens in complement-fixation tests. This serum may also be reactive with herpesvirus-induced nonvirion antigens (22); the possibility that such antigens are responsible for CF reactivity with cervical cancer patient sera is under investigation. These observations are compatible with the association of herpesvirus type 2 with cervical cancer (6–8).

Summary. A soluble membrane antigen was isolated from vaginal, vulvar and cervical cancer which reacted with antiserum prepared in a guinea pig against semipurified herpesvirus type 2. In a series of coded experiments, 20 of 21 sera from women with cervical cancer reacted in complement-fixation tests with a soluble membrane antigen from genital cancer cells, while reactions occurred in 5 of 21 sera from matched control women. Neither cancer nor control sera reacted with similar preparations from normal vaginal tissue. Antibody to the virus-induced soluble membrane antigen of cancer tissues appears, therefore, to be more prevalent in the cancer population but is also found in a matched control population.

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