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Herpes Virus-related Antigens in Herpes Simplex Virus Type 2-transformed Cells in the Course of Cervical Carcinoma

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Abstract—*Anticomplement immunofluorescence (ACIF) technique was applied to Herpes simplex virus type 2 (HSV-2)-transformed cells (333-8-9) and to cells lytically infected with HSV-2 in sera from women with cervical carcinoma. There was a correlation between the positive results with both types of cells. Long-term survivors suffering from cervical cancer showed a high percentage of positive immunofluorescence (IF) reactions in HSV-2 transformed cells (77%) as well as in cells lytically infected with HSV-2 (77%) in sera obtained before treatment. The figures were 81 and 83% respectively in sera collected 6–24 months after treatment. When the IFs of survivors and non-survivors were compared, the survivors showed more cytoplasmic staining reactions in sera obtained before treatment than in similar sera from non-survivors, whereas there was no difference in nuclear IF. The sera of survivors taken 6–24 months after treatment had a significantly higher overall fluorescence rate than similar sera from non-survivors. In late sera taken 36 months after treatment, a drop in reactivity was noted for the survivors to values comparable with those obtained from non-survivors. The controls (age-matched, healthy women and patients with malignancies other than cervical carcinoma) showed significantly less IF reactions ($P < 0.001$).*

INTRODUCTION

THE ONCOGENIC potential of HSV was indicated by the demonstration of cell transformation after exposure to u.v.-irradiated HSV-2 or HSV-1 [1, 2] and the development of sarcomas in hamsters after direct inoculation of HSV-2 [3]. Viral DNA was demonstrated in hamster tumours and in serially passaged, HSV-2-transformed (333-8-9) cell lines [4]. Viral DNA was also demonstrated in human tissue [5]. Antigens common to HSV, HSV-associated hamster tumours and human cervical cancer have been shown [6–8]. Using the indirect immunofluoresence technique, the presence of HSV antigens has been reported in exfoliated cells from cervical cancer [9] in women with cervical dysplasia and to a minor extent in women with normal cervical epithelium [10].

Many sero-epidemiological studies have recorded the prevalence of antibodies to HSV-2 antigens in cervical carcinoma patients [11–13] and most of them have reported evidence of such a relationship [14, 15]. It has also been shown that the antibody response to HSV-2 antigens varies in women in different stages of cervical cancer [16–18]. The incidence of anti-HSV-2 antibody in a number of tests was higher among long-term survivors than among patients whose cancers caused death.

The present study is an attempt to demonstrate the presence of antibodies to antigenic structures associated with HSV-2 in HSV-2-transformed cells in sera from women with cervical cancer. The purpose is also to study the relationship between the reactivity of HSV-2-transformed cells and that of lytically infected cells. Different antibodies to HSV-2-transformed cells and, in particular, to nuclear antigens in such cells may be of prognostic value.

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MATERIALS AND METHODS

Cell line

The properties of the HSV-2-transformed cell line used in this study have been described previously [1, 2]. This cell line was provided by Dr. F. Rapp of the Hershey Medical Center. Green monkey kidney (GMK) cells were used for the

HSV-2 lytically infected cells. For the infection of HSV-2, strain D64 (received from Dr. G. Plummer of Loyola University, Chicago, IL), with an infectivity titre of $10^{7.3}$ ID_{50}/ml, was used.

Study groups

Sera were obtained from women who had undergone radiotherapy at Radiumhemmet, Karolinska Hospital, Stockholm, because of invasive cervical cancer. The study includes sera from 49 long-term survivors and from 22 patients who died during the observation period. Serum samples collected at zero time, i.e. before treatment, were available from 22 of the long-term survivors and from 12 of the women whose cancers caused death. The control groups consisted of 55 age-matched, healthy women and 52 patients with malignancies other than cervical carcinoma.

Anticomplement immunofluorescence (ACIF) test

The 333-8-9 cell line was passaged and grown for 24 hr in MEM supplemented with 10% foetal calf serum (FCS). The cells were trypsinized and washed in PBS with 5% FCS for two periods. Two drops of washed cells were placed on a clean slide. The slides were dried at room temperature, fixed for 5 min in an equal volume of chilled acetone–methanol (–20°C) and then used immediately. The slides with the 18 hr post-HSV-2-inoculated cells were prepared in the same fashion. On each slide was placed one drop of HSV-2-infected cells and one drop of non-infected cells, so that the same serum was simultaneously tested on infected and non-infected cells. The ACIF technique employed by Reedman and Klein [19] to demonstrate Epstein–Barr virus antigen was applied with some modifications. Sera were diluted 1:5 in all tests and inactivated at 56°C for 30 min prior to use. Before staining, the slides were dropped with guinea pig complement (1.5 nl/ml), incubated at 37°C from 20 min and then washed, with stirring, for 30 min. The cells were stained with a suitable dilution of FITC-conjugated, anti-human BIC/BIA globulin (Hyland Laboratories, Los Angeles, CA). Serum from a woman with cervical cancer positive to cells lytically infected with HSV-2 and with positive cytoplasmic and nuclear fluorescence on 333-8-9 cells was used as a positive control serum. Human serum which was negative to 333-8-9 cells and to cells lytically infected with HSV-2 was used as a complement source and as a non-immune, HSV-2 control serum. The complement was absorbed with HEF in order to avoid unspecific reactions. All tests included positive and negative serum control and complement control. All sera were also tested on normal, primary Syrian hamster embryo fibroblast cells (HEF). The slides were coded and read blind.

Statistical treatment was undertaken with the formula calculated according to the chi-square test.

RESULTS

In order to test the specificity of the system used in this survey, most sera were also tested on cells lytically infected with HSV-2. In some cases this was not possible because of exhaustion of the serum. The action of the positive serum used as a control in all tests and obtained from a woman with cervical cancer is shown in Fig. 1 for HSV-2-transformed cells and in Fig. 2 for GMK cells lytically infected with HSV-2. The nuclear and cytoplasmic fluorescences in HSV-2 transformed cells are illustrated in Fig. 3 for the serum from a woman with advanced cervical cancer who survived for 50 months but finally died of her cancer. All sera were also tested on primary Syrian hamster embryo fibroblast cells (HEF) and on non-infected GMK cells. Sera from eight women with cervical cancer which yielded positive ACIF on the HSV-2-transformed cells were absorbed with HEF and retested on HSV-2-transformed cells. When retested there was no difference in reactivity between the absorbed and the un-absorbed sera.

Table 1 gives data on the initial staining reactions in sera obtained at zero time (i.e. before initial treatment) from 22 long-term survivors who completed the observation period of more than 60 months with no signs of residual tumours or recurrences. A positive staining reaction in HSV-2-transformed cells was obtained for 17 of the 22 women (77%). Cytoplasmic fluorescence was seen in 13 cases (61%) and nuclear fluorescence in 14 (66%). With HSV-2 cells, cytoplasmic fluorescence was observed in 17 (77%) and nuclear fluorescence in 14 (66%).

Table 2, which does not include any patients from Table 1, gives the results from 27 long-term survivors for sera collected 6–24 months after treatment. In 18 of these cases results were obtained in HSV-2-transformed cells for sera collected 36 months after treatment or later. Cytoplasmic and/or nuclear fluorescences were demonstrated in 22 out of 27 sera (81%) with HSV-2-transformed cells and in 23 sera (85%) with HSV-2-infected cells. There was a drop in reactivity with HSV-2-transformed cells for sera obtained 36 months after treatment or later; 10 (55%) showed cytoplasmic and 7 (38%) nuclear fluorescence. Because of the exhaustion of the sera collected 36 months after treatment or later, it was not possible to test them on lytically infected cells.

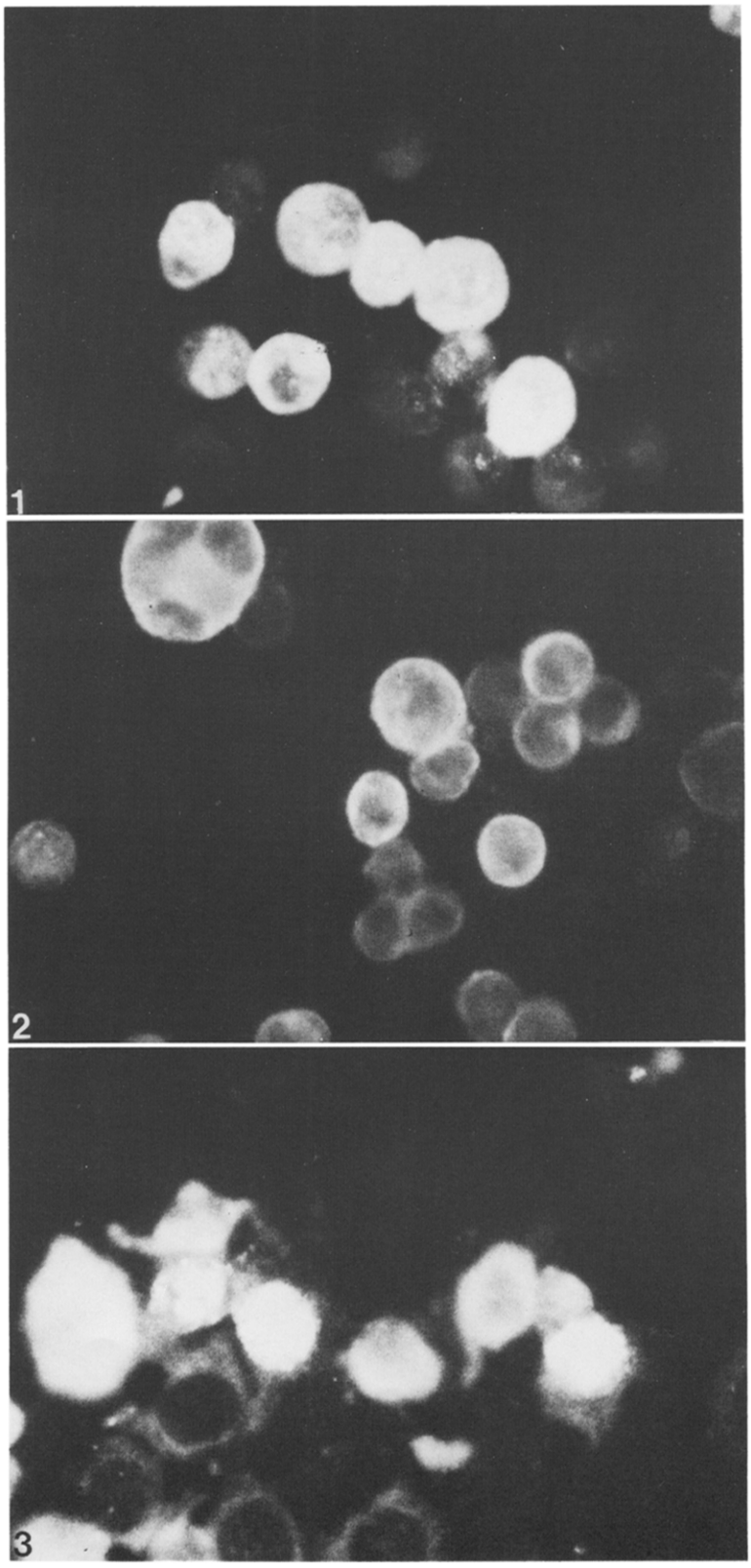

Fig. 1. Anticomplement immunofluorescence (ACIF) in HSV-2-transformed cells (333-8-9) stained by serum from a woman with invasive cervical carcinoma.

Fig. 2. Anticomplement immunofluorescence (ACIF) in GMK cells 18 hr *post-infected with HSV-2 stained by serum from a woman with invasive cervical carcinoma.*

Fig. 3. Anticomplement immunofluorescence (ACIF) assay on HSV-2-transformed cells (333-8-9) stained by serum from a woman who survived for a long time (50 months), *in spite of the advanced cervical cancer which finally caused her death.*

Table 1. Anticomplement immunofluorescence (ACIF) test of sera from survivors of cervical carcinoma in HSV-2-transformed and HSV-2-infected GMK-cells

Case No.	Stage of cancer	Zero-time sera: HSV-2-transformed cells		HSV-2-infected cells	
		C	N	C	N
1	Ia	+	—	+	+
2	Ia	—	—	—	—
3	Ia	+	—	+	—
4	Ia	+	+	+	+
5	Ia	—	+	+	+
6	IIa	—	—	—	—
7	IIa	—	+	+	+
8	IIa	+	+	+	+
9	IIa	—	—	—	—
10	IIa	—	—	—	—
11	IIa	+	—	+	—
12	IIb	+	+	+	+
13	IIb	+	+	+	—
14	IIb	+	+	+	+
15	IIb	+	+	+	+
16	IIb	+	+	+	+
17	IIb	+	+	+	+
18	III	+	+	+	+
19	III	—	+	+	+
20	III	—	—	—	—
21	III	—	+	+	+
22	III	+	+	+	+

Zero-time sera are sera obtained before initial treatment.
C = Cytoplasmic fluorescence; N = nuclear fluorescence.

Table 2. Anticomplement immunofluorescence (ACIF) test of sera collected after treatment from survivors of cervical carcinoma in HSV-2-transformed and HSV-2 lytically infected GMK-cells

Case No.	Stage of cancer	HSV-2-transformed cells: 6–24 months		>36 months		HSV-infected cells: 6–24 months	
		C	N	C	N	C	N
23	Ib	+	+	+	+	+	+
24	Ib	—	—			+	+
25	Ib	—	+	—	—	—	+
26	Ib	—	—			—	—
27	Ib	—	+	—	—	+	+
28	Ib	—	—	—	—	—	—
29	IIa	—	+	—	+	—	+
30	IIa	—	—	—	—	—	—
31	IIa	+	+	—	—	+	+
32	IIa	+	+	+	—	+	+
33	IIa	+	+	+	+	+	+
34	IIa	+	—	—	—	+	+
35	IIa	+	+			+	+
36	IIa	—	+	+	+	+	+
37	IIa	+	—	—	—	+	+
38	IIa	—	+	—	—	—	+
39	IIb	+	+	+	+	+	+
40	IIb	+	—	+	—	+	+
41	IIb	+	+	+	+	+	+
42	IIb	+	+	+	+	—	—
43	IIb	+	+			+	+
44	IIb	+	+			+	+
45	IIb	—	+			—	+
46	III	—	—			+	+
47	III	+	+	+	—	+	+
48	III	+	+			+	+
49	III	+	—			—	+

C = Cytoplasmic fluorescence; N = nuclear fluorescence.

Table 3 presents data on staining reactions in sera from 22 women who died during the observation period. The cases are numbered according to survival time. Sera were available from the period before treatment in 12 cases, and from 10 other women sera were collected 6–24 months after treatment. In the zero-time sera, 2 out of 12 (17%) showed cytoplasmic and 8 (67%) nuclear fluorescence. In sera obtained after treatment, 2 (20%) yielded cytoplasmic and 4 (47%) nuclear fluorescence. The four women in Table 3 who showed cytoplasmic IF (cases 11, 12, 21 and 22) also showed the longest survival times. There was good correlation between ACIF in HSV-2-transformed and infected cells according to the formula of Pearson and Fisher (chi-square test), both with sera from survivors (Tables 1 and 2) and from patients whose cancers caused death (Table 3).

There was a significant difference ($P < 0.01$) in the reactivity of sera obtained from long-term survivors compared with that of similar sera from non-survivors (Tables 1 and 3) in that the survivors had more cytoplasmic reactivity, whereas there was no difference in nuclear fluorescence. Sera taken 6–24 months after treatment had a significantly higher overall fluorescence rate than similar sera from non-survivors ($P < 0.001$; Tables 2 and 3), but they did not differ in the overall fluorescence rate from sera taken 36 months after treatment in survivors. On comparing the difference in reactivity between the women with cervical cancer and the control groups (Table 4), the level of significance was found to be $P < 0.001$. The controls—both the age-matched women and the patients with malignancies other than cervical carcinoma—had more reactivity with HSV-2 lytically infected cells than with the transformed cells.

DISCUSSION

The ACIF technique provided a useful approach in detecting the presence of HSV-2-related antigens in cells transformed by HSV-2 and in demonstrating antibodies to these antigens in sera from women with cervical cancer. A correlation between the positive results with both types of cells was demonstrated.

A high proportion of women with cervical cancer have been found to have antibodies to HSV-2-infected cells in a variety of tests: cytotoxic antibodies in antibody-dependent and com-

Table 3. Anticomplement immunofluorescence (ACIF) test of sera from 22 non-survivors of cervical carcinoma in HSV-2-transformed and HSV-2 lytically infected GMK cells

Case No.	Stage of cancer	Survival, months	Zero-time sera: HSV-2-transformed cells C	Zero-time sera: HSV-2-transformed cells N	Zero-time sera: HSV-2-infected cells C	Zero-time sera: HSV-2-infected cells N	Case No.	Stage of cancer	Survival, months	6–24 months: HSV-2-transformed cells C	6–24 months: HSV-2-transformed cells N	6–24 months: HSV-2-infected cells C	6–24 months: HSV-2-infected cells N
1	III	12	—	+	—	—	13	III	18	—	—	n.d.	n.d.
2	IIb	14	—	+	n.d.*	n.d.	14	III	20	—	—	n.d.	n.d.
3	IIb	17	—	—	—	—	15	III	20	—	+	n.d.	n.d.
4	IIb	18	—	—	—	—	16	IIb	24	—	—	n.d.	n.d.
5	III	18	—	—	—	+	17	IIa	24	—	—	—	+
6	III	18	—	+	—	+	18	IIa	30	—	+	—	+
7	III	19	—	—	—	—	19	III	32	—	—	—	+
8	IIa	27	—	+	—	+	20	IIb	38	—	—	—	—
9	IIa	30	—	+	+	+	21	IIb	41	+	+	+	+
10	IIa	33	—	+	—	+	22	IIb	51	+	+	—	—
11	III	39	+	+	n.d.	n.d.							
12	IIb	40	+	+	+	+							

Zero-time sera obtained before initial treatment.
C = Cytoplasmic fluorescence; N = nuclear fluorescence.
*Not done (n.d.) due to exhaustion.

Table 4. Anticomplement immunofluorescence (ACIF) test of sera from age-matched, healthy women and from patients with other malignancies than cervical carcinoma in HSV-2-transformed (333-8-9) and HSV-2 lytically infected GMK cells

Group tested	No. of sera tested	HSV-2-transformed cells C	HSV-2-transformed cells N	HSV-2-infected cells C	HSV-2-infected cells N
Age-matched, healthy women	55	16/55 (29%)	2/55 (4%)	27/55 (49%)	10/55 (18%)
Patients with malignancies other than cervical carcinoma	52	11/52 (21%)	1/52 (2%)	34/52 (65%)	17/52 (33%)

C = Cytoplasmic fluorescence; N = nuclear fluorescence.

plement-dependent cytotoxic assays [17, 18, 20] neutralization antibodies (*K*-test) and antibodies reactive to membrane antigens (MH-test) [16, 21]. It has also been shown that women with progressive cervical lesions have low or decreasing antibody titres. Cytolytic activity to surface antigens of HSV-2-transformed cells has recently been demonstrated [22].

The ACIF technique showed that women with cervical cancer had antibodies which reacted with the antigenic structure in cells transformed by HSV-2. In agreement with earlier findings, women with long survival times reacted to a greater extent than those whose cancers caused death. On comparing the staining reactions of similar sera obtained at zero time from survivors and non-survivors a significantly higher rate of cytoplasmic fluorescence was found in the survivors, whereas there was no difference in nuclear fluorescence.

Sera taken from survivors 6–24 months after treatment had a significantly higher overall fluorescence rate than similar sera from non-survivors. This is in agreement with what has been demonstrated in cells lytically infected with HSV-2 [15, 16] in that antibodies to HSV-2 became more prominent some time after therapy. In late sera there was a decline in values again. This decline in reactivity was also observed with the transformed cells.

The absence of any discrepancy between survivors and non-survivors in the nuclear fluorescence of sera obtained at zero time may be due to the fact that antibodies to nuclear antigens reflect the presence of tumour cells more than antibodies do to membrane or cytoplasmic antigens, which in turn may have a protective potential. This hypothesis may be supported by the fact that an HSV-2-specific, DNA-binding antigen has been demonstrated in the tissues of patients with severe dysplasia or carcinoma [5].

Different hypotheses have been suggested to explain the low reactivity in women with progressive cervical cancer. Frenkel *et al.* [4] assumed that the DNA templates specifying the gene product responsible for the lytic function were absent or damaged in the transformed cell, i.e. if the cell was to survive infection. According to this hypothesis, patients with a previous HSV infection have antibodies to cells lytically infected with HSV, whereas patients whose primary HSV infection results in cell transformation subsequently lack antibodies to antigenic structures specific to the lytically infected cell.

Adsorption of antibodies by the tumour cells has been proposed as one possible reason for the low reactivity in the sera of patients with progressive cervical cancer [17, 18, 20, 21]. This may also explain the increase in reactivity seen in survivors some time after treatment in the reappearance of freely circulating antibodies which would otherwise have been absorbed by tumour cells. This assumption is supported by the fact that HSV-2-specific antibodies have been eluted from cervical cancer tissue [23].

Another interpretation may be that various alterations may develop during the course of cervical cancer. A correlation may exist between the decreased expressions of virus-associated antigens which result in impaired antibody production and increased oncogenicity.

In summary, the present study, using the ACIF technique, showed that sera from patients with cervical cancer stained antigenic structures in HSV-2-transformed cells. A particular nuclear fluorescence was noted in many cases. An antigenic relationship between HSV-2-transformed and lytically infected cells was demonstrated. Serum samples obtained from different control groups showed positive immunofluorescence to a lesser extent, and only a very few cases displayed nuclear immunofluorescence. Long-term survivors showed more positive reactions than women who died during the observation period, whereas many of the latter demonstrated nuclear fluorescence.

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