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Enzyme−Linked Immunosorbent Assay Using Enzyme−Labeled Herpes Simplex Virus Type−1 and Type−2 Early Antigens for Detection of Immunoglobulin M and G Antibodies in Patients with Uterine Cervical Cancer[1)]

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Abstract: Sera from 196 individuals diagnosed as uterine cervical cancer (CaCx) and 187 healthy women as controls were examined for their IgG and IgM antibody titers against herpes simplex virus type−1 (HSV−1) and type−2 (HSV−2) early antigens (EA) by antibody−capture direct enzyme−linked immunosorbent assay (ELISA) that uses horseradish peroxidase (HRPO)−conjugated antigens. In the case of CaCx patients, IgM antibodies against HSV−1 EA was positive in 17 sera, 8 of which showed over 20 ELISA titers, while IgM antibodies against HSV−2 EA was positive in 12 sera, 5 of which were over 20 ELISA titers. On the contrary, none of the serum from healthy women possessed IgM ELISA titer exceeding 20 against HSV−2 EA, although 2 of them showed the titer between 20 to 40 against HSV−1 EA. The patterns of IgG−ELISA titer distribution between CaCx and control groups were similar for HSV−2 EA. However, there were 5 CaCx patients with titer exceeding 80, which was the upper limit for healthy controls. In contrast, IgG−ELISA against HSV−1 EA showed fewer negatives (titer under 10) and more weakly positives in healthy controls than in CaCx patients. For both HSV−1 and HSV−2, high levels of IgM antibodies against EA were found only in the early stage of the cancer (Stage 0).

Key words: Herpes simplex virus, ELISA, Early antigen, Uterine cervical cancer

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weakly positive (titer between 10 to 20) in 2 sera, however none of the serum showed exceeding 20 IgM ELISA titer against HSV−2 EA. Significant difference was observed between these 2 groups because no healthy women showed the titer over 40 against HSV−1 EA or over 20 against HSV−2 EA, while 5 CaCx patients showed IgM−ELISA titer over 40 against HSV−1 EA and 5 patients showed IgM−ELISA titer over 20 against HSV−2 EA.

Correlation between IgG & IgM ELISA titers against HSV−1 EA and HSV−2 EA

The IgG and IgM antibody titers against HSV−EAs in CaCx patient's sera which showed significant titers over the upper limit of healthy controls were compared in Fig. 4. Positive correlation was found between these 2 titers as assayed by HSV−1 EA or HSV−2 EA, and latter appeared to show higher correlation. The same serum specimens shown in Fig. 4 were compared for their IgM−ELISA titers against HSV−1 EA and HSV−2 EA and the result is shown in Fig. 5, with high correlation between these 2 titers.

Change of IgM−ELISA reactivity in CaCx patients' sera by blocking with unlabeled antigens

The same 5 CaCx patients' sera as shown Figs. 4 and 5 were pretreated by unlabeled HSV−1 EA or HSV−2 EA before tested by the IgM−ELISA using labeled EA antigens and the resulting ELISA−OD was compared with that obtained by the serum before the treatment. The result in Fig. 6 showed that the ELISA reactivity against HSV−1 EA and HSV−2 EA of 3 serum specimens decreased in parallel after blocking either by unlabeled HSV−1 EA or by HSV−2 EA. While, remaining 2

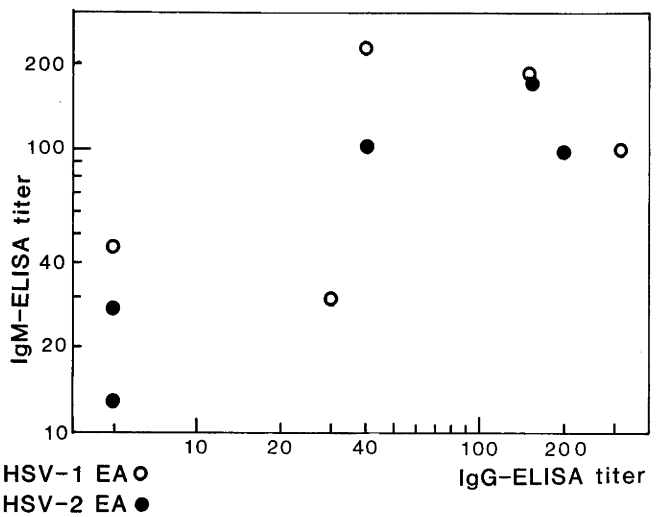

Fig. 4. Correlation between IgG−and IgM−ELISA titers in sera from CaCx patients with significant levels of IgM−ELISA titers against HSV EAs. Open circles: assayed by HSV−1 EA, closed circles: by HSV−2 EA.

After 1 hour's adsorption, the cells were washed with PBS and overlaid with the maintenance medium containing 20 μg/ml of cytosine arabinoside (Ara−C). The cells were incubated for 0, 6 or 12 hours at 37℃, and were washed with PBS followed by fixation in cold methanol for 30 min. The ACIF staining was performed according to Reedman and Kiein (1973). Briefly, fixed cells were incubated for 30 min at 36℃ and 30 min at 4℃ with a mixture of 1:10 diluted rabbit anti−HSV−2 IgG and 1:25 diluted guinea pig complement. The cells were washed 3 times with PBS, and stained with fluorescent isothiocyanate−labeled anti−guinea pig complement rabbit IgG at 1:10 dilution for 2 hours at 4℃ followed by the washing and mounting in glycerol before examined under a fluorescent microscope.

Preparation of early antigens and conjugation with HRPO: Monolayer cultures of HEp−2 cells were infected with either HSV−1 or HSV−2 at MOI of 5 PFU/cell. After 1 hour of virus adsorption, the cells were washed with PBS and incubated under the maintenance medium containing 20μg/ml of Ara−C for 6−7 hours at 37℃. Then, the cells were scraped off, washed in PBS, and suspended in RSB (0.01 M NaCl, 0.01 M Tris−HCl, 0.0015 M $MgCl_2$, pH 7.4) containing 0.2% Nonidit P−40 to swell for 5 min at 4℃. The cells were disrupted by 15 strokes in a tight−fitting Dounce homogenizer, and the nuclei were collected by centrifugation at 2,000 rpm for 10 min at 4℃. The pelleted nuclei were resuspended in PBS and sonicated for 3 min, followed by centrifugation at 7,000 rpm for 10 min. The antigen was precipitated from the supernatant by 50% saturation of ammonium sulfate and then redissolved in PBS to final protein concentration of 5 mg/ml as determined by Lowry's method (1951). The nuclei of mock infected HEp−2 cells were similarly treated and used as control. The antigens from HSV−1, HSV−2 and mock infected cells were labeled with HRPO (Sigma, type VI) by the modified periodate method of Wilson and Nakane (1978). The enzyme−conjugated antigen was precipitated by 50% saturation of ammonium sulfate, redissolved in 1 ml of PBS, and dialyzed against PBS. Fifty microliter aliquots of the labeled antigen were distributed and stored at −70℃ until use.

ELISA procedure: Antibody capture direct ELISA was performed as described by van Loon *et al.* (1981, 1983) with some modifications. Plastic Immulon microplate was coated with 75μl/well of goat−anti human IgG (Fc−specific) or goat−anti human IgM (μ chain specific) at 1:600 dilution in 0.05 M carbonate−bicarbonate buffer, pH 9.6, and incubated at 37℃ for 2 hours or 4℃ overnight in a humidified atmosphere. The plate was washed with PBS−T (0.05% Tween 20 in PBS, pH 7.2) 3 times for 3 min each. Fifty microliter of test serum at 1:10 dilution in PBS−T was added in each well and incubated at 37℃ for 1 hour. The plate was washed as above and HRPO−conjugated antigen diluted in PBS−T to final protein concentration of 40μg/ml was applied in a volume of 50μl/well. After 1 hour's incubation at 37℃, the plate was washed as above and substrate solution containing 0.5 mg/ml of o−phenylenediamine dihydrochloride and 0.02% of H_2O_2 in 0.05 M citrate phosphate buffer, pH 5.0, was applied using 50μl/well. After 20 min incubation at 37℃ in the dark, the reaction was stopped by adding

40μl/well of 4 N H_2SO_4 and the optical density at 490 nm was recorded by a MicroELISA Auto Reader model MR580 (Dynatech, USA) using 630 nm as a reference wavelength. Titers of test specimens were calculated by a computer system (Morita *et al.*, 1982), comparing the color reaction by each test specimen with those by serial dilution of a standard positive serum with known endpoint titer (Igarashi *et al.*, 1981).

Blocking test in the IgM−ELISA: To determine the specificity of each antigen in the IgM−ELISA, blocking test was performed. Anti−human IgM coated plate was reacted with the standard positive serum which had beed prereacted for 2 hours at 37℃ with diluted unlabeled antigen. Then the plate was reacted with conjugated antigen following the ELISA procedure described above.

Detection of rheumatoid factor (RF) and lupus erythematodes factor (LE): Latex agglutination tests were applied using the latex globlin manufactured by Kyowa Pharmaceutical Co. Tokyo and Japan Travenol Co. Tokyo, respectively.

RESULTS

Staining of early antigen by ACIF method

When HSV−2 infected and mock infected cells treated with Ara−C for 6 hours and stained with rabbit antiserum against HSV−2 by ACIF method were examined under a fluorescent microscope, the immunofluorescence was bright in infected cell nuclei with less intensity in the cytoplasm, and no fluorescence in the mock infected

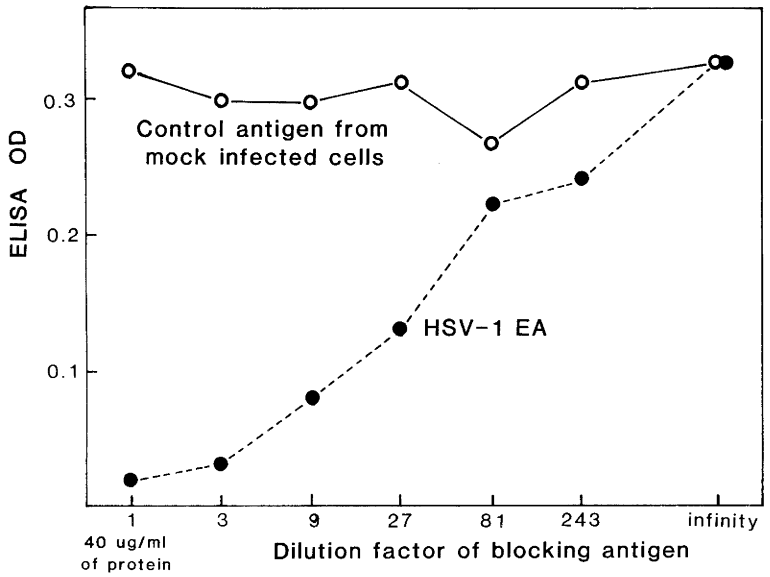

Fig. 1. Blocking test of the IgM−ELISA using HSV−1 EA. A standard positive serum at 1:10 dilution was mixed with varying concentration of unlabeled HSV−1 EA or control antigen from mock−infected HEp−2 cells, followed by the IgM−ELISA using HRPO−conjugated HSV−1 EA. The result was shown by the optical density in the ELISA color reaction.

controls were observed. The intensity of the fluorescence in nuclei and the percentage of flourescent positive cells were highest when the specimens were prepared at 6 hour postinfection with Ara−C treatment. According to the result, early antigen for the ELISA was prepared from infected and Ara−C treated HEp−2 cell nuclei 6 hours after the infection.

Blocking test

In order to determine the specificity of HRPO−conjugated antigen for the ELISA, blocking test was performed by IgM ELISA. The result with HRPO−conjugated HSV−1 EA (Fig. 1) showed that color reaction in the ELISA by this antigen was blocked when standard positive serum was pretreated by unlabeled HSV−1 EA but not by control antigen from mock infected cells.

Titer distribution of IgG−ELISA against HSV−1 EA and HSV−2 EA

Fig. 2 shows distribution of IgG−ELISA titers against HSV−1 and HSV−2 EA

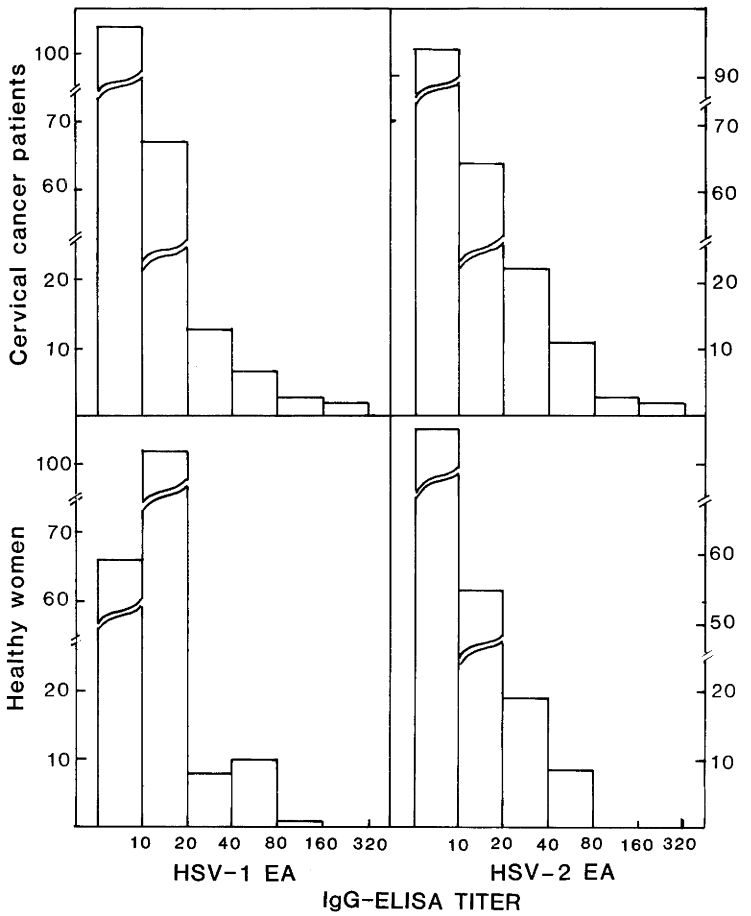

Fig. 2. Titer distribution of IgG−ELISA against HSV−1 EA and HSV−2 EA among CaCx patients and healthy controls.

among CaCx patients and control group. There were more weakly positive specimens against HSV−1 EA (titer between 10 to 20) among healthy women than CaCx patients in which more numbers were found among negatives (titer under 10). On the contrary, titer distribution curve against HSV−2 EA was similar for CaCx and control groups, although 5 cases of CaCx possessed titer over 80, which was the upper limit for the control group.

Titer distribution of IgM−ELISA against HSV−1 EA and HSV−2 EA

Fig. 3 shows distribution of IgM ELISA titers against HSV−1 EA and HSV−2 EA among CaCx patients and healthy women. In the case of CaCx patients, IgM antibodies against HSV−1 EA was positive (exceeding 10 ELISA titers) in 17 sera, IgM antibodies against HSV−2 EA was positive (exceeding 10 ELISA titers) in 12 sera. While in the case of healthy women, IgM antibodies against HSV−1 EA was

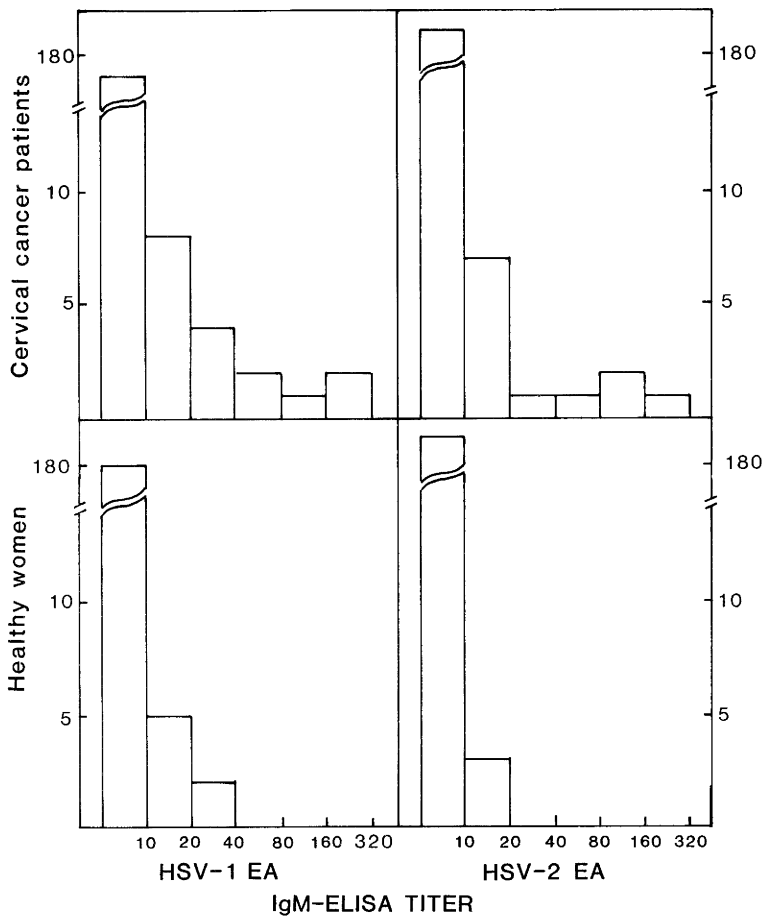

Fig. 3. Titer distribution of IgM−ELISA against HSV−1 EA and HSV−2 EA among CaCx patients and healthy controls.

INTRODUCTION

Several early antigens such as AG−4, AG−e, VP 134, etc. have been prepared from herpes simplex virus type−2 (HSV−2) infected cells or tissues for use in seroepidemiological studies on CaCx patients (Rawls *et al.*, 1977). One of these antigens, designated as AG−4 or ICP 10, has been found to be associated with active tumor progression but were negative in successfully treated CaCx patient (Aurelian *et al.*, 1973a, b, 1981). On the other hand, several investigators have reported that AG−4 antibodies persisted in CaCx patients even after their treatment (Notter & Docherty, 1976; Heise *et al.*, 1979; Arsenakis *et al.*, 1980; Arsenakis and May, 1981) and also could be found in HSV−2 infected patients (Arsenakis *et al.*, 1980; Arsenakis and May, 1981). Several reports showed the evidence of HSV−2 infection in tissues from cervical carcinoma either by detecting HSV−2 DNA (Frenkel *et al.*, 1972; Park *et al.*, 1983) or virions (Aurelian & Strandberg, 1974). However, there have been controversial views on the correlation between HSV−2 infection and CaCx.

In the present study, we describe IgM and IgG antibody titers against HSV−1 and HSV−2 EA measured by antibody−capture direct ELISA using enzyme−labeled antigens prepared from infected HEp−2 cell nuclei in order to explore antibody levels among CaCx patients as well as age−matched healthy women.

MATERIALS AND METHODS

Test sera: Serum samples from CaCx patients were obtained from 40 stage 0 (CCO), 55 stage I, 63 stage II, 10 stage III, and 28 with indistinct stage patients. The diagnosis of CCO and invasive cancer was made on histological examination of biopsy material and cases of invasive cancer were staged according to the classification of FIGO (Internatinal Federation of Gynecology and Obstetrics). Control sera were selected from the stock in Nagasaki Prefectural Institute for Public Health and Environmental Sciences and matched for age (above 30 years old), race (only Japanese) and socioeconomic status (middle class). The sera were heat inactivated at 56℃ for 30 min before use.

Cells and viruses: HEp−2 cells and MA104 cells were grown at 37℃ in Eagle's minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS) and 5% calf serum. Seed viruses of HSV−1 (strain HF) and HSV−2 (strain Savage), were prepared in MA104 cells by inoculating at input multiplicity of infection (MOI) of 0.01 PFU/cell and the cells were incubated for 48 hours at 37℃ under the maintenance medium (Eagle's medium with 2% FCS). Infected cells were collected, frozen−thawed and stored at −70℃ until use. Virus titer was assayed by plaque formation on HEp−2 cells.

Anti−complement immunofluorescence (ACIF): Monolayer cultures of HEp−2 cells in 8 chamber slides (Miles, III, USA) were infected with HSV−2 at MOI of 10 PFU/cell.

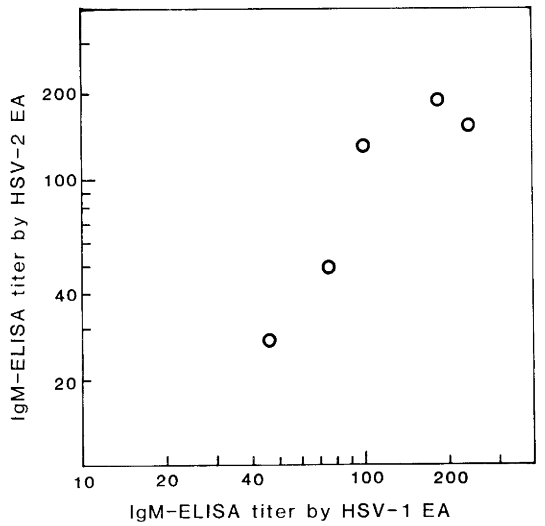

Fig. 5. Correlation between IgM−ELISA titer against HSV−1 EA and that against HSV−2 EA in 5 CaCx patients with significant IgM−ELISA titers. The same serum specimens as shown in Fig. 6 were compared for their IgM−ELISA titers against HSV−1 EA and HSV−2 EA.

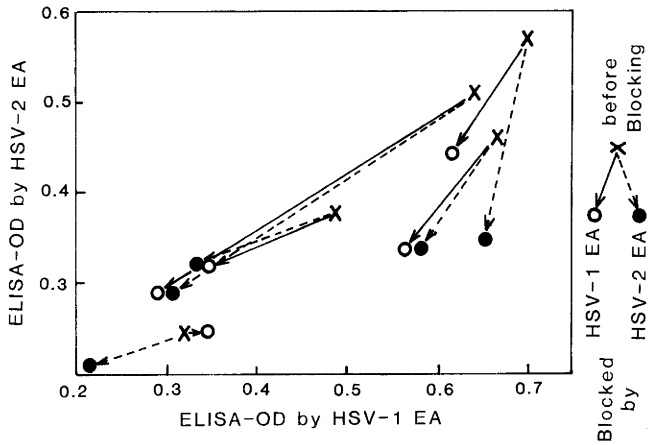

Fig. 6. Change in the IgM−ELISA reactivity against HSV−1 EA and HSV−2 EA among CaCx patients with significant IgM−ELISA titers after blocking the serum specimens with unlabeled antigens. The same serum specimens shown in Figs. 4 and 5 were preincubated either with unlabeled HSV−1 EA (open circles) or HSV−2 EA (closed circles) and tested for their reactivity in the IgM−ELISA using HRPO−labeled HSV−1 EA and HSV−2 EA. The resulting ELISA−OD values were compared with the corresponding value for each serum before being blocked as shown by the crosses.

sera showed dissociation between the ELISA reactivity against these 2 antigens after blocking with each unlabeled antigen. One of them showed more decrease in the reactivity against HSV−2 EA after blocking by HSV−2 EA, although its reactivity against both antigens decreased similarly by blocking with HSV−1 EA. Another serum did not show significant change in the reactivity against both antigens by blocking with HSV−1 EA, although its reactivity decreased by blocking with HSV−2 EA, especially against HSV−1 EA.

Stage of cervical cancer and high IgM−ELISA titer against HSV−1 EA and HSV−2 EA

Table 1 shows the number of CaCx patients examined in this study wihch were classified according to the stage of cervical cancer as well as the number of patients with high IgM−ELISA titers against HSV−1 EA and HSV−2 EA. The corresponding numbers among healthy women were also shown as controls. It is noteworthy that most of the patients with high IgM−ELISA titers against HSV−1 EA or HSV−2 EA were in stage O (CCO).

Detection of RF and LE

No serum was positive against RE and LE.

Table 1. Distribution of the specimens with high IgM-ELISA titers against HSV-1 EA and HSV-2 EA among CaCx Patients of different stages as well as among healthy controls.

		Number of specimens examined	Specimens with IgM-ELISA≥40	
			Type 1	Type 2
Cervical cancer	Stage 0	40	4	3
	Stage I	55	0	0
	Stage II	63	1	1
	Stage III	10	0	0
	Others	28	0	0
	Total	196	5	4
Healthy women		187	0	0

DISCUSSION

Several investigators have reported that sera from patients with CaCx have higher titers and higher prevalence of neutralizating antibodies against HSV−2 than control women (Rawls *et al.*, 1969; Nahmias *et al.*, 1970; Royston and Aurelian 1970). However, our results showed that the reactivity in the IgG−ELISA was not significantly different between CaCx patients and control, although there were a small number of CaCx pa-

tients with high titer exceeding the upper limit of the healthy controls. The result seems to suggest that the IgG−ELISA antibody titers against HSV−EAs measured in our study was not associated with uterine cervical cancer. Recent study by Shillitoe *et al.* (1984) reported that IgG antibodies to HSV−EA in sera of oral cancer and control groups showed similar reactive patterns. In contrast, high antibody titers in IgM−ELISA to HSV−1 EA or HSV−2 EA appeared to be associated with CaCx as shown in Fig. 3 and especially with early stage of the cancer as shown in Table 1. The result may reflect more frequent exposure of these patients to recurrent HSV infections or partial activation of certain viral antigens (Gilman *et al.*, 1980). It may be important to follow up the fluctuating IgM titers against HSV−EAs in clinical course of suspected or diagnosed CaCx cases. Although correlation was observed between the titers against HSV−1 EA and HSV−2 EA among 5 CaCx patients' sera with high IgM−ELISA titers (Fig. 5), difference in the changing reactivity in the IgM−ELISA after blocking among 2 of the 5 patients' sera (Fig. 6) indicated individual differinces and complexities in their antibody compositions against different molecules or epitopes of the EA antigens.

The antibody−capture direct ELISA technique described in this study is a simple method for detecting IgG and IgM antibodies against HSV−EAs without using hyperimmune sera and separation of each class of immunogloblins and without interference by rheumatoid factor to cause false−positive results (van Loon *et al.*, 1981; 1983). On the other hand, the method requires purified labeled antigens, otherwise false positive reactions due to anti−nuclear antigen may occur (van Loon *et al.*, 1981; 1983). In the present study, no serum was strongly positive against control antigen for any class of immunoglobulins, neither we found significant levels of RF or LE which might have given false positive results. The specificity and reactivity of HRPO−labeled antigens did not decrease during storage at −70℃ at least up to 6 months.

At present, cervical cancer is clinically diagnosed by cytology, colposcopy and punch biopsy, and no superior examination has been introduced up to the present. Also, no test has been available for screening cancer relapses or detecting new cases in their early stage. It may be that the IgM−ELISA against HSV−EAs may be applied for such purposes in the future.

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酵素標識ヘルペスウイルス1型及び2型初期抗原を用いた免疫酵素測定法による子宮頸癌患者血清の免疫グロブリンMとGの検出

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子宮頸癌患者196名と対照である健常人187名の血清中のヘルペスウイルス1型（ HSV− 1）及び2型（ HSV− 2）初期抗原に対する IgG 及び IgM 抗体価をペルオキシダーゼ標識抗原を用いた抗体捕獲微量直接 ELISA により測定した．子宮頸癌患者の17例は HSV− 1初期抗原に対して IgM 抗体が陽性（ 抗体価10以上 ）を示し，そのうち8例は抗体価20以上であった． HSV− 2初期抗原に対する IgM 抗体は12例で陽性であり，そのうち5例は抗体価20以上を示した．これに対して健常人では HSV− 2初期抗原に対して IgM 抗体価20以上を示す例はなく， HSV− 1初期抗原に対して2例が20−40の抗体価を示したのみであった． HVS−2初期抗原に対する IgG ELISA 抗体価の頻度分布は子宮頸癌患者と健常人で類似の曲線を示したが，子宮頸癌患者の5例は健常人の抗体価の上限である80以上の抗体価を示した．一方，

HSV－1初期抗原に対する IgG ELISA 抗体は健常人では陰性者（抗体価10以下）よりも弱陽性者（抗体価10－20）が多く，子宮頸癌患者では陰性者の方が多かった．初期抗原に対して高い IgM 抗体価を示した例は子宮頸患者のうちでも O 期の初期癌に多く見られた．

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