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STUDIES ON ANTIBODY SURVEILLANCE TO EB VIRUS-INDUCED ANTIGEN IN PATIENTS WITH SARCOIDOSIS AND NASOPHARYNGEAL CARCINOMA BY INDIRECT IMMUNOFLUORESCENCE[1]

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SUMMARY Titration of antibodies against viral antigen (VA) and early antigen (EA) induced by Epstein-Barr virus (EBV), in sera from patients with sarcoidosis and nasopharyngeal carcinoma (NPC), were performed by the indirect immunofluorescent technique.

The anti-VA titers of 143 sera from 70 patients with sarcoidosis and 108 sera from control groups were examined. As a positive reference, the anti-VA titers of 31 sera from NPC patients were also examined. Dissociation in the frequency distribution of anti-VA titers of normal controls and patients with sarcoidosis or NPC were found to be maximum when the limit for a positive reaction was set at 1: 640. Of the sera from sarcoidosis patients, 60.1% had anti-VA titers equal to, or greater than 1: 640. There was no significant correlation between the titers and the clinical stage of sarcoidosis, although patients with sarcoidosis, who had become clear of pulmonary X-ray findings, showed a somewhat lower positive rate of anti-VA activity than patients with pulmonary X-ray findings of sarcoidosis. Of the 108 specimens from a control group, consisting of patients with tuberculosis and other diseases and normal subjects, 21.3% had VA antibody levels equal to, or higher than 1: 640. On the other hand, the percentages of cases with anti-VA titers of 1: 640 or more, were 61.3% for NPC patients, 28.3% for patients with other types of cancer of the head and neck, 7.9% for members of families of NPC patients and 10% for normal subjects.

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Sera from 64 patients with sarcoidosis, from 28 patients with NPC and from 20 healthy persons were examined for antibodies to EA. The proportion of sera with anti-EA titers of 1: 10 or more, was significantly higher in NPC patients than in patients with sarcoidosis or in healthy persons. No significant difference was found between the EA titers of patients with sarcoidosis and healthy subjects.

INTRODUCTION

Sarcoidosis was first described by Hutchinson in 1869, but its etiological agent has not yet been found. Recently, Hirshaut et al. (1970) and Wahren et al. (1971) reported that sera of patients with sarcoidosis exhibited high antibody titers to viral antigen (VA) of Epstein-Barr virus (EBV).

EBV has been suspected as a possible viral etiological factor in Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC) and infectious mononucleosis (IM). The possibility of a causative role for this agent in BL, NPC and IM is supported mainly by the observation that sera of patients with these diseases showed high reactivities against the viral antigen of EBV. Henle, Henle and Diehl (1968) reported that paired sera from patients with IM showed a marked increase in antibody titer against EBV, as demonstrated by the indirect immunofluorescence test, and sera from patients with NPC exhibited an equally high proportion of positive reactions to EBV to sera from BL patients, although their antibody titers were slightly lower than in the latter. Kawamura et al. (1970) found that in Japan the sera from IM patients exhibited higher titers than those of cases of leukemia or other cancers, or of normal subjects. However, the titers of IM patients were lower than those of patients with NPC or BL. An anti-EBV titer of more than 1: 640 was found in the sera of 85.7% of the BL patients, 77.1% of the Chinese NPC patients in Taiwan, and 63.6% of the Japanese NPC patients examined. The reactivities of the sera of NPC and BL patients against EBV were similar.

Henle et al. (1970) and Hinuma, Sairenji and Ohta-Hatano (1970) reported that EBV infection of Raji cells and NC-37 cells results in the appearance of an early antigen (EA) in these cells, which is serologically different from VA. They found EA in the sera of patients with BL or NPC but little if any in sera from patients with other neoplasms or from healthy donors.

The present paper reports studies on the antibodies to VA and EA of EBV in sera from patients with sarcoidosis and NPC in Japan, examined by the indirect immunofluorescence.

MATERIALS AND METHODS

1. *Sera*

Two categories of human sera were examined. 1) Sera (143 samples) from 70 patients with sarcoidosis, and 108 control sera obtained from 14 patients with pulmonary tuberculosis, 40 patients with diseases other than sarcoidosis and pulmonary tuberclosis and 54 healthy persons. Sera were obtained from the Osaka Prefectural Hospital. Control persons were confirmed not to have the picture of sarcoidosis by pulmonary X-ray. 2) Thirty one sera from patients with NPC, 120 sera from patients with carcinoma and neoplasms of the head and neck other than NPC, 38 sera from members of families of patients with NPC and 40 sera from healthy persons. These sera were obtained from the Osaka Kaisei Hospital and the Hospital of the Osaka University Medical School.

The age distributions of subjects in these two categories are shown in Tables 1 and 2. All sera were stored at −20 C until tested.

2. *Cells*

The P3HR-1 subline of the P3J (Jijoye) Burkitt's cell line, was kindly supplied by Dr. Y. Hinuma, Kumamoto University, School of Medicine. Cells were cultured in Eagle's minimal essential medium with 20% calf serum, 100 IU of penicillin and 100 μg

TABLE 1. *Age distribution of cases with sarcoidosis and control subjects*

Group	Age (years)							Total
	-9	10-19	20-29	30-39	40-49	50-59	60-	
Sarcoidosis								
Before treatment	1	14	26	10	7	4	2	64
Under treatment	1	13	23	10	5	4	0	56
After clearing of X-ray changes	3	6	8	4	2	0	0	23
Total	5	33	57	24	14	8	2	143
Control								
Tuberculosis	0	3	4	4	0	2	1	14
Other diseases	0	3	6	5	11	6	9	40
Normal subjects	1 (1)[a]	11 (2)	20	11	4 (2)	4 (1)	3	54 (6)
Total	1	17	30	20	15	12	13	108

[a] Members of families of patients.

TABLE 2. *Age distribution of cases with NPC, and with cancer of the head and neck other than NPC and of normal subjects*

Group	Age (years)								Total
	-9	10-19	20-29	30-39	40-49	50-59	60-69	70-	
Nasopharyngeal carcinoma	0	2	2	3	8	9	6	1	31
Members of families of NPC cases	0	5	7	8	12	5	1	0	38
Cancer of head and neck other than NPC	0	1	8	6	23	35	28	19	120
Normal subjects	0	4	4	4	10	9	6	3	40

of streptomycin per ml at 33 C. On the 14th day, approximately 1/3 of the fluid in the culture was replaced by fresh medium. The NC-37 cell line of human hematopoietic cells was kindly supplied by Dr. T. Osato, Cancer Institute, Hokkaido University, School of Medicine. Cells were grown in RPMI-1640 medium containing 10% fetal calf serum at 37 C in a CO_2 incubator.

3. *Preparation of VA of EBV*

As described by Hinuma et al. (1967), the percentages of VA positive cells in cultures of the P3HR-1 line increased on culturing the cells at 33 C for 8-12 days. In this way 20-30% of the cells became immunofluorescent positive. These cells were washed five times with phosphate buffered saline (PBS), pH 7.4, and suspended in PBS at a final concn of approx 10^6-10^7 cells/ml. A drop of this suspension was smeared on a coverslip, dried at room temp and fixed with cold acetone for 15 min.

4. *Preparation of early antigen (EA) induced by EBV*

For assays of antibodies to EA, NC-37 cells were exposed to concd suspensions of EBV which had been prepared from P3HR-1 cultures as described by Henle et al. (1970). Cells of the P3HR-1 line were incubated at 33 C for 10-12 days, and then centrifuged at 10,400×g for 20 min. The supernatant was recentrifuged at 27,300×g for 90 min. The precipitate was suspended in 3 ml of PRMI-1640 medium containing 20% fetal calf serum. For in-

fection, cells of the NC-37 line were harvested by centrifugation at 1,000 × g for 5 min and resuspended at a concn of 1×10^7 cells in 3 ml of the above mentioned virus suspension. After 1 to 3 hr at 37 C, the cells were washed with the same medium and resuspended in fresh medium at a concn of 10^6 cells/ml. Then the cultures were incubated at 37 C for 18 hr, and acetone-fixed cell smears were prepared from them.

5. *Indirect immunofluorescent antibody technique for titration of the antibody levels to VA and EA*

A slight modification of the indirect method established by Henle and Henle (1966) was employed. For determinations of the anti-VA titer, test sera were diluted 1:40, 1:160, 1:640 and 1:2,560 with PBS, and for determinations of the anti-EA titer, sera were diluted 1:10, 1:40, 1:160 and 1:640 with PBS. These sera were used as primary antisera in the indirect immunofluorescence procedure. Coverslips with the virus antigens were first exposed to the various dilutions of the primary serum for 30 min at 37 C. Then they were thoroughly washed five times with PBS, and overlaid with anti-human γ globulin conjugated with fluorescein isothiocyanate (Eiken Chemical Co., Tokyo) and incubated in a moist chamber for 30 min at 37 C. Then they were washed five times in petri dishes containing PBS on a vibrator and mounted on slides with buffered glycerol (pH 9.5). These preparations were observed with a Tiyoda FM 200A microscope equipped with an Osram HBO 200 high pressure mercury lamp and by the ultraviolet excitor procedure. The antibody titers against Marek's disease virus (Biken C2 strain) were also examined as described by Naito et al. (1970).

6. *Hemagglutination-inhibition (HI) test*

Assay of antibodies to influenza A (A/Aichi/2/68), B (B/Kagoshima/68) and mumps (Enders) virus were performed through the courtesy of Drs. S. Kunita and T. Kurimura by Miss A. Maeda at the Osaka Prefectural Institute of Public Health, using the microtiter method.

RESULTS

1. *Distribution of anti-VA titers in sera from patients with sarcoidosis and controls*

Dissociation in the frequency distribution of the incidence of anti-VA titers in sera from sarcoidosis patients and controls was found to be max when the limit of the positive reaction was set at 1:640 (Fig. 1). As shown in Table 3, of the 143 sera of sarcoidosis patients, 60.1% had anti-VA titers of 1:640 or more, while only 21.3% of the 108 control sera had anti-VA titers of 1:640 or more. Sera of sarcoidosis cases and control subjects were each divided into three groups as shown in Table 3. There is no significant correlation between the titers and groups of sarcoidosis cases, although the group of sarcoidosis patients in whom pulmonary X-ray findings had become normal, showed a somewhat lower positive rate of anti-VA activity than the other groups.

The data were analyzed statistically by the ridit method, taking the anti-VA titers among the control groups as the identified distribution. The sera of sarcoidosis patients exhibited a higher average ridit than those of controls.

2. *Distribution of anti-VA titers in sera from patients with NPC and controls*

Table 4 shows the distribution of anti-VA titers in patients with NPC, patients with cancer of the head and neck other than NPC, members of families of NPC patients and normal subjects. The percentages of cases with anti-VA titers of 1:640 or more, were 61.3% in

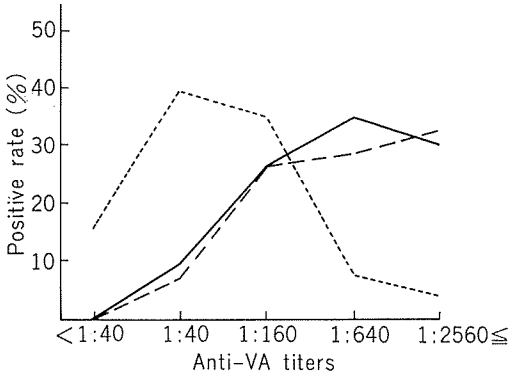

FIGURE 1. *Distribution of anti-VA titers in sera from patients with sarcoidosis and NPC and from normal subjects.* ———, *sarcoidosis (64 sera);* - - - - -, *NPC (31 sera);* ·····, *normal subjects (54 sera).*

TABLE 3. *Distribution of anti-EBV (VA) titers in sarcoidosis, other diseases and normal subjects*

Group	Anti-EBV (VA) titers					Total cases	1:640≤ positive (%)	Ridit analysis
	<1:40	1:40	1:160	1:640	1:2560≤			
Sarcoidosis	0	11	46	47	39	143	60.1	0.733±0.048
Control group	12	42	31	16	7	108	21.3	0.500±0.056
Sarcoidosis								
Before treatment	0	6	17	22	19	64	64.1	0.831±0.072
Under treatment	0	1	18	17	20	56	66.1	0.867±0.077
After clearing of X-ray changes	0	4	11	8	0	23	34.8	0.722±0.120
Control								
Tuberculosis	1	6	2	3	2	14	35.7	0.591±0.154
Other diseases	3	15	10	9	3	40	30.0	0.593±0.091
Normal subjects	8	21 (2)[a]	19 (2)	4	2 (2)	54 (6)	11.1	0.500±0.079

[a] Members of families of patients.

TABLE 4. *Distribution of anti-EBV (VA) titers in normal subjects and cases of selected disease*

Group	Anti-EBV (VA) titers					Total cases	1:640≤ positive (%)	Ridit analysis
	<1:40	1:40	1:160	1:640	1:2560≤			
Nasopharyngeal carcinoma	0	4	8	9	10	31	61.3	0.788±0.104
Members of families of NPC cases	10	15	10	3	0	38	7.9	0.366±0.098
Cancer of head and neck other than NPC	18	23	45	28	6	120	28.3	0.568±0.033
Normal subjects	7	7	22	3	1	40	10.0	0.500±0.091

patients with NPC, 28.3% in patients with cancer of the head and neck other than NPC, 7.9% in members of families of NPC patiens and 10% in normal subjects. The sera of NPC patients exhibited a higher average ridit than those of other groups.

3. *Incidence of antibody titers to EA in sera from patients with sarcoidosis and NPC and controls*

Antibodies to EA were examined in sera from 64 patients with sarcoidosis, 28 patients with NPC and 20 healthy presons. As shown in Table 5, 85.7% of the NPC patients had detectable anti-EA antibodies (1:10≤), whereas 32.8% of the sarcoidosis patients and 5.3% of the healthy persons had detectable antibodies. The percentage of sera with anti-EA titers of 1:10 or more was significantly higher in the NPC group than in group of sarcoidosis patients or healthy persons. There was no significant correlation between the anti-EA titers of sarcoidosis patients and controls.

4. *Correlation between anti-EA titers and anti-VA titers*

The anti-EA titers of sera from patients with NPC, and sarcoidosis and healthy controls, were plotted against their anti-VA titers (Fig. 2). In patients with NPC, positive anti-EA titers (1:10≤) were observed in 89% of 18 sera with positive anti-VA titers (1:640≤) and

TABLE 5. *Distribution of anti-EA titers in sera of patients NPC and sarcoidosis and normal subjects*

Group	No. of sera	Anti-EA titers					1:10≤ positive (%)
		<1:10	1:10	1:40	1:160	1:640≤	
NPC	28	4	5	10	7	2	85.7
Sarcoidosis	64	43	18	3	0	0	32.8
Normal subjects	20	19	1	0	0	0	5.3

80% of 10 sera with low anti-VA titers (≤1:160). On the other hand, in patients with sarcoidosis positive anti-EA titers were observed in 32% of 48 sera from patients with high anti-VA titers (1:640≤), and 36% of 14 sera from these with low anti-VA titers. Thus no correlation was found between the antibody levels to VA and EA.

5. *HI antibody titers and immunofluorescent antibody (FA) titers to different viral antigens*

No significant correlation was found between the distributions of HI antibody titers to influenza A and B and mumps virus antigens and FA antibody titers against Marek's disease virus antigen in the sera from sarcoidosis and NPC patients and normal subjects.

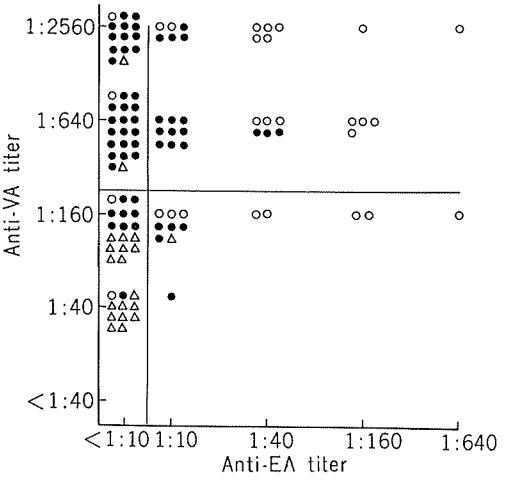

FIGURE 2. *Relationship between anti-EA and anti-VA titers in sera from patients with NPC and sarcoidosis and in healthy persons.* ○, *NPC;* ●, *sarcoidosis;* △, *normal subjects.*

DISCUSSION

Hirshaut et al. (1970) and Wahren et al. (1971) demonstrated that the percentages of sera of sarcoidosis patients with antibodies to EBV were significantly higher than in control groups. We confirmed that sarcoidosis patients in Japan also exhibited high titers of antibodies against EBV. The antibody titers to viral antigen (VA) of EBV in sera of sarcoidosis patients reported in this paper, were similar to those reported by Hirshaut et al. (1970), but were higher than those obtained by Wahren et al. (1971). The anti-VA titers of Wahren et al. were not as high as those seen in sera of cases of Burkitt's lymphoma or NPC, and the percentages of reactive Burkitt's lymphoma cells (Silfere line) were estimated as 2–5% using a known positive serum from a patient with infectious mononucleosis. We used 20–30% immunofluorescent positive cells of the P3HR-1 line as VA antigen of EBV. It is unknown whether the discrepansy is due to a racial difference. Various factors could influence the antibody titers to VA of EBV, such as the cell line and percentages of VA positive cells used as VA antigen, and the type of fluorescent microscope used for observation.

Patients with sarcoidosis were divided into 3 groups on the basis of their clinical condition. Groups 1 and 2 were patients before and during treatment and group 3 were patients who had become free of pulmonary X-ray lesions. No significant association was found between the

stage of the disease and the antibody titer to EBV, though the frequency of positive anti-VA antibodies in group 3 was somewhat lower than in the other two groups. Further studies are required on this.

Wahren et al. (1971) demonstrated that the numbers of sarcoidosis patients with antibodies to herpes simplex and cytomegalovirus were also significantly more than in control groups, whereas those of other antibodies, including antibodies to varicella zoster virus, mumps virus and measles virus, were similar to those in controls. In the present work, the members of cases with antibodies to influenza virus A type and B type and mumps virus were not found to be higher in sarcoidosis or NPC patients than in controls. It is unknown whether there is any mechanism to elevate the immunological reactivity to certain herpes virus infections.

Henle et al. (1970) reported that EA was shown to induce abortive infection in most, if not all, of invaded cell, but not of VA and thus not mature virus particles. Hinuma et al. (1970) also found a new antigen, which is similar to EA. Cells containing EA are detectable by indirect immunofluorescence tests with sera from many patients with Burkitt's lymphoma or NPC, but rarely with sera from healthy donors or patients with other diseases. Wahren et al. (1971) reported that the finding of anti-EA in sarcoidosis was relatively rare and the titers were low. We also found low titers of anti-EA, i.e., less than 1: 40 in sera of patients with sarcoidosis, although some of these sera contained high anti-VA titers. On the other hand, anti-EA was detected in 85.7% of the cases of NPC. The low titers of anti-EA in cases of sarcoidosis suggest that the made of involvement of EBV in sarcoidosis differs from that in BL or NPC.

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