

# Frequency and levels of antibodies to Epstein-Barr virus-specific DNase are elevated in patients with nasopharyngeal carcinoma

(neutralizing antibodies)

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**ABSTRACT** Sera from healthy individuals and patients with infectious mononucleosis, Burkitt lymphoma, nasopharyngeal carcinoma, or other malignancies were examined for their capacity to neutralize Epstein-Barr virus (EBV)-induced DNase activity. Sera were found that neutralized the EBV DNase but not herpes simplex virus type 1 or type 2 DNases, and vice versa. Sera from 46 of the 49 patients with nasopharyngeal carcinoma examined (94%) neutralized >6 units of EBV DNase per ml of serum. In contrast, only 19% of 47 patients with Burkitt lymphoma, 12% of 183 patients with other malignancies, 4% of 58 patients with infectious mononucleosis, and none of 101 healthy individuals had such levels of neutralizing activity. The neutralizing factor was found in the IgG fraction derived from nasopharyngeal carcinoma sera. There was no correlation between the concentration of these antibodies and the titers of IgG and IgA antibodies to the EBV capsid antigen, the early antigen complex, or the EBV-associated nuclear antigen.

The Epstein-Barr virus (EBV) is closely associated with nasopharyngeal carcinoma (NPC). Patients beyond the initial stage of this tumor generally show high titers of IgG and IgA antibodies to the EBV capsid antigen (VCA) and to the diffuse (D) component of the early antigen complex (1-8). Biopsies of the tumor regularly reveal the presence of EBV DNA (9-11), and the carcinoma cells express the EBV-associated nuclear antigen (EBNA) (12-15). The antibody spectrum and titers increase with the stage of the disease; i.e., the total tumor burden (3, 5, 8, 16). Correspondingly, the antibody titers decline gradually after effective therapy of NPC, so that the EBV-specific serologic tests may serve to monitor the success of treatment (8). Relapses or metastases are heralded by rising antibody titers months before they become clinically evident (8).

Recently, it was demonstrated that EBV is capable of inducing a virus-specific DNase upon superinfection of Raji cells or upon exposure of somatic hybrid cells to IdUrd (17). This induced DNase differs in its physical and immunogenic properties from DNases induced in appropriate cells by other herpes group viruses (17, 18). Because the DNases induced by herpes simplex virus (HSV) type 1 or 2 are capable of eliciting specific antibody responses in patients with HSV infections (19), we assayed sera from various patients and controls for the presence of antibody against the EBV-specific DNase. The serum donors included healthy individuals with or without past exposure to EBV and patients with infectious mononucleosis, Burkitt lymphoma, NPC, or other malignant diseases. The data to be presented show a high frequency of strong anti-EBV DNase activity in sera from patients with NPC. The degree of anti-EBV DNase activity does not correlate with EBV-specific antibody titers to early antigen, VCA, or EBNA.

## MATERIALS AND METHODS

**Preparation of Cellular Extract.** Raji cells were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, 100 units of penicillin per ml, 100  $\mu$ g of streptomycin per ml, 1  $\mu$ g of fungizone per ml, 10 units of mycostatin per ml, and 0.075% NaHCO<sub>3</sub> in 16-oz. glass prescription bottles (1 oz. = 29.57 ml). They were infected with cell-free EBV derived from HR-1 cultures by described procedures (17) and harvested 48 hr after infection. One milliliter of extraction buffer containing 2 mM Mg<sup>2+</sup>-ATP, 0.4 mM KCl, 3 mM dithiothreitol, and 50 mM Tris-HCl, pH 7.5, was added to 2  $\times$  10<sup>7</sup> cells. The cells were frozen and thawed four times and then centrifuged for 3 min at 4°C in a Beckman model B microfuge. The supernatant was used for determination of DNase activity.

**Enzyme Assay.** The assay procedures for EBV DNase activity were the same as described for exonuclease (18). One unit of DNase activity is defined as the amount of enzyme that converts 1  $\mu$ g of double-stranded DNA to acid-soluble material in 10 min at 37°C. The specific activity of the DNase used was 30 units/mg of protein, and the activity of DNase in mock-infected Raji cells was  $\approx$ 1 unit/mg.

**Human Sera.** The sera had been collected and tested previously for other studies and had been kept at -20°C. They included 101 sera from healthy donors (27 anti-VCA negative, 74 positive), 58 sera from infectious mononucleosis patients, 47 sera from Burkitt lymphoma patients, 49 sera from NPC patients, and 31 sera from patients with Hodgkin disease, malignant lymphomas, or other cancers.

**Determination of Anti-EBV DNase Activity.** A 0.1-unit sample of DNase from crude homogenates of EBV-superinfected Raji cells in a total volume of 5  $\mu$ l was incubated with 5  $\mu$ l of serum at room temperature for 20 min. The activity of EBV DNase was then determined as described (17). The difference of activity in the presence and absence of serum was calculated and the anti-DNase antibody activity was expressed as the units of DNase activity neutralized by 1 ml of serum.

**Determination of Antibodies Against Other EBV-Specific Antigens.** All sera were titrated for IgG antibodies and, when indicated, IgA or IgM antibodies to VCA and to the diffuse (D) and restricted (R) components of the EBV-determined early antigen complex by indirect immunofluorescence. They were titrated for antibodies to EBNA by anti-complement immunofluorescence. The techniques have been described (7, 20-22).

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Abbreviations: EBV, Epstein-Barr virus; EBNA, EBV-associated nuclear antigen; NPC, nasopharyngeal carcinoma; HSV-1 and HSV-2, herpes simplex virus types 1 and 2, respectively; VCA, virus capsid antigen.

Table 1. Specificity of antiviral DNase activity in human serum

Serum added	Antiviral DNase activity, units neutralized/ml serum		
	HSV-1	HSV-2	EBV
1	0	0	0
2	20	38	0
3	1*	0	9
4	1*	5	0

Crude cellular extract from HSV-1 (KOS)- and HSV-2 (333)-infected KB cells (18) and from EBV-infected Raji cells were used for this study.

\* Within the range of nonspecific neutralization.

**Purification of IgG from Serum.** Three milliliters of serum from patients with NPC were dialyzed overnight against 20 mM potassium phosphate buffer (pH 8.0) containing 0.02% sodium azide and then placed on a DEAE-Affi-Gel Blue (Bio-Rad) column (1.5 × 13 cm) that had been equilibrated with the same buffer. The highly purified IgG fraction was collected in the void volume by washing the column with buffer. The total recovery of anti-EBV DNase activity was nearly 100%. The purity of the IgG fraction was examined by disc electrophoresis by published procedures (23). One major and one minor protein band were observed in the gel. The major band had the same mobility as IgG. The minor band had the same mobility as transferrin.

**RESULTS**

**Demonstration of Antibody Specific for EBV-Induced DNase.** Sera from four individuals demonstrate the specificity of the neutralizing activity against enzymes derived from HSV-1-, HSV-2-, and EBV-infected cells (Table 1). Serum no. 1 had no activity against any of the virus-specific DNase activities. Serum no. 2 neutralized both the HSV-1 and HSV-2 DNases, although to different extents, but not the EBV DNase, whereas serum no. 3 had strong activity against EBV but not against HSV-1 and HSV-2 DNases.

In order to determine whether the inhibition of the EBV-induced DNase was due to antibody or to a nonspecific factor, several strongly positive sera were pooled and the IgG fraction was separated by DEAE-Affi-Gel Blue column chromatography. The anti-EBV DNase activity of serial dilutions of the purified IgG fraction was tested against the EBV-induced

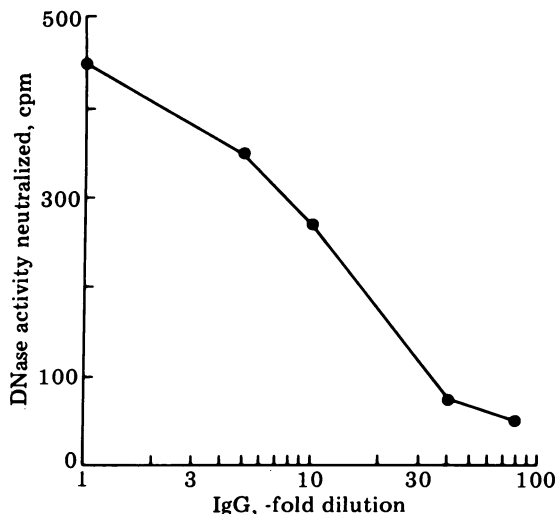


FIG. 1. Dose-response relationship of anti-EBV DNase activity and IgG fraction purified from sera of NPC patients.

Table 2. Incidence of antibodies against EBV-induced DNase in human sera

Source of sera	No. of patients	Anti-EBV DNase activity* (units neutralized/ml serum)		
		<3	3-6	>6
Healthy donors†	101	94 (93)	7 (7)	0 (0)
Infectious mononucleosis	58	52 (90)	2 (3)	4 (7)
NPC	49	2 (4)	1 (2)	46 (94)
Burkitt lymphoma	47	28 (60)	10 (21)	9 (19)
Other lymphomas‡	31	23 (73)	3 (10)	5 (17)
Leukemias§	49	37 (76)	5 (10)	7 (14)
Other carcinomas¶	103	90 (87)	3 (3)	10 (9)

\* Values are the no. of patients whose sera had the indicated activity. Numbers in parentheses indicate the percentage of sera.

† Seventy-five subjects were anti-VCA positive and 26 subjects were negative.

‡ Five patients with malignant lymphoma and 26 patients with Hodgkin disease.

§ Ten patients with chronic lymphocytic leukemia and 39 patients with acute myelocytic leukemia.

¶ Five patients with carcinoma of the tonsil and 98 patients with prostate carcinoma.

DNase. The results (Fig. 1) showed a dose-response relationship. The IgG fraction of pooled sera isolated from healthy individuals in a similar fashion showed no neutralization of EBV DNase activity (data not shown).

**Frequency and Concentration of Antibody to EBV-Induced DNase.** Table 2 and Fig. 2 summarize the data observed with sera from healthy donors and from several groups of patients. Of 101 healthy donors studied, 27 had not yet been infected with EBV; they had no antibodies to VCA or EBNA. Sera from seven of these individuals nevertheless neutralized between 1 and 2.5 units of EBV DNase. For this reason, only neutralization of ≥3 units of enzyme can be considered due to a virus-specific antibody. Taking neutralization of 6 units of EBV DNase as strong reactivity, none of the 74 sera from healthy anti-VCA-positive individuals, only 4 of 58 (7%) infectious mononucleosis sera, 9 of 47 (19%) Burkitt lymphoma sera, and 22 of 183 (12%) sera from other patients fell into this category. In contrast, 46 of the 49 (94%) sera from NPC patients neutralized >6 and as many as 18 units of the enzyme.

**Correlation of Anti-EBV DNase Activity with Other EBV-Specific Antibodies in Serum from Patients with NPC.** In Fig. 3, various EBV-specific antibody titers in sera from NPC patients are plotted against the anti-EBV DNase activity. No correlation was evident between the anti-EBV DNase activity and the other types of EBV-specific antibodies.

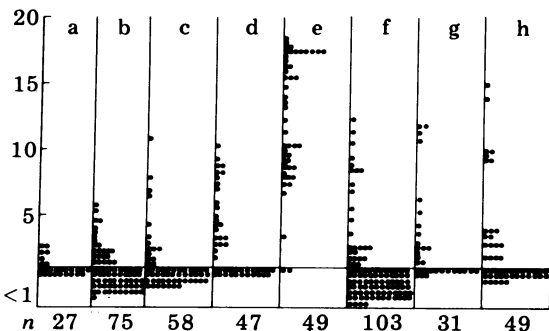


FIG. 2. Concentration of antibody to EBV-induced DNase in sera from healthy individuals and patients with different conditions. The ordinate indicates the number of EBV DNase units neutralized. a, Healthy donor, anti-VCA negative (<1:10); b, healthy donor, anti-VCA positive; c, infectious mononucleosis; d, Burkitt lymphoma; e, NPC; f, other carcinomas; g, other lymphomas; and h, leukemias.

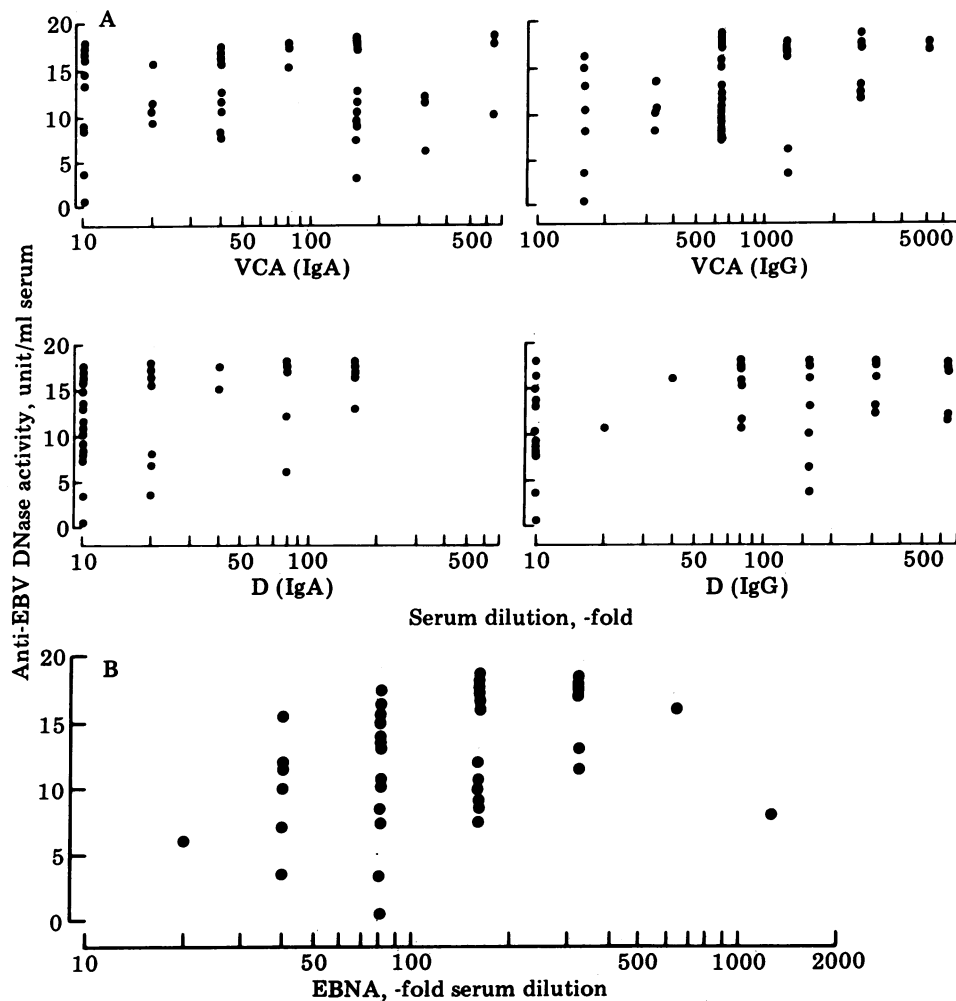


FIG. 3. Lack of correlation between the degree of anti-EBV DNase activity and the titers of IgG or IgA antibodies to VCA (A), the D (diffuse) component of the early antigen complex (A), and EBNA (B). Serum dilutions of 10 and <10 have been combined. For VCA and EBNA,  $n = 45$ ; for D (IgA),  $n = 38$ ; and for D (IgG),  $n = 40$ .

## DISCUSSION

The high incidence and high concentration of anti-EBV DNase activity in sera from patients with NPC demonstrated in this communication appear to be another outstanding feature of this malignancy and further support the close relationship between NPC and EBV. This anti-DNase activity is virus specific, as evident from Table 1. In general, most sera from healthy individuals have anti-HSV-1 DNase activity, but, as seen in Table 2, none has high titers of anti-EBV DNase activity. Burkitt lymphoma is, like NPC, intimately associated with EBV (24), but the incidence and concentration of anti-EBV DNase activity in sera from such patients are much lower than observed in NPC. If we assume that the presence of anti-EBV DNase activity is due to the continuous expression of EBV DNase, the degree of this expression would clearly be less pronounced in Burkitt lymphoma (lymphoma cells) than in NPC (undifferentiated carcinoma cells).

A question may be raised concerning the degree of purity of the EBV DNase used as antigen in our study. We have not been able to purify EBV DNase from EBV-superinfected Raji cells to the same degree as we purify HSV-1 or HSV-2 DNase from infected cells. Based on our past experience, the purity of antigen may not be a problem because the interaction of antibodies against either HSV-1 or HSV-2 DNase was similar whether purified or crude preparations were used. Furthermore, because mock-infected Raji cell control extracts have 97%

less DNase activity, the predominant species of DNase in EBV-superinfected Raji cells is virus specific (17).

We are now collecting sera from NPC patients at different stages of the disease and before and after treatment, as well as sera from high-risk populations. Study of these sera may provide information on the potential usefulness of the degree of anti-EBV DNase activity in determining the prognosis of the patients.

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