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Decreased Expression of Early Antigens in P3HR-1-EBV Superinfected Raji Cells Cultured in EBV-Seropositive Human Sera

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Antibody-induced change in the expression of Epstein-Barr virus (EBV) antigens was studied after P3HR-1-EBV superinfection of Raji cells. The superinfected Raji cells were cultured in medium containing fetal calf serum (FCS) and either EBV antibody-positive or -negative human sera which had been selected for low natural cytotoxic antibodies to Raji cells. At 24 postsuperinfective (psi) h the percentages of early antigen (EA)-positive cells were similar for cultures with FCS only or also with EBV antibody-positive or -negative sera. However, at 48 psi h decreased expression of EA-positive cells was observed in cultures with EBV antibody-positive sera, but not with EBV antibody-negative sera or with FCS only. This EA suppressing effect was dependent upon concentration of EBV antibody-positive human serum and related with anti-viral capsid antigen (VCA) or membrane antigen (MA) titers among effective sera. Antibody-mediated suppression of EA at 48 psi h was inhibited by treatment with phosphonoacetic acid which inhibits herpesvirus DNA replication and blocks late viral functions. These results are consistent with hypotheses that suppression of intracellular EA follows from modulation (or alteration) of MA by an anti-MA antibody and that a late viral function is required for the antibody-mediated inhibition of EA expression in this system.

Key words: Epstein-Barr virus; early antigen; membrane antigen; modulation; anti-EBV antibodies

To understand how the immune system controls infections with Epstein-Barr virus (EBV), we have analyzed the role of antibodies in modulating expression of EBV genome functions. EBV causes infectious mononucleosis (IM) and is associated with the pathogenesis of Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC) and the X-linked lymphoproliferative syndrome (Purtilo et al., 1976; Epstein and Achong, 1979; Henle and Henle, 1979). Chronic EBV infections can occur in some patients with nonmalignant diseases (Virelizier et al., 1978; Sairenji et al., 1995). Cellular and humoral immune mechanisms influence EBV infection and transformation. Cytotoxic T cells attack virally infected cells in vivo and retard the outgrowth of virally transformed cells in vitro (Thorey-Lawson et al., 1977; Moss et al., 1979; Klein, 1994). Virus-neutralizing antibodies can protect against reinfection or recurrent viremia (Sairenji et al., 1984, 1985a, 1985b). Antibody-dependent cellular cytotoxicity (ADCC) effectors can kill cells expressing antibody-recognized, EBV-induced antigens (Takaki et al., 1980). Natural killer cells can attack cells with other EBV neoantigens (Patarroyo et al., 1980). EBV antigens of these immune responses have also been identified

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; BL, Burkitt's lymphoma; EBV, Epstein-Barr virus; EA, early antigen; EA-SI, EA suppression index; EBNA, EBV-nuclear antigen; FCS, fetal calf serum; IM, infectious mononucleosis; MA, membrane antigen; NPC, nasopharyngeal carcinoma; PAA, phosphonoacetic acid; psi, postsuperinfective; VCA, viral capsid antigen.

Table 1. Anti-EBV characteristics of human sera

Patient's No.	Diagnosis	Anti-EBV titer		
		VCA	EA	MA
60	Normal adult	< 5	< 5	< 5
61	Normal adult	< 5	< 5	< 5
72	Normal adult	< 5	< 5	< 5
73	Normal adult	< 5	< 5	< 5
70	Normal adult	80	< 5	80
58	Normal adult	80	< 5	80
64	Normal adult	80	< 5	40
53	Normal adult	160	< 10	160
59	Normal adult	160	< 10	160
63	Normal adult	320	< 10	160
74	Normal cord blood	160	< 10	160
71	NPC	320	80	640

EA, early antigen; EBV, Epstein-Barr virus; MA, membrane antigen; NPC, nasopharyngeal carcinoma; VCA, viral capsid antigen.

with immunofluorescence assays: EBV-nuclear antigen (EBNA) (Reedman and Klein, 1973), early antigen (EA) (Henle et al., 1970), viral capsid antigen (VCA) (Henle and Henle, 1966), and MA (Klein et al., 1966).

The components of MA can be considered to play a central role in many of these mechanisms for attack upon EBV-infected or transformed cells. MA determinants were found in the virus envelope and could participate in neutralization (Sairenji et al., 1984, 1985a, 1988). MA appeared to be a target for ADCC killing (Takaki et al., 1980). The presence of high antibody titers to ADCC-determined MA correlates with survival in patients with NPC and with tumor response to chemotherapy in patients with BL (Chan et al., 1979; Pearson et al., 1979). We demonstrated that EBV release from EBV producer cells was inhibited by anti-MA antibodies (Sairenji et al., 1985a, 1988). In order to explore further how anti-EBV antibodies in human serum might influence EBV-infected cells, we sought to determine whether an antibody might modulate or change the expression of EBV-associated antigens. We found that the expression of EA in P3HR-1-EBV superinfected Raji cells was decreased when culturing the cells with EBV-seropositive human serum and that this phenomenon is phosphonoacetic acid (PAA)-sensitive.

Materials and Methods

Cell lines and medium

The cell line Raji (Pulvertaft, 1965) was the target for superinfection with EBV derived from P3HR-1 cells (Hinuma et al., 1967). RPMI 1640 medium (GIBCO, Grand Island, NY), containing 2% fetal calf serum (FCS) and 8% newborn calf serum with 100 μg/mL streptomycin and 100 IU/mL penicillin, was used for cell growth and for EBV production.

EBV preparation

Methods for optimal yields in P3HR-1 virus production have been described (Sairenji and Hinuma, 1975). P3HR-1 EBV was obtained from 14-day-old, 33˚C cultures. Cell cultures were frozen at –70˚C, thawed, centrifuged at 2,000 × *g* for 20 min to remove cellular debris and centrifuged again at 26,000 × *g* for 90 min. The pellets were resuspended in medium at a 100-fold concentration relative to the original culture medium. This virus suspension was passed through a membrane filter (0.45 μM pore size) and stored at –70˚C.

EBV superinfection

Raji cells in logarithmic growth phase (viability

98%) were used for the EA suppression assay. An amount of virus to induce 20% to 40% EA^+ cells at 48 postsuperinfective (psi) h was mixed with target cells to yield a final concentration of 2×10^6 cells/mL which were incubated at 37˚C for 90 min (Sairenji and Hinuma, 1973). After being washed twice with fresh culture medium, the cells were resuspended in fresh medium (RPMI 1640 medium containing 5% FCS and with varying concentrations of human sera) at 10^6 cells/mL and incubated in 5% CO^2 at 37˚C.

Human sera

Human sera were heat-inactivated and stored at –20˚C. The sera were screened for natural cytotoxic antibodies to Raji cells (Yang and Hewetson, 1974) by culture at 37˚C for 48 h in RPMI 1640 medium containing 10% human serum. Cell viability was assayed by trypan blue dye exclusion and sera which maintained viability at greater than 90% were used in this study (Table 1).

Titration of antibodies to EBV antigens

Anti-VCA titers were determined with acetone-fixed smears of P3HR-1 cells which had been cultured at 33˚C, using an indirect immunofluorescence technique (Henle and Henle, 1966). Anti-EA titers were determined by indirect immunofluorescence on acetone-fixed smears of P3HR-1-EBV superinfected Raji cells (Sairenji et al., 1991). Anti-MA titers were determined by indirect membrane immunofluorescence on living P3HR-1 cells cultured at 33˚C (Harada et al., 1980).

EA detection and EA suppression index (SI)

P3HR-1-EBV superinfected Raji cells were smeared and fixed with acetone for 10 min at room temperature. EA-positive cells were detected with indirect immunofluorescence (Henle et al., 1970). Serum (anti-EA and anti-VCA titers each 1:320) from a patient with NPC was used as the anti-EA indicator antibody. The fluorescent second antibody was a goat anti-human IgG (gamma-specific; Hyland Laboratory, Deerfield, IN). EA-positive (EA^+) and -negative cells were counted, in a group of at least 500 cells, under a fluorescence microscope and the percentages of EA^+ cells were determined. The EA-SI was calculated from the percentages of EA^+ cells at 48 psi h according to the following formula.

$$\text{EA-SI} = \frac{(\text{control\% without}) - (\text{test\% with})}{(\text{control\% without})}$$

where, (control% without) stands for (control % EA^+ cells without human serum) and (test% with) for (test % EA^+ cells with human serum).

Results

Effect of anti-EBV antibody-positive or -negative human sera on EA expression

EBV-superinfected Raji cells were cultured in medium (RPMI 1640 with 5% FCS) with different concentrations of EBV antibody-positive (Nos. 70 and 71) or -negative (Nos. 72 and 73) sera (Fig. 1). The percentages of EA^+ cells were determined at 24 psi h and 48 psi h. At 24 psi h the percentages of EA^+ cells were similar in all cultures with different concentrations of each serum regardless of whether or not the serum contained anti-EBV antibodies. However, at 48 psi h the expression of EA^+ cells varied significantly among the cultures, with fewer EA^+ cells being observed in the cultures with EBV antibody-positive sera, but no depression in numbers of EA^+ cells being found in cultures with EBV antibody-negative sera. In each assay showing inhibition of EA expression with a given EBV antibody-positive serum, the decrease in percentage of EA^+ cells was dependent upon the concentration of the serum. The concentration of Raji cells in the culture did not affect significantly the percentages of EA^+ cells at any concentration of human serum. Death of EA expressing, superinfected cells was apparently not a highly significant factor in this effect. In a control experiment with serum No.

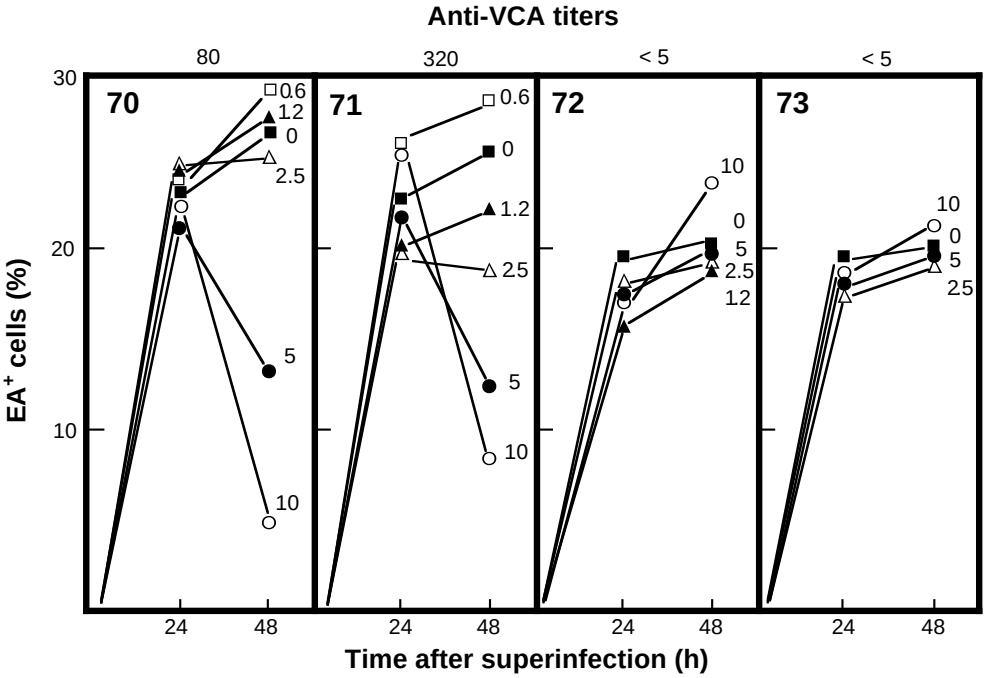

Fig. 1. Effect of anti-Epstein-Barr virus (EBV)-positive (Nos. 70 and 71) or -negative (Nos. 72 and 73) human sera on early antigen (EA) expression. EBV-superinfected Raji cells were cultured in medium containing 5% fetal calf serum and different concentrations (0, 0.6, 1.2, 2.5, 5 or 10%) of human serum. Percentages of EA^+ cells were determined 24 and 48 h after superinfection.

53, for example, high levels of EA suppression were observed at 48 h, but cell viability in 10% serum was 90% (data not shown). Cell viability in FCS or EBV antibody-negative serum was 94 and 92%, respectively. These data were consistent with the view that EA suppression was induced by an antibody(ies) to EBV-induced determinants. In order to test this EBV antibody-specificity further we analyzed the relationship between antibody titers to EBV antigens and effect in suppressing EA induction.

Relationship between EA suppression and antibody titers to EBV antigens

Normal adults' sera with high anti-VCA titers (1:320 or 1:160; Nos. 59, 53 and 63), with moderate anti-VCA titers (1:80; Nos. 58 and 64) and without detected VCA titers (<1:5; Nos. 60 and 61) were tested for effectiveness in suppressing EA at 48 psi h (Fig. 2). Effectiveness in EA suppression was quantitated by EA-SI (presented in Materials and Methods). The EA-SI for each serum correlated, in general, with its anti-VCA antibody titer.

A general concordance exists between titers to VCA and MA in our tested sera (Table 1). However, EA suppression activity was not always parallel to the immunofluorescence titers of VCA or MA. The EA suppressing antibody titers at 0.5 EA-SI were 1:160 for serum No. 74 or 1:20 for sera Nos. 70 and 71, respectively (Fig. 3). Cord blood serum (No. 74), with a modest anti-VCA titer (1:160) had the highest titer of EA-SI (1:160). This serum also had a high anti-MA reactivity as reflected in: (i) ADCC antibody titer (1:10,000); (ii) EBV neutralizing antibody titer; (1:160) and (iii) strong immunoprecipitation reactivity to membrane-associated, EBV-induced antigens (data not shown).

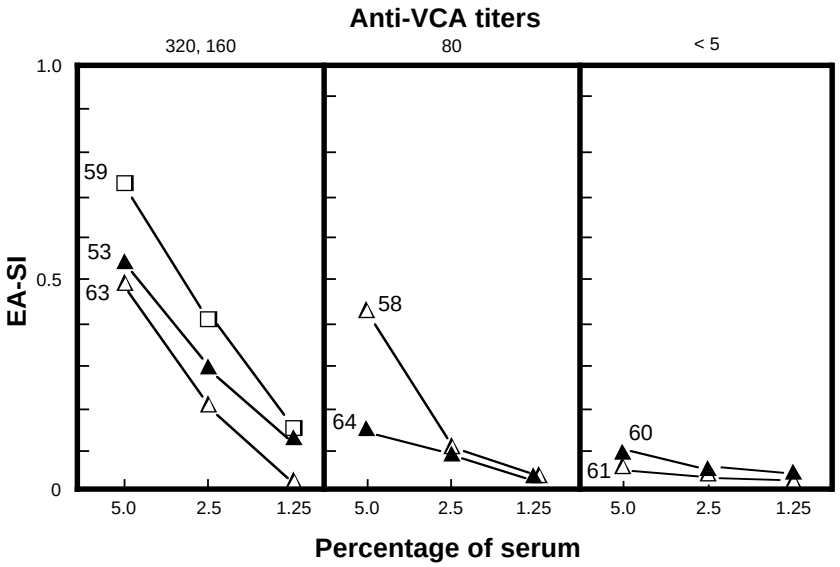

Fig. 2. Relationship of anti-viral capsid antigen (VCA) antibody titer to early antigen (EA) suppression. The EA suppression index (EA-SI) reflects the frequency of EA^+ cells in the cultures 48 h after superinfection.

Effect of PAA on EA suppression by anti-EBV antibody

Time course studies of EA suppression showed that the suppression was observed at 48 psi h but not at 24 psi h (Fig. 1). This result was consistent with the hypothesis that the EA suppression was dependent upon expression of a late gene function(s) of EBV. PAA inhibits viral-specific DNA polymerase and therefore, blocks expression of late EBV functions (Sairenji et al., 1978; Takagi et al., 1991; Tsurumi et al., 1994). If EA suppression is mediated by late viral function, it could be hypothesized that treatment of cells with PAA inhibits EA suppression. We tested varying doses of PAA on the EA suppressing activity of 2 sera (Nos. 53 and 63) (Fig. 4). The decrease in EA-SI was dependent upon PAA concentration. At a PAA concentration of 100 μg/mL, EA suppression was almost completely negated. No significant toxic effect of PAA (as judged with trypan blue dye exclusion) was observed on either virus infected or uninfected cells treated with any concentrations of PAA. These results are consistent with the view that suppression of intracellular EA expression may follow the modulation or alteration of a late EBV antigen(s) by an

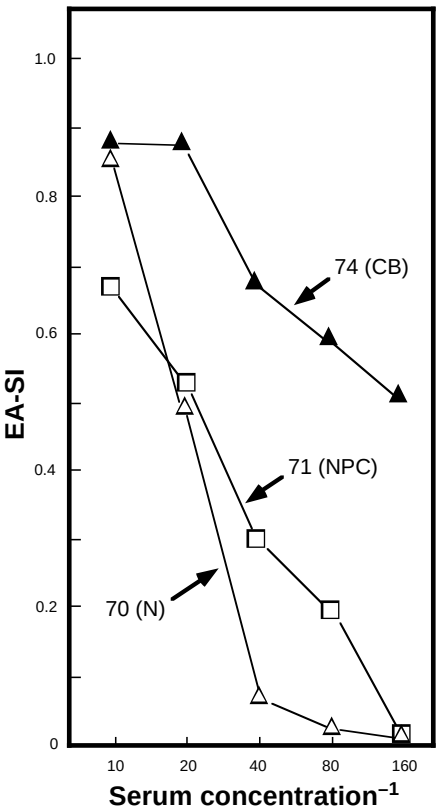

Fig. 3. Suppression index (SI) related to serum dilution. Cord blood serum (No. 74) with high anti-membrane antigen (MA) reactivity reveals a high EA-SI in comparison to nasopharyngeal carcinoma (NPC) serum (No. 71) and EBV antibody-positive serum from a normal person (No. 70).

anti-EBV antibody. Alternatively, it is also possible that a late viral function is required for antibody-mediated inhibition of EA expression.

Discussion

In order to understand one aspect of the immune response to EBV, we have tried to define the role of antibodies in affecting the course of antigen expression following EBV infection. In particular we have looked for modulation of EBV antigen expression by anti-EBV antibodies. EBV-determined MA are expressed on the cell surface of EBV-infected cells. The anti-MA antibody, like the anti-VCA antibody, is present in the sera of nearly all persons who have been infected with EBV or who have an EBV-associated malignancy. MA was originally demonstrated in BL biopsies (Klein et al., 1966). It seems likely that one of the important events leading to the growth of a BL tumor is the escape of the tumor clone from an already developed host immune response (Klein, 1978). Aoki and Old reported that antigenic modulation by the anti-MA antibody could occur in incubation of cultured BL cells in human serum containing BL antibodies to escape from such immune responses (Aoki et al., 1971). In our study we demonstrated that EBV release from EBV producer cell lines was inhibited by monoclonal antibodies against MA and we proposed it as a mechanism of immune defense for the control of EBV infection (Sairenji et al., 1985a, 1988, 1991).

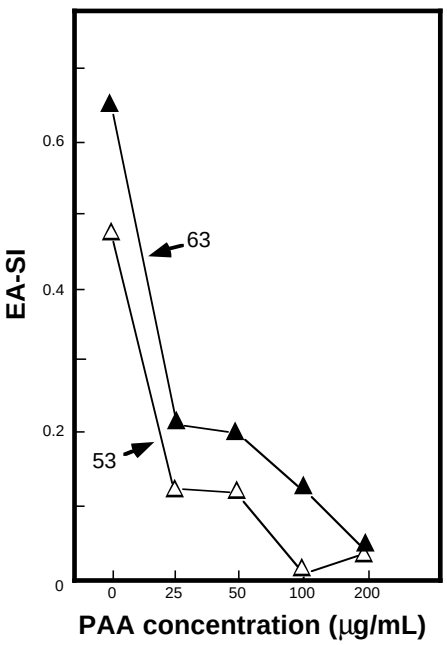

Fig. 4. Effect of phosphonoacetic acid (PAA) on early antigen (EA) suppression. Superinfected Raji cells were cultured in the medium containing 10% serum with EA suppressing activity (No. 53 or 63) and different concentrations of PAA. The EA suppression index (EA-SI) was determined 48 h after superinfection.

For this study human sera with low levels of natural cytotoxic antibodies to Raji cells were selected (Yang and Hewetson, 1974). Thus, we could observe alteration of EA antigen expression mediated by anti-EBV antibodies during the 48 h assay period following P3HR-1-EBV superinfection of Raji. We conclude that suppression of EA expression at 48 h in EBV-superinfected cells was, in fact, mediated by anti-EBV antibodies for the following reasons: first, suppression was observed in the cultures with only EBV antibody-positive sera but not with EBV antibody-negative sera (Figs. 1 and 2); second, suppression was inhibited with PAA treatment which inhibits EBV DNA polymerase (Fig. 4); and third, suppression was mediated by an immunoglobulin fraction of an EA-suppressing serum, separated by DEAE cellulose ion exchange chromatography (data not shown).

We do not know about the mechanism of EA supression in cells cultured with EBV antibody-positive serum. It may be hypothesized that an anti-MA antibody was responsible for the EA suppression. MA is a major antigen expressed on the cell surface of EBV-superinfected Raji cells to which EBV-infected patients generate a substantial antibody response (Casareale et al., 1983). MA is a target antigen(s) for ADCC (Pearson et al., 1978; Takaki et al., 1980) and for complement-dependent cytolysis (Strnad et al., 1982). It was not likely that anti-EA antibodies reacted directly with EA because EA is an intracellular antigen, and because most of the sera we tested

with the suppressing activity had negligible or undetectable levels of anti-EA antibodies. EBNA and VCA are also intracellular antigens, which would not react directly with the antibodies in culture fluids. However, there is no exact concordance between EA suppression and MA titers in some sera. For example, one cord blood serum (No. 74 with a moderate anti-MA titer of 1:160) had higher EA suppressing activity than the other sera (Nos. 70 and 71) with the same level of anti-MA titer (Fig. 3). On the other hand, serum from an NPC patient (No. 71) had a high anti-MA (1:640) titer but showed an EA suppression titer at the same level as did a normal adult's serum with an anti-MA titer at lower levels (1:80). For this discrepancy we noticed that No. 74 had a high ADCC titer (1:10,000) and a high neutralization titer (1:160), while No. 71 had a low neutralization titer (1:20) (data not shown). These data suggest that EA suppression was induced by anti-MA antibody to a specific MA component(s). Studies of anti-MA immunoprecipitation have shown that MA is actually complex with at least 3 distinguishable molecules for human anti-MA antibodies (Sairenji et al., 1988; Bertoni et al., 1989).

MA is a product of an EBV late gene after EBV DNA replication and the expression is inhibited with PAA (Sairenji et al., 1978). The EA suppression was completely inhibited by the addition of 100 μg/mL of PAA to the cell culture (Fig. 4). These results are consistent with the view that suppression of intracellular EA might follow modulation or alteration of MA by an anti-MA antibody or that a late viral function is required for antibody-mediated inhibition of EA expression in this system. In addition to this study we observed that cell culturing with EBV-seropositive sera reduced both production of infectious virus, amounts of virus DNA in the culture fluids and also intracellular EBV DNA in EBV producing P3HR-1 cells (Sairenji et al., 1991).

In conclusion, we can consider the potential significance of EA suppression to EA-positive cells by an anti-EBV antibody in human serum in vivo. EA suppression should be associated with inhibition of productive infection of EBV-infected cells and, thus, per se could constitute an immune defense against EBV.

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