Epstein-Barr Virus-Associated Antigens Activated in Human Cells by 5-Bromodeoxyuridine (36791)

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We have recently reported the activation of Epstein-Barr virus (EBV)-associated antigens in 3 human "virus-free" lymphoid cell lines following short-term treatment with 5-bromodeoxyuridine (BUDR) (1). These antigens could be detected by immunofluorescent tests with human sera containing EBV antibodies while EBV negative sera failed to react. During the 7–10 day period of treatment no EB virus particles were found in these cells which suggests that the immunofluorescence reaction involves noncapsid viral components.

In the present report evidence is presented to indicate that BUDR-activated, immunofluorescent antigens may be similar to or identical with the "early antigens" of EBV described by Henle *et al.* (2).

Materials and Methods. Cell cultures. Raji cells derived from a Burkitt tumor (3) and NC_{37} cells which originated from the peripheral blood leukocytes of a patient with pneumonia were cultured in McCoy's medium containing 20% fetal calf serum and 80 μg/ml of neomycin sulfate. Both of these cells lines are free of detectable EBV.

BUDR activation of EBV-associated antigens. Cultures of Raji or NC_{37} cells at a concentration of 10^6 cells/ml were treated with 10 μg/ml of BUDR in the presence of 0.6 μg/ml fluorodeoxyuridine (4). Treated cultures were protected from exposure to direct light to prevent cell damage (5). Half volumes of culture fluid were replaced with fresh medium and drug at 2–3 day intervals. Cell viability remained generally between 60–80% throughout the period of treatment. At 6–8 days about 8–12% of the treated cells showed bright immunofluorescence with selected human sera containing EBV antibodies. The BUDR-activated antigens in these cells are provisionally designated "early antigens" (EA).

Immunofluorescence tests (IF). The indirect method was used. Air-dried cell smears were fixed for 10 min in acetone or methanol at room temperature and stored at −20° until use. The cells were treated with sera diluted 1:10 for 1 hr at 37°. After thorough washing in saline solution the preparations were stained with fluorescein–isothiocyanate-labeled (FITC) goat anti-human IgG or IgM (Hyland Laboratories) diluted 1:8. After 1 hr at 37° the cells were washed in saline and mounted in Elvanol for ultraviolet microscopy.

Indirect membrane immunofluorescence tests were performed by treating viable cells with sera containing membrane reactive antibodies (6) diluted 1:5 for 30 min at 37°. After 3 saline washes the cells were stained with FITC-labeled goat anti-human IgG for 30 min, washed and mounted in glycerine–saline.

Complement-fixation tests (CF). BUDR-treated Raji or NC_{37} cells were pelleted and washed in Veronal-buffered saline (pH 7.4); 10% suspensions were frozen and thawed 3 times and disrupted by ultrasonic vibration. After centrifugation at 10,000 rpm for 15 min the supernatant fluid was titrated by the microtiter test for EBV-associated soluble (S) antigens with a known positive human serum B76, reactive with both virion and soluble antigens of EBV (7). Two units of antigen were used and all appropriate controls were included in each test.

Preparation of Epstein-Barr virus. Cultures of a human lymphoid cell line (AV) chronically infected with EBV (8) were maintained

TABLE I. Reactivity of Anti-EBV Positive Human Sera with BUDR-Activated Early Antigens of EBV.

Source of sera	Age	Geographic origin	No. tested	Sera reactive with early antigens[a]			
				Acetone-fixed		Methanol-fixed	
				No.	%	No.	%
Burkitt lymphoma	5–13	Uganda	62	60	97	21	34
Normal controls	5–14	Uganda	27	2	7.4	0	0
Nasophar. carcinoma	35–55	Hong Kong	22	20	90	17	72
Normal controls	30–58	Hong Kong	20	8	40	1	5
Infectious mononucleosis	18–25	U.S.	45	29	64	18	40
Normal children	1–16	U.S.	63	23	36	0	0
Normal adults	18–65	U.S.	51	20	39	4	8

[a] Positive at 1:10 serum dilution.

at 33° for 7–10 days in medium 1640 and 10% fetal calf serum. The cell-free culture fluids were filtered through a Millipore membrane of 0.45 μ porosity and were then centrifuged at 20,000 rpm for 2 hr. The virus-containing pellets were resuspended in 1/250 of the original volume of medium and stored at —70°. Infectively titrations were performed by inoculating NC_{37} cells with 10-fold dilutions of virus. After 7 days of incubation cell smears were examined for presence of EBV by immunofluorescence tests. The infectivity titer of virus concentrates was $10^{-3.5}$/0.2 ml.

Sera. Human sera were obtained from normal children and adults and from patients with infectious mononucleosis (IM) in this country. Serum samples from patients with Burkitt lymphoma (BL) in Uganda most of which were in long-term remission and from age and sex matched controls from the same region were obtained through the courtesy of Dr. Robert DePue of the Special Virus Cancer Program, National Cancer Institute. Sera from patients with nasopharyngeal carcinoma (NPC) in Hong Kong and from matched controls were kindly made available by Dr. Guy de Thé of the International Agency for Research on Cancer, Lyon. Chimpanzee sera were obtained from several primate research centers. All sera were stored at —20°.

Results. Reactivity of human sera with EA. All sera listed in Tables I and II contained CF antibody titers against EBV of 1:8 or greater. Since EBV antibody-negative sera failed to react with BUDR-activated, EBV-associated EA they were not included in these studies. The results refer to IgG antibody responses; none of the sera tested demonstrated IgM antibody reactivity with EA. As shown in Table I, 97% of 62 BL patients had antibodies reactive with acetone-fixed EA and only 34% reacted with methanol-fixed antigens. This is in sharp contrast to the low incidence of reactivity of matched controls, 7.4 and 0%, respectively. Patients with NPC showed a high frequency of serum reactivity with antigens fixed by both acetone (90%) and methanol (72%). During the acute stage or early convalescence of IM 64 and 40% of the patients' sera, respectively, reacted with acetone or methanol-fixed EA preparations. By contrast, only 36–40% of normal adults and children in Hong Kong or in this country had antibodies to EA (acetone-fixed) and only an occasional serum reacted with methanol-fixed antigens.

Reactivity of sera from subhuman primates. In view of our previous findings of a high incidence of antibodies reactive with EBV in several species of subhuman primates (8) it was of interest to determine the presence of EA antibodies in the sera of some of these animals. Results summarized in Ta-

TABLE II. Reactivity of Anti-EBV Positive Subhuman Primate Sera with BUDR-Activated Early Antigens of EBV.

Species	No. tested	Reactivity with early antigens[a]			
		Acetone-fixed		Methanol-fixed	
		No.	%	No.	%
Chimpanzee	40	32	80	3	7.5
Rhesus	20	10	50	0	0
Cynomolgus	10	4	40	0	0

[a] Positive at 1:10 serum dilution.

ble II indicate that the sera of 80% of 40 chimpanzees, 50% of rhesus and 40% of cynomolgus monkeys contained EA-reactive antibodies. Only 3 chimpanzee sera and none of the monkey sera reacted with methanol-fixed antigens. The EA reactivity of human and subhuman primate sera was identical in both BUDR-activated cell lines.

Persistence of EA reactive antibodies in man. It has been reported by other workers (9) that EA antibodies reached a peak titer during acute illness in IM patients and disappeared usually within a few months. It was also stated (2) that these antibodies were rarely found in sera of normal persons who possess anti-EBV antibodies. In view of our findings (Table I) that approximately 1/3 of normal, EBV seropositive individuals had detectable EA antibodies, the question arose whether this is a response to recurrent infection or due to persistence of antibodies. We tested 31 paired sera collected from normal adults 10 yr apart. As shown in Table III, 10 sera (32%) obtained in 1957 reacted with EA and 9 of these retained these antibodies for 10 yr. None of the sera reacted with methanol-fixed antigens.

Specificity of EA reactivity. In order to demonstrate that BUDR-activated cells contain early antigens and no viral capsid antigens (VCA) serum absorption experiments were carried out. Serum B76 containing both VCA and EA reactive antibodies was absorbed 2 times for 24 hr with 5 vol of concentrated EBV. The results, summarized in Table IV, indicated that these absorptions resulted in a marked reduction of the VCA titer from 640 to 80 but had no detectable effect on the EA titer.

TABLE III. Persistence of Antibodies Reactive with BUDR-Activated EBV-Associated Antigens in 31 Paired Human Sera.

Time of bleeding	Positive[a]		Negative	
	No.	%	No.	%
1957	10/31	32	21/31	68
1967	9/31	29	22/31	71

[a] Positive at 1:10 serum dilution.

TABLE IV. Effect of Serum Absorption with Epstein-Barr Virus.

Serum B76	Antibody titers to:	
	VCA	EA
Unabsorbed	640	160
Absorbed	80	160

Comparative studies of EA and S antigens. Both Raji and NC_{37} cells contain relatively small amounts of complement-fixing, EBV-related S antigens (7, 10, 11). Following BUDR treatment we observed an 8-fold increase in S antigen titer (1). It was of interest to determine if there is an immunologic relation between EA and S antigens. This was carried out by comparing the reactivity of sera with the respective antigens and by serum absorption studies. Results of preliminary studies summarized in Table V indicate that among normal adults and chimpanzees the anti-S reactivity of sera showed no correlation with the anti-EA response. In the case of BL and NPC patients where both anti-EA and S reactivity were present in 90–100% of the sera, the correlation was difficult to evaluate.

TABLE V. Reactivity of Sera with BUDR-Activated EA and S Antigens Associated with EBV.

Source of sera	No. tested	No. of sera with indicated reactivity			
		S+[a] EA+[a]	S+ EA−	S− EA+	S− EA−
Normal adults	42	11	21	4	6
Chimpanzees	41	14	1	21	5
Infectious mononucleosis	24	12	6	3	3
Burkitt lymphoma	20	15	4	0	1
Nasophar. carcinoma	20	18	2	0	0

[a] Positive at 1:8 serum dilution.

The immunologic difference of EA and S antigens is also supported by the results obtained by serum absorption tests. Ten milliliter aliquots of S antigen preparations containing 320–640 CF units/ml were lyophilized. The antigen titer remained unchanged following lyophilization. EA-positive human or chimpanzee sera were absorbed 2 times for 18 hr at 4°. Absorptions with S antigens failed to reduce or remove EA reactivity.

Absence of EBV-related membrane antigens following BUDR activation. Our earlier immunofluorescence studies of BUDR-activated antigens focused on the intracellular antigens of fixed cells. Gergely, Klein and Ernberg (12) claimed that EBV-related cell membrane antigens (MA) appeared as an early product of the viral genome in EBV-infected Raji cells. It was therefore of interest to determine the appearance of MA in the course of BUDR activation of NC_{37} or Raji cells. Viable cells were treated with MA-positive human sera at 7, 10 and 14 days of treatment and examined by indirect immunofluorescence tests. None of several hundred cells examined were MA positive.

Discussion. Intracellular IF antigens activated by BUDR following short-term treatment of NC_{37} or Raji cells appear to be similar to or identical with the early antigens in abortively infected Raji cells (2). This conclusion is based on the following evidence: (a) BUDR-activated cells after 7–10 days of drug treatment contained no detectable virus particles (1); (b) absorption of a VCA and EA-reactive serum with concentrated EBV had no effect on the EA titer but significantly reduced the VCA titer of the serum; (c) in agreement with the findings of Henle *et al.* (9, 13) patients with IM, BL or NPC had the highest frequency of EA antibodies; and (d) BUDR-activated EA consists of at least 2 components: one is methanol-resistant and is analogous to the D (diffuse staining) antigen reported by Henle, Henle and Klein (14), the other is denatured by methanol like the so-called R (restricted staining) antigen (14).

It should be noted that EBV particles were found by Hampar *et al.* (15) in BUDR-resistant Raji cells following prolonged treatment. The appearance of EA in BUDR-activated cells represents an early function of the viral genome; no EBV-related membrane antigens were detected on the cell surface. This is in contrast to the data reported by Gergely, Klein and Ernberg (12) describing the appearance of MA as an early product of the viral genome.

No attempt was made in this study to compare EA antibody titers of healthy persons with patients with EBV-associated illnesses. Our main objective was to determine the incidence of these antibodies in sera of human and subhuman primates. EA antibodies were detectable in about 1/3 of sera from healthy adults and children in this country but only 2/27 normal African children possessed these antibodies. These IF reactions were mainly of the R type since they were generally absent in methanol-fixed preparations. EA antibodies were of the IgG type and persisted for 10 yr or more; no EA-reactive IgM antibodies were found. These findings suggest that the anti-EA immune response may not be transitory or due

to a recent EBV infection as reported earlier (9). Antibodies against the R component of EA were present in 1/3 of sera of normal adults and in 80% of chimpanzee sera. By contrast, D-reactive antibodies were absent or rare in these sera but were found frequently in sera of patients with IM, BL and NPC. Early antigens appear to be immunologically unrelated to S antigens. The significance of the differential EA antibody reactivities (R and D) in relation to diagnosis or prognosis in EBV-associated illnesses remains to be determined.

BUDR activation in EA synthesis offers a simple, reproducible and convenient method for obtaining EA-positive cells. There are 2 major advantages compared to the published procedure employing abortive infection of Raji cells (2): (a) it eliminates the need for infectious EBV, which is difficult to prepare consistently, and (b) it yields EA-positive cells free of viral capsid antigen.

Summary. Activation of the Epstein-Barr virus (EBV) genome by 5-bromodeoxyuridine (BUDR) in "virus-free" Raji or NC_{37} cells resulted in the synthesis of early viral antigens (EA) detectable by immunofluorescence. A high proportion of sera (65–97%) from patients with infectious mononucleosis, Burkitt lymphoma and nasopharyngeal carcinoma reacted with these antigens while only 1/3 of the sera from healthy persons were reactive. Forty to 80% of sera from subhuman primates also contained anti-EA antibodies. The significance of these antibodies in man in relation to EBV infection or associated illnesses remains obscure. EA appeared to be immunologically distinct from EBV-related soluble complement-fixing antigens. BUDR activation of Raji or NC_{37} cells offers a simple and convenient method of obtaining EA preparations free of viral capsid antigens.

1. Gerber, P., Proc. Nat. Acad. Sci. U.S.A. **69**, 83 (1972).

2. Henle, W., Henle, G., Zajac, B. A., Waubke, R., and Scriba, M., Science **169**, 188 (1970).

3. Pulvertaft, R. J. V., J. Clin. Pathol. **18**, 261 (1965).

4. Erikson, R. L., and Szybalski, W., Cancer Res. **23**, 122 (1963).

5. Puck, T. T., and Kao, F., Proc. Nat. Acad. Sci. U.S.A. **58**, 1227 (1967).

6. Gerber, P., and Goldstein, G., J. Immunol. **105**, 793 (1970).

7. Gerber, P., and Deal, D. R., Proc. Soc. Exp. Biol. Med. **134**, 748 (1970).

8. Gerber, P., Whang-Peng, J., and Monroe, J. H., Proc. Nat. Acad. Sci. U.S.A. **63**, 740 (1969).

9. Henle, W., Henle, G., Niederman, J. C., Klemola, E., and Haltia, K., J. Infec. Dis. **124**, 58 (1971).

10. Pope, J. H., Horne, M. K., and Wetters, E. J., Nature (London) **222**, 186 (1969).

11. Vonka, V., Benyesh-Melnick, M., and McCombs, R. M., J. Nat. Cancer Inst. **44**, 865 (1970).

12. Gergely, L., Klein, G., and Ernberg, I., Virology **45**, 10 (1971).

13. Henle, G., Henle, W., Klein, G., Gunven, P. C., Morrow, R. H., and Ziegler, J. L., J. Nat. Cancer Inst. **46**, 861 (1971).

14. Henle, G., Henle, W., and Klein, G., Int. J. Cancer **8**, 272 (1971).

15. Hampar, B., Derge, J. G., Martos, L. M., and Walker, J. L., Proc. Nat. Acad. Sci. U.S.A. **69**, 78 (1972).

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