Epstein-Barr Virus-Induced Viral and Soluble Complement-Fixing Antigens in Burkitt Lymphoma Cell Cultures (34875)

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A partially purified viral particle antigen extracted from cultured Burkitt lymphoma cells has been used in our laboratory for the detection of complement-fixing (CF) antibodies to a herpes-like virus designated Epstein-Barr Virus or EBV (1–3). The results of a comparative serological study indicated excellent agreement between the CF, immunofluorescence and antibody-coating test in the detection of EBV antibodies (4).

Since several members of the herpes group (herpes simplex, pseudorabies, and cytamegalo viruses) elicit the production of both viral and nonsedimentable "soluble" antigens in cultures of infected cells (5–7) it was of interest to look for EBV-induced soluble antigens and to study their antigenic and physical properties. This report presents evidence for the existence of EBV-induced soluble antigens in culture fluids of EBV harboring and EBV-free lymphoid cell lines.

Materials and methods. Cell cultures. The following lymphoblastoid cell lines were grown in McCoy 5A medium (8) supplemented with 20% heat-inactivated fetal calf serum and 80 μg/ml of streptomycin sulfate: The P3J (HR1K) Burkitt tumor cell line (9) which contains variable amounts of EBV and the Raji Burkitt tumor cell line (10) which contains no detectable EBV.

Preparation of antigens. When the cultures reached a cell density of 1.5–2.0 $\times$ 10^6/ml, the cells were pelleted, washed in serum-free medium, and 300–400 $\times$ 10^6 cells were seeded in serum-free medium in 32 oz bottles which were incubated horizontally at 33°. After 10 days' incubation, the cells were sedimented at 2500 rpm for 15 min and washed in veronal-buffered saline (VBS). The cells were suspended at a 10% concentration in VBS with 0.005 *M* sodium azide, disrupted by sonication for 2 min, and fractionated by sucrose-gradient centrifugation at 30,000 rpm for 1 hr. The virus-rich fractions at a density of 1.19–1.21 g/cm^3 were used for viral (V) antigen (11) and the corresponding fractions of virus-free Raji cell extracts were designated Raji cellular control antigen.

The culture fluids were filtered through a 0.45 μ Millipore membrane and centrifuged at 30,000 rpm for 2 hr. The virus-containing pellets obtained from P3J culture fluids were resuspended in growth medium in 1/100 the original volume and used for infectivity and transformation studies ((12). The supernatant fluids were concentrated 30–50 times by Diaflo ultrafiltration using a UM 10 membrane. These preparations constitute the soluble (S) antigens.

Complement-fixation (CF) test. The microtiter technique (13) was employed using 1.8 units of guinea pig complement. Two per cent suspensions of sheep red blood cells were standardized spectrophotometrically. Sera were heat-inactivated and tested at the lowest dilution of 1:4. A known EBV positive and negative control serum was included in all tests. Two units of antigen and 4–8 units of antibody were used for titrations of antibody or antigen, respectively.

Immunofluorescence (IF) Tests. For the detection of intracellular antigens cell smears were air dried, fixed in acetone, and stained by indirect IF technique using a goat anti-human IgG fluorescein-labeled serum. The indirect membrane immunofluorescence test (MIF) was carried out with unfixed, viable cells according to the method described by Klein *et al.* (14). Appropriate positive and negative cell and serum controls were included in all tests.

Results. Properties of antigens. Prepara-

TABLE I. CF Antibodies in Human Sera to Viral and Soluble Antigens of EB Virus.

Source of sera	Diagnosis	Age range (years)	No. sera tested	Viral antigen			P3J-soluble antigen		
				Pos. sera	Titer range	GMT[a]	Pos. sera	Titer range	GMT[a]
U. S.	Healthy adults	25–45	40	32	16–256	48	22	4–32	8
	Healthy children	3–12	30	16	16–128	33	13	4–16	6
	Infectious mononucleosis	18–23	10	10	32–512	152	9	4–32	13
Africa	Burkitt lymphoma	3–12	10	10	128–8192	980	9	4–1024	94
	Normal controls	3–14	15	14	32–1024	180	13	4–512	32

[a] Geometric mean titer.

tions of viral (V) and soluble (P3J-S) antigens derived from P3J cultures contained generally 16–32 and 8–16 CF units/0.025 ml, respectively. Raji soluble antigens (Raji-S) contained 2–4 CF units/0.025 ml and Raji cell extract (Raji cell control antigen) had 32 CF units/0.025 ml reactive only with a guinea pig anti-Raji cell serum.

All antigens were stable over a pH range of 6.0–8.0 and could be stored for 6 months or longer at 4° in the presence of 0.005 *M* sodium azide without loss of antigenic activity. Antigenicity was not affected by lyophilization, heating to 56° for 30 min, or by ether treatment (20% for 30 min). Antigenic activity was, however, destroyed by treatment with 0.05% trypsin (Difco 200) at 37° for 2 hr. The antigens can be precipitated by 30% $(NH_4)_2So_4$ with 100% recovery of activity.

This affords a convenient method for concentration and preliminary purification. Results of preliminary experiments with Sephadex G-200 gel filtration indicated that the V antigen was excluded and found in the void volume while the S antigen appeared in the elution volumes corresponding to a molecular weight range of 65,000–150,000.

Studies are in progress to determine the degree of purity of these fractions.

Reactivity of human and subhuman primate sera. A total of 105 human sera have been tested and the results are summarized in Table I. None of the sera reacted with Raji cellular control antigen. The incidence of V antibodies is similar to earlier observations (1). About 70–90% of sera containing V antibodies reacted with P3J-S antigen while none of the V-negative sera was reactive. Antibody titers to P3J-S antigen were consistently about 4- to 8-fold lower than the corresponding V antibody levels. Among the relatively small number of sera of Burkitt lymphoma patients tested there was no detectable pattern of S antibody response in relation to state of disease.

It was also found (not seen in this table) that about 20–50% of P3J-V-positive sera reacted with Raji-S antigen at titers of 4–16. None of the V-negative sera were reactive.

In order to assess the specificity and significance of the reactivity with Raji-S antigen we retested V-negative and V-positive sera, and the results are summarized in Table II. None of 31 V-negative human and subhuman primate sera reacted with either S antigens or Raji cell extract antigen (gradient fractions densities 1.19–1.21). The incidence of Raji-S antibodies among V-positive normal human and subhuman primate sera ranged from 15–33% and was 50% among infectious mononucleosis and Burkitt lymphoma patients. Results of preliminary experiments indicate the presence of soluble antigens similar to Raji-S antigens in EB virus-free lymphoid cell lines NHDL-3 (15) and NC 37 (16) both of which were derived from normal human donors.

Duration of P3J-S antibodies. In order to obtain some information on the persistence of P3J-S antibodies paired sera of eight normal V-positive subjects collected 10 years apart (1957–1967) were examined. Antibody titers to V antigen ranged from 16–256 and to P3J-S antigen from 4–32. The reactivity to

TABLE II. CF Reactivity of Human and Subhuman Primate Sera with Antigens Derived from P_3J and Raji Cell Cultures.

Sera	Source	No. sera tested	Number of sera reactive with antigens			
			P_3J		Raji	
			Viral	Soluble	Control[a]	Soluble
Human	Healthy adults	15	0	0	0	0
		20	20	10	0	3
	Healthy children	10	0	0	0	0
		15	15	13	0	5
	Infectious mononucleosis	10	10	9	0	5
	Burkitt lymphoma	10	10	9	0	5
Subhuman Primates	Chimpanzee	6	0	0	0	0
	Cynomologous African green	15	15	13	0	3

[a] Gradient fractions of densities 1.19–1.21.

both antigens remained generally unchanged in paired sera indicating the persistence of both types of antibodies for long periods. Only one of the 1967 sera reacted with Raji-S antigen at 1:4 dilution. However, the early (1957) serum of this donor was anticomplementary at a 1:8 dilution.

Immunofluorescence tests. Acetone-fixed preparations of Raji, NHDL-3, and NC-37 cells were consistently negative in indirect IF tests with V-positive sera containing CF antibodies to P3J-S and Raji-S antigens. Negative results were obtained when viable cells of these three cell lines were incubated with the same sera in the indirect MIF test.

Discussion. In the present study we demonstrated soluble CF antigens in cell-free concentrated culture fluids of EBV-infected P3J and virus-free Raji cell cultures of Burkitt lymphomas. Armstrong *et al.* (17) and more recently Pope *et al.* (18) prepared crude antigens extracted from cultured Burkitt lymphoma and other lymphoid cells and found that some of the CF activity remained nonsedimentable after high-speed centrifugation for 1–2 hr. However, the antigens prepared by Armstrong *et al.* were relatively unstable on storage at 4°, and their activity was abolished by heating at 56° for 30 min. Both the V and S antigens described in this report maintained their activity on prolonged storage at 4° in the presence of 0.005 *M* sodium azide. Heating at 56° for 30 min reduced or destroyed the infectivity associated with the viral antigen (19) without affecting the antigenicity of V and S antigens. Antigenic activity was preserved after lyophilization, which offers a convenient method for storage and concentration of antigens and for serum-absorption experiments. Preliminary results indicate that V and S antigens are separable by Sephadex gel filtration, the latter appearing in fractions corresponding to a molecular weight range of 65,000–150,000.

Three lines of evidence suggest that the serum reactions with S-antigens are probably not attributable to isoantibodies:

1. V-positive subjects with S antibodies had to our knowledge no previous multiple transfusions, pregnancies, or certain allergic or hematologic disorders.

2. None of the V-negative human and subhuman primate sera reacted with S antigens.

3. Thirteen of fifteen V-positive chimpanzee and monkey sera reacted with P3J-S and 3/15 with Raji-S antigens.

These findings suggest that S antibodies are related to EBV infection in man and in subhuman primates to infection with a closely related virus. The incidence of human an-

tibodies to S antigens was highest among pahuman primate sera containing antibodies mononucleosis. The significance of this observation remains to be determined. Among the relatively small number of Burkitt lymphoma sera tested there was no detectable pattern of S antibody response in relation to clinical stages of the disease. Studies are in progress to examine this aspect in more patients. The immunologic relationship of V and S antigens is at present unknown. Preliminary results indicate that absorption of sera with V antigen resulted in an 8-fold reduction of both V and S antibody titers and serum-blocking tests wtih S antigen caused a reduction in the serum-neutralizing titer. However, this data must be interpreted with caution since relatively crude preparations of antigens were employed.

The origin and biologic significance of the S antigens is at present obscure. They seem to be related to EBV infection since only V positive sera were reactive. The presence of these antigens in virus-free lymphoid cell lines suggest the persistence of at least a part of the viral genome in these cells. However, there is no direct evidence available to indicate that S antigens are coded for by the viral genome. Repeated attempts during the past 2 years to rescue the viral genome in Raji cells have been unsuccessful (19). The proposed similarity (18) between the S antigens and the tumor antigens present in cells transformed by papova or adenoviruses is not supported by the available evidence:

1. There was no detectable pattern of S antibody response in Burkitt lymphoma patients in relation to tumor regression and relapse, and

2. No intracellular antigens were detectable in Raji cells by IF tests with S antibody.

Critical studies on the relationship of EBV and S antigens are at present limited by the lack of suitable *in vitro* techniques.

Summary. Epstein-Barr virus-infected Burkitt lymphoma cell cultures contain viral (V) and virus-induced, nonsedimentable, soluble (S) antigens which react in complement-fixation tests with certain human and subhuman primate sera containing antibodies to the V antigen. An apparently related soluble antigen was demonstrated in concentrated cell-free fluids of virus-free Raji cell cultures. Serologic evidence is presented to relate S antigens to EBV infections in man and subhuman primates. The release of S antigen by virus-free Raji and other lymphoid cell lines suggests the possible presence of a portion of the viral genome in some of these cells. Data on the physical properties of V and S antigens are presented.

1. Gerber, P., and Birch, S. M., Proc. Nat. Acad. Sci. U.S.A. **58**, 478 (1967).

2. Gerber, P., and Rosenblum, E. N., Proc. Soc. Exp. Biol. Med. **128**, 541 (1968).

3. Gerber, P., Hamre, D., Moy, R. A., and Rosenblum, E. N., Science **161**, 173 (1968).

4. Report from the Special Virus-Cancer Program, J. Nat. Cancer Inst. **42**, 623 (1969).

5. Hayward, M. L., Brit. J. Exp. Pathol. **30**, 520 (1949).

6. Hamada, C., and Kaplan, A. S., J. Bacteriol. **89**, 1328 (1965).

7. Benyesh-Melnick, M., Vonka, V., Probstmeyer, F., and Wimberly, I., J. Immunol. **96**, 261 (1966).

8. McCoy, T. A., Maxwell, M., and Kruse, P. F., Proc. Soc. Exp. Biol. Med. **100**, 115 (1959).

9. Hinuma, Y., and Grace, J. T., Proc. Soc. Exp. Biol. Med. **124**, 107 (1967).

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

11. Toplin, I., and Schidlovsky, G., Science **152**, 1084 (1966).

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

13. Sever, J. L., J. Immunol. **88**, 320 (1962).

14. Klein, G., Clifford, P., Klein, E., and Stjernsward, J., Proc. Nat. Acad. Sci. U.S.A. **55**, 1628 (1966).

15. Gerber, P., and Monroe, J. H., J. Nat. Cancer Inst. **40**, 855 (1968).

16. Jensen, E. M., unpublished.

17. Armstrong, D., Henle, G., and Henle, W., J. Bacteriol. **91**, 1257 (1966).

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

19. Gerber, P., unpublished observations.

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