PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 166, 522–527 (1981)

Depression of Mitogen-Induced Lymphocyte Blastogenesis by Baboon Endogenous Retrovirus-Associated Components[1] (41101)

OWEN S. WEISLOW,* OSCAR U. FISHER, JR.,* DANIEL R. TWARDZIK,† ALFRED HELLMAN,† AND ARNOLD K. FOWLER†

NIH Intramural Research Support Program and †Carcinogenesis Intramural Program, National Cancer Institute, Frederick Cancer Research Center, Frederick, Maryland 21701

Abstract. Freeze/thaw (F/T) extracts of gradient purified concentrates of baboon endogenous retrovirus (BEV) depress phytohemagglutinin (PHA)-induced blastogenic transformation of human, baboon, and mouse lymphocytes. Extracts of normal fetal dog thymus cells (vehicle for virus replication), pelleted virus cores, spent tissue culture medium, and virus suspension buffers were not depressive. Sephacryl S-200 chromatography of BEV F/T extracts yielded two peaks of suppressive activity; peak A, eluted in the region of relatively high protein concentration with the 45,000-dalton ovalbumin marker, was highly labile, but not cytotoxic. Peak B, eluted between cytochrome *c* and DNP alanine markers, contained no detectable protein and appeared to consist of two components; one cytotoxic and highly labile and a second, noncytotoxic and relatively stable. A polyvalent antiserum to BEV proteins precipitated components of the BEV F/T extract and peaks A and B, but failed to abrogate immunodepression.

Immunodepression is a classic manifestation of leukemia virus infection in the mouse (1). Although many factors may be responsible for this nonspecific immunological unresponsiveness, structural components of leukemia viruses or other products of virus-infected leukemic cells might contribute to this phenomenon (2). Evidence of viral-component-induced immunodepression was recently obtained in studies of murine (3) and feline (4) retroviruses. Although proteins related to type C viruses of murine or primate origin have been demonstrated in human tissues (5, 6) and primate-related retroviruses reportedly have been isolated from human leukemic cells (7–10), there exist no published reports of primate retrovirus component-induced immunodepression in human or nonhuman primate systems. In the present communication, we present evidence of depressed PHA-induced lymphocyte blastogenic transformation by components associated with the baboon endogenous type C retrovirus, note the interspecies cross-reactivity of this depression for human, baboon, and mouse cells, and begin the purification and characterization of the responsible viral-associated elements.

Materials and Methods. *Animals.* BALB/c female mice, 8–10 weeks old, were employed where murine lymphoid cells were required. All mice were supplied by the Frederick Cancer Research Center's animal production unit. Mice were housed in plastic suspension cages measuring 45.5 × 24 × 20.50 cm and covered with filter tops, 10 mice per cage. Bedding of hardwood shavings was changed weekly. Animals were provided a commercial diet (Lab Blox) *ad libitum;* water was constantly available. Animal rooms were constantly monitored for changes of temperature and humidity and the photoperiod was automatically controlled. Animals were routinely monitored and found to be free of any organisms which could interfere with this study.

Cell preparation and cultures. Heparinized baboon (*Papio cynocephalous*) peripheral bloods were obtained from the breeding colony at the Southwest Foundation for Research and Education, San Antonio, Texas, with the kind assistance

[1] The U.S. Government's right to retain a nonexclusive royalty-free license in and to the copyright covering this paper, for governmental purposes, is acknowledged.

of Dr. R. Heberling. Human peripheral blood was obtained from volunteers on the Frederick Cancer Research Center staff. Ficoll-purified peripheral lymphocytes (2×10^5) from human or baboon and splenic lymphocytes from BALB/c mice were cultured in 200 μl of RPMI 1640 containing 5% heat-inactivated fetal calf serum. BEV F/T extract viral cores or supernates, prepared in sterile distilled water as indicated below, were diluted in RPMI 1640 and added in 25-μl aliquots. After 1 hr at 37°, PHA-P (Difco; 6.25 μg for human and baboon or 1.5 μg for mouse) in 25 μl RMPI 1640 was added to appropriate cultures. Sterile distilled water, 25 μl, or 5 μg of BEV F/T viral cores served as negative extract controls. All cultures were reincubated, human cells for 3 days, baboon cells for 5 days, and mouse cells for 2 days to allow for peak PHA stimulation which was monitored by incorporation of [^{3}H]thymidine (1 μCi/culture). After an appropriate period of exposure to [^{3}H]thymidine (4–6 hr for mouse or 18 hr for human and baboon) all cultures were washed and collected on a multiple automated cell harvester and counted in a Searle liquid scintillation counter.

Viral extracts. BEV was concentrated 1000× from supernates of infected fetal canine thymus (FCf_2th) or human rhabdomysarcoma (A204) cell cultures by double banding on sucrose density gradients. After pelleting, the virions were resuspended in 1 ml of PBS or sterile distilled water, and disrupted by five cycles of freezing (15 min in alcohol–dry ice) and thawing (37° water bath) followed by centrifugation at 100,000*g* for 1 hr. The resulting F/T supernatants and pellets (virus cores) were frozen separately at −70°. Control preparations of uninfected fetal canine thymus cells or culture fluids were prepared two ways: (i) cell-free culture supernatant fluids were centrifuged at high speed (100,000*g*/1–2 hr) and the pelleted material was utilized in assay after freezing and thawing as were pelleted (400*g*/10 min) uninfected canine thymus cells. BEV-infected supernatants or cells prepared in this manner were always immunodepressive while control uninfected materials were never depressive; (ii) spent supernatant media were dialyzed against distilled water, concentrated by lyophilization, and tested after freezing and thawing. Again, only BEV-infected supernatant fluids were immunodepressive when prepared in this manner. Attempts to isolate a control product from culture supernatants of uninfected cells by sucrose density gradient techniques never yielded sufficient quantities of material for testing in our hands. The same culture medium was used to grow both infected and uninfected cells.

Partial purification and characterization of F/T extracts. To identify the suppressor factors in BEV F/T extract supernatants, 500 μl of each supernatant was subjected to gel filtration through Sephacryl S-200 (Pharmacia) overnight at 4°, in PBS diluted 1:20 without Ca^{2+} or Mg^{2+}. Fractions, 300 μl, were immediately examined for protein concentration using a colorimetric determination of bound Coomassie Brilliant blue-250 dye (11) and for immunodepressive potential by direct addition (25 μl) to lymphocyte cultures. Cytotoxicity of viral extracts and fractionated suppressor factors for lymphocytes were determined by erythrocin B dye exclusion at various intervals after the start of culture and at the time of lymphocyte harvest for [^{3}H]thymidine incorporation. Sensitivity of viral extract supernatants to proteases was determined by digestion with trypsin (Sigma T8003 type I 2.5 μg/ml) for 6 hr at 37° followed by centrifugation at 30,000 rpm for 1 hr to remove any potentially insoluble polypeptides generated by the protease treatment. Digested supernatants were then subjected to gel filtration chromatography on Sephadex G-100 (Pharmacia) and the resulting fractions were tested for immunosuppressive activity. Iodination of the viral extract supernatant and fractionated suppressor factors was accomplished with the chloramine-T method of Greenwood *et al.* (12). Sudan black B (13) was utilized for the identification of lipids. A multivalent antiserum prepared in goats to disrupted (F/T) preparations of BEV (anti-BEV) was utilized in an effort to block immunodepression by these factors. Two methods were used: (i) anti-BEV was added in different dilutions di-

TABLE I. INHIBITION OF PHA-INDUCED LYMPHOCYTE BLASTOGENESIS BY FREEZE–THAW EXTRACTS OF BEV

Lymphocyte treatment	Human	Baboon[a]	Mouse
Unstimulated	418 ± 61 —	307 ± 100 —	1,502 ± 230 —
PHA stimulated	27,222 ± 1816 (100)[b]	3639 ± 154 (100)	44,223 ± 2388 (100)
Distilled H_2O control + PHA	25,900 ± 1100 (95)	3505 ± 215 (96)	43,799 ± 4614 (99)
BEV F/T extract cores (5 μg)[c] + PHA	24,700 ± 2300 (91)	3327 ± 180 (91)	48,356 ± 3145 (109)
BEV F/T extract supernate[d] + PHA			
5 μg	3,544 ± 120 (13)	258 ± 123 (7)	4,033 ± 321 (9)
2.5 μg	7,571 ± 559 (28)	954 ± 96 (26)	11,031 ± 1533 (25)
1.25 μg	14,043 ± 1660 (51)	1994 ± 188 (54)	29,232 ± 1216 (66)
0.625 μg	17,678 ± 656 (65)	2320 ± 288 (64)	40,356 ± 2095 (91)
0.3125 μg	24,347 ± 3490 (89)	2990 ± 197 (82)	36,595 ± 1795 (83)

[a] Baboon peripheral blood donated by Dr. R. Heberling, Southwest Foundation for Research and Education, San Antonio, Tex.

[b] Mean of triplicate determinations ± 1 SD. Numbers in parentheses represent the percentage of the PHA-stimulated, positive control response remaining.

[c] BEV viral cores, obtained after F/T and 100,000*g* centrifugation for 1 hr, resuspended in sterile distilled water.

[d] Soluble BEV components obtained after F/T and 100,000*g* centrifugation for 1 hr and separation from pelleted viral cores.

rectly to the test cultures or (ii) anti-BEV was used to precipitate the various extracts directly. In the latter case, the precipitated material was removed by centrifugation and the remaining supernatant was tested for suppression.

Results. *Immunodepression by virus extract supernatants.* Freeze/thaw viral extracts were tested for suppressor activity as indicated under Materials and Methods. Table I summarizes the results of a representative experiment. Depression of the PHA response to 50% of control levels was obtained with as little as 1 μg of F/T virus extract supernatant per 2×10^5 human lymphocytes (first column). Addition of F/T supernatants up to 24–30 hr after PHA depressed the incorporation of [^{3}H]thymidine, while later additions had no effect (data not shown). Subsequent experimentation indicated that this depression did not result from a temporal shifting of peak PHA stimulation nor loss of cell viability (as determined by dye exclusion when cultures were terminated). Moreover, increasing the PHA concentration had little or no effect on suppression, suggesting that competition for PHA-binding sites was not a factor in depressing the response. Virus cores, obtained after the first cycle of 5 F/T and high-speed centrifugation (pellets), did not depress PHA stimulation when added to cultures in protein concentrations identical to the F/T supernates. Concentrated, spent supernatant media from uninfected fetal dog thymus cells (used to produce BEV), as well as concentrated dog thymus cell extracts, were also not suppressive when tested in concentrations identical to those employed with the F/T viral supernatants (data not shown).

To determine the species specificity of the suppressive component(s), twofold dilutions of F/T viral extracts were also tested for suppression of baboon (*Papio cynocephalus*) and mouse (BALB/c) lymphocyte PHA stimulation. These results are shown in columns 2 and 3 of Table I. BEV F/T extract supernatants were equally suppressive in all of the three species tested. Subsequent experiments have confirmed these findings and support earlier observa-

tions that suppressor factors from both murine and feline retroviruses lack species specificity (14, 15).

Partial purification of immunodepressive factors. In an effort to identify the factor(s) responsible for suppression of lymphocyte stimulation, BEV F/T extract supernatants were subjected to gel filtration through Sephacryl S-200 in PBS. Each fraction (0.3 ml) was examined for protein concentration by the colorimetric determination of bound Coomassie Brilliant blue-250 dye (11) and for immunodepressive potential by direct addition (25 μl) to lymphocyte cultures subsequently stimulated by PHA. Protein and immunodepression profiles of a representative BEV F/T extract are illustrated in Fig. 1. Although molecular weight determinations are estimated in this separation system, two peaks of suppressive activity were obtained; one in the region of the 45,000-dalton marker, an area of relatively high protein concentration, (peak A), and a second (peak B), migrating in a region of apparently low molecular weight (between cytochrome *c* and DNP–alanine) and no detectable protein.

Characterization of immunodepressive factors. Our attempts to further characterize the factor(s) responsible for depression of PHA stimulation have been frustrated by the apparent lability and low concentration of the specific components involved. Table II briefly summarizes those characteristics of the freeze/thaw supernatant, peak A, and peak B that have been examined. All three of these fractions were immunodepressive. The F/T supernatant appeared to lose this activity when stored at 4° for 1 week or after 2–3 weeks at −70°. Lyophilized material also lost activity after storage at −70° for 2–3 weeks. Depression of PHA stimulation by F/T supernate did not result from the killing of effector cells and while this fraction reacted strongly with antiserum to Tween–ether disrupted BEV (anti-BEV), its immunosuppressive activities were not blocked. Although F/T supernatants contained 300 to 600 μg of protein/ml as determined by Coomassie blue, and iodinated efficiently with chloramine T (12), their ability to depress PHA stimulation was not influenced by pretreatment with trypsin.

Peak A, obtained from the gel filtration column (Fig. 1), had to be tested within 24 hr of its separation; lyophilization failed to extend this period of activity. Peak A also lost activity during subsequent purification attempts on Sephadex G-75 columns preventing further characterization. Like the F/T supernatant, peak A contained a substantial quantity of protein that iodinated well by the chloramine-T method and was

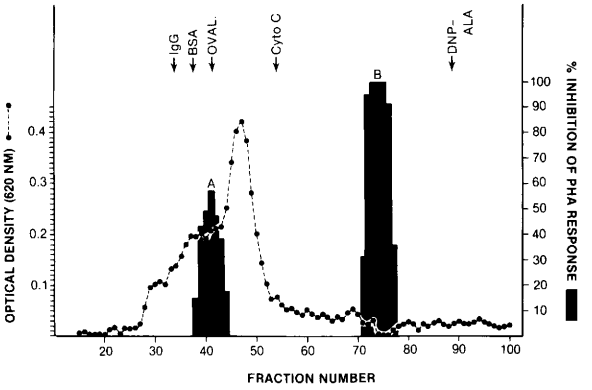

FIG. 1. Protein elution and mitogenesis suppression profile from Sephacryl 200 gel filtration column separation of BEV freeze–thaw extract supernatant. Five hundred microliters of BEV freeze–thaw extract supernatant prepared as indicated in the text collected over night at 4°, in PBS diluted 1:20 without Ca^{2+} or Mg^{2+}, in 300-μl fractions. Each fraction immediately examined for protein concentration using a colorimetric determination of bound Coomassie blue dye and for suppression of PHA response of human lymphocytes as indicated in the text.

TABLE II. CHARACTERISTICS OF FRACTIONATED BEV COMPONENTS

Characteristic	F/T extract supernatant	Peak A	Peak B
Immunodepressive[a]	Yes	Yes	Yes
Cytotoxic[a]	No	No	No[b]
Labile[a]	Yes	Yes	No
Reacts with antiserum to BEV[a]	Yes	Yes	Yes
Immunodepression Blocked by anti-BEV[a]	No	No	No
Reacts with Commassie Blue[a]	Yes	Yes	No
Iodinates with chloramine T[a]	Yes	Yes	No
Immunodepression sensitive to trypsin[c]	No	NT	No
Contains a sudanophilic component[a]	NT[d]	NT	Yes

[a] As described in the text.

[b] As indicated in the text, peak B contained noncytotoxic and cytotoxic, but labile components.

[c] Crude immunosuppressive freeze/thaw extracts were digested with trypsin (2.5 μg/ml) for 6 hr at 37° and centrifuged at 30K for 1 hr. Digested supernatants were then subjected to gel filtration chromatography on Sephadex G-100 and pooled fractions were tested for immunosuppressive activity.

[d] Not tested.

precipitable with anti-BEV, but its noncytotoxic immunodepressive capacity was not blocked by this antiserum. Peak B contains no detectable protein, does not react with Coomassie blue or iodinate effectively, and proteolytic enzymes do not influence its immunodepressive potential. Surprisingly, peak B was precipitated by anti-BEV serum in immunodiffusion analyses, but its blockage of PHA stimulation was also not effected by this treatment. Cytotoxicity studies with peak B were equivocal. Immediately after gel filtration, dye exclusion studies showed that peak B was highly cytotoxic beginning 2 hr after the start of culture. After 1–2 weeks of storage at −70°, peak B was still immunodepressive, but no longer cytotoxic. In this regard, it is of interest that immunodepressive F/T extracts of Rauscher murine leukemia virus (RLV), fractionated on Sephacryl S-200, yield profiles similar to the BEV F/T extract illustrated in text-Fig. 1 (14). However, in the peak B region, RLV F/T usually shows two peaks of immunodepression, one associated with cytotoxicity and one which is not cytotoxic. Thus, peak B of BEV F/T extracts probably consists of a highly labile cytotoxic factor and an immunodepressive, noncytotoxic factor of unknown chemical composition. Sudan black B staining of peak B following polyacrylamide gel electrophoretic analysis demonstrated a Sudanophilic molecule migrating at the same R_f as a 70,000-dalton protein (data not shown). It is thus possible that peak B represents a lipid-containing moiety whose apparent molecular weight on Sephacryl S-200 is lowered by its retention in the column, perhaps as a result of nonspecific adsorption to the gel matrix.

Discussion. We have demonstrated that substances associated with concentrates of a purified primate retrovirus can inhibit polyclonal mitogen-induced lymphocyte blastogenesis *in vitro*. In addition, we have shown that lymphocytes from at least three species, human, baboon, and mouse, are sensitive to the immunodepressive effects of BEV extracts; this lack of species specificity is also found with immunodepression induced by murine and feline retroviruses (14, 15). Preliminary experiments have suggested that human mixed lymphocyte reactions are also depressed by BEV extracts (data not shown). Subsequent reports will discuss the cellular specificity of this suppression.

The chemical composition of the suppressive factor(s) remains unresolved, its further purification and subsequent identification being complicated by high lability and low concentration. Other investigators have suggested that, at least with feline retroviruses, immunodepression may be mediated by the viral envelope protein p15E (4); we have, however, no evidence for or against the involvement of a BEV p15E-like component in the immunodepression reported herein. The preliminary data

suggest that the suppressive factor or factors do not carry antigenic determinants for any of the major structural components of BEV; i.e., antisera to BEV virus did not block suppression. Moreover, these factors may not be proteins as our initial studies with trypsin also failed to block immunodepression. Additional proteolytic enzyme preparations, on solid supports to limit the need for additional column separation, will have to be employed before reaching any firm conclusion. The possibility of a concentrated buffer, media, or normal dog thymus cell contaminant has been effectively ruled out by appropriate controls. We have not excluded, however, the possible concentration of a BEV-infected cell component (immunosuppressive) into the virion during maturation and release. The Sudanophilic nature of a component in peak B points to the possible participation of a lipid moiety derived perhaps from the viral envelope. The suppression of lymphocyte stimulation by lipoproteins is well established (16, 17). Whatever the biochemical nature of these factors, their presence in retrovirus-infected animals or synthesis by retrovirus-infected cells may account, at least in part, for the nonspecific immunodepression noted in a variety of species both *in vivo* and *in vitro*. Retroviruses of human origin have yet to be identified with certainty and the reports of human isolates related to primate retroviruses remain in question (10). Nevertheless, noncoordinate expression of viral components with immunoregulatory properties, however, related to those described in the present communication, could contribute to the well-established breakdown of immune surveillance mechanisms in human neoplastic disease or participate in homeostatic mechanisms regulating normal immune function.

This work was supported in part by Contract N01-C0-75308 with the National Cancer Institute, NIH, Bethesda, Md. 20205.

1. Cerny, J., and Essex, M., *in* "Naturally Occurring Biological Immunosuppressive Factors and their Relationship to Disease" (R. H. Neubauer ed.), p. 233. CRC Press, Florida (1979).
2. Weislow, O. S., and Wheelock, E. F., J. Immunol. **114**, 211 (1975).
3. Fowler, A. K., Twardzik, D. R., Reed, C. D., Weislow, O. S., and Hellman, A., Cancer Res. **37**, 4529 (1977).
4. Mathes, L. E., Olsen, R. G., Heberbrand, L. C., Hoover, E. A., and Schaller, J. P., Nature (London) **274**, 687 (1978).
5. Strand, M., and August, J. T., J. Virol. **14**, 1584 (1974).
6. Sherr, C. J., and Todaro, G. J., Science **187**, 855 (1975).
7. Gallagher, R. E., and Gallo, R. C., Science **187**, 350 (1975).
8. Nooter, K., Aarssen, A. M., Bentvelzen, P., de Groot, F. G., and van Pel, F. G., Nature (London) **256**, 595 (1975).
9. Kaplan, H. S., Goodenow, R. S., Epstein, A. L., Gartner, S., Decleve, A., and Rosenthal, P. N., Proc. Nat. Acad. Sci. USA **74**, 2564 (1979).
10. Okabe, H., Gilden, R. V., Hatanaka, M., Stephenson, J. R., Gallagher, R. E., Gallo, R. C., Tronick, S. R., and Aaronson, S. A., Nature (London) **260**, 264 (1976).
11. Sedmak, J. J., and Grossberg, S. E., Anal. Biochem. **79**, 544 (1977).
12. Greenwood, F. C., Hunter, W. M., and Glover, J. S., Biochem. J. **89**, 114 (1963).
13. Ouchterlony, O., and Nilsson, L. A., *in* "Handbook of Experimental Immunology" (D. M. Weis, ed.), Vol. I, p. 19.37. Blackwell, London (1973).
14. Hellman, A., Fowler, A. K., Twardzik, D. R., Weislow, O. S., and Reed, C. D., Advan. Exp. Med. Biol. **121B**, 99 (1979).
15. Heberbrand, L. C., Olsen, R. G., Mathes, L. E., and Nichols, W. S., Cancer Res. **39**, 443 (1979).
16. Curtiss, L., and Edgington, T. S., J. Immunol. **116**, 1452 (1976).
17. Morse, J. H., Witte, L. D., Goodman, D. S., J. Exp. Med. **146**, 1791 (1976).

Received July 22, 1980. P.S.E.B.M. 1981, Vol. 166.