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In Vivo Activation of the Baboon Endogenous Virus (41057)

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Abstract. Activation of humoral immune responsiveness to the baboon endogenous type C virus (BaEV) and its viral-specific envelope glycoprotein gp71, as well as activation of gp71 itself, was experimentally accomplished in baboons by inoculating fetal bovine serum (FBS) or horse serum (HS) plus Freund's complete adjuvant (FCA) or by cellular and viral antigens plus FCA, but not by FCA alone. Activated gp71 and anti-gp71 existed both as free and as immune complexes in baboon sera. Specificity of observed reactivities was supported by use of highly purified antigens in the radioimmunoprecipitation (RIP) and radioimmunoassay (RIA), employing iodinated FBS as antigen in the RIP assay, and absorption studies of BaEV-positive sera. These data suggest *in vivo* activation of BaEV or its structural component gp71 by an immune response to nonviral antigens.

A number of investigators have focused on *in vitro* activation of endogenous type C viruses or viral-specific information in normal and transformed cells. Activation may occur spontaneously, or following hormonal, chemical, physical, mitogenic, allogeneic, and viral stimuli (1–7). However, only limited experimental evidence exists concerning *in vivo* activation of endogenous, particularly xenotropic, type C viruses, and the mechanisms involved in the activation process. We report here the experimental activation of humoral immune responsiveness to the baboon endogenous virus (BaEV) and its viral-specific envelope glycoprotein gp71, as well as activation of gp71 itself. Induction was accomplished in baboons by inoculation of fetal bovine or horse serum plus Freund's complete adjuvant (FCA) or by cellular and viral antigens plus FCA, but not by FCA alone. *In vitro* studies demonstrated that activated gp71 and anti-gp71 existed both as free and as immune complexes in the baboon sera.

Materials and Methods. *Radioimmunoprecipitation.* Sera or plasmas (0.2 ml) were diluted 1:50 in 0.05 *M* Tris (pH 7.5), 0.1 *M* NaCl, 1 m*M* EDTA (TNE buffer) containing 1% bovine serum albumin (BSA) and assayed in duplicate. Five-hundredth-milliliter of ^{125}I-labeled purified BaEV, iodinated by the technique of Hunter and Greenwood as modified by Hutchinson and Ziegler (8, 9), was added and the mixture was incubated at 37° for 1 hr. After that time, 0.2 ml of goat anti-human IgG (supplied by Viral Oncology Program, NCI) diluted 1:2 in TNE was added and incubated at 37° for 1 hr and at 4° overnight. After addition of 2 ml of TNE, precipitates were collected by centrifugation at 100*g* for 10 min, washed three times with 2 ml TNE, and counted in a γ-counter (Packard, Downers Grove, Ill.). Supernatant fluids were also saved for counting. Precipitation was expressed as percentages of binding in the precipitate relative to the combined counts in the precipitate plus supernatant minus background; 0 represents no precipitation or below background. A positive response was considered to be ≥3.4% (based on a survey of 80 normal baboons).

Purification, iodination, and determination of BaEV antigens. Thirty milligrams of BaEV was exposed to five freeze–thaw cycles (−90 to 0°) and centrifuged at 100,000*g* for 45 min. The supernatant was lyophilized and redissolved in 1 ml 10 m*M* Tris–HCl buffer, pH 7.2, containing 5% glycerol. This was followed by a sequential column chromatography on phosphocel-

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lulose and Sephadex G-100. The material eluted at the BSA marker was designated gp71. The 100,000*g* pellet was resuspended in 1 ml 10 m*M* Tris–HCl buffer, pH 7.2, containing 1% Triton X-100 and subjected to a phosphocellulose and DEAE-cellulose column chromatography for the purification of p30 (9) and p12. Gp71, p30, and p12 were radiolabeled with ^{125}I. Two micrograms of each protein was exposed to 500 μCi ^{125}I and 10 μg of chloramine T. Each reaction was suppressed by 200 μM of tyrosine and the iodinated proteins were separated by Sephadex G-25 column chromatography. Approximately 60,000 cpm gp71, ~100,000 cpm p30, and ~100,000 cpm p12 were subjected to a sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis at 40 V for 16 hr. Each protein demonstrated a single band on the gel at the respective molecular weight region. For antibody and antigen demonstration, 5 ml of each serum was dialyzed against 10 m*M* Tris–HCl buffer (pH 7.2) and lyophilized. The dried material was resuspended in 200 μl of the same buffer. Results of antibody determinations are shown using 5 μl of each sample to precipitate 15,000 cpm of ^{125}I gp71, 20,000 cpm of ^{125}I p30, and 18,000 cpm of ^{125}I p12. Antigen quantitation was done by a competitive RIA [(10), (1 ng of gp71 or 200 pg of p30 or 500 pg of p12 can be detected by this assay)]. Results of antigen determinations show the presence of nanograms of viral protein per 20 μl of concentrated serum.

Clq solid-phase and monoclonal rheumatoid factor (mRF) solid-phase assays. The solid-phase (SP) assays were modified after that described by Yoshinoya and Pope (11). Five-tenths milliliter of isolated Clq (5 μg/ml) or mRF (1 μg/ml) was incubated in polystyrene tubes (12 × 75 mm, Falcon 2054, Falcon Labware, Division of Becton, Dickinson & Co., Oxnard, Calif.) overnight at 4°. After washing three times with 1.0 ml of PBS (5 m*M* sodium phosphate, 0.15 *M* sodium chloride, pH 8.0), 0.5 ml of PBS plus 1% bovine serum albumin (PBS–BSA) was incubated in the tubes for 3 hr at 4°. After three additional washes with PBS–BSA, 0.025 ml of test sample, which had been previously incubated in 0.2 *M* EDTA (pH 7.4) (1:2, v/v) at 37° for 30 min, was added, and the final volume was adjusted to 0.5 ml with PBS–BSA. The tubes were incubated at 4° for 1 hr. After washing, 0.5 ml of specific ^{125}I-F(ab')$_2$ rabbit anti-human Fc (1 μg/ml) was added and incubated for 1 hr at 4°. After aspiration and washing, the tubes were counted in an automatic well-type γ-counter. The results were expressed as nanograms of anti-Fc bound per tube. Coated tubes to which only buffer was added served as the background controls. All radioimmunoassays were performed in duplicate.

Viruses. BaEV (strain M7) was kindly supplied by the Viral Oncology Program, NCI. It had been grown in A204 cells in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS). Purification was achieved by banding it twice on isopycnic sucrose gradients formed in zonal ultracentrifuges. The virus containing cuts were pelletized, resuspended in TNE buffer, and stored at −70°.

Herpesvirus hominis type 1 (HVH-1 strain Mayo) was grown in Vero cells in minimal essential medium Eagle (MEME) supplemented with 5% horse serum (HS). It had been obtained from the Research Resources Branch, NIAID. It had an infectivity titer of $10^{8.5}$ $TCID_{50}$/ml.

Herpesvirus saimiri (HVS, strain S 295 C) was grown in owl monkey kidney cells (OMK) in MEME supplemented with 10% FBS. Both HVS and OMK had been supplied by Dr. M. D. Daniel, New England Regional Primate Research Center. As HVH-1 it had been frozen and thawed once and clarified by low-speed centrifugation (2000 rpm for 30 min). It had an infectivity titer of $10^{6.5}$ $TCID_{50}$/ml and as HVH-1 was stored at −70°.

Results. Twenty-two normal adult female baboons (*Papio cynocephalus*) were immunized with 4 ml of various inocula plus 4 ml of FCA as listed in Table I. Three weeks postinoculation (p.i.) they received a 4-ml booster injection without FCA (except when FCA was the only inoculum). Humoral immune responses of these baboons following the booster injection as assayed by radioimmunoprecipitation (RIP) are given in Table I.

TABLE I. PRE- AND POSTIMMUNIZATION IMMUNE REACTIVITY OF BABOONS TO BaEV AS DETERMINED BY RADIOIMMUNOPRECIPITATION

Expt	Inoculum	Immune reactivity to BaEV (% binding)		
		Animal	Preimmunization	Postimmunization
I	HVS(FBS) + FCA	1	0	55.3
		2	0	55.2
		3	0.5	60.9
II	V-HVS(FBS) + FCA	1	0	62.3
		2	0	62.1
		3	0	62.1
III	HVS(FBS)	1	10.7	33.2
		2	0	7.6
		3	4.4	35.4
IV	FBS + FCA	1	0	66.7
		2	0	65.0
V	OMK(FBS) + FCA	1	0	64.6
		2	0	63.9
VI	FCA	1	0	0
		2	0	0
		3	0	0
VII	HVS(HS) + FCA	1	0	26.7
		2	0	31.7
VIII	HS + FCA	1	4.4	45.9
		2	0	41.6
IX	HVH(HS) + FCA	1	0.6	40.9
		2	0	30.5

Note. HVS, *Herpesvirus saimiri;* FBS, fetal bovine serum; FCA, Freund's complete adjuvant; uv-HVS, ultraviolet-inactivated HVS; OMK, owl monkey kidney cells; HS, horse serum; HVH, *Herpesvirus hominis* type 1.

Infectious ($10^{6.5}$ $TCID_{50}$/ml) and ultraviolet (uv)-inactivated HVS in FCA elicited a significant humoral response in all six animals (Experiments I and II). Since these experiments demonstrated that elicited immune reactivity to BaEV did not depend on live HVS, we attempted to determine which components of the inocula were responsible for the activation. HVS had been grown in OMK cells in MEME supplemented with 10% FBS. When HVS was inoculated without FCA, a significant antibody response was also observed (Experiment III); however, without the adjuvant effect of FCA the response was lower than in Experiments I and II. Inoculation of both FBS and OMK plus FCA (Experiments IV and V) induced positive responses equal to those observed in the original experiments (I and II), while FCA alone produced no response (Experiment VI).

Because all inocula used in Experiments I-V contained 10% FBS (0.4 ml), positive anti-BaEV immune reactivity could have been partly or totally directed toward trace amounts of FBS retained in the purified BaEV preparations used in the RIP assay. We therefore substituted HS for FBS as an antigen alone and in the preparation of an HVS inoculum (Experiments VII and VIII). In addition, clarified HVH-1, grown in Vero cells in MEME supplemented with 5% HS was used (Experiment IX). In these last three experiments (VII–IX), lower percentages of binding to BaEV were found than in Experiments I, II, IV, and V. Nonetheless, significant increases in immune reactivity to BaEV were demonstrated p.i.

We examined a number of experimental sera from baboons of experiments I and II, which had been concentrated, for the presence of precipitating antibodies to purified

BaEV structural polypeptides gp71, p30, and p12. The same sera were also examined by a competitive radioimmunoassay (RIA) for the presence of free gp71, p30, and p12 antigens (Table II). Increased levels of gp71 and anti-gp71 could be shown in sera postactivation. No p30 or p12 antibodies or free antigens were detected. Employing Sephadex G-100 column chromatography gp71 was found as free antigen and also complexed to antibody. A 30-ml sample of postactivation serum contained 60 ng of free gp71. After dissociation of immune complexes by glycine–HCl, 80 ng of gp71 was detected.

Further evidence for the presence of antigen–antibody complexes in postactivation sera was obtained by Clq solid-phase and monoclonal rheumatoid factor solid-phase RIAs (11). Immune complexes were absent in preinoculation sera and their presence postactivation corresponded closely with reactivity detected in RIP assays (Table III). In addition, immune complexes were demonstrated by these RIAs following sucrose density gradient fractionation with subsequent dissociation with 4 *M* urea.

Discussion. Positive immune responses to BaEV and viral-specific antigens observed in baboons could have originated from several sources. The introduction of an exogenous type C virus via the inocula has to be considered as a possibility. Recently an oncornavirus was isolated from OMK cells, but this virus (OMC-1) is unrelated to BaEV (12). In addition, no BaEV related oncornaviruses have been found in Vero cells and it is unlikely that they are present in FBS or HS.

Cross-reactivity between herpesviruses and oncornaviruses has been reported (13) but this could not account for the positive immune reactivity obtained with inocula containing no herpes viruses.

Although it has been reported that FBS by itself is capable of inducing murine type C viruses (14), the possibility of detecting immune reactivity solely due to FBS was refuted by several experiments: (a) highly purified antigens (sucrose gradient, Sephadex G-100 and phosphocellulose column chromatography) were used to demonstrate anti-gp71 binding and free gp71 (Table II); (b) employing iodinated FBS as antigen in the RIP assay, we found that in Experiments I–V an average of 31.8% of BaEV reactivity was attributable to anti-HBS antibodies but only 3.3% in Experiments VII–IX; (c) three absorptions of a BaEV-positive serum with an FBS saturated uninfected human rhabdomyosarcoma (A204) cell culture in which BaEV

TABLE II. PRESENCE OF ANTIBODIES TO PURIFIED BaEV-POLYPEPTIDES AND FREE POLYPEPTIDES IN EXPERIMENTAL BABOON SERA PRE- AND POSTIMMUNIZATION (EXPERIMENTS I AND II)

Serum sample	Antibody to (% binding)			Amount of free antigen (ng/20 μl)		
	gp71	p30	p12	gp71	p30	p12
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	28	0	0	10	0	0
6	35	0	0	20	0	0
7	30	0	0	10	0	0
8	20	0	0	3	0	0
9	15	0	0	3	0	0
10	15	0	0	3	0	0
11	42	0	0	10	0	0
12	30	0	0	5	0	0
13	40	0	0	10	0	0

Note. 1–4, preimmunization; 5–13 postimmunization.

TABLE III. DETECTION OF IMMUNE COMPLEXES PRE- AND POSTIMMUNIZATION AND THEIR CORRELATION WITH PRECIPITATING ANTIBODIES TO BaEV

Serum sample	Expt	Percentage binding	
		Clq	RIP
1	IX	7	0
2		59	40.9
3	VIII	6	2.2
4		42	57.3
5	V	<1	0
6		53	59.6
7	I	<1	0
8		42	42.6

Note. 1,3,5,7, preimmunization; 2,4,6,8, postimmunization.

was grown reduced BaEV reactivity by 41.5%, but a fourth absorption did not further reduce the percentage binding. Continued absorptions (five times) of this serum with BaEV infected canine thymus cells (Cf2th) reduced the percentage binding to BaEV to preinoculation levels.

In vivo activation of immune reactivity to BaEV or its viral-specific structural component gp71 is highly possible. Our data point to an incomplete activation of BaEV. It has been shown *in vitro* that activated type C viruses may remain cell associated. Antigenically stimulated mouse T and B cells display gp71 on their surface from where they may be released or are accessible for the host to mount an immune response (15, 16). Another question regarding the activation of endogenous type C viruses or their structural antigens is whether they are "switched on" by infectious or immunologic processes. *In vivo* studies with Marek's disease herpes virus, guinea pig herpes virus, Epstein–Barr virus, and *in vitro* experiments with HVH (6, 7, 17–22) lend support for a cocarcinogenic effect of two viruses and for the involvement of an infectious process. Evidence supporting the participation of immunologic factors in virus induction has been presented as well (14, 23, 24). Although infection and immune responses are not mutually exclusive (infection usually leads to immune response), the data reported herein suggest an *in vivo* activation of type C virus or structural components by an immune response to nonviral antigens, since serum proteins (FBS, HS) were the common denominators of the immunizing inocula.

Although no direct oncogenic capability has been demonstrated as yet for BaEV, this potential has been suggested by its association with human leukemia and baboon lymphomas (25–27). If not directly oncogenic, however, xenotropic type C viruses may, when activated, have an immunosuppressive effect, allowing neoplasia to escape immune surveillance, or they may induce persistent immunologic responses culminating in blast transformation of lymphoid cells (28–30). As a recent report indicates (31), it is also conceivable that frequent *in vivo* induction of type C virus products through immunization with nonviral antigens might actually prevent development of neoplasia.

Other functions have been proposed for xenotropic type C viruses: e.g., a normal physiological role (32), a function in evolution of species (33, 34), cellular differentiation (35), and involvement in autoimmune disease (36). The demonstration of the experimental activation of an immune response to BaEV or its viral envelope protein gp71, free gp71, and immune complexes in the endogenous host may suggest the derepression of viral genes and may provide the means to investigate the putative role of xenotropic viruses in the above-mentioned hypotheses.

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