

EPSTEIN-BARR VIRUS

HETEROPHILE RESPONSES IN SQUIRREL MONKEYS INOCULATED WITH VIRUS-TRANSFORMED AUTOLOGOUS LEUKOCYTES*

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Epstein-Barr virus (EBV)¹ is considered, largely on the basis of serologic and epidemiologic evidence, to be the etiologic agent of infectious mononucleosis (IM). In IM a variety of EBV-specific antibodies appear during the course of illness (1-4), and absence or presence of EBV antibodies correlates with susceptibility or resistance to the disease (5, 6). Furthermore, in IM, EBV is recoverable from washed leukocytes (7) and from oropharyngeal sites during the acute illness and for long periods thereafter (8).² The causal role of EBV in other disease states, such as Burkitt lymphoma, nasopharyngeal cancer, and sarcoidosis, in which there are elevated EBV antibody titers is less certain.

Animal experimentation has been undertaken to investigate the etiologic role of EBV in IM, to define the oncogenic potential of EBV, to study the pathogenesis of EBV infections, and, perhaps, to detect differences in EBV strains. Thus far, animal experiments reported in the literature have consisted of heterotransplantation of human lymphoblastoid cell lines. In neonatal or immunosuppressed rodents, lymphoblastoid cell lines have produced tumor masses, but have not induced EBV antibodies (9). Rhesus and marmoset monkeys inoculated with EBV-infected lymphoblastoid lines have not developed disease or detectable antibodies (10).

The experimental approach in our laboratory has been to evaluate the effects of inoculation of autologous leukocytes converted into continuous cell lines after *in vitro* exposure to EBV (11). The present report concerns the serologic responses of three squirrel monkeys that received a series of inoculations of

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¹ Abbreviations used in this paper: BHK, baby hamster kidney cells; CF, complement fixation; EBV, Epstein-Barr virus; GPK, guinea pig kidney; IF, immunofluorescence; IM, infectious mononucleosis; SLCL, simian lymphoblastoid cell lines.

² Miller, G., and J. C. Niederman. Prolonged oropharyngeal excretion of Epstein-Barr virus following infectious mononucleosis. *N. Engl. J. Med.* Inpress.

autologous EBV-converted lymphoblasts. The results suggest that EBV induces the appearance of the heterophile antigen of infectious mononucleosis on the cell membrane of transformed monkey cells.

Materials and Methods

Virus and Cell Lines.—Simian lymphoblastoid cell lines (SLCLs) were derived after exposure to EBV. The virus was in the form of a cell extract prepared from a human lymphoblastoid cell line (883L) established from the blood of a patient with transfusion-induced infectious mononucleosis. Techniques for extracting virus and for establishing and maintaining SLCL have been published (11).

Animals and Inoculation Schedules.—Female squirrel monkeys (*Saimiri sciureus*) were purchased from the Tarpon Zoo, Tarpon Springs, Fla. All the monkeys initially lacked EBV antibodies detectable by the immunofluorescent and complement-fixation methods. Each monkey was housed in a separate cage in the same room. Each animal received five or six inoculations of $1-2 \times 10^8$ living (trypan blue negative) autologous EBV-transformed cells over a 30-day period. Cell inocula were prepared by low-speed (800 g) centrifugation of cells from 100 ml of tissue culture medium, washed three times with Puck's saline A, and resuspended in Puck's saline.

7 mo (27 wk) after the beginning of the initial series of inoculations, another inoculation was given to each animal to determine whether the observed antibody responses required living cells or could be induced with products of disrupted cells. Animal no. 5-96 was challenged with 1.6×10^8 washed living autologous cells; no. 5-97 received a filtrate prepared from 4.2×10^8 washed autologous cells, suspended in Puck's saline, disrupted by repeated freezing and thawing, and passed through a 0.8- μ m Millipore filter (Millipore Corporation, Bedford, Mass.); no. 5-98 was given cell ghosts prepared from 6×10^8 autologous cells. Cell ghosts consisted of material found in the pellet after cells treated with three cycles of freezing and thawing had been centrifuged at 800 g. 7 wk later, no. 5-97 was challenged with a cell-free filtrate prepared from 2×10^8 cells.

A control animal was observed and bled in parallel with the other three animals, but was not inoculated. All inoculations were by the intravenous route. The inoculations are summarized in Table I.

TABLE I
Protocol for Inoculation and Challenge of Squirrel Monkeys with Epstein-Barr Virus

Animal no.	Primary inoculations			Challenge inoculation(s)	
	Material inoculated	Number of inoculations	Time after first inoculation	Material inoculated	Titer*
5-96	Live autologous EBV-transformed cells	6	27	Live autologous EBV-transformed cells	3.0
5-97	Live autologous EBV-transformed cells	6	27, 34	Filtrate of autologous EBV-transformed cells	2.5
5-98	Live autologous EBV-transformed cells	5	27	Ghosts of autologous EBV-transformed cells	2.75

* Log_{10} transforming units₅₀ per 0.2 ml of cell lysate (5-96), cell filtrate (5-97), and cell ghosts (5-98) prepared from 10^7 cells/ml.

Infectious EBV in the Inocula.—The titers of virus present in whole cells, filtrates, and ghosts of SLCL were quantitated by transformation assay on human umbilical-cord leukocytes. Primary cord leukocyte cultures with 10^6 cells/ml were started in 15×150 -mm glass test tubes (0.8 ml/tube). The medium used was RPMI 1640 plus 20% fetal calf serum. A 0.2-ml aliquot of material to be tested for infectivity was added to each tube. Three replicate cultures were inoculated for each serial decimal dilution. Transformation was recognized by the appearance of clusters of rapidly growing, enlarged leukocytes and by production of acid. End points were calculated by the Reed Muench formula.

Observations and Serology.—Animals were observed daily. EBV and heterophile antibody levels and white cell and differential counts were measured weekly. EBV antibodies were measured by indirect immunofluorescent and complement-fixation techniques (11); antigen for both tests was derived from Burkitt lymphoma cell strain HRIK. In the indirect immunofluorescent method, fluorescein-conjugated rabbit antisera prepared against human or rhesus monkey gamma globulin proved satisfactory in tests with squirrel monkey sera. Heterophile agglutinins directed against sheep red blood cells were detected in a microtiter adaptation of the Paul-Bunnell method. Differential absorptions of sera were performed with guinea pig kidney (GPK) (Forssman) antigen and beef erythrocyte antigen (both purchased from Difco Laboratories, Inc., Detroit, Mich.).

Virus Recovery Attempts.—At five intervals from 9 to 22 wk after inoculation, peripheral blood was obtained from each animal. Leukocytes were prepared as described and cocultivated with human placental fibroblasts (11). More than 30 individual cultures were started from each monkey. Cultures were observed for 60 days for the appearance of transformed leukocytes.

RESULTS

The four animals have been observed for 8 mo. None has developed palpable tumor or hematologic abnormalities detectable by differential peripheral leukocyte counts.

Serologic Responses after Initial Series of Inoculations.—The three monkeys that received multiple inoculations of autologous cells developed heterophile agglutinins and EBV-specific antibodies (Figs. 1–3). Heterophile agglutinins appeared after a single inoculation (day 6) in one animal, after two inoculations (day 13) in a second, and not until three inoculations (day 16) in the third. At the time of peak titers, agglutination was present after absorption with guinea pig kidney antigen. Some early and all late sera also contained heterophile agglutinins that were absorbed by guinea pig kidney antigen. As in infectious mononucleosis, agglutinins were entirely removed by beef erythrocyte antigen. Heterophile agglutinins disappeared 2–8 wk after inoculations stopped.

EBV-specific antibodies detectable by complement fixation (CF) and immunofluorescence (IF) appeared after the heterophile agglutinins in two of three animals and after at least two inoculations; but, with the exception of a persistent low IF antibody titer in one animal (no. 5-96), the EBV antibodies were transient and disappeared 4–18 wk after the inoculations stopped.

Serologic Responses after Challenge after a 7-mo Interval.—Reinoculation of either autologous intact cells or cell ghosts caused the reappearance of heterophile agglutinins; but filtered cell lysate, given on two separate occasions, failed to stimulate the heterophile response. The heterophile agglutinin that

resulted from inoculation of intact cells was absorbed both by guinea pig kidney and by beef erythrocyte antigen, whereas the heterophile agglutinins present after inoculation with cell ghosts were partially resistant to treatment with guinea pig kidney antigen.

EBV-specific antibodies (both CF and IF) were also stimulated in anamnestic fashion (6 days) in the two animals given intact cells or cell ghosts. Only a low-titer IF antibody response followed inoculation of autologous cell filtrate.

Virus Recovery Attempts.—To determine whether the animals had become

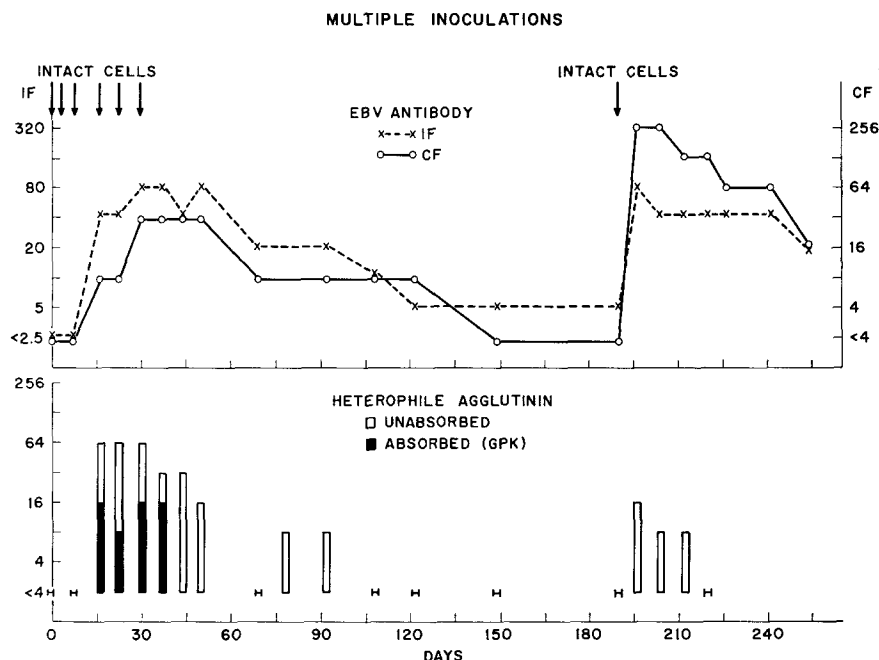


FIG. 1. Squirrel monkey no. 5-96. Heterophile and EBV-specific antibody responses after repeated inoculations of autologous EBV-transformed lymphoblasts; followed by challenge 7 mo later with autologous EBV-transformed whole cells. († indicates a heterophile agglutinin titer less than 1:4.)

persistently infected with EBV after the initial series of intact cell inoculations, more than 30 leukocyte cultures from each animal were observed for spontaneous transformation. No continuous cultures were established. The technique was capable of detecting transformed cells in the peripheral blood, however (we have observed spontaneous transformation of leukocytes in cultures prepared very soon [18 h] after inoculation of autologous transformed cells).

DISCUSSION

Heterophile Antibody Responses.—Although the results of the studies described must be considered preliminary because of the small number of ex-

perimental animals used, the data nonetheless suggest that the appearance of heterophile antigen(s) accompanies leukocyte transformation by EBV. The new heterophile antigen(s) are presumably located on cell membranes, because cell extracts containing infectious EBV without membranes did not stimulate a heterophile agglutinin whereas preparations rich in cell membranes induced the heterophile antibody. Another explanation, which seems less likely, is that the heterophile antigen is a soluble cytoplasmic antigen whose immunogenicity is enhanced by the presence of cell membranes.

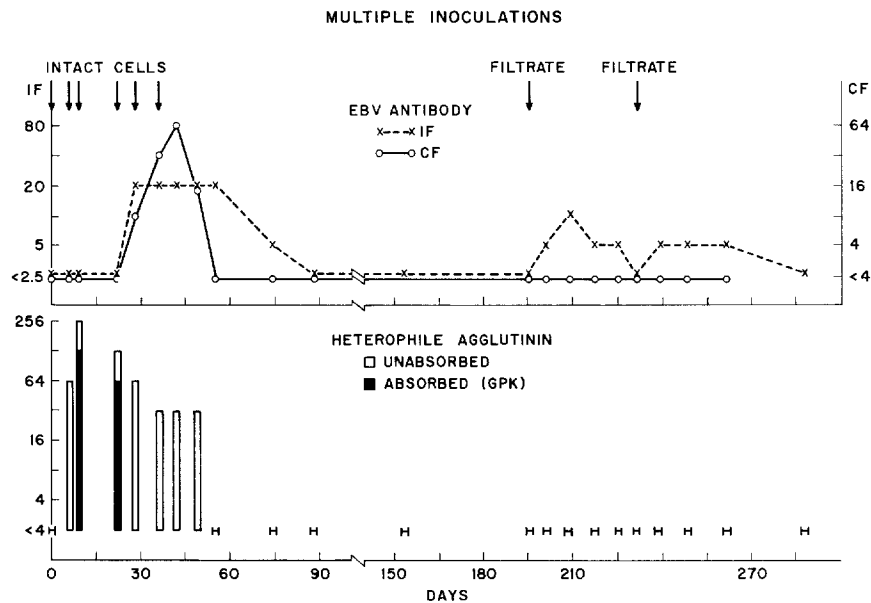


FIG. 2. Squirrel monkey no. 5-97. Heterophile and EBV-specific antibody responses after repeated inoculations of autologous EBV-transformed lymphoblasts; followed by two challenges 7 and 9 mo later with filtrate of autologous transformed cells containing infectious EBV. (H indicates a heterophile agglutinin titer less than 1:4.)

The new heterophile antigen may be a product of the viral genome or it may be a host cell antigen that is revealed in the process of transformation. Burger has shown that baby hamster kidney cells (BHK) display Forssman antigen activity on their surfaces not only when transformed by polyoma and Rous sarcoma viruses but also after brief exposure to proteases. Burger concludes that the Forssman antigen is present in BHK cells before virus transformation (12). Similarly, the heterophile antigen peculiar to infectious mononucleosis may be present on normal lymphoid cells, but not expressed until changes are brought about in the cell membrane after transformation by EBV.

The heterophile antigens present in EBV-transformed cells are not Forssman

antigens; rather, differential absorption of the monkey sera with guinea pig kidney and beef erythrocyte antigens suggests that two other types of heterophile agglutinins are present. Sera from each inoculated monkey contain agglutinins with an absorption pattern characteristic of infectious mononucleosis sera, i.e., agglutinins are completely removed by beef erythrocytes but not by guinea pig kidney antigens. However, some early and all late monkey sera with agglutinins demonstrate a pattern similar to that seen in serum sickness, i.e., complete absorption by both guinea pig kidney and beef erythrocytes. There

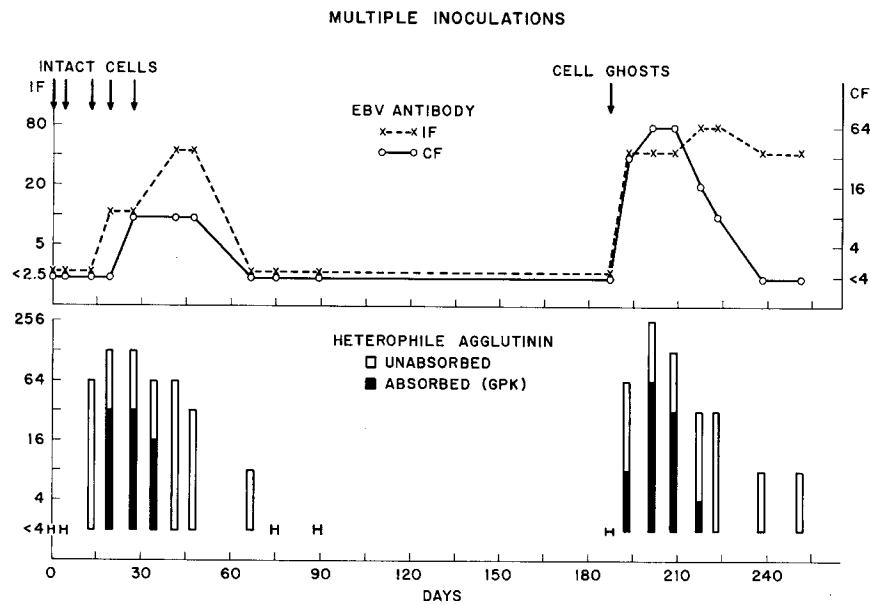


FIG. 3. Squirrel monkey no. 5-98. Heterophile and EBV-specific antibody responses after repeated inoculations of autologous EBV-transformed lymphoblasts; followed by challenge 7 mo later with ghosts of disrupted autologous transformed cells. (H indicates a heterophile agglutinin titer less than 1:4.)

may be two different heterophile antigens associated with transformation of the simian leukocytes by EBV, or, alternatively, the animals may have developed a true serum sickness as a result of repeated exposure to the small amounts of fetal bovine serum used in the cell culture media and not removed by repeated washing.

Infection or Immunization with Nonreplicating Antigen?—An important question, Did the inoculated cells or their associated EBV replicate in the animals?, is not conclusively answered. However, most of the evidence suggests that both the EBV-specific antibody responses and the heterophile agglutinins were due to immunization with a considerable antigenic mass. The rapidly

declining EBV antibody levels after cessation of inoculations and the inability to reisolate EBV from the blood leukocytes favor immunization without infection. Furthermore, in an experiment not reported in this series, squirrel monkeys that had received one inoculation of similar numbers of autologous or homologous intact EBV-infected cells failed to develop EBV antibodies or IM-type heterophile antibodies.

The appearance of heterophile antibodies does not seem to require cellular replication. For example, IM-type heterophile agglutinin has been induced in human volunteers by inoculation of sheep erythrocytes (13).

Perhaps it is not surprising that we were unable to infect squirrel monkeys under these experimental conditions. The squirrel monkey appears to be naturally insusceptible to infection with EBV: we have detected no EBV antibodies in 20 animals of this species obtained from a variety of sources, including a primate research center, a commercial animal dealer, and a zoo, where opportunities for exposure to EBV presumably exist.

Oncogenic Potential of EBV-Transformed Cells.—As noted above, most of the evidence suggests that the experimental animals were not infected with EBV. The question of the oncogenicity of EBV if the animals were infected is still unresolved. Our data are incomplete, for the animals are still under observation and have not yet been autopsied. Furthermore, experimental viral tumor induction requires coordination of a large number of complex variables pertaining to both host and virus. Failure to observe any tumors at this point in the experiment might be explained, in part, by the finding that the inoculated EBV-transformed cells contain a strong neoantigen, the heterophile antigen. A likely hypothesis is that the EBV-induced heterophile antigen plays an important role in the surveillance of the transformed cells.

SUMMARY

Epstein-Barr virus (EBV)-transformed autologous lymphoblasts were repeatedly inoculated into three squirrel monkeys. Each animal developed the heterophile antibodies of infectious mononucleosis and EBV-specific antibodies. After serologic responses had disappeared or markedly declined, the animals were challenged with either whole cells, cell filtrate, or cell ghosts. Animals challenged with living cells and cell ghosts developed agglutinin responses; the recipient of filtrate did not. The results suggest that EBV induces the appearance of the infectious mononucleosis heterophile antigen on the transformed cell membrane.

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