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Malignant Lymphoma in Cottontop Marmosets after Inoculation with Epstein-Barr Virus

(immunofluorescence/primate/tumor)

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ABSTRACT **Neoplasia resembling human malignant lymphoma, reticulum cell sarcoma type, occurred in cottontop marmosets inoculated with materials containing Epstein-Barr virus. One of four monkeys that received autologous cells transformed *in vitro* by Epstein-Barr virus developed lymphoma in mesenteric lymph nodes 7.5 months after inoculation. Three of four marmosets inoculated with cell-free Epstein-Barr virus developed lymphoma. The latent period for detectable tumor formation after addition of virus was 31-46 days. Immunosuppressive drugs given with the virus accelerated the course of disease. Nevertheless, malignant lymphoma occurred in an animal given only cell-free virus. Six of eight marmosets inoculated with the virus demonstrated antibodies to the virus. Four marmosets not exposed to the virus, including two that received immunosuppressive drugs, developed neither tumors nor antibodies to Epstein-Barr virus. Virus antigen detectable by immunofluorescence was found in 5% of cells shed from one tumor maintained in organ culture. These results imply that Epstein-Barr virus is capable of inducing malignant lymphoma in at least one primate species. Additional evidence is required before its oncogenic capacity in this host can be accepted without reservation.**

Epstein-Barr virus (EBV) is regularly associated with lymphoproliferative disease. In infectious mononucleosis, a wide range of evidence suggests that the association is causal (1). In Burkitt lymphoma and nasopharyngeal carcinoma, EBV is associated with the tumor, and most patients with these diseases exhibit high titers of specific antibodies (2-4). EBV genome can be detected by nucleic acid hybridization in tumor-cell lines and in tumor biopsies in which viral particles are not found (5-6). Induction of experimental lymphoproliferative disease by EBV is essential, if the classical Koch-Henle postulates are to be fulfilled in identification of the agent responsible for these conditions. The investigations here described were undertaken with this requirement in mind.

A significant biologic property of EBV is its capacity to cause human and certain other primate leukocytes to change into lymphoblastoid cell lines, which proliferate continuously *in vitro* (7-10). The crucial question, whether this represents a malignant change, has recently been examined experimentally in primates. No tumors were observed in three adult squirrel monkeys (*Saimiri sciureus*) given multiple inoculations of autologous lymphoblasts transformed by EBV, although the animals developed both EBV-specific and heterophile antibodies (11). Similarly, no tumors were found in five gibbons (*Hylobates lar*) inoculated with autologous lymphoblasts converted by EBV; three of these animals received immunosuppressive drugs (12).

In the experiments to be reported, we have used cottontop marmosets (*Saguinus oedipus*) to examine further the possible oncogenic potential of EBV. In earlier work, Melendez and his associates showed that marmosets are susceptible to induction of lymphomas by two simian herpes viruses, *H. saimiri* and *H. ateles* (13, 14). Cottontop marmosets do not have naturally occurring antibodies to EBV, yet their leukocytes are susceptible to infection and establish cell lines after exposure to the virus (15).

METHODS

Animals. Adult and juvenile marmosets from Columbia, S.A., were used. They were housed in individual cages, and examined at weekly intervals for palpable tumors.

EBV Inoculum. Two types of inoculum were used: autologous cells transformed by EBV and cell-free virus. To obtain infected autologous cells, we transformed peripheral blood leukocytes of marmosets into continuous lymphoblastoid cell lines after exposure to EBV in the form of a filtered cell-free extract of line 883L, which originated from a patient with infectious mononucleosis (16). The lines were carried *in vitro* for about 5 months before being returned to the autologous host. The source of the cell-free virus used for inoculation was filtered (0.8 μm) supernatant fluid of line B95-8, a continuous line of marmoset leukocytes infected by EBV established by exposure to virus from line 883L. Infectivity of cell-free virus was assayed *in vitro* by transformation of leukocytes from human umbilical cord (17). A single EBV stock with a titer of $10^{4.4}$ 50% transforming units per ml was used for inoculations.

Immunosuppression. Half the animals in each experiment were given azathioprine (1 mg/day) and prednisilone (0.05 mg/day) intramuscularly. Inoculations were begun 3 days before administration of EBV and continued daily, except Sunday, for 3 weeks afterwards. The animals remained well during immunosuppressive therapy, and their leukocyte and platelet counts were stable.

Virus Inoculations. Autologous EBV-transformed cells were washed and resuspended in Puck's saline A at a concentration of 1.2 to 3.0 $\times$ 10^8 cells per ml. The total inoculum for each animal was 1 ml, divided into 3 aliquots given intravenously, intraperitoneally, and subcutaneously. 1 ml of cell-free virus was inoculated similarly in other animals.

Serology. The animals were bled weekly for 2 months and then biweekly. Sera were tested for EBV "viral capsid antibodies" by the indirect immunofluorescence method (18), with the B95-8 cell line as antigen and Raji cells, which do

Abbreviation: EBV, Epstein-Barr virus.

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FIG. 1. A marmoset that received EBV and no immunosuppressive drugs (642). Multiple tumor nodules located in mesenteric lymph nodes. The spleen is normal.

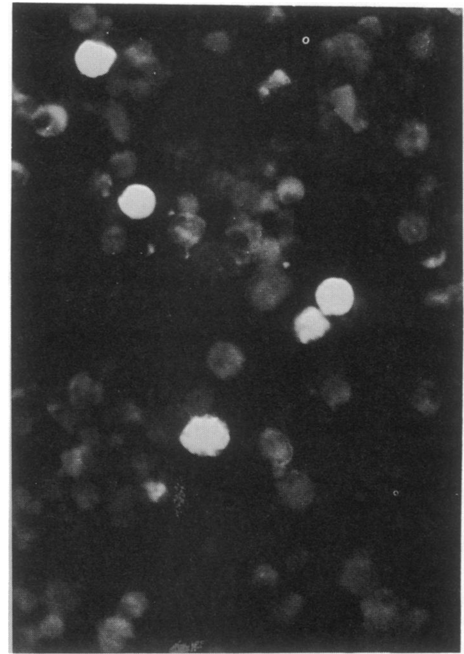

FIG. 3. Indirect immunofluorescence test for EBV antigen in cells shed from an explant of mesenteric tumor maintained in organ culture for 3 weeks. Original magnification, × 250.

not contain EBV nucleocapsids, as the negative control antigen. Fluorescein-conjugated antibodies against gamma globulin of rhesus monkey made in a goat were used. Line B95-8 was also used as antigen for detection of EBV complement-fixing antibodies. Normal marmoset leukocytes maintained in culture for 5 days without exposure to EBV were prepared similarly for use as a negative control complement-fixing antigen. In selected sera, antibodies against EBV "early antigens," demonstrable by superinfection of the Raji line, were measured by Dr. Werner Henle (University of Pennsylvania), and tests for neutralizing antibodies against *Herpes saimiri* were performed by Dr. Luis Melendez (Harvard University).

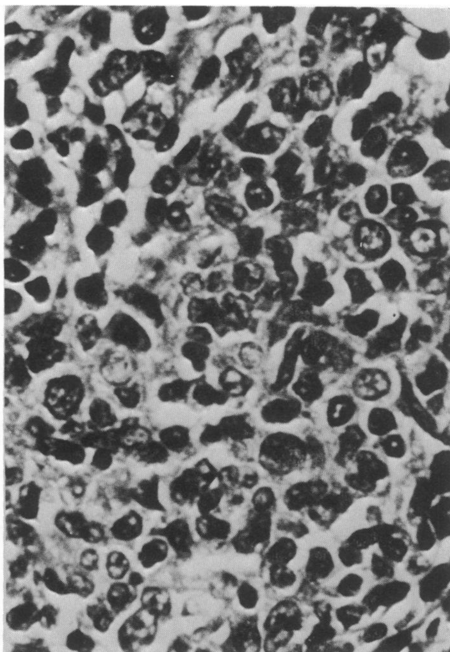

FIG. 2. Histologic appearance of tumor cells from a mesenteric lymphoma. Hematoxylin and eosin stains. Original magnification, × 400.

Attempts to Demonstrate Virus or Viral Antigen. Fragments of tumor were examined by electron microscopy, and imprints taken directly from tumors were studied for EBV antigens detectable by indirect immunofluorescence (19). As an attempt to detect cytopathic agents, tissue fragments were cocultivated with monolayer cultures of VERO and JR-1 lines of grivet monkey kidney, as well as with strains of human embryo fibroblasts and squirrel monkey lung cells established in our laboratory. In an attempt to derive continuous lymphoid-cell lines, tissue fragments were maintained as organ cultures on stainless steel grids (20). Spread preparations of cells shed from organ cultures were serially examined for EBV antigens by immunofluorescence.

RESULTS

Incidence of Tumors. The two experiments conducted involved a total of 12 marmosets. Eight were given EBV; four were held in adjacent cages, bled and handled in parallel, but not inoculated. Tumors, documented pathologically, developed in four of the eight in the experimental group, but in none of the four controls. Both immunosuppressed and non-immunosuppressed animals showed multiple tumor masses at autopsy (Tables 1 and 2).

Administration of EBV-transformed cells into four marmosets was followed by the appearance of an abdominal mass in one (625), first observed 222 days after inoculation (Table 2). The animal died during the night on the 226th day, and at postmortem analysis, there was considerable autolysis of tissues. However, two obvious tumors, 5 cm and 3 cm in diameter, were seen in the mesentery, adherent to the adjacent

TABLE 1. *Incidence of lymphomas after inoculation of autologous cells transformed by EBV or cell-free EBV in cottontop marmosets*

			EBV inoculated		Uninoculated controls	
Exp.	Material inoculated	Dose*	Immuno-suppressed	Not immuno-suppressed	Immuno-suppressed	Not immuno-suppressed
1	Autologous EBV-converted cells	1.2–3.0 $\times 10^8$ cells	1/2†	0/2‡	0/1	0/1
2	Cell-free EBV	$10^{4.4}$ 50% transforming units	2/2	1/2	0/1	0/1
		Total	3/4	1/4	0/2	0/2

* Dose: one-third of the inoculum was given by each of the following intravenously, intraperitoneally, and subcutaneously.
† No. of developing lymphomas per no. tested.
‡ One of the two animals in this group died of pneumonia (122 days).

bowel. Three other animals, two inoculated and one control, have had autopsies, from 4–11 months after inoculation. No gross or histologic evidence of tumor was found. One inoculated and one uninoculated marmoset that have remained normal are still under observation.

In the second experiment, all four animals that received cell-free virus developed palpable abdominal tumors; autopsies have been performed on three of them (Table 2). The time of onset was considerably shorter than after inoculation of transformed cells: the two animals (639 and 641) that received EBV and immunosuppressive drugs died after 31 and 34 days, respectively; the third animal, which received no drugs (642), was moribund and was killed on day 49. In each animal the major tumors occupied the mesenteric lymph nodes (Fig. 1). One or two large tumor masses up to 3 cm in diameter were surrounded by numerous smaller nodules. Tumor nodules involved the intestinal wall in two animals, and the liver and lung in one each. The two that received immunosuppressive drugs also had markedly enlarged multinodular spleens. A fourth animal with abdominal masses is still being followed. No tumors have been noted in any of the control animals.

Histopathology. In all instances the tumor tissue contained a nearly homogeneous population of cells, which were randomly oriented. There were large zones of necrosis and hemorrhage. The nucleus of the major cell type was large and reticular in appearance due to marginated chromatin. Many nuclei contained multiple nucleoli, and mitotic figures were frequently observed. The cytoplasm comprised one-third to one-half of the cell diameter and was often vacuolated (Fig. 2).

The same cell type was found displacing nonlymphoid tissue in several organs (see Table 2). The liver of animal 639 demonstrated diffuse necrosis and was widely infiltrated with tumor cells in periportal areas as well as in the center of liver lobules. There was extensive involvement of the gut of two animals, involving all layers, and in number 642 there were areas of intestinal mucosal ulceration and fibrin deposition. In this same animal, which had not received immunosuppressive drugs, mediastinal tumor masses were evident grossly, and in adjacent areas the lung parenchyma was solidified by reticular tumor cells.

The spleens of immunosuppressed animals that had been inoculated with virus (639 and 641) showed identical histologic patterns consisting of complete disruption of normal architecture by necrosis and hemorrhage. No evidence of tumor was found in histologic material from two animals (621 and 626) that received transformed cells and one control marmoset (628).

Serologic Studies. All of the marmosets lacked EBV antibodies, detectable by immunofluorescence and complement fixation tests, before initiation of the experiment. EBV antibodies appeared in all the animals inoculated with autologous transformed cells and in two of the four given cell-free virus. Neither of the two animals that were immunosuppressed and inoculated with virus had detectable antibodies in the last available serum before death obtained 19 and 31 days after inoculation. None of the marmoset sera positive for EBV capsid antibody by immunofluorescence reacted with Raji cells; similarly none of the sera positive for antibody by complement fixation reacted with an antigen prepared from cultured normal marmoset leukocytes. These results confirm the specificity of the serologic reactions.

One animal (625), which died of a tumor 226 days after inoculation, demonstrated EBV antibody titers considerably higher than the three comparable ones with no tumors. Antibody to "early antigen" was also highest in this animal. All control animals failed to develop EBV antibodies. *Herpes saimiri* antibodies were not found in any of the marmoset sera, nor were heterophile agglutinins for sheep erythrocytes detected.

Attempts to Demonstrate Virus or Viral Antigen in Tumors and Organs. These studies are still in progress. No viral particles were seen on electron microscopic examination of tumors from two animals (625 and 642). No consistent cytopathic effects were observed for several weeks after cocultivation of organ and tumor fragments with various monolayer cell cultures. Attempts to establish lymphoblastoid cell lines from the tumors of three animals that died (625, 639, and 641) were unsuccessful, probably because all the materials cultured were obtained some hours after death; the tumors showed extensive necrosis on histologic examination and presumably contained few viable cells.

However, fresh cells from a mesenteric tumor of an animal (642) that was killed have proliferated in organ culture and contain EBV "viral capsid antigen" demonstrable by the indirect immunofluorescence technique. A rare cell (<0.1%)

TABLE 2. *Results of inoculations of autologous EBV-transformed cells or cell-free EBV in individual cottontop marmosets*

Animal	Inoculum*	Immunosuppressed	Outcome	Day of death	Maximum EBV antibody titers (reciprocal)		Pathologic findings	
					IF†	CF‡	Gross	Microscopic
Exp. 1								
625	Autologous EBV-converted cells	Yes	Death	226	320 (222)§	≥512 (222)	Mesenteric tumor	Lymphoma
626	Autologous EBV-converted cells	No	Death	122	80 (28)	128 (48)	Pneumonia Fractured femur	Pneumonia
621	Autologous EBV-converted cells	Yes	Killed	319	40 (49)	64 (164)	Normal	Normal
623	Autologous EBV-converted cells	No	Living	—	80 (77)	128 (48)	—	—
628	None	Yes	Killed	327	<10 (279)	<4 (279)	Normal	Normal
627	None	No	Living	—	<10 (279)	<4 (279)	—	—
Exp. 2								
639	Cell-free EBV	Yes	Death	31	<5 (19)	<4 (19)	Splenomegaly Mesenteric tumors Liver nodules	Lymphoma: Liver infiltrated Spleen & tumor necrotic
641	Cell-free EBV	Yes	Death	34	<5 (31)	<4 (31)	Splenomegaly Mesenteric tumors Gut invasion	Lymphoma: Liver normal Spleen & tumor necrotic
642	Cell-free EBV	No	Killed	49	20 (31)	8 (38)	Mesenteric tumors Gut invasion Lung nodules Mediastinal tumors	Lymphoma: Lung & gut infiltrated
640	Cell-free EBV	No	Living		40 (45)	<4 (38)		
643	None	Yes	Living		<5 (31)	<4 (31)		
644	None	No	Living		<5 (31)	<4 (31)		

* Inoculum: for dose and route of inoculation see Table 1 and *Methods*.
† *IF*, EBV antibodies detectable by the indirect immunofluorescence method, for viral capsid antigen.
‡ *CF*, EBV antibodies detectable by complement fixation.
§ No. in parentheses indicates day of observation.

in impression smears taken directly from the tumor showed immunofluorescent staining with human and marmoset sera positive for EBV antibody and not with corresponding antibody-negative sera. The proportion of antigen-containing cells harvested from organ cultures of this tumor has increased from <0.1% on day 4 to about 5% of cells on day 21 (Fig. 3). Cells shed from organ cultures of lymph node and spleen of two animals (621 and 628) without tumor did not display EBV immunofluorescence antigen.

DISCUSSION

The present experiments demonstrate the capacity of materials containing EBV to induce in marmosets malignant lymphoma, reticulum cell sarcoma type. If evidence accumulated in the future confirms that EBV itself is the tumorigenic agent, this system will provide a direct experimental demonstration of the oncogenic potential of a putative human tumor virus.

Considerable indirect evidence supports the contention that EBV represents the tumorigenic factor in these experiments. Although the total number of animals studied is small, only those inoculated with virus developed tumors. Furthermore, these animals developed lymphomas, the same class of tumor with which EBV has been associated in humans. In the first experiment, in which transformed cells constituted the inoculum, the animal with tumor (625) had a higher EBV antibody titer than was found in the animals without tumors similarly inoculated. This result suggests a parallel to human disease; antibody titers to EBV are higher in patients with Burkitt lymphoma than in control groups (4).

In the second experiment, two animals died with lymphoma but failed to demonstrate EBV antibodies. It seems likely that insufficient time had elapsed for elaboration of detectable antibodies. Antibodies first appeared on days 31 and 45 in the two other EBV-inoculated marmosets. Werner and coworkers have found the first evidence of EBV antibody 5 weeks after inoculation of gibbons with virus (21).

From one tumor obtained from an animal that was killed (642), proliferating cells with EBV antigen have been recovered. Extremely low amounts of viral capsid antigen were detected in imprints from the tumor and no viral particles were found; as the tumor was maintained *in vitro* the proportion of cells with viral antigen increased. Similarly, biopsies of Burkitt tumor do not contain viral capsid antigens or nucleocapsids, but tumor cells express these antigens and produce viral particles after explants are cultivated *in vitro* (22).

No evidence has been obtained suggesting that the cell-free virus stock used in the second series of tests in which all the animals developed palpable tumors contains viral agents other than EBV. Infectivity of the stock assessed *in vitro* by transformation of human cord leukocytes is eliminated by EBV human sera positive for EBV antibody and unaf-

fected by antibody-negative sera. Only herpes-type particles have been seen on examination of fixed thin sections of marmoset cells that were the source of the virus or in negatively stained preparations of semipurified virus (17). Furthermore, in purification experiments, transforming activity *in vitro* was found in gradient fractions that contained enveloped herpes-type particles (Miller, G., Heston, L. & Lipman, M., unpublished results). The stock is free of mycoplasmas, and it does not induce cytopathic effects in any monolayer cell culture tested.

The role of other agents acting independently or in concert with EBV in the induction of tumors remains to be evaluated. Kufe *et al.* have reported that 16 of 21 Burkitt tumors contained polysomal RNA that was complementary to [^{3}H]DNA transcribed from RNA of Rauscher leukemia virus (23). They also found that 9 of 11 Burkitt tumors biopsied contained a 70S RNA component that was associated with RNA-dependent DNA polymerase activity (24). These results suggest that a C-type virus may be present in Burkitt lymphoma. The same investigators have failed to find biophysical evidence for RNA tumor viruses in six lymphoid cell lines derived from normal individuals or patients with infectious mononucleosis. Included among the latter group was line 883L, which was the original human source of virus for the present experiments. It is possible, however, that EBV from line 883L may have activated endogenous RNA tumor viruses in marmosets.

It should be possible to characterize further the oncogenic role of EBV in marmosets by the use of cloned purified virus and by neutralization tests with sera from patients with infectious mononucleosis before their illness and during convalescence. Furthermore, it would be expected that several different EBV strains could induce lymphomas in marmosets. Such strains might include representatives isolated from Burkitt lymphoma as well as EBV obtained from throat washings that have not been passaged *in vitro* (25).

Production of lymphomas in various species of laboratory animals would provide additional evidence for the oncogenic role of EBV. In this respect, we have found that newborn and juvenile rhesus monkeys develop neither EBV antibody responses nor detectable illness after inoculation of the same virus stock that was tumorigenic in marmosets (Miller, G., van Wagenen, G. & Horstmann, D. M., unpublished results). Marmosets may be exceptional in certain of their reactions to EBV since in contrast to human cells, their cells release large amounts of infectious EBV (11). Moreover, as noted above, marmosets are susceptible to induction of lymphoma by two simian herpes viruses, *H. saimiri* and *H. ateles*, which are not oncogenic in their natural hosts, squirrel and spider monkeys.

Since the mechanism of tumorigenesis in marmosets by herpes viruses from other species is undefined, one must entertain several alternative hypotheses. EBV infection may provide an oncogenic stimulus by indirect means, for example, by activating endogenous viruses, or by depressing immunological surveillance mechanisms. EBV may stimulate lymphocyte replication in a "nonspecific" manner analogous to the blastogenic responses to mitogens such as phytohemagglutinin. The EBV inoculum may also contain other oncogenic agents that we have failed to detect. The most direct explanation for the results, however, is that, *in vivo*, EBV transforms marmoset lymphoid elements into malignant cells.

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