LYMPHOMA IN COTTON-TOP MARMOSETS AFTER INOCULATION WITH EPSTEIN-BARR VIRUS: TUMOR INCIDENCE, HISTOLOGIC SPECTRUM ANTIBODY RESPONSES, DEMONSTRATION OF VIRAL DNA, AND CHARACTERIZATION OF VIRUSES*

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In certain New World monkeys Epstein-Barr (EB) viral infection is accompanied by the development of lymphoproliferative disease. Four of eight cotton-top marmosets (tamarins) inoculated with Epstein-Barr virus (EBV) (the Hawley strain) derived from a patient with post-transfusion heterophile-positive mononucleosis, developed malignant lymphoma (1). The EB, Burkitt lymphoma virus strain induced malignant lymphoproliferative disease in an owl monkey, and a cell line recovered from this tumor contained viral DNA (2, 3). Another mononucleosis strain of EBV, Kaplan, was subsequently found to induce lymphosarcoma in cotton-top marmosets (4). The likelihood that EBV was etiologically responsible for the lesions was increased by the demonstration of the EB nuclear antigen in the majority of cells of an imprint prepared from a tumor biopsy (5). Additional evidence for the etiologic role of EBV was the demonstration of the EBV genome directly in tumor cell DNA by the technique of molecular hybridization (6). Finally, in vivo neutralization tests done on small scale demonstrated that leukoviremia and mesenteric adenopathy could be prevented by preincubation of the inoculum with EBV antibody-positive human serum and not by incubation with antibody-free human serum (7).

The present report describes results of inoculations of a larger group of cotton-top marmosets with EBV and delineates the spectrum of histologic responses. Particular emphasis was placed on attempts to demonstrate EB virus and viral DNA in the pathologically involved lymph nodes and to characterize biologic and antigenic properties of virus obtained from the experimental lesions.

* Supported by grants from American Cancer Society (VC-107), U. S. Public Health Service (CA-12055, CA-16638, CA-04568), and contracts NO1CP33336 and NO1CP33272 from the National Cancer Institute; Damon Runyon-Walter Winchell Cancer Fund (DRG-1147); and John A. Hartford Foundation (SFB118) (methodenforschung zur Früherkennung des Krebses).

‡ Investigator of the Howard Hughes Medical Institute.

§ Recipient of Career Award 5-K6-AI-22, 683 from the National Institutes of Health, U. S. Public Health Service.

'Abbreviations used in this paper: EA, early antigen; EBNA, Epstein-Barr nuclear antigen; EBV, Epstein-Barr virus; VCA, viral capsid antigen.'
Table I

Summary of Inocula

| Exp. | Description of inoculum                           | Transforming titer/0.2 ml | Volume ml |
|------|---------------------------------------------------|---------------------------|-----------|
| 1    | Autologous EBV-converted cells (1-3 x 10^8 cells)* | NA                        | 1.0       |
| 2    | Cell-free EBV; B95-8 strain; E68 stock†           | 10^4             | 1.0       |
| 3    | EBV from throat washing — Marmoset adapted       | 10^5             | 1.0       |
|      | Direct from patient‡                              | 10^4             | 1.0       |
| 4    | EBV from cloned cells; F-12 clone 29             | 10^2             | 3.0       |
| 5    | Cell-free EBV; B95-8 strain; K41 stock¶          | 10^4             | 5.0       |
| 6    | Cell-free EBV; B95-8 strain; K86 stock¶          | 10^4             | 8.5       |
| 7    | Cell-free EBV; L39-2 stock**                     | 10^4             | 5.5       |

* Reference 1.  
† Reference 8.  
‡ Reference 9.  
¶ Passed in vitro for 11 mo.  
* Cells revived from freezer after 13 mo.  
** From a cell line obtained from a tumor of animal 667 in experiment 5.

Methods

Marmosets. Wild cotton-topped tamarins, Saguinus oedipus, were a mixture of juveniles and adults, males and females. They were regularly infected with two parasites, microfilaria and burrowing acanthocephalid nematodes, Prosthenorcis elegans, found in the terminal small intestine. Death from intestinal perforation, peritonitis, and abscess formation due to Prosthenorcis occurred in several animals before inoculation with EBV. During observation of several months to 3 yr certain animals developed clinical and radiologic evidence of pathologic fractures which responded to treatment with vitamin D and ultraviolet light.

EBV and Control Inocula. Several different types of EBV inocula, listed in Table I, were employed in seven experiments involving 20 animals. Inocula were filtered through a 0.8 μm Millipore filter and were shown to contain biologically active EBV as determined by the assay based on immortalization of human umbilical cord leukocytes (8). The virus titer ranged from 10^5 to 10^4 TDs/0.2 ml. 16 animals (experiments 1, 2, 4-7) received the Hawley strain (8). The other four animals received virus from throat washings of two other patients, one with mononucleosis and one an inapparent excretor who was an immunosuppressed renal transplant recipient (9).

Six uninoculated and four mock-inoculated animals were observed in parallel with inoculated animals. Two of these received spent medium from the "Raji" Burkitt lymphoma line which does not contain EB virions; one animal received extracellular fluid from the "P3J-HR-1" Burkitt lymphoma line which contained EB virions biologically active only when measured by induction of early antigen in nonproducer human lymphoblastoid cell lines and inactive by transformation (10). A fourth animal received fresh tissue culture medium RPMI 1640 supplemented with fetal calf serum and antibiotics.

Each inoculation was made on a single day by a combination of the intravenous, intraperitoneal, and intramuscular routes. One animal (experiment 6) was also given EBV by means of spray into the pharynx.

Assays for EB Virus. The number of viral particles was estimated by electron microscope counts of negatively stained preparations concentrated 100-fold by ultracentrifugation (11). Biologically active virus was measured by morphologic transformation of human umbilical cord leukocytes (8). Virus of the transforming biotype was also measured by stimulation of DNA synthesis in human umbilical cord leukocytes, as judged by the incorporation of [3H]TdR (12). Virus from the P3J-HR-1 line of Burkitt lymphoma cells was assayed by the appearance of "early antigen" (EA) in superinfected Raji cells. The number of EA-positive cells was determined 3 days after addition of virus, by indirect immunofluorescence using an anti-EA positive human reference serum (13).
**Derivation of Cell Lines.** Leukocytes from heparinized peripheral blood were separated by addition of dextran at a final concentration of 2%, and the leukocytes were placed in stoppered tubes at a concentration of $1-2 \times 10^6$/ml. Cell lines from spleen and lymph nodes were established by placing minced fragments in a tube with medium. Alternatively, the fragments were placed on a 2-cm circle of teabag paper atop stainless steel grids. The grid was put in 3 ml of medium in a 35-mm Petri dish which was incubated in a humidified atmosphere of 5% $\text{CO}_2$ and air.

**Detection of EBV-Associated Antigens in Lymph Nodes and in Cell Lines.** Two antigens were sought, the viral capsid antigen (VCA) and the EB nuclear antigen (EBNA). Reference human sera with and without anti-VCA and anti-EBNA were used. The techniques of indirect immunofluorescence (14) and of anti-complement immunofluorescence (15) were employed.

**Detection of EBV DNA.** Tumors were tested by the technique of DNA/DNA reassociation. Cellular DNA was extracted from tumor tissues (16). EBV DNA was isolated from P3HR-1 virus particles, purified by centrifugation in CsCl, and labeled in vitro by the nick-repair method (17). Reassociation kinetics were performed as described previously (18).

The method of cRNA/DNA membrane hybridization was used to test for the presence of the EBV genome in cell lines. $3\text{H}$-cRNA with a sp act of about $10^6$ cpm per $\mu$g was prepared from two EB virus producer lines, B95-8 and P3J-HR-1; sera simultaneously titered on the two cell lines demonstrated equivalent titers. Antibodies to early antigen were measured by reaction with Raji cells which had been superinfected with P3J-HR-1 virus.

**Neutralizing Antibodies.** Virus-neutralizing antibodies were determined by the technique of inhibition of stimulation of DNA synthesis (19). Antibodies were measured against three EBV strains, the homologous strain with which the animals were inoculated and two BL-derived strains.

**Results**

**Incidence of Lesions (Table II).** 6 of 20 animals developed large tumors of mesenteric lymph nodes. In three instances the tumors were highly necrotic. In three instances (642, 655, 667) the cytology was well preserved and resembled diffuse malignant lymphoma of the reticulum cell or immunoblastic type of man. One animal (656) developed extensive lymphoreticular hyperplasia evident at the autopsy performed when the animal was moribund. In two other animals (650, 644) there was extensive lymphoreticular hyperplasia in mesenteric lymph nodes removed by surgical biopsy 8 wk after inoculation; however, these lesions regressed. At autopsy 9 and 18 mo after inoculation, respectively, the lymph nodes were normal. Seven marmosets demonstrated no pathologic evidence of lymphoproliferative disease at autopsies 17 wk to 22 mo after inoculation. One animal died 8 days after inoculation from abscess formation, intestinal perforation, and peritonitis due to Prosthennorctis. Three animals are still living and show no evidence of disease.

Of six animals that developed malignant lymphoma, one received autologous EBV-converted cells and five received cell-free virus. Four of the six animals with tumors had received immunosuppressive drugs. Lymphoma was observed in an animal given virus obtained from a single cell clone. Transient hyperplasia was observed in one of two animals given EBV obtained directly from a throat washing and never placed in vitro (9).
### Table II

A Listing of 20 Cotton-Top Marmosets Inoculated With Transforming EB Virus

| Exp. | Animal no. | Immunosuppressive drugs | Duration of observation after inoculation | EB virus recovery (wk)* | Maximum antibody titer to VCA | Maximum antibody titer to EA | Histologic result (lymph nodes) |
|------|------------|--------------------------|-------------------------------------------|------------------------|-----------------------------|-----------------------------|---------------------------------|
| 1    | 625        | 3                        | 32 wk                                     | -                      | 640                         | 160                         | Necrotic tumors                 |
| 626  | 0          | 0                        | 17 wk                                     | ND                     | 80                          | <10                         | Normal                          |
| 621  | 3          | 21                       | 45 wk                                     | -                      | 40                          | 20                          | Normal                          |
| 623  | 0          | 0                        | 36 mo                                     | -                      | 80                          | 80                          | Normal                          |
| 2    | 639        | 3                        | 31 days                                   | -                      | <5                          | ND                          | Necrotic tumors                 |
| 641  | 3          | 21                       | 34 days                                   | -                      | <5                          | ND                          | Necrotic tumors                 |
| 640  | 0          | 0                        | 22 mo                                     | -                      | 20                          | 5                           | Normal§                         |
| 642  | 0          | 0                        | 49 days + (7)                             | 20                     | 5                           | Immunoblastic lymphoma       |
| 3    | 645        | 3                        | 74 days                                   | -                      | 10                          | ND                          | Normal                          |
| 646  | 3          | 0                        | 8 days                                    | -                      | <10                         | ND                          | Abscess                         |
| 649  | 3          | 0                        | >28 mo                                    | -                      | <10                         | ND                          | Not done (living)               |
| 650  | 3          | 0                        | 9 mo                                      | -                      | 20                          | ND                          | Hyperplasia§                    |
| 4    | 654        | 0                        | 16 mo                                     | -                      | 20                          | <5                          | Normal                          |
| 655  | 0          | 0                        | 49 days + (7)                             | 10                     | <5                          | Immunoblastic lymphoma       |
| 5    | 643        | 0                        | 17 mo                                     | -                      | <10                         | <5                          | Normal                          |
| 644  | 0          | 0                        | 18 mo + (9)                               | 20                     | <5                          | Hyperplasia                   |
| 657  | 3          | 14                       | 57 days + (8, 9)                           | 80                     | 80                          | Immunoblastic lymphoma       |
| 660  | 3          | 14                       | >24 mo                                    | ND                     | 20                          | <5                          | Not done (living)               |
| 6    | 656        | 0                        | 38 days + (5)                             | 80                     | <5                          | Extensive hyperplasia†        |
| 7    | 671        | 2                        | >19 mo + (3, 4, 5)                         | 40                     | 5                           | Not done (living)             |

* After inoculation.
§ Animal died.
† Hyperplasia present at biopsy approximately 2 mo after inoculation but not at autopsy.
¶ Present at autopsy when a moribund animal was sacrificed.

The entire spectrum of pathologic responses including no infection, inapparent infection (development of antibodies only), transient hyperplasia, and malignant lymphoma occurred in experiment 5 in which four animals received the identical inoculum.

6 of 10 uninoculated or mock inoculated animals were autopsied. All failed to demonstrate lymphoreticular lesions. None developed antibodies and no virus was recovered from their lymph nodes, spleen, or peripheral blood. Of the 10 control marmosets 4 had received 3 wk therapy with immunosuppressive drugs.

**Histological Findings by Light Microscopy.** In uninoculated animals or in those that remained normal after inoculation, the lymph nodes were small, with well-defined germinal centers and interfollicular zones (Fig. 1). The follicles contained few cells in mitotic division. The borders of the follicles were crowded with closely packed small lymphocytes. In an occasional animal, irregularity of follicle size and confluence of interfollicular lymphoid accumulations indicated mild focal hyperplasia which was not considered abnormal.

Hyperplasia of a pronounced degree was a prominent finding in the lymph nodes of three inoculated animals (Fig. 2). This change varied from a slight increase in size and irregularity of germinal centers with increase and crowding of lymphocytes in focal areas of nodes, to massive confluence of follicles with...
abundant mitotic figures, accompanied by dense lymphocytic cellularity of the interfollicular zones. Mitoses were present in the latter areas as well as numerous small foci of large reticular of histiocytic cells giving rise to a "starry sky" appearance. Although occasionally such changes suggested the histological appearance of malignancy, uniformity of cellular and nuclear size and shape and some preservation of node architecture made such an interpretation unlikely. Normal, hyperplastic and even malignant lymphoid nodules in the same animal were not uncommon (Fig. 3). In the germinal centers of hyperplastic nodes the predominant cells exhibited large, vesicular, uncleaved nuclei. Mitotic figures were abundant.

In the six animals with tumors, the gross lesions presented as nodules, 0.5-2.0 cm in diameter, in the mesenteries of the small intestines. Some of these nodules were in close proximity to the gut and were locally invasive (Fig. 4). Histologically the tumor masses were often partially or totally necrotic. Where the tissues were preserved the component cells were arranged in irregular cords and masses, obliterating the basic lymph node architecture. (Figs. 4 and 5). The replacement of nodal architecture occurred through enlargement and confluence of follicular structures. Nodes with marked follicular and interfollicular hyperplasia were present along with the definitely malignant tumor nodules. Acute inflammatory changes consisting of edema, hemorrhage, and accumulations of polymorphonuclear leukocytes were also observed in several sections of tumor. In one animal, with inflammatory tissue changes and hyperplastic nodes, cross-sections of a parasitic nematode were observed.

The tumor cells were arranged without polarization or organoid structure. The majority were large, polygonal in shape, with abundant cytoplasm and with
FIG. 2. Histology of hyperplastic marmoset lymph nodes (marmoset no. 650): (a) × 40, (b) × 250. The nodes are enlarged (10-15 mm) and congested. There is increase in number, size, and irregularity of follicles. Follicular cells have large vesicular, predominantly uncleaved, nuclei (b). Nucleoli are often present. Mitoses are frequent. In the interfollicular regions there are, besides the masses of small lymphocytes, many large nucleated cells similar to the large cells of the follicles. The architecture of the node is intact, with an intact capsule.

FIG. 3. Histology of lymph nodes with combined hyperplasia and lymphoma (marmoset no. 655): (a) × 40, (b) × 250. The nodes are intensely hyperplastic with large cortical follicles and crowding of interfollicular lymphocytes (a). In other nodules the follicular structure of the node is lost and is replaced by diffuse masses of cells with large vesicular nuclei (b). There are a fair number of even larger cells with cleaved or partitioned nuclei. Large irregular nuclei. The nuclei always contained one or more clearly observed nucleoli. They were predominantly uncleaved. Mitotic figures were plentiful in all tumors. Admixture of small and medium-sized lymphocytes in varying numbers was present. Large, clear cells of macrophage type were often found scattered through intensely hyperplastic interfollicular areas.
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Fig. 4. Histology of lymph nodes with malignant lymphoma (marmoset no. 642): (a and b) \( \times 40 \), (c) \( \times 100 \), and (d), \( \times 1,000 \). A nodule involving the bowel wall is shown (a). Interspersed among zones of coagulation necrosis (b) are solid cores of large, irregularly shaped cells showing little orientation (c). These cells have abundant cytoplasm and large, irregular nuclei with prominent nucleoli (d). Their cytoplasmic borders form a mosaic pattern. Mitotic figures are abundant, some with abnormal configurations. There are a fair number of cleaved, or folded, nuclei, but these comprise a minority of those present.

Ultrastructure of Tumor Cells (Fig. 6). Two types of cells different in nuclear and cytoplasmic detail were found in the lymphoma of one marmoset (642). The nucleus of one cell type (Fig. 6a and b) had prominent heterochromatin and nuclear pores; whereas there was sparse heterochromatin and few pores in the nucleus of the other cell type (Fig. 6c and d). Many of the features of the cell with coarse heterochromatin were preserved in cell lines cultivated in vitro from the tumors.

Antibody Responses

Antibodies to Viral Capsid Antigen (Fig. 7). Of 20 animals given transforming EBV 14 developed antibodies to VCA. Four of the six animals without
FIG. 5. Histology of diffuse malignant lymphoma compatible with immunoblastic sarcoma (marmoset no. 667): (a and b) x 40, (c) x 250, and (d) x 1,000. An encapsulated nodule in the mesentery is devoid of normal lymph node structure (a). It contains diffuse masses of large lymphoid cells. Areas of the node display a "starry sky" appearance (b). The masses of tumor cells are closely packed without orientation. Individual cells have large, vesicular nuclei with prominent nucleoli (c and d). Some nuclei have a plasmacytoid arrangement of chromatin. There are scattered mitotic figures. The majority of the cell nuclei are uncleaved, though irregular.

detectable anti-VCA died or were sacrificed within the first 7 wk after inoculation; of these seronegative animals which died early, three had tumors and one had an abscess. The remaining two seronegative animals survived for many months and failed to develop clinical or pathologic signs of lymph node disease. They presumably were never infected by the virus.

Antibodies were first detected about 2-3 wk after inoculation of autologous cells and from 4 to 12 wk after inoculation of cell-free virus (5). Once anti-VCA appeared in the serum it persisted thereafter for the remainder of the observation period. In only one instance was a significant change in titer observed. An
animal subsequently found to have lymphoma showed a rise in antibody titer between the 3rd and 6th mo after inoculation.

The maximum titer of anti-VCA was higher in animals given autologous EBV-converted cells than in those given cell-free virus (Fig. 7). The animal that
developed lymphoma after injection of autologous cells demonstrated the highest anti-VCA titer (1:640). Among the group given virus the two highest anti-VCA titers were observed in animals that developed lymphoma or extensive hyperplasia.

**Antibodies to early antigen and EB nuclear antigen.** Sera from 14 marmosets were tested for antibody to "early antigen" at the time when anti-VCA was maximum. Three of four animals inoculated with cells showed anti-EA; the highest anti-EA titer was seen in the animal with lymphoma. Among marmosets given cell-free virus, only one animal, which developed lymphoma, had a high anti-EA titer. The antibodies observed were directed against the diffuse D component of the early antigen. A low level of antibody to EBNA (1:10) was detected only in one animal with lymphoma (no. 667).

**Neutralizing antibody.** Preinoculation and late postinoculation sera of two animals were tested for their ability to inhibit stimulation of DNA synthesis by three transforming strains of EBV (Fig. 8). Preinoculation serum failed to neutralize either the Hawley EBV strain with which the animals were inoculated or two other virus strains from Burkitt lymphoma. One animal with inapparent infection and a moderate anti-VCA antibody response developed neutralizing antibodies against the Hawley strain and the Olare strain. The other animal with lymphoma and a high anti-VCA titer developed neutralizing antibodies to all three strains. When preillness serum from the animal with lymphoma was mixed with the three viruses, the extent of DNA stimulation by the viruses was comparable to that produced by their admixture with human antibody-negative serum or with medium (Fig. 8). Late convalescent serum obtained 224 days after inoculation produced about a 100-fold inhibition of DNA stimulation by the Nyevu (BL) virus, about a 10-fold reduction in stimulation of DNA synthesis by the Olare (BL) virus, and a 10,000-fold reduction in stimulation of DNA synthesis by the homologous virus (Hawley) with which the animal was inoculated.

**Recovery of and Characterization of Virus**

**Direct examination of lymph nodes.** We failed to detect virus particles in thin sections of lymph nodes from three animals with lymphoma and one control animal. Imprint smears prepared from five lymph nodes, three with lymphoma and two with hyperplasia, did not show evidence of viral capsid antigen. EB nuclear antigen was seen in 100% of cells of one lymphoma and in a smaller proportion of cells from a node with hyperplasia (Fig. 9).

**EBV DNA in tumors.** EBV DNA was detected in lymphomas of three animals. (Table IV). It was found in both a biopsy and autopsy specimen from

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**Fig. 6.** Ultrastructure of cells found in lymphoma of CTM 642. Two types of cells were found. The nucleus of the cell type shown in (a) and (b) has dense chromatin and nuclear pores are easily recognized (arrowheads). There are both free ribosomes and sparse rough endoplasmic reticulum. The surface of the cells is highly elaborated with microvilli and pseudopods. Neither Golgi apparatus nor lysosomes are prominent. Cells are surrounded by a matrix of collagen. The cells in (c) and (d) have distinctly different nuclear and cytoplasmic characteristics. The chromatin is dispersed and noncondensed, and few nuclear pores are identified. The cytoplasm has few free ribosomes; instead there is massive elaboration of rough endoplasmic reticulum. The channels (d) appear swollen with a granular material. Microvilli formation is not as prominent as in cells shown in (a) and (b). Bars represent 1 μm.
Animals Given
Autologous
EBV-converted
Cells

Animals Given
Cell Free
Virus

Titer
of Anti-
VCA
and
Anti-EA

640
320
160
80
40
20
10
5
<5

Lymphoma Normal Lymphoma Hyperplasia Normal

Fig. 7. Maximum antibody titers to EB VCA and EA in inoculated marmosets with different histologic responses. (●) anti-VCA; (○) anti-EA.

one animal (642). There were approximately 8–30 genome equivalents of EBV DNA per cell. A barely detectable level of EBV DNA (about three genomes/cell) was found in the lymph node of an animal with hyperplasia. No EBV DNA was detected in a lymph node from an animal (646) that died of an abscess.

Cell Lines. Lymphoblastoid lines were established from mesenteric lymph nodes, spleen, mediastinal lymph nodes, or blood leukocytes of six animals from 3 to 9 wk after inoculation (Table III). In one instance a virus shedding line was recovered from an animal (no. 655) that never developed detectable antibodies to VCA. All but one of the lines produced virus. About 5–10% of the cells made viral capsid antigen. Typical EBV-herpes virions were found in 100-fold concentrated extracellular fluids from the three lines studied. A total of eight cell lines from five animals were found to release biologically active extracellular EBV with titers of 1,000–30,000 transforming units per 0.2 ml. All viable cells in each line contained EBNA.

CytoLOGIC FEATURES OF CELL LINES DERIVED FROM TUMORS. The cell lines adhered loosely to glass and plastic. Receptors for serum complement (EAC') or the Fc fragment of IgG (EA-7S) were not detectable in a cell line from a marmoset tumor (20).

Leukocyte-Associated Viremia. Continuous lymphoblastoid cell lines were derived from peripheral blood leukocyte cultures of two animals, one with hyperplasia in the 9th wk after inoculation and one with inapparent infection in the 3rd through 5th wk after inoculation. In the latter instance (see Fig. 10) cell lines were not obtained after the 5th wk. Antibody to viral capsid antigen was
not detected until the 12th wk after inoculation. In this experiment another marmoset given an equal number of virions from the nontransforming P$_{38}$-HR-1 virus strain failed to develop leukoviremia or antiviral antibodies.

**EBV-DNA in Cell Lines (Table IV).** EBV DNA was found in two cell lines derived from lymphomatous nodes placed in culture 7 and 9 wk after inoculation, respectively. The amount of hybridization indicated an "average" of 140 and 205 genome equivalents per cell, a finding consistent with both cell lines being virus producers.

**Neutralization of Viruses Released from Two Lymphoma Cell Lines.** In one experiment the ability of antibody-positive and antibody-negative human sera to neutralize morphologic transformation was measured. In a second experiment the ability of such sera to inhibit stimulation of DNA synthesis in human umbilical cord blood leukocytes was examined. In both experiments the viruses obtained from the lymphomas were neutralized by antibody-positive human sera and not by antibody-negative human sera (Table V).
Fig. 9. EB nuclear antigen in direct imprint of lymph node from marmoset 667 with lymphoma.

Table III
Establishment of Cell Lines from Inoculated Animals and Demonstration of EBV in Extracellular Fluids

| Animal | Exp. | Diagnosis       | Sources of cell lines       | Weeks after inoculation when cell line was obtained | Extracellular EB virions released from cell lines |
|--------|------|-----------------|----------------------------|-----------------------------------------------|-----------------------------------------------|
|        |      |                 |                            |                                               | No. particles/ml of 100 x concentrated supernatant fluid |
| 642    | 2    | Lymphoma        | Mesenteric node; spleen    | 7                                             |                                               |
| 655    | 4    | Lymphoma        | Mesenteric node (biopsy)   | 7                                             | $>10^4$                                       |
| 644    | 5    | Hyperplasia     | Mesenteric node (biopsy);  | 9                                             | $3 \times 10^8$                               |
|        |      |                 | peripheral blood           |                                               | $10^9$                                       |
| 667    | 5    | Lymphoma        | Mesenteric node (biopsy);  | 8, 9                                          | $2 \times 10^8$                               |
|        |      |                 | mediastinal node           |                                               | $10^8$, $10^9$, $10^{10}$                      |
| 656    | 6    | Extensive hyperplasia | Mesenteric node; spleen   | 6                                             | $5 \times 10^8$                               |
| 671    | 7    | Inapparent infection | Peripheral blood          | 3, 4, 5                                       | $10^4$, $10^5$                               |

Discussion
Parallels Between Experimental and Human Infection. The lymph nodes of cotton-top marmosets inoculated with EBV are the seat of lymphoreticular proliferations ranging from mild to intense hyperplasia and in six instances to definite lymphoma formation. The tumors, on the basis of their predominant cellular characteristics, would formerly have been classified as reticulum cell or histiocytic lymphomas. However, recent advances have stressed the probability that immunologically or virally transformed lymphoid cells of follicular origin are involved in lymphoma formation. Composed mainly of large polygonal cells with oval vesicular, uncleaved nuclei, with distinct nucleoli and with
TABLE IV
Demonstration of EBV DNA in Lymph Nodes and in Cell Lines of EBV-Inoculated Cotton-Top Marmosets

| Animal | Histologic diagnosis     | EBV genome equivalents/ cell | cpm ³H-cRNA hybridized/50 µg cell DNA$ | EBV genome equivalents/ cell |
|--------|---------------------------|------------------------------|---------------------------------------|------------------------------|
| 625    | Lymphoma (necrotic)       | 30                           | 655 Lymphoma (immunoblastic)           | 4,377 140                     |
| 639    | Lymphoma (necrotic)       | 30                           | 667 Lymphoma (immunoblastic)           | 7,356 205                     |
| 642    | Lymphoma (immunoblastic)  | 8; 10|||                                |
| 656    | Hyperplasia               | 3                            |                                       |                              |
| 646    | Normal                    | 0                            |                                       |                              |

* Technique of DNA/DNA reassociation.
† Technique of cRNA/DNA membrane hybridization.
§ The cpm hybridized to 50 µg HEp2 DNA was 430; to 50 µg Raji cell DNA 2,206.
|| One determination on a biopsy, one on an autopsy 1 mo apart.

abundant cytoplasm, these tumors would fit either the large, uncleaved follicular lymphoma or the immunoblastic sarcoma categories of human lymphomas as classified by Lukes and Collins (21). The presence in some areas of the involved nodes of intense lymphoid hyperplasia with scattered "reactive" phagocytes simulates a histologic pattern found in Burkitt's lymphoma in man.

Several aspects of the pathogenesis of the experimental infection of marmosets mimics natural infection of man by EBV. Infection of the marmoset results in permanent acquisition of antibodies that persist for the remainder of the life of the animal. Virus can be readily recovered from blood leukocytes of infected animals for several weeks after inoculation and from lymph nodes as late as 9 wk after inoculation. These findings are compatible with continued viral persistence after the initial infection.

Virus particles are not found in the marmoset tumors per se which nonetheless contain the EBV nuclear antigen and the EBV genome. Once the tumor cells are placed in culture, mature EBV is found in the cells which proliferate in vitro. This finding is analogous to Burkitt lymphoma. Viral DNA and EBNA are found directly in tumor biopsies and mature virus in the derivative cell lines (22).

An additional parallel with human infections is that marmosets with lymphoma and hyperplasia had higher anti-VCA and anti-EA titers than those whose infections were clinically inapparent (23). This finding presumably reflects a larger number of virus-infected cells in the tumor-bearing individuals. The more rapid appearance and higher titer of anti-viral antibodies after injection of transformed cells than after virus also probably reflects a larger antigenic mass.

Some differences between infection of humans and marmosets with EBV are
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Fig. 10. Leukoviremia and development of antibody to viral capsid antigen in a marmoset (no. 671) which developed inapparent infection after inoculation of EBV derived from a marmoset lymphoma cell line. For comparison leukoviremia and anti-VCA responses failed to occur in a marmoset (no. 672) given the nontransforming P3HR-1 EBV strain. Anti-VCA was measured against two antigens (O) P3HR-1; (●) B95-8.

also notable. In the marmoset the peripheral lymphoid system is not usually involved. We have been unable to detect the heterophile antibody response in marmosets, and the anti-EBNA response is weak or absent. These important differences in serologic responses may reflect differences in events leading to detection and elimination of virus-transformed cells. The latent period between infection and tumor is short in the marmoset (several weeks to months) and longer in the case of Burkitt lymphoma. Furthermore, the ratio of lymphoma to infection, even in nonimmunosuppressed marmosets, is high.

Characteristics of the Virus Found in the Tumors. The virus identified in the experimentally infected marmosets is human EBV on the basis of a combination of biochemical, antigenic, and biologic markers. All of the sequences in the B95-8 EB virus, which was the original inoculum for most of the experiments, have now been shown to be present in the prototype human P3HR-1 viral DNA by techniques of DNA reassociation kinetics, although the P3HR-1 virus
apparently contains sequences not found in B95-8 (24). Both marmoset lymphomas and derivative cell lines contain the EBV genome detectable by molecular hybridization. The herpes-like virus derived from the tumors shares all of the biologic properties of the EB virus in the inoculum: it immortalizes and stimulates cellular DNA synthesis in human lymphocytes, and in one instance (experiment 7) caused experimental infection (though not tumors) of marmosets on further passage. The EBV recovered from the experimental lesions shows serologic cross-reaction with the virus inoculated in viral capsid antigens, nuclear antigens, and viral surface antigens.

Neutralization tests between marmoset sera and various EBV strains (Fig. 9) showed that antibodies developed against both mononucleosis and BL strains, but with greater activity against the former. Further work is needed to determine whether sera obtained from experimentally infected marmosets will differentiate EBV strains.

There is no evidence that there is a special strain of EBV which is oncogenic in marmosets or in man, nor has this hypothesis yet been experimentally excluded. Two mononucleosis-derived strains (Hawley and Kaplan) induce malignant tumors in marmosets. The strain that we employed in the majority of experiments induced the entire spectrum of pathologic lesions. Virus was recovered from a tumor, propagated in vitro, and then returned to another marmoset that developed inapparent infection with leukoviremia, but without detectable lymph node enlargement. This finding suggested that a virus found in a tumor is not invariably oncogenic.

One EBV strain from Burkitt lymphoma P2J-HR-1, is incapable of inducing experimental disease or even infection, as evidenced by appearance of antibodies in marmosets (Fig. 10) (25). Current laboratory stocks of this strain cannot cause transformation of human or primate cells in vitro. This finding suggests that cell transformation is an important early event in the pathogenesis of experimental nonhuman primate infection, as well as lymphoproliferation.
Special Susceptibility of the Cotton-Top Marmoset to Viral Oncogenesis. The cotton-top marmoset is highly susceptible to oncogenesis by herpes viruses from other primate species (26). Thus the tumors induced by EBV might be an example of "crossed species virulence." However, unlike Herpes saimiri EBV is associated with lymphoproliferative disease in both its natural and experimental hosts. There are also marked differences in the nature of the experimental disease induced in the marmoset by EBV and "crossed species" oncogenic viruses such as Herpes saimiri. In the latter case all the inoculated animals develop a rapidly fatal malignant lymphoma and occasionally leukemia. EBV infection of marmosets produces a quite different picture in which the outcome is variable, and there is a spectrum of pathologic responses.

Another hypothesis is that marmosets are deficient in immunologic mechanisms necessary for surveillance and elimination of virus-transformed cells. It may be relevant that marmosets are chimeric twins. However, three of four animals given autochthonous EBV-converted cells remained normal and thus were able to eliminate the cells transformed in vitro. The fourth animal developed a tumor only after a long latent period; the lesion could have been due to retransformation of cells in vivo by virus released from the inoculated cells.

Marmosets develop antibodies to viral capsid antigen slowly after inoculation of virus (5). Some animals with lymphoma failed to develop antibodies even 8-10 wk after inoculation at a time when virus could be detected in lymphoid organs. A similar tardiness of anti-viral antibody responses has been noted in cotton-top marmosets inoculated with Herpes saimiri viruses (27). In this connection, several human families have recently been described in which failure to develop antibodies after primary EBV infection is accompanied by disseminated fatal lymphoreticuloproliferative disease (28).

The increased susceptibility of the marmoset may derive from special features of the EBV-transformed cell per se as well as from abnormal host-tumor cell interactions. Unlike human cells transformed in vitro by EBV, marmoset transformants are productive of relatively large amounts of mature extracellular virions (8). Virus production by marmoset cells may permit virus spread and more extensive transformation of susceptible cells in the experimentally infected host.

Marmoset cells transformed in vitro or in vivo are cytologically different from EBV-converted human cells. Marmoset cells adhere to glass or plastic and have a higher frequency of polykarocytosis than do EBV-converted human cells. Marmoset transformants, including cell lines derived from malignant lymphoma, express neither the receptors for complement nor the receptors for the Fc fragment of immunoglobulin G; C' receptors are present on human cell lines (20). Cells with the C' and Fc receptors are present in marmoset peripheral blood in approximately the same proportion as in human blood. These findings and other experimental evidence suggest that the marmoset cell loses some of its B-lymphocyte surface characteristics after immortalization. Such cell surface differences may enhance the oncogenicity of the transformed marmoset cells.

Summary

6 of 20 cotton-top tamarins (Saguinus oedipus) inoculated with Epstein-Barr virus (EBV) developed diffuse malignant lymphoma resembling reticulum cell...
or immunoblastic sarcoma of man. Hyperplastic lymphoreticular lesions were induced in three additional animals; in two instances the hyperplastic lesions regressed. Inapparent infection with development of antibody occurred in eight animals. In two animals there was no evidence of EBV infection. One animal died in the first week after inoculation of parasitic infection. 10 animals uninoculated or mock-inoculated developed neither lymphoproliferative disease nor antibody.

The malignant lymphoma appeared to arise from a cell with an uncleaved vesicular nucleus found in the center of the germinal follicle. The prominent cytologic features of this cell were extensive formation of rough endoplasmic reticulum and elaboration of the cytoplasmic membrane with microvilli. Cell lines derived from these tumors did not have receptors for complement, IgFc, or sheep erythrocytes, and the cell lines adhered to glass and plastic.

EB nuclear antigen was found in imprints of two lymph nodes, one with lymphoma and one with hyperplasia. EB virus DNA was detected directly in the tumors of three animals and in cell lines from two lymphomas. Typical herpes virus particles were found in supernatant fluids from cell lines obtained from lymph nodes with tumors and hyperplasia, as well as in lines derived from blood leukocytes of marmosets with inapparent infection. These virus preparations had the biologic property characteristic of EBV, namely, stimulation of cellular DNA synthesis and immortalization of human lymphocytes. The virus derived from two cell lines was neutralized by reference human sera with EBV antibody and not by antibody-negative human sera. The virus derived from the experimental lesions is thus indistinguishable from human EBV.

The marmoset has enhanced susceptibility to oncogenesis by EB virus. Among identified factors which may play a role in the heightened tumorigenicity of EB virus in this species are the increased production of virus by transformed cells and the absence of membrane receptors for complement or IgFc on transformed cells.

We are grateful for help from G. W. Smith with photomicrography, from M. Lipman with electron microscopy, and for cheerful technical aid from S. Smith, V. Thompson, and W. Webb.

Received for publication 16 August 1976.

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