somes on the nascent pre-mRNA might be expected to interfere with proper folding of the RNA and therefore with splicing in vivo. Furthermore, the intrinsic rate of self-splicing is not high. It occurs with a half-life of about 1 minute at saturating concentrations of guanosine in vitro (*5*), a time that might be substantial relative to the mRNA half-life. It should be noted that the rate and the absolute efficiency of splicing in vivo are unknown. Because the α-donor gene is present on a multiple-copy plasmid, even inefficient splicing might give full complementation of β-galactosidase activity.

Thus RNA splicing, generally considered to be a eukaryotic phenomenon, can take place in a prokaryote. In addition to the artificial example reported here, there may be naturally occurring cases; there is now evidence for splicing of phage T4 thymidylate synthase mRNA (*15*), and some archaebacterial transfer RNA's contain insertions that may be intervening sequences (*16*). It is not yet clear why RNA splicing is not a more common feature of gene expression in *E. coli*, and whether it might be more common in other prokaryotes.

On a practical level, a simple color assay can now be used as a screen for splicing-deficient mutants generated by either random or site-specific mutagenesis. The system also should allow selection for second-site revertants, which are most valuable for identifying or confirming secondary and tertiary structures that facilitate splicing (*17*). Such studies are essential for establishing the structure-function relationships of self-splicing RNA.

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Individual Tumors of Multifocal EB Virus–Induced Malignant Lymphomas in Tamarins Arise from Different B-Cell Clones

Abstract. *Cotton-top tamarins were inoculated with sufficient Epstein-Barr virus to induce multiple tumors in each animal within 14 to 21 days. The tumors consisted of large-cell lymphomas that contained multiple copies of the Epstein-Barr virus genome and generated Epstein-Barr virus–carrying cell lines showing no detectable consistent chromosomal abnormality. Hybridization of tumor DNA with immunoglobulin gene probes revealed that each lymphoma was oligo- or monoclonal in origin and that individual tumors from the same animal arose from different B-cell clones. Thus the virus induced multiple transformation events in tamarins in vivo to cause malignant tumors resembling the Epstein-Barr virus–associated lymphomas of patients with organ transplants.*

The strong links between Epstein-Barr virus (EBV) and endemic Burkitt's lymphoma suggest that the virus activates the complicated chain of events that cause the tumor (*1, 2*). EBV was recently implicated in the etiology of the lymphomas seen with undue frequency in immunosuppressed recipients of human allografts (*3, 4*). These lymphomas in transplant patients have been controversial, since most were thought to be polyclonal and therefore hyperplastic, and some have regressed (*5*). However, Southern blot hybridization to detect clonal immunoglobulin (Ig) gene rearrangements (*6*) in lymphomas from cardiac transplant recipients has shown monoclonality at first presentation (*7*), and thus that the tumors are best regarded as truly malignant.

The demonstration that inoculation of EBV caused lymphomas in owl monkeys and cotton-top tamarins was evidence supporting EBV oncogenicity (*8*). But, on the basis of conventional tests, the disease in tamarins was recently dismissed as a polyclonal lymphoproliferation (*9*), which is not a malignant process.

In research to develop a subunit vaccine against EBV (*10*), it was necessary to elaborate a virus challenge that would induce multiple lymphoid tumors in 100 percent of unprotected cotton-top tamarins. Because of this and the disputed clonality of the resultant tumors, we have reinvestigated the tamarin lesions with reference to EBV carcinogenicity and similarities to the tumors of organ graft patients.

Cotton-top tamarins (*Saguinus oedipus oedipus*) were bred successfully in captivity (*11*). Suspensions of EBV were prepared from B95-8 cells (*12*); culture supernatants were concentrated by 2 hours of centrifugation at 100,000*g* and the pellet was carefully resuspended in 1 percent of the original fluid volume by drawing in and out through four graded needles of 26 to 19 gauge. The suspension was passed through a Millipore filter (pore size, 1.2 μm) and titrated in fetal cord blood lymphocytes (*13*); the titrations included a virus preparation of known titer and were repeated three times to take account of variations in the sensitivity of cells from different cord blood samples. Virus suspensions with more than 10^5 lymphocyte-transforming doses per milliliter were selected since smaller doses did not regularly cause tumors.

We gave all the animals $10^{5.3}$ lymphocyte-transforming doses. In experiment 1 inoculations were given in 4 ml of culture fluid, divided equally among intravenous, intraperitoneal, and intramuscular routes; in experiments 2 and 3

inoculations were given in 7 ml, with two-thirds intraperitoneally and one-third intramuscularly in the thigh. (The latter site allowed progress of the disease to be followed easily in the inguinal nodes.) Macroscopically the lesions resembled those reported previously (*8, 9*), but they arose earlier (2 to 3 weeks after injection) and were larger. Firm, whitish lymph nodes up to 20 mm in diameter (normal diameter is 1 to 2 mm) were found in the groin, axilla, abdomen, mediastinum, and submandibular region, together with white tumor masses (not infiltrates) in the spleen, liver, kidneys, gut wall, adrenals, and thoracic inlet. Each animal displayed most of these lesions; tumor distribution did not vary with the route of inoculation. Tamarins with progressing symptoms were killed for ethical reasons when terminal disease developed (Table 1); lesions in the remaining animals regressed over 8 to 14 weeks.

Histological examination of autopsy material showed that the diseased nodes and organs were filled by large-cell malignant lymphomas conforming with the large noncleaved (follicular center cell) and immunoblastic types of the new working formulation on non-Hodgkin's lymphomas (Fig. 1a) (*14*). Tumors in some nodes, the intestine, and other sites showed distinctive "geographic necrosis" (Fig. 1b). Information could not be obtained on the lesions that regressed, but before resolution they were clinically indistinguishable from those that progressed.

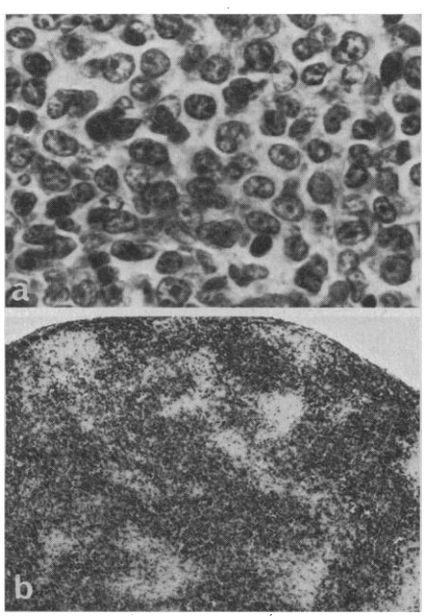

Fig. 1. (a) Tamarin tumor showing a malignant lymphoma of the large-cell type. Stain, hematoxylin and eosin; magnification, ×480. (b) Tamarin lymphoma in lymph node with areas of geographic necrosis. Stain, hematoxylin and eosin; magnification, ×75.

Table 1. Epstein-Barr virus–induced disease in cotton-top tamarins given $10^{5.3}$ lymphocyte-transforming doses.

Experiment	Injection routes	Number of animals with lesions	Number of animals killed	Number of days between inoculation and death
1	Intravenous, intraperitoneal, and intramuscular	2 of 2	1 of 2	22
2	Intraperitoneal and intramuscular	2 of 2	2 of 2	25, 28
3	Intraperitoneal and intramuscular	4 of 4	1 of 4	38

We stored multiple samples of each tumor at −70°C until thawing them for analysis. DNA was extracted from two tumors from each animal and digested with Bgl II and Bam HI restriction endonucleases. Fragments were separated on a 0.6 percent agarose gel, transferred to nitrocellulose, and hybridized to the ^{32}P-labeled insert of the cloned large internal repeat of B95-8 virus (Bam HI-W) (*15*). Assuming on average ten large internal repeats per virus genome, we estimated the number of copies per cell by comparing the signals with those of reference lanes containing 0.2, 2, 20, 200, and 2000 pg of the 3.1-kb repeat, respectively. The apparent number of virus genomes per cell varied from 2 to 25 for individual tumors (Table 2).

Other frozen portions of the same tumors and of additional tumors were thawed and DNA was extracted, digested with restriction enzymes, size-fractionated, Southern-blotted, and hybridized with the human J_H Ig gene probe (*7*). Besides the germ-line unrearranged band, each tumor showed one, or a few, rearranged Ig-specific bands (Fig. 2), indicating the proliferation of only one or several B-lymphocyte clones in each tumor. A polyclonal proliferation tested by these methods would not give an observable signal other than the germ-line band (*7*). Immunoglobulin gene rearrangements also provide markers characteristic of the clone or clones in an individual tumor, and since the rearrangements varied by site in a given animal, different lymphocyte clones were involved in each tumor.

Fresh material from various tumors of each dead animal was cultured as a single-cell suspension (*12*). Lymphoid cells grew in every case and were positive when stained (*16*) for the EBV nuclear antigen (EBNA) (Table 2). Tests for cord blood transformation (*13*) on 7-day supernatant culture fluids showed that seven of nine tumor-derived lines produced infectious EBV (Table 2). The Ig gene rearrangements in a selected tumor from each of two animals showed complete and almost complete concordance with those of the derived cell line, confirming the successful culture of the tumor clones. Cytogenetic studies were carried out on 20 metaphase spreads (*17*) from every cell line, but no consistent chromosomal rearrangements were detected (Table 2).

Our results show that an appropriate amount of EBV ($10^{5.3}$ lymphocyte-transforming doses) induced multiple lesions in all the tamarins, confirming earlier observations (*9*). The virus has been given intravenously, intraperitoneally, and intramuscularly in the past (*8, 9*), but even when intravenous inoculation was omitted (Table 1), generalized disease resulted, with lesions in many visceral

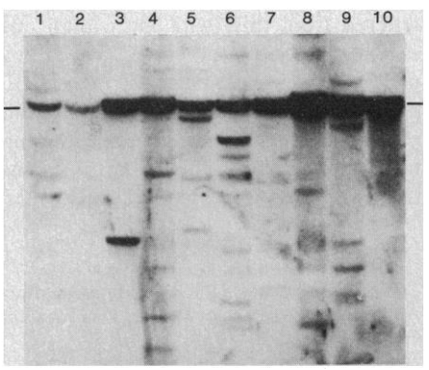

Fig. 2. Southern blot hybridization analysis of tamarin tumors. DNA was extracted from the tumor (*6*), digested with Bgl II restriction enzyme, and electrophoresed in an 0.8 percent agarose gel. The DNA was then transferred onto activated nylon membranes and hybridized against a radioactively labeled DNA probe fragment specific for the human Ig heavy-chain–joining (J_H) region (*7*). The filters were washed in 0.1× standard saline citrate at 58°C, dried, and exposed to x-ray film at −70°C for 1 to 7 days. The dashes indicate the germ line or unrearranged heavy-chain J_H gene of 7.5 kb, as determined by analysis of normal tamarin tissue. All other bands denote clonally rearranged Ig genes. Lanes 1 to 3, Bu57 pelvic node, thymic node, and mesenteric node 2; lanes 4 to 6, Bu41 renal retroperitoneal node, anterior retroperitoneal node, and mesenteric node 2; lanes 7 to 9, Bu24 mesenteric node 1, mesenteric node 2, and gastric node; and lane 10, normal tamarin kidney.

organs and peripheral lymph nodes. A large volume of inoculum was used to ensure as wide a dispersal of virus as possible. The lesions were undoubtedly caused by EBV, since they arose 2 to 3 weeks after inoculation, have never been seen in scores of uninoculated animals observed over 6 years, contained the EBV genome, and generated EBNA-positive tumor-derived cell lines that produced infectious virus (Table 2).

The histological evaluation, which was based on new criteria (*14*), showed multifocal, large-cell malignant lymphomas in all the dead animals with monomorphism and nuclear features (Fig. 1a) distinct from reactive lymphoid hyperplasia and the polymorphous immunoblastic proliferations of nonmalignant polyclonal lymphoproliferative disorders. The tumors resembled lymphomas in human cardiac allograft recipients, a resemblance increased by the presence of "geographic necrosis" (Fig. 1b).

The diagnosis of malignancy was upheld by the finding of clonal B-lymphocyte populations in the lesions (Table 2), a situation like that in the lymphoproliferations of cardiac transplant recipients (*7*). In these patients the lymphoid tumors constituted multifocal lymphomas with different monoclonal B-cell populations proliferating at various sites (*18*). Although some of the tamarin lesions differed from this, with more than one clonal population at some sites, they paralleled the situation in the fatal EBV-associated lymphoproliferative disorder of a patient with severe combined immunodeficiency (*19*). The tamarin lesions that regressed were probably also lymphomatous, as in the lymphomas of organ graft patients (*5*).

The observation that different tumors in the same tamarin arose from different cell clones (Table 2) is particularly interesting. If EBV caused the tumors, as seems beyond question, then it must have induced multiple transformation events in every animal, each event giving rise to separate and distinct clonally derived proliferations in the various organs. Identical processes appear to induce the EBV-associated tumor masses of immunosuppressed lymphoma patients (*18*), and these, like those in the tamarins, are distinct from polyclonal or hyperplastic processes when studied by refined genetic methods for determining clonality (*6*). The same methods have also verified that the cell lines (Table 2) were representative of their tumors of origin.

No specific chromosome abnormalities were detected in the tumor cell lines similar to those considered important in cellular oncogene activation in human lymphomas (*2*). However, whether or not more subtle changes were present, the speed with which tumors followed EBV inoculation shows that this agent can potently, rapidly, and directly activate the chain of events leading to the development of malignant lymphomas. Such a direct role is not surprising in light of recent findings on transformation by EBV in vitro, often categorized merely as "immortalization"; yet tests have shown that besides this phenomenon some cells are changed by the virus from the outset so as to possess many attributes of malignant transformation (*20*).

Table 2. Properties of EBV-induced tumors removed from cotton-top tamarins at autopsy. Abbreviations: NT, not tested; O, oligoclonal; M, monoclonal; and ND, no gene rearrangements detected.

Animal	Tumor sample	Clonality by Ig gene	Number of EBV genomes per cell	Cell line cultured	EBNA in cells	EBV in culture fluid	Consistent chromosome change
Bu41	Mesenteric node 2	O	20	Yes	Yes	Yes	No
	Renal retroperitoneal node	O	25	Yes	Yes	No	No
	Anterior retroperitoneal node	O	NT	NT	NT	NT	NT
Bu57	Thymic node	M	5	Yes	Yes	Yes	Yes*
	Pelvic node	O	NT	Yes	Yes	Yes	No
	Mesenteric node 2	M	NT	Yes	Yes	Yes	No
	Renal node	NT	15	Yes	Yes	Yes	No
Bu24	Gastric node	O	NT	Yes	Yes	Yes	No
	Mesenteric node 1	O	5	NT	NT	NT	NT
	Mesenteric node 2	O	15	NT	NT	NT	NT
Bu36	Mesenteric node	M	NT	Yes	Yes	No	No
	Left axillary node	ND	NT	Yes	Yes	Yes	No
	Mediastinal node	NT	2	NT	NT	NT	NT
	Mediastinal	NT	15	NT	NT	NT	NT

*3q+ in 80 percent of cells (source not known).

In conclusion, our findings indicate that the tamarin tumors are appropriate experimental analogs for the lymphomas that arise in human organ graft recipients and support a direct role for EBV in tumorigenesis in vivo.

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