JOURNAL OF VIROLOGY, Oct. 1985, p. 60–65
0022-538X/85/100060-06$02.00/0

Vol. 56, No. 1

Herpesvirus Sylvilagus Infects Both B and T Lymphocytes In Vivo

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Received 1 April 1985/Accepted 21 May 1985

Herpesvirus sylvilagus infection of cottontail rabbits (*Sylvilagus floridanus*) was studied as a model of herpesvirus-induced lymphoproliferative disorders. Leukocytosis, splenomegaly, proliferation of T cells and virus production by lymphocytes characterized this infectious mononucleosis-like disease. Approximately two copies of circular herpesvirus sylvilagus genomes per cell were detected in spleen cells at 2 weeks postinfection, and circular genomes could still be observed after 4 months. Circular viral genomes were found in both B and T lymphocytes. Small amounts of linear viral DNA (0.1 to 0.3 copies per cell) were also detected in both B and T cells. These results indicated that the virus did not replicate in the majority of lymphocytes in vivo. Herpesvirus sylvilagus infection in cottontail rabbits could be useful as a model for studying the complex virus-host relationships of lymphotropic herpesviruses and perhaps as an animal model for Epstein-Barr virus infection in humans.

Lymphotropic herpesviruses are characterized by their ability to infect cells of the immune system, establish latency, and cause lymphoid neoplasias. In addition, these viruses can transform lymphoid cells, and multiple copies of the viral genome can be detected in transformed cells. The most studied virus of this group is Epstein-Barr virus (EBV); EBV infects and transforms human B lymphocytes (20, 28, 35). The development of EBV-containing B-cell lymphomas in allograft recipients (5, 13, 32) and immunosuppressed individuals (39) is of considerable clinical significance. Herpesvirus saimiri and herpesvirus ateles are nonpathogenic in their natural host, although it is possible to isolate viruses from normal animals (9). When introduced into marmosets (8, 17, 18) or rabbits (7), these viruses are highly oncogenic, causing T-cell lymphomas. Marek's disease virus of chickens is less selective in the cells it infects. Both T and B lymphocytes are infected (34), and a paralytic disease due to lymphocyte infiltration into the peripheral nervous system ensues (26). Birds that survive this initial disease rapidly develop disseminated T-cell lymphomas (26). In turkeys infected with this virus, the lymphomas bear B- (23) or T-cell markers (27). Circular viral genomes have been demonstrated in tumor cell lines or in vitro-transformed cell lines or both for all of these viruses (1, 21, 30, 31, 42). In lymphocytes transformed by lymphotropic herpesviruses, circular episomal viral DNA is indicative of latency, and linear viral DNA indicates active virus production.

Herpesvirus sylvilagus infection in cottontail rabbits (*Sylvilagus floridanus*) is characterized by leukocytosis, splenomegaly, lymphadenopathy, and circulating, virus-infected mononuclear cells (15, 41). Virus can be isolated from the oral cavities and from circulating lymphocytes of infected animals (16, 37) and can be grown in kidney-derived epithelial cells (14). The presence of neutralizing antibody in trapped wild animals indicates that this agent is a natural pathogen of cottontail rabbits (36), and the failure to infect any other rabbit species suggests that the host range of this virus is highly restricted (14). A lymphoproliferative disorder characterized by extensive lymphocytic infiltrates in the kidney, liver, and myocardium occurs in 10 to 25% of animals experimentally infected with herpesvirus sylvilagus (15). These observations suggest that this virus is a lymphotropic herpesvirus.

We have recently reported that spleen cells from infected rabbits contained circular herpesvirus sylvilagus genomes (22). We have extended those observations and now report that both B and T lymphocytes are infected and carry circular viral DNA and that T-cell proliferation occurs in response to infection.

MATERIALS AND METHODS

Animals. Wild cottontail rabbits (*S. floridanus*) were trapped live in Massachusetts with the permission of the Massachusetts Division of Fish and Game. Animals were quarantined for 2 weeks and then kept in animal facilities on standard laboratory rabbit rations. Before any experimental procedure, animals were anesthetized with 4 mg of xylazine and 30 mg of ketamine per kg of body weight given intramuscularly. Animals used in these studies were negative when screened for anti-herpesvirus sylvilagus antibody before infection as determined by immunofluorescence and virus neutralization.

Tissue culture and virus stocks. Two primary kidney epithelial cell lines were isolated in this laboratory from newborn cottontail rabbits. These cell lines, designated NCRK1 and NCRK2, were used in growing virus pools and in plaque assays for determining virus titers and infectious centers. Herpesvirus sylvilagus seed stocks were the same virus isolates as described previously (14), and virus pools of 10^7 PFU/ml were grown in the NCRK2 cell line. Virus stocks were maintained at −80°C until use.

Virus plaque and infectious center assays. Virus titers were determined by plaque formation on monolayers of NCRK cells. We enumerated infected leukocytes by plating spleen cells from infected animals on monolayers of NCRK cells and overlaying them with media containing 0.75% agarose. After 7 days, we enumerated plaques by fixing the monolayers with 10% Formalin and staining them with crystal violet.

Infection of animals and isolation of leukocytes. Animals were infected with 5×10^6 PFU of virus by intraperitoneal

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TABLE 1. Herpesvirus sylvilagus infection in cottontail rabbits: lymphoid response[a]

Days postinfection	WBC[b] (10^3/mm^3)	Spleen cells[c] (10^6)	% B cells[d]	% T cells[e]	Infectious centers per 10^6 cells[f]
Uninfected	4.2	54	54	47	0
6	10.8	320	46	55	50
9		1,000	40	56	125
11	8.5	750	21	74	210
13	5.7	820	39	77	225
16	10.1	850	27	67	130
17	7.2	740	26	70	117
23	8.7	620	36	62	88
110	6.8	175	33	64	15

[a] Animals were infected with 5×10^6 PFU of herpesvirus sylvilagus and then sacrificed on the days indicated. Peripheral blood and spleens were harvested and processed.

[b] Circulating leukocyte (WBC) counts were determined with a Coulter counter.

[c] We determined spleen cell counts by staining the cell suspension with Turks stain and counting with a hemacytometer.

[d] We determined B cells by staining spleen cells with fluorescein-conjugated goat anti-rabbit IgM.

[e] We determined T cells by reacting spleen cells with L11/135, a mouse monoclonal antibody raised against a New Zealand rabbit T-cell line, and then staining them with fluorescein-conjugated goat anti-mouse IgG.

[f] We determined infectious centers by layering 10^6 spleen cells on a monolayer of NCRK2 cells and overlaying them with agarose. Plaques were enumerated after 7 days.

injection. At the time of sacrifice, animals were bled by cardiac puncture to obtain peripheral blood leukocytes, and the spleens were removed and immediately minced through a wire screen in tissue culture medium. A sample of whole blood was analyzed on a Coulter counter to determine the leukocyte count. Peripheral blood leukocytes were obtained by mixing heparinized whole blood with 1 volume of RPMI medium at 37°C which contained 3% dissolved gelatin. After $1 \times g$ sedimentation (for 1 h at 37°C), the upper layer was removed and washed twice in RPMI medium. Contaminating red cells were removed by lysis in 155 mM NH_4Cl-10 mM $KHCO_3$-1 mM EDTA (pH 7.4). Spleen cells were washed twice and subjected to the NH_4Cl lysis procedure. Cells which were not needed for the plaque assays were frozen with a Planar programmed liquid nitrogen cell freezer. Previously frozen cells were separated on Ficoll-Hypaque gradients (density, 1.093) to remove dead cells before use.

Determination of B- and T-lymphocyte populations. We enumerated B lymphocytes by staining spleen cells with fluorescein-conjugated goat anti-rabbit immunoglobulin M (IgM) (Cappel Laboratories, Cochranville, Pa.). We quantitated T lymphocytes by staining them with L11/135, a mouse monoclonal antibody developed against a New Zealand rabbit lymphoid cell line (19) (a gift from Susan Jackson, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.), and then reacting the cells with fluorescein-conjugated, affinity-purified goat anti-mouse IgG heavy chain (Southern Biotechnologies Assoc., Birmingham, Ala.). We obtained purified lymphocyte populations by sorting gradient-purified, stained spleen cells (2×10^7) on a FACS IV fluorescence-activated cell sorter (Becton Dickinson and Co., Paramus, N.J.). For double-color immunofluorescence, lymphoid cells (4×10^7 to 8×10^7) were stained with fluoresceinated goat anti-rabbit IgM and unconjugated L11/135. L11/135 was counterstained with a biotin-labeled goat anti-mouse IgG Fab fragment (Zymed Laboratories), and the cells were reacted with phycoerythrin-conjugated avidin (Becton Dickinson). Cells were sorted on the basis of their staining with either one or both of the fluorochromes.

Gardella gel technique. We recently described a modification of the Eckhardt electrophoretic technique for detecting bacterial plasmids to permit detection of circular and linear extrachromosomal herpesvirus genomes in mammalian cells (10). Briefly, 10^6 viable mononuclear cells were suspended in 0.1 ml of TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA [pH 8.2]) containing 15% Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.), 2 Kunitz units of RNase (type 1A; Sigma Chemical Co., St. Louis, Mo.) per ml, and 0.01% bromophenol blue. Cell suspensions (75 μl) were loaded into wells of a vertical 0.8% agarose gel prepared in TBE buffer. Lysis buffer (TBE buffer containing 5% Ficoll, 1% sodium dodecyl sulfate, 1 mg of pronase per ml, and 0.05% xylene cyanol green) was then carefully layered over the cell sample layers. Electrophoresis was started at 0.8 V/cm for 3 h and then increased to 7.5 V/cm for 14 h. Gels were stained with ethidium bromide (1 μg/ml) and photographed under UV light. The DNA in the gel was then nicked with hydrochloric acid, denatured in alkali, neutralized, and transferred to nitrocellulose filters, and the filter was hybridized with a ^{32}P-labeled, cloned *Bam*HI F fragment of herpesvirus sylvilagus genomic DNA (22). An additional probe was developed to increase the sensitivity of our hybridization assays. *Sma*I fragment Y of the herpesvirus sylvilagus genome was cloned in pUC12. This 0.5-kilobase fragment is a unit of the repetitive element and is repeated at least 15 to 20 times in the herpesvirus sylvilagus genome.

RESULTS

Clinical findings on cottontail rabbits after infection with herpesvirus sylvilagus. Experiments were performed to characterize the lymphoid response to herpesvirus sylvilagus infection. Lymphoid responses were monitored in eight rabbits infected with 5×10^6 PFU of virus (Table 1). Elevated mononuclear blood cell counts (up to 2.5 times normal) were observed in seven animals at the time of sacrifice. Intense mononuclear cell proliferation was also evident by increased numbers of spleen cells isolated from infected animals. A sixfold increase in the number of spleen cells recovered was observed as early as 6 days after infection. Spleen size continued to increase (to greater than 10-fold) for the next 2 weeks but appeared to regress to almost normal by 110 days after infection.

Antibodies are not available which are specific for cottontail rabbit lymphoid cell populations. We tested a commercial goat anti-rabbit IgM and the monoclonal antibody L11/135 (two reagents which mark B and T lymphocytes, respectively, in the New Zealand rabbit) on cottontail rabbit lymphocytes. Greater than 90% of spleen cells were stained by these two reagents, and the percentage of lymphocytes reactive with either antibody was similar to that observed for New Zealand rabbit lymphocytes (19; data not shown). To verify that the cells which were identified were indeed T and B lymphocytes, we determined specific mitogen reactivities on enriched populations. We separated uninfected spleen cells by panning them on plastic dishes coated with either anti-IgM or L11/135 (19). Adherent and nonadherent cells were analyzed for the ability to proliferate in response to the T-cell mitogens concanavalin A and phytohemagglutinin or the B-cell mitogen anti-IgM. Cells adherent to L11/135-coated plates responded very well to both T-cell mitogens

but not to anti-IgM, indicating that L11/135 marker was present on T lymphocytes (Table 2). Cells adherent to anti-IgM-coated plates responded very well to anti-IgM but not to the T-cell mitogens, indicating that these were B lymphocytes. The responses of both nonadherent populations to both T- and B-cell mitogens indicated that these populations were not pure.

Using the reagents tested above, we estimated the percentages of B and T lymphocytes in infected animals by immunofluorescence staining (Table 1). The percentage of T cells increased from approximately 50% to greater than 70% during the course of infection, and the B-cell population declined to approximately 20%. Thus, the increase in T lymphocytes was 3 to 4 times greater than that of B lymphocytes. Although mononuclear cell proliferation subsided by 110 days, the ratio of B and T cells was similar to that observed early in infection. It is not known if infection with herpesvirus sylvilagus causes a permanent change in the ratio of these cells or if they eventually recover.

Virus production in lymphoid cells. The exuberant cellular response to herpesvirus sylvilagus infection observed in spleen cells suggested this lymphoid organ as a site of viral replication. Examination of spleen cells by infectious center assay indicated that peak virus recovery occurred in week 2 after infection, with 0.02% of the cells replicating infectious virus. The number of virus-infected cells declined as the infection progressed, but it was still possible to detect virus in spleen cells from an animal 110 days postinfection. Evidence that virus detected by infectious center assay was cell associated is as follows. (i) Cells were washed several times before plating, which would dilute out free virus. (ii) Attempts to isolate virus from the plasma of infected rabbits were unsuccessful.

Experiments were performed to identify which population(s) of lymphocytes was replicating the virus. Staining spleen cells from infected animals with anti-herpesvirus sylvilagus antibody and analyzing the cells on a FACS IV indicated that the number of infected cells expressing viral antigens on their surfaces was lower than the detection limit of 1% over background, which is in agreement with the virus recovery experiments described above. Lymphocytes were stained with B- or T-cell-specific antibody and separated on a FACS IV into antibody-positive and antibody-negative populations. Sorted cells were analyzed for purity and studied in infectious center assays. The purity of each lymphocyte preparation was greater than 95%. Table 3 shows the results of six independent cell separations. Infectious centers were detected in both purified B- and T-lymphocyte populations. These results indicated that both populations of lymphocytes contained cells which were replicating herpesvirus sylvilagus.

TABLE 2. Mitogen responses of cottontail rabbit lymphocytes: ability of goat anti-rabbit IgM and L11/135 to specifically label B and T lymphocytes[a]

Lymphocytes	Proliferative response (cpm)[b]			
	Control	PHA (2 μg/ml)	ConA (4 μg/ml)	IgM (500 μg/ml)
Unfractionated	2,988	27,920	40,417	5,286
L11/135 nonadherent[c]	1,340	14,707	2,857	6,386
L11/135 adherent[d]	180	20,020	24,755	536
Anti-Igm nonadherent[c]	2,360	38,850	2,390	5,723
Anti-IgM adherent[d]	213	1,227	1,073	10,060

[a] We obtained purified B and T cells by panning spleen cells on plastic dishes with either goat anti-rabbit IgM or L11/135 antibody adsorbed to the surface (19).

[b] We put purified lymphocytes (2×10^5) into culture in 96-well flat bottom plates with mitogens; 24 h before harvest, the cultures were pulsed with 1.0 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Mass.). Cultures were harvested with a Microharvester (Bellco Glass Inc., Vineland, N.J.) and the filter disks were counted in a liquid scintillation spectrometer. Results are the mean incorporated counts of triplicate cultures from a representative experiment. PHA, Phytohemagglutinin; ConA, concanavalin A.

[c] We obtained nonadherent cells by incubating the cells in RPMI medium on the plate for 2 h at room temperature then gently washing them.

[d] We removed adherent cells by adding excess antibody, incubating the plates for 30 min at 37°C, and then gently scraping them with a rubber policeman.

TABLE 3. Identification of lymphocyte populations infected by herpesvirus sylvilagus

Day postinfection (cell type)	Plaques per 10^6 unfractionated cells[a]				
	Unseparated[b]	IgM[c]		L11/135[c]	
		+	−	+	−
11 (Spleen)	41	34	11	18	10
16 (Spleen)	67	24	19	ND[d]	ND
16 (PBL)[e]	14	10	4	ND	ND
17 (Spleen)	30	28	9	24	7

[a] Plaques were determined by infectious center assay on 10^6 purified B or T lymphocytes. The number of plaques was multiplied by the percentage of the appropriate population to determine the number of plaques per 10^6 unfractionated cells.

[b] Unseparated cells were cells which were stained but not separated.

[c] Cells were stained with the appropriate fluorescein-conjugated antibody and separated by FACS IV into antibody-positive and antibody-negative populations.

[d] ND, Not done.

[e] PBL, Peripheral blood lymphocytes.

Detection of circular and linear viral DNA in lymphoid cells. To investigate the presence of viral genomes during the course of infection, we examined lymphoid cells for herpesvirus sylvilagus DNA content. Several samples of spleen lymphocytes from rabbits infected for different periods of time were analyzed for the presence of circular and linear viral DNA by the Gardella gel technique. This technique is a simple in situ electrophoretic method which can separate linear and circular viral DNA molecules from intact cells (10). These experiments indicated that the amount of viral DNA associated with spleen lymphocytes was maximal during weeks 2 and 3 after infection, although we were able to detect circular viral genomes as long as 110 days postinfection (data not shown). To determine the presence and physical state of viral genomes in specific lymphoid cell populations, we separated spleen cells by FACS IV and assayed them by the Gardella gel technique.

Lymphocytes from two animals (days 11 and 16 postinfection) studied in the plaque assay (Table 3) were separated by FACS IV as described above. Purified B and T cells (10^6) were tested for the presence of viral genomes by the Gardella gel technique. Figure 1 is an example of such a gel, showing the fluorograph of the stained gel (panel A) and the autoradiograph after hybridization with a herpesvirus sylvilagus-specific DNA probe (panel B). Approximately equal amounts of circular and linear herpesvirus sylvilagus DNA were detected in IgM^+-$L11/135^-$ (B-cell) populations and in IgM^--$L11/135^+$ (T-cell) populations (Fig. 1B, lanes 2 through 5; day 11 animal). The hybridization observed at the origins was probably due to trapped or replicating viral DNA molecules, although it could not be ruled out that viral DNA was also integrated into host chromosomes. These results were consistent with those presented in Table 3 and sup-

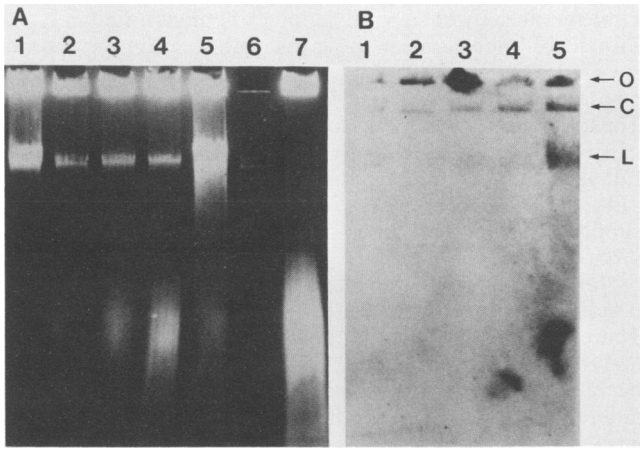

FIG. 1. Detection of circular (C) and linear (L) viral DNA in purified B and T lymphocytes. FACS IV-sorted spleen cells (10^6) were analyzed for the presence of circular and linear genomes by electrophoresis (10, 22) as described in the text. DNA was transferred to nitrocellulose filters and hybridized with ^{32}P-labeled pSYB9 containing *Bam*HI fragment F of herpesvirus sylvilagus DNA and exposed to X-ray film (22). (A) Fluorogram; (B) autoradiogram after hybridization of lanes 1 through 5. Lanes: 1, IgM^+ cells; 2, $L11/135^-$ cells; 3, IgM^- cells; 4, $L11/135^+$ cells; 5, unsorted spleen cells; 6, 10 ng of herpesvirus sylvilagus virion DNA; 7, Raji cells. O, Gel origin.

ported the interpretation that both T- and B-lymphocyte populations were infected with herpesvirus sylvilagus, and that the latent form of the virus was present in both cell types.

Detection of viral genomes in purified B and T lymphocytes. During the course of analyzing spleen cells by two-color immunofluorescence, it was observed that approximately 10% of the spleen cells showed double staining with IgM and L11/135. This large number of cells expressing both B- and T-cell markers could explain the observed results indicating infection of both B and T lymphocytes. However, it was still possible that only B or T cells were infected if the doubly stained cells were a subpopulation of the infected cell type. To exclude this possibility, we analyzed FACS IV-purified B-, T-, and doubly stained cell populations on a Gardella gel. Spleen cells from an animal 16 days postinfection were stained with anti-IgM and L11/135 for two-color immunofluorescence and sorted by FACS IV. Table 4 shows the proportion of each cell population in the spleen and its purity after separation. One million cells of the three populations were analyzed on Gardella gels. All of the cell populations contained circular as well as linear viral DNA (Fig. 2).

We estimated the amount of herpesvirus sylvilagus DNA observed in each of these cell populations by comparing the amount of viral DNA in each lane with that in the reconstitution experiment shown in Fig. 2, lanes 6, 7, and 8, which contained known amounts of herpesvirus sylvilagus DNA (Fig. 2B). This indicated that unseparated spleen cells contained approximately two copies of circular viral DNA per cell (lane 1), and in each of the purified cell populations (lanes 2, 3, and 4) about one-half copy of circular viral DNA per cell was observed. Small amounts of linear viral DNA, about 0.1 to 0.3 copy per cell, were also detectable in B- and T-lymphoid subpopulations (Fig. 2B, lanes 2 and 4). It should be noted that the double-staining population had considerably more linear viral DNA, about two copies per cell (lane 3). However, in this doubly stained population much more partially degraded linear cellular DNA was visible in the fluorograph of the gel (Fig. 2A, lane 3), which is usually attributed to large numbers of dead cells in the starting population. This could mean that the hybridization observed in the linear DNA region was the result of degradation of circular DNA molecules or, alternatively, that the cells were dying because they were undergoing lytic infection and the hybridization observed was due to virion DNA. The amount of viral DNA estimated in each of the purified populations does not appear to account for all of the viral DNA observed in unseparated cells. This could mean that cells which were not analyzed, either by rejection because of light scatter properties (2) or lack of staining with the reagents used (Table 4), contained much larger amounts of viral DNA than those observed in purified lymphocyte populations. Alternatively, it may indicate that the FACS IV did not randomly sort uninfected and infected lymphocytes

TABLE 4. Two-color immunofluorescence analysis of herpesvirus sylvilagus-infected cottontail rabbit lymphocytes stained with L11/135 and goat anti-rabbit IgM[a]

Cell population	% of cells expressing phenotype			
	L11/135$^+$	IgM^+	L11/135$^+$-IgM^+	L11/135$^-$-IgM^-
Unseparated	65	20	9	6
L11/135$^+$	98.7	0.1	1.2	
L11/135$^+$-IgM^+	5	5	90	
IgM^+	0.1	90.9	9.0	

[a] Gradient-purified spleen cells (4×10^7) from an animal 16 days postinfection were stained with fluorescein-conjugated goat anti-rabbit IgM and L11/135. Cells were counterstained with biotin-labeled goat anti-mouse IgG and finally reacted with avidin-conjugated phycoerythrin. Cell populations were determined by two-color immunofluorescence and sorted according to phenotype. A FACS IV equipped with a single argon laser was used for fluorescence analysis and sorting.

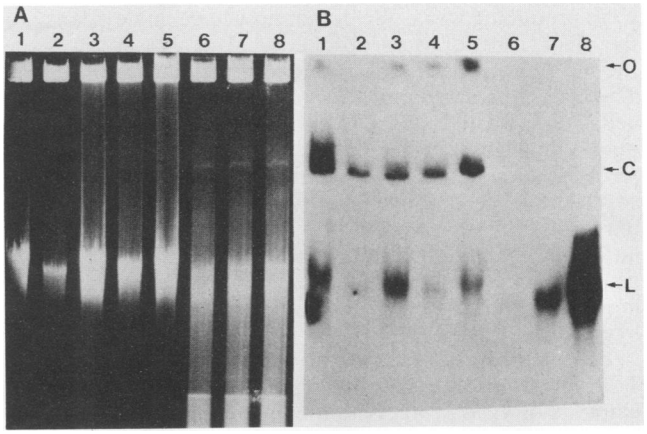

FIG. 2. Circular and linear viral DNA in spleen cells separated by FACS IV by using two-color fluorescence. Conditions for gel electrophoresis were as described in the text. After transfer to nitrocellulose, the gel replica was hybridized to a ^{32}P-labeled mixture of pSYB9 and pSYS0.5. (A) Fluorogram of ethidium bromide-stained gel; (B) autoradiogram. The following cells (10^6 per lane) were used. Lanes: 1, unseparated spleen cells after Ficoll gradient purification; 2, IgM^--L11/135$^+$ cells; 3, IgM^+-L11/135$^+$ cells; 4, IgM^+-L11/135$^-$ cells; 5, cells processed by FACS IV laser but not selected during the sort; 6 through 8, Raji cells mixed with 8, 80, and 800 pg of herpesvirus sylvilagus DNA, respectively. O, Gel origin; C, circular viral DNA; L, linear viral DNA.

(i.e., FACS IV rejection of infected lymphocytes because they were dead). In either case, cells which were processed by FACS IV but were not sorted into a specific population (lane 5) contained approximately the same amounts of viral DNA as did unseparated cells.

DISCUSSION

The aim of these studies was to further characterize events occurring early after infection of cottontail rabbits with herpesvirus sylvilagus and to identify which lymphoid cell population was infected. It was observed that intense T-cell proliferation occurred, which may be analogous to that seen in EBV-induced infectious mononucleosis (25). It is thought that in infectious mononucleosis T cells are responsible for the elimination of EBV-infected B cells (40) and are the cause of many of the clinical manifestations observed (38).

Although the antibodies used to identify B and T cells appeared to define functional populations for cottontail rabbit lymphocytes, it was observed, surprisingly, that a population of cells stained for both B- and T-cell markers. There is controversy in the literature with respect to rabbit lymphocytes expressing both B- and T-cell markers (3, 33). We believe that the population of doubly staining cells represents a real subpopulation of lymphoid cells because of the following. (i) They have been present in all two-color immunofluorescent analyses of cottontail rabbit lymphocytes. (ii) IgM-positive cells enriched to greater than 90% by panning contained significant numbers (>30%) of L11/135-positive cells. (iii) Doubly stained cells appear to harbor significantly more virus than either of the purified B- or T-cell populations. (iv) We have other monoclonal antibodies which appear to stain this population specifically (unpublished data). Preliminary studies with New Zealand rabbit peripheral blood indicate that there is a similar proportion of doubly stained cells, which is in contrast to the conclusions of Jackson et al. (19) but is consistent with a reinterpretation of Fig. 4 in their report.

At the peak of lymphoid hyperplasia, 0.02% of spleen cells were producing virus. We do not now have any evidence for the mechanism of virus production. One possibility is that the virus produced is the result of reactivated latent virus. This would be analogous to virus producing EBV lymphoid cell lines in which all cells carry latent viral genomes but only a small percentage of the cells show any evidence of virus production (4, 24). A second possibility is that there are two types of cells, those latently infected and those producing virus. The observation that purified B- and T-cell populations contained predominantly the latent form of the viral genome, whereas the double-stained population had much more of the linear DNA, could offer evidence to support this hypothesis.

The results presented clearly demonstrate that both B- and T-lymphocyte populations are infected, produce small amounts of virus, and contain circular viral genomes. Similarly, Marek's disease virus and guinea pig herpeslike virus have also been shown to infect B and T cells (12, 34). In contrast to guinea pig herpeslike virus (11) and human cytomegalovirus (29), herpesvirus sylvilagus does not appear to infect macrophages, as evidenced by a proportional decrease in viral DNA detected in plastic adherent cells (unpublished data).

Circular viral genomes have been demonstrated in tumor cells and in vitro-transformed cell lines of EBV (1), Marek's disease virus (30), herpesvirus saimiri (31, 42), and herpesvirus ateles (21). This common feature among widely different viruses suggests an important role for this form of the viral genome. Circular viral genomes demonstrated in transformed cell lines appear to be insensitive to antiviral drugs such as acyclovir (6) or phosphonoacetic acid (28) under conditions in which linear viral DNA is totally inhibited. These drugs have no effect on the transforming capabilities of EBV (35). Treatment of nonproducer cell lines with the tumor promoter agent results in the recovery of infectious virus (43). These observations, taken together, strongly support the idea that the circular genome is a latent form of virus.

Herpesvirus sylvilagus infects both B- and T-lymphocyte populations, in contrast to EBV, which only infects B cells. Therefore it is possible that the virus-associated lymphoproliferative disorder which has been described in rabbits may be of either T- or B-cell origin or both. Apart from this difference, herpesvirus sylvilagus infection has many similarities to EBV. Both viruses have restricted host ranges with a tropism for natural host lymphocytes. The transmission of these viruses appears to be through oral-pharyngeal secretions. Both viruses cause a mononucleosis syndrome characterized by lymphoid hyperplasia which is predominantly T cell in nature. Infected lymphocytes contain latent virus as indicated by the presence of circular viral genomes. Because of these similarities, herpesvirus sylvilagus infection in the cottontail rabbit appears to be an appropriate animal model for EBV to study the course of lymphotropic herpesvirus infection, development of viral latency, cellular immune response, consequences of viral reactivation, viral oncogenesis, and antiviral chemotherapy.

This report further extends our previous report of circular viral genomes in infected spleen cells (22) by demonstrating virus infection and latency in both B- and T-lymphocyte populations. This is the first demonstration of the circular form of a lymphotropic herpesvirus genome in cells undergoing recent infection. Herpesvirus sylvilagus infection of cottontail rabbits provides a unique opportunity to study, in vivo, initial permissive infection and the development of latency in lymphoid cells of a lymphotropic herpesvirus.

ACKNOWLEDGMENTS

We thank Maria Medveczky for her technical assistance, Marci McFadden for performing the FACS IV analysis and sorting, and Kathy Beauregard for preparing the manuscript.

This work was supported in part by Public Health Service grant no. AI-18255 from the National Institutes of Health, institutional grant no. IN129C from The American Cancer Society to the University of Massachusetts Medical School, and biomedical research support grant no. SO7RR05712 from the National Institutes of Health to the University of Massachusetts Medical School. J.L.S. is an established investigator of the American Heart Association.

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