A NONIMMUNOSUPPRESSIVE HELPER VIRUS ALLOWS HIGH EFFICIENCY INDUCTION OF B CELL LYMPHOMAS BY RETICULOENDOTHELIOSIS VIRUS STRAIN T

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Reticuloendotheliosis viruses (REVs)[1] are a group of avian retroviruses that infect chickens, turkeys, and ducks (1). The prototype virus of this group is reticuloendotheliosis virus strain T, REV-T(REV-A), a mixture of a replication-defective virus (REV-T) that induces acute neoplastic disease and a nondefective helper virus (REV-A) capable of inducing an immunosuppressive runting disease (2–4). REV-A and other nondefective REVs can be distinguished from REV-T by their ability to replicate in vitro in fibroblasts and their inability to induce acute neoplastic disease in vivo (5, 6). Some nondefective REVs, chick syncytial virus (CSV) and REV-A, induce a bursal-dependent B cell lymphoma that is indistinguishable from avian leukosis virus (ALV)-induced lymphoid leukosis (6, 7). These tumors develop after a long latent period, are monoclonal, and are characterized by proviral insertion within the c-*myc* locus (8, 9). In contrast, REV-T causes an acute neoplastic disease known as reticuloendotheliosis because the prominent cell in the original neoplastic lesion was morphologically identified as reticuloendothelial (10). These tumors develop rapidly, appear to be polyclonal and are thought to require the expression of the v-*rel* oncogene carried by REV-T (11, 12).

Despite the original description of the disease, the identity of the tumor induced by v-*rel* remains unclear. In situ characterization of the in vivo–derived tumor tissue has not been reported. In vitro studies suggest the REV-T-transformed cells are of early lymphoid origin (13–15), but the absence of specific markers that define this phenotype has prevented conclusive identification. There are two reports of REV-T-derived cell lines that express IgM (14, 16) and it is possible that several phenotypically distinct cells may serve as target cells for v-*rel*-induced tumors.

REV-A is known to cause thymic and bursal atrophy as well as immunosuppression (2, 17, 18). Since the bursa is the major compartment of B lymphocyte development in the chicken, we reasoned that one consequence of REV-A

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[1] *Abbreviations used in this paper:* ALV, avian leukosis virus; CEF, chicken embryo fibroblasts; CSV, chick syncytial virus; EV-4, endogenous viral locus 4; PAP, peroxidase–antiperoxidase; REVs, reticuloendotheliosis viruses; REV-A, nondefective helper virus; REV-T, replication-defective virus; TBS, Tris-buffered saline.

infection and the subsequent disruption of this organ might be a reduction in B cell proliferation and differentiation. If REV-T induces lymphoid tumors as suggested by studies of in vitro–derived REV-T cell lines, it is possible that REV-A replication influences the spectrum of lymphoid tumors that develop by reducing the pool of IgM^+ B lymphocytes that are available for REV-T infection. It has been reported that both immunosuppression and bursal atrophy are less severe in CSV-infected chicks (6, 18). We speculated, therefore, that if CSV provided the helper virus functions required for REV-T replication, the pool of cells available for infection by REV-T might contain significantly more IgM^+ lymphocytes. Since IgM^+ tumor cell lines have been isolated after REV-T(REV-A) infection, albeit rarely, we wanted to test the prediction that REV-T(CSV) infection would lead to high frequency production of IgM^+ B cell lymphomas.

Materials and Methods

Cells and Viruses. SC chicken embryo fibroblasts (CEF) were cultured in plastic dishes (Nunc, Roskilde, Denmark) in $ET_{10}Ca_{10}$: DME (Flow Laboratories, Inc., McLean, VA) containing 10% tryptose phosphate broth and 10% calf serum (Hazelton, Lenexa, KS) with antibiotics.

REV-A was rescued by calcium phosphate transfection of CEF with pSW253 provided by Dr. H. Temin (12). Transfected cells were cultured and transferred twice. Medium was harvested at 3-h intervals from confluent plates, clarified by centrifugation at 250 *g*, passed through a 0.2-μm Nalgene filter, and stored at −70°C.

CSV(CN19691)-infected line O cells were provided by Dr. R. L. Witter, Regional Poultry Laboratories, East Lansing, MI). Culture medium from these cells was used to infect SC CEF and CSV stocks were prepared as for REV-A.

A REV-T nonproducer cell line developed in the laboratory of Dr. H. Bose by in vitro infection of spleen cells (14) was grown in $ET_{10}Ca_{10}Ck_2$ ($ET_{10}Ca_{10}$ plus 2% chick serum [Gibco Laboratories, Grand Island, NY]). Stocks of REV-T(CSV) were made by infecting the REV-T nonproducer line with CSV and harvesting virus as above after several cell transfers. REV-T(REV-A) was harvested from a clone of the bone marrow cell line isolated in the laboratory of Dr. H. Bose (19).

Chickens and Virus Infections. Embryonated SC eggs were purchased from Hyline International Hatcheries, West Des Moines, IA, and incubated with humidity at 39°C. On day 1 after hatch, chicks were infected via intrajugular injection with 10^5 IU of REV-A, CSV, or REV-T per bird. The chicks were housed by the Animal Resource Center, University of Texas Health Science Center at Dallas, in rooms isolated from control or ALV-infected chicks. CSV and REV-A-infected chicks were housed in separate cages in the same isolation unit. Food and water were provided ad libitum.

For repopulation studies, recipient chicks were injected intraperitoneally with 3 mg Cytoxan (cyclophosphamide; Mead Johnson, Syracuse, NY) daily for 4 d after hatching to eliminate the resident B cells (20). On the sixth day after hatch, sibling donor chicks were killed by cervical displacement and their bursae were surgically removed. Bursae were then rinsed in DME plus antibiotics and minced with scissors. A single cell suspension, which was >95% positive for Ig expression, was prepared from bursal pieces and washed once with medium before resuspension in 2 ml of REV-T(CSV) per 5×10^7 cells (multiplicity of infection [moi] of 0.05 for REV-T). Cells were incubated with virus for 15 min on ice followed by 45 min at 37°C to permit virus absorption and penetration. After one wash with medium, 5×10^6 of the infected bursal cells were injected via the jugular vein into cytoxan-treated recipients.

Sample Collection. Hematocrit samples were obtained from the wing vein and plasma was collected from the jugular vein and prepared as previously described (21). The bursa, spleen, liver, and heart were excised and weighed as whole organs. The uppermost bilateral lobes of the kidney and seven lobes of the thymus were excised for weighing in

lieu of the total organs since complete recovery of these organs is difficult and prone to error. After the organs were weighed, samples for histology were fixed in 10% neutral buffered formalin and samples for immunohistochemistry were snap-frozen in 2-methylbutane at −70°C.

Cell Line Isolation. Cell lines were isolated by preparing single-cell suspensions from nodules in the liver and random sections of tissue from the spleen, thymus, and bursa. These suspensions were diluted into Hahn's medium (22) and cultured at 37°C with 10% CO_2 for 48 h before transferring cultures at a 1:5 dilution into $ET_{10}Ca_{10}Ck_2$. Spleen and liver cultures were transferred at a 1:10 dilution every 24–48 h thereafter. Bursa and thymus cultures required more time before the initial transfer; however, after the second or third transfer, these lines also required daily transfer. Liver suspensions were tested at the initial isolation for IgM expression by immunofluorescence, and all lines were assayed by the fourth or fifth transfer. Cellular DNA was isolated by the sixth cell transfer.

Virus Titrations. REV-A and CSV stocks were titrated by endpoint dilution onto SC CEF cultures as described previously (21). The REV-A titer was 2×10^6 IU/ml and the CSV titer was 1.5×10^6 IU/ml. We have experienced difficulty in establishing a reliable and quantitative in vitro assay for REV-T. Consequently, we determined the titer of infectious REV-T relative to the infectious titer of REV-A or CSV by comparing the amount of REV-T RNA with that of the helper virus. Viral RNAs were measured by hybridization to ^{32}P-pKW101 (v-*rel*) and ^{32}P-pSW253 (REV-A). Specific activities and the size of the probes were taken into consideration. In the REV-T(REV-A) stock, the titer of REV-A was 1.5×10^4 IU/ml, while the relative titer of REV-T was 8×10^4 IU/ml. In the REV-T(CSV) stock, the titer of CSV was 5×10^5 IU/ml, while the relative titer of REV-T was 10^5 IU/ml.

Infectious virus present in the plasma samples of infected chicks was also titrated by endpoint dilution onto SC CEF cultures. The reverse transcriptase reaction used in this assay has been described by Waite and Allen (23).

Immunohistochemical Analysis. Antibodies used to distinguish between REV-A and CSV infection were obtained from Dr. R. L. Witter (24). The REV-A specific mAb, 11C100, was used at a final dilution of 1:400, whereas the mAb capable of detecting both REV-A and CSV, 11A25, was used at a final dilution of 1:200. mAbs Hy-19 and Hy-16, which detect chicken IgM heavy chain and chicken IgG, respectively, were developed in this laboratory by Dr. Tim Baba.

Cells used in indirect immunofluorescence assays were washed twice in 10 mM PO_4, 150 mM NaCl, pH 7.5 (PBS) and resuspended to ~10^6 cells/ml. To prepare cytospins, 10^5 cells were centrifuged at 90 *g* and were fixed briefly in acetone before adding either Hy-19 or Hy-16 as primary antibody. Slides were incubated at 4°C overnight, washed three times in cold PBS, and wiped dry before adding fluorescein-labeled goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). After a 90-min incubation at 4°C, slides were washed three times in cold PBS, mounted in buffered glycerol, and examined by fluorescence microscopy.

Frozen tissue was embedded in OCT medium (Lab-Tek Products, Naperville, IL) and sectioned on a cryostat at 8 μm. Sections were dried and fixed in ice-cold acetone for 5–10 min. Once dried, slides were stored at −20°C until use.

Acetone-fixed frozen tissue sections were stained with Hy-19 and Hy-16 using a peroxidase–antiperoxidase (PAP) technique. Tissues were blocked with 50% FCS in PBS containing 0.2% sodium azide and an equal volume of primary antibody was added. After a 30-min room temperature (RT) incubation, the slides were washed in 20 mM Tris, 140 mM NaCl, pH 7.5 (TBS) three times for 5 min each at RT. After a brief fixation in 10% neutral buffered formalin, rabbit anti–mouse antibody (Dakopatts, Santa Barbara, CA) was added for a 20-min incubation. After three TBS washes, monoclonal mouse PAP (Dakopatts) was added for another 20 min. After three TBS washes, the slides were developed in 3% ammonium acetate, pH 5.5, containing 450 μg/ml diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and 0.0045% H_2O_2. Slides were dried, mounted, and examined by light microscopy.

Histology. Formalin-fixed samples were embedded in paraffin for histological exami-

TABLE I
Comparison of REV-A and CSV-infected Chicks

Chick	Time*	*n*	Body weight	Tissue analyzed						
				Spleen	Liver	Bursa	Kidney	Heart	Thymus	Hematocrit
	wk		*g*				%			
Uninfected	1	13	58	0.08	3.6	0.33	0.31	0.81	ND	37.5
	2	9	103	0.12	2.8	0.60	0.31	0.79	ND	33.4
	4	7	241	0.20	2.4	0.80	0.34	0.67	0.25	33.7
REV-A-infected	1	5	54	0.17	5.0	0.17	0.30	0.70	ND	31.5
	2	9	71	0.13	3.7	0.21	0.30	0.58	ND	29.3
	4	11	117	0.15	3.9	0.22	0.29	0.57	0.15	18.0
CSV-infected	1	4	64	0.14	4.3	0.25	0.33	0.72	ND	33.5
	2	10	94	0.16	3.7	0.39	0.33	0.70	ND	34.3
	4	7	187	0.18	2.8	0.37	0.27	0.48	0.19	30.6

SC chicks from Hyline were infected on day 1 after hatch with 10^5 IU of REV-A or CSV via the jugular vein. Body weights are expressed as the average weights in grams. Organ weights are represented as the ratio of organ to body weight × 100. Hematocrits are averages of percent packed cell volume.

* The time analysis was performed in weeks after infection.

nation and sectioned on a microtome at 5 μm. Transformed follicles were identified as described previously (25) except that bursae were serially sectioned at 200-μm intervals throughout the entire organ and stained with methyl green pyronin (Sigma Chemical Co.) under conditions specified by the manufacturer. Hematoxylin and eosin staining was provided by the university pathology laboratory.

Analysis of Cellular DNA. Cellular DNA was prepared from red blood cells or cultured cell lines derived from tumors as previously described (21). Eco RI and Bgl II enzymes were purchased from Boehringer Mannheim, Indianapolis, IN. Digestion conditions were as specified by the manufacturer. Analysis of DNA by Southern transfer and hybridization conditions have been previously described (21). pBB12, a plasmid containing a 1,300-bp fragment of *gag* sequences derived from the Schmidt-Ruppin A strain of avian sarcoma virus (26) was used to locate endogenous viral sequences. pKW101, a plasmid containing the 967 bp Eco RI fragment of v-*rel* sequences (27) was provided by Dr. H. Temin and used to locate REV-T-specific integration sites and c-*rel* sequences.

Results

Analysis of REV-A and CSV Infection in the SC Chick. Previous reports indicate that REV-A infection results in immunosuppression and bursal atrophy. Since CSV is reported to be less pathogenic than the other nondefective members of the reticuloendotheliosis virus family, we compared the effects of REV-A and CSV infection in the SC chick. 1-d-old SC chicks were infected with 10^5 IU of either REV-A or CSV. Hematocrits, organs, and plasmas were collected from chicks at 1, 2, and 4 wk after infection to examine the progression of disease. A comparison of total body weights indicates that REV-A-infected chicks were runted relative to control chicks (Table I). While slight splenomegaly and hepatomegaly were observed, the bursa exhibited severe atrophy. By 4 wk after infection, hematocrit values were low, indicating the presence of anemia in later stages of REV-A disease. These findings agree well with previous observations of REV-A-induced runting, anemia, and bursal atrophy (2, 28). In contrast to REV-A infection, the consequences of CSV infection appeared relatively minor. Although atrophy of the bursa was detected, there was reduced runting and negligible anemia in the CSV-infected chick.

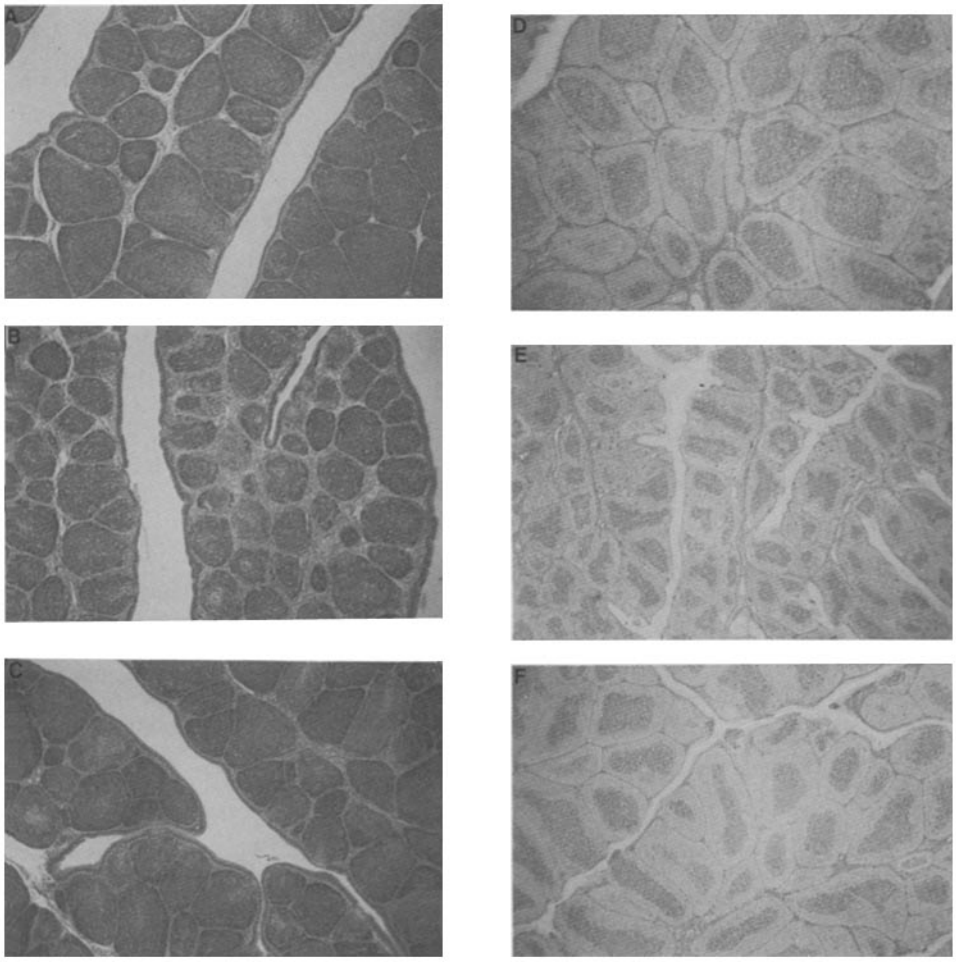

FIGURE 1. Comparison of normal, REV-A, and CSV-infected bursal tissues. Bursal tissue from 2-wk-old normal, REV-A, and CSV-infected chicks was fixed in 10% neutral buffered formalin and processed for histological examination. Paraffin blocks were sectioned at 5 μm and slides were stained with hematoxylin and eosin (*A*, *B*, and *C*). Bursal tissue from 4-wk-old chicks was snap-frozen in 2-methylbutane at −70°C and sectioned at 8 μm. Slides were stained to reveal IgM expression using the PAP assay (*D*, *E*, and *F*). (*A* and *D*) Normal bursa, (*B* and *E*) REV-A-infected bursa, (*C* and *F*) CSV-infected bursa.

To further distinguish the effects of these two viruses on the bursa, we prepared sections of infected bursal tissue for histological and immunohistochemical analysis. Bursae from four birds infected with either REV-A or CSV were examined at 1 and 2 wk after infection. Hematoxylin and eosin staining of the bursal follicles revealed that a majority (>80%) of the follicles in the REV-A-infected bursa were reduced in size (Fig. 1, *A* and *B*). Expansion of the interfollicular cell mass was evident throughout the organ. The cortico-medullary boundaries of the follicles were aberrant and individual cells appeared more eosinophilic and vacuolated with chromatin condensation at the nuclear membrane. This appearance is characteristic of dead or dying cells. In contrast, only a minority (<10%) of follicles in the CSV-infected bursa were similarly affected

so that the tissue as a whole appeared nearly normal (Fig. 1 *C*). Bursae from several REV-A or CSV-infected chicks were stained to reveal IgM using the PAP assay (Fig. 1, *D* and *E*). The normal bursa was characterized by even staining throughout the tissue with more intense staining in the medulla. The anti-IgM staining pattern in REV-A-infected bursae was patchy and irregular with tissue from birds killed 4 wk after infection more obviously affected. In contrast, analysis of CSV-infected tissue revealed normal distribution of IgM in the bursa (Fig. 1 *F*).

As one of the functions of the bursa is to seed the spleen with IgM^+ cells, the functional integrity of the bursa can be examined by determining the IgM staining pattern of the spleen. The PAP analysis, therefore, was extended to splenic tissue from normal, REV-A, and CSV-infected 2-wk-old chicks. In control spleens, the B lymphocyte areas surrounding the Schweigger-Seidel sheaths stained positively for IgM while a few individual plasma cells stained intensely (Fig. 2 *A*). In contrast, CSV- and ALV-infected spleens contained greater numbers of intensely staining plasma cells (Fig. 2, *C* and *D*). This increase in the number of plasma cells coincides with the appearance of an active immune response to virus (29, 30). Germinal centers were not observed as they require 3–4 wk to develop. In distinct contrast to splenic tissue from ALV- and CSV-infected chicks, REV-A-infected spleens exhibited an IgM staining pattern similar to that of uninfected birds (Fig. 2 *B*). The absence of an increase in intensely staining plasma cells in the REV-A-infected spleen coincided with the morphological atrophy of the bursa. Our results indicate that REV-A disrupts the ability of the bursa to seed the spleen with maturing plasma cells and that this immunosuppressive effect is distinct from the induction of suppressor T cell activity that follows REV-A infection (17) and may be related to a diminished ability of REV-A-infected chicks to mount a humoral response against T-independent antigens (18).

Helper Virus Replication in Bursal Lymphocytes. The differential effects of REV-A and CSV infection of bursal tissue might result from more extensive replication of REV-A in the SC chick. To evaluate this possibility, the amount of virus present in the plasma of REV-A and CSV-infected chicks was determined by end-point dilution. REV-A-infected birds maintained a viremia of 10^3 to 10^4 IU/ml of plasma throughout the 4-wk time period examined, while CSV-infected chicks had 50- to 500-fold lower levels of virus circulating during the same period (Table II). These results indicate that REV-A infection leads to greater levels of circulating virus. To evaluate the extent of viral infection in the bursa, we used mAbs to assay frozen sections of bursal tissue for the presence of viral antigens. As expected, analysis with 11C100, specific for REV-A, detected antigen only in REV-A-infected tissue (data not shown). The analysis with 11A25, a reagent capable of reacting with both REV-A and CSV, demonstrated that both REV-A- and CSV-infected tissue stained equally (Fig. 3). This result suggests that despite greater levels of circulating infectious REV-A, there is no difference in the amount of viral antigen present in bursal tissue infected with either virus and suggests that increased viral expression is not the basis for the toxic effect of REV-A on the bursa. However, a more detailed and quantitative analysis is necessary to substantiate this hypothesis.

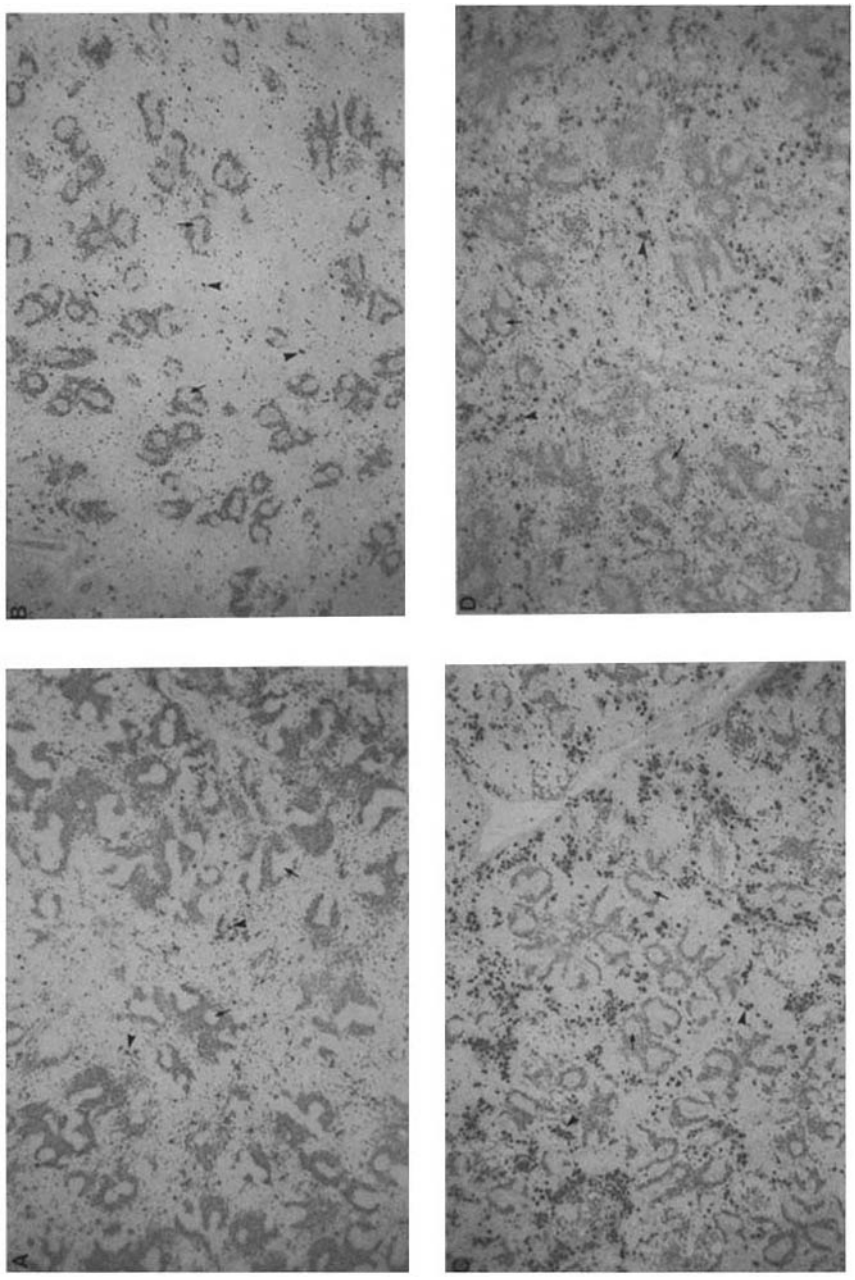

FIGURE 2. IgM expression in normal, REV-A, CSV, and ALV-infected splenic tissues. Spleens from 2-wk-old chicks were snap-frozen in 2-methylbutane at −70 °C and sectioned at 8 μm. Slides were stained to reveal IgM expression using a PAP assay. (*A*) Normal spleen, (*B*) REV-A-infected spleen, (*C*) CSV-infected spleen, and (*D*) ALV-infected spleen. Arrows mark Schweigger-Seidel sheaths and arrowheads indicate plasma cells.

TABLE II
Viremia in REV-A and CSV-infected Chicks

Virus	Time	*n*	Average viremia
	wk		*IU/ml*
REV-A	1	5	1.6×10^4
	2	9	1.6×10^4
	4	4	1.0×10^3
CSV	1	5	5.0×10^2
	2	10	4.0×10^2
	4	6	2.5×10^0

1 ml of blood was collected from REV-A and CSV-infected chicks at 1, 2, and 4 wk after infection in 1 ml of Alsever's solution to prevent clotting. Plasma was collected aseptically after centrifugation at 800 *g* to remove cellular constituents and stored at −70°C until use. Plasma samples were assayed for virus by end-point titration on SC CEF using the assay for reverse transcriptase as an indicator of virus replication. Viremias are expressed as averages in infectious units per milliliter.

Virus Expression in the Transformed Follicle. REV-A and CSV are known to cause lymphoid leukosis, a disease that is bursal dependent and characterized by the early development of a preneoplastic lesion designated the transformed follicle (25). To examine the presence of transformed follicles in REV-A- and CSV-infected bursae, formalin-fixed bursal tissue was serially sectioned and stained with methyl green pyronin. No more than two transformed follicles per bursa were observed in either REV-A$^-$ or CSV-infected tissue (Fig. 4). Data from analysis of ALV-infected chicks suggest that the maximum number of transformed follicles are seen by 4 wk after infection (25). Significantly, equivalent numbers of transformed follicles were seen in both infected tissues. This assay was repeated using frozen tissue sections and adjacent serial sections containing transformed follicles were stained by the PAP assay to detect the presence of viral antigens and IgM. Transformed follicles from both infected birds contained viral antigen (data not shown), indicating that either REV-A or CSV replication can occur within proliferating bursal lymphocytes without resulting in cell death. Further, consistent with normal B lymphocyte function, these transformed follicles exhibited IgM expression (data not shown). Therefore, although REV-A infection results in either destruction or depletion of the bursal population, it seems unlikely that this is a direct consequence of viral replication within bursal lymphocytes.

REV-A and CSV as Helper Viruses for REV-T-induced Disease. Having established the consequences of REV-A and CSV infection in the day old chick, we compared these two viruses as helper viruses for REV-T-mediated tumor induction. 1-d-old SC chicks were infected with either REV-T(REV-A) or REV-T(CSV) and killed at 1 wk. Analysis of body and organ weights showed a significant increase in spleen and liver weights of birds infected with either REV-T(REV-A) or REV-T(CSV) (Table III). This increase appeared to correlate with the tumor mass observed at autopsy. Moreover, REV-T(CSV)-infected spleens were significantly larger than those from REV-T(REV-A)-infected birds. There was no difference in the bursal weights between chicks infected with either virus;

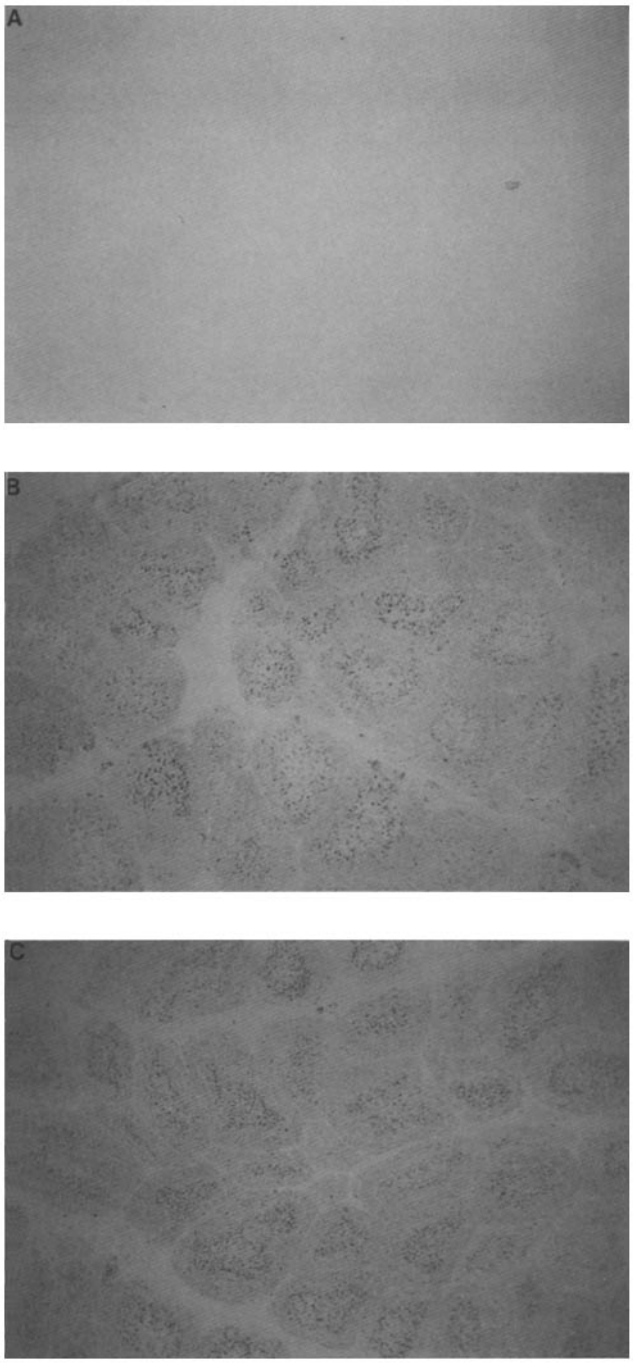

FIGURE 3. Expression of REV-A and CSV viral antigens in infected bursal tissue. Frozen sections of bursal tissue from 4-wk-old chicks were stained in a PAP assay with mAb 11A25, which recognizes both REV-A and CSV antigens. (*A*) Normal bursa, (*B*) REV-A-infected bursa, and (*C*) CSV-infected bursa.

however, both were decreased in comparison to uninfected controls. Anemia was observed in chicks infected with either virus, suggesting tumor involvement in the bone marrow. The large increase in the size of REV-T(CSV)-infected

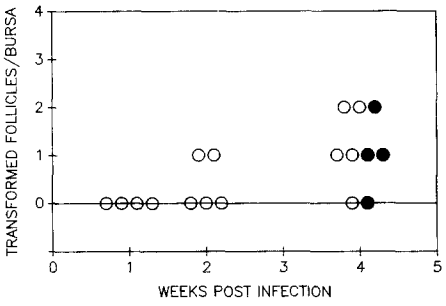

FIGURE 4. Incidence of transformed follicles in REV-A and CSV-infected chicks. SC chicks were infected with 10^5 IU REV-A or CSV on day 1 after hatch and killed at 1, 2, and 4 wk after infection. Bursae were fixed in 10% neutral buffered formalin and processed for histological examination. Serial sections were prepared throughout the entire bursa at 200 μm intervals, stained with methyl green pyronin, and examined for the presence of transformed follicles. Each symbol represents the number of transformed follicles in a single bursa. Open symbols (○) represent CSV-infected bursae and closed symbols (●) represent REV-A-infected bursae.

TABLE III

Comparison of REV-T(REV-A) and REV-T(CSF)-infected SC Chicks

Chicks	*n*	Body weight	Tissue analyzed						
			Spleen	Liver	Bursa	Kidney	Heart	Thymus	Hematocrit
		g				%			
Uninfected	13	58	0.08	3.6	0.33	0.31	0.81	0.17	37.5
REV-T(REV-A)-infected	13	54	0.28	5.4	0.20	0.35	0.72	0.11	29.1
REV-T(CSV)-infected	10	56	1.02	7.2	0.21	0.39	0.75	0.13	29.4

SC chicks from Hyline were infected with REV-T(REV-A) or REV-T(CSV) on day 1 after hatch and were killed 1 wk later for analysis. Samples for hematocrits were obtained from the wing vein before chicks were killed. Body weights are represented as the average weight in grams. Organ weights are expressed as the ratio of organ to body weight × 100. Hematocrits are averages of percent packed cell volume.

spleen and liver suggested that tumor development resulted from REV-T infection of a population of cells that is not present, or at least less susceptible to infection and proliferation, in the REV-T(REV-A)-infected chicks.

We analyzed spleen, liver, bursa, and thymus tissue from infected birds for the presence of tumors. To detect the majority of tumors present in the affected organs, we serially sectioned each tissue in at least four distinct areas ~200 μm apart and examined them with hematoxylin and eosin, methyl green pyronin, anti-IgM, and anti-IgG staining. Six REV-T(REV-A) and four REV-T(CSV)-infected birds were analyzed. Due to the extensive range in size and number of tumors present in the affected organs, it was difficult to quantitate precisely the number of individual tumors per bird. However, the majority of tumors (~90%) identified by hematoxylin and eosin staining in the REV-T(REV-A)-infected liver were negative for IgM expression, whereas the majority of tumors (~90%) in the REV-T(CSV)-infected liver were positive for IgM expression (Fig. 5). While analysis of the spleen and bursa was more difficult due to the background of IgM^+ cells in these organs, the same general observation was apparent (data not shown). The number of tumors present in the thymus was too few to be informative. None of the tumors in any tissue were positive for IgG expression.

The difference between the phenotype of these two tumors was pursued by developing cell lines from tumor tissue. Twenty-seven cell lines were developed from tumors of 13 REV-T(REV-A)-infected birds and 16 cell lines were made from tumors of 9 REV-T(CSV)-infected birds. Lines derived from REV-T(REV-A)-induced tumors were never more than 30% positive for IgM expression, while lines derived from REV-T(CSV)-induced tumors were 50 to 100% positive

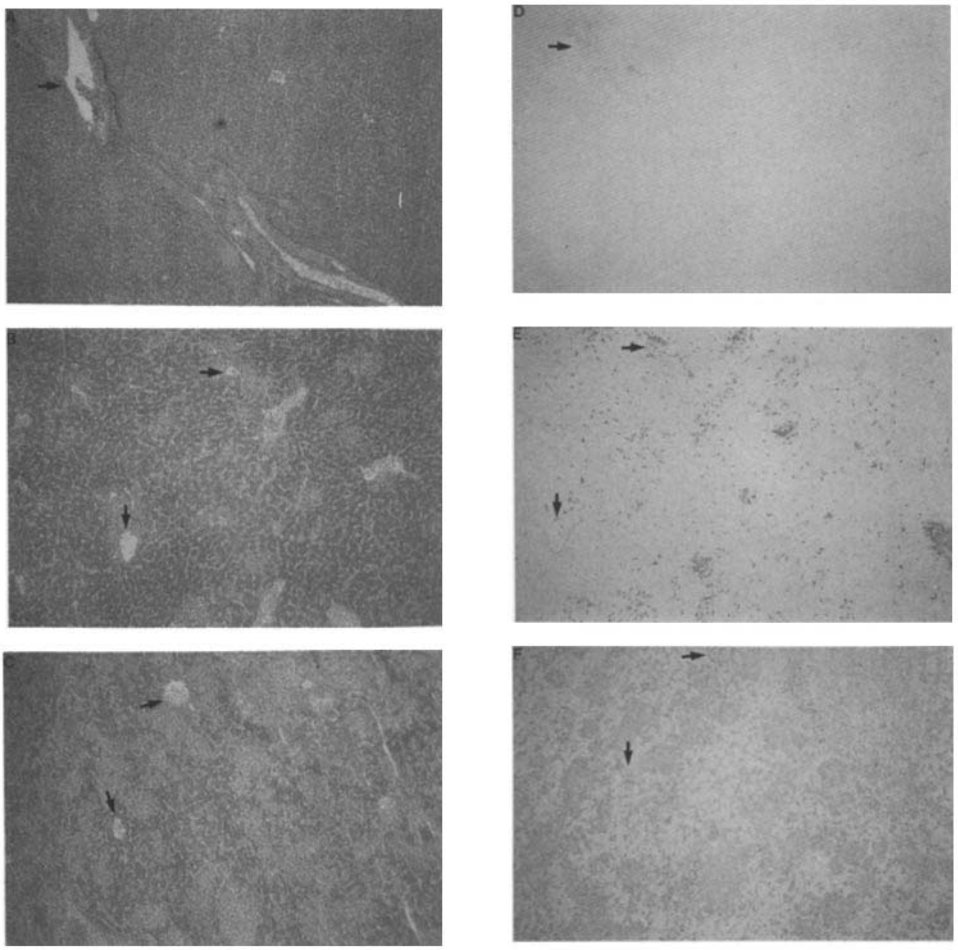

FIGURE 5. Expression of IgM in REV-T(REV-A)- and REV-T(CSV)-induced tumors. Adjacent serial sections of normal, REV-T(REV-A)-, and REV-T(CSV)-infected livers prepared from tissue frozen at 1 wk after infection were stained (i) with hematoxylin-eosin or (ii) with an anti-IgM monoclonal antibody. Hematoxylin-eosin stains of (*A*) normal liver, (*B*) REV-T(REV-A)-infected liver, and (*C*) REV-T(CSV)-infected liver can be compared with PAP stains for IgM of (*D*) normal liver, (*E*) REV-T(REV-A)-infected liver, and (*F*) REV-T(CSV)-infected liver. Arrows indicate nontumor markers that allow correct orientation of the adjacent sections.

for IgM expression (Table IV). Clones from these lines were established in soft agar and analyzed for IgM and IgG expression. Of 34 REV-T(REV-A)-generated clonal lines tested, all 34 were negative for IgM expression. In contrast, of the 32 REV-T(CSV)-induced clonal lines assayed, 29 were IgM positive. All of the clones were negative for IgG expression. Therefore, the cell line analysis correlates well with the in vivo analysis showing that REV-T(REV-A) infection induces primarily IgM negative tumors while REV-T(CSV) infection induces primarily IgM positive tumors.

The REV-T(CSV)-derived IgM$^+$ Tumor Cell Can Be of Bursal Origin. In the day old chick, the major population of proliferating B cells is located within the bursa. Since REV-T(CSV) infection produced predominately IgM$^+$ B cell tumors,

TABLE IV
Comparison of REV-T(REV-A) and REV-T(CSV) Tumor-derived Cell Lines

Phenotype	Number of REV-T(REV-A) lines	Number of REV-T(CSV) lines
Uncloned		
IgM$^-$	17	0
1–30% IgM$^+$	10	0
50–100% IgM$^+$	0	15
Cloned		
IgM$^-$	34	3
IgM$^+$	0	29
Total birds*	13	9
Total uncloned	27	15
Total cloned	34	32

Tumor tissue from REV-T(REV-A) and REV-T(CSV)-infected birds was minced with scissors and cultured in Hahn's medium. Cultures were transferred at 1:5 or 1:10 dilutions 24–48 h after the initial plating and passaged every 1–2 d thereafter. Cell lines were assayed for IgM expression after four or five transfers. Clones from these lines were established in soft agar and analyzed for IgM expression after amplification.

* The number of individual birds from which uncloned cell lines were derived.

we wished to determine whether or not bursal lymphocytes could serve as target cells for REV-T-induced tumors. We performed a bursal repopulation experiment that used the segregating endogenous viral locus 4 (EV-4) of the SC chick as a marker to differentiate between donor and recipient cells (26). EV-4$^-$ chicks were used as recipients while EV-4$^+$ chicks were used as donors. Recipient SC chicks were treated with cytoxan and repopulated with CSV-infected donor bursal B cells. 5.5 d later, three morbid recipient birds were killed and autopsied for the presence of tumors. For each bird, five separate tumor nodules from the liver, three separate portions of the spleen, and a portion each of bursa and thymus were removed to prepare cell suspensions for cell line development. A small sample of the liver suspension was assayed for IgM expression by immunofluorescence. To minimize selection, uncloned cell lines were analyzed between the fourth and sixth transfer after isolation for IgM expression, presence of EV loci, and viral integration. Analysis of the liver cell suspensions prepared at isolation showed that all samples were 50–100% positive for IgM. As the liver suspensions were prepared from tumor nodules and were probably clonal, the high percentage of IgM$^+$ cells was expected. Cell lines grew out of all tissue samples taken, including the thymus preparations. When these lines were tested for IgM expression at the fourth transfer, all lines, whether derived from liver, spleen, bursa, or thymus, were >99% IgM$^+$ (data not shown). This result demonstrates that IgM$^+$ tumor was present in all tissues and, therefore, capable of metastasis and proliferation in multiple microenvironments.

Because helper virus is present in REV-T(CSV), a spreading infection is

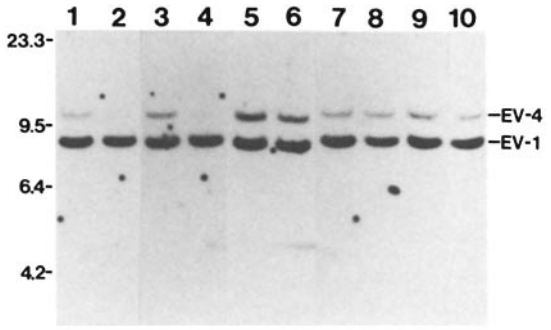

FIGURE 6. Analysis of the endogenous viral loci present in tumors isolated after transplantation of REV-T(CSV)-infected bursal lymphocytes. Cellular DNA was prepared from cell lines developed from tumors of cytoxan-treated birds repopulated with REV-T(CSV)-infected bursal lymphocytes. DNAs were digested with Eco RI and analyzed on 0.7% agarose gels, blotted to nitrocellulose, and hybridized with pBB-12 [^{32}P]DNA to identify the endogenous viral loci present. (*1*) RBC DNA from a donor bird, (*2*) RBC DNA from a recipient bird, (*3–10*) DNAs from cell lines derived from tumors of a recipient bird. The cell lines were derived from liver nodules (*3–6*), bursa (*7*), thymus (*8*), and spleen (*9* and *10*). Molecular size markers are indicated in kilobases at the left and EV loci are designated at the right. The EV-4 fragment is 10 kb and the EV-1 fragment is 8.7 kb.

established once the infected bursal cells have divided after transplantation. Consequently, the DNA from the cell lines had to be analyzed for the EV-4 locus to positively identify the lines as of donor origin. DNA samples digested with Eco RI were analyzed by Southern transfer and hybridization with pBB12 to detect EV loci. 18 cell lines from 2 birds were analyzed, along with donor and recipient RBC DNA. Of the 18 lines, 16 had EV-4 loci, demonstrating they were of donor cell origin (Fig. 6). This experiment demonstrates that REV-T is capable of infecting cells of bursal origin and inducing IgM$^+$ tumors that appear at the time of tumor initiation to be both bursal independent and capable of in vitro proliferation.

The cell lines were analyzed for unique REV-T integration sites to determine whether the tumors from which these lines were developed were identical. DNA was digested with Bgl II, which cuts once inside REV-T but outside v-*rel* sequences (31). Bgl II digestion and hybridization to v-*rel*, therefore, identifies a single unique fragment for each exogenous REV-T integration. The pKW101 *rel*-specific probe used also hybridizes to three fragments of c-*rel* (32). When DNA samples from the tumor lines were analyzed for *rel*-specific sequences, integration specific bands were detected in every line (Fig. 7). The multiple bands observed in lines developed from bursal, thymic, and splenic tumor tissue probably represent multiple, independent tumors as these cell lines were not cloned. Consistent with this interpretation, the hybridization of v-*rel* to the integration specific fragments is less intense than to fragments of c-*rel* (which serves as an internal standard for a single copy gene), indicating significant heterogeneity in the tumor population. Lines developed from liver nodules had single integration-specific fragments that hybridized with intensities equivalent to that of fragments of c-*rel*, indicating that these lines were probably clonal with respect to REV-T integration. 27 different patterns of integration were found in 29 different lines isolated from 3 birds (data not shown), indicating that multiple REV-T-infected bursal B lymphocytes gave rise to tumors in this system.

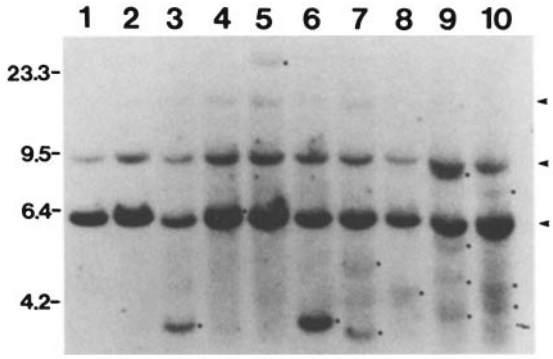

FIGURE 7. Analysis of v-*rel* sequences in tumors isolated after transplantation of REV-T(CSV)-infected bursal lymphocytes. Cellular DNA was prepared from cell lines developed from tumors of cytoxan-treated birds repopulated with REV-T(CSV)-infected bursal lymphocytes. DNAs were digested with Bgl II and analyzed on 0.7% agarose gels, blotted to nitrocellulose, and hybridized with pKW101 [^{32}P]DNA to identify the REV-T integration site. (*1*) RBC DNA from a donor bird, (*2*) RBC DNA from a recipient bird, (*3–10*) DNAs from cell lines derived from tumors of a recipient bird. The cell lines were derived from liver nodules (*3–6*), bursa (*7*), thymus (*8*), and spleen (*9* and *10*). Molecular size markers are indicated in kilobases at the left and REV-T-specific integration sites are marked with asterisks. Sizes of REV-T-specific integration fragments range from >23 kb to 3.7 kb. c-*rel* fragments are 15, 9, and 6 kb and are indicated with arrowheads at the right.

Discussion

High-Frequency Induction of IgM$^+$ B Cell Lymphomas. Experiments described in this report demonstrate that by using CSV to provide the helper functions for REV-T replication, the majority of the tumors induced after infection of day old chicks express IgM. The tumors within a single bird are polyclonal, suggesting that initiation and tumor development occur efficiently within a number of cells. We have also demonstrated that bursal B cells infected by REV-T(CSV) are able to develop as a disseminated IgM$^+$ tumor. Dissemination to a variety of microenvironments occurs without requiring an extended period of tumor progression, indicating that the initial tumor is bursal independent. These same tumors proliferate indefinitely as in vitro cell lines.

These experiments provide the first evidence that expression of v-*rel* can induce IgM$^+$ B cell tumors with a high efficiency. Previous studies characterizing in vitro–derived cell lines with a variety of heterosera, including several directed against both B and T lymphocytes of the chicken, have suggested that REV-T(REV-A) induces a poorly defined lymphoid tumor perhaps within the B cell lineage (13–15). The issue of tumor phenotype is somewhat confused as the original tumor was described as reticuloendothelial perhaps within the macrophage–dendritic cell lineage (2, 3, 10). Definitive markers capable of identifying these tumors and relating their phenotype to that of a normal cellular compartment have not yet been identified. In retrospect, it is significant that two REV-T-induced cell lines expressing IgM have been isolated (14, 16). Our in situ analysis of tumors produced after infection with REV-T(REV-A) revealed that <10% of these tumors expressed IgM. None of the cell lines prepared from these tumors (isolated on a completely random basis) expressed IgM. While analysis of tumor tissue has not been reported, these results are consistent with previous in vitro observations (13–15).

Our observation that altering the helper virus that provides the viral proteins for REV-T replication also changes the type of tumor induced by v-*rel* expression appears to be the first report of such a phenomenon. Other helper viruses are

able to influence the course of tumor development after infection by an acute transforming retrovirus but the actual type of tumor that develops remains unchanged. The development of Abelson disease can be markedly influenced by the specific helper virus with which the animal is infected, but only the incidence and rate of disease onset are altered (33). Similarly, the type of Friend disease is specified by the infecting strain of SFFV, either $SFFV_a$ or $SFFV_p$, as determined by the different *env* regions (34). In each instance, different strains of helper murine leukemia virus (MuLV) can influence the course of the disease. In contrast to these examples, REV-A and CSV have a direct influence on the actual type of tumor that REV-T induces. As discussed below, we believe this influence is due to the cytotoxic effect REV-A replication has on the IgM^+ B cell population within the bursa.

REV-A Induces Extensive Bursal Atrophy. Previous work has shown that REV-A induces the appearance of a suppressor T cell that correlates with a state of immunosuppression that is independent of bursal function (17, 18, 35). While the basis for this phenomenon has not been determined, it would appear to differ from the mechanism by which REV-A influences the spectrum of REV-T-induced tumors. The IgM^- tumor induced by REV-T(REV-A) appears to result from the generalized atrophy that affects the bursa. Our analysis of the bursa demonstrates not only that the size of the bursa is reduced but also that the tissue within this organ is disrupted and the expression of IgM is aberrant. It may be relevant that acute REV-A infections are known to be cytotoxic to fibroblasts in vitro (5). The interfollicular tissue, a potential source of fibroblast and stromal cell–derived growth factors, is markedly altered. While we have no direct evidence, it seems likely that the B cell population, which is normally undergoing extensive proliferation and differentiation, has ceased division and is stationary or dying. Under these conditions, while REV-T may be able to infect the bursal lymphocyte population, activation of v-*rel* expression and tumor induction would be unlikely. In contrast, while the bursa in the CSV-infected chick is smaller than in the uninfected chick, both the follicular structure and the cells within the follicles appear healthy and normal. It would appear that CSV enables REV-T to induce primarily IgM^+ tumors by not destroying the bursal lymphocyte and thereby enlarging the target cell population to include proliferating, maturing B cells.

Further work will be required to elucidate the mechanism by which REV-A exerts its pathogenic effect on the bursa. It is significant, however, that both viruses appear able to replicate with equal efficiency in bursal tissue, as evidenced by the expression of viral antigen. Furthermore, the expression of REV-A antigens within bursal lymphocytes does not appear to be cytotoxic by itself as transformed follicles, equally frequent in both REV-A- and CSV-infected chicks, express such proteins in roughly equal quantities. It also seems unlikely that immune elimination of REV-A-infected tissue is responsible for atrophy of the bursa, since at the height of the humoral response during an ALV infection, ALV DNA sequences are eliminated rapidly from both the bone marrow and the peripheral white blood cell population while they are selectively maintained in the bursa (36). We favor the possibility that REV-A infection results in destruction of the bursal stroma such that stromal–lymphocyte interactions

TABLE V
Comparison of REV-T(CSV) and ALV-derived IgM+ B Cell Lymphomas

REV-T(CSV)-induced	ALV-induced
Develop within 1–2 wk	Development requires 3–6 months
Apparent single hit kinetics	Multiple hit kinetics
No preneoplastic lesion	Identifiable preneoplastic lesion
Primary tumor is bursal independent	Primary tumor is bursal dependent
Tumor progression not required for metastasis to nonbursal sites	Tumor progression required for metastasis to nonbursal sites
Adaptation to in vitro growth not required	Adaptation to in vitro growth required

and/or production of essential growth factors required for B cell proliferation and survival are absent. It is possible that one of the REV-A glycoproteins binds to bursal cells or a specific growth factor thereby blocking an interaction required for bursal lymphocyte proliferation. It has been proposed that a 26-amino-acid peptide found in a number of retroviral transmembrane glycoproteins has immunosuppressive activity (37). A similar sequence has been located in gp20 for REV-A (38).

The Target Cell and v-rel-induced Neoplastic Disease. The observation that REV-T can induce tumors that are predominantly IgM+ demonstrates that the spectrum of cells in which v-*rel* is able to induce neoplastic disease is larger than originally thought. While two IgM+ cell lines have been seen before, the fact that the frequency with which they can be induced is so dramatically altered by changing the helper virus illustrates that access to a given cell type plays a significant role in defining which cells are target cells for v-*rel*-induced tumorigenesis. While access to the IgM target cell may have been uniquely provided by the helper virus in this system, a formally similar situation has been studied with Abelson virus–induced disease. The range of cell phenotypes that can be transformed in vitro by A-MuLV includes pre-B, immature, and mature B lymphocytes, erythroid precursors, macrophages, and mast cells (39–41). Not all of these cells, however, serve as targets in vivo. Pre-B and immature B lymphocytes serve as the most frequent target cells for Abelson-induced tumors (42, 43). In contrast, mast cells and macrophages serve as targets infrequently and Abelson-induced erythroid tumors have not been observed (39, 44). These data support the conclusion that a variety of factors beyond the ability of expressed v-*abl* sequences to function in a permissive environment are important in determining whether or not a cell serves as a target for Abelson-induced tumor development.

Defining the range of target cells has important implications for identifying the cellular genes that are involved in v-*rel*-mediated tumor development. Of particular interest to this laboratory is the genetic analysis of B cell lymphoma development in the chicken. The IgM+ bursal-derived tumor induced by expression of v-*rel* differs significantly from the IgM+ bursal-derived tumor isolated after ALV infection. This ALV-induced tumor is characterized by elevated levels of c-*myc* resulting from viral integration within the normal cellular locus (45, 46). While these two tumors have developed from apparently similar target cells and appear phenotypically identical, their development, as outlined in Table V, is quite distinct and indicates that significant differences exist in the genetic

pathways used in the development of these two lymphomas. A molecular comparison of the sequences expressed in the two tumors should identify genes that function specifically in one or the other of the pathways, thereby providing information that is important in dissecting the functions of v-*rel* and c-*myc* during neoplastic development in the avian B lymphocyte.

Summary

We have documented the effect of two nondefective helper viruses, reticuloendotheliosis virus A (REV-A) and chick syncytial virus (CSV) infection on bursal tissue. REV-A infection results in bursal atrophy, destroying both its structural and functional integrity. In contrast, the bursae in CSV-infected chicks, while reduced slightly in size, appear both structurally and functionally normal. REV-A-induced bursal atrophy is not a result of viral replication in the B-lymphocyte as (*a*) both viruses are capable of inducing, with equal efficiency, the formation of preneoplastic lesions containing proliferating B lymphocytes and (*b*) it appears that equivalent amounts of viral antigen are expressed in the bursae of chicks infected with either virus.

We have examined the phenotype of tumors induced by the replication-defective virus REV-T when replicated by the two different helper viruses, REV-A and CSV. In REV-T(REV-A)-infected chicks, the majority of tumors that develop are negative for IgM expression. In contrast, the majority of tumors induced by REV-T(CSV) infection are IgM^+. This finding is confirmed by recovery of IgM^- cell lines from REV-T(REV-A)-infected chicks and IgM^+ cell lines from REV-T(CSV)-infected chicks. In addition, repopulation studies show that bursal-derived cells that are IgM^+ serve as target cells for REV-T(CSV)-induced lymphomas. This study demonstrates, therefore, that REV-T can induce IgM^+, B cell lymphomas with high efficiency. We conclude that infections by the helper viruses, REV-A and CSV, differ dramatically in their effects on the composition of the population of cells that serve as targets for REV-T-induced neoplasia.

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