Reticuloendotheliosis Virus REV-T(REV-A)-Induced Neoplasia: Development of Tumors within the T-Lymphoid and Myeloid Lineages

CAROLYN F. BARTH,^{1†} DONALD L. EWERT,² WALTER C. OLSON,² AND ERIC H. HUMPHRIES^{1‡*}

Department of Microbiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235-9048,¹ and The Wistar Institute, Philadelphia, Pennsylvania 19104²

Received 13 June 1990/Accepted 14 September 1990

Infection of 1-day-old chicks with reticuloendotheliosis virus strain T induces a neoplastic disease that kills the chicks 7 to 14 days postinfection. In association with reticuloendotheliosis-associated virus (REV-A), reticuloendotheliosis virus T (REV-T) induces tumors that are predominantly immunoglobulin M (IgM) negative. We examined a variety of REV-T(REV-A)-induced tumors and tumor-derived cell lines and concluded that the principal IgM-negative tumors that develop in REV-T(REV-A)-infected chicks are neither pre-B or pre-B-pre-T but rather mature T lymphoid and myeloid. Without exception, the immunoglobulin heavy- and light-chain loci were in germ line configuration. Furthermore, the cell lines expressed neither sterile transcripts of the heavy- or light-chain immunoglobulin genes nor elevated levels of *c-myc*, two characteristics associated with murine pre-B lymphomas. Cell lines were also examined by using monoclonal antibodies for expression of a variety of cell surface markers expressed on B lymphocytes and/or T lymphocytes and/or myeloid cells. These reagents defined two types of IgM-negative tumor cell lines, one $Cl\alpha^+$ CT-3⁺ (T lymphoid) and the other $Cl\alpha^+$ CT-3⁻. By using the same approaches, tumor development was examined following REV-T(REV-A) infection at 1 and 3 weeks post-hatching of cyclophosphamide-treated chicks shown to be devoid of B-lymphoid cells. Again, the tumors that developed were either $Cl\alpha^+$ CT-3⁺ (T lymphoid) or $Cl\alpha^+$ CT-3⁻. Furthermore, the frequency and rate with which IgM-negative tumors developed in cyclophosphamide-treated chicks were not different from those observed in normal chicks. In 3-week-old cyclophosphamide-treated chicks, the presence of $Cl\alpha^+$ CT-3⁻ tumors bearing hematopoietic lineage markers, such as CLA-3 and SM19, are most likely to have been derived from cells within the myeloid lineage.

Reticuloendotheliosis virus strain T is an avian retrovirus complex consisting of a replication-competent helper virus, reticuloendotheliosis-associated virus A (REV-A), and a replication-defective acute transforming virus, reticuloendotheliosis virus T (REV-T) (11, 12). REV-T contains the viral oncogene *v-rel*, which codes for a fusion protein consisting of sequences derived from both the 5' and 3' ends of the viral envelope gene, as well as the turkey cellular protooncogene *c-rel* (28, 32). REV-T(REV-A) possesses acute in vivo and in vitro transforming activity. The first studies characterizing the tumor induced by REV-T(REV-A) described the disease as histologically similar to lymphomatosis (26). This early conclusion seems justified, in part, by the isolation of a tumor-derived lymphoblastoid cell line that appeared to express immunoglobulin (15). In contrast, independent in vivo studies by Olson (18) and Twiehaus et al. (29) provided both histological and cytological evidence that the disease was reticuloendothelial. The proliferating population was histocytoid or primitive mesenchymal in appearance and was located around blood vessels, along the sinusoids in the liver, and in lymph follicles. Of particular interest was the general observation that tissue in both the thymus and bursa had undergone significant atrophy (17). In these organs, the tumor cell population appeared to expand at the expense of lymphoid tissue. Consistent with these

observations, it has been observed that both mitogen-mediated T-cell proliferation and T-cell-independent humoral responses are suppressed in REV-A-infected chickens (24, 25, 31).

To define the nature of the REV-T(REV-A) target cell, several investigators have characterized cell lines established by in vitro transformation (5, 16). On the basis of low-level expression of terminal deoxynucleotidyl transferase and staining by absorbed antibursal and antithymic heterosera, it was proposed that in vitro transformation of splenic cells produced immature lymphoid cell lines, possibly pre-B or pre-B-pre-T. Characterization of the λ light- and μ heavy-chain loci in several of these cell lines revealed that some lines possessed aberrant, apparently nonfunctional heavy-chain rearrangements while other lines retained germ line configurations for both heavy- and light-chain loci (10). It was proposed that these latter cell lines might be immature pre-B lymphoid in phenotype.

Recently, we used a different approach to reexamine the type of tumor induced by REV-T. REV-T was rescued from a transformed nonproducing cell line by superinfection with chicken syncytial virus (CSV) to generate the virus complex REV-T(CSV). Tumors resulting from infection of day-old chicks with REV-T(CSV) were examined and shown to be largely (>95%) immunoglobulin M (IgM) positive and probably bursal in origin (2). Subsequent analysis of cell lines derived from the REV-T(CSV) tumors demonstrated that they contained rearranged and diversified heavy- and light-chain loci and secreted IgM (3). These studies firmly established that one of the target cells transformed by *v-rel* is a mature (secreting) IgM-positive B lymphocyte.

* Corresponding author.

† Present address: Imperial Cancer Research Fund, Lincoln's Inn Field, London, England WC2A 3PX.

‡ Present address: Mary Babb Randolph Cancer Center, School of Medicine, West Virginia University, Morgantown, WV 26506.

In contrast, our study also demonstrated that the primary REV-T(REV-A)-induced tumor was IgM negative, developed as a smaller mass, and was histologically comparable to the disease originally described as reticuloendotheliosis (2). To define the IgM-negative REV-T(REV-A) target cell further, we examined tumor tissues produced under a variety of *in vivo* conditions. Tumor tissues and cell lines derived from tumor tissue were characterized for expression of a variety of hematopoietic cell markers. DNAs from tumor-derived cell lines were analyzed to determine the configuration of the heavy- and light-chain immunoglobulin loci. Our data indicate that most of the IgM-negative tumors were not pre-B lymphoid in nature but that they arose from cells within the myeloid and T-lymphoid lineages.

MATERIALS AND METHODS

Cells and viruses. SC chicken embryo fibroblasts were cultured in plastic dishes (Nunc, Roskilde, Denmark) in Dulbecco modified Eagle medium (Flow Laboratories, Inc., McLean, Va.) containing 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) and 10% calf serum (Hazleton, Lenexa, Kans.) with antibiotics (ET₁₀C₁₀). All cell lines established from tumor tissue were maintained in ET₁₀C₁₀ supplemented with 2% chicken serum (ET₁₀C₁₀Ck₂) (GIBCO Biologicals, Grand Island, N.Y.).

Normal bursal, splenic, and thymic lymphocytes were isolated from fresh tissues taken from 1-, 2-, and 4-week-old chicks. The tissues were minced, expressed through a nylon mesh bag (Bally Ribbon Mills, Bally, Pa.), and purified over a Ficoll gradient (Isolymp; Gallard-Schlesinger, Carla Place, N.Y.), and single-cell suspensions were washed twice with ET₁₀C₁₀Ck₂ and used for immunological analysis. Cultured blood monocytes were prepared from marrow from femurs of 1-week-old chicks. Mononuclear cells were isolated from the marrow suspension by Ficoll gradient and seeded at a density of 5×10^6 cells per 90-mm-diameter dish in Dulbecco modified Eagle medium containing 8% fetal bovine serum, 2% chicken serum, and 10% conditioned medium from primary chicken embryo fibroblast cultures. Daily for the next 3 days, the nonadherent population was transferred to a fresh dish and new medium was added as required. Cells were harvested for analysis on day 4.

REV-T(REV-A) was prepared from the 2A1 clone of the K1 bone marrow cell line as previously described (2). REV-A and REV-T were titrated either as infectious units by endpoint dilution on CEF or as RNA hybridizing to REV-A and REV-T viral DNA probes as previously described (2).

Chickens, virus infections, and cell line isolation. Embryonated SC eggs were purchased from Hyline International Hatcheries, West Des Moines, Iowa, and incubated with humidity at 39°C. On day 1 after hatching, chicks were infected via intrajugular injection with 0.2 ml of REV-T(REV-A) containing 10^5 IU of REV-T per ml. The chicks were housed by the Animal Resource Center, University of Texas Southwestern Medical Center at Dallas, in rooms isolated from control or avian leukosis virus-infected chicks. To prepare B-lymphocyte-deficient chicks, chicks were injected intraperitoneally with 3 mg of cyclophosphamide (Cytoxan; Mead Johnson, Syracuse, N.Y.) daily for 4 days after hatching. Food and water were provided *ad libitum*.

Cell lines derived from tumor tissues were isolated by preparing single-cell suspensions from tumor nodules isolated from livers and from random sections of tissue taken from spleens as previously described (2). Clonal cell lines were prepared by cloning in soft agar (3).

Histological and immunohistochemical analyses. Samples for histological analysis were fixed in 10% neutral buffered Formalin. Formalin-fixed samples were embedded in paraffin and sectioned on a microtome at 5 μ m. Hematoxylin and eosin stains were provided by the university pathology laboratory. Samples for immunohistochemical analysis were snap frozen in 2-methylbutane at -70°C. Frozen tissue was embedded in OCT medium (Lab-Tek Products, Naperville, Ill.) and sectioned on a cryostat at 8 μ m. Sections were dried and fixed in ice-cold acetone for 5 to 10 minutes. Once dried, slides were stored at -20°C until use. Acetone-fixed frozen tissue sections were stained with monoclonal antibodies (MAbs) by using a peroxidase-antiperoxidase technique as previously described (3).

MAbs. All MAbs isolated in our laboratory are designated by the prefix HY. The specificity of these MAbs and the antigens they recognize was examined by using flow cytometry; immunohistochemical analysis on bursal, thymic, and splenic tissues; and immunoprecipitation as previously described (3). The following MAbs recognize previously described antigens: HY1 (CT-1), HY16 (IgG), HY19 (IgM), HY32 (CIa), and HY40 (class I major histocompatibility complex [MHC]) (3). HY30 has been defined as recognizing Bu-2 as previously described (13). HY7, which immunoprecipitates 200- and 180-kDa proteins from T and B cells, respectively, and was previously described as similar to anti-CLA-1 (3), is now known to recognize a distinct antigen. Anti-Bu-1 (30) was provided by Michael Ratcliffe (McGill University, Montreal, Quebec, Canada). MAbs to CLA-1, CT-3, CT-4, CT-8, TCR-1, TCR-2, and TCR-3 (6-9, 27) were provided by Max Cooper and Chen-Lo Chen (University of Alabama at Birmingham). CT-3, CT-4, and CT-8 have been described as homologs of the CD-3, CD-4, and CD-8 markers present in mice and humans. Similarly, TCR-1, TCR-2, and TCR-3 are T-cell receptors described for chickens. Anti-HNK-1 was obtained from the American Type Culture Collection (20). MAb 86B5 (19) recognizes an antigen expressed by cells of all hematopoietic lineages but detects <20% of bursal B lymphocytes. A full description of the marker defined by 86B5 is being prepared for publication. MAb CLA-3 (19) defines an antigen expressed by cells of the lymphoid and myeloid lineages but not cells of the erythroid lineage. CLA-3-positive cells with myeloid morphology constitute <25% of the mature erythrocyte-depleted bone marrow population. MAb 5M19 recognizes an antigen expressed by cells of both lymphoid and myeloid lineages. Approximately 70% of peripheral blood leukocytes and erythrocyte-depleted spleen cells are 5M19 positive, including all T lymphocytes and myeloid cells, but <7% of mature B lymphocytes are 5M19 positive. Bursal B lymphocytes do not express the 5M19-defined antigen. Characterization of the antigen recognized by 5M19 is currently being prepared for publication.

Analysis of nucleic acids. DNAs were prepared from cultured cell lines as previously described (14). All restriction enzymes were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Digestion conditions were as specified by the manufacturer. Analysis of DNA by Southern transfer and hybridization conditions have been previously described (1). Total cellular RNA was prepared and examined by Northern (RNA) analysis as described previously (3).

Nucleic acid probes. Specific DNA fragments were isolated from parental plasmids by appropriate restriction enzyme digestion, acrylamide gel electrophoretic separation, and elution from the gel with 0.3 M NaOH (3). DNA fragments

TABLE 1. Hematopoietic cell markers

MAb	Designated antigen ^a	% of positive cells (staining intensity) ^b					
		Thymus	Bursa	Spleen	Cultured mono-cytes	Bone marrow	Peripheral blood lymphocytes
Hy1	CT-1	90 (+3)	<5	10-15 (+1/+2)	0	0	<5 (+2)
Hy7		>95 (+3)	>95 (+3)	>95 (+2/+3)	>95 (+1/+2)	40-50 (+3)	>95 (+3)
Hy16	IgG	0	5 (+2)	<5	0	0	0
Hy19	IgM	5-10 (+2/+3)	90 (+2/+3)	30 (+2/+3)	0	<5	15 (+2)
Hy30	Bu-2	10-15 (+3)	>95 (+3)	35 (+3)	80-90 (+1/+3)	15 (+3)	15-20 (+3)
Hy32	CIa	10 (+1/+2)	90 (+1/+2)	50 (+1/+3)	50	30 (+1/+3)	ND ^c
Hy40	Class I MHC	>95 (+3)	>95 (+3)	>95 (+3)	>95 (+3)	>95 (+3)	ND
Anti-HNK-1	HNK-1	<5 (+1/+2)	75 (+3)	<5 (+1/+2)	0	0	0
Anti-Bu-1	Bu-1	5-10 (+3)	95 (+3)	15-25 (+3)	0	<5	15-20 (+3)

^a Indicates a previously identified antigen that is recognized by a MAb isolated in our laboratory.

^b +1, Weak staining but above background; +2, moderate staining; +3, staining as strong as or stronger than that of positive controls. Reactivity is based primarily upon fluorescence-activated cell sorter analysis of single-cell suspensions prepared by fractionation on Ficoll gradients. Analysis on cytosols by immunoperoxidase was also considered. Cultured monocytes were prepared as cytosols and analyzed by immunoperoxidase. 0%, no significant staining; <5%, low but undetermined positive population. A range of positive values indicates significant variability among individuals, tissue age, or preparation. The results were collected from 2- to 5-week-old chicks.

^c ND, Not done.

and plasmids were labeled with ³²P by nick translation (Bethesda Research Laboratories, Inc., Bethesda, Md.). Probes to identify *v-rel*, *Cμ₁-Cμ₂*, and *CA* have already been described (3). Endogenous *c-myc* and *c-myb* RNAs were identified by Northern analysis by using a 1-kb exon 3-specific fragment isolated from *c-myc* and an *EcoRI-SalI*-specific 400-bp *myb* fragment derived from pVM2, respectively (23).

RESULTS

Expression of hematopoietic cell markers on REV-T(REV-A) tumors and tumor-derived cell lines. In the initial analysis of REV-T(REV-A) tumors, MAbs were used to detect the presence of specific antigens expressed by cells of the chicken hematopoietic lineage. The tumors were examined for the expression of several markers that have been studied extensively, including CT-1, IgG, IgM, CIa, and the class I MHC. Other, less thoroughly characterized markers, including Bu-1, Bu-2, HNK-1, and HY7, were also used. This latter set of markers is expressed by different popula-

tions within the myeloid and lymphoid lineages (Table 1). Frozen liver tissue taken from tumor-bearing chicks infected with REV-T(REV-A) were serially sectioned and analyzed by an immunoperoxidase assay for reactivity by using the MAbs listed in Table 2. Representative serial analyses are presented in Fig. 1. Three antigens, the class I MHC, CIa, and that recognized by HY7, were expressed uniformly and abundantly. Expression of Bu-2 was more variable; a spectrum of staining intensity was seen not only among tumors but among the individual cells making up a single tumor. Staining of apparently normal liver tissue, particularly in areas associated with the circulatory system, was prominent with αCIa, αIgG, and HY7. As reported previously (3), this background staining was observed in normal liver tissue with HY7 and with αIgG in liver tissue from chicks infected with REV-A alone. Staining with the rest of the MAbs, including αCT-1, αIgM, αIgG, αBu-1, and αHNK-1, revealed that these antigens were not expressed on the REV-T(REV-A)-induced tumor. A parallel analysis of tumors induced following infection with REV-T(CSV) demonstrated that, with the single exception of αIgM staining, the staining properties of

TABLE 2. Comparison of antigenic profiles of REV-T(REV-A)- and REV-T(CSV)-induced tumors and cell lines^a

MAb	Designated antigen	Staining intensity			
		Tumors		Cell lines	
		REV-T(REV-A)	REV-T(CSV)	REV-T(REV-A)	REV-T(CSV)
HY1	CT-1	—	—	—	—
HY7		+3	+3	+3	+3
HY16	IgG	—	—	—	—
HY19	IgM	—	+3	—	+3
HY30	Bu2	+1/+3 (v) ^b	+3 (v) ^c	+1/+3 (v) ^b	+3 (v) ^c
HY32	CIa	+3	+3	+3	+3
HY40	Class I MHC	+3	+3	+3	+3
Anti-HNK-1	HNK-1	—	—	—	—
Anti-Bu-1	Bu-1	—	—	—	—

^a The MAbs listed were used to stain tumors from seven chicks (prepared as serial sections from frozen tissue) or 12 cell lines (prepared by cytocentrifugation) in an immunoperoxidase assay. Results for REV-T(REV-A) cell lines represent a compilation of four lines stained for all nine markers plus an additional eight lines stained with all MAbs except anti-HNK-1 and anti-Bu-1. The results obtained with REV-T(CSV) cell lines are based on six lines stained for all antigens plus an additional three lines stained with all MAbs except anti-HNK-1 and anti-Bu-1. For definitions of staining intensities, see Table 1, footnote b. —, No staining.

^b These tumors showed extensive variations in staining with HY30, ranging from negative to strongly positive within a single tumor.

^c Staining for any particular tumor was uniform, but between different tumors, even within a bird, staining showed considerable variation.

FIG. 1. Expression of avian hematopoietic markers on REV-T(REV-A)-induced tumors. Adjacent serial sections of REV-T(REV-A)-infected liver tissue were stained with hematoxylin and eosin (A) or MAbs to HY7 (B), IgM (HY19) (C), Bu-2 (HY30) (D), Cla (HY32) (E), and a dendritic cell marker (HY34) (F) (3).

both the REV-T(CSV)- and REV-T(REV-A)-induced tumors were indistinguishable. As reported earlier (2), the tumors found in REV-T(REV-A)-infected birds were, in most cases, smaller and developed fewer large nodules than the mature B-cell tumors induced by REV-T(CSV). Moreover, REV-T(REV-A) tumor development was most pronounced along the blood vessels and sinusoids of the liver, an observation originally made by Olson (18) in his description of the reticuloendothelial tumor.

We extended the analysis of markers to clonal cell lines derived from REV-T(REV-A)-induced tumors. In agreement with the analysis of the tumors and the clonal nature of the cell lines, the staining with each antibody was uniform for any cell line, except α Bu-2. Within any clonal population positive for Bu-2 expression, as few as 10% or as many as 50% of the cells expressed this antigen. This staining with α Bu-2 was in contrast to the α Bu-2 staining on REV-T(CSV)-induced tumors reported previously (3), in which,

although variation from tumor to tumor was observed, clonal populations were uniformly reactive. Staining of the cell lines revealed that the reactivities of the cell lines and the tumors from which they were derived were identical.

With the MAbs described in Table 2, immunohistochemical analysis of REV-T(REV-A)-induced tumors and cell lines derived from tumors revealed that the phenotype of the cell lines isolated reflected the phenotype of most of the tumors examined. This observation indicates that it is unlikely that major selection occurs during cell line isolation and that examination of the cell lines provides a reasonable profile of the tumors. These results also demonstrate that with the markers listed in Table 1, tumors induced by REV-T(REV-A) can be distinguished from the IgM⁺ mature B-cell REV-T(CSV) tumors only by the absence of IgM. The presence of Cla, combined with the absence of CT-1 and IgM, indicates that REV-T(REV-A) tumors are neither early T nor mature B in nature but are hematopoietic in origin.

REV-T(REV-A)-induced cell lines retain the germ line configuration of heavy- and light-chain immunoglobulin genes. To examine the possibility that REV-T(REV-A) tumor-derived cell lines contain heavy- or light-chain (but not both) gene rearrangements, Southern analysis was performed on clonally derived cell lines. Independently derived REV-T(REV-A) cell lines were identified by analysis of REV-T proviral integration sites. Of 22 lines isolated from 10 chickens, 18 possessed unique patterns of integrated viral sequences. As in REV-T(CSV)-derived cell lines, several unique lines were isolated from a single chicken, indicating the oligoclonality of the tumor population. Most of the clones analyzed contained between one and four proviral copies of REV-T (data not shown). These REV-T(REV-A)-induced cell lines were analyzed for heavy-chain immunoglobulin gene rearrangement. Polymorphisms at *Pvu*II sites used to detect heavy-chain rearrangement have been identified previously (3). Hybridization of *Pvu*II-digested cell line DNA with C μ ₁-C μ ₂ [³²P]DNA revealed that all of the 18 IgM⁻ REV-T(REV-A)-induced cell lines had retained the germ line configuration of the C μ locus. Six representative lines (Fig. 2, lanes 2 to 7) showed absence of heavy-chain rearrangement, while an IgM-positive REV-T(CSV) B-cell line (lane 8) showed rearrangement of one allele. Rearrangement of the light-chain gene was analyzed by using a *Bam*HI site present in the intron between variable and joining regions of the λ locus. Removal of this site during V-to-J region rearrangement allowed detection of a fragment of increased size hybridizing to a C λ probe. Figure 3 shows representative REV-T(REV-A)-induced cell lines (lanes 2 to 8) which have not rearranged their λ chain alleles. A bursal DNA sample (lane 1) showed rearrangement of approximately half of the light-chain alleles, for comparison. All 18 lines analyzed possessed the germ line configuration of the λ chain alleles, indicating that in all REV-T(REV-A) tumor-derived lines, the IgM⁻ status is a function of germ line configuration for both the heavy- and light-chain loci.

Two parameters that have been used to define murine early B cells before rearrangement of their immunoglobulin loci are the presence of elevated levels (100 to 200 copies per cell) of *c-myc* RNA (4) and detection of sterile transcripts (21, 22). Northern analysis of total cellular RNAs from eight clonal REV-T(REV-A) cell lines was carried out by using [³²P]DNA probes from *v-rel*, *c-myc*, *c-myb*, C μ ₁-C μ ₂, and C λ . The results demonstrated that *c-myc* and *c-myb* were expressed at relatively low, housekeeping levels, 1 to 10 copies per cell, in all lines (data not shown). Furthermore, synthesis of sterile heavy- or light-chain transcripts was not

FIG. 2. REV-T(REV-A)-induced cell lines retain the germ line configuration of μ heavy-chain immunoglobulin alleles. (A) Cellular DNAs from clonal lines derived from REV-T(REV-A)-induced tumors were digested with *Pvu*II and analyzed by Southern transfer and hybridization to C μ ₁-C μ ₂ [³²P]DNA. Molecular size markers are indicated in kilobases at the left, and germ line (G) and rearranged (R) loci are marked at the right. Lanes: 1, SC erythrocyte DNA; 2 to 7, REV-T(REV-A)-induced cell lines 21B5, 22L2, 31K1, 34S1, 35S1, and 36S3, respectively; 8, IgM-positive REV-T(CSV)-induced cell line 11S3, which served as a control for rearrangement. The C μ ₂-C μ ₃-homologous fragment is indicated with an asterisk, and the C μ ₃-C μ ₄-homologous fragments are indicated with arrows. (B) Restriction map of the μ locus indicating pertinent polymorphic *Pvu*II recognition sites and genomic locations of the probes. Two *Pvu*II sites located in C μ exons are present in the cDNA sequence; however, it is not known whether the *Pvu*II sites associated with rearrangement (P*) are in V or D sequences. p? denotes the possible presence of a *Pvu*II site in the intron between C μ ₂ and C μ ₃; however, intronic polymorphism could account for the fragment size variation as well. Probe sequences are denoted over homologous regions of the gene by squares joined by broken lines. An asterisk preceding the name of a probe denotes radioactivity.

observed. On the basis of the presence of a processed 2.3-kb light-chain mRNA in control IgM⁺ B-cell lines, sterile transcripts at the level of approximately 10 copies per cell ought to have been visible (data not shown). Expression of both 2.1- and 5.3-kb REV-T mRNAs served as internal controls for mRNA quality.

REV-T(REV-A) target cell present in both cyclophosphamide-treated and untreated 3-week-old chicks. Previous in

FIG. 3. REV-T(REV-A)-induced cell lines retain the germ line configuration of λ light-chain immunoglobulin alleles. (A) Cellular DNAs from clonal cell lines derived from REV-T(REV-A)-induced tumors were digested with *Bam*HI and analyzed by Southern transfer and hybridization to λ C [32 P]DNA. Molecular size markers are indicated in kilobases at the left. Lanes: 1, bursal DNA; 2 to 8, REV-T(REV-A)-induced cell lines 33S1, 32M4, 22L2, 23L1, 23S3, 35S2, and 36S3, respectively. (B) Map of *Bam*HI (B) and *Sal*I (S) restriction sites in the λ light-chain locus of SC chickens. The genomic location of the λ C probe is shown. G, Germ line locus; R, rearranged locus.

vitro experimental analysis has suggested that a major target cell of REV-T(REV-A) is an early lymphoid cell, perhaps pre-B or pre-B-pre-T. The results presented above provided no evidence for a distinctive marker indicative of the early (IgM^-) B or early T lineage. In the face of these negative results, we asked whether cyclophosphamide-treated chicks (deficient in mature and precursor B cells) or older chicks (deficient in bursal stem cell activity) developed REV-T(REV-A)-induced neoplasia and, if so, how similar such tumors were to those that develop in normal chicks infected on the day of hatching. To address this issue, 14 cyclophosphamide-treated chicks were injected with either REV-T(REV-A) or REV-T(CSV) on day 5 posthatching and observed for tumor development. On day 9 postinfection, several birds appeared morbid (Table 3). At this time, all birds were sacrificed and autopsied for tumors. Autopsy revealed no significant differences between the two types of infection at a gross level, as all birds had enlarged livers and spleens containing small, white, nodular tumors. Liver tissue samples were collected, frozen, and processed for immunohistochemical analysis. Multiple sets of serial sections were analyzed by immunoperoxidase assay by using the previously described panel of MAbs. All tumors were neg-

ative for IgM expression whether obtained from chicks infected with REV-T(CSV) or chicks infected with REV-T(REV-A) (Table 3). Staining with MAbs provided results identical to those obtained by analysis of normal chicks infected with REV-T(REV-A) on the day of hatching (Fig. 1; Tables 2 and 3). To control for the efficiency of cyclophosphamide treatment, the bursae of all chicks were examined at autopsy. This analysis revealed that all cyclophosphamide-treated chicks had bursae reduced at least 10-fold from normal size. In addition, two bursae from uninfected, cyclophosphamide-treated chicks sacrificed at the same time as infected chicks (in the same experiment) were analyzed by α IgM staining and shown to lack IgM^+ cells. Five additional cyclophosphamide-treated and nine normal chicks were similarly infected at 21 days post-hatching. At 14 days later, all birds were sacrificed and analyzed as described above. The experiment yielded identical results, indicating that the tumors induced by REV-T(REV-A) following infection of 1- or 21-day-old chicks, treated with cyclophosphamide or not, were indistinguishable. The same tumor phenotype was observed following REV-T(CSV) infection of cyclophosphamide-treated birds. Most importantly, in both 1- and 3-week-old cyclophosphamide-treated chicks, the tumor mass present at the time of sacrifice was approximately equal to that observed in infected normal chicks, indicating no obvious reduction in target cell frequency. In view of the nature of the spreading infection in these birds, we consider this observation significant.

The tumor phenotype that developed in 3-week-old cyclophosphamide-treated birds was pursued further by developing 12 clonal, tumor-derived cell lines from three different birds. These lines, distinct on the basis of their REV-T integration pattern, were analyzed by reactivity with the MAbs as described in Table 2, footnote a, and found to be identical to the tumors from which they were derived (data not shown). All of the lines contained light- and heavy-chain loci in the germ line configuration (data not shown). The absence of mature B cells in cyclophosphamide-treated chicks, the inability of cells from such chicks to reestablish the B-lymphoid lineage, and the absence of bursal stem cell activity in older chicks make it highly unlikely that the REV-T(REV-A) tumors that developed in these experiments arose from either pre-B or pre-B-pre-T cells.

Two types of IgM^- tumors induced by REV-T(REV-A). The analysis presented above indicates that it is unlikely that REV-T(REV-A)-induced IgM^- tumors are within the B-lymphoid lineage. To define these tumors, 12 clonal IgM^- tumor-derived cell lines were examined for expression of cell surface antigens found on T-lymphoid and myeloid cells (Table 4). Five lines were derived from infection of normal 1-day-old chicks, while seven were isolated following infection of 3-week-old cyclophosphamide-treated chicks. The analysis revealed two distinct phenotypes. While all of the lines expressed CLA-3, ClA, and 5M19, eight lines expressed CT3 and were defined as T cell in origin and four lines did not. Among the lines that were CT-3 $^+$, most expressed TCR-2, while staining with CT-4 and CT-8 was variable. The four CT-3 $^-$ lines expressed none of the surface T-cell markers. On the basis of the presence of CLA-3, ClA, and 5M19 and the absence of 86B5, these IgM^- lines are most likely of myeloid origin. To pursue this conclusion further, 36 more cell lines were derived from splenic tissue removed from normal and cyclophosphamide-treated chicks following infection with REV-T(REV-A) 1 and 3 weeks after hatching. These lines were isolated as clones from a primary culture established from a splenic tumor explant. The lines were

TABLE 3. Analysis of REV-T(REV-A)-induced tumors in 3-week-old normal and cyclophosphamide-treated chicks^a

Treatment	Virus	Age at infection (days)	No. of days postinfection to sacrifice ^b	No. infected	No. with tumors ^c	No. of cell lines tested by cytospot ^d
None	AT	1	7-10	16	16 (7)	12 (4)
Cyclophosphamide	AT	5	9	5	5 (3)	0
None	AT	21	14	9	8 (6)	2 (1)
Cyclophosphamide	AT	21	14	5	4 (0)	12 (3) ^e
Cyclophosphamide	CT	5	9	9	9 (5)	0

^a Normal and cyclophosphamide-treated chicks were infected with (REV-T)(REV-A) (AT) or REV-T(CSV) (CT) virus. 2×10^4 IU of REV-T (T) was injected per bird. The dose of REV-A (A) was approximately 10^3 IU per bird, while the dose of CSV (C) was approximately 10^5 IU per bird. Tumors from selected birds were analyzed by staining multiple sets of serial sections of tumor tissue with the nine MAbs listed in Table 1. One-day- and three-week-old normal chicks infected with REV-T(REV-A) had a low percentage (<10%) of tumors staining positively for IgM. All of the remaining tumors were immunoglobulin negative.

^b At the time of sacrifice, most birds were weak and moribund.

^c The number of birds analyzed immunohistochemically is given in parentheses.

^d Clonal cell lines were established from tumor tissue isolated from the different types of infected chicks. The numbers of such lines analyzed for staining with all nine MAbs listed in Table 1 are shown. The numbers in parentheses denote the numbers of individual birds from which the cell lines were derived. All of the cell lines examined were IgM negative.

^e All 12 lines contained germ line heavy- and light-chain immunoglobulin loci.

stained for expression of CLA-3, IgM, CT-3, and ClA (Table 5). The data demonstrate that the two distinct phenotypes defined in Table 4 were observed in both normal and cyclophosphamide-treated chicks. On the basis of this experiment, we conclude that the CT-3⁻ ClA⁺ cell line is derived from a cell(s) within the myeloid lineage.

DISCUSSION

Previously published studies have described two distinct neoplastic diseases induced by reticuloendotheliosis virus strain T (2). One of these tumors has been analyzed in detail and been shown to possess the properties of a mature IgM-positive B-cell lymphoma. This tumor, induced preferentially by REV-T(CSV), consists of B cells that secrete IgM synthesized from light-chain alleles with various degrees of diversification (3). The second neoplasm, induced preferentially by REV-T(REV-A), consists of IgM-negative cells and has been described variously as reticuloendothelial or lymphoblastoid (18, 26, 29). As recently described, these two types of neoplasm coexist in a single bird, with one or the other predominating, as determined by the infecting helper virus (2). This observation is consistent with the fact that

previous studies of REV-T(REV-A) in vitro-derived cell lines have described them almost exclusively as IgM negative (5, 10, 16), while a recent analysis has described several new lines that are derived from IgM-positive mature B cells (33).

The focus of the current study was further characterization of the IgM-negative tumor originally described as reticuloendotheliosis. The reticuloendothelial system consists of cells of monocytic and macrophage character, implying that reticuloendothelial cells are actually of myeloid origin. The diagnosis of REV-T-induced reticuloendotheliosis was based upon histological analysis of lesions and a study of the cytology of proliferating cells in situ (18, 29). Both studies described the cells as irregular and undifferentiated in appearance, with the burden of the tumor located along the blood vessels and sinusoids of the liver. Several studies since then have analyzed REV-T(REV-A)-transformed IgM⁻ cell lines established following infection in vitro (5, 10, 16). On the basis of an analysis of their surface phenotype, as revealed by differentially absorbed heterosera, as well as the presence of deoxynucleotidyl transferase activity, these lines were considered likely to be of early lymphoid origin.

TABLE 4. Expression of T-lymphoid and myeloid markers on REV-T(REV-A)-induced IgM⁻ cell lines

Cell line ^a	Presence, absence, or % of staining ^b							
	CLA-1	CLA-3	ClA	5M19	86B5	CT-3	CT-4	CT-8
21B5	—	+	+	+	—	—	—	—
22L2	—	+	+	80	—	—	—	—
34S1	—	+	+	30	—	—	—	—
35S2	—	+	+	+	—	—	—	—
36S3	+	+	+	+	+	35	—	—
17L1	+	+	+	+	+	+	—	—
17L2	+	+	+	75	+	+	—	10
17L3	+	+	+	+	+	+	—	10
17S1	+	+	+	+	+	80	20	25
17S2	+	+	+	+	+	+	—	10
17S3	+	+	+	+	+	+	—	—
24S1	+	+	+	+	70	80	—	15

^a The 12 cell lines are distinct clones as determined by integration of REV-T proviral DNA at the time of their isolation. Lines 21B5, 22L2, 34S1, 35S2, and 36S3 were isolated from tumor-bearing chicks following infection of normal chicks at hatching. The other lines were derived from tumors isolated from cyclophosphamide-treated chicks infected 3 weeks after hatching. Six distinct clones were isolated from a single bird (17L1, L2, L3, S1, S2, and S3). Cell lines were analyzed by immunofluorescence and flow cytometry or by indirect immunoperoxidase reactivity by using the indicated MAbs.

^b +, Greater than 90% positive; —, no staining over the background. Numbers indicate percentages determined by flow cytometry. Seventeen percent of line 24S1 stained with α TCR-1. No other reactivity with α TCR-1 or α TCR-3 was observed. Six REV-T(CSV)-induced IgM⁺ lines were CLA-1⁻ 5M19⁻ CT-3⁻.

TABLE 5. Distribution of T-lymphoid and myeloid lines isolated following REV-T(REV-A) infection of normal and cyclophosphamide-treated chicks at 1 and 3 weeks of age^a

Age and treatment	No. of cell lines		
	Total	T lymphoid	Myeloid
1 wk			
None ^b	7	2	5
Cyclophosphamide	16	12	4
3 wk			
None	3	2	1
Cyclophosphamide	10	7	3

^a Lines judged T lymphoid were $\text{Cl}\alpha^+$ IgM^- CT-3^+ . Lines judged as myeloid were $\text{Cl}\alpha^+$ IgM^- CT-3^- . Staining with $\alpha\text{Cl}\alpha$ -3 was variable but usually positive. B-lymphoid (IgM^+) lines were not detected. Three to five chicks were infected for each group, and one to four clones from each chick were characterized. While past experience has shown that greater than 90% of the clones from a single chick possess distinct integration sites, some cell lines isolated may represent duplicates of clones previously isolated from the same chick.

^b Chicks not treated with cyclophosphamide were infected 1 day after hatching.

Analysis of the immunoglobulin heavy- and light-chain loci demonstrated they were unrearranged, suggesting the possibility that these lines had yet to undergo functional diversification (10). We reexamined the IgM^- tumor by using recently developed MABs that react with different hematopoietic cell populations. We also studied the development of these tumors in chickens devoid of early B-lymphocytic precursors. The results indicated that, in contrast to the in vitro analysis, the two principal IgM^- tumors were T lymphoid and myeloid in origin.

The data in this report support several conclusions. On the basis of staining with $\alpha\text{CT-3}$, the IgM^- tumor-derived cell lines represent at least two distinct tumors. The CT-3^+ tumor is variably positive for CT-8, usually expresses TCR-2, and is probably derived from the major T-cell population found in the periphery. The other tumor is $\text{Cl}\alpha$ -3⁺ CT-3^- and is likely to be of myeloid origin. This conclusion is based on the following three observations: (i) the presence of surface $\text{Cl}\alpha$ in the absence of other mature B- or T-cell markers; (ii) the development of these tumors in cyclophosphamide-treated and 3-week-old chicks (Table 5); and (iii) the presence of the 5M19 marker, an antigen found on all peripheral myeloid cells but less than 7% of IgM^+ B cells and not on bursal B lymphocytes. On the basis of the combined antigenic phenotype of these cells, they do not resemble immature B cells in the bursa, which are $\text{Cl}\alpha^+$ IgM^+ and 5M19 negative. Equally important is the observation that both tumors and cell lines are CT-1^- and unlikely to be early or thymus-derived T lymphoid. The only other cell population that is both $\text{Cl}\alpha^+$ and 5M19⁺ and lacks IgM or T-cell markers are cells of the myeloid lineage. Thus, in the absence of a clear myeloid-cell-specific marker, such as mac-I for murine macrophages, classification of tumors as derived from the myeloid lineage can be made only by a constellation of markers.

It is necessary to point out that use of surface markers present on a transformed tumor cell to define the target cell that gave rise to that tumor or transformed cell must be qualified by the possibility that neoplastic development can alter the expression of such markers. The absence or presence of a marker on a tumor cell does not ensure the corresponding absence or presence on the normal cell from which the tumor arose. In CT-3^+ tumors, the frequent

coordinate presence of CT-8 and TCR-2 makes such a consideration less troublesome. Additional investigation, including analysis of the configuration of T-cell receptor genes in CT-3^+ tumors, will clarify and extend this issue. Similarly, the definition of genes expressed in the myeloid cell population would be useful in further defining the properties of the myeloid tumor.

A final concern in our analysis is the extensive use we made of characterization of tumor-derived cell lines to determine the phenotype of the tumor. The data in Table 2 indicate that for the antigens evaluated, tumors and tumor-derived cell lines were indistinguishable. Obvious in vitro selection or antigen modulation did not occur for these markers. The need for quantitative evaluation and/or practical difficulties with other monoclonal reagents, including an apparently normal cell background, limited the utility of direct in vivo analysis. One major complicating factor was the small size of the average REV-T(REV-A) tumor. It is, therefore, possible that our analysis using one or more of the other MAB reagents was confounded by inapparent in vitro-induced antigen modulation. Equally, it is possible that in vitro-induced differentiation occurred. Relevant to this point, we detected evidence for population heterogeneity in cloned lines (Table 4). While it seems unlikely, in vitro infection of uninfected target cells may have occurred during development of some cell lines, resulting in the appearance of cell lines originally not present as tumor tissue.

While the tumors defined in this and other studies may be the principal tumors observed following REV-T(CSV) or REV-T(REV-A) infection, other tumors may develop either at a reduced frequency or in different experimental protocols. For example, the nature of the in vitro-derived lines and their relationship to the two tumors described in this study remain unknown. It is possible that additional cells have functioned as target cells in vitro. Should they represent additional distinct target cells for v-rel-induced transformation, it is unclear whether or not they develop as tumors in vivo. It is also possible that CT-3^+ CT-4^+ T-cell lymphomas, as well as early, immature IgM^+ B-cell tumors with stem cell properties, will be isolated. Target cell frequency, the relative rate of tumor cell growth, and the frequency of mitotic cells within different populations all play important roles in v-rel-induced neoplastic development. It is of particular importance to define the genotype of cells in which v-rel expression can result in tumor development or in vitro transformation. Permissive and nonpermissive cells will help to identify genes that are required for or inhibit v-rel-mediated neoplastic development.

ACKNOWLEDGMENTS

We thank Willie Young and Rae Allen for expert technical assistance. We also acknowledge the dedicated secretarial assistance of Susan Campbell with preparation of the manuscript.

This work was supported by Public Health Service grants AIO7324-01A2, CA41450, and CA39000 from the National Institutes of Health.

LITERATURE CITED

1. Baba, T. W., and E. H. Humphries. 1984. Avian leukosis virus infection: analysis of viremia and DNA integration in susceptible and resistant chicken lines. *J. Virol.* 51:123-130.
2. Barth, C. F., and E. H. Humphries. 1988. A nonimmunosuppressive helper virus allows high efficiency induction of B-cell lymphomas by reticuloendotheliosis virus strain T. *J. Exp. Med.* 167:89-108.
3. Barth, C. F., and E. H. Humphries. 1988. Expression of v-rel induces mature B-cell lines that reflect the diversity of avian

- immunoglobulin heavy- and light-chain rearrangements. *Mol. Cell. Biol.* **8**:5358–5368.
4. **Bender, T. P., and W. M. Kuehl.** 1987. Differential expression of the c-myc proto-oncogene marks the pre-B cell/B cell junction in murine B lymphoid tumors. *J. Immunol.* **139**:3822–3827.
 5. **Beug, H., H. Muller, S. Grieser, G. Doederlein, and T. Graf.** 1981. Hematopoietic cells transformed in vitro by REV-T avian reticuloendotheliosis virus express characteristics of very immature lymphoid cells. *Virology* **115**:295–309.
 6. **Chan, M. M., C.-L. H. Chen, L. L. Ager, and M. D. Cooper.** 1988. Identification of the avian homologues of mammalian CD4 and CD8 antigens. *J. Immunol.* **140**:2133–2138.
 7. **Chen, C.-L. H., L. L. Ager, G. L. Gartland, and M. D. Cooper.** 1986. Identification of a T3/T cell receptor complex in chickens. *J. Exp. Med.* **164**:375–380.
 8. **Chen, C.-L. H., J. Cihak, U. Losch, and M. D. Cooper.** 1988. Differential expression of two T cell receptors, TcR1 and TcR2, on chicken lymphocytes. *Eur. J. Immunol.* **18**:539–543.
 9. **Chen, C.-L. H., and M. D. Cooper.** 1987. Identification of cell surface molecules on chicken lymphocytes with monoclonal antibodies, p. 137–154. *In* A. Toivanen and P. Toivanen (ed.), *Avian immunology: basis and practice*. CRC Press, Inc., Boca Raton, Fla.
 10. **Chen, L., M. Y. Lim, H. R. Bose, Jr., and J. M. Bishop.** 1988. Rearrangements of chicken immunoglobulin genes in lymphoid cells transformed by the avian retroviral oncogene v-rel. *Proc. Natl. Acad. Sci. USA* **85**:549–553.
 11. **Hoelzer, J. D., R. B. Franklin, and H. R. Bose.** 1979. Transformation by reticuloendotheliosis virus: development of a focus assay and isolation of a nontransforming virus. *Virology* **93**:20–30.
 12. **Hoelzer, J. D., R. B. Lewis, C. R. Wasmuth, and H. R. Bose, Jr.** 1980. Hematopoietic cell transformation by reticuloendotheliosis virus: characterization of the genetic defect. *Virology* **100**:462–474.
 13. **Huffnagle, G. B., M. J. H. Ratcliffe, and E. H. Humphries.** 1989. Bu-2, a novel avian cell surface antigen on B cells and a population of non-lymphoid cells, is expressed homogeneously in germinal centers. *Hybridoma* **8**:589–604.
 14. **Humphries, E. H., R. Allen, and C. Glover.** 1981. Clonal analysis of the integration and expression of endogenous avian retroviral DNA acquired by exogenous viral infection. *J. Virol.* **39**:584–596.
 15. **Keller, L. H., R. Rufner, and M. Sevoian.** 1979. Isolation and development of a reticuloendotheliosis virus-transformed lymphoblastoid cell line from chicken spleen cells. *Infect. Immun.* **25**:694–701.
 16. **Lewis, R. B., J. McClure, B. Rup, D. W. Niesel, R. F. Garry, J. D. Hoelzer, K. Nazerian, and H. R. Bose, Jr.** 1981. Avian reticuloendotheliosis virus: identification of the hematopoietic target cell for transformation. *Cell* **25**:421–431.
 17. **Mussman, H. C., and M. J. Twiehaus.** 1971. Pathogenesis of reticuloendothelial virus disease in chicks—an acute runting syndrome. *Avian Dis.* **15**:483–502.
 18. **Olson, L. D.** 1967. Histopathologic and hematologic changes in moribund stages of chicks infected with T-virus. *Am. J. Vet. Res.* **28**:1501–1507.
 19. **Olson, W. C., and D. L. Ewert.** 1990. Markers of B lymphocyte differentiation in the chicken. *Hybridoma* **9**:331–350.
 20. **Peault, B., C.-L. H. Chen, M. D. Cooper, M. Barbu, M. Lipinski, and N. M. Le Douarin.** 1987. Phylogenetically conserved antigen on nerve cells and lymphocytes resembles myelin-associated glycoprotein. *Proc. Natl. Acad. Sci. USA* **84**:814–818.
 21. **Perry, R. P., D. E. Kelley, C. Coleclough, and J. F. Kearney.** 1981. Organization and expression of immunoglobulin genes in fetal liver hybridomas. *Proc. Natl. Acad. Sci. USA* **78**:247–251.
 22. **Perry, R. P., and D. E. Kelley.** 1979. Immunoglobulin messenger RNAs in murine cell lines that have characteristics of immature B lymphocytes. *Cell* **18**:1333–1339.
 23. **Pizer, E., and E. H. Humphries.** 1989. RAV-1 insertional mutagenesis: disruption of the c-myc locus and development of avian B-cell lymphomas. *J. Virol.* **63**:1630–1640.
 24. **Rup, B. J., J. L. Spence, J. D. Hoelzer, R. B. Lewis, C. R. Carpenter, A. S. Rubin, and H. R. Bose, Jr.** 1979. Immunosuppression induced by avian reticuloendotheliosis virus: mechanism of induction of the suppressor cell. *J. Immunol.* **123**:1362–1370.
 25. **Scofield, V. L., and H. R. Bose, Jr.** 1978. Depression of mitogen response in spleen cells from reticuloendotheliosis virus-infected chickens and their suppressive effect on normal lymphocyte response. *J. Immunol.* **120**:1321–1325.
 26. **Sevoian, M., R. N. Larose, and D. M. Chamberlain.** 1964. Avian lymphomatosis. VI. A virus of unusual potency and pathogenicity. *Avian Dis.* **8**:336–347.
 27. **Sowder, J. T., C.-L. H. Chen, L. L. Ager, M. M. Chan, and M. D. Cooper.** 1988. A large subpopulation of avian T cells express a homologue of the mammalian TcR receptor. *J. Exp. Med.* **167**:315–322.
 28. **Stephens, R. M., N. R. Rice, R. R. Hiebsch, H. R. Bose, Jr., and R. V. Gilden.** 1983. Nucleotide sequence of v-rel: the oncogene of reticuloendotheliosis virus. *Proc. Natl. Acad. Sci. USA* **80**:6229–6233.
 29. **Theilen, G. H., R. F. Zeigel, and M. J. Twiehaus.** 1966. Biological studies with RE virus (strain T) that induces reticuloendotheliosis in turkeys, chickens and Japanese quail. *J. Natl. Cancer Inst.* **37**:731–743.
 30. **Veromaa, T., O. Vainio, E. Eerola, and P. Toivanen.** 1988. Monoclonal antibodies against chicken Bu-1a and Bu-1b alloantigens. *Hybridoma* **7**:41–48.
 31. **Witter, R. L., L. F. Lee, L. D. Bacon, and E. J. Smith.** 1979. Depression of vaccinal immunity to Marek's disease by infection with reticuloendotheliosis virus. *Infect. Immun.* **26**:90–98.
 32. **Wong, T. C., and M. M. C. Lai.** 1981. Avian reticuloendotheliosis virus contains a new class of oncogene of turkey origin. *Virology* **111**:289–293.
 33. **Zhang, J., W. Bargmann, and H. R. Bose, Jr.** 1989. Rearrangement and diversification of immunoglobulin light-chain genes in lymphoid cells transformed by reticuloendotheliosis virus. *Mol. Cell. Biol.* **9**:4970–4976.