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Specificity in the Immunosuppression Induced by Avian Reticuloendotheliosis Virus

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Several parameters of the cellular and humoral immune responses of chickens infected with reticuloendotheliosis virus (REV-T), an avian defective acute leukemia virus, or with its helper virus, reticuloendotheliosis-associated virus (REV-A), were evaluated. Spleen cells from chickens infected with REV-T (REV-A) or REV-A exhibited depressed mixed lymphocyte and mitogen responses in vitro. Allograft rejection was delayed by 6 to 14 days in birds infected with REV-A. The specific antitumor cell immune response was also studied by a ^{51}Cr-release cytotoxicity assay. Lymphocytes from chickens infected with low numbers of the REV-T-transformed cells exhibited significant levels of cytolytic reactivity against the ^{51}Cr-labeled REV-T tumor cells in vitro. The mitogen response of lymphocytes from these injected birds was similar to that of uninjected chickens. In contrast, lymphocytes from chickens injected with higher numbers of REV-T-transformed cells exhibited suppressed mitogen reactivity and failed to develop detectable levels of cytotoxic activity directed against the REV-T tumor cells. These results suggest that the general depression of cellular immune competence which occurs during REV-T (REV-A) infection could contribute to the development of this acute leukemia by inhibiting the proliferation of cytotoxic cells directed against the tumor cell antigens. The cytotoxic effect observed after the injection of chickens with non-immunosuppressive levels of REV-T-transformed cells appears to be specific for the REV-T tumor cell antigens since cells transformed by Marek's disease virus or avian erythroblastosis virus were not lysed. In marked contrast, birds whose cellular immune responses were suppressed by infection with REV-A were capable of producing a humoral immune response to viral antigens. Detectable levels of viral antibody, however, did not appear until 12 to 15 days after REV-A infection. Since REV-T (REV-A) induced an acute leukemia resulting in death within 7 to 14 days, it appears unlikely that the ability of chickens to make antiviral antibody influences the development of lethal reticuloendotheliosis.

The infection of animals with oncogenic viruses has frequently been associated with impaired immune functions (10, 33). Since the cellular immune system has been shown to play an important role in the restriction of tumor cell proliferation (20, 29), the suppression of cellular immune function may contribute to the development of neoplastic diseases. In some cases, the immune response to the tumor cell antigens is specifically impaired, whereas the response to nontumor cell antigens is unaltered (11). In other cases, a general depression of immune competence may occur (44).

Avian reticuloendotheliosis virus, REV-T (REV-A), is a replication-defective acute leukemia virus (22) that transforms immature lymphocytes that express bursal cell determinants (24, 30). Oncogenic stocks of REV-T (REV-A) contain a replication-competent nontransforming virus called reticuloendotheliosis-associated virus (REV-A), which is present in 1,000-fold excess (21). The infection of immunocompetent chickens with REV-T (REV-A) results in a neoplastic state which culminates in the death of the birds between 7 and 14 days after infection (40). In an effort to understand the extreme virulence of this leukemia virus, we examined the cellular and humoral immune response of birds infected with REV-T (REV-A) or its helper virus (REV-A). REV-T (REV-A) or its helper virus induce a rapid and severe suppression of the mitogen-stimulated T-cell proliferative response of the infected birds (5, 38, 39). REV-T (REV-A) or REV-A induces or activates a suppressor cell

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population in the spleens of infected birds within 3 days after infection. Although the suppressed cells fail to proliferate, their cytotoxic effector function is normal (7). Phytohemagglutinin (PHA)-stimulated lymphocytes from REV-T (REV-A)-infected chickens displayed normal cytotoxic responses against allogeneic target cells (6). These observations indicate that the suppressing agent inhibits only the proliferation of lymphocytes.

REV-T–transformed nonvirus-producing (NP) cells have been isolated after in vitro infection which have not been coinfected by the helper virus (22). These REV-transformed NP cells fail either to produce infectious virus or release noninfectious particles (22). These REV-T–transformed NP cells correspond to the in vivo target cell for this acute leukemia virus (30). These cells are tumorigenic and induce lethal reticuloendotheliosis when injected into birds histocompatibly matched at the B-locus. These REV-T–transformed NP cells also induce or activate a suppressor cell population (38). A correlation was observed between the number of cells required to induce immunosuppression and the development of lethal reticuloendotheliosis (38). Chickens injected with lower doses of REV-T–transformed NP cells did not develop immunosuppression or reticuloendotheliosis, suggesting that in the absence of immunosuppression the host immune defense mechanisms may rapidly eliminate the transformed cells, allowing the recovery of the animal. We therefore evaluated the specific immune response of immunosuppressed chickens to REV-T tumor cells. Chickens injected with immunosuppressive doses of histocompatibly matched REV-T–transformed cells failed to develop detectable levels of cells capable of specific cytotoxic responses against ^{51}Cr-labeled REV-T–transformed cells in vitro. Birds infected with REV-T (REV-A) or its helper virus also exhibited depressed mixed lymphocyte responses, and allograft rejection in chickens infected with REV-A was also inhibited.

In contrast to the general depression of the cellular immune response observed in REV-T (REV-A)– or REV-A–infected chickens, we determined that the humoral immune response against REV-T (REV-A) viral antigens is unimpaired.

MATERIALS AND METHODS

Viruses and cell lines. REV-T (REV-A) was obtained from the culture fluid of a REV-T–transformed bone marrow cell line (15). REV-A was isolated from the leukemia virus stocks by endpoint dilution (21). REV-A was propagated in leukosis-free (Spafas, Norwich, Conn.) chicken embryo fibroblast cultures.

The REV-T–transformed bone marrow cell line was maintained in RPMI 1640 medium supplemented with 2% fetal calf serum and 10% calf serum (KC Biological, Inc., Lenexa, Kans.). This cell line, isolated from a chicken injected with REV-T (REV-A), chronically yields REV-T at a rate of approximately 2×10^3 50% lethal doses per ml per day (15). These REV-T (REV-A) virus preparations are free from avian leukosis virus (15) and Marek's disease virus (R. L. Witter, personal communication).

In vitro REV-T–transformed virus-producing and NP cell lines were established by infecting Hyline SC (B_2B_2) spleen cells with oncogenic REV-T (REV-A) preparations in vitro (21). Single spleen cell suspensions (10^7 cells per ml) were exposed to REV-T (10^7 focus-forming units [FFU]) and REV-A (10^4 PFU) in the presence of 2 μg of Polybrene per ml for 1 h. The virus inoculum was removed, and the spleen cells were then suspended in RPMI 1640 medium containing 0.35% Noble agar, 15% fetal calf serum, 1% beef embryo extract, 1% chicken serum, 500 U of penicillin, 250 U of streptomycin, 0.028% sodium bicarbonate, and 0.01% gentamicin, and plated in 60-mm petri dishes. These agar plates were incubated at 37°C in a CO_2 atmosphere. Isolated transformed clones were picked 8 to 14 days after infection and adapted to growth in RPMI 1640 medium supplemented with 15% fetal calf serum. The individual clones were screened for virus production by the reverse transcriptase assay (44). Supernatant fluids from these clones were also tested for their ability to induce reticuloendotheliosis in day-old chickens. A clone that did not release particles which had reverse transcriptase activity and did not produce supernatants which could induce reticuloendotheliosis in chickens was designated a NP clone. A clone that released particles that had high reverse transcriptase activity and produced lethal reticuloendotheliosis was designated a virus-producing clone.

Avian erythroblastosis virus (AEV)-transformed erythroblastic cells were isolated from the spleens of birds which had been injected with AEV strain R (provided by R. L. Witter, Regional Poultry Laboratories, East Lansing, Mich.). The MSB cells, which are T-lymphoblasts transformed by Marek's disease virus, were also provided by R. L. Witter.

AEV-, REV-T–, and Marek's disease virus-transformed cells were maintained in RPMI 1640 medium supplemented with 2% fetal calf serum, 8% calf serum, penicillin, and streptomycin.

Experimental animals. Hyline SC (B_2B_2) and FP ($B_{15}B_{21}$) chickens were purchased as fertile eggs from Hyline International (Johnston, Iowa). These birds are F_1 progeny of inbred lines.

Chickens infected with RE group viruses were housed in the same animal room as uninfected control birds, since significant horizontal transmission of these viruses does not occur (3).

Mixed lymphocyte cultures. Spleen cells to be used in a two-way allogeneic mixed lymphocyte assay were obtained from SC (B_2B_2) and FP ($B_{15}B_{21}$) strain chickens. Mixed cultures consisted of 4×10^6 splenic lymphocytes in 0.5 ml of serum-free RPMI 1640 medium from each strain. Control cultures contained 4×10^6 spleen cells in 0.5 ml of RPMI 1640 from either strain. Triplicate cultures of each sample were incubated at 37°C in a 10% CO_2-humidified atmosphere and 0.5 μCi of [^{3}H]thymidine (TdR) was added after 5 days. After an 18-h pulse with the radiolabel, samples

were washed, trichloroacetic acid precipitated, and counted as described above for PHA assays. Results from control and mixed lymphocyte cultures are expressed as mean counts per minute ± standard error.

Allogeneic skin grafts. Five-week-old FP ($B_{15}B_{21}$) chickens were grafted with SC (B_2B_2) skin. FP birds were anesthetized with 0.3-ml injection of pentobarbitol sodium (50 mg/ml, Abbott Laboratories, North Chicago, Ill.). Pentobarbitol sodium anesthesia was supplemented with ether during the grafting procedure as needed. The graft site was cleaned with 75% ethanol. Circular pieces of skin approximately 2 cm in diameter were removed from beneath the wings of SC chickens and placed on gauze pads moistened with sterile Hanks balanced salt solution. An area of skin approximately 2 cm in diameter was removed from an area beneath the wing of each FP chicken and was replaced with the SC donor graft tissue. Grafts were secured with 8 to 10 stitches of ethicon 4-0 nylon suture thread. The placement of skin grafts under the wings lessened the possibility that the graft could be disturbed or damaged by the recipient or by other chickens housed in the same cage. Grafts on individual chickens were examined daily for evidence of necrosis. Since chicken allogeneic skin grafts are normally rejected 7 to 12 days after transplantation (10, 12), any grafts which became necrotic in less than 5 days were disregarded in the results of these experiments.

Preparation of target cells for cytotoxicity assay. Cultured cells within 48 h of subculture were washed, and 10^7 cells were suspended in 0.2 ml of serum-free RPMI 1640. These cells were incubated with 0.1 mCi of ^{51}Cr ($Na_2\ ^{51}CrO_4$; 1 mCi/ml; New England Nuclear Corp., Boston, Mass.) at 37°C in a humidified CO_2 atmosphere for 3 h. Cells were washed three times with RPMI 1640 medium and suspended to 3×10^6 cells per ml.

Cell-mediated cytotoxicity assay. The procedure for the cell-mediated cytotoxicity assay was similar to those described by others (9, 41). Splenic lymphocytes or Ficoll-Hypaque-separated peripheral blood lymphocytes were washed and suspended in serum-free RPMI 1640 medium. Varied concentrations of these effector cells in 0.5 ml of medium were mixed with 0.1 ml of labeled target cells (3×10^6 cells per ml) in plastic culture tubes (10 by 75 mm). Unlabeled tumor cells were also tested for their ability to inhibit the release of ^{51}Cr from REV-T–transformed cells. In these experiments, 10^6 unlabeled inhibitor cells in 0.1 ml of RPMI 1640 medium were added to each effector cell/^{51}Cr-labeled target mixture. In one experiment, cultures were incubated at 37°C for 6 h in a humidified CO_2 atmosphere. In other experiments, cultures were incubated for 18 h. At the end of the incubation period, the culture tubes were centrifuged at 800 rpm for 10 min. Supernatant fluids were transferred to separate tubes, and radioactivity was determined in a Beckman 7000 gamma scintillation counter. The maximum release of ^{51}Cr was determined by counting the supernatant fluids of labeled target cells lysed by incubation in 1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) for 18 h. Spontaneous release, which was always less than 45% of maximum release, was determined by the incubation of the ^{51}Cr-labeled targets with 0.5 ml of RPMI medium.

The percent specific release of ^{51}Cr for each sample was calculated by the following formula: % specific release = cpm released from target cells incubated with effector cells − spontaneous-release cpm/maximum-release cpm − spontaneous-release cpm.

Collection of serum for study of the kinetics of expression of humoral immune response. Two-week-old SC (B_2B_2) chickens were injected with 10^3 FFU of REV-T or 10^6 PFU of REV-A or both collected as supernatants from REV-T–transformed cell cultures (REV-T) or chicken embryo fibroblast cultures (REV-A). Immunized birds were injected with 0.25 mg of UV-inactivated virus per bird also obtained as cell culture supernatants. Uninfected birds were housed in separate cages and used as controls. All birds were bled from the jugular on day 0 and at 3-day intervals thereafter. Plasma was collected by centrifugation within 24 h of bleeding.

Radioimmunoassay for anti-REV-T (REV-A) viral antigens. REV-T (REV-A) viral preparations were made by the clarification of supernatants collected from REV-T–transformed cells, followed by ultracentrifugation at 19,000 rpm for 120 min. Resuspended pellets were further purified by Sepharose 4B column chromatography. The column-purified viral preparations were diluted to a concentration of 1 mg/ml in 0.15 M phosphate-buffered saline (PBS; pH 7.2) and subsequently diluted 1:25 for overnight binding to polyvinyl 96-well microtiter culture plates (Flow Laboratories, McLean, Va.) at 37°C in a CO_2-humidified incubator. The following morning, the wells were blocked with 2% horse serum in PBS for 1 h at 37°C in a humidified CO_2 incubator. Appropriate dilutions of chicken sera were added, and the plates were incubated for 1 h at 37°C as described above. After this incubation, the plates were washed three times in PBS-0.2% horse serum. Approximately 50,000 cpm of ^{125}I-labeled rabbit anti-chicken immunoglobulin G (IgG) was added to each well, and the plates were incubated for 1 h at 37°C in a humidified CO_2 incubator. After four washes in PBS-0.2% horse serum, the wells were counted in a Beckman gamma counter (model 7000).

Preparation of ^{125}I-labeled rabbit anti-chicken immunoglobulin. The IgG-enriched fraction of rabbit anti-chicken IgG was obtained commercially from Miles Laboratories, Elkhart, Ind. The rabbit anti-chicken IgG was immunoaffinity purified by adsorption to a chicken IgG-Sepharose column. The chicken IgG-Sepharose was prepared by covalent coupling of a sodium sulfate precipitate (obtained from chicken serum by successive precipitations of 16, 16, and 18% sodium sulfate) (28) to CNBr-activated Sepharose 4B (Sigma) by published procedures (35). The rabbit anti-chicken IgG was bound to chicken IgG-Sepharose for 1 h at room temperature and then eluted by washing the column with glycine-HCl (pH 2.2). Fractions were collected in the presence of 1 M K_2PO_4 for rapid neutralization. Fractions were monitored spectrophotometrically at 280 nm, and peak protein fractions were pooled, frozen, and lyophilized. Iodination of immunoaffinity-purified rabbit anti-chicken IgG was accomplished by reaction with chloramine T essentially as described by Greenwood et al. (17). Unincorporated ^{125}I was removed by passing the reaction mixture over Dowex in a siliconized disposable column.

Radioimmunoprecipitation of ^{125}I-labeled REV-T (REV-A) virus. Virus for ^{125}I labeling was column purified from supernatants of cultures of REV-T–transformed bone marrow cells (KBMC) as described

above. Virus was labeled with 1 mCi of ^{125}I in the presence of the Iodo-Gen reagent (Bio-Rad Laboratories, Richmond, Calif.) by incubation at room temperature for 30 min. The reaction mixture containing the Iodo-Gen beads was clarified by centrifugation (2,500 rpm for 5 min), and the supernatant containing ^{125}I-labeled virus was cleared of unincorporated ^{125}I by Sephadex G-25 column chromatography in a siliconized disposable column. Fractions were eluted with PBS and counted in a Beckman 7000 gamma counter. Fractions possessing maximum radioactivity appearing in the void volume were pooled and used directly in radioimmunoprecipitation.

For radioimmunoprecipitation, 4.5×10^5 cpm of ^{125}I-labeled REV-T (REV-A) was incubated overnight at 4°C in the presence of 10 μl of undiluted serum (either rabbit or chicken). Samples immunoprecipitated with chicken serum were further incubated for 4 h at 4°C with rabbit anti-chicken IgG to allow the collection of immune complexes by Formalin-fixed *Staphylococcus aureus* cells (PANSORBIN; Calbiochem-Behring, LaJolla, Calif.). Briefly, 100 μl of a washed preparation of Formalin-fixed *S. aureus* cells was added to each sample and incubated at 4°C for 45 min. The *S. aureus* pellets were collected by centrifugation (2,500 rpm for 10 min) and washed three times with RIP buffer (10 mM potassium phosphate [pH 7.2], 0.1% Triton-X 100, 0.1% sodium dodecyl sulfate [SDS], 0.5% Nonidet P-40, 0.5 M KCl, 0.25 mM phenylmethylsulfonyl chloride). After the final wash, the pellets were resuspended and boiled (3 min) in SDS-polyacrylamide gel electrophoresis sample buffer (2.5 M urea, 3.33% SDS, 3.33% 2-mercaptoethanol). SDS-polyacrylamide slab gel electrophoresis was carried out essentially as described by Laemmli (26) on 7 to 15% gradient slab gels. Autoradiography was carried out on dried gels for a 5-day exposure time.

Mitogen stimulation assays. PHA-P (Difco Laboratories, Detroit, Mich.) was used in a serum-free culture system as described previously (39). Briefly, splenic lymphocytes from REV-T (REV-A)- or REV-A-infected birds (3×10^6 cells from peripheral blood or 2×10^6 cells from spleens) were suspended in 0.5 ml of RPMI 1640 medium and were incubated with the optimum amount of PHA (0.1 ml of a 1:400 dilution). The cells were then incubated at 37°C in a 10% CO_2-humidified atmosphere, and 0.5 μCi of [^{3}H]TdR (6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added after 48 h. After an 18-h pulse with the radiolabel, the cells were washed with PBS and precipitated with cold 6% trichloroacetic acid. The precipitates were collected on Whatman glass filters (GF/A) and immersed in Bray scintillation fluid, and radioactivity was counted in a Packard liquid scintillation counter.

RESULTS

REV-T-induced suppression of the mixed lymphocyte response. To determine whether REV-T (REV-A) infection induces a general depression of the cellular immune response, we examined the mixed lymphocyte reaction and allograft rejection. The mixed lymphocyte reaction is a measure of lymphocyte proliferation in response to stimulation by allogeneic lymphoid cells and may be used to assess cellular immune competence (32). Splenic lymphocytes from REV-T (REV-A)- and REV-A-infected (6 days after infection) or uninfected FP ($B_{15}B_{21}$) chickens were cultured for 96 h with spleen cells from uninfected SC (B_2B_2) birds. Cocultures of SC and uninfected FP spleen cells exhibited significantly increased uptake of [^{3}H]TdR compared with the uptake when these cells were incubated separately (Table 1). In contrast, when spleen cells taken from the REV-T (REV-A)- or REV-A-infected FP chickens were incubated with SC spleen cells, the mixed lymphocyte response was significantly suppressed. Also shown are the PHA-induced responses of spleen cells from these chickens. Spleen cells from REV-T (REV-A)-infected FP birds or chickens infected by the helper virus were incapable of responding to PHA. These results show that the mitogen and mixed lymphocyte responses, two in vitro assays of immune competence, are suppressed by REV-T infection.

REV-A-induced inhibition of allograft rejection. The infection of chickens with REV-T (REV-A) or REV-A results in the induction of a population of splenic suppressor cells early after infection (38, 39). Chickens injected with REV-T (REV-A) die within 7 to 14 days, whereas chickens injected with REV-A survive well beyond this time period. Therefore, REV-A infection can be used to assess in vivo parameters of general immune responsiveness. The rejection of allogeneic grafts in chickens is a thymus-dependent response (2) which is controlled by the B locus, the major histocompatibility locus of the chicken (10, 12). Allograft rejection can be used as an in vivo assay for cellular immune competence. Skin from SC (B_2B_2) chickens was grafted under the wings of FP birds ($B_{15}B_{21}$). Some of the skin-grafted chickens were injected with REV-A (0.1 ml of stock containing 10^6 PFU/ml). Skin grafts from all chickens were examined daily. The day of rejection was determined as the first day on which the graft became completely necrotic. Since allograft rejection by chickens normally occurs 7 to 12 days after grafting (10, 12), any chickens which rejected grafts before day 5 were discounted from these experiments. The period for graft rejection was prolonged by 6 to 14 days in chickens injected with REV-A (Table 2). Also shown are the PHA responses of Ficoll-Hypaque-separated peripheral blood lymphocytes from these chickens 7 days after infection. The depression of the PHA response by REV-A was correlated with delayed graft rejection in REV-A-injected chickens.

Suppression of cell-mediated cytotoxicity against REV-T-transformed cells. Chickens infected with REV-T (REV-A) or REV-A prepara-

TABLE 1. REV-T-induced suppression of the mixed lymphocyte response

Expt	Cell mixtures[a]	[^{3}H]TdR uptake[b] (cpm)	PHA-stimulated [^{3}H]TdR uptake[b]	
			+PHA	−PHA
1[c]	$FP_{uninfected}$	278 ± 73	46,238 ± 3,672	375 ± 105
	$FP_{REV\text{-}T\ infected}$	81 ± 10	113 ± 25	301 ± 40
	$SC_{uninfected}$	445 ± 70	82,813 ± 10,662	757 ± 134
	$SC_{uninfected}$ + $FP_{uninfected}$	5,990 ± 534	—	—
	$SC_{uninfected}$ + $FP_{REV\text{-}T\ infected}$	59 ± 6	—	—
2[c]	$FP_{uninfected}$	825 ± 48	ND	
	$FP_{REV\text{-}T\ infected}$	102 ± 20	ND	
	$SC_{uninfected}$	125 ± 17	ND	
	$SC_{uninfected}$ + $FP_{uninfected}$	8,348 ± 582	—	—
	$SC_{uninfected}$ + $FP_{REV\text{-}T\ infected}$	931 ± 20	—	—
3[d]	$FP_{uninfected}$	341 ± 41	49,619 ± 4,108	540 ± 82
	$FP_{REV\text{-}A\ infected}$	157 ± 12	103 ± 27	376 ± 67
	$SC_{uninfected}$	889 ± 100	46,673 ± 2,979	429 ± 42
	$SC_{uninfected}$ + $FP_{uninfected}$	8,111 ± 657	—	—
	$SC_{uninfected}$ + $FP_{REV\text{-}A\ infected}$	56 ± 5	—	—

[a] Pooled spleen cells (3×10^6) from FP birds mixed with 3×10^6 pooled spleen SC cells in a 5-day assay.
[b] Means ± standard error of three determinations. —, None. ND, Not determined.
[c] Chickens injected with 0.1 ml of REV-T stock containing approximately 2×10^3 50% lethal doses per ml.
[d] Chickens injected with 0.1 ml of REV-A stock containing approximately 10^7 PFU/ml.

tions exhibit a general depression of cellular immune competence as assessed by the mitogen and mixed lymphocyte responses in vitro and the allograft rejection response in vivo. Chickens injected with high doses of REV-T-transformed NP cells exhibited suppressed PHA-induced blastogenic responses, whereas birds injected with lower doses of tumor cells had normal PHA responses (38). A general suppression of the cellular immune response may contribute to the development of reticuloendotheliosis by preventing the expansion of a population of cytotoxic cells directed specifically against REV-T tumor cell antigens. To determine whether REV-T (REV-A)-induced immunosuppression prevents the development of a cellular cytotoxic response against REV-T tumor cells, we tested peripheral blood lymphocytes from uninjected chickens and chickens injected with suppressive or non-immunosuppressive numbers of tumor cells in a cell-mediated cytotoxicity assay.

Immunocompetent chickens (5 to 8 weeks old) were injected with various amounts of tumor cells (Table 3). Two different independently derived REV-T clones were used in these studies. RECC-UT 310 is a NP cell line and RECC-UT 316 is a clone that releases low levels of REV-T and REV-A. Spleen or peripheral blood lymphocytes taken from these birds 7 to 11 days after injection were mixed with ^{51}Cr-labeled REV-T-transformed tumor cell targets in an 18-h ^{51}Cr-release cytotoxicity assay. The results shown in Table 3 indicate that birds injected with immunosuppressive levels of REV-T tumor cells failed to develop a detectable cytotoxic response against these tumor cells. In these experiments, the cellular immunocompetent status of the infected birds was determined by a mitogen assay. Chickens injected with levels of tumor cells

TABLE 2. REV-A-induced inhibition of allograft rejection

Bird no.	Virus injection[a]	PHA-induced [^{3}H]TdR uptake[b]		Time of graft rejection (days)[c]
		+PHA	−PHA	
1	−	ND		12
2	−	ND		11
3	−	15,763 ± 564	791 ± 31	11
4	−	10,982 ± 789	674 ± 31	8
5	−	15,823 ± 1,084	625 ± 46	10
6	+	1,000 ± 57	284 ± 48	19
7	+	3,459 ± 124	549 ± 48	22
8	+	399 ± 37	349 ± 39	25
9	+	11,553 ± 630	386 ± 19	10
10	+	363 ± 27	494 ± 24	20
11	+	957 ± 46	531 ± 48	24
12	+	1,483 ± 65	1,114 ± 291	22

[a] Chickens were injected with 0.1 ml of REV-A stock containing approximately 10^7 PFU/ml.
[b] PHA response of peripheral blood lymphocytes 7 days after virus injection. Means ± standard error of three determinations. ND, Not determined.
[c] The day of graft rejection was determined as the day on which graft was completely necrotic. Chickens which rejected grafts before 5 days were not included in experimental results.

TABLE 3. Suppression of cell-mediated cytotoxicity against REV-T-transformed cells

Expt. no.	Bird no.	Source of effector cells	% Specific ^{51}Cr release with effector cells of:				PHA-induced [^{3}H]TdR uptake[a] (cpm)	
			10^7	6×10^6	3×10^6	10^6	+PHA	−PHA
1[b]	1-1	Uninjected	−1	2	—	3	13,567 ± 4,909	278 ± 32
	1-2	Uninjected	1	−8	—	7	17,125 ± 1,452	325 ± 21
	1-3	RECC-UT310 (10^4)	3	−5	—	−1	2,188 ± 315	199 ± 21
	1-4	RECC-UT310 (10^4)	4	6	—	9	1,251 ± 75	201 ± 30
	1-5	RECC-UT310 (10^3)	32	16	—	6	27,692 ± 2,899	125 ± 15
	1-6	RECC-UT310 (10^3)	41	22	—	8	13,920 ± 334	485 ± 13
2[c]	2-1	Uninjected	−3	−4	−3	—	12,756 ± 3,420	276 ± 15
	2-2	Uninjected	−2	−2	−2	—	14,408 ± 1,639	206 ± 56
	2-3	RECC-UT316 (5×10^8)	−4	−2	−1	—	234 ± 49	269 ± 59
	2-4	RECC-UT316 (5×10^8)	−3	−3	−2	—	437 ± 18	315 ± 71
	2-5	RECC-UT316 (5×10^6)	52	50	56	—	16,939 ± 645	425 ± 28
	2-6	RECC-UT316 (5×10^6)	45	5	9	—	15,700 ± 504	555 ± 105
3[d]	3-1	Uninjected	2	8	3	—	18,895 ± 1,942	621 ± 56
	3-2	Uninjected	−3	1	1	—	26,608 ± 1,976	519 ± 48
	3-3	RECC-UT316 (2×10^8)	13	18	5	—	26,246 ± 239	397 ± 30
	3-4	RECC-UT316 (2×10^8)	5	18	7	—	12,408 ± 521	877 ± 83
	3-5	RECC-UT316 (5×10^7)	−4	2	1	—	189 ± 9	261 ± 25
	3-6	RECC-UT316 (5×10^7)	24	29	10	—	26,707 ± 1,469	708 ± 72

[a] Spleen or peripheral blood lymphocytes taken from individual birds were tested in a PHA stimulation assay. Mean ± standard error of three determinations.

[b] ^{51}Cr-labeled RECC-UT310 cells (10^5) added to various effector cell numbers from control uninjected spleen cells or spleen cells from 7-day RECC-UT310-injected chickens. Birds 1-3 and 1-4 were injected with 10^4 tumor cells. Birds 1-5 and 1-6 were injected with 10^3 tumor cells.

[c] ^{51}Cr-labeled RECC-UT316 cells (10^5) added to peripheral blood lymphocytes from uninjected or 9-day RECC-UT316-injected chickens. Birds 2-3 and 2-4 were injected with 5×10^8 tumor cells. Birds 2-5 and 2-6 were injected with 5×10^6 tumor cells.

[d] ^{51}Cr-labeled RECC-UT316 cells (10^5) added to peripheral blood lymphocytes from uninjected or day-11 RECC-UT316-injected chickens. Birds 3-3 and 3-4 were injected with 2×10^8 tumor cells. Birds 3-5 and 3-6 were injected with 5×10^7 tumor cells.

which did not induce immunosuppression did exhibit cytotoxic responses to the tumor cells.

Specificity of the cytolytic response to REV-T-transformed cells. The immune response in animals to virus-induced tumor cells often involves the generation of cytolytic T lymphocytes specific for the tumor cell surface antigens (4, 27, 41). To determine whether the cytolytic response observed in chickens injected with non-immunosuppressive doses of REV-T-transformed cells is specific for REV-T tumor cell antigens, we also tested the ability of these REV-T-induced cytolytic cells to lyse unrelated tumor cell lines. Marek's disease lymphoblastoid cell lines (MSB cells) are T lymphoblasts transformed by Marek's disease virus, a member of the herpesvirus group (1). AEV is a replication-defective acute leukemia of the retrovirus group which is antigenically (31) and genetically (23) unrelated to the reticuloendotheliosis virus group and transforms erythroblasts. Cytolytic lymphocytes induced by REV-T-transformed cells were not capable of killing ^{51}Cr-labeled MSB- or AEV-transformed cells (Fig. 1).

Since some of the REV-T tumor cell lines never exhibited high amounts of specific ^{51}Cr release (data not shown), the absence of cytolytic activity against the MSB or AEV tumor cells might reflect a resistance of these cells to lysis. To assess whether other tumor cells are recognized by REV-T-tumor-cell-induced cytotoxic cells, we also tested the ability of unlabeled MSB- or REV-T-transformed cells to specifically inhibit the lysis of ^{51}Cr-labeled REV-T tumor cells. Coincubation with unlabeled REV-T-transformed cells inhibited the cytotoxic response against ^{51}Cr-labeled REV-T tumor cells, whereas coincubation with MSB cells did not cause any decrease in the release of the isotope (Fig. 2). These results indicate that the cytotoxic response is directed specifically against the REV-T-transformed cells.

Antiviral humoral immune response in REV-A- and REV-T (REV-A)-infected birds. To determine whether the REV-T (REV-A) or REV-A infection of chickens results in the suppression of the humoral immune response, we examined the ability of REV-T (REV-A)- or REV-A-infected chickens to produce antiviral antibod-

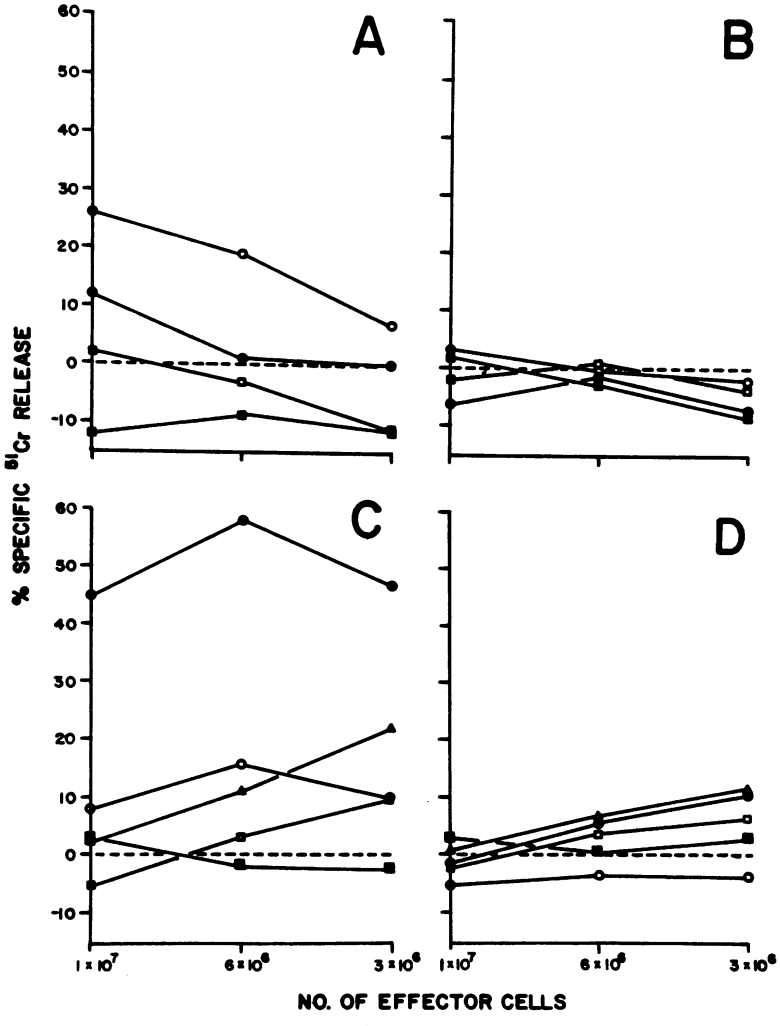

FIG. 1. Specificity of the cytotoxic response against REV-T tumor cells. The response of peripheral blood lymphocytes against REV-T, MSB, or AEV tumor cells was measured. Sources of peripheral blood lymphocytes were uninjected (□,■) or REV-T tumor cell-injected (○, ●, △) chickens. In one experiment (A and B), ^{51}Cr-labeled REV-T tumor cells (A) or MSB tumor cells (B) were used as target cells. In the other experiment (C and D), target cells were ^{51}Cr-labeled REV-T tumor cells (C) or AEV tumor cells (D).

ies. Two-week-old chickens were infected with either REV-A (10^6 FFU) or REV-T (10^3 FFU of REV-T and 10^6 PFU of REV-A), and at different time intervals after infection, serum was collected and assayed for antiviral antibodies. In addition, some birds were immunized by the injection of 0.25 mg of UV-inactivated REV-A per bird and reinjected weekly. The presence of antiviral antibodies in the serum of uninfected, infected, and immunized birds was determined in a radioimmunoassay in which Sepharose 4B column-purified REV-T (REV-A) was adsorbed to microtiter plates. Undisrupted virion preparations were employed in the radioimmunoassay to screen for the presence of antibodies directed against core or envelope proteins. Appropriate dilutions of chicken serum were then added to the plates, and the level of bound antiviral antibody was detected by the addition of immunoaffinity-purified ^{125}I-labeled rabbit anti-chicken immunoglobulin. The cellular immune status of infected and immunized chickens was tested by assaying the PHA response of Ficoll-Hypaque-separated lymphocytes 9 days after infection. Birds infected with REV-A produced antibody against the viral antigens (Fig. 3). Significant levels of antibodies to viral antigens could be detected in chickens immunized or infected with REV-A by 15 days after infection. The levels of antiviral antibody in REV-A–

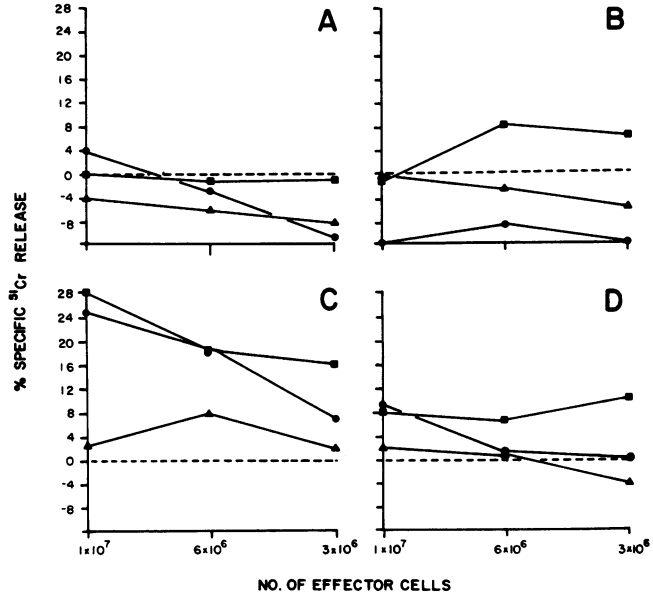

FIG. 2. Inhibitory effect of unlabeled REV-T or MSB tumor cells on the specific cytotoxic response of peripheral blood lymphocytes against ^{51}Cr-labeled REV-T tumor cells. The percent specific release of ^{51}Cr was determined without unlabeled inhibitor cells (●), with unlabeled REV-T tumor cells added as inhibitors (▲), or with MSB cells added as inhibitors (■). Peripheral blood lymphocytes as a source of cytotoxic cells were obtained from uninjected (A and B) or REV-T tumor cell-injected (C and D) chickens.

infected chickens were comparable to those of immunized birds. In spite of the fact that chickens infected with REV-A produced significant levels of antiviral antibody, lymphocytes obtained from these REV-A–infected birds were impaired in their ability to respond to PHA (Table 4).

Since REV-T (REV-A) induces a rapidly lethal lymphoproliferative disease resulting in death within 7 to 14 days after infection, it was

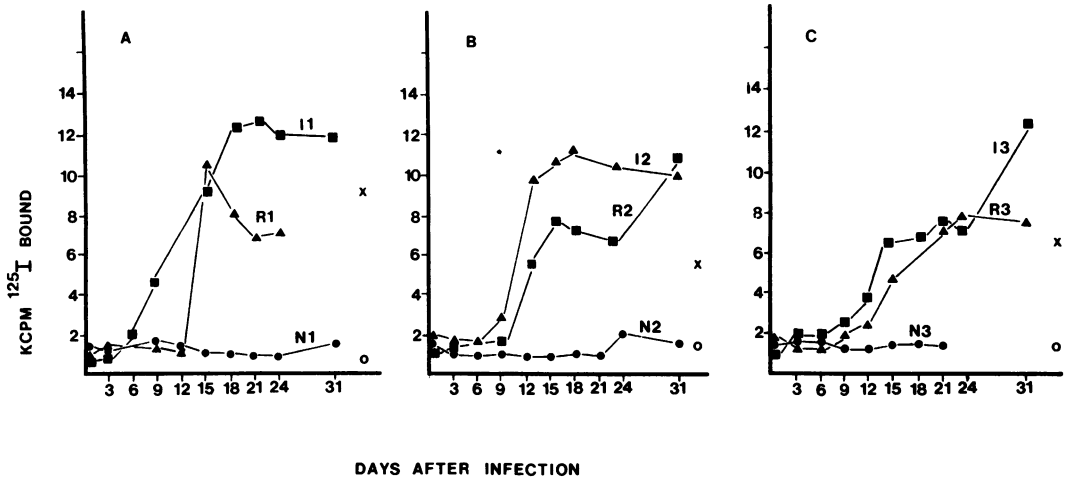

FIG. 3. Radioimmunoassay demonstrating levels of antiviral antibody produced in birds infected with REV-A. Graphs A, B, and C represent three independent experiments with sera collected from three sets of birds. Symbols: ■, birds immunized with UV-inactivated REV-A; ▲, birds infected with REV-A; ●, uninfected birds; X, immunized sera known to immunoprecipitate antiviral antibody; O, no sera added to plates. Each point represents the mean of three determinations; the standard error was less than 10%. All points plotted represent a 250-fold dilution of the chicken sera tested.

TABLE 4. REV-A-induced inhibition of PHA response[a]

Bird	Treatment	PHA stimulation index[b]
N1	Uninfected control	5.70
N2	Uninfected control	11.47
N3	Uninfected control	12.30
R1	REV-A infected	0.79
R2	REV-A infected	0.98
R3	REV-A infected	0.40
I1	UV inactivated REV-A injected	2.47
I2	UV inactivated REV-A injected	3.42
I3	UV inactivated REV-A injected	2.63

[a] PHA response of peripheral blood lymphocytes 9 days after virus infection.

[b] Stimulation index = (stimulated uptake/unstimulated uptake).

unlikely that birds infected with REV-T (REV-A) would be able to produce a humoral immune response to antiviral antigens. Birds infected with REV-T (REV-A) did not produce antiviral antibody within 7 to 14 days after infection, the latent period for lethal reticuloendotheliosis (data not shown).

To further demonstrate that the antiviral activity determined in the radioimmunoassay (Fig. 3) was directed against REV-T (REV-A) antigens, column-purified viral preparations were radiolabeled with ^{125}I and immunoprecipitated with serum collected from a REV-A-infected bird (R2, Fig. 3) at different time intervals after infection. The bird infected with REV-A made antibody to the major core protein (p29) of REV-A which could be detected 18 days after virus infection (Fig. 4). Control immunoprecipitations were carried out with normal rabbit and normal chicken sera, in addition to antiviral antisera raised in rabbits and immunized chickens (Fig. 4, lanes a through e).

DISCUSSION

Retroviruses, which suppress the immune response, provide excellent model systems to define the role of cell-mediated immunity in protection against neoplastic disorders. REV-T is an avian retrovirus which causes an acute lymphomatosis within 7 to 14 days after infection (40) and induces a severe immunosuppression of the mitogen-stimulated blastogenic response in infected chickens (5, 39). Another in vitro assay for general cellular immune competence, the mixed lymphocyte response, also measures cell proliferation (32) in response to alloantigens. The mixed lymphocyte reaction is suppressed when one of the cell populations in the reaction is obtained from REV-T (REV-A)- or REV-A-infected chickens. Although lymphocytes from REV-T (REV-A)-infected chickens were incapable of proliferating in response to mitogens, the PHA-induced cytotoxic response against allogeneic erythrocytes is similar to that of normal chickens (6). The mitogen-induced cytotoxic response to allogeneic erythrocytes does not require cell division (19), suggesting that REV-T (REV-A) infection blocks only the proliferative phase of lymphocyte stimulation. A general inhibition of lymphocyte proliferation in response to stimulation by tumor cell antigens would be expected to influence the progression of REV-T-induced tumorigenesis. Lymphocytes from chickens injected with high numbers of REV-T-transformed cells failed to exhibit detectable levels of cytolytic activity against the REV-T tumor cells in vitro. REV-T-induced immunosuppression may prevent the emergence of adequate numbers of cytolytic lymphocytes to protect against the rapidly proliferating REV-T-transformed cells in vivo.

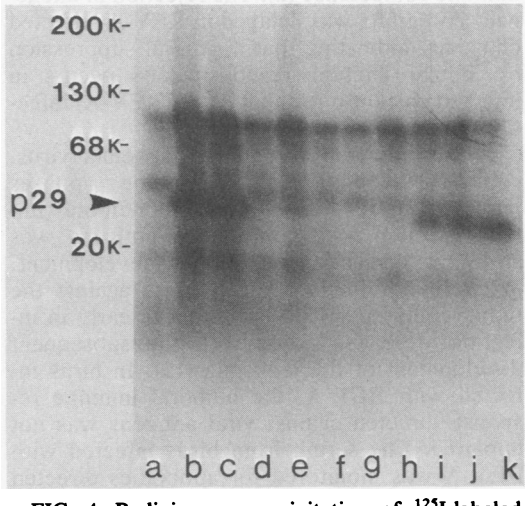

FIG. 4. Radioimmunoprecipitation of ^{125}I-labeled REV-T (REV-A) by antiviral antibodies produced by chickens infected with REV-A. The major core polypeptide, p29, of REV-T (REV-A) is indicated by the arrow. The precipitating antibody added to each lane was as follows: lane a, normal rabbit sera; lane b, rabbit anti-REV-T (REV-A); lane c, rabbit anti-REV-A; lane d, normal chicken sera; lane e, chicken anti-REV-A produced in a bird immunized by weekly injections of 0.25-mg of column-purified virus; lane f, chicken sera collected on day 0 from bird infected with REV-A (Fig. 3, graph B); lane g, chicken sera from REV-A-infected bird, day 6; lane h, chicken sera from REV-A-infected bird, day 12; lane i, chicken sera from REV-A-infected bird, day 18; lane j, chicken sera from REV-A-infected bird, day 24; lane k, chicken sera from REV-A-infected bird, day 31. Molecular weight markers are expressed in kilodaltons.

Mice injected with the Moloney strain of murine sarcoma virus also develop a suppressor cell population which inhibits the proliferation, but not the cytolytic effector function, of T cells from infected animals (25). The suppressor cells were capable of inhibiting the generation of the secondary cytolytic T cell response to Moloney sarcoma virus tumor cell antigens in vitro (16). The peak activity of these suppressor cells occurred 8 to 12 days after infection, when the cytolytic activity of mice to the tumor cells was also highest, indicating that the suppressor cells are induced after cytolytic T cells have proliferated. Although the Moloney sarcoma virus-induced suppressor cells probably do not effect the primary cytolytic response to the tumor cell antigens, the inhibition of the secondary cytolytic response could allow the continued proliferation of remaining tumor cells.

The inhibition of lymphocyte proliferation in REV-T (REV-A)–infected chickens also limits the development of immune responses to foreign antigens in vivo. The allogeneic skin graft rejection response is a thymus-dependent response which has been correlated with general cellular immune competence (2). The rejection of allogeneic skin grafts was delayed in REV-A–infected chickens, indicating that a general suppression of cellular immune responsiveness occurs in infected chickens during REV-T (REV-A) infection.

In cats infected with feline leukemia virus, cellular (34) and humoral (14) immune responses may be suppressed. The lack of humoral immune response to tumor cell antigens was strongly correlated with tumor development. Weak humoral immune responses against the tumor-cell-associated viral antigens early in infection were associated with the subsequent development of the leukemia (13). In birds infected with REV-A, the humoral immune response directed against viral antigens was not impaired. The serum from birds infected with REV-A was monitored for antibodies directed against the virus with undisrupted virions that detected antibodies against envelope and core polypeptides. However, REV-T (REV-A) is an acute leukemia virus which induces neoplastic disease after a short latent period (40). Chronic leukemia viruses, such as feline leukemia virus, require extended periods of latency before the formation of tumors (18, 36). Antiviral antibodies were not observed in the serum of REV-A–infected birds until 12 to 18 days after infection. Since REV-T infection results in death within 7 to 14 days, it is unlikely that the humoral immune response could affect the progression of this acute leukemia. In addition, birds infected with REV-T (REV-A) were unable to produce antiviral antibody before the progression of the disease led to death.

Cellular immunosuppression may play a significant role in the development of avian acute leukemias. Chickens injected with the known avian acute leukemia viruses or their helpers also develop a rapid suppression of the ability of their splenic lymphocytes to undergo PHA-induced blastogenesis (37, 42), indicating that immunosuppression is a common feature of acute leukemia virus infection. These findings and the results presented here suggest that a rapidly induced general depression of cellular immune competence may play an important role in the development of acute neoplastic diseases induced by REV-T (REV-A), as well as that of the other avian acute leukemia viruses.

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