Lymphocyte subpopulations in chickens with regressing or progressing tumors induced by Rous sarcoma virus

by

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LIST OF ABBREVIATIONS

CD - cluster of differentiation

CD4 - antigen on helper T cells

CD8 - antigen on cytotoxic/suppressor T cells

IA - major histocompatibility complex class II antigen

MAb - monoclonal antibody

MHC - major histocompatibility complex

PBL - peripheral blood lymphocyte

RSV - Rous sarcoma virus

T4 - human homologue for CD4

T8 - human homologue for CD8

INTRODUCTION

Rous sarcoma virus (RSV) is a retrovirus that causes a sarcoma in chickens. In susceptible birds, tumor either regress or progress. Regression and progression of RSV-induced tumors are affected by both major histocompatibility complex (MHC)-linked genes and non-MHC-linked genes (Collins and Zsigray 1984).

Few distinctions have been found that give insight into the dissimilarities between birds with different tumor response phenotypes. Powell *et al.* (1987) found that tumor cells in regressing RSV-induced tumors expressed B-L (MHC class II) antigens, while tumor cells from progressing tumors did not. Perry *et al.* (1978) observed moderate to marked areas of lymphocyte infiltration and lymphocytes that were frequently in contact with tumor cells in regressing Rous sarcomas, whereas progressing tumors had less lymphocyte infiltration and lymphocyte-tumor cell interaction. The migration of peripheral blood lymphocytes (PBL) from chickens with regressing RSV-induced tumors was found to be inhibited by exposure to soluble tumor extract to a greater degree than PBL from chickens with progressing tumors (Cotter *et al.* 1976).

The objective of this study was to gain an increased understanding of the mechanisms of the immune response to RSV in chickens. CD4-positive cells (helper T cells), CD8-positive cells (cytotoxic/suppressor T cells) and IA-positive cells (B cells and activated T cells) play important roles in the immune response. Altered ratios of lymphocyte subsets have been associated with many diseases (Bach and Bach 1981). The specific

objectives of this study were to determine if peripheral blood lymphocytes from regressor and progressor chickens differ in their expressions of CD4, CD8, and IA antigens during the course of tumor growth over time or tumor size, and to determine if there were differences in lymphocyte subsets between birds of different genotypes.

LITERATURE REVIEW

Rous sarcoma virus (RSV) is a retrovirus that causes a sarcoma, a malignant tumor of mesenchymal origin, in chickens. It was discovered by Rous (1911), who described the histology of sarcomas transferred by tumor graft into the breast muscle of the chicken as "spindle cells, usually in bundles, with a slight vascularizing framework". The neoplastic tissue was reported as gristly and grayish white, but occasionally soft or gelatinous. It was discovered by Rous (1911) that the sarcoma could be transmitted by an agent separable from the tumor cells. This agent, RSV, was the first tumor virus isolated from a solid tumor.

Rous Sarcoma Virus Structure

RSV is a RNA virus of the family Retroviridae. The structure of the genome of RSV has been described. It can consist of 4 genes: *gag*, encoding the structural proteins of the virion; *pol*, encoding the reverse transcriptase; *env*, encoding the envelope glycoproteins; and *src*, encoding the oncogene that is the hallmark of RSV. These genes are flanked on both ends by long terminal repeats (LTR) (Wang and Hanafusa 1988) (Figure 1). The viral oncogene, *src*, was derived from a cellular oncogene and encodes a 60 kd phosphoprotein, $pp60^{v\text{-}src}$. This protein is a tyrosine kinase. The expression of $pp60^{v\text{-}src}$ produces many structural and functional changes, thus leading to cell transformation (Svoboda 1988).

The genomes of different strains of RSV have been characterized. RSV may have originally been formed by the insertion of *src* into an avian

leukosis virus (ALV) (Bishop 1982) (Figure 1). Schmidt-Ruppin RSV (SR-RSV) and Prague RSV (PR-RSV) both have genomes containing the four complete retroviral genes. In the genome of Bryan high-titer RSV (BH-RSV), the *src* gene has inserted and the *env* sequence is missing, making the virus replication-defective (Wang and Hanafusa 1988). Many acutely transforming viruses are replication-defective because they have an incomplete genome, having lost all or part of *env* or *gag* while acquiring *src*. Replication-defective RSV require an ALV as a helper virus for the production of infectious virus (Bishop 1982). These helper viruses are called Rous-associated viruses (RAV). BH-RSV has the helper virus RAV-1, which provides the missing *env* gene products. ALV and RSV are divided into five subgroups: A, B, C, D, and E. The subgroup specificity is determined by differences in the envelope glycoproteins, 37 kd spikes (gp37) and 85 kd knobs (gp85). BH-RSV (RAV-1) has a subgroup A specificity (Svoboda 1988).

Genetics of Resistance and Response to RSV

The gp85 is important in determining resistance of a bird to RSV-induced tumorigenesis. Because gp85 is different for every subgroup and is involved in the interaction with cellular receptors, the presence of a given receptor may confer susceptibility, while the absence may result in resistance to infection. Susceptibility is dominant over resistance (Payne 1985). Lines of chicken embryo fibroblasts (CEF) resistant to subgroup A were used by Crittenden (1968) to determine the level of genetic cellular resistance. It was found that although resistance to infection was almost absolute, absorption of the virus was not affected. The genetic block

seemed to occur at either the level of penetration of the virus into the cell or at the subsequent uncoating of the virus particle.

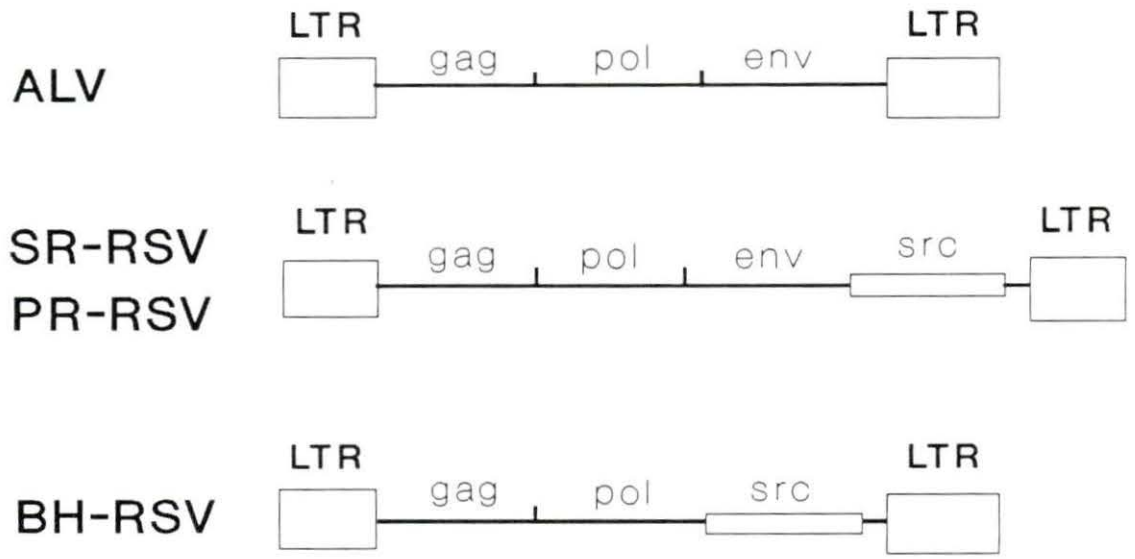


Figure 1. Genomes of avian leukosis virus (ALV) and different strains of Rous sarcoma virus (RSV). Adapted from Wang and Hanafusa (1988) and Bishop (1982)

SR-RSV, Schmidt-Ruppin RSV; PR-RSV, Prague RSV; BH-RSV, Bryan high-titer RSV; LTR, long terminal repeat.

In birds susceptible to the induction of RSV-induced tumors there is a second level of genetic control; this level affects the progression or regression of the tumor. A progressor is defined as a bird which, following virus inoculation, developed a large tumor that persisted or increased in size throughout the period of observation. A regressor refers to a chicken that developed a tumor that completely disappeared or

noticeably decreased in size during the experimental observation period following virus inoculation (Collins and Zsigray 1984). Regression and progression are affected both by major histocompatibility complex (MHC)-linked genes and non-MHC-linked genes (Collins and Zsigray 1984).

The MHC in chickens is linked to the B blood group system, which serves as a useful genetic marker (Schierman and Nordskog 1961). The three class model of the chicken MHC proposed by Pink *et al.* (1977) is still accepted. The three subregions are B-G, B-F, and B-L. The B-G antigens (class IV antigens) are found primarily on red blood cells. B-F antigens are chicken MHC class I antigens. They are found on almost all nucleated cells, including chicken erythrocytes. B-L antigens are found on B cells, activated T cells, and cells of the monocyte/macrophage lineage. The B-L antigens are the MHC class II antigens, also known as the IA or IA-like antigens.

The MHC in chickens has been associated with disease resistance (Bacon 1987). Chickens from the Iowa State S1 White Leghorn line (used in this thesis) with the B^1/B^1 blood type were significantly more resistant to Marek's disease (Steadham *et al.* 1987) and fowl cholera (Lamont *et al.* 1987) than birds of the B^{19}/B^{19} blood type.

MHC-linked genes have been associated with Rous sarcoma regression and progression. Schierman *et al.* (1977) used the MHC-congenic lines of G-B1 and G-B2 chickens to look at regression and progression. They found that all G-B1 birds were progressors and most G-B2 and F_1 birds were regressors. This indicated that the regression of RSV-induced tumors was a dominantly inherited trait associated with the B haplotype. They designated the MHC-

linked gene conferring the ability to regress RSV-induced tumors *R-Rs-1*, and the allele allowing progression *r-Rs-1*. Collins *et al.* (1977) also found differences in B haplotypes associated with RSV tumor regression. They determined that birds from the RPRL line 6_1 (genotype B^2/B^2) primarily regressed the tumors, while birds from line 15_1 (genotype B^5/B^5) almost invariably died with the tumors. Heinzelmann *et al.* (1981b) found that targets of B^2/B^2 and B^5/B^5 RSV-infected chicken embryo fibroblasts (CEF) and uninfected B^5/B^5 CEF were lysed by lymphocytes from B^2/B^2 chickens bearing RSV-induced tumors. They hypothesized that cross-reactivity between B_5 antigen and tumor-specific antigen make it difficult for the B^5/B^5 chicken to recognize the RSV-induced tumor as foreign.

Complementation characterized by enhanced regression in heterozygotes has been observed for both MHC-linked and non-MHC-linked genes. B^{23}/B^{26} birds had a greater anti-tumor (regression) response than either of the homozygous haplotypes B^{23}/B^{23} or B^{26}/B^{26} (Brown *et al.* 1982). The G-B1 (B^{13}/B^{13}) and G-B3 (B^{15}/B^{15}) lines of chickens were found to have almost 100% progression of RSV-induced tumors, whereas most birds from the G-B2 (B^6/B^6) line regressed the tumor (Cutting *et al.* 1981). A complementation effect was demonstrated between the two progressor G-B1 and G-B3 parental lines, whose F_1 progeny predominantly regressed the tumor. Backcross data indicated that both complementing MHC-linked and non-MHC-linked genes were necessary in tumor regression (Cutting *et al.* 1981).

Non-MHC-linked genes influence the regression or progression of RSV-induced tumors. Two independent autosomal loci, *Ly-4* and *Th-1*, which determine surface alloantigens on partly overlapping subsets of T cells,

are important. Chickens of the genotype *Ly-4*^{a}/*Ly-4*^{a}, *Th-1*^{a}/*Th-1*^{a} (abbreviated as *aa/aa*) had a significantly higher ability to regress tumors than the other three double homozygous genotypes (*aa/bb*, *bb/aa*, *bb/bb*) (Gilmour *et al.* 1983). In addition to *Ly-4* and *Th-1*, a locus, *Bu-1*, determining a surface alloantigen on B lymphocytes, was examined (Gilmour *et al.* 1986). Although the *Th-1* genotype had no association with tumor regression, *Ly-4*^{a}/*Ly-4*^{a}, *Bu-1*^{b}/*Bu-1*^{b} and *Ly-4*^{b}/*Ly-4*^{b}, *Bu-1*^{a}/*Bu-1*^{a} genotypes had higher levels of regression than *aa/aa* or *bb/bb* genotypes. Higher levels of regression to RSV-induced tumors were concluded to be associated with the interaction between the *Ly-4* and *Bu-1* loci and the complementation observed.

Many other factors also affect regression and progression. Regression of RSV-induced tumors is influenced by age of the bird; the incidence of regression is higher in older chickens than in younger ones (Cotter *et al.* 1973, Heinzelmann *et al.* 1981a). Virus strain and subgroup also have an effect on sarcoma regression (McBride *et al.* 1981). The B77 strain (subgroup C) produced regressing tumors in G-B1 birds, while strain PR-RSV (subgroup C) caused progressing tumors in the same line of birds. Brown and co-workers (1984) found that both the B haplotype of the bird and the subgroup of the virus influence the anti-tumor response.

Immune Response to RSV-Induced Tumors

The role of the humoral immune system in the response to RSV-induced tumors is controversial. The role of antibody was tested by McArthur *et al.* (1972) by using bursectomized (Bx) chickens, and no differences in

incidence or growth of the tumor were found between Bx and control chickens. In contrast, McBride *et al.* (1978) found that genetically resistant chickens became susceptible to progressive tumor growth following bursectomy. Other studies found that although bursectomy had little or no effect on the response to RSV-induced tumors, thymectomy did have an effect. Yamanouchi *et al.* (1971) found that thymectomy inhibited regression of primary tumors and the induction of resistance to RSV challenge in Japanese quails, but no differences were observed in quails that were bursectomized. Thymectomized chickens were found to have a higher incidence of tumors, larger tumors, and higher tumor mortality than control chickens, but Bx chickens only had a difference in tumor size (Lam and Linna 1976). These results indicate that although the role of the humoral immune response is not well understood, cellular immunity seems to play a role in the RSV-induced tumor response.

Effects of thymectomy were not the only criteria used to evaluate the role of the cell-mediated immune system in the regression and progression of Rous sarcomas. Stimulation of peripheral blood leukocytes (PBL) with supernatant fluids of infected cell cultures and extracts of tumor cells was demonstrated in tumor-bearing birds (Israël and Wainberg 1977). The blastogenic response was observed during regression of the tumors. Whitfill and co-workers (1984) stimulated PBL from regressor and progressor lines of chickens with concanavalin A (Con A) and phytohemagglutinin-P (PHA-P). They found no differences in dose dependency of blastogenesis with Con A, but at high concentrations of PHA-P regressor PBL were stimulated more than progressor PBL. Also, regressor leukocytes exhibited

sensitization to sarcoma antigens not observed with the progressor leukocytes. Cell-mediated cytotoxicity was also used as an indication of cell-mediated immunity. Spleen cells and thymus cells from regressor Japanese quails were cytotoxic to cultivated Rous sarcoma cells, while no cytotoxic effects were observed using lymphoid cells from progressor quail (Hayami *et al.* 1972). Wainberg *et al.* (1974) found spleen cells from RSV-induced tumor-bearing chickens to be more reactive against autochthonous than allogeneic neoplastic target cells *in vitro*. This was observed in chickens with progressively growing sarcomas. Compelling evidence for the major role of cell-mediated immunity in RSV-induced tumor regression was shown by Whitfill *et al.* (1986). They demonstrated that the transfer of sensitized histocompatible blood lymphocytes and peritoneal macrophages from regressor donor birds to progressor recipients resulted in the eradication of growing tumors in age-matched chickens.

Lymphocyte Subsets: Phenotypes and Functions

The cellular immune system can be divided into subsets of cells involved in tumor regression. Subsets of lymphocytes with different functional roles have been described. The functional subsets bear different surface proteins. Monoclonal antibodies (MAbs) are used to detect the specific cell surface proteins on the functional subsets. B and T lymphocytes are two of the important subsets. B lymphocytes, which synthesize immunoglobulins, are an important facet of the cellular immune system. They are characterized by the cell surface MHC class II antigens (IA antigens) and immunoglobulins (Sell 1987). Two of the major functional

subpopulations of T cells are helper T cells and cytotoxic/suppressor T cells (Parnes 1989). Lymphocyte subsets can be distinguished by cluster of differentiation (CD) antigens. Helper T cells are identified by the CD4 antigen. In humans, this antigen is designated T4, and the MAb OKT4 identifies this subset; in the mouse, the MAb L3T4 designates this population. OKT4 binds to 55-60% of the peripheral blood T cell population and identifies cells necessary for the generation of cytotoxic T cells (Reinherz *et al.* 1979b). This MAb also characterizes T cells that provide the signals necessary for the differentiation of B cells into immunoglobulin-producing cells (Reinherz *et al.* 1979a). Helper T cells are restricted by class II MHC antigens (Swain 1983); they are activated by the recognition of a foreign antigen in conjunction with self MHC class II antigens (Sell 1987).

The cytotoxic and suppressor T cell population, which are generally class I MHC restricted (Swain 1983), is defined by the CD8 antigen. T8, the human homologue of CD8, is recognized by the MAb OKT5. The symbols Ly2 and Lyt2 are used to designate the same population in mice. Approximately 20% of human peripheral blood T cells are bound by OKT5. OKT5-bound cells are minimally responsive to soluble antigens and have a poor proliferative response to PHA. The cytotoxic function of this subpopulation was established using cell-mediated lympholysis, and the suppressor function was demonstrated by the suppressive effect of Con A stimulated cells on autologous cell proliferation in mixed lymphocyte culture (MLC) (Reinherz *et al.* 1980).

Helper and cytotoxic T cells have been described in the chicken (Chan *et al.* 1988; Lillehoj *et al.* 1988). MAbs developed by Lillehoj *et al.* (1988) against the avian homologue of CD8 antigen stained 18% of peripheral blood lymphocytes (PBL); the MAbs against the CD4 homologue bound 19% of PBL. The Con A-induced proliferation of spleen cells was reduced after pretreatment with anti-CD8 antibody and complement (C). Pretreatment with anti-CD4 antibody and C resulted in substantially less inhibition of Con A-induced proliferation than treatment with anti-CD8 antibody and C. Treatment of spleen cells with anti-CD8 and C, but not anti-CD4 antibody and C, resulted in a significant reduction in the cytotoxic activity. This agreed with the established functions of helper T cells and cytotoxic/suppressor T cells. The anti-CD4 MAbs immunoprecipitated a 55 kd band; anti-CD8 MAbs immunoprecipitated 33-35 kd proteins under reducing conditions.

MAbs produced by Chan *et al.* (1988) against avian CD4 and CD8 bound 45% and 15% of PBL, respectively. The removal of CD8-positive cells inhibited the cytotoxic activity induced by allogeneic lymphocyte stimulation, and the removal of CD4-positive lymphocytes inhibited pokeweed mitogen-induced interleukin-2 (IL-2) production. The anti-CD8 antibody immunoprecipitated molecules of 34 kd under reducing conditions, and anti-CD4 antibody identified a 64 kd protein.

The anti-CD8 MAbs produced by Lillehoj *et al.* (1988) and Chan *et al.* (1988) seem to bind similar subsets. However, the percentages of PBL bound by the anti-CD4 antibodies and the molecular weights of the immunoprecipitated proteins were different.

A simple scheme for the roles of helper T cells and cytotoxic T cells in the recognition and destruction of virus-infected cells involves the recognition of virus antigen in conjunction with MHC antigens (Figure 2). The T cell receptor on the surface of helper T cells recognizes the virus antigen together with class II MHC antigens on the surface of antigen presenting cells (APC). This, along with the release of interleukin-1 (IL-1) by the APC stimulates the helper T cell to release interleukin-2 (IL-2). IL-2 stimulates T cells, and induces cytotoxic T cell precursors recognizing the same viral antigen with MHC class I antigens on the APC to differentiate into activated cytotoxic T cells capable of killing virus-infected cells. The helper T cell is also affected by the IL-2, and becomes activated. This system is a model for killing tumor cells as well as virally-infected cells (Sell 1987).

Both helper (CD4) and cytotoxic (CD8) T cells have a role in tumor regression. Flamand *et al.* (1990) found that both CD4-positive and CD8-positive T cells were important *in vivo* for induction of immunological control on tumor growth. The generation of cytotoxic T cells is necessary for the regression of a tumor. The CD4-positive cells may act as helpers be releasing factors that activate cytotoxic cell precursors after recognition of tumor-derived antigens displayed by antigen presenting cells. It was shown (Mills and North 1983) that the passive transfer of non-cytotoxic T cells sensitized to the tumor does not cause regression of the tumor until a sufficient number of cytotoxic T lymphocytes have been produced in the draining lymph nodes in T cell-deficient recipients. They postulated that the infusion of sensitized non-cytotoxic lymphocytes gave

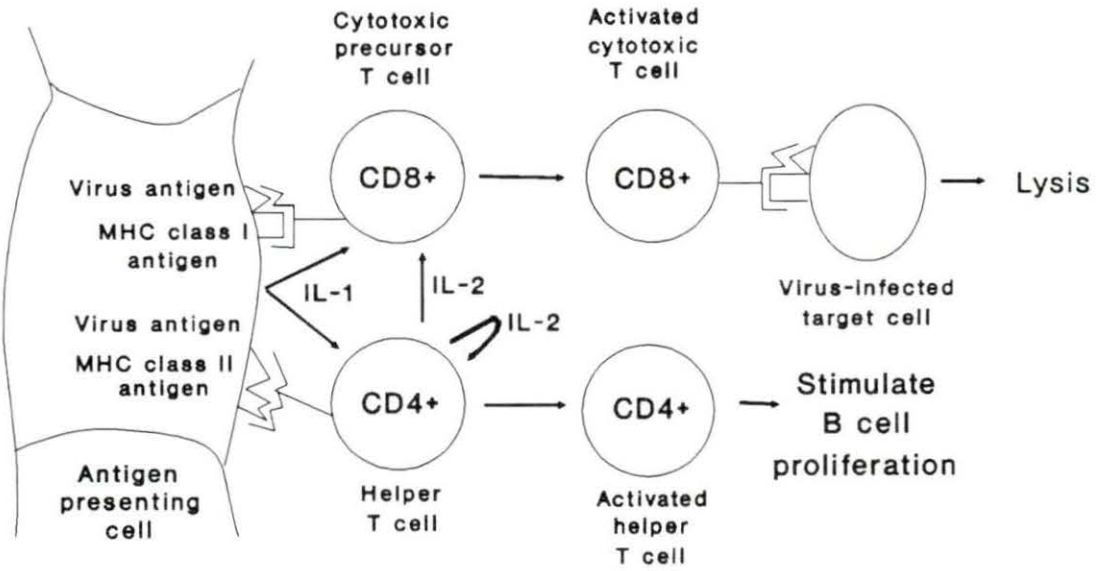


Figure 2. Simplified scheme of cellular interactions involved in recognition and killing of virus-infected cells. Adapted from Sell (1987, Chap. 10)

the tumor-bearing recipient the ability to generate cytotoxic T lymphocytes against the tumor. Helper T cells have been shown to act as inducers for the generation of tumor-specific cytotoxic T lymphocytes (Schild *et al.* 1987, Yamamoto *et al.* 1987). Kosugi *et al.* (1987) demonstrated that the

generation of tumor-specific cytotoxic T lymphocytes was dependent upon helper factors released from activated helper T cells. Different functions of the T cell subsets were found using Moloney sarcoma virus (MSV)-induced tumors as a model (Bateman *et al.* 1987). They found that L3T4$^+$ cells were necessary for the protection against MSV-induced tumors in normally resistant unprimed mice, while Lyt-2$^+$ cells were not. A second experiment evaluating the role of the two subsets of T cells in the protection of unprimed mice through adoptive immunization. It was found that although Lyt-2$^+$ T cells were able to confer full tumor resistance, the population of L3T4$^+$ positive T cells failed to do so. Other workers (Ershler *et al.* 1989) suggested that the functional importance of the helper T cell may be in early tumor cell recognition and destruction, but after the tumor has become established, the helper T cell has a lesser role in the restraint of tumor growth.

MATERIALS AND METHODS

Virus and Injections

The virus used was a mixture of BH-RSV and the helper virus RAV-1. It will be referred to throughout this thesis as RSV. It was kindly donated by Dr. L. Crittenden (U.S. Department of Agriculture, Agriculture Research Service, Regional Poultry Research Laboratory, East Lansing, MI). The stock virus was obtained from supernatants from line 0 primary chicken embryo fibroblasts, which lack endogenous viral genes, infected with BH-RSV(RAV-1) (Crittenden, personal communication 1990). The stock solution of the virus was kept at -70C. The virus stock was diluted with PBS pH 7.2 to a concentration of 200 FFU (focus forming units)/50 μl. The working dilution was kept in an ice water bath until used. Each chicken was injected with 50 μl (200 FFU) sub-dermally in the wingweb of the wing not containing the wingband.

Tumor Scoring Criteria

The tumor forms in the wingweb at the point of injection. The wingweb of each bird was examined visually and palpated weekly following the injection of the virus. Any observable tumor was assigned a tumor score based on the criteria described in Table 1 (Collins *et al.* 1977). The control birds and any bird without an observable tumor were given a tumor score of zero.

After a period of 7 or 8 weeks, each chicken was categorized as either a regressor or a progressor. A chicken was designated as a regressor if

the last tumor score observed was a zero, or if a decrease in the tumor score was observed between the last two weeks evaluated. Any chicken that died prior to termination of the experiment, and any chicken that showed an increase in the tumor score over the last 2 weeks or maintained the same tumor score over the last 2 weeks of observation was designated as a progressor.

Table 1. Criteria used for describing the stages of RSV-induced tumor growth (Collins *et al.* 1977)

Tumor Score	Description of Tumor
0	No palpable tumor.
1	Small tumor 0-0.5 cm in diameter.
2	Tumor 0.5-1.5 cm in diameter.
3	Tumor 1.5 cm in diameter to 1/2 the wingweb.
4	Tumor 1/2 the size of the wingweb to slightly less than the wingweb.
5	Tumor fills the wingweb.
6	Tumor fills the wingweb, engulfs the wing. Bird is moribund.

Experimental Animals

Chickens were from the S1 line of White Leghorns at the Iowa State Poultry Center at Iowa State University. The S1 line was derived from a cross of two inbred commercial lines (Nordskog *et al.* 1973). Since 1978,

it has been maintained as sublines homozygous for major histocompatibility complex (MHC) haplotype (B^1/B^1 or B^{19}/B^{19}) and for the humoral immune response to the synthetic antigen glutamic acid-alanine-tyrosine (GAT), high (H) or low (L) (Nordskog and Cheng 1988). Thus, a bird was of the genotype 1H, 1L, 19H, or 19L.

Chicks were kept in battery brooders until 4 weeks of age, when they were moved to suspended wire cages where they were kept throughout the experiment. Food and water were available *ad libitum*.

Chicks were hatched from each of the four genotypes. Full-sibling groups with at least 10 chicks were selected, and the 4-week-old chicks were injected with RSV. All birds formed tumors, which were scored weekly for 7 weeks, and each bird was designated as a regressor or a progressor. Families within the four groups were then assigned a designation of progressor, regressor, or mixed, depending on the designation of the birds within the family. A family was designated as mixed if there was an approximately equal number of progressors or regressors within the family.

Full-siblings and half-siblings of the tested birds were used to set up matings to produce chicks for the research presented in this thesis. Five groups of birds were selected based on haplotype and response to RSV-induced tumors. The groups selected were: 1H regressor, 1H mixed (both progressors and regressors), 1L mixed, 19H mixed, and 19L progressor. Because the birds in the 1H mixed group were later found to be predominantly regressors, the two groups were combined after trial 2 and were subsequently referred to as 1H regressors. Four trials of the peripheral blood lymphocyte (PBL) analysis experiment were done; trial 4

contained only uninoculated birds. Trials 1, 2, and 4 were performed on the progeny of the siblings of the original tested birds. The birds used in trial 3 were progeny of siblings of the birds used in the other three trials. Table 2 contains the numbers and genotypes-family tumor response of the birds selected for use in each trial.

Table 2. Numbers of birds in each trial of the experiment divided by genotype and family designation according to tumor response

		Genotype-family tumor response				
	RSV	1H-Reg[a]	1H-Mix[b]	1L-Mix	19H-Mix	19L-Prog[c]
Trial 1	+	10	5	5	5	5
	-	1	0	1	2	1
Trial 2	+	8	7	5	5	5
	-	0	1	1	2	1
Trial 3	+	14	0	4	5	3
	-	1	0	1	1	2
Trial 4	+	5	0	5	5	5

[a]Reg = regressor.

[b]Mix = mixed.

[c]Prog = progressor.

The number of birds in a trial was selected as being the maximum number of samples easily handled in a day in the laboratory. More birds from the 1H genotype were selected than the other genotypes because a

balanced number of progressor and regressor birds was desired, and the genotypes that had a mixed response of progression/regression to RSV-induced tumors tended to have a higher percentage of progressors than regressors at the conditions used in this study. Birds were distributed randomly in the groups by sex. Trial 3 had only 31 birds because 4 of the birds in the experiment did not produce tumors. The data from these 4 birds were discarded.

Lymphocyte Collection

Blood samples were collected from all birds before the injection of the virus and at weekly intervals for 7 weeks following the injection. Approximately 3 ml of blood was collected from the jugular vein of each bird each week by using a heparinized syringe.

Whole blood samples were placed in glass test tubes siliconized with Sigmacote (Sigma Chemical Co., St. Louis, MO) and centrifuged for 10 minutes at 60 x *g*. The leukocytes suspended in the serum were pipetted off by using a siliconized pasteur pipette. These cells were then pelleted by centrifugation at 600 x *g*. If too few cells (determined by visual inspection of the pelleted cells in the tube) were collected, the remaining portion of the whole blood sample was recentrifuged at 60 x *g*, and the additional leukocytes were pipetted off and pooled with those collected from the first centrifugation. The collected leukocytes were pelleted at 600 x *g*, the serum was discarded, and the cells were then resuspended in 1 ml of the wash buffer composed of 1.1 x Dulbecco's phosphate buffered saline (DPBS) (Sigma Chemical Co., St. Louis, MO), 2% fetal calf serum

(FCS), and 0.1% NaN_3. The cells were again pelleted, the wash buffer was discarded, and approximately 1 ml of fresh wash buffer was added. The cells were resuspended in the wash buffer by using a siliconized pasteur pipette, diluted by using trypan blue dye, and the concentration of viable cells was determined for each sample by using a hemacytometer. The volume containing 1 x 10^6 cells was determined, and 1 x 10^6 cells from each sample were placed in each of 4 siliconized test tubes. Approximately 1 ml of the wash buffer was then added, the cells were pelleted by centrifugation, and the wash buffer was discarded.

Labelling of Lymphocytes with MAbs

Monoclonal antibodies (MAbs) to the antigens CD4, CD8, and IA were a gift from Dr. Hyun Lillehoj (United States Department of Agriculture - Agriculture Research Service, Beltsville, MD). MAbs used for trials 1, 2, and 4 were in ascites fluid; those used for trial 3 were in culture supernatant. Working dilutions of the Mabs were based on the recommendations by Hyun Lillehoj (personal communication 1989, 1990). MAbs were diluted by using the wash buffer. The ascites fluids were used at dilutions of 1:10, 1:10, and 1:50 for CD4, CD8, and IA, respectively; the supernatants were used at dilutions of 1:5, 1:10, and 1:10 for CD4, CD8, and IA, respectively.

Each of the 4 tubes of leukocytes prepared from each blood sample was labelled stained with anti-CD4, anti-CD8, or anti-IA MAb or used as the background control. All tubes labelled with a MAb received 100 μl of the antibody dilution, and the control tubes received nothing. All tubes were

shaken gently and then incubated at 5C for 30 minutes. The cells were washed twice following incubation using approximately 1 ml of the wash buffer. After cells were pelleted and the wash buffer was drained off the second time, the secondary antibody was added.

The secondary antibody was a fluoroisothiocyanate (FITC)-labelled antibody. Trial 1 used affinity purified FITC-labelled goat anti-mouse IgG (ICN Immunobiologicals, Lisle, IL). All other trials were done using FITC-labelled anti-mouse IgG $F(ab')_2$ fragments absorbed with human serum (Sigma Chemical Co., St. Louis, MO). Secondary antibodies were used at a dilution of 1:100. All washed and drained tubes received 100 μl of the secondary antibody. They were incubated at 5C for 30 minutes following a gentle shaking. After incubation, the cells were washed three times by using approximately 1 ml of the wash buffer.

The cells were fixed following the last wash. Equal volumes (usually 100 μl) of buffer (1.1 x DPBS + 0.1% NaN_3) and a 2% solution of electron microscopy (EM) grade formaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in buffer were added to each tube. This resulted in a final fixing solution containing 1% formaldehyde. The cells were then resuspended by shaking, covered with aluminum foil, and stored at 5C until analysis was executed on the flow cytometer.

Flow Cytometric Analysis

Antibody-labelled cells from trials 1, 2, and 4 were analyzed on an EPICS 752 flow cytometer (Coulter Electronics Corporation, Hialeah, FL); cells from trial 3 were analyzed on an EPICS Profile I flow cytometer

(Coulter Electronics Corporation, Hialeah, FL). Lymphocytes were distinguished from other white blood cells on the basis of their forward angle light scatter (related to cell size) versus right angle scatter (related to cellular granularity) (Muirhead *et al.* 1985). An electronic analysis gate was set on the cluster of lymphocytes containing the fluorescent cells to eliminate background fluorescence. Green fluorescence was determined on a 256 channel histogram in linear fluorescence units. Percentages of positive cells were reported as the percentage of cells from the negative control subtracted from the test sample to give the net percentage of positive cells.

Tumor-Infiltrating Lymphocyte Isolation

Collection of tumor-infiltrating lymphocytes (TIL) was adapted from a procedure by Trail and Yang (1985). RSV-induced tumors at various stages of tumor growth were excised using a scalpel from the wingwebs of birds killed by cervical dislocation. The excised tumor was placed in calcium- and magnesium-free Hank's buffered salt solution (HBSS) (Sigma Chemical Company, St. Louis, MO) on ice. Feathers attached to the tumor were removed and the weight of the tumor was recorded. The tumor was then minced into small pieces by using a scalpel. The minced pieces were suspended in approximately 10 ml of calcium- and magnesium-free HBSS with 0.125% collagenase (Sigma Chemical Company, St. Louis, MO) and homogenized in a glass homogenizer for approximately 1 minute. The resulting mixture was stirred slowly for 1 hour. A steel strainer (mesh size 80) was used to obtain a single-cell suspension. Buffer consisting of 1.1 x DPBS, 2% FCS,

and 0.1% NaN_3 was used to dilute the suspension approximately 1:3; the cells were then pelleted by centrifugation at 600 x *g* for 15 minutes. The pelleted cells were resuspended in 20 ml of buffer and pelleted at 600 x *g* twice. The cells were resuspended in 5 ml of buffer, layered on 15 ml of Ficoll-Paque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) and centrifuged at 400 x *g* for 30 minutes. The interface band containing viable tumor cells and lymphocytes was harvested. Cells were washed twice using 5 ml of the buffer. Washed cells were layered onto a discontinuous Percoll (Sigma Chemical Co., St. Louis, MO) gradient. The gradient was formed by diluting 9 ml of Percoll with 1 ml of 10 x HBSS. This stock solution was further diluted to 20, 40, and 65% of the stock. Two ml of each density were layered in the order of decreasing density to form the gradients. The cells were either suspended in 2 ml buffer on top of the 20% density, or resuspended in the 2 ml of 65% Percoll used for the bottom layer. Gradients were centrifuged for 30 minutes at 400 x *g*, and cells at the interface between the 40 and 65% Percoll gradients were collected. These cells were washed twice with 1 ml of buffer and then resuspended in 1 ml buffer. The volume of 1 x 10^6 cells was determined and the lymphocytes were labelled with MAbs.

Variations in the above procedure were done to test the efficacy of the modifications. These included deleting the Ficoll-Paque centrifugation, performing the collagenase digestion at room temperature, and collecting the tumors into HBSS at room temperature.

Immunohistochemistry of Tumor Sections

Cross-sections less than 3 mm thick were excised from the tumors collected for TIL analysis. A spleen from a normal chicken was used as a positive control. Sections were fixed in 10% neutral buffered formalin and imbedded in paraffin blocks. Sections 4 μm thick were cut and placed on poly-L-lysine-coated slides. Tissue sections were deparaffinized and rehydrated in a series of baths: 2 for 5 minutes in xylene, 2 for 3 minutes in 100% alcohol, 2 for 3 minutes in 95% alcohol, 1 for 3 minutes in 70% alcohol, 1 for 3 minutes in distilled water. Sections were trypsinized using different concentrations of trypsin for varying time periods. Solutions of 0.1%, 0.05%, and 0.025% solutions of trypsin (Sigma Chem Co., St. Louis, MO) and CaCl were used at incubation periods of 0, 10, 15, 20, and 30 minutes at 37C. Slides were warmed in 0.05M pH 7.6 tris twice for 5 minutes. Sections were exposed to 3% H_2O_2 at room temperature for 10 minutes, followed by 2 washes for 5 minutes. Sections were stained using methods described by Bourne (1983). Tissue sections were blocked using normal horse serum (NHS) (obtained locally) in tris/PBS (1:20) for 20 minutes at room temperature. Excess serum was removed by blotting, and a MAb specific for CD3, a cell surface marker on all T cells, obtained from Dr. Chen-Lo Chen (University of Alabama at Birmingham, Birmingham, AL) was applied to the tissue section. The section was incubated in a humidity chamber for approximately 20 hours at room temperature. The slides were washed 3 times with 1% NHS in 0.05M tris, which was used for all subsequent washes. Incubation for 30 minutes at room temperature in horse anti-mouse biotinylated secondary antibody (Vector Laboratories Inc., Burlingame, CA)

diluted 1:200 in tris/PBS was the next step. Following 3 washes, the tissue sections were incubated with streptavidin peroxidase (BioGenics Laboratories, San Ramon, CA) diluted 1:100 in tris/PBS for 40 minutes at room temperature. Sections were washed, 3-amino-9-ethylcarbazole (AEC) solution (Polysciences Inc., Warrington, PA) was added to the tissue slides, and slides were incubated until a reddish color appeared or for 20 minutes. Tissue sections were counterstained with Gill's stain and NH_4^+ for approximately 30 seconds each. Slides were coverslipped, mounted, and observed by using a light microscope.

Statistical Analysis

Data were analyzed using general linear models procedures (SAS^R Institute Inc. 1985). Differences between means were determined using Duncan's multiple range test. Standard errors of least squares means were obtained.

RESULTS

Differences in Tumor Response Between Trials

Percentages of peripheral blood lymphocytes (PBL) positive for CD4, CD8, and IA cell surface markers were measured in samples from individual birds. Trials differed in the percentages of progressor and regressor birds within a trial (Table 3) and in the mean tumor score of a trial. The mean tumor scores averaged over time of trials 1, 2, and 3 were 1.76, 2.02, and 0.93, respectively; trials 1 and 2 had significantly higher ($p<0.05$) mean tumor scores than trial 3. Differences in the tumor scores among trials within weeks were also observed (Table 3). Trials 1 and 2 did not have significantly different mean tumor scores for progressing tumors between weeks 0 and 6, or for regressing birds at every week except week 2. Significantly lower ($p<0.05$) mean tumor scores were found in trial 3 for most weeks in both tumor response groups than trials 1 and 2. The data from trial 3 were not included in subsequent analyses. Explanations for this are given in the discussion. Figure 3 shows the differences in tumor growth between progressor and regressor birds from trials 1 and 2, with mean tumor scores for each week.

Comparison of PBL Subsets Between Sexes

Data from all birds in trials 1, 2, and 4 were analyzed for differences associated with sex. Females made up 61% and males made up 39% of these birds. At week 6, female birds had higher percentages of CD4-positive and CD8-positive PBL than male birds. All other weeks showed no

Table 3. Mean tumor scores for progressor and regressor birds within weeks among trials[1]

TRP[2]	Trial	n	%[3]	Week 0	1	2	3	4	5	6	7
Pro-	1	12	40	0	$0.3\pm.15^{a}$	$3.3\pm.14^{a}$	$4.1\pm.21^{a}$	$4.6\pm.34^{a}$	$4.6\pm.58^{a}$	$4.6\pm.51^{a}$	$4.6\pm.34^{a}$
gressor	2	19	63	0	$0.3\pm.12^{a}$	$3.1\pm.11^{a}$	$3.7\pm.16^{a}$	$3.9\pm.24^{a}$	$4.0\pm.30^{a}$	$3.7\pm.28^{a}$	$3.2\pm.31^{bc}$
	3	5	19	0	$0.0\pm.23^{a}$	$1.6\pm.22^{b}$	$1.8\pm.32^{b}$	$2.4\pm.40^{b}$	$2.4\pm.40^{b}$	$3.0\pm.51^{a}$	$3.0\pm.45^{c}$
Re-	1	18	60	0	$0.0\pm.12^{a}$	$2.4\pm.12^{a}$	$3.1\pm.17^{a}$	$2.9\pm.21^{a}$	$1.8\pm.27^{a}$	$1.6\pm.24^{a}$	$1.0\pm.17^{a}$
gressor	2	11	37	0	$0.1\pm.16^{a}$	$3.2\pm.15^{b}$	$3.0\pm.22^{a}$	$2.7\pm.27^{a}$	$1.6\pm.35^{a}$	$1.0\pm.31^{ab}$	$0.1\pm.15^{ab}$
	3	21	81	0	$0.0\pm.11^{a}$	$1.3\pm.11^{c}$	$1.4\pm.16^{b}$	$1.0\pm.20^{b}$	$0.8\pm.25^{b}$	$0.5\pm.22^{b}$	$0.4\pm.21^{b}$

[1]Means shown are least squares mean $\pm$ standard error of the least squares mean.

[2]Tumor response phenotype.

[3]Percentage of total birds within a replicate having the indicated tumor response.

[a,b,c]Mean tumor scores not sharing superscripts within a week within a tumor response are significantly different ($p<0.05$).

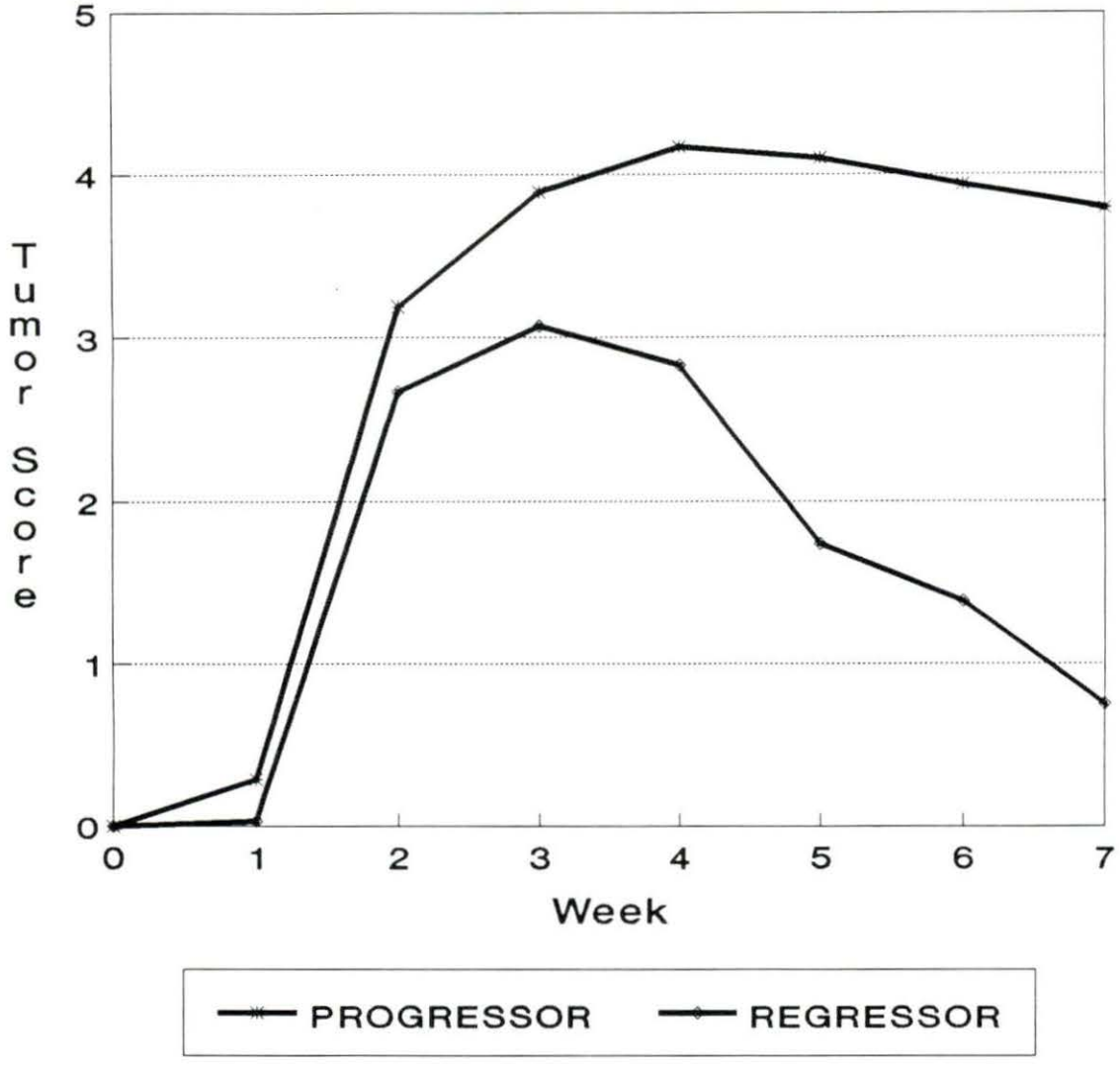


Figure 3. Mean tumor scores by week of progressor and regressor birds

significant differences in PBL subsets between sexes (Table 4). No significant differences were observed between birds of different sexes bearing tumors of the same size (Table 5).

Effects of RSV Inoculation

PBL from uninoculated birds in trials 1, 2, and 4 were compared at each week with PBL from birds in trials 1 and 2 inoculated with RSV. Uninoculated birds had higher percentages of CD4-positive PBL than uninoculated birds at all weeks except two; the difference was significantly higher ($p<0.05$) at weeks 0, 1, 4, and 7 compared to inoculated birds (Figure 4). Only at weeks 5 and 6 post-inoculation did inoculated birds have significantly higher ($p<0.05$) percentages of CD4-positive PBL than uninoculated birds.

A higher percentage of CD8-positive PBL was found for uninoculated birds than inoculated birds at all weeks except two (Figure 5). Significantly higher ($p<0.05$) percentages of CD8-positive PBL were found in uninoculated birds than in inoculated birds at weeks 0, 2, and 7; inoculated birds had a significantly higher ($p<0.05$) percentage of CD8-positive PBL than uninoculated birds only at week 1. No significant differences were found between the groups at weeks 3, 4, 5, or 6.

Uninoculated birds had higher percentages of IA-positive PBL than inoculated birds at all weeks except one; IA-positive PBL were found at significantly higher ($p<0.05$) percentages in uninoculated birds than inoculated birds at weeks 1, 2, and 4 post-inoculation (Figure 6). No significant differences were observed at other weeks.

Table 4. Percentages of PBL subsets in birds of different sexes within weeks[1]

Week	Lymphocyte Subsets					
	CD4		CD8		IA	
	Female	Male	Female	Male	Female	Male
0	5.1±.31	4.4±.38	10.8±.69	10.0±.86	9.5±.43	9.7±.53
1	3.7±.28	3.0±.35	14.7±1.1	14.1±1.4	7.0±.42	7.0±.54
2	3.3±.28	2.8±.35	15.4±1.0	16.5±1.3	9.0±.58	9.2±.73
3	3.4±.23	3.0±.29	17.8±1.2	15.7±1.5	9.3±.56	10.4±.70
4	3.5±.30	2.9±.36	15.0±.79	11.7±.96	9.0±.94	8.3±1.1
5	6.4±.52	6.4±.64	18.0±1.5	16.4±1.8	10.3±.68	11.2±.84
6	6.0±.30[a]	4.6±.35[b]	15.2±.86[a]	12.1±1.0[b]	10.7±.50	9.7±.58
7	9.8±1.4	8.1±1.7	14.1±.75	12.5±.91	10.0±.60	9.8±.75

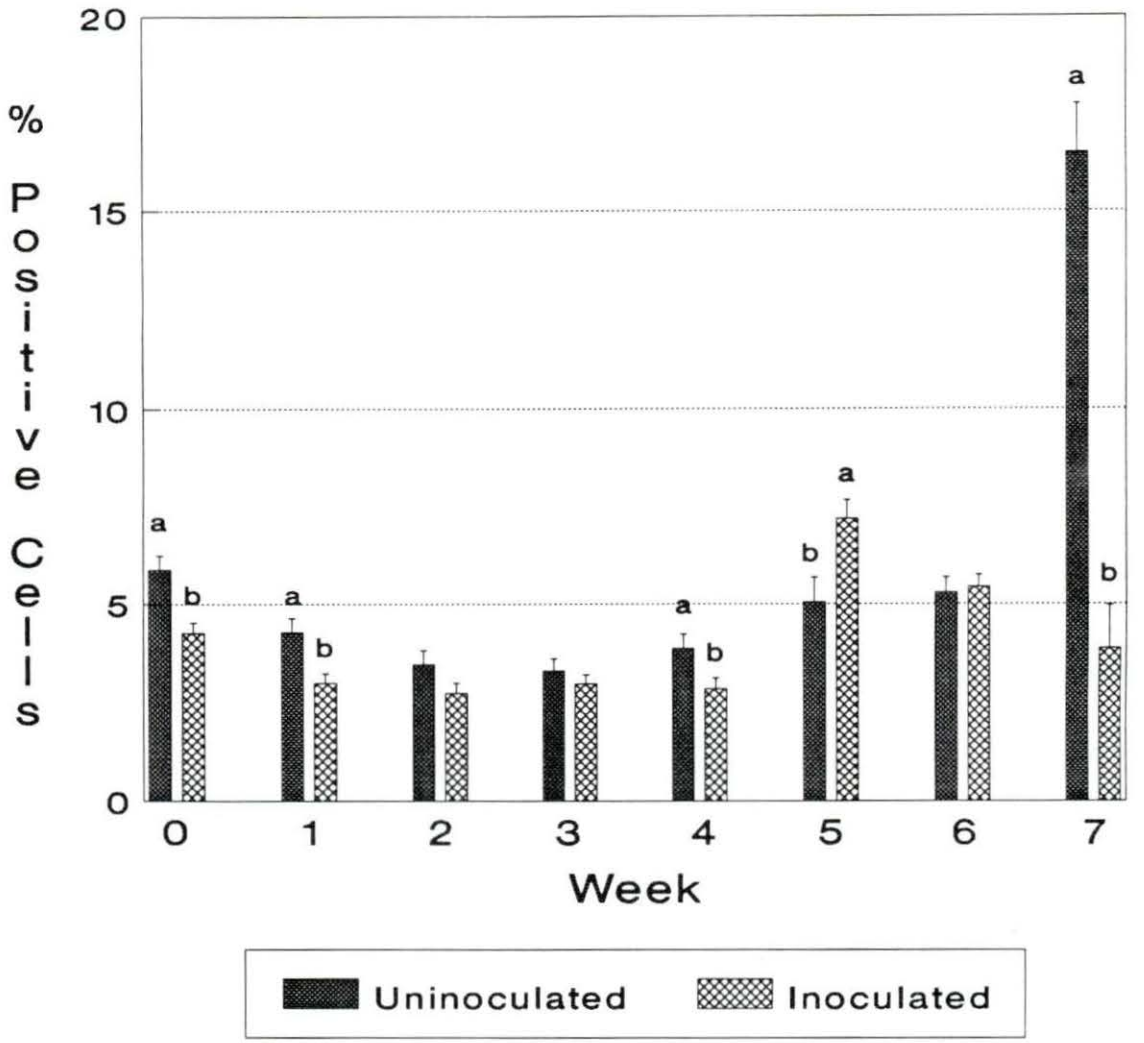
[1]Means shown are least squares mean ± standard error of the least squares mean.

[a,b]Means in a row within a lymphocyte subset with different superscripts are significantly different ($p<0.05$).

Table 5. Percentages of PBL subsets in birds of different sexes within tumor scores[1]

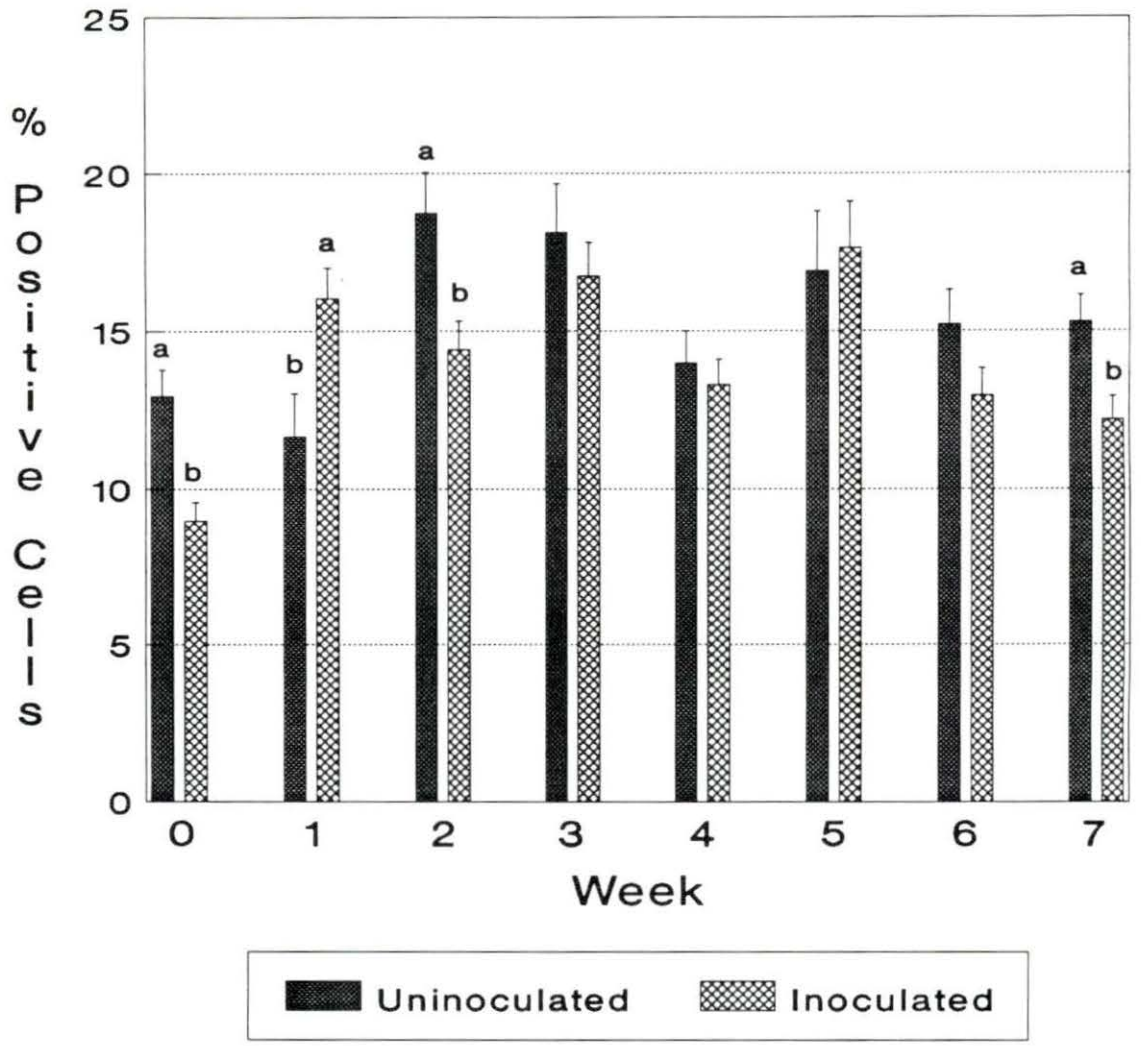
Tumor Score	Lymphocyte Subsets					
	CD4		CD8		IA	
	Female	Male	Female	Male	Female	Male
0	5.7±.31	4.6±.40	15.5±.51	13.1±.64	9.8±.29	9.7±.37
1	4.7±.63	4.7±.69	14.7±2.3	14.7±2.5	9.3±1.1	8.8±1.3
2	2.8±.65	3.7±.56	14.7±1.6	14.2±1.4	8.6±1.5	9.4±1.2
3	3.9±.34	3.9±.39	14.0±.92	14.5±.94	8.4±.53	8.8±.54
4	4.1±.52	4.5±.71	15.0±1.0	15.8±1.4	8.7±.56	9.4±.74
5	4.5±.63	2.7±1.3	15.1±1.7	9.08±3.5	8.0±.67	10.4±1.4
6	3.8±.48	2.8±.90	10.3±2.1	9.36±3.9	7.7±1.5	6.7±2.7

[1]Means shown are least squares mean ± standard error of the least squares mean.



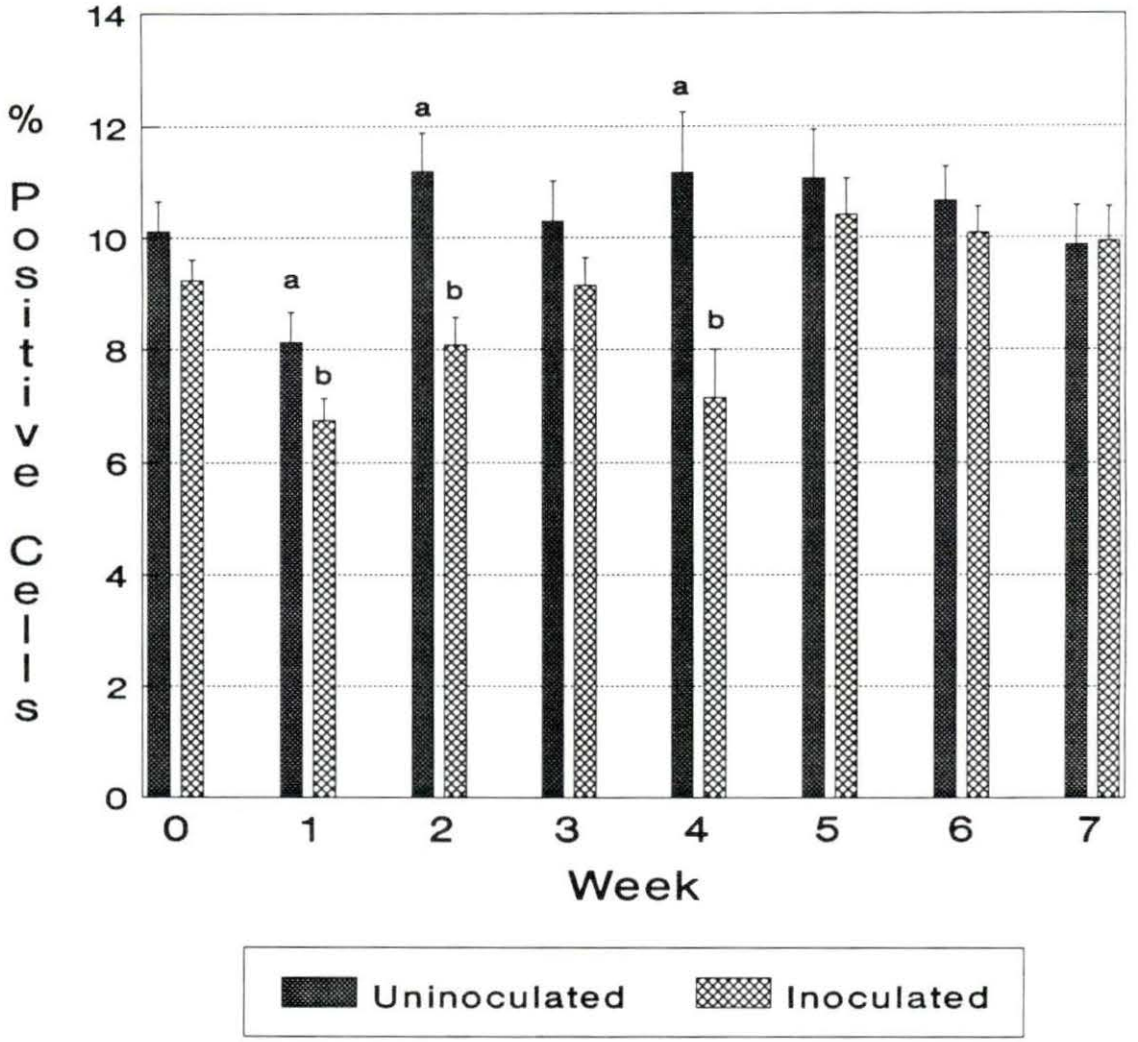
[a,b]Means within a week with different letters are significantly different.

Figure 4. Weekly percentages of CD4-positive peripheral blood lymphocytes in uninoculated and inoculated birds. The n values for uninoculated birds were 30 at all weeks; the n values for the inoculated birds were 60, 60, 60, 50, 48, 46, and 42, at weeks 0, 1, 2, 3, 4, 5, 6. and 7, respectively



[a,b]Means within a week with different letters are significantly different.

Figure 5. Weekly percentages of CD8-positive peripheral blood lymphocytes uninoculated and inoculated birds. The n values for uninoculated birds were 30 at all weeks; the n values for the inoculated birds were 60, 60, 60, 60, 50, 48, 46, and 42, at weeks 0, 1, 2, 3, 4, 5, 6, and 7, respectively



[a,b]Means within a week with different letters are significantly different.

Figure 6. Weekly percentages of IA-positive peripheral blood lymphocytes in uninoculated birds. The n values for uninoculated birds were 30 at all weeks; the n values for the inoculated birds were 60, 58, 57, 59, 48, 48, 46, and 41, at weeks 0, 1, 2, 3, 4, 5, 6, and 7, respectively

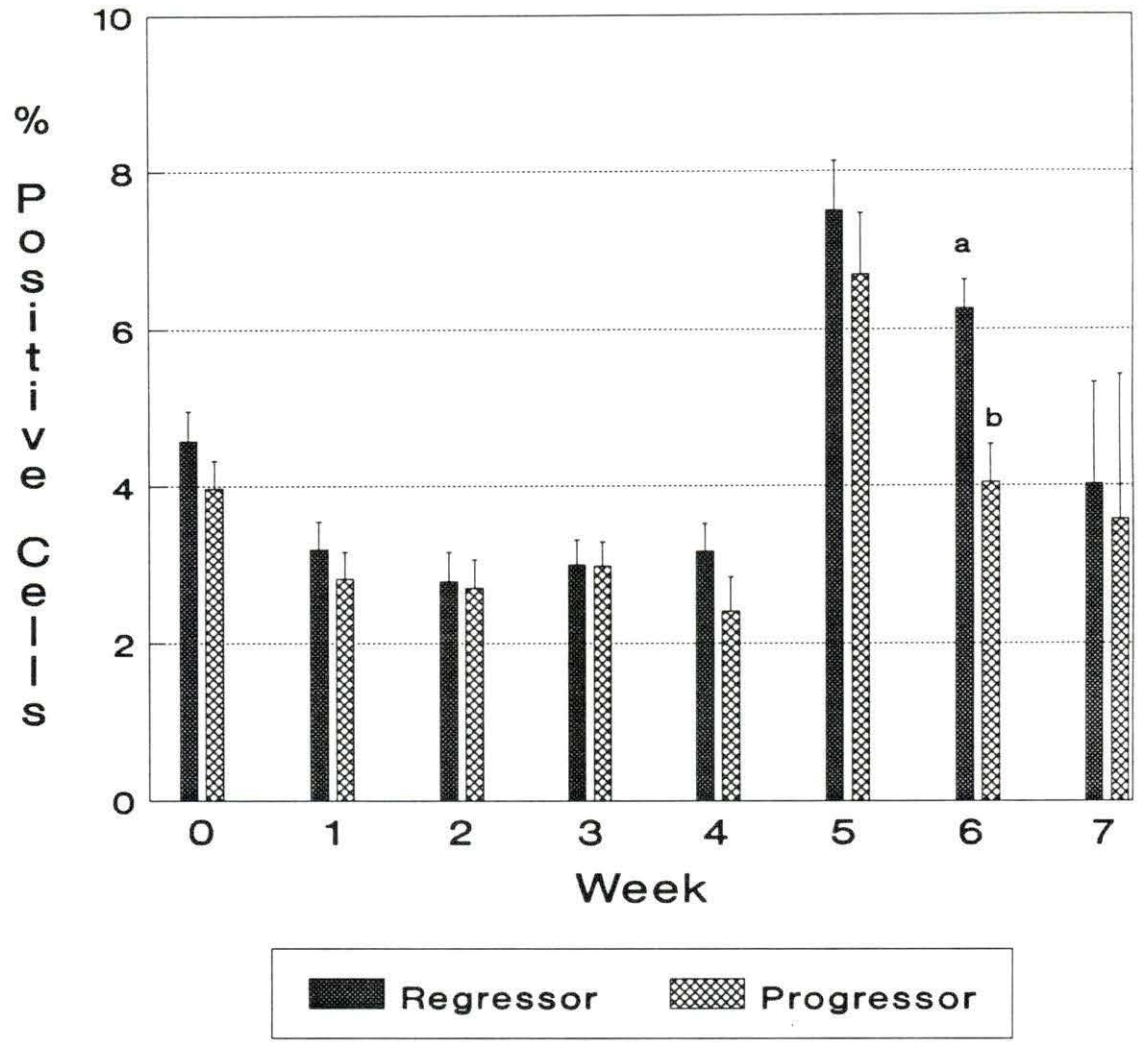
Comparison of Tumor-Regressor and -Progressor Phenotypes

RSV-inoculated birds were designated as a regressor or progressor bird based on the bird's tumor response phenotype. Percentages of PBL subsets averaged over time were compared between regressor and progressor birds in trials 1 and 2. Regressor birds had significantly higher ($p<0.01$) percentages of CD4-, CD8-, and IA-positive cells than progressor birds. Regressor birds had mean percentages of cells positive for CD4, CD8, and IA of 4.3, 15.1, and 9.4, respectively; mean CD4-, CD8-, and IA-positive PBL percentages were 3.5, 12.9, and 8.0, respectively, for progressor birds.

PBL subsets from regressors and progressors were also compared at weekly time intervals. Regressor birds had higher percentages of CD4-positive cells than progressor birds at all weeks (Figure 7). At week 6, regressor birds had a significantly higher ($p<0.05$) percentage of CD4-positive PBL than progressor birds.

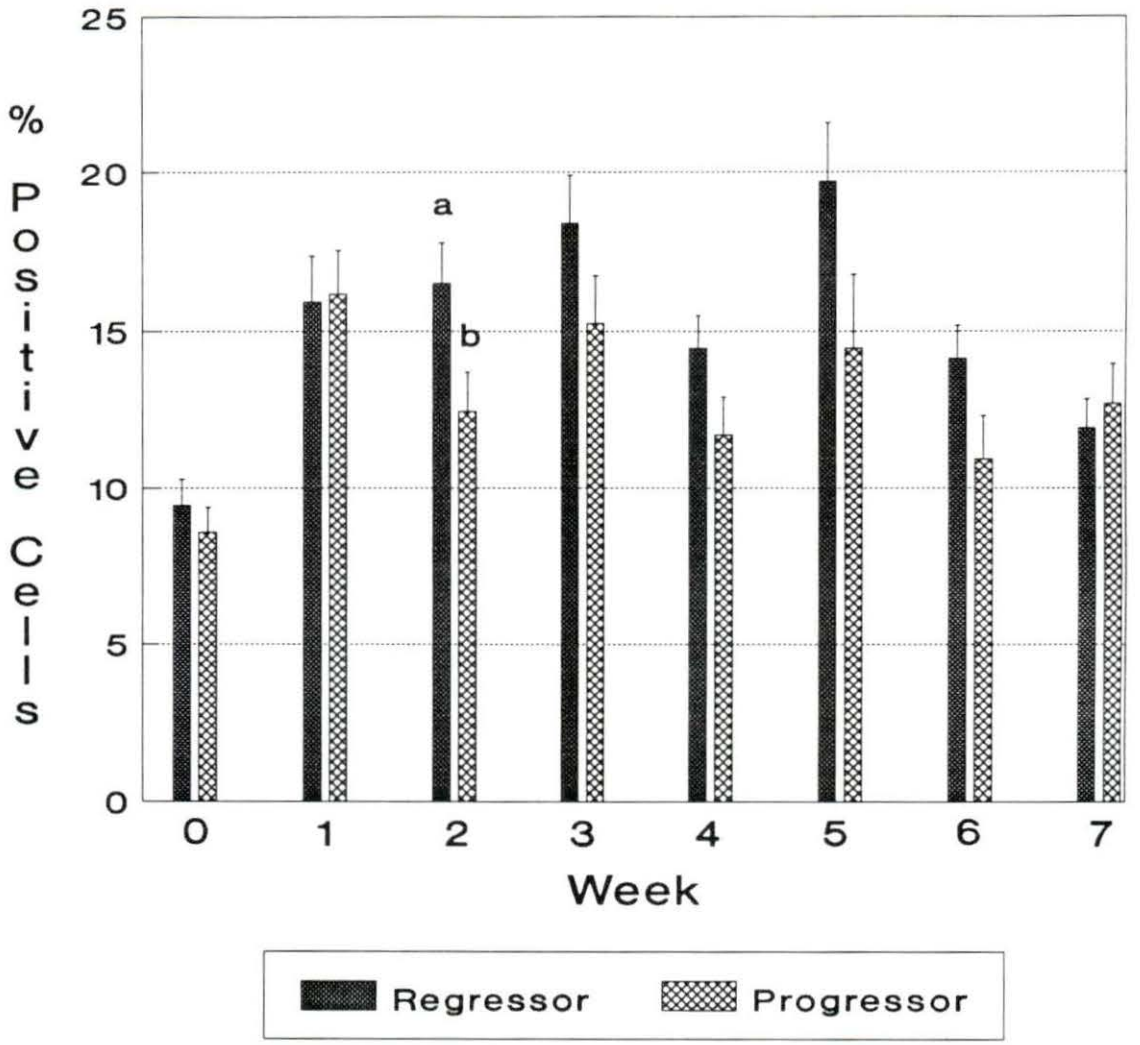
Regressor birds had higher percentages of CD8-positive cells than progressor birds at all weeks except two; a significantly higher ($p<0.05$) percentage of CD8-positive PBL was found in regressors than in progressors at week 2 (Figure 8).

A higher percentage of IA-positive cells was observed in regressor birds than progressor birds at all weeks except one (Figure 9). Regressor birds had a significantly higher ($p<0.05$) percentage of IA-positive PBL at weeks 3 and 5 than progressor birds. No significant differences of PBL subsets were observed between progressor and regressor birds at the other weeks.



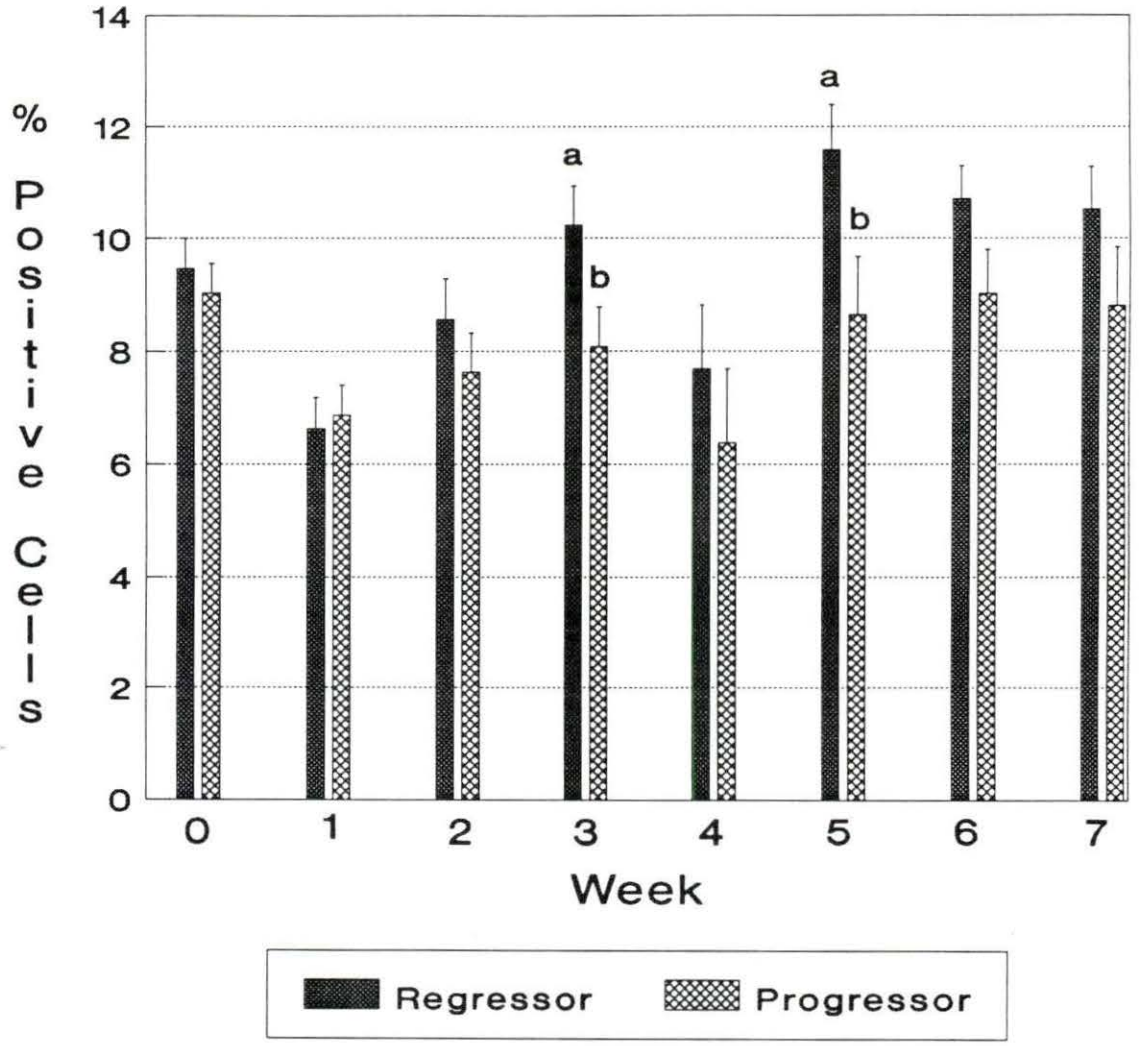
[a,b]Means within a week with different letters are significantly different.

Figure 7. Weekly percentages of CD4-positive peripheral blood lymphocytes in progressor and regressor birds. The n values for progressor birds and regressor birds were 30 and 29, respectively, for weeks 0 through 3; after week 3, the n values for regressor birds and progressor birds were 29 and 19, respectively



[a,b]Means within a week with different letters are significantly different.

Figure 8. Weekly percentages of CD8-positive peripheral blood lymphocytes in progressor and regressor birds. The n values for progressor and regressor birds were 30 and 29, respectively, for weeks 0 through 3; after week 3, the n values for regressor birds and progressor birds were 29 and 19, respectively



[a,b]Means with a week with different letters are significantly different.

Figure 9. Weekly percentages of IA-positive peripheral blood lymphocytes in progressor and regressor birds. The n values for progressor and regressor birds were 30 and 29, respectively, for weeks 0 through 3; after week 3, the n values for regressor birds and progressor birds were 29 and 19, respectively

The kinetics of tumor growth varies between birds. This difference in kinetics can be resolved by comparing PBL subsets between regressor and progressor birds with tumors of approximately the same size. Data from RSV-injected regressor and progressor birds with tumors of the same size from trials 1 and 2 were analyzed for differences in lymphocyte subsets.

Regressor birds had higher percentages of CD4-positive cells at all tumor sizes than progressor birds (Figure 10). No significant differences in CD4-positive PBL were found between progressor and regressor birds with tumors of similar size.

Differences in the percentages of CD8-positive PBL were found between regressor and progressor birds with tumors of the same size (Figure 11); regressor birds had higher percentages than progressor birds at all tumor scores except one. Regressor birds had significantly higher ($p<0.05$) percentages of CD8-positive PBL than progressors with tumors at scores 2, 3, and 4.

Regressor birds had a higher percentage of IA-positive PBL at all tumor scores and a significantly higher ($p<0.05$) percentage of IA-positive PBL at the tumor score 3 than progressor birds (Figure 12).

To separate the effects of the descending phase of tumor growth from the ascending phase, a data set was generated which eliminated information from the descending phase of tumor growth gathered from birds with tumors that decreased in size. Phenotypically progressor or regressor birds bearing tumors of the same size that were increasing or stable in size were compared. Although regressor birds had higher percentages at four tumor

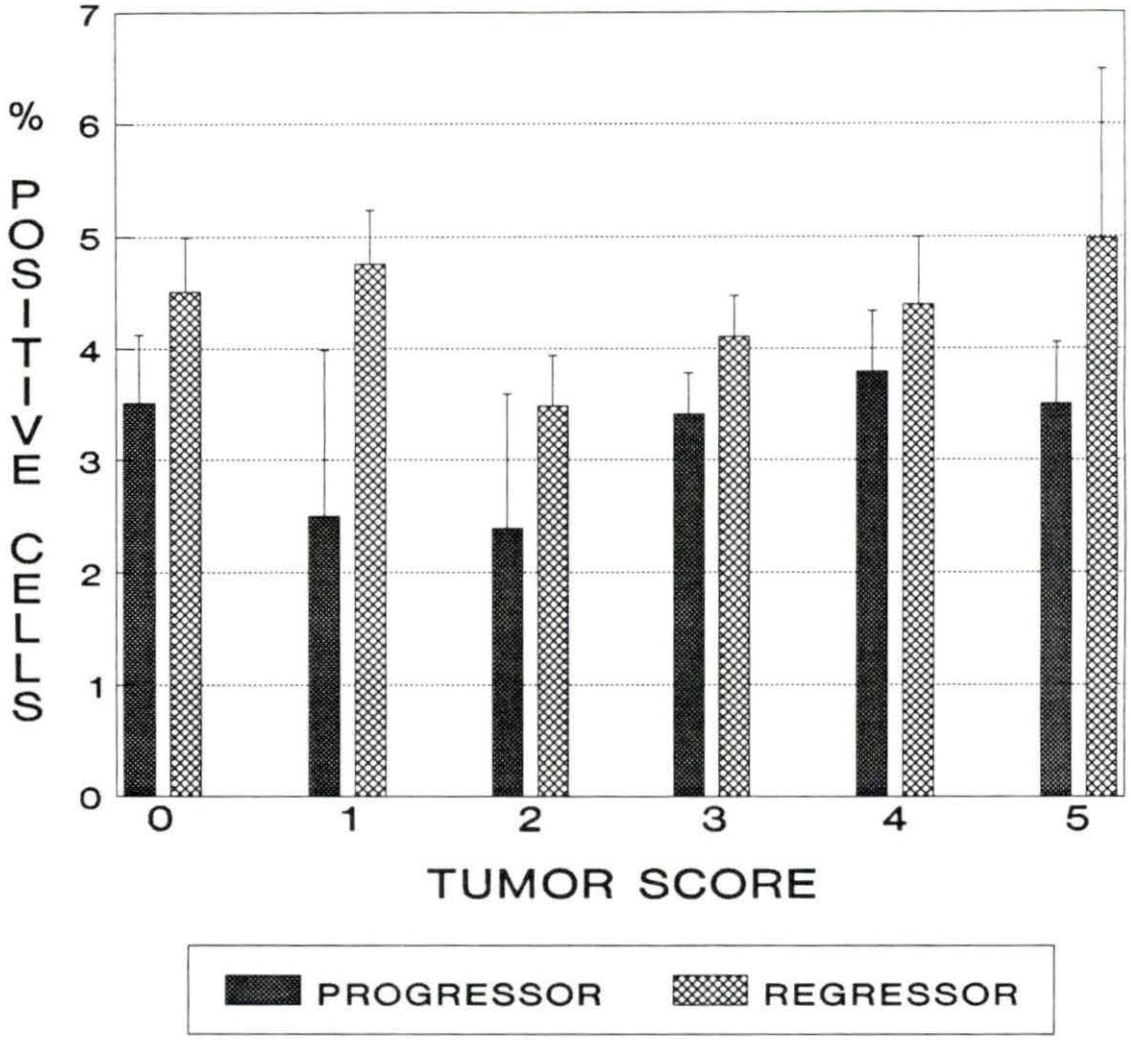
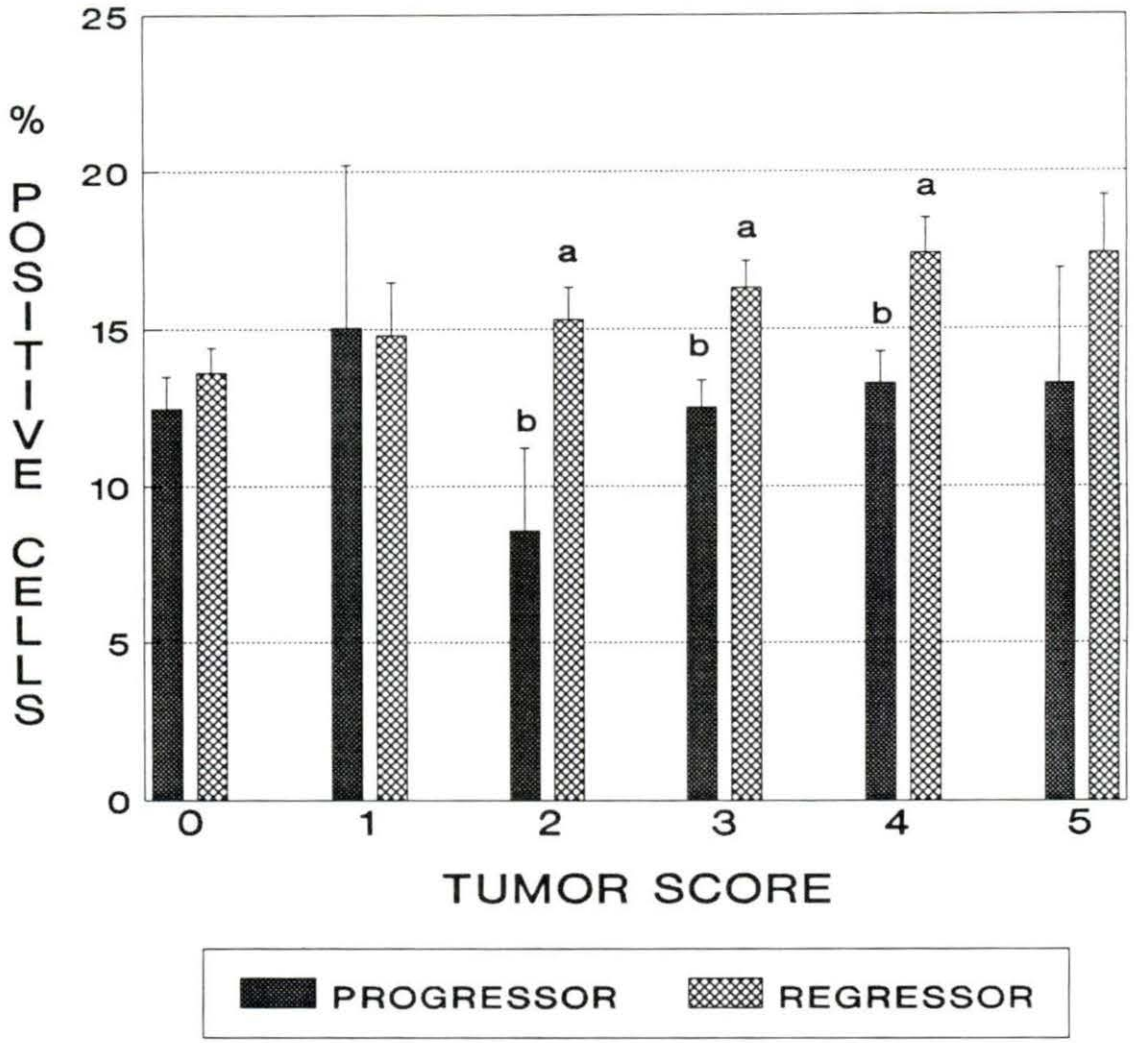
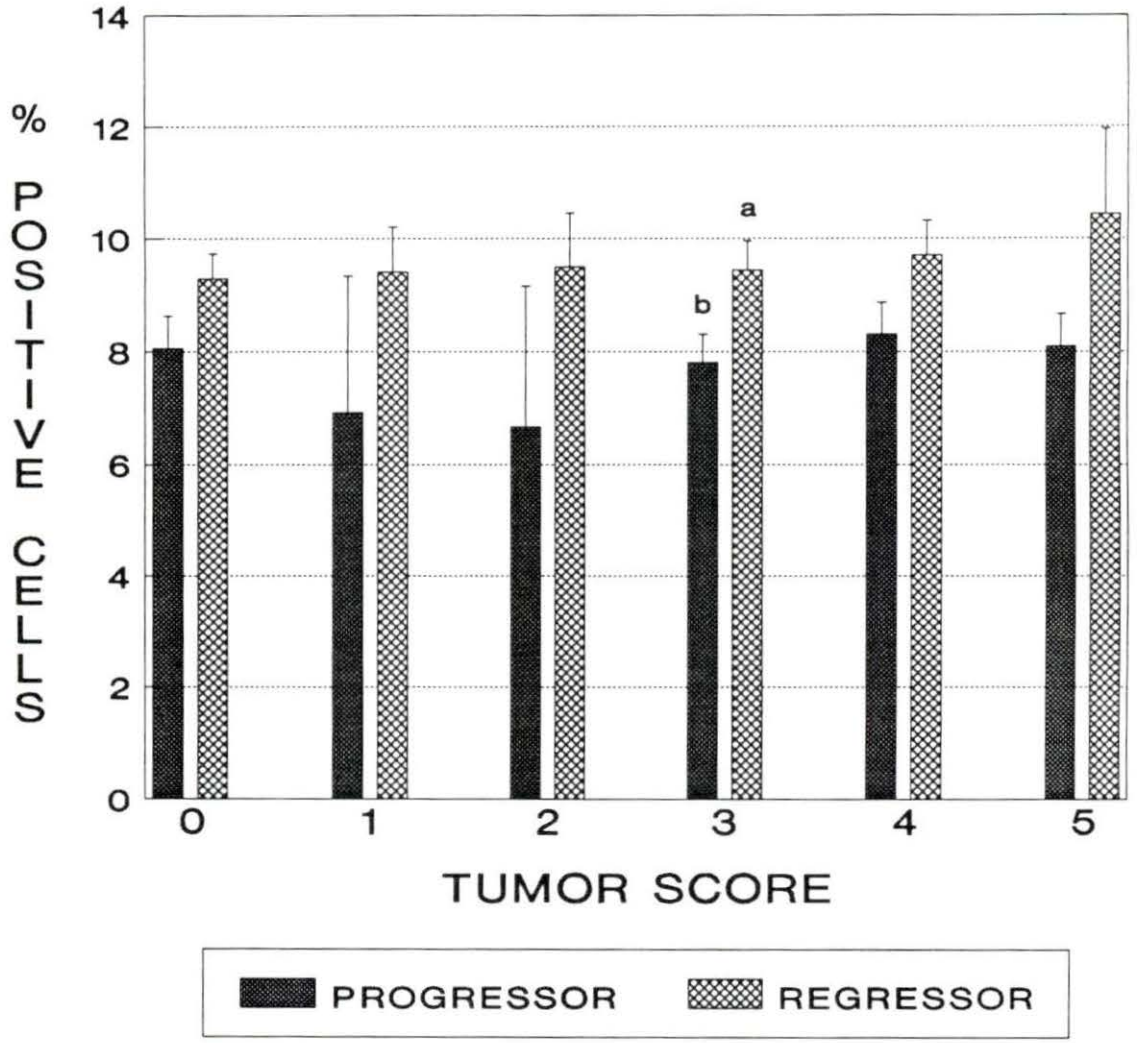


Figure 10. Percentages of CD4-positive peripheral blood lymphocytes in progressor and regressor birds by tumor score. The n values at tumor scores of 0, 1, 2, 3, 4, and 5 for progressor birds were 58, 2, 4, 53, 39, and 29, and for regressor birds were 96, 19, 27, 53, 32, and 4, respectively



[a,b]Means within a tumor score with different letters are significantly different.

Figure 11. Percentages of CD8-positive peripheral blood lymphocytes in progressor and regressor birds by tumor score. The n values at tumor scores of 0, 1, 2, 3, 4, and 5 for progressor birds were 58, 2, 4, 53, 39, and 29, and for regressor birds were 96, 19, 27, 53, 32, and 4, respectively



[a,b]Means with a tumor score with different letters are significantly different.

Figure 12. Percentages of IA-positive peripheral blood lymphocytes in progressor and regressor birds by tumor score. The n values at tumor scores of 0, 1, 2, 3, 4, and 5 for progressor birds were 57, 2, 52, 37, and 29, and for regressor birds were 94, 18, 26, 52, 32, and 4, respectively

scores, no significant differences in percentages of CD4-positive PBL were found between regressor and progressor birds with tumors at the same tumor score (Figure 13). Regressor birds had significantly higher ($p<0.05$) percentages of CD8-positive PBL than progressor birds at tumor scores of 2, 3, and 4 (Figure 14). No significant differences were observed between regressor and progressor birds with tumors of the same size for percentages of IA-positive peripheral lymphocytes, but regressor birds had higher percentages than progressor birds at all tumor scores (Figure 15).

Differences in PBL Subsets Among Genotypes

Data from all uninoculated birds in the four trials were evaluated for differences in PBL subsets among genotypes. Birds of genotype 1H had a significantly higher percentage of CD4-positive PBL than birds of the other genotypes (Table 6). The percentage of CD8-positive PBL was significantly lower ($p<0.05$) in birds of genotype 1L than in birds of the other three genotypes. 19H birds had a significantly higher ($p<0.05$) percentage of IA-positive PBL than all other genotypes; 1H birds had a significantly higher ($p<0.05$) percentage of peripheral lymphocytes bearing the IA antigen than 1L and 19L birds, although a significantly lower ($p<0.05$) percentage than the 19H birds.

Tumor response phenotypes were confounded with the genotypes of the birds. Genotypes 1H, 19H, 1L, and 19L had 4:11, 1:1, 9:1, and 9:1 ratios of progressor birds:regressor birds, respectively. Only the 19H genotype had an even ratio. Inoculated 19H birds from replicates 1 and 2 were

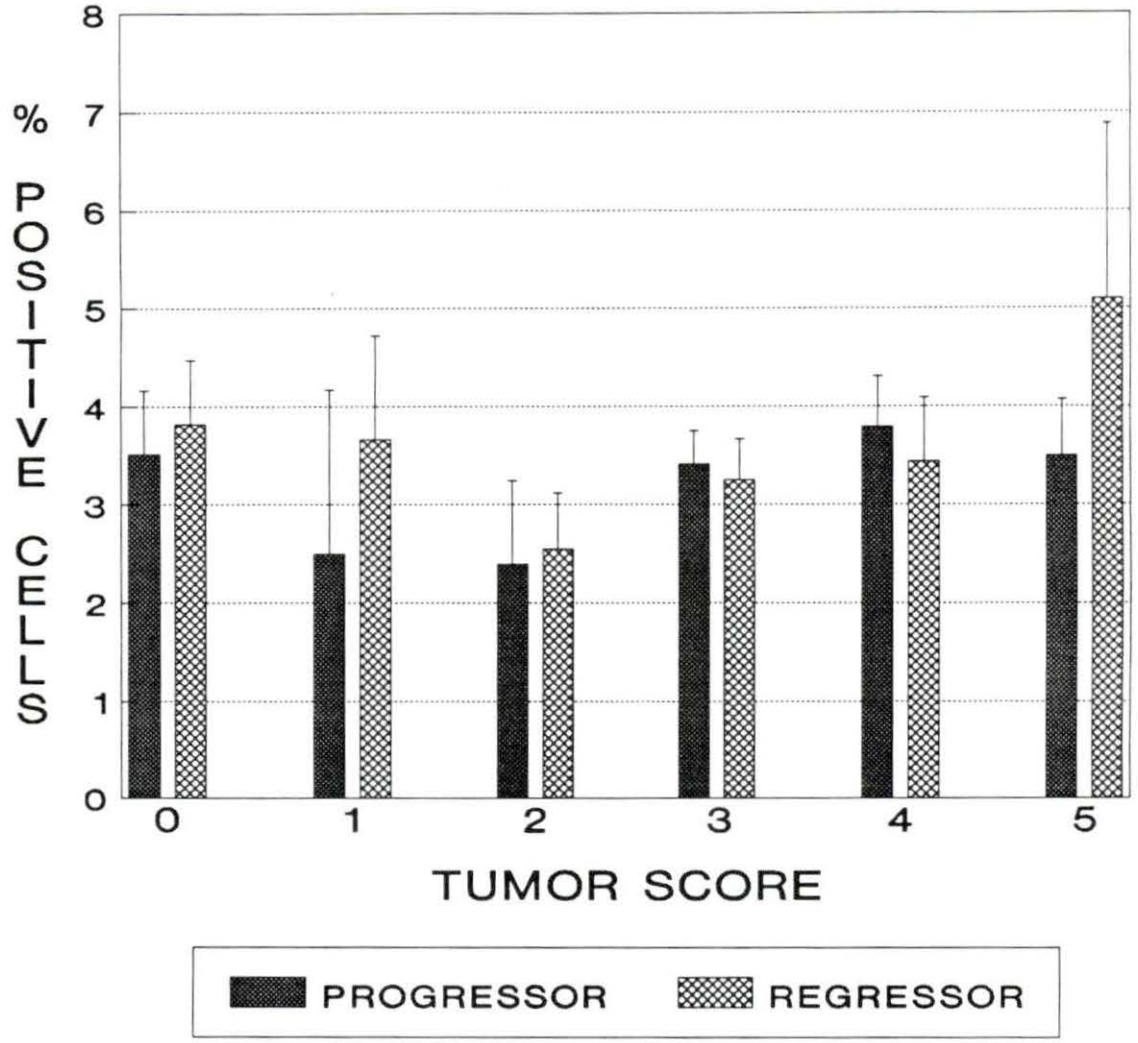
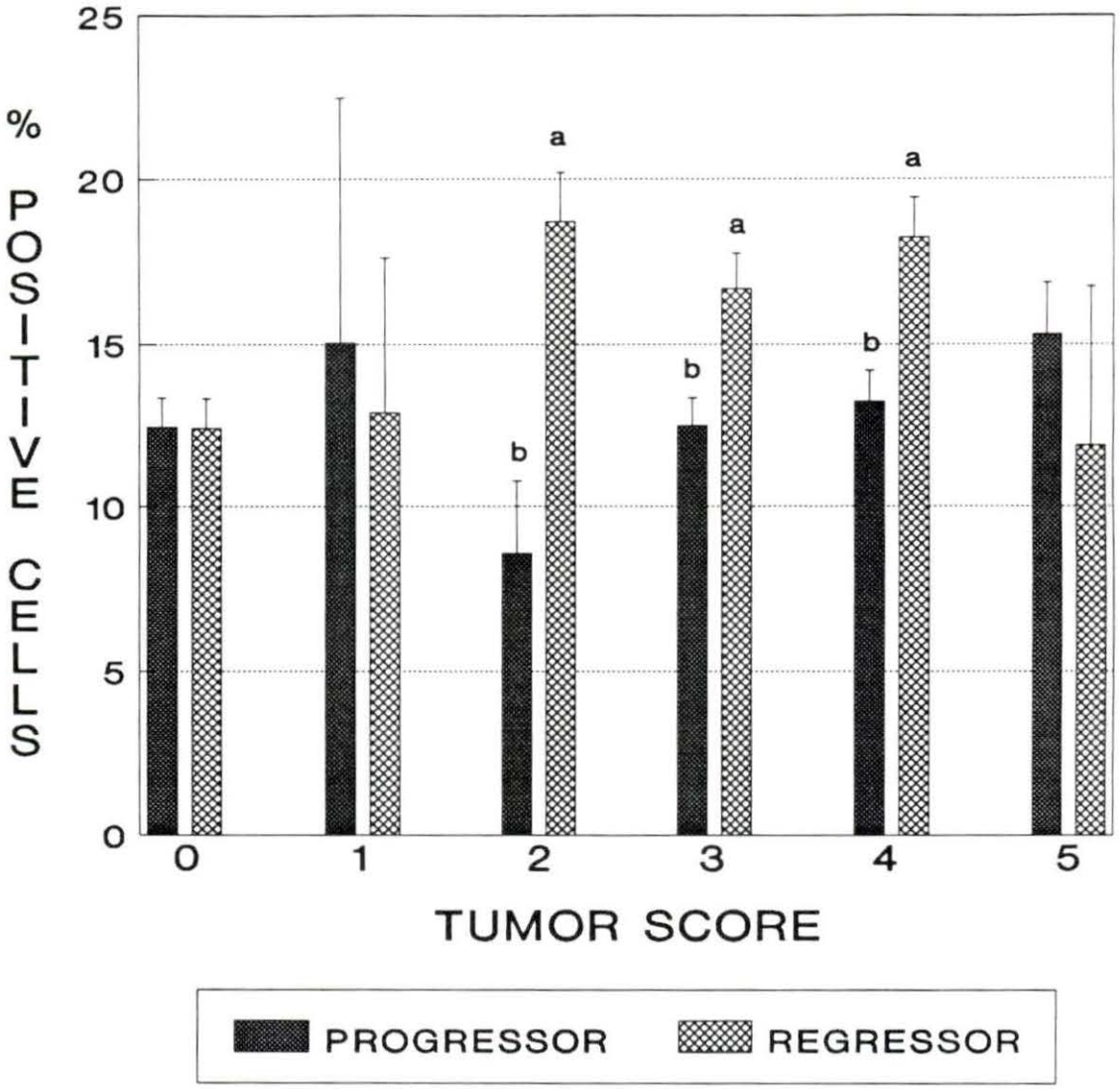


Figure 13. Percentages of CD4-positive peripheral blood lymphocytes in progressor and regressor birds bearing tumors increasing in size by tumor score. The n values at tumor scores of 0, 1, 2, 3, 4, and 5 for regressor birds were 57, 5, 9, 35, 25, and 3, and for progressor birds were 58, 2, 4, 53, 39, and 29, respectively



[a,b]Means within a tumor score with different letters are significantly different.

Figure 14. Percentages of CD8-positive peripheral blood lymphocytes in progressor and regressor birds bearing tumors increasing in size by tumor score. The n values at tumor scores of 0, 1, 2, 3, 4, and 5 for regressor birds were 57, 5, 9, 35, 25, and 3, and for progressor birds were 58, 2, 4, 53, 39, and 29, respectively

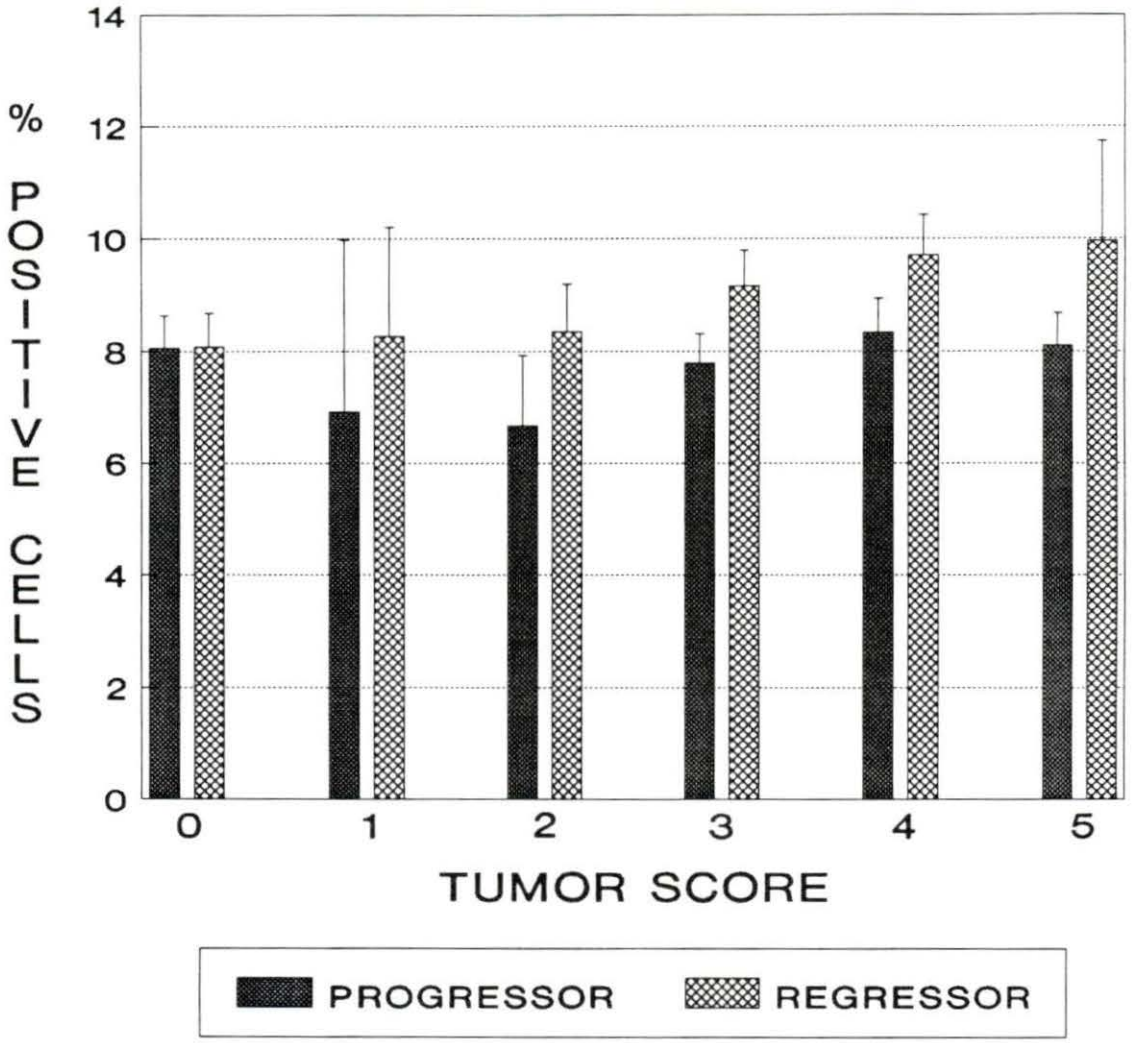


Figure 15. Percentages of IA-positive peripheral blood lymphocytes in progressor and regressor birds bearing tumors increasing in size by tumor score. The n values at tumor scores of 0, 1, 2, 3, 4, and 5 for progressor birds were 57, 2, 4, 52, 37, and 29, and for regressor birds were 55, 5, 9, 34, 25, and 3, respectively

evaluated for differences in PBL subsets between the regressor birds and progressor birds. No significant differences were found in percentages of CD4-positive PBL between regressor and progressor 19H birds at any week (Figure 16). Higher percentages of CD8-positive cells were observed for regressor birds than progressor birds at all weeks except two; regressor birds had significantly higher ($p<0.05$) percentages of CD8-bearing PBL than progressor birds at weeks 4 and 5 (Figure 17). No significant differences were observed at the other weeks. Percentages of IA-positive PBL were not significantly different between progressor and regressor 19H birds (Figure 18).

Table 6. Comparison of mean percentages of peripheral blood lymphocyte subsets in uninoculated birds among genotypes[1]

Genotype	n	PBL Subset		
		CD4	CD8	IA
1H	64	$7.3\pm.68^{a2}$	$16.3\pm.84^{a}$	$10.4\pm.57^{b}$
19H	78	$5.0\pm.61^{b}$	$14.4\pm.76^{a}$	$12.9\pm.51^{a}$
1L	64	$4.6\pm.68^{b}$	$11.2\pm.84^{b}$	$8.23\pm.57^{c}$
19L	70	$5.3\pm.64^{b}$	$15.8\pm.80^{a}$	$8.61\pm.54^{c}$

[1]Means are least squares mean $\pm$ standard error of the least squares mean.

[2]Means within a column with different superscripts are significantly different ($p<0.05$).

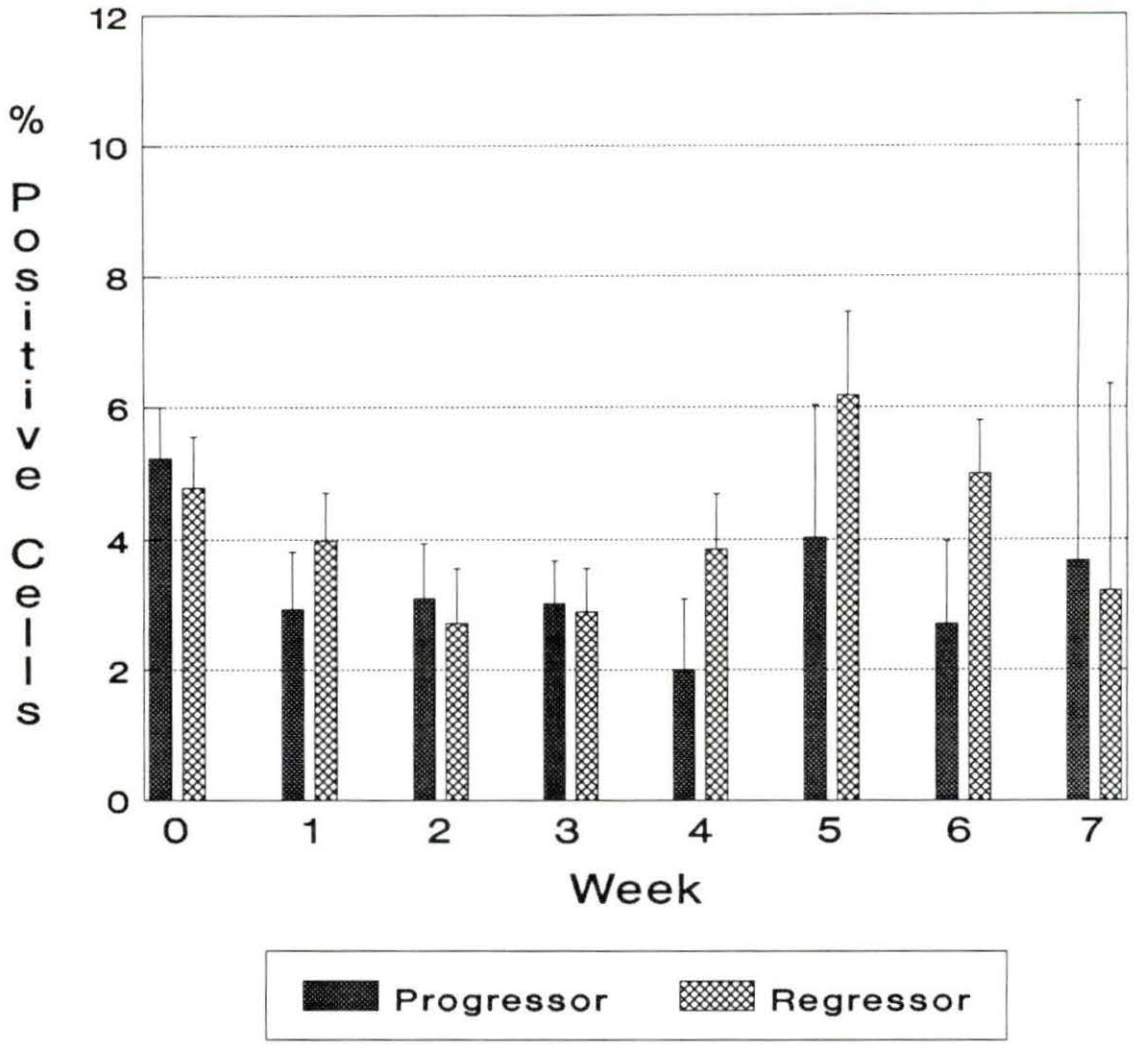
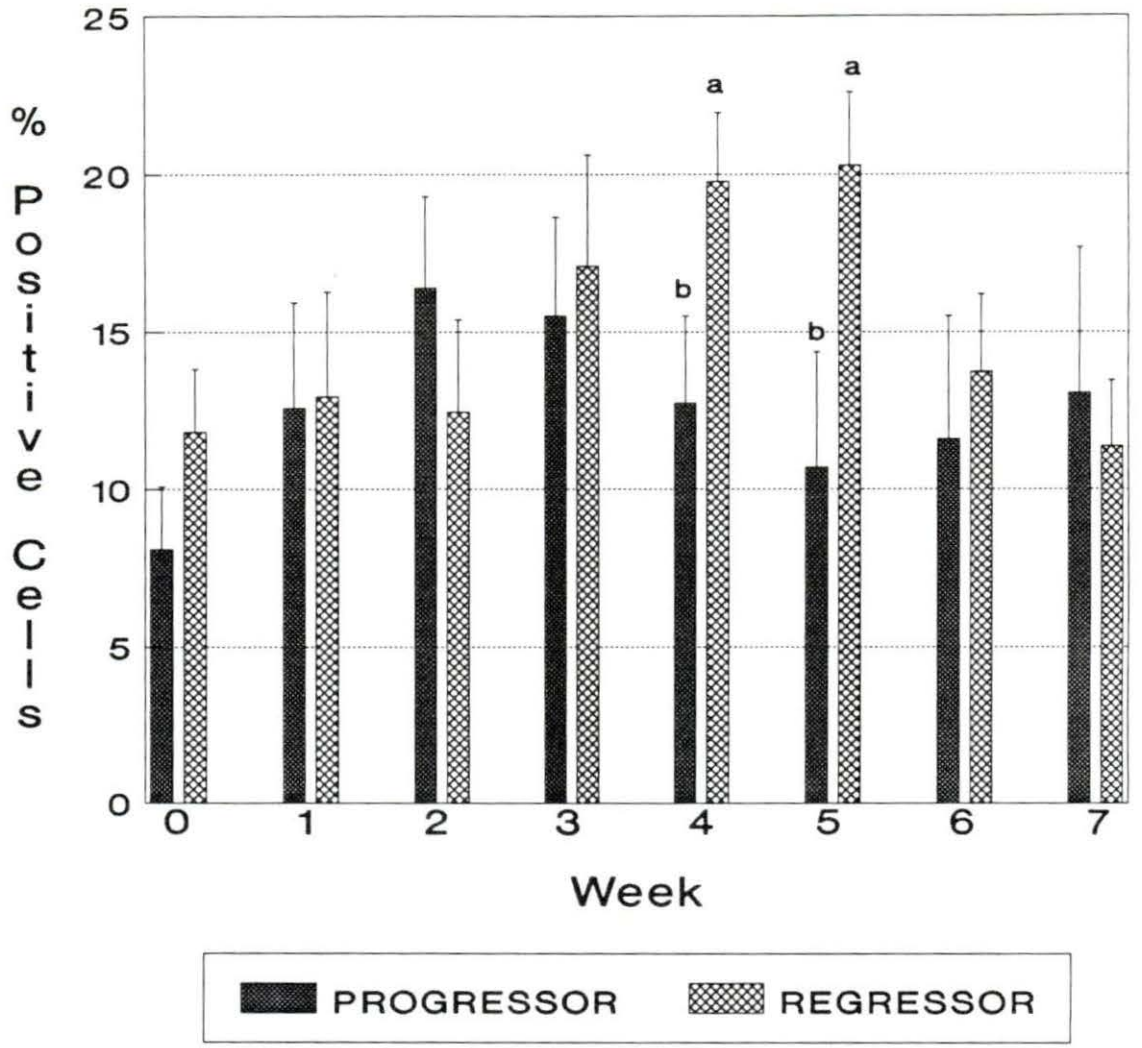


Figure 16. Weekly percentages of CD4-positive peripheral blood lymphocytes in progressor and regressor birds of genotype 19H. The n values for regressor birds were 5 at all weeks, and the progressor birds had n values of 5, 5, 5, 5, 3, 2, 2, and 1 at weeks 0, 1, 2, 3, 4, 5, 6, and 7, respectively



[a,b]Means within a week with different letters are significantly different.

Figure 17. Weekly percentages of CD8-positive peripheral blood lymphocytes in progressor and regressor birds of genotype 19H. The n values for regressor birds were 5 at all weeks, and the progressor birds had n values of 5, 5, 5, 5, 3, 2, 2, and 1 at weeks 0, 1, 2, 3, 4, 5, 6, and 7, respectively

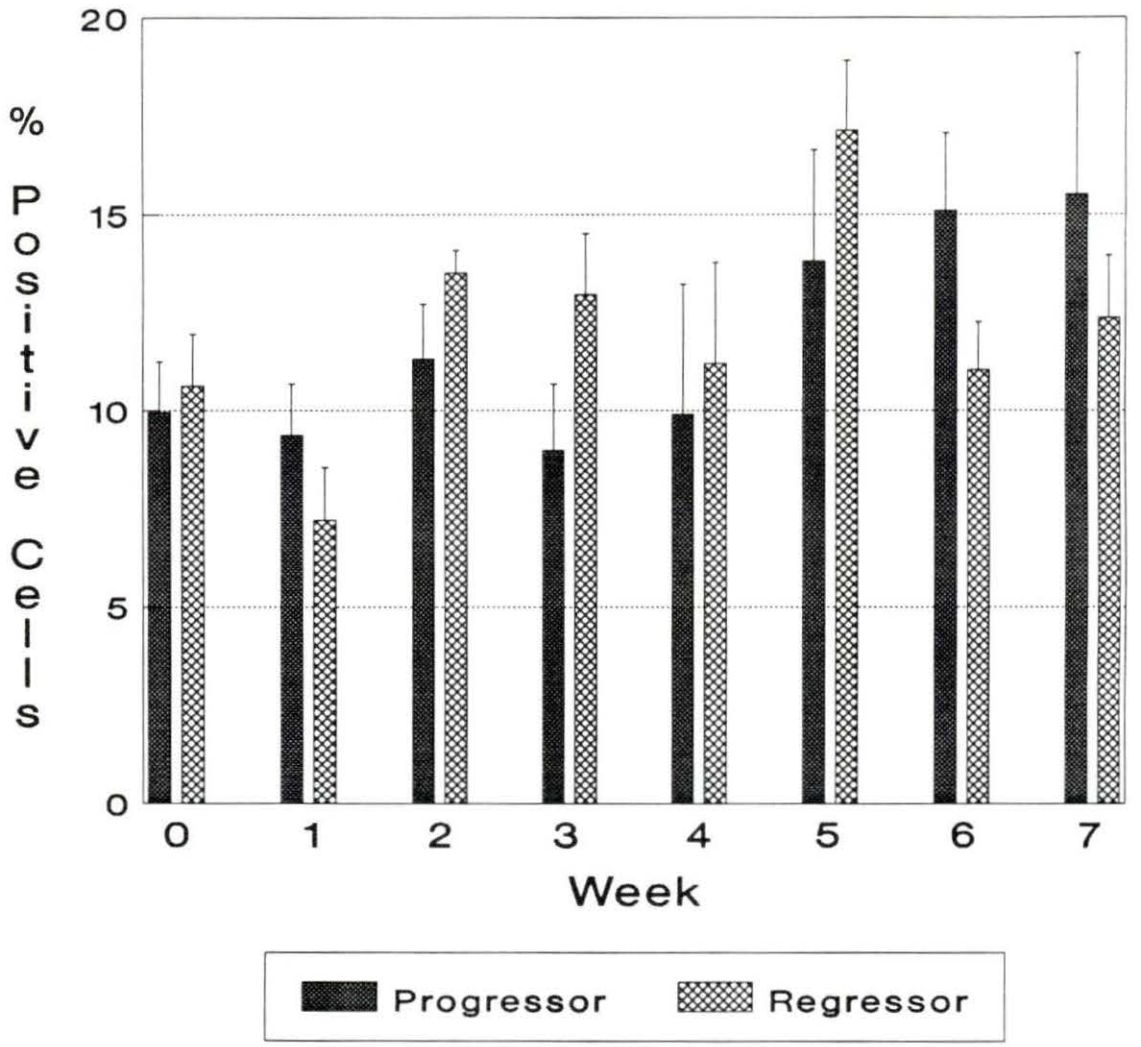


Figure 18. Weekly percentages of IA-positive peripheral blood lymphocytes in progressor and regressor birds of genotype 19H. The n values at weeks 0, 1, 2, 3, 4, 5, 6, and 7 for regressor birds were 5, 5, 4, 5, 5, 5, 5, and 5 and for progressor birds were 5, 5, 5, 4, 3, 2, 2, and 1, respectively

Isolation of Tumor-Infiltrating Lymphocytes

The isolation of tumor infiltrating lymphocytes from RSV-induced tumors was not consistently successful. Too few cells were obtained for analysis from tumors weighing less than 1.5 grams. The few viable cells remaining after Ficoll-Paque separation severely limited the number of cells available; occasionally this step was not done. The collection of the tumor into ice-cold HBSS and performing the collagenase digestion in an ice bath sometimes increased the viability of the cells as determined by the Ficoll-Paque gradient. TIL isolation was not consistently successfully accomplished on enough tumors to give sufficient results for analysis (data not shown).

Immunohistochemistry

An unsuccessful attempt was made to establish a positive control by using a chicken spleen and a MAb (CD3) that binds all T cells. None of the trypsinization or primary antibody incubation time variations gave a positive result. Recent evidence by other researchers has indicated that formalin-fixed, paraffin-embedded tissues are not satisfactory for use in this protocol (K. A. Schat, Cornell University, Ithaca, NY, personal communication).

DISCUSSION

The role of cellular immunity in Rous sarcoma virus-induced tumor response has been established (Hayami *et al.* 1972, Whitfill *et al.* 1986, Yamanouchi *et al.* 1971). However, few distinctions have been found that give insight into the dissimilarities between birds with different tumor response phenotypes. Powell *et al.* (1987) found that tumor cells in regressing RSV-induced tumors expressed B-L (IA-like) antigens, while tumor cells from progressing tumors did not. Perry *et al.* (1978) observed moderate to marked areas of lymphocyte infiltration and lymphocytes that were frequently in contact with tumor cells in regressing Rous sarcomas, whereas progressing tumors had less lymphocyte infiltration and lymphocyte-tumor cell interaction. PBL from chickens regressing RSV-induced tumors were found to be inhibited from migrating by exposure to soluble tumor extract to a greater degree than PBL from chickens with progressing tumors (Cotter *et al.* 1976).

CD4-positive cells (helper T cells), CD8-positive cells (cytotoxic/suppressor T cells) and IA-positive cells (B cells and activated T cells) play important roles in the immune response. Altered ratios of lymphocyte subpopulations have been associated with many diseases (Bach and Bach 1981). The ratio of helper T cells:cytotoxic/suppressor T cells was increased in patients with multiple sclerosis, autoimmune hemolytic anemia, and myasthenia gravis. A decrease in the percentage of helper T cells and a reduced helper T cell:cytotoxic/suppressor T cell ratio are characteristic of acquired immune deficiency syndrome (AIDS) (Fahey *et al.*

1984) and cervical intraepithelial neoplasia (Turner *et al.* 1988); both diseases are associated with viral infections and immunosuppression. Peripheral blood mononuclear cells isolated from patients with non-Hodgkin's lymphoma had a significant reduction in the numbers of B cells and T cells compared to cells from healthy controls (Murray *et al.* 1980). Melendro *et al.* (1983) found that patients with active systemic lupus erythematosus, a connective tissue disease, had lower percentages of T8 cells than patients with the inactive disease, but that patients with the active disease had decreased percentages of T4 cells. Monoclonal antibodies to T lymphocyte subsets in chickens have recently been developed and characterized (Lillehoj *et al.* 1988, Chan *et al.* 1988), enabling the comparison of the different subsets between birds with regressing and progressing RSV-induced sarcomas.

The objective of this study was to gain an increased understanding of the mechanisms of the immune response to RSV. The specific objectives were to determine if PBL from regressor and progressor chickens differ in their expressions of CD4, CD8, and IA antigens during the course of tumor growth by week or tumor score, and to determine if differences in lymphocyte subsets exist among birds of different genotypes.

Inoculated birds from trial 3 had significantly different kinetics of tumor formation, a smaller mean tumor score, and a lower percentage of progressor birds than trials 1 and 2 (Table 3). These differences were caused by a decrease in titer of the virus used in trial 3; the cause of the decrease in virus titer is unknown. The differences were in agreement with other results that found that lower doses of virus result in

relatively higher frequencies of regression (Collins and Zsigray, 1984). Data from the inoculated birds in trial 3 were not included in the analysis because the genetic mechanisms of regression and progression vary at different virus doses.

No differences in PBL subsets were observed between male and female birds at any week, except week 5, or at any tumor score (Tables 4 and 5). One of the few differences between sexes noted in the literature was that female birds had fewer incidences of metastasis than males, which was postulated to be due to a hormonal influence (Collins *et al.* 1986).

Differences in tumor growth kinetics between birds progressing and regressing RSV-induced tumors were shown in Figure 3. Tumor regression generally occurred between weeks 3 and 5. Regressing tumors reached a highest mean tumor score of 3. Differences between regressors and progressors were evident after week 2. These observations were important considerations in the interpretation of the results.

Inoculation of birds with RSV caused a reduction in the percentages of CD4-, CD8-, and IA-positive cells in the peripheral blood compared with uninoculated birds at most weeks (Figures 4, 5, and 6). The extremely high percentage of CD4-positive cells in the uninoculated birds at week 7 was believed to be due to a technical error (mislabelled tube of antigen used) that week for trial 4, because no percentages of CD4-positive PBL that high were observed for any other birds in the other trials at week 7 or any other week. This reduction in percentages of PBL subsets is consistent with reports on human connective tissue diseases that found lower percentages of T4 and T8 PBL in diseased patients than in normal controls

(Melendro *et al.* 1983). The reduction could be due to the migration of cells bearing these antigens to the site of RSV injection and tumor formation. The increase in the percentage of cytotoxic/suppressor T cells in the peripheral blood of inoculated birds compared to control birds between weeks 0 and 1 could be an indication of an early proliferative response in the effector cell populations caused by the virus inoculation.

Higher percentages of CD4-, CD8-, and IA-positive cells averaged over time were found in regressor birds than progressor birds. Regressor birds tended to have higher percentages of PBL positive for CD8 and IA than progressor birds at weeks 2 through 6 (Figures 8 and 9). This occurred during the time that the fate of the tumor was becoming evident, and may indicate that these subsets are more readily available or have a greater inducibility in regressor birds compared to progressors.

Peripheral lymphocyte subsets between birds bearing progressing and regressing tumors at the same tumor stage were compared to account for differences in the kinetics of tumor growth between birds. Although differences were not significant, birds with a regressor tumor phenotype had higher percentages of CD4-, CD8-, and IA-positive cells at almost all tumor scores (Figures 10, 11, and 12). These results may be masked by differences in tumor growth kinetics when data were analyzed by week. The higher percentages of CD8-positive and IA-positive cells in regressor birds than progressor birds at tumor scores of 2, 3, and 4 may contribute to the regression of the tumors in some birds. It should be noted that most tumors from regressor birds reached a score of 3 before regression occurred.

Because the data set used for Figures 10-12 contained data from tumors both increasing and decreasing in size, a data set was generated to compare the ascending stages of tumor growth between regressors and progressors (Figures 13-15). Progressors had higher percentages of CD4-positive cells and CD8-positive cells at some tumor scores. Regressor birds had significantly greater percentages of CD8-positive cells than progressor birds at tumor scores of 2, 3, and 4. Chandler and Yang (1981) found no differences in total T or B cell levels in the peripheral blood of dogs with progressing or regressing canine transmissible venereal sarcoma (CTVS). Higher percentages of tumor infiltrating T cells were found in regressing tumors than progressing tumors. The percentages of T_{μ} lymphocytes, which express the IgMFc receptor and have a suppressive effect on pokeweed mitogen-driven B cell proliferation, and T_{γ} lymphocytes, which express the IgGFc receptor and induce the proliferation of B cells in response to pokeweed mitogen, were analyzed in the peripheral blood of dogs with CTVS (Trail and Yang 1985). Dogs with progressing tumors had significantly higher percentages of T_{γ} lymphocytes in peripheral blood than dogs with early-regressing tumors, which were defined as tumors rapidly decreasing in volume. If IgGFc receptors identify a cytotoxic or suppressor T cell population, these results are in agreement with the data presented here. Regression may be controlled by the ability of a bird to generate large numbers of cytotoxic/suppressor T cells, helper T cells, and possibly B cells or activated T cells, or by the increased availability of these cells in birds capable of regressing RSV-induced tumors.

To avoid the confounding of genotypes with the effects of RSV injection on lymphocyte subsets (demonstrated previously), the data were analyzed for genotypic differences in lymphocyte subsets between uninoculated birds only. Differences among genotypes were found in the uninoculated birds. 1H birds had higher percentages of CD4-positive PBL than the other genotypes and had more IA-positive PBL than 1L and 19L birds. Birds of this genotype were more resistant to Marek's disease (Steadham *et al.* 1987) and fowl cholera (Lamont *et al.* 1987), as well as containing the only regressor family observed in the preliminary screening. The 1H birds were also found to be the only one of the four genotypes that had both a high interleukin-2 activity and a high mitogen response to Con A (Knudtson *et al.* 1990). The higher percentages of subsets containing the helper T cells and B cells, which are both involved in the generation of an antibody response, may be explained by the selection of these birds for high antibody response to GAT. However, as these birds are also tend to be more resistant to both viral and bacterial infections, the availability of these subsets may contribute to general immune responsiveness. Birds of the 1L genotype were found to have a mixed population of progressor and regressor birds in the preliminary screening, but were predominantly of the progressor phenotype in the experimentally inoculated birds. Uninoculated birds of this genotype were found to have lower percentages of all three subsets than the other genotypes. These birds were selected for low antibody response to GAT, and the decreased availability of the various peripheral lymphocyte subsets may lower the general immune responsiveness. Genotype differences in percentages of CD4- and CD8-positive cells were

also found in two congenic inbred lines of chickens by Hála *et al.* (1990). Although they postulated that T cell subset differences might be responsible for the RSV response differences between the lines, they did not examine subsets during the course of an RSV tumor response, as did the current study.

Because genotype influenced tumor response, and differences among genotypes were found for the percentages of PBL subsets, a more powerful analysis of the relationship of PBL subsets with tumor response was conducted by comparing the equal numbers of regressor and progressor birds within the 19H genotype. These birds were of the same genotype, but of different tumor response phenotypes, thus allowing a more valid assessment of the relationship of tumor response phenotype and lymphocyte subsets between regressor and progressor birds. These regressor birds tended to have higher percentages of CD8-positive and IA-positive cells than regressor birds at weeks 3, 4, and 5, during which regression occurred. This gives further evidence for the association of the cytotoxic/suppressor T cells and activated T cells and B cells with tumor regression.

Although many immunological diseases in humans have been evaluated for percentages and/or ratios of various lymphocyte subsets (Bach and Bach 1981), few cancers have been examined for differences in lymphocyte subpopulations. McCluskey *et al.* (1983) found that patients with advanced malignant breast cancer have fewer helper T cells and an increased number of cytotoxic/suppressor T cells in peripheral blood compared to normal controls, patients with benign breast cancer, or malignant breast cancer in the early stages. Abnormalities in circulating lymphocyte subsets were

found in patients with untreated lung cancer (Ginns *et al*. 1982). Patients with adenocarcinoma had a decreased percentage of cytotoxic/suppressor T cells in the peripheral blood. Bernengo *et al*. (1983) found reduced percentages of helper T cells in melanoma patients with metastases compared to normal controls; cytotoxic/suppressor T cells were significantly reduced in long-surviving, non-metastatic, and metastatic melanoma patients compared to controls. A decrease in helper T cells was consistent between the different tumors, but the differences in percentages of cytotoxic/suppressor T cells were contradictory. Reduced numbers of CD4-positive cells could either shift the ratio of helper:cytotoxic/suppressor T cells toward suppression, or, through the decrease in lymphoproliferative signals mediated by lymphokines, such as IL-2, released by helper T cells. Increased numbers of CD8-positive cells could also shift the immune response toward suppression, while a decrease may be due more to a reduction in the numbers of cytotoxic T cells.

A major disadvantage in human cancer research is the difficulty of obtaining patients bearing tumors at the same stage of growth. The experimental model described in this thesis has many advantages for cancer research. One advantage is that the kinetics of tumor growth can be standardized by evaluating tumors at different stages of growth and to some extent by the dose of the virus. Because the exact time of exposure to the cancer-inducing agent is known, data can be collected in the early stages of cancer growth, before the tumor is detectable. This system is a model for the comparison of factors involved in the spontaneous regression versus progression of tumors.

This study found increased percentages of helper T cells, cytotoxic/suppressor T cells, and IA-positive lymphocytes in regressor birds compared to progressor birds. The greater availability of these subsets in birds able to regress the tumor compared to birds that progress the tumor may influence the tumor response. The presence of significant differences in lymphocyte subsets among the uninoculated birds of different genotypes, combined with the association of genotype and tumor response, also support this conclusion.

Further elucidation of the roles of lymphocyte subsets in tumor regression could be obtained in future studies by separating the functional subsets in the cytotoxic/suppressor T cell population. The role of the B cells could also be determined using anti-immunoglobulin antibody. These subsets could be analyzed within the tumor tissue as well as the peripheral blood, if the tumor-infiltrating lymphocytes could be isolated consistently and without loss of specific subsets, and immunohistochemistry was accomplished on frozen sections. The evaluation of tumor infiltrating lymphocyte subsets between progressing and regressing RSV-induced tumors may allow greater understanding of the mechanisms of regression.

SUMMARY

Rous sarcoma virus (RSV) causes a sarcoma in chickens. The regression or progression of the tumor is dependent upon both major histocompatibility complex (MHC)-linked genes and non-MHC-linked genes (Collins and Zsigray 1984). Although the role of the cellular immune system has been established to be important in the regression of RSV-induced tumors, few differences between regressor and progressor birds have been described in the literature. The objective of this study was to gain an increased understanding of the mechanisms of the immune response to RSV.

The specific objectives were to determine if PBL from regressor and progressor chickens differ in their expressions of CD4 (helper T cell antigen), CD8 (cytotoxic/suppressor T cell antigen), and IA (MHC class II antigen) during the course of tumor growth by week or tumor score, and to determine if differences in lymphocyte subsets exist among birds of different genotypes.

Increased percentages of helper T cells, cytotoxic/suppressor T cells, and IA-positive lymphocytes were found in regressor birds compared to progressor birds. The greater availability of these subsets in birds able to regress the tumor compared with birds that progress the tumor may influence the tumor response. Significant differences in lymphocyte subsets among the uninoculated birds of different genotypes, and differences in progressor and regressor birds within a genotype supported this conclusion.

This study describes an experimental model for cancer research that has many advantages. The kinetics of tumor growth can be controlled, and data can be collected in the early stages following tumor induction.

Future studies could further elucidate the roles of the different lymphocyte subpopulations in tumor regression. The cytotoxic/suppressor T cell population should be separated and the individual functional roles evaluated. Tumor infiltrating lymphocyte subsets in regressing birds should be analyzed and compared to allow greater understanding of the mechanisms of regression.

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