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CROSS-REACTIVITY BETWEEN MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGEN AND ROUS SARCOMA VIRUS-INDUCED TUMOR ANTIGEN IN CHICKENS

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TABLE OF CONTENTS

LIST OF TABLES

ABSTRACT

CROSS-REACTIVITY BETWEEN MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGEN AND ROUS SARCOMA VIRUS-INDUCED TUMOR ANTIGEN IN CHICKENS

by

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University of New Hampshire, December, 1980

Cross-reactivity between major histocompatibility complex (MHC) antigen and Rous sarcoma virus (RSV)-induced tumor associated antigen (TAA) in chickens was investigated. The B erythrocyte alloantigen locus was the genetic marker for the MHC. B2B2 and B5B5 chickens from crosses of highly inbred lines 6-1 and 15-1 and B24B24 chickens from noninbred line UNH 105 were used.

B2B2 chickens rendered partially tolerant to B5 antigen through multiple intraperitoneal inoculations of either viable or lysed white blood cells (WBC) or viable red blood cells (RBC) from B5B5 chickens had a significantly higher incidence of tumor progression than untreated, phosphate buffered saline treated, or B2B2 WBC inoculated B2B2 chickens. The criteria of tolerance were absence of antibody to the cell type inoculated and acceptance of skin allografts from B5B5 donors by B2B2 chickens. The higher incidence of tumor progression did not result from a

non-specific effect of graft versus host activity (GVH) because GVH activity was present only in chickens inoculated with B5B5 viable WBC. B5 antigen-TAA cross-reactivity was indicated because B2B2 chickens partially tolerant to B5 antigen were also tolerant to RSV-induced tumor, as shown by an increased incidence of tumor progression. Since WBC and REC share B-F antigens, and both were involved in producing the partial tolerance, the B-F region of the MHC was implicated in the cross-reactivity.

Cross-reactivity was also observed when lymphocytes from B2B2 chickens bearing RSV-induced tumors lysed *in vitro* targets of B2B2 and B5B5 RSV-infected chicken embryo fibroblasts (CEF) and B5B5 normal CEF, but did not lyse B2B2 and B24B24 normal CEF. Lymphocytes from normal B2B2 chickens did not lyse any of the five types of CEF targets. Cross-reactivity was observed yet again when absorption with B2B2 RSV-infected CEF significantly lowered the titer of B2B2 anti-B5B5 alloantisera. Alloantisera absorption studies also showed that both RSV-infected and uninfected CEF shared alloantigens, in particular B-F allcantigens, with syngeneic RBC's further supporting the idea that B-F antigen is involved in the cross-reactivity.

Cross-reactivity was not observed in the IgG from chickens bearing RSV-induced tumors. Moreover, use of three techniques of immunization of B2B2 chickens with B5B5 cells did not increase the incidence of tumor regression in the immunized chickens, and provided no evidence of

cross-reactivity. Although humoral immunity was observed in these two studies, cell-mediated immunity may be relatively more important in tumor regression and the focus of cross-reactivity.

Based upon these findings it is hypothesized that B5B5 RSV-induced tumor bearing hosts respond poorly to tumor partially because B5 antigen cross-reacts with TAA. B5B5 individuals, therefore, may have difficulty recognizing RSV-induced tumor as foreign and this severely limits the development of an effective anti-tumor immunity. Either TAA or a TAA-B2 antigen complex may cross-react with B5 antigen. Cross-reactivity involving cell-mediated immunity may make the difference between tumor regression and progression.

INTRODUCTION

The major histocompatibility complex (MHC) is a tightly linked group of genes which in general code for cell surface antigens (Klein 1975). When the MHC was defined in the mouse, first as a blood group locus and later as a locus controlling tissue graft rejection (Gorer et al. 1948) the MHC's role in immunology was not yet imagined. With the discovery of the profound controlling effect that MHC genes have on immune responses the MHC took a much larger role in immunological thought (Benacerraf and Katz 1975). Further light was shed on MHC function when a series of experiments starting with Zinkernagel and Doherty (1974) showed that cytotoxic T cells lysed only target cells which bore the same foreign antigen and MHC antigen as the immunizing cells. This phenomenon was termed MHC restriction. Furthermore, it was found that immune cell cooperation required MHC homology (Benacerraf and Katz 1975). It has recently been suggested that MHC restriction is at the heart of all major MHC functions including those of immune response genes (Wettstein and Frelinger 1980).

In humans MHC haplotypes have been associated with several disease states including acute leukemia, Hodgkins disease, and trophoblastic tumors (Bodmer 1978). Immunity to tumors has been shown in many cases to be mediated by

tumor-associated antigen which elicits and acts as a target for, an immune response which leads to tumor destruction (Each 1974). In chickens all members of the Avian tumor virus group induce tumors which share common antigenic determinants (Hall et al. 1979). Furthermore, the MHC has a major influence on the fate of Rous sarcoma virus (RSV)-induced tumors in chickens (Collins et al. 1977, Schierman et al. 1977). Therefore, the chicken, through the use of RSV-induced tumors, provides an excellent model system for investigating how MHC genetics and tumor antigens interact to contribute to the immune response toward tumors.

Different types of experimental tumors in mice express antigens which cross-react with allogeneic MHC antigens and these determinants are suitable targets for in vivo anti-tumor immunity (Parmiani et al. 1979). Since immune responses may be modulated by histocompatibility antigen-foreign antigen interactions on the cell surface and since some tumors exhibit allogeneic MHC antigens, certain tumors may survive in hosts with particular MHC haplotypes.

In this thesis I have endeavored to study, in a well defined genetic system, how cell surface antigens may interact to produce anti-tumor immune reactions. Based upon this research I hypothesize that particular MHC defined chickens which normally have progressively growing RSV-induced tumors until death, do so in part because host MHC antigen cross-reacts with tumor associated antigen. As a result of this cross-reactivity recognition of the tumor

as foreign by the host is severely limited and a weak immune response is elicited toward the tumor. My objective was to search for cross-reactivity and investigate antigen phenomena using three general approaches: a) to generate tolerance in chickens toward MHC antigen from chickens genetically predisposed to tumor progression and investigate the response of tolerant chickens to RSV-induced tumors; b) to detect cross-reactivity through the use of antisera and cytotoxic lymphocytes; c) to immunize animals with MHC antigen from chickens genetically predisposed toward tumor progression and investigate the response of the immune chickens to RSV-induced tumors.

Cross-reactivity of the type referred to here may be the reason tumors survive in many hosts. If the mechanism can be understood the extent of the phenomenon may be evaluated and a therapeutic approach developed.

CHAPTER I

REVIEW OF THE LITERATURE

ROUS SARCOMA VIRUS (RSV)

Rous (1911) first isolated an avian sarcoma virus from chickens and demonstrated viral etiology for neoplastic disease. Rous sarcoma virus (RSV) was designated a type C RNA tumor virus (Bernhard 1960) and found to contain at least three genes coding for components of the virion (Hanafusa 1975). The *gag* gene coded for four nonglycosylated internal structural proteins, *pol* coded for RNA-dependent DNA polymerase, and *env* was the gene for the envelope glycoproteins (Vogt and Hu 1977). A fourth gene, *src*, which coded for a phosphoprotein with protein kinase activity, was required for the initiation and maintenance of oncogenic transformation (Brugge and Erikson 1977).

Bryan and Harris strains of RSV were found to be defective in the synthesis of glycoprotein gp85 and were dependent on helper virus for the formation of infectious virus (Scheele and Hanafusa 1971). Schmidt-Ruppin, Carr-Zilker, and Prague strains of RSV were helper independent (Hanafusa 1975).

RSV along with lymphoid leukosis virus have been classified into subgroups based on several criteria: a) ability to infect chicken embryo cells bearing specific resistance factors for the various subgroups; b) ability to specificially inhibit secondary infection of infected cells by a second member of the same subgroup, but not by viruses of other subgroups; c) susceptibility to neutralization by antiviral serum against any member of that subgroup, but not by antiserum against members of other subgroups (Wainberg and Phillips 1976). In addition to envelope subgroup specific antigen, RSV was found to contain group specific antigens generally thought to be an internal component of all avian leukosis virus and not involved in virus neutralization (Wainberg and Phillips 1976).

HOST RESISTANCE TO RSV INFECTION

Inherited resistance to infection with specific subgroups of RSV was referred to as a first line of host defense and this resistance appeared to be effective whether the chickens were exposed to high or low doses of virus (Crittenden *et al.* 1967, Payne and Biggs 1970). The resistance was a block to the early stages of virus infection (Piraino 1967, Crittenden 1968). Other host genes may affect the efficiency of later steps in the viral replication cycle or the efficiency of transformation by the virus (Crittenden *et al.* 1972). Evidence that the host

cell may affect the nature of the viral progeny has been given by Hanafusa et al. (1970).

IMMUNITY TO RSV-INDUCED TUMORS

Crittenden et al. (1972) referred to genetic resistance as a second line of defense. Infection was required to initiate this defense mechanism and resistance at this level was characteristic of the intact host. Host age at the time of RSV inoculation affected the regression of Rous sarcomas in chickens, with younger individuals having lower frequencies of regression (Duran-Reynals et al. 1953, Cotter et al. 1973). Strength of RSV inoculum also affects tumor regression (Gyles et al. 1967, Radzichovskaja et al. 1968).

Tumor Associated Surface Antigens

Tumor associated antigen (TAA) has been presumed both to elicit, and to act as a target for, an immune response which leads to tumor destruction (Bach 1974). The second line of defense referred to above may involve the immune response toward transformation antigens on Rous sarcoma cells. Up to four different kinds of antigens were reported to be expressed on RSV-transformed cells (Hayni et al. 1977, Bauer et al. 1977a, Bauer et al. 1977b, Ignatovic et al. 1978): a) a subgroup-specific determinant of the virus-envelope glycoprotein gp85 expressed on the surface of

productively infected, transformed as well as untransformed cells; b) a group-specific determinant of gp85 expressed only on the surface of virus transformed cells; c) embryonic antigens, detectable on virus and chemically transformed and on primary normal embryonic cells and; d) a RSV-induced tumor specific surface antigen, not a structural constituent of the virus, and induced *in vivo* by all members of the Avian tumor virus group.

Mechanisms of Immunity to RSV-Induced Tumors

Hall *et al.* (1979) have postulated that determinants of the virus envelope antigen were more important than TSSA in provoking relevant immune responses in chickens with RSV-induced tumors; TSSA may be of greater importance when in conjunction with these VEA determinants.

Chickens bearing avian virus-induced tumors exhibited cell mediated immune responsiveness toward antigens associated with those neoplasms (Wainberg *et al.* 1979). Thymectomy, but not bursectomy, was stimulatory to tumor growth and abolished sensitized lymphocyte immune responsiveness suggesting that thymus mediated effector mechanisms and not antibody was involved in the immune response to RSV-induced tumors (Cotter *et al.* 1975, Wainberg *et al.* 1979). Leukocyte migration inhibition studies (Cotter *et al.* 1976) and lymphocytotoxicity studies (McGrail *et al.* 1978) in chickens bearing regressing Rous sarcomas suggested that the thymus dependent cells become

sensitized tc and subsecuently effect the destruction of tumor cells. Hematclcgical studies suggested that lymphocytes may have a primary role in the immune respcnse tcward RSV-induced tumors with heterophils acting in a secondary manner (Smith et al. 1974). Serum blocking and unblocking factors in cell mediated immunity may ccntribcte to tumor develcpment (McGrail 1977)

Genetics of Immunity tc RSV-Induced Tumors

A general heritable nature of regression of Rous sarcomas has been shown by Greenwood et al. (1948), Gyles et al. (1967), and Cotter et al. (1973). Furthermore, the incidence of tumor regression can be significantly modified by selection (Gyles et al. 1971, Carte et al. 1972).

A more definitive analysis of the genetics of RSV-induced tumcr regression came with the studies of Ccllins et al. (1977) and Schierman et al. (1977) who associated regression with the E locus, a marker for the major histoccmpatibility complex (MHC) of the chicken. Ccllins et al. (1977) showed that in F2 generation segregants of a cross of inbred lines 6-1 and 15-1 (B2B2, B2B5, and B5B5), 5, 26, and 93 percent, respectively, died of RSV-induced tumors by 70 days post-virus inccculatien. Neither the E ncr I alloantigen loci ncr sex had any effect cn tumor grcwth. The F2 generatien studies invclved ten sires, approximately 85 dams, and some 690 prcgeny in seven hatches. Schierman et al. (1977) studied RSV-induced tumor

regression in inbred lines G-B1 and G-B2 and crosses of these lines. Their experiments involving 90 progeny from a single sire indicated that RSV-induced tumor regression was controlled by a dominant gene for regression linked to the MHC, designated R-Rs-1, with the allele for progressive tumor growth in homozygous chickens designated r-Rs-1. The difference between the results of Collins et al. (1977) and Schierman et al. (1977), a quantitative response versus a dominant gene effect, respectively, has not been explained but may be due to the use of genetically different chickens, the greater number of individuals used by Collins et al. (1977) and/or to Collins et al. (1977) using Bryan high titer RSV (subgroup A) and Schierman et al. (1977) using Schmidt-Ruppin RSV (subgroup B). McBride et al. (1980) found that different RSV strains, of identical subgroup specificities, gave significantly different tumor growth patterns in the same inbred line of chickens. This result may be due to the existence of fundamentally different tumor antigens from those previously described and indicates a profound influence of virus strain on the fate of RSV-induced tumors. Chickens with different MHC genotypes may respond differently to certain virus-induced tumor antigens.

Recent studies have also indicated that the B complex has a profound effect on RSV-induced tumor regression even in noninbred chickens (Collins et al. 1979). Line UNH 105 was a noninbred line of New Hampshires recently derived from

a commercial line. The lowest level of regression was associated with the B24 allele, the highest with B26, and B23 gave an intermediate response. More recent studies have shown that the B23B26 genotype gave a significantly higher incidence of RSV-induced tumor regression than B23B23 and B26B26 as well as the other three possible genotypes. This provides evidence of heterosis or genetic complementation of the genes controlling tumor regression (Brown et al. unpublished).

Genes not linked to the MHC may be involved in RSV-induced tumor regression. Crosses of lines 6-3 and 100 gave progeny segregating at the C, D, E, I, and L allocantigen loci (Collins unpublished). L genotype was significantly associated with the incidence of tumor regression in females, but not in males. Loci C, D, E, and I had no detectable influence on the incidence of tumor regression. The L locus was not linked to B (Briles 1964). Marks et al. (1979) using lines 6-1, 6-3, and 7-2 and F1, F2, and reciprocal backcross progenies of these lines, indicated that a locus (or loci) other than B or L has a role in RSV-induced tumor regression. Alternatively, they suggested that the immune response region of the MHC differs in lines 6 and 7 even though the serological and/or graft vs. host regions have not been shown to differ. Lines 6-1, 6-3, and 7-2 were homozygous for serologically defined shared blood group alleles B2, C5, L1, and r. The findings of Marks et al. (1979) were supported by those of Collins

et al. (1980) where lines 100, 7-2, 6-3, and 6-1, all homozygous B2, differed significantly in the incidence of RSV-induced tumor regression with 6.5, 45.5, 68.9, and 91.8 percent, respectively, regressed tumors.

Three recent studies shed additional light on the genetics of tumor regression. Collins and Briles (1980) utilized B complex recombinants and have evidence that the B-F region of the MHC was involved in RSV-induced tumor regression. Watanabe et al. (1980) have shown that the F1 cross of inbred lines G-B1 and G-B3, each of which normally has progressive tumors, now regresses tumors. Backcross data indicated that the B complex and a gene not linked to B were responsible for the gene complementation. Finally, Collins and Gilmour (unpublished) have shown that Th-1 and Ly-4 genotypes affected tumor regression in the F4 generation of line 6-3 crossed to line 7-2. Th-1 and Ly-4 are not linked to each other or to the MHC.

THE MAJOR HISTOCOMPATIBILITY COMPLEX

The major histocompatibility complex (MHC) has been found in all mammals studied and in the chicken but is best characterized in the mouse (Paul and Benacerraf 1977).

Mouse

The MHC has been found to be a group of tightly linked genes first discovered in the mouse as a blood group locus and a factor in skin graft survival and designated H-2 (Gorer et al. 1948). The H-2 complex codes for cell surface antigens which differ between individuals and are recognized in graft rejection (Klein 1979). The complex is also involved in a variety of immunological phenomena. The MHC is located in the middle of chromosome 17, sharing the chromosome with the T, t group of loci which may be a functional unit similar to H-2 but involved in embryonic differentiation (Klein 1979). The current H-2 map has been divided into six regions (K, I, S, G, D, and T) with the I region divided into five subregions (A, E, J, F, and C) (Klein 1979). A non-conservative map includes at least 18 loci in the regions with a distance of 1.5 cM between the extremes of the complex (Klein 1979).

H-2 loci have been grouped into three classes designated arbitrarily by Roman numerals I through III. Class I loci (H-2K and H-2D) code for membrane bound glycoproteins 44,000 mw which are noncovalently associated in the membrane with a 12,000 mw polypeptide designated β2-microglobulin (Cunningham 1977). Class I molecules are the H-2 molecules most involved in graft rejection and can be found on most tissues of the body (Klein 1979).

Class II genes, located in the I region, are of two types, I region associated (Ia) and immune response (Ir) genes (Klein 1979). Two Ia genes have been well defined, one in the A subregion and the other in the E/C subregions (Cullen et al. 1976). Ia genes code for membrane bound glycoproteins consisting of two noncovalently associated polypeptide chains α (35,000 mw) and β (28,000 mw) and an unknown number of carbohydrate chains (Cullen et al 1976). Ia antigens have been detected primarily on T and B lymphocytes and macrophages (Delovitch and McDevitt 1975, Press et al. 1976, Schwartz et al. 1977) and have been shown to be important for the successful collaboration among immunocompetent cells (Pierce et al. 1976, Uhr et al. 1979). Ia antigens may be the products of Ir genes (Uhr et al 1979).

H-2 linked Ir genes (mapped to the A, E, C, or E subregions) and immune suppressor (Is) genes (mapped to the J subregion) were first characterized as dominant genes expressed on immunocompetent T and B cells leading to high or low immune responses to various classes of antigen ranging from synthetic polypeptides to foreign antigens (Benacerraf and Katz 1975). Of particular interest was that susceptibility to Gross leukemia virus oncogenesis was influenced by an H-2 linked gene (Rgv-1) (Lilly 1966, Tennent and Snell 1968). For certain antigens two complementing MHC linked Ir genes were required for responsiveness (Benacerraf and Dorf 1976). I-region linked

complementing loci may be involved in resistance to murine leukemia (Lonai et al. 1980). The α and β genes of Ia antigens may be the complementing genes and a gene dose effect may be responsible for differential immune responses in certain cases (Dorf et al. 1979). Most studies of Ir genes have shown dominant inheritance of high responsiveness; however, in the H-2 controlled immune response to TNP-MSA (Rathbun and Hildeman 1969) and to mouse liver F antigen (Silver and Lane 1977) high responsiveness could be inherited as a recessive trait. Wicker et al. (1980) have shown that the dominance-recessive pattern of Ir genes can change as the genetic background of the H-2 haplotype varies. Berzofsky et al. (1979) have shown that genes mapping in different I subregions independently regulated antibody and T cell proliferative responses to chemically discrete regions of the same protein antigen, Sperm Whale myoglobin.

Class III genes, located in the S region, have been found to code for serum proteins Ss and Slp, both 200,000 mw and consisting of three covalently linked polypeptide chains (Shreffler 1976). These molecules were found to be the C4 component of the classical complement pathway. Moreover, DaSilva et al. (1978) have associated the C3 component of the classical complement pathway with the H-2 complex.

The G region codes for the appearance of an antigen on erythrocytes (Klein 1979).

It is interesting to note that the H-2 complex genes exhibit a very high degree of genetic polymorphism compared to other mouse genes (Klein 1979). The relationship of the polymorphism to function is unknown. Also, certain H-2 haplotypes have been found to be more frequently associated with other genes on chromosome 17 than would be expected on the basis of gene frequencies and the frequency of recombination between genes; this has been referred to as linkage disequilibrum (Hammerberg and Klein 1975).

Human

The MHC in man (HLA complex) has been found to be very similar to that of the mouse (Albert and Gotze 1977). Class I genes are in the A, B, and C regions, class II genes in the D and Dr regions and class III genes map between the B and D regions. Strong linkage disequilibrium has been observed within the HLA complex (Albert and Gotze 1977). HLA has been associated with many disease states in man including, rheumatoid arthritis, multiple sclerosis and acute leukaemia (Bodmer 1978). HLA is most closely associated with the disease ankylosing spondylitis where 90 percent of the diseased individuals carry the B27 HLA allele compared to seven percent of healthy individuals (Ivanyi 1980).

Chicken

The structure of the MHC (B complex) in the chicken has not been as well established as that of the mouse or man. The B blood group system was discovered by Briles et al. (1950) and shown to be a marker for the MHC by Schierman and Nordskog (1961). The B complex has been located on one of the medium sized microchromosomes (15-18) (Bloom and Cole 1978) and there has been evidence for recombination of genes within the chicken MHC (Schierman and McBride 1969, Hala et al. 1976, Briles and Briles 1977, Hala et al. 1977, Pink et al. 1977, Schierman et al. 1977, and Pevzner et al. 1978).

Hala et al. (1977) proposed that the B complex be divided into at least three regions; B-F, B-I, and B-G. The B-F and B-I regions coded for surface antigens on white blood cells (WBC) and the B-F and B-G regions for surface antigens on red blood cells (RBC). The B-F gene products were associated with B2-microglobulin--like molecules making the B-F antigens similar to K and D molecules in mice (Ziegler and Pink 1978). B-F antigens on RBC were 20% as numerous as those found on WBC (Ziegler and Pink 1978). B-L region antigens appeared to be identical to Ia antigens in the mouse (Ziegler and Pink 1976, Hala et al. 1977, Ewert and Cooper 1978, and Ewert et al. 1980). B-F and B-L region antigens were involved in histocompatibility reactions, but the B-G region appeared to have no effect on these reactions (Hala et al. 1976, Hala et al. 1977, Pink

et al. 1977, and Simonsen et al. 1977). Immunological tolerance to WBC surface antigens, but not REC surface antigens, mediated allogeneic skin graft acceptance (Billingham et al. 1956, Schierman and Nordskog 1964).

Serum hemolytic complement level has been found to be controlled by a dominant gene associated with the B complex (Chanh et al. 1976).

Pevzner et al. (1978) showed recombination between genes coding for immune response and the serologically defined regions of the B complex. Antibody response of chickens to (T,G)-A-L (Gunther et al. 1974), GAT10 and GA (Benedict et al. 1975) and DNP (Balcarova et al. 1974) was associated with certain B haplotypes, suggesting possible close linkage with immune response genes. Control of immune responsiveness to Salmonella pullorum, (Pevzner et al. 1978), spontaneous autoimmune thyroiditis (Bacon et al. 1974, Wick et al. 1974), and erythroblastosis (Bacon et al. 1979) was linked to the MHC of the chicken. Susceptibility to Marek's disease, which has a Herpes virus as an etiological agent, was linked to the MHC (Hanson et al. 1967, Brewer et al. 1969, Pazderka et al. 1975). The B complex has a profound effect on the fate of RSV-induced tumors (discussed previously) (Collins et al. 1977, Schierman et al. 1977). Gebriel et al. (1979) has shown genetic linkage between the immune response to GAT and the fate of RSV-induced tumors in a particular subpopulation of S1 inbred Leghorns.

Recently, Simonsen et al. (1980) suggested that linkage disequilibrium of B-F and B-G genes in outbred chicken populations may be relatively stronger than linkage disequilibrium in man.

MHC RESTRICTION

Studies in the mouse have given the strongest clue to the function of the MHC; that of H-2 restriction or associative recognition of antigens (Klein 1979). Cytotoxic T cells primed to virus-infected cells (Zinkernagel and Doherty 1974), hapten-modified cells (Shearer et al. 1975) or minor histocompatibility antigens (Bevan 1975) were capable of lysing only target cells which bore the same foreign antigen and MHC antigen as the immunizing cells. The proliferative response of T cells to antigen presented on the macrophage surface required histocompatibility at the H-2I region of the two cell types (Shevach and Rosenthal 1973). Also, cooperation between helper T cells and B cells exhibited H-2 restriction (Katz and Benacerraf 1975). In a series of experiments Zinkernagel et al. (1977, 1978) demonstrated that the thymus epithelium dictated the H-2 context of antigen recognition by lymphocytes. In the chicken, cytotoxic splenocytes primed against RSV-induced tumors showed significantly greater killing of autochthonous RSV-induced tumor cell targets than of allogeneic RSV-induced tumor cell targets (Wainberg et al. 1974).

Teivanen and Teivanen (1977) have shown that an interaction of histocompatible or semi-allogeneic B and T cells was necessary for the formation of germinal centers in chickens.

To explain H-2 restriction two general types of hypotheses have been proposed; dual recognition and altered self hypotheses (Doherty 1976). The dual recognition hypothesis states that T cells exhibit two separate receptors on their surfaces, one with specificity for H-2 antigen and the other with specificity for non--H-2 antigens. The altered self hypothesis suggests that T cells bear a single receptor capable of recognizing antigens in association with H-2. Hale (1980) showed that a close spatial relationship exists between a serologically defined portion of the G protein of Vesicular stomatitis virus (VSV), the H-2K(k) molecules and those antigens (virus and cell specific) recognized by anti-VSV cytotoxic T lymphocytes, suggesting that MHC antigens and foreign antigens closely associate on the cell surface.

It has been postulated that Ia molecules, through the phenomenon of associative recognition, regulate the interaction of T cells with macrophages and B cells and lead to Ir gene responses (Benacerraf and Germain 1978). If I and H-2K/D antigens perform similar functions (i.e. associative recognition) then Ir genes which regulate specifically the ability of cells to present antigens to antigen-specific T cells should map to both I and K/D regions (Wettstein and Frelinger 1980). Wettstein and

Frelinger (1980) showed that H-2K/D region gene specifically regulate the immunogenicity of a panel of non--H-2 histocompatibility antigens. This study suggested that the MHC linked immune response phenomenon was due to an associative recognition, perhaps, because different foreign antigens having variable affinities for particular MHC antigens.

ALIEN HISTOCOMPATIBILITY ANTIGENS ON TUMOR CELLS

Transplantation Studies

Immunity to DBA/2 (H-2(d)) or C3Hf (H-2(k)), but not to AKR (H-2(k)) tissue induced a resistance against the challenge of the syngeneic ST2 sarcoma in BALB/c (H-2(d)) mice (Invernizzi and Parmiani 1975, Parmiani and Invernizzi 1975, Invernizzi et al. 1977b). The immunity was resistant to 400R and could be passively transferred by lymphoid cells to syngeneic mice. Martin et al. (1976, 1977) found that immunization of C3Hf, but not (C3Hf X A) F1 mice with normal B10.A or A tissues induced transplantation immunity to subsequent challenges of C3Hf lung tumors expressing H-2(a)-like determinants. The extra-H-2 antigens on the tumors were mapped into the K region of H-2(a) which seems to be different than the K region of C3Hf mice (Gipson et al. 1978, Martin et al. 1976). The transplantation studies indicated that different types of experimental tumors may express antigens cross-reacting with allogeneic

H-2 antigens and that these determinants were suitable targets for an efficient *in vivo* anti-tumor immunity (Parmiani *et al.* 1979).

Serological Studies

Using the C-1 fibrosarcoma of BALB/c strain (H-2(d)) Meschini *et al.* (1977), Invernizzi *et al.* (1977a), and Carbone *et al.* (1978) showed with antisera absorption studies that the tumor contained the H-2(k) alien antigen. Operationally nonospecific anti--H-2 allocantiserum contained complement-dependent cytotoxic activity to apparently H-2 unrelated murine leukemia targets (Garrido *et al.* 1976, 1977). Roman and Bonivida (1979) showed that SJL (H-2(s)) reticulum cell sarcomas expressed extra--H-2(d) and H-2(b) antigens detectable by complement dependent cytotoxicity of anti--H-2 allocantisera on tumor cells; this activity could be absorbed by normal H-2(d) and H-2(b) cells. Pellegrino *et al.* (1976) showed the presence of extra HLA antigens on SV40 transformed human fibroblasts.

Cytotoxic Lymphocyte Studies

Parmiani *et al.* (1979) showed that cell mediated lympholysis defined determinants cross-reacting with H-2(k) existed on BALB/c sarcoma C-1, although they were less immunogenic than the H-2(d) original determinants. Normal SJL (H-2(s)) lymphocytes sensitized *in vitro* to syngeneic reticulum cell sarcomas generated effectors capable of

lysing not only syngeneic SJL tumors but also H-2(d) or H-2(b) allogeneic lymphoblasts induced by concanavalin A stimulation of neoplastic cells (Roman and Bonavida 1979). Moreover, SJL lymphocytes sensitized to H-2(b) or H-2(d) cells efficiently lysed neoplastic SJL cells in vitro. Russell et al. (1979) showed in BALB/c mice that cytotoxic T lymphocytes to the BALB/c myeloma tumor MOPC-167 caused essentially no lysis of eight other BALB/c tumors, including three myeloma tumors, but extensively lysed six out of nine tumors of DBA/2 mice. Their observations suggested that MOPC-167 expressed an alloantigen normally absent in BALB/c mice but present in DBA/2 mice and in many DBA/2 tumors.

Prat et al. (1978) and Robinson and Schirrmacher (1979) showed that amplification of previously undetected public H-2 antigens on tumor cells took place on lymphomas of SJL (H-2(s)) and DBA/2 strains of mice. Therefore, shared public antigens may be the cause of some cross-reactivity.

Nature of Cross-Reacting Antigens

Invernizzi and Parmiani (1975) and Parmiani and Invernizzi (1975) concluded that cross-reactivity of tumor antigens with allogeneic MHC antigens was due to either modification of existing histocompatibility antigens or products of derepressed silent histocompatibility genes. Perry and Greene (1980) provided an alternative explanation for cross-reactivity between tumor specific transplantation

antigen on chemically induced tumor cells and allogeneic H-2 antigens. They suggested that specificity of T cell recognition with regard to tumor antigen and alloantigen reflects the differential associative context in which these determinants are recognized. They concluded that interaction of tumor antigen and host macrophage I-A determinants may create a complex antigenic structure which resembles anticens coded by the K or I-A subregions of the foreign H-2 haplotype, at the level of T cell recognition.

H-2-restricted cytotoxic lymphocytes specific for minor histocompatibility antigens (Bevan 1977), Sendai virus (Finberg et al. 1978), H-Y antigen (Von Boehmer et al. 1979), and Herpes simplex virus (Pfizermaier et al. 1980), selectively cross-react with allogeneic MHC determinants. Pfizenmaier et al. (1980) concluded that H-2D(k) plus Herpes simplex virus antigenic determinants may evoke new determinants similar to those expressed by allelic variants of MHC products, or that cross-reactivity may be due to cross-reaction of the anti-foreign receptor on the cytotoxic T lymphocyte. Ivanyi et al. (1980) showed that in BALB/c mice syngeneic immunization with normal lymphoid cells induced alloreactive antibodies of high cytotoxic titer. They hypothesized that virus modified H-2(d) antigenic determinants had triggered alloreactive B-cell clones to produce anti--H-2 antibodies.

Simonsen (1955) showed that tolerance to chicken erythrocytes in the turkey led to increased susceptibility of the normally resistant mature turkey to RSV. Subsequent studies have revealed that human group A erythrocytes, sheep erythrocytes, and chicken tissue extracts all have the property of altering the susceptibility of turkeys. All have in common a Forssman-like heterophile antigen (Harris and Simons 1958). In these studies the phenomenon being observed may have been a host-tumor antigen cross-reactivity.

Evidence has been presented which indicates that cell surface antigens are important to the immune response both as targets and regulators. Furthermore, tumor cells present altered MHC antigens. Whether or not these changes affect the outcome of any non-experimental neoplasms is unknown.

CHAPTER II

MATERIALS AND METHODS

ANIMALS

F2, F3, F4, and F5 generation crosses of lines 6 subline 1 (6-1) and line 15 subline 1 (15-1), designated (6-1 X 15-1), two highly inbred (F > 0.99) Single Comb White Leghorn lines developed and maintained at the Regional Poultry Research Laboratory (RPRL) of the United States Department of Agriculture, East Lansing, Michigan, were used in this research. Line 6-1 was homozygous for susceptibility to subgroup A lymphoid leukosis (LL) virus and resistant to Marek's disease (MD). Line 15-1 was segregating for cellular susceptibility to subgroup A LL virus and was susceptible to MD (Stone 1975 and personal communication, Sommes 1975). Parental and F2 generation chickens were blood typed in the laboratory of W. E. Briles (Department of Biological Sciences, Northern Illinois University) and F2 progeny were known to be segregating for genes at the B, D, and I alloantigen loci. Parents of the F3, F4, and F5 generations and all offspring except those from homozygous matings were typed for B system antigens, the marker for the MHC. F3 through F5 generation progeny

were not blood typed for the D and I antigens because neither D nor I genotype, nor sex, significantly affected tumor growth (Collins et al. 1977). Line 6-1 was homozygous B2 and line 15-1 homozygous B5. Line 100 (F > 0.78), a Single Comb White Leghorn line developed and maintained at the RFRI, homozygous B2 (parents blood typed by W. E. Briles), was used as a blood source in one immunization experiment. Noninbred line UNH 105 B24B24 chickens from homozygous B24B24 parents (parents blood typed by W. E. Briles) served as a source of a third MHC haplotype for the cytotoxicity and alloantisera experiments and as a negative control in the skin graft experiments.

Animals used as blood donors were either homozygous B5 or B2 and at least 16 weeks of age. In partial tolerance experiments (6-1 X 15-1) F3, F4, and F5 generation chickens were used as experimental animals and blood sources for each experiment were chickens from the previous generation. Animals within an experiment were always of the same generation, but animals in different experiments of the same type may have come from more than one generation. In immunization experiments (6-1 X 15-1) F5 generation chickens were used as experimental animals and F4 and line 100 chickens served as blood donors. In cytotoxicity and alloantisera experiments (6-1 X 15-1) F4 generation chickens were used in alloantisera production while F5 chickens were used as the source of effector cells and most target cells. Up to five different sires were represented in each

experiment but progeny of a given sire were randomized in approximately equal numbers to each treatment group.

Chickens were vaccinated at hatching with Marek's disease vaccine (live turkey Herpesvirus, chicken tissue culture origin, cell-free, Sterwin Laboratories Inc., Millsboro, Delaware) and at ten days of age with Newcastle-bronchitis vaccine (live virus, chicken embryo orgin, Sterwin Laboratories Inc., Millsboro, Delaware). In the cytotoxicity, allantisera, and immunization experiments each chicken also received 0.2 mg gentimicin sulfate (Garasol, American Scientific Laboratories, Madison, Wisconsin) mixed with the Marek's disease vaccine in a 0.2 ml dose subcutaneously at hatching to decrease chick mortality due to a recurrent respiratory problem at the University of New Hampshire (UNH) Poultry Research Farm.

Chickens were brooded from hatching to four to six weeks of age in conventional, electrically-heated brooding batteries located in windowless houses at the UNH Poultry Research Farm. Chickens were moved to semi-isolated facilities for virus inoculation and were maintained in conventional holding batteries until the end of the experiment. Chickens used as blood sources and for allantisera production were kept in separate semi-isolated facilities.

All chickens were fed, ad libitum, commercially prepared, all mash, medicated (0.004% amprolium and bacitracin methylene disalicylate to aid in the development

of immunity to coccidiosis under conditions of slight exposure) chick starter feed.

CELLS

Blood was drawn aseptically via either cardiac puncture or the brachial vein in the wing into heparinized (50 units/ml whole blood) syringes. Peripheral white blood cells (WBC) were separated from red blood cells (RBC) on a Ficoll-Hypaque gradient (Archambault et al. 1976). Cells were counted, checked for viability using a trypan blue dye exclusion test (Hudson and Hay 1976) and suspended in phosphate buffered saline (PBS) to the proper dilution. WBC were lysed completely (assayed by the trypan blue dye exclusion test) by suspending the cells in sterile double-distilled water for 30 minutes at room temperature. WBC preparations contained from zero to five percent RBC and RBC preparations contained from zero to one percent WBC.

Secondary cultures of chicken embryo fibroblasts (CEF) (two to three days in culture) were derived from 10 to 11 day old embryos of line 105 and the F5 generation of (6-1 X 15-1) (procedure from Vogt 1965). CEF were cultured in Falcon 3013, 25 square centimeter tissue culture flasks with RPMI 1640 medium supplemented with 5% fetal calf serum, 100 units/ml penicillin, 100 ug/ml streptomycin and freshly added L-glutamine (2mM) at 37C in a 5% carbon-dioxide atmosphere. The total medium was designated as RPMI 1640+.

Spleens were removed aseptically and placed in RPMI 1640+ medium. Splenocyte suspensions were prepared by chopping the spleen into small pieces, drawing the pieces into a syringe (18-gauge needle) and gently expressing them to disaggregate the spleen. Large spleen fragments were allowed to settle and the supernate was centrifuged at 400 x g for two minutes. The supernate was collected, centrifuged at 400 x g for five minutes and the pelleted cells washed twice in PBS. The splenocytes were resuspended in RPMI 1640+ medium and used immediately in the cytotoxicity assay. Cell viability was tested by the ability of the cells to exclude trypan blue dye.

Tumors were removed aseptically, necrotic tissue discarded and healthy tissue placed in RPMI 1640+ medium. Tumor cell suspensions were prepared in the manner of spleen suspensions and used immediately in the fluorescent antibody assay.

BLOOD AND CELL INOCULATIONS

Partial Tolerance Experiments

Recipient (6-1 X 15-1) B2B2 chickens in blood inoculation experiments were inoculated intraperitoneally (IP) with either 0.5 ml blood from (6-1 X 15-1) B5B5 chickens or PBS on days zero (hatching) and two and with 0.3 ml on days eight, 14, 20, and 23. In all other experiments inoculation IP with 0.3 ml of the appropriate

dilution of viable WBC or RBC (100 million, 1 million, or 1 hundred thousand cells) from (6-1 X 15-1) B5B5 chickens, 1 million lysed WBC from B5B5 chickens, or 1 million viable WBC from (6-1 X 15-1) B2B2 chickens was made on days zero, two, seven, 14, and 21.

Immunization Experiments

Recipient (6-1 X 15-1) B2B2 chickens were inoculated either subcutaneously, IP, or intravenously (IV) with either viable WBC or RBC from (6-1 X 15-1) B5B5 chickens, (6-1 X 15-1) B2B2 chickens, or line 100 B2B2 chickens. Subcutaneous inoculations were made in the dorsal neck surface and the ventral surface beside the breast bone at 21 days of age. The subcutaneous inoculum consisted of 1 million cells in 0.3 ml of an emulsion of PBS and Freunds complete adjuvant (Herbert 1978). IP inoculations were made with 5 million cells in 0.2 ml PBS at 21 and 28 days of age. IV inoculations were made in the brachial vein of the left wing with 5 million cells in 0.1 ml PBS at 28 and 41 days of age.

TESTS FOR TOLERANCE

Chickens in experiments involving inoculation of whole blood and in those involving inoculations of 100 million and 1 million WBC and RBC from (6-1 X 15-1) B5B5 chickens were tested on day 27 for antibody toward cells from B5B5

chickens using slide hemagglutination tests (Hudson and Hay 1976). Sera were obtained from blood drawn from the brachial vein. Test cells were suspended at two percent in PBS. The positive control consisted of a known antisera to cells from B5B5 chickens. A Coombs test (modification of Coombs et al. 1945) was used to test for incomplete antibody in the serum samples from chickens inoculated with 100 million and 1 million RBC from B5B5 chickens. Serum samples had been frozen at -22C for approximately six months. One tenth milliliter of two percent RBC in PBS was mixed with 0.1 ml test serum and incubated at 37C for one hour. RBC's were washed three times in PBS and 0.1 ml rabbit anti-chicken IgG (Miles Laboratories Inc., Elkhart Indiana) added. Activity of the rabbit anti-chicken IgG was confirmed by its ability to block the binding of fluorescin-conjugated rabbit anti-chicken IgG (Miles Laboratories Inc., Elkhart Indiana) on the surface of WBC. The mixture was incubated for ten minutes, centrifuged for two minutes at 400 x g, and observed for clumped cells. Washed, untreated RBC were mixed with rabbit anti-chicken IgG and showed no agglutination.

Allograft tolerance was tested by observing the fate of dorsal skin grafts on (6-1 X 15-1) B2B2 recipients. Skin donor chickens were killed and portions of dorsal skin approximately three centimeters square immediately removed and floated on RPMI 1640 medium. Graft recipients, 4-weeks old, were plucked of dorsal feathers, swabbed with tincture

of iodine, and anesthetized with 17mg/100gm body weight of ketamine intramuscularly in the thigh. Two graft beds, approximately 0.5 centimeters square were prepared on each recipient. Skin from (6-1 X 15-1) B5B5 donors was placed in one graft bed and skin from either (6-1 X 15-1) B2B2 or line 105 B24B24 donors fitted into the second bed. Each graft was covered with sterile gauze and taped securely. Recipient chickens were individually caged and monitored for acute graft rejection on days five, seven, nine and 14 after grafting. A dry, brittle, black graft was interpreted as a positive indication of rejection (Polley et al. 1960).

MEASUREMENT OF GRAFT VS. HOST (GVH) ACTIVITY

A spleen weight assay was used to assess GVH activity resulting from cell inoculation. Samples of control and cell recipient chickens were sacrificed on day zero, eight, 15, 22, and 28, body weight recorded and the spleen removed and weighed immediately. GVH response was calculated according to the following spleen index (Ford 1978):

$$\text{Spleen index} = \frac{\text{Spleen weight}}{\text{Body weight}} \Big/ \text{mean} \; \frac{\text{Spleen weight}}{\text{Body weight}} \; \text{controls}$$

An index of 1.3 or greater was considered significant (Ford 1978).

ALLOANTISERA PRODUCTION

Adult half-sib B2B2 and B5B5 (6-1 X 15-1) chickens were given IV inoculations in the brachial vein of one ml of 500 million cells/ml, Ficoll-Hypaque separated WBC from the reciprocal genotype. After two weeks they were re-immunized with cells from the same source used in the primary immunization. Six days after the second immunization serum was collected and tested for agglutination of both syngeneic and donor WBC and RBC. Blood was drawn aseptically via the brachial vein.

ABSORPTION OF ALLOANTISERA

Alloantisera diluted 1:10 in PBS were specifically absorbed by secondary culture monolayers (approximately 3-4 million cells/monolayer) of (6-1 X 15-1) B2B2, B5B5, and line 105 E24E24 CEF, including both Rous sarcoma virus (RSV)-infected and uninfected cultures. The cell-alloantisera mixture was incubated at 37C for one hour with gentle shaking. Absorption was repeated three times.

AGGLUTINATION ASSAY

The alloantisera were tested for ability to specifically agglutinate (6-1 X 15-1) F5 generation B2B2 and B5B5 RBC (2% in PBS). Sera from chickens in immunization

experiments were tested for agglutinating antibody toward the cell type inoculated. Control chickens were tested for agglutinating antibody toward B5B5 RBC except in the line 100 experiments where line 100 WBC were used. The sera were tested in a doubling dilution series starting with a 1:2 dilution. Controls of cells in PBS were used throughout.

FLUORESCENT ANTIBODY ASSAY

One tenth milliliter of a 1% cell suspension was mixed with 0.1 ml of 1:16 PBS diluted rabbit anti-chicken IgG (Miles Laboratories Inc., Elkhart Indiana) and shaken gently at 37C for ten minutes. Cells were washed in PBS and resuspended in 0.1 ml PBS, 0.1 ml test serum added and shaken gently at 37C for ten minutes. Cells were washed twice in PBS and resuspended in 0.1 ml PBS, 0.1 ml FITC conjugated rabbit anti-chicken IgG (Miles Laboratories Inc., Elkhart Indiana) added and shaken gently at 37C for ten minutes. Cells were washed three times in PBS and resuspended in 0.1 ml PBS, a wet mount prepared and cells observed for fluorescence with a Reichert fluorescence microscope (exciting filter #KG-2/BG-12, barrier filter #1.5/OG1 1/GG-9). One hundred cells were counted.

CYTOTOXICITY ASSAY

Lymphocyte cytotoxicity was tested using a modification of the 51Cr uptake microcytotoxicity assay (Moore et al. 1975, McGrail et al. 1978). Effector cells were splenocytes from six week old chickens with RSV-induced tumors (tumor scores of three or four at time of spleen harvest) and from chickens uninoculated with RSV. Target cells were (6-1 X 15-1) F5 generation B2B2, B5B5, and line 105 B24B24 CEF including RSV-infected B2B2 and B5B5 CEF. Aliquots of ten thousand target CEF, suspended in 0.1 ml RPMI 1640+ medium were seeded into appropriate wells of a Falcon 3040 microtest II tissue culture microtiter plate. RSV-infected CEF were in separate plates from uninfected CEF. After 48 hours incubation at 37C, the medium was discarded and 0.1 ml RPMI 1640+ containing one million lymphocytes added to appropriate wells. Each effector-target cell combination was repeated in five wells. Two sets of target cells (five wells of each of the five CEF types per set), incubated in medium only, were used as controls. The microtiter plates were incubated at 37C for 48 hours, the medium with dead cells discarded and the wells washed thoroughly with PBS. Na2(51)CrO4 (sodium chromate, specific activity = 50-400 mCi/mgCr) was added to each well at a concentration of 0.5 uCi in 0.1 ml RPMI 1640+ medium. The plates were incubated three hours at 37C, the medium discarded and the plates washed three times with PBS. The

remaining target cells were trypsinized, transferred to individual BEEM capsules and counted for one minute in a Packard tri-carb liquid scintillation counter (Model 3320) with an attached autogamma spectrometer.

The percent cytotoxicity for each effector-target cell combination was calculated as follows:

$$\frac{\text{Mean } ^{51}\text{Cr c.p.m. control} - \text{Mean } ^{51}\text{Cr c.p.m. test sample}}{\text{Mean } ^{51}\text{Cr c.p.m. control}} \times 100$$

Negative cytotoxicity values were interpreted as complete absence of target cell lysis.

VIRUS AND VIRUS INOCULATION

A highly purified pseudotype of Bryan High-titer Rous sarcoma virus, subgroup A, designated BH RSV (RAV-1) abbreviated RSV-1, supplied by L. E. Crittenden of the RPRL, and stored in liquid Nitrogen, was used in this research. The stock virus was diluted in Hanks balanced salts solution containing 5% fetal calf serum, 100 units/ml penicillin, 100 ug/ml streptomycin, and 100 ug/ml hyalauronidase. Virus inoculated chickens received 0.05 ml of a 1 : 1000 dilution of stock virus (approximately 10 pock-forming units on the chorioallantoic membranes of susceptible embryos) intradermally, in the left wingweb at four weeks of age in partial tolerance, cytotoxicity and allantisera experiments and six weeks of age in immunization experiments.

RSV-1--infected CEF were attained by using a modification of the technique of Hanafusa (1969). Approximately 50% confluent monolayers were overlayed with 0.1 ml of DEAE-dextran in RPMI 1640+ (240 ug/ml) and 0.5 ml of RSV-1 at a 1 : 1000 final dilution in RPMI 1640+. After adsorption of virus for one hour at 37C the cultures were washed and re-incubated in RPMI 1640+ for 48 hours prior to use in the allcantiserum or cytotoxicity assays. Virus infected and uninfected cultures were handled at separate times and in separate facilities prior to cytotoxic assays.

TUMOR MEASUREMENT

Tumors appeared on the site of virus inoculation at approximately ten days post inoculation (PI) and were subjectively scored for size at weekly intervals between 14 and 70 days PI. Tumor scores were based upon the following criteria (Collins et al. 1977).

Score	Criterion
0	No palpable tumor
1	Tumor > 0 and ≤ 0.5 cm diameter
2	Tumor > 0.5 cm and ≤ 1.2 cm diameter
3	Tumor > 1.2 cm and ≤ 1/2 wingweb area
4	Tumor > 1/2 but ≤ total wingweb area
5	Tumor fills wingweb
6	Tumor extends beyond wingweb

Based upon the criteria below a tumor profile index (TPI) was assigned (Collins et al. 1977).

TPI	Criterion
1	Complete regression by 28 days.
2	Complete regression by 56 days.
3	Complete regression by 70 days, or a decreasing slope, or complete regression by 56 days followed by recurrence.
4	General upward trend, or plateau; slight regression after 56 days.
5	Terminal tumor prior to 70 days.

A necropsy was performed upon chickens which died during an experiment.

STATISTICAL ANALYSES

Analysis of variance was used to statistically examine the data with statistical significance determined at $P \leq 0.05$ (Snedecor and Cochran 1967). A mean separation

test appropriate to equal and unequal subclass numbers, was used (Dixon and Duncan 1975).

Partial Tolerance Experiments

Tumor experiments were arranged in a completely randomized design with the dependent variable being TPI and the independent variable being type of inoculum. Error mean squares of experiments within the same experiment type were tested for homogeneity of variance and in all cases the error mean squares proved to be homogenous allowing pooling of experiments. The pooled experiments were arranged in a randomized block design with experiments treated as blocks. The dependent variable was TPI and the independent variable was type of inoculum.

Cytotoxicity and Alloantisera Experiments

Percent cytotoxicity and percent fluorescing cells were analysed in the same way. Percentages were converted to an angle (angle = $\arcsin\sqrt{\text{percent}}$) and in the case of percent cytotoxicity were coded to a positive value prior to the analysis of variance (Snedecor and Cochran 1967). The experiments were arranged in a completely randomized design. In the cytotoxicity experiment the dependent variable was the transformed percent cytotoxicity and the independent variable was the type of target cell. Data concerning lymphocytes from normal chickens were analysed separately from that of tumor bearing chickens. In the fluorescence

experiment the dependent variable was the transformed percent fluorescing cells and the independent variable was days postinoculation. Data on each of the four target cell types were analysed separately.

In allantisera experiments titers of <2 were taken as 1 in the analysis. Titers were transformed by taking log(titer) before analysis of variance (Lutz 1978). The experiments were arranged in a completely randomized design. The dependent variable was the transformed titer value and the independent variable was the type of absorbing CEF combined with one of the BBC targets. Data were not analysed between allantisera types.

Immunization Experiments

Tumor experiments were arranged in a completely randomized design with the dependent variable being TPI and the independent variable being the type of inoculum.

CHAPTER III

RESULTS

This research may be divided into three segments: a) Partial tolerance experiments; b) Cytotoxicity and alloantisera experiments; and c) Immunization experiments. All of the studies were designed to test the hypothesis that (6-1 X 15-1) B5B5 chickens have RSV-1--induced tumors that grow progressively to death, in part because host MHC antigen cross-reacts with tumor associated antigen.

PARTIAL TOLERANCE EXPERIMENTS

(6-1 X 15-1) B2B2 and B5B5 chickens were used in these studies. B2B2 chickens were always the host chickens in which partial tolerance to B5 antigen was elicited. Line UNH 105 B24B24 chickens were used as a negative control in the skin graft experiments.

The mechanism of the partial tolerance was not important to these experiments. What was important was to observe whether or not cells from B5B5 individuals elicited a state of specific immune paralysis which in turn would affect RSV-1--induced tumor growth.

The criteria of partial tolerance were absence of complete and incomplete antibody toward the cell type used to induce partial tolerance and acceptance of skin allografts from B5B5 donors by B2B2 chickens. Antibody against the cellular types was not detected in any tested chickens at 27 days of age. Dorsal skin graft results are given in Table 1. In uninoculated B2B2 chickens 30 percent of 15 grafts from B2B2 donors were still healthy on day 14, whereas all 15 grafts from B5B5 donors were rejected by day seven. Where WBC or RBC from B5B5 chickens were inoculated, grafts from B2B2 donors served as graft acceptance controls and grafts from B24B24 donors as graft rejection controls. Twenty-six to 81 percent of the grafts of B2B2 donors were healthy on day 14. For grafts of B24B24 donors from zero to 13 percent were healthy on day seven but all were rejected by day nine. With inoculations of viable WBC, RBC, and lysed WBC, from B5B5 chickens, 41, 34, and 35 percent, respectively, of grafts from B5B5 donors were healthy on day seven compared to zero percent for those receiving no cell inoculations. On day 14, 28 and 21 percent of grafts from B5B5 donors on chickens inoculated with viable and lysed WBC from B5B5 individuals, respectively, were healthy compared to zero percent for the grafts from B5B5 donors on chickens inoculated with B5B5 RBC and on uninoculated chickens. These results indicated a partial state of tolerance toward B5 cell surface antigens existed in treated B2B2 chickens 14 days after skin grafting. Furthermore, partial tolerance

Table 1. Skin graft results from (6-1 X 15-1) B2B2 graft recipients receiving an inoculum containing 10^6 viable WBC, RBC, or lysed WBC from (6-1 X 15-1) B5B5 chickens.

Type of cell inoculum	Number of grafts[a]	B genotype of graft donor	Healthy skin grafts (%) on day: 5	7	9	14
Uninoculated	15	5/5	30	0	0	0
	15	2/2	47	40	30	30
Viable WBC	32	5/5	68	41	31	28
	16	2/2	81	81	81	81
	16	24/24	38	13	0	0
Viable RBC	35	5/5	43	34	0	0
	17	2/2	82	71	47	41
	18	24/24	22	1	0	0
Lysed WBC	34	5/5	44	35	21	21
	19	2/2	37	32	26	26
	15	24/24	20	0	0	0

[a]Two grafts per chicken.

was specific to B5 antigen since grafts from B5B5 chickens were frequently accepted but grafts from B24B24 chickens never were.

Table 2 gives the response of partially tolerant and control chickens to RSV-1--induced tumors presented in two ways: 1) as mean TPI and; 2) as the percent distribution of animals according to TPI. B2B2 chickens were inoculated with B5 antigen in the manner shown in the previous set of experiments to produce partial tolerance to B5 antigen.

With respect to mean TPI, particulate control inoculations of B2B2 WBC or RBC into B2B2 hosts gave mean TPI's of 3.0 and 2.8, respectively, and each was not significantly different from the mean TPI of 3.1 for the uninoculated B2B2 chickens. B2B2 chickens inoculated with B5B5 blood had a mean TPI of 3.7 which was significantly higher than the mean TPI's of 2.8 and 3.1 for PBS treated and uninoculated B2B2 chickens, respectively. B2B2 chickens inoculated with 100 million B5B5 viable WBC had a mean TPI of 3.9 which was significantly higher than the mean TPI of 3.1 for uninoculated B2B2 chickens. B2B2 chickens inoculated with 100 million B5B5 viable RBC, however, had a mean TPI of 3.4 which was not significantly different from that of 3.9 for B2B2 chickens inoculated with 100 million B5B5 viable WBC and that of 3.1 for uninoculated B2B2 chickens. B2B2 chickens inoculated with 1 million B5B5 viable WBC or RBC had mean TPI's of 4.3 and 3.8, respectively, which were not significantly different from

Table 2. Mean TPI[a] of (6-1 X 15-1) B2B2 recipients with inocula containing various types and numbers of cells.

B Genotype of cell donor	Type of cell inoculum	No. of cells inoculated	No. of experiments	No. of animals	Percent distribution of animals according to TPI: 1	2	3	4	5	Mean TPI[c]
2/2	Viable WBC			22	14	27	23	23	14	3.0^{A}
	Viable RBC	10^6	2	19	5	21	42	11	21	2.8^{A}
	Uninoculated			29	7	28	24	34	7	3.1^{A}
5/5	Blood			36	6	6	25	44	19	3.7^{A}
	PBS	b	2	28	14	36	21	18	11	2.8^{B}
	Uninoculated			29	7	28	34	14	17	3.1^{B}
5/5	Viable WBC			28	0	7	32	22	39	3.9^{A}
	Viable RBC	10^8	3	37	3	19	35	22	22	3.4^{AB}
	Uninoculated			41	7	17	42	29	5	3.1^{B}
5/5	Viable WBC			26	0	0	19	31	50	4.3^{A}
	Viable RBC	10^6	3	24	0	17	13	42	29	3.8^{A}
	Uninoculated			28	7	25	32	36	0	3.0^{B}
5/5	Viable WBC			23	0	9	17	31	43	4.1^{A}
	Viable RBC	10^5	3	24	0	8	25	17	50	4.1^{A}
	Uninoculated			27	11	37	22	19	11	2.8^{B}
5/5	Lysed WBC	10^6	3	27	0	11	22	30	37	3.9^{A}
	Uninoculated			31	10	42	19	26	3	2.7^{B}

[a] Tumor profile index of RSV-induced tumors

[b] See Materials and Methods

[c] Means within a given experiment type having no superscripts in common are significantly different, $P \leq 0.05$.

each other but each of which was significantly different from the mean TPI of 3.0 for uninoculated B2B2 chickens. B2B2 chickens inoculated with 100 thousand B5B5 viable WBC or RBC had mean TPI's of 4.1, each, and each was significantly different from the mean TPI of 2.8 for uninoculated B2B2 chickens. B2B2 chickens inoculated with 1 million lysed WBC had a mean TPI of 3.9 which was significantly higher than the mean TPI of 2.7 for uninoculated B2B2 chickens.

With respect to percent distribution of animals according to TPI it was shown that the increase in mean TPI exhibited by B2B2 chickens partially tolerant to B5 antigen was due to a decrease in the number of chickens with TPI's of 1 and 2 (completely regressing categories) and an increase in the number of chickens with TPI's of 4 and 5 (progressing categories), when compared to uninoculated control B2B2 chickens. For example, B2B2 chickens inoculated with 1 million B5B5 viable WBC showed zero percent of the chickens in TPI categories of 1 and 2 while 50 percent were in TPI category 5 compared to 7 and 25 percent of uninoculated B2B2 chickens in TPI categories 1 and 2, respectively, and zero percent in category 5.

To determine whether or not a severe GVH response was occuring in the test chickens during antigenic exposure a GVH (spleen weight) assay was utilized. In this assay a mean spleen index $\geq$ 1.3 was interpreted as a significant spleen enlargement over spleens of uninoculated control

Table 3. Graft vs. host response to inoculations of 10^6 WBC or RBC from (6-1 X 15-1) B5B5 or B2B2 donors into (6-1 X 15-1) B2B2 chickens.

B Genotype of cell donor	Type of cell inoculum	Number of chickens sacrificed each day	Mean spleen index (days) 0	8	15	22	28
5/5	Viable RBC	10	0.99	1.22	1.05	1.13	1.09
5/5	Lysed WBC	10	1.07	1.27	1.22	1.17	1.27
2/2	Viable WBC	10	1.01	1.20	1.16	1.10	1.22
5/5	Viable WBC	10	1.06	1.27	1.51	1.24	1.82

[a] Mean spleen index $\geq$ 1.3 was interpreted as a significant spleen enlargement over spleens of uninoculated control chickens (Ford 1978).

chickens (Ford 1978). Table 3 gives the GVH response of B2B2 chickens to inoculation with 1 million cells. Neither RBC or lysed WBC from B5B5 chickens, nor WBC from B2B2 chickens gave significant spleen enlargement over uninoculated B2B2 chickens. Inoculations of viable WBC from B5B5 chickens, however, gave significant spleen enlargement over uninoculated chickens on days 15 and 28, indicating a GVH response.

In summary, these experiments showed that crosses of lines 6-1 and 15-1 having a B2B2 MHC genotype when made partially tolerant to viable WBC, RBC, or lysed WBC from corresponding chickens of B5B5 MHC genotype, exibited a greater frequency of progressing RSV-1--induced tumors than untreated B2B2 chickens. Cell inocula consisting of 100 million, 1 million, or 100 thousand cells gave similar results. B2B2 control chickens inoculated with PBS, or with cells from B2B2 chickens, gave results not significantly different from uninoculated B2B2 chickens indicating that increased tumor growth did not result from a non-specific effect resulting from the cell inoculations.

A non-specific effect of chronic GVH on tumor growth would not appear to be the explanation for the results because: a) Lysed WBC and viable RBC contributed to increased tumor growth but not to GVH; b) differential skin graft results were observed indicating specificity in the partial tolerance; c) tumor growth patterns were changed significantly and in a manner that would not appear to be

attributable to a mild effect of GVH.

CYTOTOXICITY AND ALLOANTISERA EXPERIMENTS

These experiments were designed to detect cross-reactivity between B5B5 antigen and RSV-1--induced tumor associated antigen. B2B2 and B5B5 chickens were from the F4 and F5 generations of a cross of lines 6-1 and 15-1. B24B24 chickens were from line UNH 105.

Twenty six-week old B2B2 chickens, ten uninoculated with RSV-1 and ten bearing RSV-1--induced sarcomas (two weeks post-RSV-1--inoculation), were tested for cytolytic lymphocytes (CL) against B2B2 and B5B5 chicken embryo fibroblast (CEF) targets, both uninfected and infected with RSV-1. Six of the B2B2 RSV-1--inoculated chickens and six of the uninoculated chickens were also tested for CL against B24B24 CEF targets.

Table 4 gives mean cytotoxicity percentages for the effector-target cell combinations. Lymphocytes from chickens uninoculated with RSV-1 (normal chickens) gave mean cytotoxicity percentages toward the five target CEF types ranging from -1.4 to 0.0; none significantly different. Lymphocytes from chickens with RSV-1--induced tumors gave mean cytotoxicity percentages of 49.0, 28.3, and 37.8 for targets of B2B2 and B5B5 RSV-1--infected and B5B5 uninfected CEF, respectively, all three significantly different from the cytotoxicity percentages of -9.0 and -8.8 for targets of

Table 4. Mean cytotoxicity for lymphocytes from (6-1 X 15-1)F5 B2B2 chickens with and without RSV-1--induced tumors tested against each of the five target CEF[a] types.

Target CEF		Mean cytotoxicity (percent)[bc]	
B Genotype	RSV-1 status	normal chickens	tumorous chickens
2/2	not infected	-0.4^{A}	-9.0^{A}
5/5	not infected	0.0^{A}	31.8^{B}
24/24	not infected	-0.8^{A}	-8.8^{A}
2/2	infected	-0.5^{A}	49.0^{B}
5/5	infected	-1.4^{A}	28.3^{B}

[a] Chicken embryo fibroblasts.

[b] Splenocyte preparations from 20 B2B2 chickens, ten with tumors and ten without, were tested for cytotoxicity against each of the five target CEF types except for targets of B24B24 CEF where only six chickens from each group were used.

[c] Means within one column having no superscripts in common were significantly different, $P \leq 0.05$.

non-RSV-1--infected B2B2 and B24B24 CEF, respectively. Negative and zero percent cytotoxicities indicated no lymphocyte-mediated lysis of target cells. Positive percent cytotoxicities indicated target cell lysis by lymphocytes.

Table 5 gives RBC agglutination titers of CEF absorbed alloantisera. Specific B5B5 anti-B2B2 and B2B2 anti-B5B5 allcantisera were produced and tested prior to the start of the experiment (see Materials and Methods, Allcantisera Production, page 33). Samples of allcantisera were absorbed with CEF and the resulting absorbed alloantisera tested for the ability to agglutinate B2B2 or B5B5 RBC. Three separate allcantisera absorptions with subsequent tests for agglutination were used to calculate mean titers. Anti-B5B5 allcantisera produced in B2B2 chickens, agglutinated B5B5 RBC, with mean titers from 10.0 to 21.3, but not B2B2 RBC, with mean titers all < 2. Likewise, anti-B2B2 alloantisera produced in B5B5 chickens, agglutinated B2B2 RBC, with mean titers from 26.7 to 42.7, but not B5B5 RBC, with mean titers all < 2. Absorption of anti-B5B5 alloantisera with RSV-1--infected or uninfected B5B5 CEF removed all agglutination activity from the alloantisera giving mean titers of < 2, each, and each was significantly different from the mean titer of 21.3 for unabsorbed anti-B5B5 allcantisera, when tested against B5B5 RBC. Absorption of anti-B2B2 allcantisera with RSV-1--infected or uninfected B2B2 CEF removed all agglutination activity from the alloantisera giving mean titers of < 2, each, and each was

Table 5. RBC agglutination titers of alloantisera absorbed with CEF[a].

Alloantisera	Absorbing CEF: B Genotype	Absorbing CEF: RSV-1 status	Mean titer for agglutination of RBC of genotype:[bc] B2B2	Mean titer for agglutination of RBC of genotype:[bc] B5B5
B2B2 anti-B5B5	2/2	not infected	<2[A]	21.3[B]
	5/5	not infected	<2[A]	<2 [A]
	24/24	not infected	<2[A]	16.0[BC]
	2/2	infected	<2[A]	10.0[C]
	5/5	infected	<2[A]	<2 [A]
	No absorption		<2[A]	21.3[B]
B5B5 anti-B2B2	2/2	not infected	<2 [A]	<2[A]
	5/5	not infected	26.7[B]	<2[A]
	24/24	not infected	42.7[B]	<2[A]
	2/2	infected	<2 [A]	<2[A]
	5/5	infected	32.0[B]	<2[A]
	No absorption		32.0[B]	<2[A]

[a] Chicken embryo fibroblasts.

[b] Mean titers based upon three observations.

[c] Mean titers within the same alloantisera category having no superscripts in common are significantly different, $P \leq 0.05$.

significantly different from the mean titer of 32.0 for the unabsorbed anti-B2B2 alloantisera, when tested against B2B2 RBC. None of the other CEF-alloantisera combinations resulted in significantly reduced agglutination titers when tested against their specific RBC except that absorption of anti-B5B5 alloantisera with RSV-1--infected B2B2 CEF gave a mean titer of 10.0 which was significantly lower than the 21.3 mean titer of unabsorbed anti-B5B5 alloantisera, when tested against B5B5 RBC. The latter result indicates cross-reactivity between RSV-1--infected B2B2 CEF and B5 antigen.

Antisera from B2B2 chickens with RSV-1--induced tumors were tested for cross-reactivity with B5B5 cells using a fluorescent antibody test. Table 6 gives the fluorescent antibody test of serum from B2B2 chickens with RSV-1--induced tumors for IgG against B5B5 cells. Ten-week old uninoculated B2B2 chickens served as negative controls and gave low mean percent fluorescing cells; 0.4, 0.8, 1.2, and 1.8 for sarcoma cells, WBC, RBC and CEF, respectively. At all days PI tested, B2B2 RSV-1--induced tumor bearing chickens contained IgG that bound to B5B5 RSV-1--induced sarcoma cells with mean percent fluorescing cells ranging from 60 to 78 percent and with no significant differences between age groups, but all significantly different from the mean percent fluorescing cells (0.4) found when serum from chickens uninoculated with RSV-1 was added to sarcoma cells. The fluorescence in sarcoma cells served as positive

Table 6. Fluorescent antibody test of serum from (6-1 X 15-1)F5 B2B2 chickens with RSV-1--induced tumors for IgG against (6-1 X 15-1) F5 B5B5 cells.

Day PI[a]	Number of serum samples	Mean percent fluorescing B5B5 cells[d]			
		sarcoma cells	WBC	RBC	CEF[c]
Uninoculated[b]	5	0.4^{A}	0.8^{A}	1.2^{A}	1.8^{A}
14	5	70^{B}	1.4^{A}	0.2^{A}	1.6^{A}
28	5	77^{B}	0.8^{A}	2.6^{A}	1.4^{A}
42	5	60^{B}	2.4^{A}	0.4^{A}	0.8^{A}
56	5	78^{B}	2.4^{A}	1.8^{A}	1.8^{A}
70	5	63^{B}	1.0^{A}	1.6^{A}	0.6^{A}

[a] Post-RSV-1--inoculation.

[b] 10 weeks old.

[c] chicken embryo fibroblasts.

[d] Means within the same cell type having no superscripts in common are significantly different, $P \leq 0.05$.

controls because the same serum samples were always tested for IgG binding to each of the four target cell types. Binding of IgG to B5E5 WBC, REC, or CEF was not observed. All samples from 14 to 70 days PI gave mean percent of fluorescing cells not significantly different than those associated with the serum samples from uninoculated chickens. The data indicated that the IgG was binding to the sarcoma cells because of affinity for tumor associated antigen and not affinity for B5 antigen.

In summary, the alloantisera absorption studies showed that the RSV-1--infected and uninfected CEF shared antigen with syngeneic REC. Absorption with RSV-1--infected B2B2 CEF significantly lowered the B5E5 REC agglutination titer of B2B2 anti-B5E5 alloantisera suggesting cross-reactivity between RSV-1--infected cells and B5B5 REC. B2B2 cytotoxic lymphocytes primed against RSV-1--induced tumor cells specifically lysed RSV-1--infected B2B2 CEF and both RSV-1--infected and uninfected B5B5 CEF; again cross-reactivity was indicated. However, no cross-reactivity was observed in IgG from chickens bearing RSV-1--induced tumors.

IMMUNIZATION EXPERIMENTS

If B5 antigen cross-reacts with RSV-1--induced tumor associated antigen it was hypothesized that it should be possible to immunize a chicken against B5 antigen and that

this should enhance the immune response toward an RSV-1--induced tumor. The following experiments were designed to test this hypothesis. In all cases the chickens immunized were (6-1 X 15-1) F5 generation B2B2 chickens. The immunizing cells were (6-1 X 15-1) F4 generation B5B5 WBC and REC, B2B2 WBC (as an inoculation control) and line 100 WBC and REC. Line 100 chickens have a B2B2 genotype but their RSV-1--induced tumors grow progressively in a manner similar to those of (6-1 X 15-1) B5B5 chickens (Collins et al. 1980).

A subcutaneous immunization technique with the cells emulsified with Freund's adjuvant was tried initially. Two similar experiments were conducted. The chickens were immunized once at 21 days of age, allantisera titers were obtained at 42 days of age and RSV-1--inoculation made at 42 days of age. The titers were used to give an indication of the effectiveness of immunization. It was hoped that the three week interval between immunization and RSV-1--inoculation would allow a strong secondary immune response to develop toward the tumor if cross-reactivity was present.

Table 7 gives the results following subcutaneous immunization. In both experiments 1 and 2 antisera titers resulted from immunization. In experiment 1, however, the mean titer values were 69 and 90 for B5B5 WBC and RBC, respectively, while in experiment 2 the corresponding mean titers were only 26 and 30. In experiment 1 mean TPI's for

Table 7. Mean antisera titers and TPI's[a] of (6-1 X 15-1)F5 B2B2 chickens immunized subcutaneously at 21 days of age with (6-1 X 15-1)F4 B5B5 cells followed by RSV-1--inoculation at 42 days of age.

Experiment	Immunizing cell	Number of animals	Mean titer[bcd] of antisera	Percent distribution of animals according to TPI: 1	2	3	4	5	Mean TPI[d]
1	Viable WBC	6	69^{A}	0	67	17	17	0	2.5^{A}
	Viable RBC	5	90^{A}	20	40	20	0	20	2.6^{A}
	Uninoculated	5	$<2^{B}$	0	20	0	80	0	3.6^{A}
2	Viable WBC	12	26^{A}	17	17	8	50	8	3.2^{A}
	Viable RBC	12	30^{A}	17	25	8	25	25	3.2^{A}
	Uninoculated	12	$<2^{B}$	8	50	17	17	8	2.6^{A}

[a] Tumor profile index

[b] Antisera collected at 42 days of age

[c] Antisera were titered for activity toward the immunizing cell type; samples from uninoculated controls were titered for activity toward (6-1 X 15-1)F4 B5B5 RBC's.

[d] Means within the same experiment with no superscripts in common are significantly different, $P \leq 0.05$.

chickens immunized with B5B5 WBC, RBC, or uninoculated were 2.5, 2.6, and 3.6, respectively. Although the mean TPI's of the immunizing cell types did not differ significantly from each other there was some indication that the immunization was successful because the mean TPI's of both cell inoculated groups were approximately two-thirds that of the uninoculated group. On the other hand, the mean TPI of the uninoculated group (3.6) was higher than would be expected in untreated (6-1 X 15-1) B2B2 chickens (see partial tolerance experiments). Moreover, the sample size was small. In Experiment 2 results appeared to be opposite to those of experiment 1. The mean TPI's of B5B5 WBC and RBC immunized groups (3.2) were higher than the mean TPI of the uninoculated group (2.6), although the differences were not significant. The differences in mean TPI's in experiment 1 may have been influenced by sample size. But antisera titers in experiment 1 were higher than those in experiment 2 and this could have contributed to the different results in the two experiments.

To attempt to improve the immunization results intraperitoneal inoculations were tried. Furthermore, two inoculations, one at 21 days and an other at 28 days were utilized in an attempt to develop a stronger immune response toward the tumor than that which resulted from subcutaneous immunizations. RSV-1--inoculation was at 42 days of age.

Table 8. Mean antisera titers and TPI's[a] of (6-1 X 15-1)F5 B2B2 chickens immunized intraperitoneally at 21 and 28 days of age with various types of (6-1 X 15-1)F4 cells followed by RSV-1--inoculation at 42 days of age.

Immunizing cell	Number of animals	Mean titer[bcd] of antisera	Percent distribution of animals according to TPI: 1	2	3	4	5	Mean TPI[d]
B5B5 Viable WBC	16	7[A]	31	13	31	13	13	2.6[A]
B5B5 Viable RBC	11	9[A]	55	18	9	0	18	2.1[A]
B2B2 Viable WBC	16	<2[B]	38	19	19	6	19	2.5[A]
Uninoculated	13	<2[B]	23	31	23	8	8	2.6[A]

[a] Tumor profile index

[b] Antisera collected at 42 days of age.

[c] Antisera were titered for activity toward the immunizing cell type; samples from uninoculated controls were titered for activity toward (6-1 X 15-1)F4 B5B5 RBC's.

[d] Means with no superscripts in common are significantly different, $P \leq 0.05$.

Table 8 gives the results of intraperitoneal immunization. The antiserum titers, tested at 42 days of age, showed that anti-E5B5 antibody had been formed giving mean titers of 7 and 9 for E2E2 chickens inoculated with E5B5 viable WBC and RBC, respectively. Each was significantly different from the titers of < 2 for B2B2 chickens inoculated with viable B2B2 WBC or uninoculated chickens. None of the differences among the mean TPI's as a result of using different immunizing cells were statistically significant. All groups were at the level of tumor response expected for untreated chickens.

In the third immunization technique intravenous inoculations were tried because subcutaneous and IP inoculations did not lead to altered tumor growth. Immunizations were made at 28 and 41 days of age and RSV-1--inoculation at 42 days of age. The inoculation at 28 days was intended to elicit a primary immune response and the one at 41 days to produce a secondary immune response. It was hypothesized that a strong secondary immune response would develop before tumor formation and that this would alter tumor growth. The antisera was titered at 49 days of age to check the immune response after RSV-1--inoculation.

Table 9 gives the results of the intravenous immunizations. The mean titers showed a strong immune response to the cells after RSV-1--inoculation. However, the mean TPI's showed that tumor growth in the immunized individuals was not different from that of uninoculated

Table 9. Mean antisera titers and TPI's[a] of (6-1 X 15-1)F5 B2B2 chickens immunized intravenously at 28 and 41 days of age with various types of cells followed by RSV-1--inoculation at 42 days of age.

Experiment	Immunizing cell[b]	Number of animals	Mean titer[cdf] of antisera	Percent distribution of animals according to TPI: 1	2	3	4	5	Mean TPI[f]
1	B5B5 Viable WBC	13	315[A]	23	23	39	15	0	2.5[A]
	B5B5 Viable RBC	14	363[A]	36	21	36	0	7	2.2[A]
	B2B2 Viable WBC	11	<2[B]	18	18	36	9	18	2.9[A]
	Uninoculated	10	<2[B]	20	20	40	10	10	2.7[A]
2	B5B5 Viable WBC	10	416[A]	30	20	30	10	10	2.5[A]
	Uninoculated	7	<2[B]	0	57	29	14	0	2.6[A]
3	B5B5 Viable WBC	11	548[A]	18	36	46	0	0	2.3[A]
	B5B5 Viable RBC	7	411[A]	0	29	71	0	0	2.7[A]
	B2B2 Viable WBC	8	<2[B]	0	25	50	25	0	3.0[A]
	Uninoculated	7	<2[B]	14	44	14	14	14	2.7[A]
4	Line 100 Viable WBC	11	32[A]	45	9	18	18	9	2.4[A]
	Line 100 Viable RBC	15	20[A]	33	13	33	13	7	2.4[A]
	Uninoculated	15	<2[B]	47	7	13	20	13	2.5[A]

[a] Tumor profile index

[b] All (6-1 X 15-1)F4 cells except in experiment 4 where Line 100 cells were used.

[c] Antisera collected at 49 days of age.

[d] Antiserawere titered for activity toward the immunizing cell type; samples from uninoculated controls were titered for activity toward (6-1 X 15-1)F4 B5B5 RBC except in experiment 4 where Line 100 RBC were used.

[f] Means within the same experiment with no superscripts in common are significantly different, $P \leq 0.05$.

chickens. Experiments 1, 2, and 3 gave mean TPI's ranging from 2.2 to 3.0. Experiment 4 was designed to investigate whether line 100 cells would act similarly to (6-1 X 15-1) B5B5 cells and produce cross-reactivity between host and tumor. Line 100, which is B2B2, normally has a high incidence of progressing tumors as do B5B5 chickens (Collins et al. 1980). However, the effect of immunization was no different in experiment four than in the previous three experiments.

In summary, three different techniques of immunization of (6-1 X 15-1) F4 generation B2B2 chickens with B5B5 cells were used to attempt to improve the immune response toward RSV-1--induced tumors. In no case, however, was tumor growth significantly altered. The first subcutaneous immunization experiment suggested that immunization might assist in eliminating the tumor, but the second subcutaneous immunization experiment, the intraperitoneal immunization experiments and the intravenous immunization experiments did not support this result. Immunization with line 100 cells, also, did not alter tumor growth.

CHAPTER IV

DISCUSSION

PARTIAL TOLERANCE EXPERIMENTS

(6-1 X 15-1) B2B2 chickens partially tolerant to B5 antigen had a significantly higher incidence of RSV-1--induced tumor progression than untreated B2B2 chickens. It appears likely that B5 antigen cross-reacted with tumor associated antigen and thereby severely limited B2B2 host recognition of the tumor as foreign.

Partial tolerance may involve blocking factors and/or suppressor cells (Brent et al. 1976). The absence of both complete and incomplete antibody at four weeks of age toward the cells inoculated indicated that antibody did not mediate the partial tolerance observed. The partial tolerance produced was specific because partially tolerant B2B2 chickens frequently accepted B5B5 skin grafts but always rejected B24B24 grafts.

The B genotype has a major influence on the ability of the chicken to regress RSV-induced tumors (Collins et al. 1977, Schierman et al. 1977). B2B2, B2B5, and B5B5 chickens from an F2 generation cross of lines 6-1 and 15-1 had mean TPI's of 2.9, 3.8, and 4.8, respectively (Collins

et al. 1977). The data presented here indicated a close association between B5 cell surface antigen and progression of RSV-1--induced tumors. Mortality from RSV-1--induced tumors was higher in B2B2 chickens partially tolerant to B5B5 cells than in normal B2B2 chickens. The B-F region antigen(s) was the only MHC antigen found on both WBC and RBC (Hala et al. 1977). The involvement of both WBC and RBC antigens in tumor growth suggests, therefore, that the class of molecules coded for by the B-F region may have contributed to increased tumor growth.

Not all B2B2 chickens made partially tolerant to B5 antigen showed the same high degree of tumor progression as observed in B5B5 chickens by Collins et al. (1977). A few B2B2 chickens inoculated with cells from B5B5 individuals had low TPI's (i.e. 1 or 2) which lowered the mean TPI. Partial tolerance may be stronger in some individuals than in others. None of the treated chickens tested had detectable antibody toward the B5 cell surface antigens. Skin grafts from B5B5 donors were rejected, however, by 72 and 79 percent of the partially tolerant chickens inoculated with viable and lysed WBC, respectively, from B5B5 chickens. Variation in the degree of partial tolerance would be expected to increase the variation among the TPI's. Furthermore, tolerance may have been present at 4 to 5 weeks of age but may have disappeared prior to the end of the experiment (14 weeks of age) allowing some of the B2B2 hosts to recognize the tumor as foreign and belatedly to mount an

effective anti-tumor immune response. B2B2 hosts having progressive tumors may have a strong state of tolerance to B5 antigen which, if present for only a short period of time, might permit establishment of the tumor and prevent its ultimate rejection. In the mouse strong neonatally induced transplantation tolerance is much more difficult to establish when donor and recipient differ at the K region of the MHC than when they differ at the D and/or I regions (Bolan et al. 1978). In the chicken the total subregion structure of the B2 and B5 haplotypes is not known. Therefore, these two haplotypes could be similar in regions that would otherwise promote strong tolerance. Alternatively, the gene coding for the cross-reacting antigen may be located in a region of the MHC which does not promote strong tolerance.

Both RBC and WBC cell surface antigens contributed to altered tumor growth in partially tolerant B2B2 tumorous hosts, but tolerance to RBC antigens had no effect on acceptance of skin grafts. Possibly this difference between the tumor and skin graft results may be explained by the difference between the density of the B-F antigens on the RBC versus the WBC. B-F antigens on the RBC are only 20 percent as numerous as those on the WBC (Ziegler and Pink 1978). The lower antigenic dose on the RBC may be insufficient for skin graft acceptance but adequate to inhibit the immune system during growth of an RSV-induced tumor.

Simonsen (1955) showed that tolerance to chicken RBC in the turkey led to increased susceptibility of the normally resistant mature turkey to RSV. Subsequent studies showed that human group A erythrocytes, sheep erythrocytes, and chicken tissue extracts each have the property of altering the susceptibility of turkeys to RSV. All have in common a Forssman-like heterophile antigen (Harris and Simons 1958). The B5 antigen--RSV-induced tumor relationship may be a similar example which, in the B5B5 chicken leads to tumor progression.

General antigen competition or a non-specific effect of chronic GVH does not appear to provide an explanation for the partial tolerance observed because: a) Tolerant B2B2 chickens rapidly rejected skin grafts from B24B24 chickens, while accepting skin grafts from B5B5 chickens indicating specificity in the partial tolerance; b) The response to the second antigen (i.e. RSV-1--induced tumor) persisted for ten weeks, a situation not common with antigenic competition (Gross and Erdinger 1974); c) Lysed WBC and viable RBC contributed to increased tumor growth but not to GVH; and d) Tumor growth patterns were significantly changed; whereas in normal B2B2 chickens many completely regressed their tumors, under partial tolerance many chickens died with tumor.

B5 antigen may cross-react with antigenic determinants on tumor associated antigen, thereby severely limiting recognition of RSV-1--induced tumor as foreign. Rapid tumor

growth could overcome a small immune response to the tumor and lead to the death of the host. B2B2 individuals regress RSV-1--induced tumors presumably due, in part, to a strong recognition of the tumor as foreign. (6-1 X 15-1) F2 generation B5B5 chickens had a significantly higher incidence of tumor progression than B2B2 chickens (Collins et al. 1977). Since tumors in both B2B2 and B5B5 individuals were induced by the same subgroup (A) and quantity of virus, these tumors share common subgroup specific and group specific determinants as well as the non-virion tumor specific surface antigen. If B5 antigen cross-reacts with any of these common antigenic determinants, B2B2 individuals tolerant to the B5 antigen should show increased tumor progression. The mechanism of the partial tolerance is not important to this hypothesis. Required is that cells from B5B5 chickens elicit a state of specific immune paralysis which in turn affects RSV-1--induced tumor growth.

CYTOTOXICITY AND ALLOANTISERA EXPERIMENTS

The MHC of the chicken has been shown to code for at least three antigens, the B-G found on RBC, the B-I found on WBC, and the B-F found on both RBC and WBC (Bala et al 1977). The alloantisera used in the present experiments were raised against, and agglutinated, WBC and presumably contained both anti--B-F and anti--B-L antibodies. By

testing these antisera against RBC, which have no B-L determinants, agglutination was a result of the anti--B-F antibodies. The B-F antigen is associated with B2-microglobulin--like molecules making B-F gene products similar to K and D molecules in mice (Ziegler and Pink 1978). The allcantisera absorption studies showed that the RSV-1--infected and uninfected CEF shared allcantigens, in particular B-F region antigens, with syngeneic RBC.

Since B2B2 cytotoxic lymphocytes (CL) primed against RSV-1--induced tumor specifically lysed RSV-1--infected B2B2 CEF and both RSV-1--infected and uninfected B5B5 CEF, cross-reactivity between B5 antigen and tumor associated antigen (TAA) was indicated. Cell-mediated lympholysis (CML) assays have been used to show cross-reactivity between H-2 antigens and tumor associated antigen in the mouse. Parmiani et al. (1979) showed that CML-defined antigenic determinants which cross-reacted with H-2(k) were present on BALB/c sarcoma C-1, although they were less immunogenic than the H-2(d) original determinants. Normal SJL (H-2(s)) lymphocytes sensitized in vitro to syngeneic reticulum sarcomas generated effectors capable of lysing not only syngeneic SJL tumors but also H-2(d) or H-2(k) allogeneic Con A blasts or neoplastic cells (Roman and Bonavida 1979). Moreover, SJL lymphocytes sensitized to H-2(b) or H-2(d) cells efficiently lysed neoplastic SJL cells in vitro. Russell et al. (1979) showed in BALB/c mice that cytotoxic T lymphocytes (CTL) to the BALB/c myeloma tumor MOPC-167

caused essentially no lysis of eight other BALB/c tumors, including three myeloma tumors, but extensively lysed six out of nine tumors of DBA/2 mice. Their observations suggested that MOPC-167 expressed an alloantigen normally absent in BALB/c mice but present in DBA/2 mice and in many DBA/2 tumors.

The lysis of B5B5 CEF by B2B2 CL primed toward B2B2 RSV-1--induced tumor was due to cross-reactivity between B5 antigen and tumor associated antigen not to a common B5-B2 antigen specificity. Prat et al. (1978) and Robinson and Schirrmacher (1979) showed that amplification of previously undetected public H-2 antigens on tumor cells took place on lymphomas of SJL (H-2(s)) and DBA/2 strains. Furthermore, cross-reactivity which cytotoxic T cells display on different H-2 haplotypes may be attributable to clones against shared determinants and not to low affinity binding of cytotoxic T cells activated by private H-2 antigen (Schnaql and Boyl 1980). Therefore, shared public antigens may be the cause of some cross-reactivity. Although in the chicken B2 and B5 MHC antigens may share public specificities, priming the B2B2 CL against autologous RSV-1--induced tumor prevented immunity against shared public specificities.

Alloantisera absorption studies, also, indicated B5-TAA cross-reactivity. The data showed that absorption with RSV-1--infected B2B2 CEF significantly lowered the B5B5 RBC agglutination titer of B2B2 anti-B5B5 antisera suggesting

cross-reactivity between RSV-1--infected cells and B5B5 RBC. Further evidence for cross-reactivity was sought by investigating the possibility that IgG in serum from B2B2 chickens with regressing RSV-1--induced tumors, detected by fluorescent antibody technique to bind to B5B5 RSV-1--induced sarcoma cells, may also bind to B5B5 RBC, WBC or CEF. If B2B2 anti-TAA IgG would bind to B5B5 cells without TAA being expressed on the cells then B5-TAA cross-reactivity would be indicated. Serum samples were collected at two week intervals until 70 days post--RSV-1 inoculation, but in no case was cross-reactivity detected. Thus, two different approaches to serological detection of cross-reactivity gave different results possibly due to antisera reacting to different antigenic determinants. Evidence from studies on mutants in mice suggested that serologically defined determinants and CL defined determinants were two distinct epitopes on the same molecule (Nathenson et al. 1977). It may not be unusual, therefore, that in the chicken strong cross-reactivity was observed with CL technique but not with serological methods. Although it has been frequently observed that animals bearing RSV-induced tumors mount both cellular and humoral anti-tumor immune responses there is good reason to believe that the cellular immune system may play the more effective role (Wainberg and Phillips 1976, Israel and Wainberg 1977, Hall et al. 1979). Cross-reactivity involving cell-mediated immunity may make the difference between tumor

regression or progression.

IMMUNIZATION EXPERIMENTS

B2B2 chickens partially tolerant to B5 antigen demonstrated increased growth of RSV-1--induced tumors, but immunization of B2B2 chickens with B5 antigen, although giving antibody titers toward the B5 antigen, did not result in decreased growth of tumors. Therefore, the immunization experiments did not support the evidence from the partial tolerance, cytotoxicity, and allcantisera experiments; that B5 antigen cross-reacts with TAA. Transplantation techniques have been used successfully in mice to immunize against tumors. Immunity to DBA/2 (H-2(d)) or C3Hf (H-2(k)), but not to AKR (H-2(k)) tissue, induced in BALB/c (H-2(d)) mice a resistance against challenge of syngeneic ST2 sarcoma (Invernizzi and Parmiani 1975, Parmiani and Invernizzi 1975, Invernizzi et al. 1977b). Immunization of C3Hf, but not (C3Hf X A)F1 mice with normal B10.A or A tissue, induced transplantion immunity to subsequent challenge of C3Hf lung tumor expressing H-2(a)-like determinants (Martin et al. 1976, Martin et al. 1977).

The immunizations may have failed to influence tumor growth because they did not elicit an adequate immune response. A strong humoral immune response, but an inadequate cell-mediated immune response, may have been present. Transplantation of skin grafts and subsequent

graft rejection may be required to obtain a strong cell-mediated immunity that would effect the tumor and therefore, indicate B5-TAA cross-reactivity.

GENERAL DISCUSSION

Invernizzi and Parmiani (1975) and Parmiani and Invernizzi (1975) concluded that cross-reactivity of tumor antigens with allogeneic MHC antigens was due to either modification of existing histocompatibility antigens or to products of derepressed silent histocompatibility genes. Perry and Greene (1980) suggested that specificity of T cell recognition with regard to tumor antigen and alloantigen reflects the different associative context in which these determinants are recognized. They concluded that interaction of tumor antigen and host macrophage I-A determinants may create a complex antigenic structure which resembles antigen encoded by the K or I-A subregions of the foreign H-2 haplotype, at the level of T cell recognition.

H-2 restricted cytotoxic lymphocytes specific for minor histocompatibility antigens (Bevan 1977), Sendai virus (Finberg et al. 1978), H-Y antigen (von Boehmer et al. 1979), and Herpes simplex virus (Pfizenmaier et al. 1980), selectively cross-reacted with allogeneic MHC determinants. Pfizenmaier et al. (1980) concluded that H-2D(k) plus Herpes simplex virus antigenic determinants may evoke new antigenic determinants similar to those expressed by allelic

variants of MHC products, or that cross-reactivity may be due to cross-reaction of the anti-foreign receptor on the CTL. Ivanyi et al. (1980) showed that in BALB/c mice syngeneic immunization with normal lymphoid cells induced alloreactive antibodies with high cytotoxic titer. They hypothesized that virus-modified H-2(d) antigenic determinants had triggered allcreactive B-cell clones to produce anti--H-2 antibodies. Hale (1980) demonstrated the existence of a close spatial relationship between a serologically defined portion of the G protein of vesicular stomatitis virus (VSV), the H-2K(k) molecules and those antigens (virus and cell specific) recognized by anti-VSV CTL's.

If cross-reactivity between B5 antigen and tumor specific antigen was direct this would explain the high degree of tumor progression observed in B5B5 chickens. Direct cross-reactivity would make it difficult for B5B5 chickens to recognize RSV-1--induced tumor as foreign and would severly limit the development of an effective anti-tumor immunity. On the other hand, the cross-reactivity observed may have been due to a B2--RSV-1 antigen complex which cross-reacted with a B5 antigen. In this case cross-reactivity would be observed only in the context of the B2 haplotype. Thus, in the B5B5 host there may be a cell surface antigen which, acting in the manner of the B2 antigen, would react with the tumor antigen to form a complex. This complex in turn, would cross-react with a B5

MHC antigen and lead to non-recognition of the tumor as foreign and subsequent tumor progression.

If cross-reactivity of B5 antigen with RSV-1--induced tumor antigen constitutes a common mechanism of tumor progression this phenomenon would go far toward explaining the variety of host responses to RSV-1--induced tumors observed by Collins et al. (1980) in several highly inbred lines. Since all members of the avian tumor virus group induce tumors with common antigenic determinants (Hall et al. 1979), the response of chickens to avian leukosis virus may also be mediated by a similar mechanism.

To determine more clearly the relationship of the observed cross-reactivity to tumor progression in B5B5 chickens, immunochemical studies of the exact nature of B2, B5, and the cross-reacting antigen are required.

QUESTIONS RAISED BY THIS RESEARCH

1. What is the nature of the cross-reacting antigens? Biochemical definition of the antigens is required and monoclonal antibody would be useful in the isolation procedure. Knowing the chemical structures of the molecules involved would help to determine whether or not the cross-reactivity was direct or due to a B2--RSV-1 antigen complex. If an antigen complex is involved it is important to determine if a similar complex can form in the B5B5 host. If a complex cannot form then

cross-reactivity is an artifact of the system being studied and has no role in tumor progression in normal B5B5 chickens. Information on the relationship between tumor associated antigen and MHC antigen would be gained in any case.

2. Would long-term (> 90 days) transplantion tolerance to B5 antigen in (6-1 X 15-1) B2B2 chickens lead to complete RSV-1--induced tumor progression? Repeated IV inoculations have been shown to elicit long-term tolerance in chickens (Schierman and Nordskog 1964). Anti-tumor immunity may not develop before the tumor kills the host in chickens long-term tolerant to B5 antigen. Long-term tolerance of B2B2 chickens to B5 antigen may show all tolerant chickens dying of RSV-1--induced tumor. This result would eliminate variation among IPI's and give strong evidence for B5-TAA cross-reactivity.
3. Would immunization of (6-1 X 15-1) B2B2 chickens with B5B5 skin grafts enhance the cell-mediated immune response against RSV-1--induced tumors? Cell-mediated immunity may be more important than humoral immunity in RSV-1--induced tumor regression (Hall et al. 1978). Graft rejection is considered to be mediated, primarily, by cellular effector mechanisms (Klein 1979). Immunization with B5 grafts, leading to a strong cell-mediated immunity, therefore, may affect tumor growth and indicate B5-TAA cross-reactivity.

4. How strong is MHC restriction in the chicken? According to the theory of MHC restriction B2B2 cytolytic lymphocytes should only kill B2B2 RSV-1--infected cells and not B5B5 RSV-1--infected cells. In this thesis B2B2 cytolytic lymphocytes were observed to kill both of the above mentioned cells and this was used as evidence in support of cross-reactivity. However, if MHC restriction is weak in the chicken then such killing would not be an uncommon situation and would do little to support the hypothesis of B5-TAA cross-reactivity. In any case, the fact that B2B2 cytolytic lymphocytes primed to RSV-1--induced tumor will kill normal B5B5 cells was the strongest evidence of cross-reactivity.
5. Do the MHC regions contain multiple loci and would this complicate cross-reactivity detection? Multiple loci in syngeneic individuals with different patterns of gene expression may lead to variable results.
6. Would cross-reactivity patterns change with the use of a different RSV subgroup or clone? McBride et al. (1980) observed significant variation in the incidence of tumor regression between chickens from the same highly inbred line inoculated with different RSV subgroups and clones; no significant variation within RSV type was observed. In this case different antigens may be expressed on tumor cells depending on the virus type.

7. If cross-reactivity is a mechanism of tumor progression in nature is it general or limited to a few specific cases? The B5 and B2 MHC antigen system may present a unique case in which cross-reactivity with BSV-1 tumor associated antigen is present. On the other hand if tumor associated antigen chemical structure is closely related to general MHC antigen chemical structure and/or if TAA-MHC antigen complexes are necessary for anti-tumor immunity to develop, then TAA-MHC antigen cross-reactivity may be a general phenomenon.

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Collegiate Institutions Attended	Dates	Degrees
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Honors or Awards:

Selected for participation in the International Union against Cancer, Sixth Training Course in Cancer Research held at The Jackson Laboratory, Bar Harbor, Maine; Sept. 9-21, 1979.

Sigma Xi - UNH Chapter; 1979 - present.

Project assistant at UNH, NIH grant (CA-17680); 1978 - present.

Teaching assistart in genetics at UNH; 1977.

Graduated with honors in the major of Biology from Colby College, Waterville, Maine; May 1977.

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Publications:

Heinzelmann, E. W., Zsigray, R. M., and Collins, W. M.: Increased growth of RSV-induced tumors in chickens partially tolerant to MHC alloantigens. Immunogenetics (In Press).

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