Genetic associations of interleukin-2 levels in the chicken

by

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LIST OF ABBREVIATIONS

α-MM - α-D-mannopyranoside

Bx - chicken MHC allele

CM - conditioned medium

Con A - concanavalin A

CTL - cytotoxic T lymphocyte

Ea-B - chicken B blood group gene locus

GAT - poly (glutamic acid^{60}-alanine^{30}-tyrosine^{10})

IFN-γ - gamma interferon

IL-1 - interleukin-1

IL-2 - interleukin-2

MHC - major histocompatibility complex

MLC - mixed lymphocyte culture

MLR - mixed lymphocyte reaction

MRC - mitogen-red blood cell complex

NK - natural killer cell

PBL - peripheral blood lymphocyte

PHA - phytohemagglutinin

RBC - red blood cell

RSV - Rous sarcoma virus

T_H - helper T lymphocyte

T_S - suppressor T lymphocyte

INTRODUCTION

Interleukin-2 (IL-2) is a soluble immunomodulator that plays a central role in cell-mediated immunity by augmenting the proliferation and activity of T lymphocytes. Stimulation of these cells requires not only activation by an antigen, but activation by an antigen in association with the gene products of the major histocompatibility complex (MHC). Non-MHC loci also affect the immune response. The genetic control of the immune response, therefore, is mediated by gene loci encoded within and outside the MHC.

There have been a paucity of reports describing the genetic control of IL-2 production. It has been demonstrated that MHC loci does not affect levels of IL-2 production (for a review, see Farrar et al., 1982). Non-MHC gene loci, however, have been shown to influence levels of production of and response to IL-2 in mice (Wofsy et al., 1981; Kawamura et al., 1986).

A great deal of work has been aimed at determining the mechanism of action of IL-2 in the mammalian immune system. Studies of avian IL-2, however, have not received the same attention. The paucity of reports concerning avian IL-2 has been primarily due to the absence of an efficient and effective assay to measure avian IL-2 activity. Recently, Kromer et al. (1984) have made the study of avian IL-2 possible by developing an assay to measure avian IL-2 activity.

The purpose of this study was to utilize the avian IL-2 assay to determine whether levels of IL-2 production are genetically controlled. The diversity of genetic stocks available at the Iowa State University

Poultry Research Center, which differ at their MHC and/or non-MHC regions, are a useful tool for determining whether any genetic influences that may be present can be attributed to MHC or non-MHC effects. Therefore, relative levels of IL-2 production of the various genotypes were measured to determine whether MHC and/or non-MHC influences mediate levels of IL-2 production. In addition, once levels of IL-2 production have been determined, relative abilities to produce IL-2 were compared with their known responses to different antigenic challenges to determine whether levels of IL-2 production correlate with the ability to mount an immune response to an antigenic challenge.

LITERATURE REVIEW

The discovery that lymphocyte culture supernatants contained soluble mitogenic factors was made possible by the previous observation that plant lectins promoted the proliferation of T cells *in vitro* (Kasakura and Lowenstein, 1965; Gordon and MacLean, 1965). Since 1965, many mitogenic factors for lymphocytes have been reported including: thymocyte stimulating factor (TSF), thymocyte mitogenic factor (TMF), T cell growth factor (TCGF), co-stimulator, killer cell helper factor (KHF), and secondary cytoxic T cell inducing factor (SCIF) (Farrar et al., 1978; Gillis et al., 1978; Shaw et al., 1978; Wagner and Rollinghoff, 1978). The plethora of mitogenic factors that had similar functions and were being reported under different names created a great deal of confusion in the field. In 1979, researchers met at the Second International Lymphokine Workshop and determined that each of these mitogenic factors were identical and resolved to designate this soluble mitogenic factor interleukin-2 (IL-2) (Aarden et al., 1979).

Cellular Functions of IL-2

IL-2 is described as a soluble immunoenhancing factor produced primarily by helper T cells (T_H) following activation with antigen or mitogen in the presence of interleukin-1 (IL-1) (Farrar et al., 1982). Some of the immunoenhancing properties of IL-2 include: the promotion of mitogen- or antigen-stimulated T cell proliferation, the maintenance of cytotoxic T cell (CTL) lines, and the augmentation of T cell proliferation in mixed lymphocyte reactions (for review, see Farrar et al., 1982). The production of and response to IL-2 is antigen dependent

(Smith, 1984). However, levels of production of and receptor expression for IL-2 have been shown to have some degree of genetic regulation (Wofsy et al., 1981; Kawamura et al., 1986).

Immunoenhancing properties of IL-2

Following the observation by Morgan et al. (1976) that conditioned media from lectin-stimulated mononuclear cells contained a soluble factor that would support the growth of lectin-activated T cells, researchers began to investigate the function of this soluble factor (IL-2) in T cell proliferation. IL-2 was determined to be necessary for the long-term growth and clonal expansion of CTLs since these cells do not grow in the absence of IL-2 while the addition of exogeneous IL-2 will promote their growth (Gillis and Smith, 1977a; Gillis and Smith, 1977b). Wagner and Rollinghoff (1978) later provided evidence that IL-2 producing cells were distinct from IL-2 responding cells. They observed that Ly 1^+ (T_H) cells were responsible for production, whereas antigen-primed Ly 23^+ (CTL) cells responded to IL-2.

Concurrently, investigators observed that another soluble factor, IL-1, which is released by macrophages, also appeared to play a role in the proliferation of T cells as well as the enhancement of IL-2 production. To differentiate and determine the role of these soluble factors in T cell activation, an experiment using resting splenic T cells as well as blast T cells cultured with or without concanavalin A (Con A) in the presence or absence of IL-1 and/or IL-2 was performed (Larsson et al., 1980b). They found that the growth induction of resting T cells was greatly enhanced by the addition of both Con A and IL-1 compared to

either added alone. The addition of IL-2 alone or IL-2 and IL-1 to the resting T cell cultures did not induce their growth. However, the addition of IL-2 with Con A did promote T cell growth. Conversely, the addition of Con A and IL-1 did not enhance the proliferation of the blast cells. In addition, the presence of IL-2 in any combination or alone induced the growth of the T cell blast cultures. A similar experiment using phytohemagglutinin (PHA) showed that IL-2 production was dependent on the presence of both the lectin and IL-1 (Smith, 1980). Therefore, there appeared to be two signals required for IL-2 production: lectin or antigen attachment to the T cell and the simultaneous activation of these T cells by IL-1.

The immunoenhancing properties of IL-2 have been defined by its role in the in vitro T cell culture systems. After it was determined that the soluble factor found in the culture supernatants played an intricate role in T cell proliferation, investigators began to use the supernatant as a tool to study the processes involved in T cell responses.

Following the observation by Morgan et al. (1976) of a soluble factor that would promote T cell proliferation, Gillis and Smith (1977b) showed that this factor would support the long-term growth of CTLs while maintaining specificity for the antigen which initially activated them. Early studies using primary mixed lymphocyte cultures (MLC) suggested Ly 1^+ (T_H) cells amplify Ly 23^+ (CTL) cells via a soluble factor (Altman and Cohen, 1975; Plate, 1976).

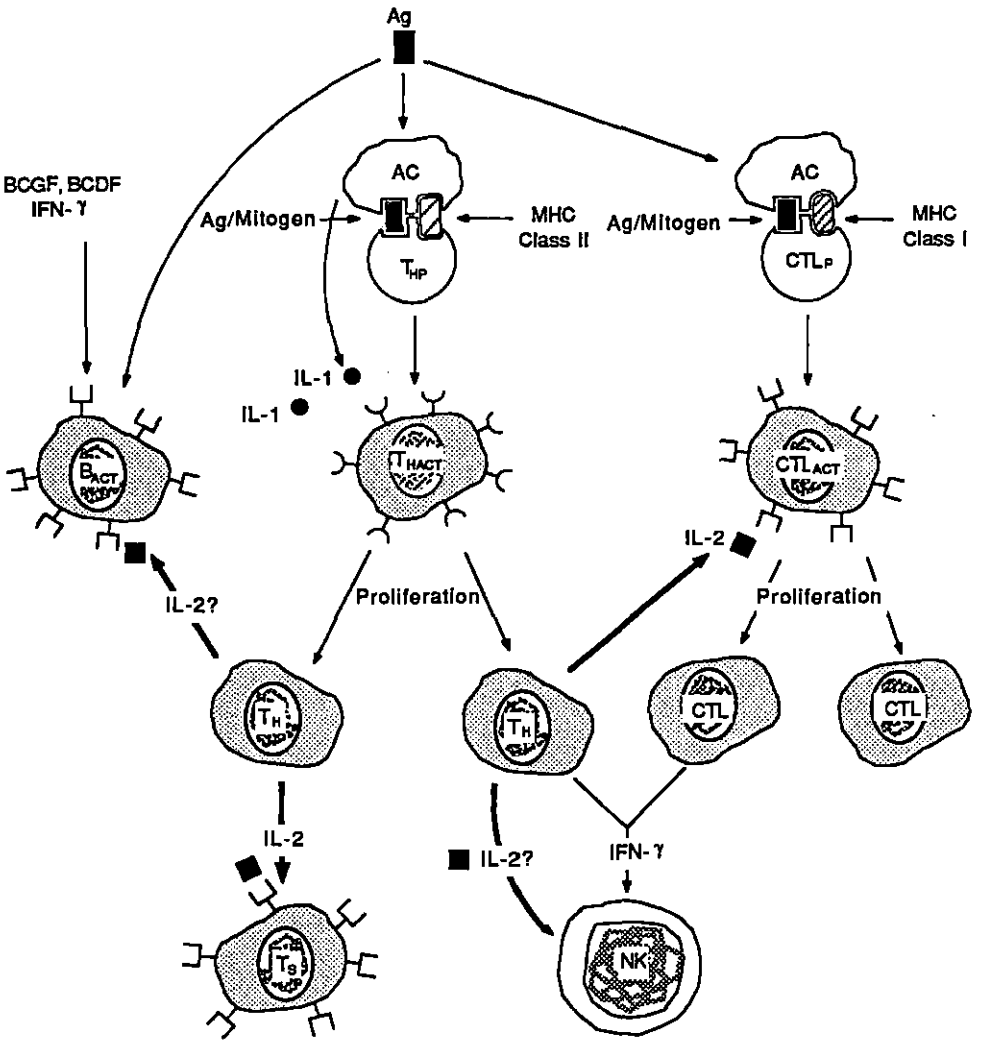
Wagner and Rollinghoff (1978), using secondary MLCs, provided definitive evidence which established the role of IL-2 and identified the

cells which produced and responded to it. Their experiment was based on three previous observations: 1) secondary MLCs contained a soluble factor that enhanced the activation of CTLs (Uotila et al., 1978; Ryser et al., 1978), 2) the *in vitro* addition of Con A, a polyclonal activator of T cells, to *in vivo*-sensitized alloimmune T cells enhanced secondary CTL responses with specificity to the alloantigen used for the primary sensitization (Heininger et al., 1976; Bonavida, 1977), 3) CTLs specific for H-2K or H-2D (Class I) alloantigens were stimulated in a secondary MLC by cells sharing only the I region (Class II) alloantigens (Alter et al., 1976). With these observations in mind, Wagner and Rollinghoff (1978) conducted a study using supernatants from Con A activated T cells or I region incompatible MLCs and found that both were effective in inducing H-2K or H-2D specific CTL responses. Since it had already been demonstrated that Ly 1^+ (T_H) cells recognize the I region whereas Ly 23^+ (CTL) cells recognize the K and D regions, they postulated that activated Ly 1^+ T cells produced IL-2 and previously stimulated Ly 23^+ cells responded to IL-2 (Cantor and Boyse, 1975; Nagy et al., 1976). Therefore, two T cell subclasses were necessary for an IL-2-dependent T cell response, the activated-T_H cell which was responsible for production of IL-2, and the antigen-primed CTL which responded to IL-2.

Once the role of the soluble factors, IL-1 and IL-2, and the cells involved in T cell proliferation had been defined, the events leading to a cell-mediated response could be determined. The role of IL-2 in the enhancement of CTL proliferation occurs as follows (Figure 1) (Farrar et al., 1982; Robb, 1984): 1) An accessory cell (e.g., macrophage)

Figure 1. Cellular interactions influenced by IL-2

Ag, antigen; AC, accessory cell; MHC, major histocompatibility complex; T_{HP}, precursor of helper T cells; T_{HACT}, activated helper T cell; T_H. helper T cell; IL-1, interleukin 1; IL-2, interleukin 2; CTL_P, cytotoxic T lymphocyte precursor; CTL_{ACT}, activated cytotoxic T lymphocyte; NK, natural killer cell; T_S, suppressor T cell; IFN-γ, gamma interferon; BCGF, B cell growth factor; BCDF, B cell differentiating factor; B_{ACT}, activated B cell.


Ag
AC
AC
BCGF, BCDF
IFN-γ
Ag/Mitogen
MHC
Class II
Ag/Mitogen
MHC
Class I
IL-1
IL-1
IL-2
IL-2?
Proliferation
Proliferation
IL-2
IL-2?
IFN-γ
CTL
CTL
NK

attaches to an antigen or mitogen. 2) A precursor T_H cell becomes activated to respond to IL-1 after prior recognition of the antigen or mitogen along with the class II determinant on the accessory cell. 3) IL-1 is released by the accessory cell and the activated T_H cell undergoes blastogenesis and proliferates to a T_H cell that secretes IL-2. 4) Concurrently, a precursor CTL cell has combined with another accessory cell via recognition of the antigen or mitogen along with the class I determinant. This cell has now been activated to produce receptors for IL-2. 5) Upon stimulation by IL-2, the CTL undergoes blastogenesis and production of mature CTLs occur which have specificity for the antigen that activated it.

Shortly after it was demonstrated that IL-2 augmented CTL responses, additional immumoenhancing properties of IL-2 on other cell types were reported. IL-2 was also shown to activate the other functional T cell subtypes which include: T_H (except those that secrete IL-2), T suppressor (T_S) and T delayed hypersensitivity (T_{DH}) cells (Fishbein et al., 1983; Palacios, 1982; Farrar et al., 1982). In mice, enhancement of natural killer (NK) cell cytoxicity activity by the addition of IL-2 was observed (Henney et al., 1981). Follow-up studies, using cultures free of gamma interferon (IFN-γ), demonstrated IL-2 induced IFN-γ production by both T_H and CTL cells which, inturn, promoted NK activity (Weigent et al., 1983; Kasahara et al., 1983; Trinchieri et al., 1984). In addition to the enhancement of T cell proliferation and activity, IL-2 was able to substitute for T_H cells in T cell-dependent antibody formation (Farrar et al., 1978; Farrar et al., 1982). However, the mechanism of B cell

activation by IL-2 is unclear. It has been demonstrated that activated B cells grow poorly in IL-2 containing supernatants, B cells and B cell blasts do not absorb IL-2 activity in the murine system, and clonal expansion of hapten-specific B cells required soluble factors distinct from IL-2 (Smith and Ruscetti, 1981; Leibson et al., 1981; Pike et al., 1982). Despite these observations, other studies have suggested that activated B cells express receptors, similar to those of activated T cells, which were able to bind to IL-2 and promoted B cell proliferation (Zubler et al., 1984; Mingari et al., 1984; Muraguchi et al., 1985). There was also evidence that IL-2 promotes Ia expression on resting B cells which increased the antibody forming cell's ability to bind to macrophages thus enhancing the antigen presenting phase of antibody formation (Roehm et al., 1984).

Therefore, IL-2 has been demonstrated to play a pivotal role in the proliferation of cells involved in both the cell-mediated and humoral immune responses. In addition, it was shown to augment the production of IFN-γ, which promotes NK activity during viral infections. Moreover, the amount of proliferation of these cells has been shown to be mediated by the levels of IL-2 present (Gillis et al., 1978).

Regulation of IL-2

As described earlier (Figure 1), activation by antigen or lectin is necessary for production of and response to IL-2. Once these cells have been activated, however, the extent of their proliferation has been shown to be dependent on the levels of IL-2 present (Gillis et al., 1978; Smith et al., 1980). This concept, regulation by an antigen-nonspecific factor

(IL-2) of T cell clonal expansion (which is antigen specific), presented an enigma to immunologists until the IL-2 receptor system was realized (Smith, 1984). The influence of suppressor factors on IL-2 production has provided useful information in defining the role of IL-2 in the maintenance of immunological homeostasis (for review, see Asherson et al., 1986). Additional studies have also shown that genetic regulation of IL-2 and its receptor were a factor in the levels of T cell proliferation (Kawamura et al., 1986; Wofsy et al., 1981).

The IL-2 receptor has been described as having high- and low-affinity binding sites (Robb et al., 1984b). The high affinity receptor (K_d = 5-20 pM) was shown to be expressed on T cells following activation by antigen or lectin and was able to promote DNA synthesis resulting in T cell proliferation following induction by IL-2 (Robb et al., 1984a; Smith and Cantrell, 1985; Reske-Kunz et al., 1986). Conversely, T cell activation by lectin or antigen did not induce proliferation via the low-affinity receptor; its physiological role has not been determined at this time (Smith and Cantrell, 1985; Reske-Kunz et al., 1986). Binding studies, using AMT-13 (monoclonal antibody to the murine IL-2 receptor) and anti-Tac (monoclonal antibody to the human IL-2 receptor), have shown that both the high- and low-affinity receptors interacted with these monoclonal antibodies resulting in the decreased availability of the IL-2 binding sites (Osawa and Diamantstein, 1985; Robb et al., 1984a). However, the most remarkable observation that arose from these studies was that IL-2 regulated its own receptor (Robb et al., 1984a; Smith and Cantrell, 1985; Reske-Kunz et al., 1986). The effect of IL-2 on its

receptor has been a subject of controversy.

Smith and Cantrell (1985) used purified IL-2 with preactivated human peripheral blood mononuclear cells and observed that IL-2 lowers the number of high-affinity binding sites. They suggested that regulation by IL-2 provided a "fail-safe" mechanism of control over T cell clonal expansion. Because high-affinity IL-2 receptors were only expressed after antigen activation, they believed the presence of IL-2 insured that the number of antigen-specific activated T cells decreased as the antigen was being cleared.

Conversely, Reske-Kunz et al. (1986), using recombinant IL-2 in cloned T cell cultures, found that IL-2 upregulates the levels of high-affinity receptors. The authors suggested that IL-2 played a role in the amplification and prolonged expression of IL-2 receptors. Therefore, high levels of activated T cells could be maintained following antigenic challenge. This model was supported by other studies which demonstrated that IL-2 upregulated IL-2 receptor expression and continued presence of the antigen was not required for the continued proliferation of progeny cells (Malek and Ashwell, 1985; Reske-Kunz et al., 1984). Reske-Kunz et al. (1986) further postulated that clonal expansion continued until contraregulatory mechanisms began to act.

Asherson et al. (1986) have described a contramechanism which maintained the balance between suppression and immunity which was based on the level of IL-2 present. They have suggested that a helper-suppressor circuit existed in which suppressor function dominated when IL-2 was rate limiting, but helper function dominated when abundant IL-2

was present. Therefore, as the level of IL-2 was decreased due to the absorption by the responding T cells, T_S cells (which required less IL-2 to be activated or had a higher affinity for IL-2) removed the remaining IL-2, thus polyclonal activation was terminated.

Many suppressor factors have been reported which influence different phases of IL-2-dependent T cell proliferation (for review, see Asherson et al., 1986). Because the amplification of IL-2 production was IL-1 dependent, inhibitors of IL-1 (e.g., glucocorticoids) consequently inhibited IL-2 production (Smith et al., 1980). IL-2 was shown to be inhibited by high levels of prostaglandin E_2 released by macrophages (Rappaport and Dodge, 1982). A number of reports have described soluble factors contained in supernatants of Con A stimulated-lymphocytes that reduced the production of IL-2, but did not block the interaction between IL-2 and its receptor (Kramer and Koszinowski, 1982; Malkovsky et al., 1982; Kromer et al., 1985). Immunosuppressive drugs, namely, cyclosporin A and dexamethasone also inhibited IL-2 production (Larsson, 1980). However, at low concentrations, cyclosporin A acted by inhibiting the responsiveness to IL-2. Recently, another soluble factor released by Con A stimulated rat T_S cells has been described that inhibited DNA synthesis of proliferating T cells which resulted in failure in production of and response to IL-2 (Chiba et al., 1985). Therefore, influence at the macrophage, the T_H cell, and/or the responding T cell level has been demonstrated.

Genetic regulation of IL-2 production and of expression of the IL-2 receptor has also been demonstrated. Wofsy et al. (1981) have shown that

the murine lpr gene was responsible for decreased production of IL-2 in mice that possessed this gene. Kawamura et al. (1986) have shown that there is genetic regulation of IL-2 receptor levels in mice.

The lpr gene of MRL/MP-lpr/lpr mice has been shown be the cause lymphadenopathy, antibody production to nucleic acids and immune complex glomerulonephritis (which are characteristics of systemic lupus erythmatosis, SLE) in mice (Grant, 1982). To determine the effect of this gene on IL-2 production, Wofsy et al. (1981) transfered the lpr gene into C57BL/6J mice. They found that Con A activated T cells of mice that were homozygous for the lpr gene have a 10-fold decrease in their ability to produce IL-2 compared to the control mice (C57BL/6J-lpr^-/lpr^- or C57BL/6J-lpr^+/lpr^-). Furthermore, the spleen cells from the mice containing the lpr gene had a 100-fold decrease in their ability to respond to IL-2 following Con A stimulation in vitro.

In a recent study, Kawamura et al. (1986) have provided the first evidence of genetic regulation of IL-2 receptor expression in unstimulated murine splenic T cells. They found that there was a 2-fold increase of IL-2 receptor expression in B10.D2 mice compared to DBA/2 or BALB/c mice. The genetic control of receptor expression was not Mls- (mixed-lymphocyte stimulating locus, located on chromosome 1) or H-2-linked. There appeared to be more than one gene responsible. One of the genes has been mapped to chromosome 7, while another has been tentatively placed on chromosome 4.

Biochemical and Molecular Properties of IL-2

The IL-2 Assay

The quick and quantitative microassay for detection of IL-2 activity developed by Gillis et al. (1978) used culture supernatants from mitogen-stimulated murine or rat spleen cells. The IL-2 activity from these supernatants was measured by its ability to promote the proliferation of murine (C57BL/6) tumor specific cytotoxic T lymphocyte lines (CTLL 1 and CTLL 2), which was measured by the incorporation of ^{3}H-thymidine. The mitogens used to stimulate the spleen cells included: Con A, PHA, Pokeweed mitogen (PWM) and Escherichia coli lipopolysaccharide (LPS). They found that the Con A-stimulated spleen cells contained the greatest amount of IL-2 activity followed by PHA, PWM and LPS, respectively. In addition, they found that a 48 hour incubation period with 5 ug/ml Con A produced the highest level of IL-2 activity.

Larsson et al. (1980b) demonstrated that accessory cells were necessary for mitogen-dependent T cell activation. In a recent study, however, Roosnek et al. (1985a) demonstrated that IL-1 with phorbol myristate acetate (PMA) abolishes the need for accessory cells to activate human T cells in vitro. In addition, they demonstrated that thiols, 2-mercaptoethanol (2-ME) and reduced glutathione (GSH), which provided sulfhydral groups, substituted for accessory cells after the accessory cells have been depleted from the medium (Roosnek et al., 1985b). By varying the culture conditions, Roosnek et al. (1985b) have defined the parameters by which mitogen-dependent T cell proliferation promoted IL-2 production in vitro. At low concentrations of Con A (1-5

ug/ml), accessory cells were necessary for IL-2 production. At moderate (7.5-20 ug/ml) or high (greater than 20 ug/ml) concentrations of Con A, however, thiols or the Con A alone, respectively, were able to promote IL-2 production without the presence of accessory cells.

Another consideration of the assay was the choice of lectin used to activate the T cells to produce IL-2. Yamakido et al. (1985) compared the levels of IL-2 produced from human peripheral blood lymphocytes following activation by PHA-M (mucopolysaccharide), PHA-P (protein), Con A, PWM (polyclonal activator of both T and B cells) or LPS (polyclonal activator of B cells). They found that the IL-2 production was greatest from cells stimulated by PHA-P and PHA-M followed by Con A, PWM, and LPS, respectively. It should be noted that a 30-fold greater concentration of PHA-M was required to achieve the same levels of IL-2 production as PHA-P. The IL-2 levels achieved by stimulation with PHA-M, PHA-P, Con A and PWM were high compared to the levels obtained from LPS stimulation. The results agree with those of Gillis et al. (1978) who also found that the B cell polyclonal activator, LPS, would not promote IL-2 production.

After the development of the assay, it was immediately apparent to investigators in the field that the assay needed to be standardized so the results could be compared (Aarden et al., 1979). Consequently, a number of laboratories agreed to prepare and provide partially purified IL-2 samples with defined units of biological activity to be used as standards. One unit of IL-2 activity was defined as the log of the dilution that corresponded to 50% of the maximal proliferation of the

standard (for review, see Gillis et al., 1978). The IL-2 activity of an unknown test sample was then calculated by dividing the log dilution at 50% maximal proliferation of the unknown by the standard.

The assay developed by Gillis et al. (1978) to detect the levels of mammalian IL-2 activity was not effective, however, in detecting avian IL-2 activity until a number of modifications were made (Kromer et al., 1984). First, cloned lines of murine lymphocytes, such as those used by Gillis and his associates, did not respond to avian IL-2 (Schauenstein et al., 1982). This meant that a heterogeneous population of avian splenocytes or peripheral blood lymphocytes would have to be used instead of the homogeneous lines available to investigators studying mammalian IL-2. The use of Percoll differential centrifugation has permitted the separation of the mitogen-stimulated lymphoblasts from the rest of the non-stimulated and non-T cells that may be present (Kurnick et al., 1979). Second, mitogen-red blood cell complexes (MRC) instead of free mitogen have been used to stimulate avian IL-2 producing lymphocytes. This modification stemmed from an observation by Powell (1980) who reported that mitogen-induced proliferation of chicken lymphocytes was enhanced by the addition of red blood cells. Kromer et al. (1984) observed that there was a 3-fold increase of proliferation when the MRCs were used, compared to the same concentration of free mitogen. Finally, crude supernatants from lectin-activated chicken T cells had to be used as standards instead of the purified preparations avaialible to researchers of mammalian IL-2 (Frederickson and Sharma, 1985).

Molecular and biochemical properties of IL-2

The development of the microassay for IL-2 enhanced the ability of immunologists to biochemically characterize IL-2 (Gillis et al., 1982). Human, mouse, and bovine IL-2 have been studied extensively and were found to be 14,000-25,000 molecular weight glycoproteins (mature mouse IL-2 existed as a 30,000 molecular weight homodimer) with isoelectric points (pI) between 4.4 and 7.8 (Mier and Gallo, 1982; Gillis et al., 1982; Watson et al., 1979; Cerretti et al., 1986). Chicken IL-2 was found to have a comparable molecular weight of 13,000 daltons and a pI of 5.9 (Schnetzler et al., 1983; Vainio et al., 1986).

The assay to detect functionally active IL-2 as well as molecular cloning techniques were employed to clone and sequence the IL-2 gene (for review, see Smith, 1984). The genes of human, mouse and bovine IL-2 were found to code for mature polypeptides of 133, 149 and 135 amino acids, respectively (Holbrook et al., 1984; Kashima et al., 1985; Reeves et al., 1986; Cerretti et al., 1986). The calculated molecular weights of human, mouse and bovine IL-2 were 15,420.5, 17,233.5 and 15,464 daltons, respectively. The calculated molecular weights compared to the weights initially reported using electrophoretic analysis of purified preparations. The heterogeneity of size and charge originally observed from electrophoretic analysis of each species' IL-2 has been attributed to post-translational modification in the attachment of the carbohydrate (Robb et al., 1981; Robb et al., 1984b).

The gene for human IL-2 has been shown to exist as a single copy based on restriction endonuclease digestion followed by Southern blotting

analysis of genomic clones of IL-2 genes from normal and malignant lymphoid cells (Holbrook et al., 1984). The 4 exons and intravening sequences consisted of 4,930 base pairs. The amino acid sequence homology between mouse and human, human and bovine, or mouse and bovine was 60%, 65%, or 50%, respectively (Cerretti et al., 1986).

The similarity of sizes, charges and amino acid sequences of IL-2 between different mammalian species has permitted some degree of interspecies activity (Cerretti et al., 1986). Human IL-2 induced the proliferation of both murine and bovine T cell lines (Gillis et al., 1982; Brown and Grab, 1985). However, bovine IL-2 did not induce the proliferation of murine IL-2 dependent T cell lines (Cerretti et al., 1986). Although the size and charge of chicken IL-2 was comparable to mammalian IL-2, interspecies activity did not take place (Schauenstein et al., 1982; Vainio et al., 1986). Vainio et al. (1986) suggested that the lack of stimulatory effects between chicken and mammalian IL-2 demonstrated the long phylogenic distance between mammals and birds. They postulated that the study of the mechanism of IL-2 activity in chicken T cells would be a good starting point in studying the evolution of the molecules that mediate the T cell response.

IL-2 and Immune Disorders

Decreased production of and response to IL-2 has been reported for a variety of infectious disease systems. These included diseases resulting from Trypanosoma cruzi (Chaga's disease), Plasmodium berghei (malaria), Histoplasma capsulatum (systemic mycoses), Mycobacterium leprae (leprosy) and Leishmania major (oriental sore) infections (Harel-Bellan et al.,

1983; Lelchuk et al., 1984; Watson et al., 1985; Mohagheghpour et al., 1985; Cillari et al., 1986). Many primary (present at birth) as well as secondary (acquired) immunodeficiency diseases which have led to an autoimmune state have also exhibited low levels of IL-2 production (for a review see Kromer et al., 1986; Welte and Mertelsmann, 1985). In addition, low levels of IL-2 have been observed in young and old individuals, T cell malignancies and in the state of immunological tolerance (Gilman et al., 1982; Smith, 1984; Malkovsky et al., 1985).

Smith and Talal (1982) have suggested that IL-2 played a role in immunological homeostasis. Their model demonstrated that a certain level of IL-2 had to be present to augment the activity of cells that remove virally or chemically altered cells. Therefore a paucity of IL-2 in autoimmune or lymphoproliferative diseases had permitted the survival of altered or diseased cells.

Recently, the notion that low IL-2 levels were responsible for autoimmunity has been challenged (Kromer et al., 1986). Hefeneider et al. (1984) were the first to suggest high levels of IL-2 production were responsible for autoimmunity. This theory has been supported by a number of observations. First, some autoimmune diseases (e.g., spontaneous autoimmune thyroiditis) produced high levels of IL-2 compared to age and MHC-matched controls (Schauenstein et al., 1985). Second, the defect in Con A-induced IL-2 production originally reported in autoimmune mice was corrected <u>in vitro</u> by the addition of a second mitogen, phorbol myristate acetate (Santoro et al., 1983). Finally, limiting dilution analysis has shown that autoimmune mice had normal to increased numbers of IL-2

producing cells, but their frequencies were lower (Hefeneider et al., 1984; Miller and Stutman, 1982). Hefeneider et al. (1984) demonstrated that sites of the immune response (spleen and mesenteric lymph nodes) contained normal to increased numbers of IL-2 producing cells as well as an extremely high level of non-IL-2 producing cells. They postulated that the paucity of IL-2 production was due to a "crowding" effect created by the adsorption and subsequent proliferation by the non-IL-2 producing cells of the IL-2 present. Therefore, the paucity of IL-2 production was not the mechanism responsible for the autoimmune state, rather, hyperproduction of IL-2 had enhanced the proliferation of autoreactive lymphocytes (Kromer et al., 1986).

The model of hyperproduction of IL-2 was also supported in age-related immune dysfunctions (Kromer et al., 1985). In immunocompetent individuals, immunological homeostasis was maintained by the balance between IL-2 production and suppressor factors (Asherson et al., 1986). Globerson et al. (1982) have shown that inhibitory regulation declined with age. Kromer et al. (1985) postulated that the decreased levels of suppressor factors observed in aged individuals have permitted high levels of IL-2 production; thus, "forbidden" immune reactions were allowed to take place. Malkovsky et al. (1985) have suggested that suppressor factors of IL-2 in young individuals have created an immunological tolerant state permitting the distinction of "self" and "non-self" antigens. In addition, they reported that immunological tolerance was alleviated by the addition of exogeneous IL-2.

Hyperproduction of IL-2 due to the absence of host suppressor factors, however, could not account for low levels of IL-2 production observed in all autoimmune states, such as Nezelof syndrome and severe combined immunodeficiency disease (Kromer et al., 1986; Flomenberg et al., 1983; Lopez-Botet et al., 1982). Moreover, other immune disorders have displayed their own mechanism of inhibiting IL-2 dependent immunity. The infectious agents of T. cruzi, P. berghei, H. capsulatum and M. leprae inhibited the production of IL-2 by suppressing the macrophages' ability to produce IL-1 (Harel-Bellan et al., 1983; Lelchuk et al., 1984; Watson et al., 1985; Mohagheghpour et al., 1984). The progression of leishmaniasis was due to the hypersecretion of prostaglandin E_2 by macrophages which inhibited the production of IL-2 (Cillari et al., 1986). Jenski and Ledford (1985) reported that MCDV-12 (a transplantable Rauscher murine leukemia retrovirus) tumors secreted their own IL-2 suppressor which inhibited IL-2 dependent immunity in close proximity to the tumor. In addition, Greene et al. (1986) have shown that the insertion of the trans-activator gene of the human T-lymphotrophic virus type II (HTLV-II) into some human leukemic T cell lines induced both IL-2 and IL-2 receptor gene expression.

Therefore, a number of different immune disorders which affected IL-2 production have been reported. In addition, the mechanisms inhibiting IL-2 dependent immunity differed depending on the nature of the disorder. Because of the various methods in which immune disorders can affect immunological homeostasis, Kromer et al. (1986) have suggested caution when considering IL-2 as a therapeutic agent.

Genetic Control of the Immune Response in the Chicken

Knowledge of the genetic control of the immune response in the avian immune system has not approached the level currently known of the mammalian systems. As a result, studies of the avian immune system have relied on models developed primarily within the mammalian systems. The genetic control of the immune response can simply be divided into two levels (for a review, see Clark, 1983). One level of genetic control involves the interaction and recognition of class I and II MHC gene products. The other level involves the contributions of non-MHC gene products in antigen recognition as well as complementing the immune response.

Studies of the genetic control of the avian immune system have primarily focused on MHC genetic control. The MHC of the chicken was discovered in 1961 when it was shown that the serologically-detected Ea-B blood group locus was associated with the ability to reject grafts (Schierman and Nordskog, 1961). Later, Miggiano et al. (1974) showed that the Ea-B locus was also associated with the mixed lymphocyte reaction. Based on the studies, it was suggested that the B system was the phylogenic homolog of the murine H-2 and human HLA (Pazderka et al., 1975). Hence, the chicken MHC has been denoted as the B complex.

The requirement for MHC class I and II interaction and recognition in the immune response of mammalian species has been well-documented (for a review, see Clark, 1983). Similar evidence, however, was not provided within avian species until recently. Maccubin and Schierman (1986) have provided the first evidence of avian MHC restriction of cellular

cytotoxicity as first described by Zinkernagel and Doherty (1974). In addition, Vainio et al. (1984) used MHC recombinant lines for adoptive transfer of bursal cells to demonstrate that genes located in the class II region of the chicken encode the products controlling T-B cell interactions.

The MHC of the chicken has been shown to encode for at least three different MHC antigens (Pink et al., 1977). One antigen is the class I antigen, denoted as B-F in the chicken system, that is present on both leukocytes and erythrocytes and is homologous to the murine H2-K and H-2D antigens. Another is the class II antigen, denoted as B-L, that is present on some leukocytes and is homologous to the murine I region. The chicken MHC also encodes for a class IV antigen, denoted as B-G, that is found only on erythrocytes. There is no known mammalian homolog for the class IV antigen.

As stated previously, non-MHC genetic control has also been shown to effect the immune response. Non-MHC genetic control has been demonstrated at two levels in the chicken immune system. One level involves the ability of the chicken to genetically recognize and respond to an antigen. An example of this level of genetic control in the chicken was demonstrated by Gebriel and Nordskog (1983) when they showed that tv loci, that codes for cellular resistance to Rous sarcoma virus (RSV), mapped outside the MHC. Another level of genetic control involves the role these genes play in the immune response. Linkage of the abilty to respond to RSV-induced tumors to the MHC has been well-documented. (Schierman et al., 1977; Gebriel and Nordskog, 1983). But, the

influences of non-MHC gene loci have also been shown to be important in the control of RSV-induced tumor response (Cutting et al., 1981; Gilmour et al., 1983). In addition, non-MHC as well as MHC genetic control has been demonstrated in the ability to respond to Con A (Miggiano et al., 1976; Pink and Miggiano, 1977; Morrow and Abplanalp, 1981).

The study of the genetic control of the immune response in the chicken, therefore, has attempted to employ models already established for mammalian systems. Most of these models for the genetic control of the immune response in mammalian species have also been valid for chickens. But, in some instances (e.g., the lack of a mammalian MHC class IV homolog) the genetic models have not directly applied. Because of the phylogenic distance between aves and mammals, differences in the genetic control of the immune response may be present.

Summary

IL-2 is defined as a soluble immunoenhancing factor released primarily by lectin- or antigen-activated T_H cells in the presence of IL-1. IL-2 plays a pivotal role in the augmentation of T cell responses, IFN-γ production and NK activity, and may influence T dependent B cell responses. The regulation of IL-2 production and activity is influenced by the inducing antigen, by suppressor factors, by genotype, and by IL-2 itself. The balance between suppressor factors and IL-2 production dictate immunological homeostasis in IL-2-dependent immune responses. Many immune disorders occur as a result of the imbalance between the levels of IL-2 production and suppression.

The assay to detect IL-2 activity, which is based on the ability of supernatants from lectin-activated mononuclear cells to support the growth of lectin-activated T cells *in vitro*, has been instrumental in characterizing IL-2. Although the sizes and charges of avian and mammalian IL-2 are similar, interspecies activity does not occur. Due to the phylogenic distance between avian and mammalian species, the study of chicken IL-2 may be an initial step in analyzing the evolution of the molecules involved in T cell proliferation. Moreover, since the ability to recognize and respond to a specific antigenic challenge is genetically controlled, the study of the genetic control of factors governing immune response in the chicken may provide some insight into the evolution of genetic regulation of the immune response.

MATERIALS AND METHODS

Description of the Lines

One partially inbred line (S1) and eight inbred lines of chickens (Gallus domesticus) were used in this study. The birds were randomly housed in 10-ft.^{2} floor pens with fifteen to twenty birds per pen. The birds of each genotype (genetically distinct groups) within the S1 or inbred lines were randomly sampled at each collection time because it was not possible to obtain a blood sample from each genotype during each sampling session. In addition, equal numbers of samples from each of the genotypes were gathered during each week to insure that the weekly sampling effects would not favor one genotype over another.

Inbred lines

Inbred lines of Leghorn, Spanish, and Fayoumi breeds, with inbreeding coefficients ranging from 81 to 97%, were used in this study (Gebriel and Nordskog, 1983). The eight inbred lines each have one to three of 8 different MHC alleles fixed within their genomes (Table 1). Major histocompatibility type was determined serologically. Schierman and Nordskog (1961) showed that the serologically detected Ea-B blood group locus, which controls the expression of specific erythrocyte antigens, also serves as an MHC marker. Hence, the chicken MHC has been denoted as the B complex. For convenience, the Ea-B^X allele has been expressed as Bx (Altman and Katz, 1979). The B5.1, B15.1 and B21.1 MHC alleles have similar, but not identical, serological properties to the standard B5, B15 and B21, respectively (Gebriel and Nordskog, 1983). All inbred lines have been closed breeding populations for at least 20 years.

Lines 8, 9, and 19 are highly (approximately 91, 89, and 90%, respectively) inbred Leghorn lines possessing the B15.1 MHC allele. The B13 MHC allele is also deliberately maintained within line 19. The lines have a common ancestory originating from early inbred lines established at Iowa State University (formerly Iowa State College) in the 1930s (Stephenson, 1949; Somes, 1984). Line 9, however, was briefly crossed with the Spanish line and later backcrossed to line 9 (Somes, 1984).

Table 1. Iowa State University inbred lines and their major histocompatibility haplotypes

Line	Breed	Year of origin	Wright's coefficient of inbreeding (percent) 1978	MHC allele[a]
8	Leghorn	pre1940	91	B15.1
9	Leghorn	pre1940	89	B15.1
19	Leghorn	pre1940	90	B13, B15.1
GH	Leghorn	1954	90	B1, B13, B15.1
GHs	Leghorn	1965	95	B6, B13
HN	Leghorn	1954	97	B12, B15
M	Fayoumi	1954	81	B5.1, B15.1
Sp	Spanish	1954	92	B21.1

[a]Bx designates the B^x MHC allele.

Line GH is a highly (approximately 90%) inbred White Leghorn line that is comprised of three sublines. Each subline is fixed for either the B1, B13, or B15.1 MHC allele. The line originated from a commercial line developed by Ghostley Poultry Breeders (Somes, 1984). In 1958, a GH

female was accidently mated to an HN male producing F_1 progeny (Lee, 1980). Subsequent generations have been derived from F_2 and HN males to GH female backcross progeny (Schierman, 1962). The GH line originally segregated randomly for the MHC alleles B13 and B15.1. In 1974, the B1 allele was transfered into the line and all three alleles have been deliberately maintained in the line since.

Line GHs is another highly (approximately 95%) inbred White Leghorn line that has the B6 or B13 MHC allele fixed within each of its two sublines. In 1965, Dr. L. W. Schierman brought a sample of the GH line to the New York Medical College (Lee, 1980). Ten years later, a sample was returned to Iowa State University. Subsequent MHC-typing revealed that the birds maintained the MHC alleles B6 and B13 (Lee, 1980). GHs-B6 and GHs-B13 will be referred to as G-B2 and G-B1, respectively, and the GHs line as the G line, to be consistent with the literature from other investigators that use this line.

Line HN is also a highly (approximately 97%) inbred White Leghorn line. Line HN has the B15 and B12 MHC allele fixed within separate sublines. The line was obtained as a "pure" Kimber line from Heisdorf and Nelson, Inc. (Somes, 1984).

Lines M and Sp are the other two highly (approximately 81 and 92%, respectively) inbred lines. Line M is a Fayoumi line that is composed of two sublines that are fixed for either the B5.1 and B15.1 MHC alleles. Line Sp is a Spanish (Castellana negra) line that is fixed for the B21.1 MHC allele. The Fayoumi breed was imported from Egypt to Iowa State University in 1949. Nordskog and Phillips (1960) have shown that line M

was particularly resistant to lymphomas.

A number of genetic studies have been conducted using the inbred lines. Singh and Nordskog (1981) reported that the gene frequencies of the genetically-controlled polymorphic proteins, alkaline phosphatase, esterase I and esterase II, varied among seven of the inbred lines, whereas hemoglobin, albumin, and transferrin did not. Using these inbred lines, Nordskog and Gebriel (1983) demonstrated that cellular resistance to RSV maps outside the MHC whereas RSV regression (i.e., immune response) was influenced by the MHC. Lamont and Cowan (1985) found a genetic association of the ability of the inbred lines to phagocytize colloidal carbon in vivo. Palmer et al. (1981) have demonstrated significant line (non-MHC) differences in the immune response to Con A, whereas MHC differences were not significant.

The G line has been widely used by many laboratories to examine genetic associations of immune response. The G-B1 (GHs-B13) genotype has been used, in part, in studies which have defined the immune response genes to Con A. The immune response to Con A was shown to be controlled by two genes, Con A1 (Mr-1) gene which was not linked to the MHC and the Con A2 gene which was MHC linked (Miggiano et al., 1976; Pink and Miggiano, 1977; Morrow and Abplanalp, 1981). Maccubbin and Schierman (1986) used the G-B1 (GHs-B13) and G-B2 (GHs-B6) genotypes to demonstrate MHC-restriction in in vitro cellular cytoxicity to virus-infected cells in the chicken, as first shown with mice by Zinkernagel and Doherty (1974).

S1 line

The S1 line is a partially inbred population with an inbreeding coefficient of approximately 0.40 (Cheng et al., 1985). The S1 line was developed in 1965 from two inbred lines of commercial White Leghorns. The S1 line originally segregated for the MHC alleles B1, B2, B19, and B21 (Cheng, 1985). Prior to 1978, the S1 line was randomly bred for the MHC alleles B1, B2, and B19. B21 was intentionally dropped from the line. In 1978, the S1 line was sublined based on high or low response to GAT within each haplotype (B1 or B19) (Pevzner et al., 1978). The B2 haplotype, with intermediate immune response to GAT, was maintained as a control in the S1 line (Cheng, 1985). Between 1978 and 1982, the birds were randomly bred within each of the 5 sublines. In 1982, nine genetic groups (i.e., genotypes) were formed based on high or low immune response to GAT, regression or progression of RSV-induced tumors and the MHC alleles (B1 and B19), plus the B2 genotype (Gebriel and Nordskog, 1983). The nine genotypes have been deliberately segregated and are maintained as nine sublines within the S1 line (Table 2).

The S1 line is especially important in genetic studies involving MHC controlled immune responses because a recombination event has occured within the MHC. Benedict et al. (1975) demonstrated that the genetic control of the immune response to GAT in the chicken was associated with the MHC. Pevzner et al. (1978), using the S1 line, have demonstrated that the B1 was a low responder whereas the B19 genotype was a high responder to GAT. Backcrosses of B1B19 heterozygotes to the B1B1 and B19B19 parental homozygotes, however, produced progeny that typed as B1

or B19 and had a high or low response, respectively, to GAT. Pevzner et al. suggested that a recombination event had occurred between the immune response region and the Ea-B locus which resulted in the high GAT response in B1 birds and low response in B19 birds.

Table 2. Genotypes of the Iowa State University partially inbred[a] S1 line in 1965, 1978 and 1982 to the present

1965	1978	1982-present
B1	B1-Hi[b]	B1-Hi-Reg[c] B1-Hi-Prog
	B1-Lo	B1-Lo-Reg B1-Lo-Prog
B19	B19-Hi	B19-Hi-Reg B19-Hi-Prog
	B19-Lo	B19-Lo-Reg B19-Lo-Prog
B2	B2-Int	B2-nd-nd[d]
B21	(extinct)	(extinct)

[a]Average inbreeding coefficient of the 9 genotypes developed in 1982 is approximately 0.40.

[b]Hi, Lo, and Int represent high, low, and intermediate immune response to GAT.

[c]Reg and Prog denote regression and progression of RSV induced tumors.

[d]Immune response to GAT and RSV were not determined for the B2 genotype in 1982.

In recent years, the S1 line has been utilized to investigate genetic association of the immune response to a variety of different antigenic challenges. Lamont et al. (1987) have shown that the B1

genotype was significantly more resistant to Pasteurella multocida (the etiological agent of fowl cholera) than the B19 genotype, and there was not an association with GAT or RSV immune response. The ability to phagocytize colloidal carbon in vivo, however, was associated with response to RSV induced tumors (Lamont and Cowan, 1985). Steadham et al. (1987) demonstrated that resistance to Marek's disease virus (an oncogenic herpesvirus) was significantly greater in the B1 than in the B19 genotype. Moreover, they have shown that high responders to GAT within each MHC haplotype were significantly more resistant to Marek's disease virus than low responders to GAT. Using an F_2 population produced from inter se matings of the S1 sublines, however, only B1-Ir-GAT-high birds were significantly more resistant to Marek's disease virus.

Experimental Design

Preliminary study

The preliminary study was conducted to examine the possibility of genetic differences in levels of IL-2 production and to determine the repeatability of measurements taken at different times. One haplotype (MHC allele) from each of the inbred lines was tested, except the GH line in which two haplotypes were used. Therefore, a total of nine genotypes (line-MHC allele combinations) were used in the study. One male and female of each genotype was used to give a total of 18 birds in the study. The birds were divided into three groups of six birds each (Group 1 = GHs-B6, 19-B15.1 and HN-B15; Group 2 = Sp-B21.1, M-B5.1 and GH-B1; Group 3 = 8-B15.1, 9-B15.1 and GH-B13). One group was sampled each day.

One blood sample was obtained from each of the six birds in a group in the morning and again in the afternoon. Morning and afternoon blood samples were obtained again one week later from each of the birds (Table 3).

Table 3. Groups sampled for the preliminary study

	Day					
Time	1	2	3	8	9	10
morning	1[a]	2[b]	3[c]	1	2	3
afternoon	1	2	3	1	2	3

[a]Group 1 = genotypes GHs-B6, 19-B15.1, and HN-B15; 1 male and female per genotype.

[b]Group 2 = genotypes Sp-B21.1, M-B5.1, and GH-B1; 1 male and female per genotype.

[c]Group 3 = genotypes 8-B15.1, 9-B15.1, and GH-B13; 1 male and female per genotype.

Inbred lines study

A survey of the inbred lines was conducted to determine if differences in levels of IL-2 production were attributable to MHC or non-MHC effects. Ten birds, seven to eight months of age, from each of the genotypes were examined. Five males and females of each genotype were used to determine if sex differences influenced IL-2 production. All the blood samples were collected, processed and cultured during a four week period.

S1 line study

The experiment using the S1 line was performed to determine whether differences in levels of IL-2 production correlated with allelic and/or

immune response differences already observed in the line. Ten birds, six to seven months of age) consisting of five males and females from each of the nine genotypes in the S1 line were tested for levels of IL-2 production. All the blood samples were collected, processed and cultured during a three week period.

IL-2 Assay Procedure

The assay used to quantitate the levels of chicken IL-2 production in this study was a modification by Frederickson and Sharma (1985) of the assay first developed by Kromer et al. (1984).

Preparation of blood samples

Ten to twenty milliliters of sterile heparinized (100 IU heparin/ml, Sigma No. H-3125, Lot No. 63F-0450) blood were collected in a syringe via wing vein or jugular vein puncture. The blood was aliquoted into 3 to 4 ml volumes in sterile 12x75 mm polystyrene snap-cap tubes (Falcon). Slow-spin differential centrifugation (60 x g, 10 min., room temperature) was performed three times to separate the peripheral blood lymphocytes (PBL) from the red blood cells (RBC). The lymphocytes were washed twice (600 x g, 5 min., room temperature) in Hank's balanced salt solution (HBSS, Gibco). The cells were resuspended in culture medium and live cells were counted via trypan blue (Sigma) exclusion using a hemacytometer.

Red blood cells were separated from the remaining PBLs by means of centrifugation (700 x g, 15 min., room temperature) through Ficoll-Paque (Pharmacia). The RBCs were washed twice in HBSS and resuspended in culture medium and counted.

Preparation of Con A

A 10 mg/ml Con A (3X crystallized, Miles 79-003, Lot 79L) stock solution was prepared in saturated sodium chloride before each study. The working concentration of 2.0 ug/ml Con A was prepared from the stock solution.

Culture conditions

The culture medium was serum-free RPMI 1640 (Gibco) supplemented with L-glutamine (50 mM, Gibco), penicillin (100 U/ml, Gibco) and streptomycin (100 ug/ml, Gibco). The cells were cultured at 40°C in a humidified atmosphere containing 5% CO_2.

Production of conditioned media (CM)

The PBLs were cultured at a concentration of 3.3×10^6 cells/ml in the presence of 2.0 ug/ml Con A and 2.0×10^6 autologous RBCs/ml in 24-well tissue culture cluster plates (Costar) at 2 ml volumes. After a 48 hour incubation period, the supernatants were placed into 50 ml conical centrifuge tubes (Corning). The IL-2 containing supernatants (CM) were freed from the cells by centrifugation (600 x g, 10 min., room temp.). The CM was aliquoted into 1 to 2 ml volumes in sterile vials and stored at -20°C until assayed for IL-2 activity.

Preparation of responder cells

The responder cells used in the assay were PBLs from line 19-B15.1 birds because they yielded high numbers of responding cells, and thus more tests could be performed. The peripheral blood lymphocytes (3.3×10^6 cells/ml) were incubated in the presence of 2.0 ug/ml Con A. The cells were incubated for 72 hours in volumes of 50 ml in 75 cm^2 or

100 ml in 150 cm^2 tissue culture flasks (Corning) standing upright. The cells were centrifuged (600 x g, 8 min., room temperature), resuspended and incubated in RPMI 1640 containing 0.05 M methyl α-D-mannopyranoside (α-MM, Sigma M-6882) for 15 to 20 minutes at 40°C. The α-MM competitively removed the remaining Con A from the cell surface. After one wash in HBSS, the population of Con A-activated blast cells were enriched by means of density centrifugation (600 x g, 10 min., room temperature) using a 20-60-100% discontinuous Percoll (Pharmacia 17-0891-01, Lot LD 00359) gradient. The interface between the 20-60% layers contained the enriched population of lymphocytes that responded to IL-2. The blasts were washed twice in HBSS and adjusted to a concentration of 2.0 x 10^6 cells/ml in RPMI 1640 containing 0.05 M α-MM.

Functional determination of IL-2 activity in CM

A serial 1:2 dilution of the CM to be tested was performed in triplicate in flat bottomed 96-well microtiter plates (Costar) at a volume of 100 ul/well. One hundred microliters of the responder cell suspension were added to the CM. The control wells consisted of 100 ul of cells, CM, or culture media plus an additional 100 ul of culture media (RPMI 1640 with α-MM) to bring the total volume to 200 ul. A reference standard (CM from GH-BI5.1, bird # 11129) was used with each test. After an 18 hour incubation period at 40°C in 5% CO_2, the cells were pulsed for 5 hours with 1 uCi [methyl-^{3}H] thymidine (^{3}H-Tdr, Amersham TRK.120). The cells were collected onto glass fiber filter paper (Whatman 934-AH) using a cell harvester (Dynatech Model CH-103). The filter paper containing the cells were placed into scintillation vials (Kimble) containing 10 ml

of scintillation cocktail (2,5-diphenyloxazole (PPO); 1,4-bis(2-(5-phenyl-oxazoyl)) benzene (POPOP) in toluene; Fisher). The levels of radioactivity were measured by a Packard TRI-CARB 300 scintillation counter.

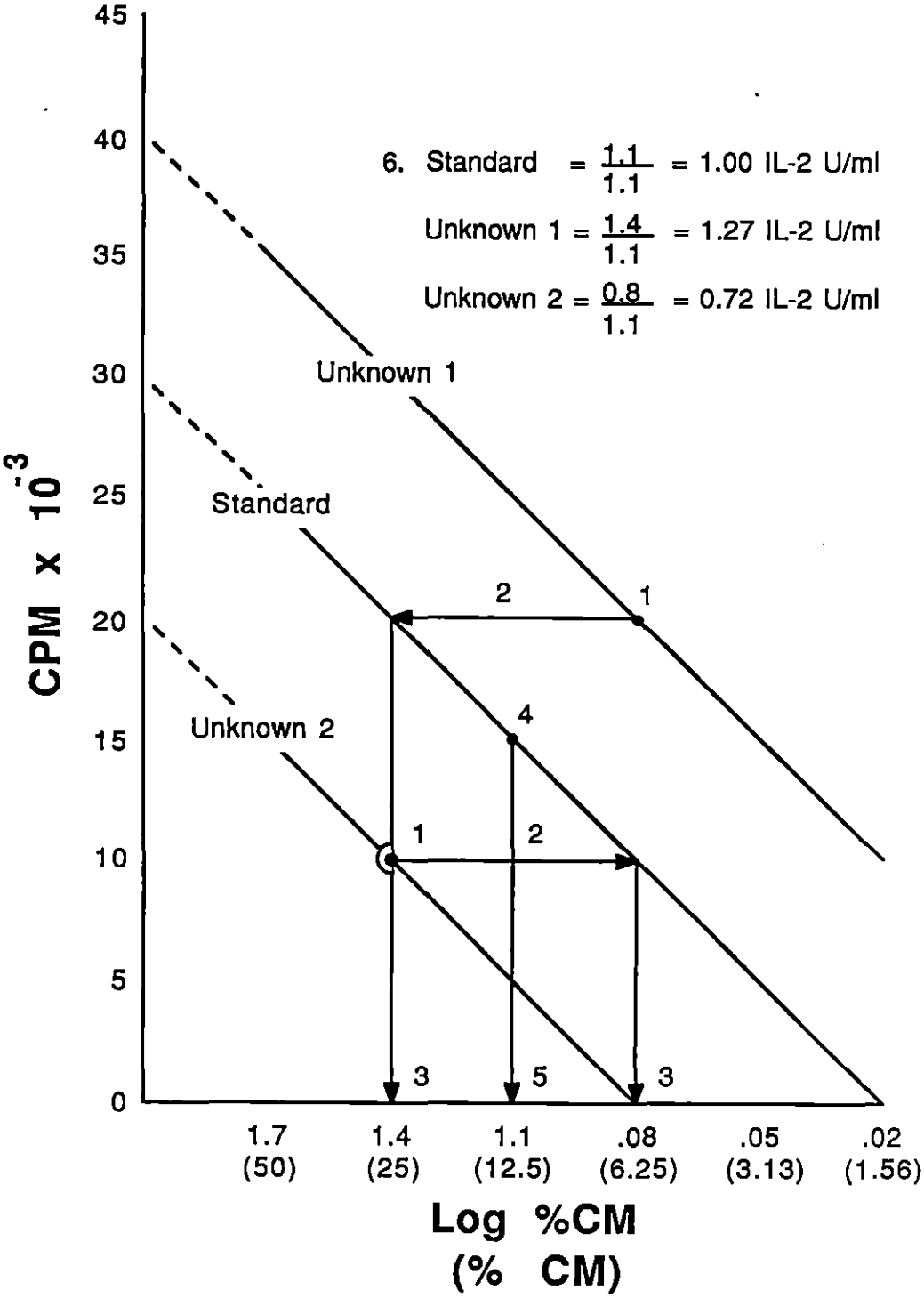
Mathematical determination of IL-2 activity

The value for each dilution of the test sample was expressed as the mean counts per minute (cpm) of the triplicates after subtraction of the background cpm. Background cpm = (cpm cells + medium) + (cpm CM + medium) - cpm medium. The cpm values for each dilution were used to plot a response regression line plotting cpm versus log %CM for each sample (Figure 2). The 50% maximal proliferation value was determined for each sample. The 50% value was then transposed to the reference standard regression line and the corresponding log %CM was determined. The 50% maximal proliferation and its corresponding log %CM was also determined for the reference standard. One unit of IL-2 was represented as the log %CM giving 50% maximal proliferation of the reference standard. Therefore, the units of IL-2 for the test samples were calculated by dividing the log %CM of the test sample by the log %CM of the reference standard at 50% maximal proliferation (Frederickson and Sharma, 1985).

Two alternative methods were also used for expressing the data. The IL-2 activity was also calculated by dividing the cpm of the test sample at 25% CM by the 25% CM of the reference standard. Gross cpm at 25% CM was also used as an indication of IL-2 production.

Figure 2. Determination of levels of IL-2 activity

1. Determine the cpm corresponding to 50% maximal proliferation for the unknowns on their respective regression lines. 2. Transpose the cpm values to the reference standard's regression line. 3. Determine the corresponding log %CM. 4. Determine the cpm corresponding to 50% maximal proliferation of the reference standard. 5. Determine the log %CM corresponding to 50% maximal proliferation of the reference standard. 6. IL-2 activity is calculated by dividing the log %CM of the unknown by the log %CM of the reference standard. The IL-2 activity of the reference standard is arbitrarily set at 1.00 IL-2 U/ml.


6. Standard = 1.1/1.1 = 1.00 IL-2 U/ml
Unknown 1 = 1.4/1.1 = 1.27 IL-2 U/ml
Unknown 2 = 0.8/1.1 = 0.72 IL-2 U/ml
Unknown 1
Standard
Unknown 2
CPM x 10^-3
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
1.7 (50)
1.4 (25)
1.1 (12.5)
.08 (6.25)
.05 (3.13)
.02 (1.56)
Log %CM
(% CM)

Statistical analysis

The analysis of the preliminary study was conducted using an F-test of significance which was divided into three parts. The first part of the analysis was a test of significance for differences in levels of IL-2 production between genotypes and of sex. The second part of the analysis tested for significant differences in levels of IL-2 production of samples collected in the morning and afternoon for each bird. The third part of the analysis tested for significant differences of levels of IL-2 in samples collected one week apart from the same birds.

The statistical analysis of the inbred study was approached two different ways. An F-test of significance was used to test for significant differences of levels of IL-2 production between MHC haplotypes, lines, and sexes for all birds used in the study. In addition, individual F-tests of significance of differences in levels of IL-2 production between MHC haplotype or line differences were conducted on available matched data sets because of the many missing cells in the previous analysis.

The S1 line was analyzed by an F-test to test for differences in levels of IL-2 production between birds that differed at their MHC allele (B1 and B19), immune response to GAT, and/or their ability to regress RSV induced tumors. The B2 birds were not used in the analysis because data were not available for their immune response to GAT and RSV.

The three different methods of calculating levels of IL-2 production were compared by determining how well the linear relationship of the IL-2 values obtained using each method correlated with each other.

RESULTS

Levels of IL-2 production were determined by the relative ability of supernatants from Con A-activated PBLs to promote the proliferation of an enriched population of Con A-stimulated lymphoblasts *in vitro*. An enriched population of activated lymphoblasts was obtained by centrifugation of Con A-stimulated lymphocytes through a Percoll discontinuous density gradient to eliminate non-activated cells. The efficacy of the Percoll separation procedure in enriching the population of lymphoblasts was examined. The cells between the 20% and 60% Percoll layers had a significantly ($P < 0.01$) higher response to IL-2 than did Con A-activated cells that were not separated (Table 4).

Table 4. Effectiveness of the Percoll separation procedure in enriching populations of Con A-activated lymphoblasts

Cell Source[3]	N[4]	^{3}H-Tdr uptake[1,2] (Mean cpm ± SEM)
Non-separated	3	12,095 ± 2,183[b]
20-60% Percoll interface	3	18,637 ± 1,809[a]
60-100% Percoll interface	3	1,265 ± 630[c]

[1]Incorporation of ^{3}H-thymidine in the presence of 25% conditioned media.

[2]Means bearing different letters are significantly different, $P < 0.01$.

[3]One hundred microliters containing 2.0×10^6 cells/ml.

[4]Number of birds used from the 19-B15.1 genotype, cultured in triplicate.

A reference standard conditioned medium (CM) was used with each assay to account for assay-to-assay variation. The reference standard was used 47 times throughout the entire study. The average log %CM giving 50% maximal proliferation was 1.12 ± 0.08.

Preliminary Study

The preliminary study was designed to examine the possibility of genetic differences in levels of IL-2 production and to determine whether measurements taken at different times were reproducible. The analysis of variance for differences between inbred genotypes indicated that there were significant differences ($P < 0.001$) in levels of IL-2 production between the nine different genotypes used in the study. Sex differences over the nine genotypes, however, did not significantly influence levels of IL-2 production (Table 5).

The IL-2 levels measured from samples collected in the morning and afternoon from the same bird did not differ significantly. In addition, the influence of sex within each genotype did not significantly influence levels of IL-2 production from samples collected in the morning and afternoon.

Differences in levels of IL-2 activity from samples obtained one week apart approached significance ($0.10 > P > 0.05$). In order to minimize the effects of week-to-week sampling differences in subsequent studies, a collection schedule was devised which insured that equal numbers of sample were obtained from each of the genotypes during each week of their respective studies.

Table 5. Analysis of variance for genetic and sampling time differences on levels of IL-2 production

Source	df	MS	F
Genotype[a]	8	2.548	35.39**
Sex	1	0.004	0.75
Error 1[b]	8	0.072	
Time[c]	1	0.024	0.86
Sex*Time	1	0.028	1.00
Error 2[d]	16		
Between Weeks	1	0.113	3.69[e]
Error 3[f]	35	0.031	
Total	71		

[a]Genotype = line-MHC allele combinations.

[b]Error 1 = genotype by sex interaction.

[c]Time = morning or afternoon samples.

[d]Error 2 = Pooled error from genotype by time and sex by genotype by time interactions.

[e]$0.10 > P > 0.05$.

[f]Error = residual.

**$P < 0.001$.

Therefore, the results of the preliminary study indicated that there were genetic influences on levels of IL-2 production. Differences between sexes and between morning and afternoon sampling did not influence levels of IL-2 activity. As a result, the influence of sex on levels of IL-2 production were not considered in the analyses of variance of the inbred lines. In addition, differences in levels of IL-2 production from samples collected in either the morning or afternoon

could confidently be considered insignificant. Differences in levels of IL-2 production that may occur as a result of weekly sampling were accounted for by gathering equal numbers of samples from each of the genotypes during each week of the study.

Inbred Lines Study

Each of the fourteen genotypes (line-allele combinations) of the inbred lines was examined for its ability to produce IL-2. An analysis of variance for the overall effects of line and MHC allelic differences indicated that both significantly influenced levels of IL-2 production (Table 6). Major histocompatibility complex allelic differences were not as significant as line differences in influencing levels of IL-2 production. The influence of sex was not significant, which confirmed the results of the preliminary study. Hence, differences between sexes were not considered in additional analyses of the inbred lines.

The mean square (MS) for the line*MHC allele interaction was zero because too many data cells were missing from the analysis. There are eight different lines and eight MHC alleles within the inbred lines (Table 1), therefore an ideal analysis would contain 64 data cells. The inbreds lines, however, contain only fourteen different genotypes. As a result, only 14 of the 64 data cells were filled in the analysis. Moreover, because the effects of line and MHC allele influences were confounded in some instances in the analysis (Table 6), it was not valid to consider the effects of line differences as being strictly non-MHC influences.

Table 6. Analysis of variance for the general effects of MHC and line differences on levels of IL-2 production

Source	df	MS	F
Line	5	1.96	21.78**
MHC Allele	5	0.23	2.56*
Sex	1	0.13	1.44
Line*MHC Allele	1	0.00	0.00[a]
Error	127	0.09	
Total	139		

[a]The F value for the line*MHC allele interaction was 0.00 due to the paucity of data cells in the analysis.

*$P < 0.05$.

**$P < 0.001$.

As indicated earlier, there are eight different lines and eight MHC alleles which comprise the 14 genotypes within the inbred lines, therefore the analysis should have used 7, 7, and 13 degrees of freedom (df), respectively, to test for significance. The analysis, however, used 5 df to test significance for both line and MHC allele differences and 1 df to test for the line*MHC allele differences because that was the number of independent tests that were possible within the 14 data cells available.

Independent (non-confounding) tests for within line (non-MHC) or MHC allelic effects on levels of IL-2 production were performed to evaluate

the influence of the many missing cells of the analysis of variance for general effects. All the independent tests of variance showed that the MHC allele did not influence levels of IL-2 production (Table 7). The effects of the MHC allele approached significance within three lines, but their influence was not as significant as the general effects of the MHC allele indicated in Table 6. The independent tests of significance for line (non-MHC) influences within a MHC type, demonstrated that the line differences significantly influenced levels of IL-2 production (Table 8). Thus, the analyses of variance in Table 8 for the effects of line (non-MHC) on levels of IL-2 production agreed with those of Table 6. Therefore, the significance of MHC allele but not line effects were affected by the missing data cells from the analysis of variance in Table 6. Moreover, the non-MHC influences seem to be more important in controlling levels of IL-2 production than does the MHC.

The line-MHC allele interactions could only be tested in paired comparisons between the data from the 19-B13, 19-B15.1, GH-B13, and GH-B15.1 genotypes. As shown in Table 9, the influence of the MHC allele was not significant whereas line (non-MHC) differences significantly influenced levels of IL-2 production. These results agreed with the previous analyses as to the significance of line and MHC allele major effects. Line by MHC allele interactions were not significant.

Independent tests of significance for the effects of either line (non-MHC) or MHC allelic differences on levels of IL-2 production could not incorporate all the different genotypes used from the inbred lines. All the genotypes used in the inbred study have been ranked in descending

Table 7. Analyses of variance for MHC allele within line effects on levels of IL-2 production

	MHC alleles (line)[a]	df	MS	F
1.	B6, B13 (GHs)	1	0.354	3.09[b]
	Error	18	0.115	
2.	B13, B15.1 (19)	1	0.005	0.07
	Error	18	0.080	
3.	B1, B13, B15.1 (GH)	2	0.286	2.47[c]
	Error	27	0.116	
4.	B12, B15 (HN)	1	0.196	2.41[c]
	Error	18	0.081	
5.	B5.1, B15.1 (M)	1	0.031	0.06
	Error	18	0.055	

[a]The source of variation in each analysis is the difference between MHC allele effects within a line.

[b]$0.10 > P > 0.05$.

[c]$0.25 > P > 0.10$.

Table 8. Analyses of variance of line (non-MHC) within MHC allele effects on levels of IL-2 production

	Line (MHC allele)[a]	df	MS	F
1.	GHs, GH, 19 (B13)	2	2.509	15.09**
	Error	27	0.166	
2.	8, 9, 19, GH, M (B15.1)	4	1.180	14.42**
	Error	45	0.082	

[a]The source of variation in each analysis is the difference between line (non-MHC) effects within the MHC allele.

**$P < 0.001$.

order based on their mean levels of IL-2 production from ten observations per genotype (Table 10). The results in Table 10 support analyses of variance presented earlier (Tables 7 and 8) for the inbred study. Lines ranked similarly as to their relative levels of IL-2 production, regardless of MHC type. The rankings of the genotypes that shared the same MHC allele (B13 or B15.1), however, were spread throughout the range of mean IL-2 U/ml values obtained in the study. The data presented in Table 10 affirmed the importance of non-MHC rather than MHC allelic effects in influencing levels of IL-2 production in the inbred lines tested.

Table 9. MHC versus non-MHC effects on levels of IL-2 production

	MHC allele		
Line[a]	B13	B15.1	$\bar{X}_{line}$
19	1.72[b]	1.79	1.76 **
GH	0.89	0.96	0.93
$\bar{X}_{allele}$	1.31	1.38	

[a]Line represents non-MHC effects.

[b]Mean IL-2 U/ml from 10 observations.

**$P < 0.001$ for line effects.

Table 10. Mean levels of IL-2 production of the inbred genotypes

Genotypes (Line-MHC allele)	Means[1] (IL-2 U/ml)
19-B15.1	1.79[a]
9-B15.1	1.76[a,b]
19-B13	1.72[a,b]
M-B15.1	1.66[a,b]
Sp-B21.1	1.60[a,b,c]
M-B5.1	1.58[b,c]
8-B15.1	1.42[c]
HN-B12	1.21[d]
HN-B15	1.01[d,e]
GH-B15.1	0.96[e]
GH-B13	0.89[e]
GHs-B13	0.81[e,f]
GH-B1	0.64[f,g]
GHs-B6	0.54[g]

[1]Means bearing different letters are significantly different, $P < 0.05$.

S1 Line Study

The sublines (genotypes) of the partially inbred S1 line were examined for their relative abilities to produce IL-2. The B2 genotype was not considered in the analysis because data were not available for its immune responses to GAT and RSV.

The analysis of variance of primary factors affecting levels of IL-2 production in the S1 line (Table 11), indicated that the influence of the allele coding for Ea-B type (B1 or B19) was highly significant ($P < 0.001$). The association between the immune response to GAT and levels of IL-2 production approached significance ($0.10 > P > 0.05$). The effects of the ability to regress or progress RSV-induced tumors and of sex, however, were not significant. The results of the S1 line study contrasted with the inbred lines study which demonstrated that the effects of the MHC allele was not significant in the inbred lines.

The primary factors, their first order and their second order interactions were also analyzed to determine whether levels of IL-2 production differed significantly when the effects of more that one factor were considered simultaneously (Table 12). Again, the effects of the MHC allele on levels of IL-2 production were significant ($P < 0.001$). Immune response to GAT also significantly ($P < 0.05$) influenced levels of IL-2 production, but to a lesser extent. The first order interaction between the MHC allele and immune response to GAT, however, was not significant. Although the general effect of RSV response did not significantly influence levels of IL-2 production, its first and second order interactions were highly significant ($P < 0.001$).

Table 11. Analysis of variance of the primary factors in the S1 line affecting levels of IL-2 production

Source	df	MS	F
MHC allele[a]	1	25.25	93.52**
GAT[b]	1	0.74	2.74[c]
RSV[d]	1	0.11	0.41
Sex	1	0.15	0.70
Error	75	0.27	

[a]MHC alleles B1 or B19.

[b]High or low immune response to GAT.

[c]$0.10 > P > 0.05$.

[d]Regression or progression of RSV induced tumors.

**$P < 0.001$.

Table 12. Analysis of variance of factors affecting levels of IL-2 production in the S1 line

Source	df	MS	F
MHC allele[a]	1	25.22	140.11**
GAT[b]	1	0.75	4.16*
RSV[c]	1	0.11	0.61
Sex	1	0.004	0.02
MHC allele*GAT	1	0.06	0.33
MHC allele*RSV	1	3.65	20.28**
GAT*RSV	1	1.66	9.22**
MHC allele*GAT*RSV	1	2.20	12.22**
Error	71	0.18	

[a]MHC alleles B1 and B19.

[b]High and low immune response to GAT.

[c]Regression and progression of RSV induced tumors.

*$P < 0.05$.

**$P < 0.001$.

Contingency tables were constructed to ascertain the relationships between RSV responses and the MHC allele and immune response to GAT on levels of IL-2 production (Table 13). The influence of the MHC allele and immune response to GAT were significant whereas the effects of the immune response to RSV were not, as previously indicated. The first order interaction between MHC allele and immune response to GAT shows that high responders to GAT within each MHC haplotype produced significantly higher levels of IL-2. In addition, the B1 haplotype produced significantly higher levels of IL-2 than the B19 haplotype regardless of GAT response. All interactions between RSV response and MHC allele and/or immune response to GAT, however, were inversely related.

Table 14 shows that the genotypes bearing the B1B1 allele produced higher levels of IL-2 than did birds bearing the B19B19 allele within the S1 line. These data agreed with the analysis of variance (Table 12) which demonstrated that the influence of the Ea-B allele on levels of IL-2 production was highly significant.

Therefore, levels of IL-2 production in the S1 line were significantly influenced by the genes controlling Ea-B type and immune response to GAT (Ir-GAT). The gene controlling regression or progression to RSV was not by itself influential in affecting levels of IL-2 production. The gene controlling the response to RSV induced tumors in concert with the genes controlling the immune response to GAT and/or Ea-B type, however, significantly influenced levels of IL-2 production.

Table 13. Contingency tables of the first order interactions of factors effecting levels of IL-2 production

1.

	MHC allele		
Ir-GAT	B1	B19	$\bar{X}_{GAT}$
High	2.26[a]	1.08	1.67 *
Low	2.01	0.94	1.48
$\bar{X}_{allele}$	2.14 **	1.01	

2.

	MHC allele		
R-Rs-1	B1	B19	$\bar{X}_{RSV}$
Regressor	1.88	1.19	1.54
Progressor	2.39	0.83	1.61
$\bar{X}_{allele}$	2.14 **	1.01	

3.

	Ir-GAT		
R-Rs-1	High	Low	$\bar{X}_{RSV}$
Regressor	1.78	1.29	1.54
Progressor	1.56	1.66	1.61
$\bar{X}_{GAT}$	1.67 *	1.48	

[a]Mean IL-2 U/ml from 10 individuals, triplicate cultures.

*$P < 0.05$.

**$P < 0.001$.

Table 14. Mean levels of IL-2 production of the S1 sublines (genotypes)

Genotype (Ea-B-Ir-GAT[2]-RSV[3])	Mean[1] (IL-2 U/ml)
B1-Hi-Reg	2.31[a]
B1-Hi-Prog	2.20[a]
B1-Lo-Reg	1.45[b]
B1-Lo-Prog	2.57[a]
B19-Hi-Reg	1.24[b,c]
B19-Hi-Prog	0.93[c,d]
B19-Lo-Reg	1.14[b,c]
B19-Lo-Prog	0.74[d]

[1]Means bearing different letters are significantly different, $P < 0.05$.

[2]High or low immune response to GAT.

[3]Regression or progression of RSV-induced tumors.

Comparison of IL-2 Calculation Methods

Due to the inconsistency in the literature, two alternative methods of calculating levels of chicken IL-2 production were compared to the method used throughout this study. The values reported and used for analysis throughout this study were obtained by dividing the log %CM at 50% maximal proliferation of the unknown test sample by the log %CM at 50% maximal proliferation of the reference standard (see materials and methods). Alternatively, levels of IL-2 can be expressed as: 1) the ratio of the cpm of the test sample at 25% CM to the cpm at 25% CM of the reference standard, and 2) gross cpm at 25% CM of the test sample. Each of the three methods were used to calculate levels of IL-2 production for all the birds used throughout the study. As shown in Table 15, the linear relationship of the values (i.e., correlation) obtained using each method were highly comparable. Therefore, studies that utilized any of these methods could validly compare results with another study that also used any the three methods of IL-2 calculation.

Table 15. Correlations of methods used to calculate levels of chicken IL-2 production

Test	N[a]	r[b]
Method 1[c] vs. Method 2[d]	230	0.94
Method 1 vs. Method 3[e]	230	0.89
Method 2 vs. Method 3	230	0.87

[a]N represents a combined total of 230 birds obtained from both the inbred and S1 studies.

[b]r = correlation coefficient.

[c]Method 1 = IL-2 U/ml values used throughout this study which were obtained by dividing the log %CM at 50% maximal proliferation of the test sample by the log %CM at 50% maximal proliferation of the reference standard.

[d]Method 2 = IL-2 U/ml values obtained by dividing the cpm of the test sample at 25% CM by the cpm of the reference standard at 25% CM.

[e]Method 3 = cpm of the test sample at 25% CM.

DISCUSSION

IL-2 is a soluble immunoenhancing factor that augments T cell responses, IFN-γ production, and NK activity and may participate in T-dependent B cell responses (Farrar et al., 1982). Since IL-2 plays a pivotal role in the immune response, a great deal of work has been aimed at determining the mechanisms of its action in mammals. Similar efforts, however, have not been made in the study of avian IL-2. The paucity of reports has been primarily due to the lack of an efficient and effective assay to detect avian IL-2 activity. Recently, Kromer et al. (1984) have determined the optimal conditions to measure avian IL-2 activity which has provided avian immunologists a means of studying avian IL-2.

The objective of this study was to utilize the avian IL-2 assay to determine whether there are genetic influences controlling levels of IL-2 production. Before the birds were assayed for relative levels of IL-2 production, the assay was standardized to insure valid comparisons of genetically different individuals. In addition, after levels of IL-2 had been determined, ability to produce IL-2 was compared with known responses to different antigenic challenges.

Avian IL-2 Assay

The lymphoproliferative assay used to detect IL-2 activity measures the ability of supernatants (containing IL-2) from lectin-activated mononuclear cells to promote the proliferation of a responding population of lectin-activated lymphocytes <u>in vitro</u> (Gillis et al., 1978). A number of modifications have been made to the mammalian IL-2 assay system first described by Gillis and his associates so it could be used to detect

avian IL-2 (Kromer et al., 1984). First, an enriched population of responding lectin-activated chicken lymphocytes are used instead of the readily available mammalian cloned cytotoxic T cell lines. Second, erythrocytes are added to the lectin-activated mononuclear cells to optimize lymphoproliferation. Finally, an avian IL-2 reference standard has been used in this study because chicken lymphocytes do not respond to mammalian IL-2 (Kromer et al., 1984; Vainio et al., 1986).

Ideally, an assay for IL-2 activity would use supernatants that contain only IL-2 and the responding cell population would be comprised solely of CTLs. The assay system used in this study, however, measures the ability of crude preparations of mitogen-stimulated PBL supernatants to promote the proliferation of an enriched population of lymphoblasts. Because both the supernatants and responding cell populations are not homogeneous, the assay can only provide an indirect estimation of IL-2 activity. Peripheral blood lymphocyte cultures are composed of a heterogeneous population of cells including T cells, B cells, macrophages, and polymorphonuclear leukocytes. These cell populations have the capability of producing other immunoenhancing factors that also augment lymphoproliferation (Farrar et al., 1982). The influence of B cells is probably negligible since a T cell mitogen, Con A, is used in the assay system. Macrophages, however, produce IL-1 which promotes the proliferation of the IL-2-producing T_H cells (Larsson et al., 1980a). Thymocytes also produce IFN-γ that affects lymphoproliferation (Farrar et al., 1981). In addition, the responding cell populations contain a heterogeneous mixture of lymphoblasts since the Percoll separation

procedure does not distinguish between lymphocyte subsets. Therefore, due to the lack of well-defined supernatants and responding cell populations, the assay used in this study measures IL-2 "like" activity based on its defined mechanism of action.

Another source of non-IL-2 associated lymphoproliferation may occur as result of residual Con A contained in either the crude supernatant preparations or responding cell cultures. The influences of any residual Con A that may be present seems unlikely, however, since α-MM is used in the assay system. Frederickson and Sharma (1985) have shown that α-MM, a competitive inhibitor of Con A, completely abrogates the influence of Con A. In this study, the responding lymphocytes were pre-incubated in the presence of α-MM, washed twice, and resuspended in media containing α-MM. The conditioned media (CM, supernatants containing IL-2) were diluted in media containing α-MM. As a result, functional determination of IL-2 activity is performed in media containing α-MM. Moreover, because the responding cell population is composed of blast cells, further proliferation can only be mediated by the addition of IL-2 and not by Con A (Smith et al., 1979).

Responding cell population

Studies that determine mammalian IL-2 activity traditionally utilize a cloned cytotoxic T cell line as their source of responding cells (Smith, 1984). Cloned murine or human T cell lines, however, do not respond to avian IL-2 (Kromer et al., 1984; Vainio et al., 1986). As a result, cultured chicken lymphocyte preparations are used as the source of responding cells. The establishment and maintenance of continuous

chicken T cell lines have been difficult which have made them hard to obtain (Kromer et al., 1984). A Percoll enrichment step has been incorporated into the avian IL-2 assay system as means of obtaining purified chicken T cell populations.

The effectiveness of the Percoll separation procedure in enriching populations for Con A-activated lymphoblasts was examined (Table 4). The results agree with those of Kromer et al. (1984) in that Percoll-enriched T cells were more sensitive to IL-2 stimulation that non-separated populations of cultures PBLs. This does not exclude the possibility that continuous chicken T cell lines may be a more effective means of detecting IL-2 activity.

Recently, Vainio et al. (1986) have generated a long-term chicken T cell line that responds better to IL-2 than Con A-activated lymphoblasts. They did not, however, enrich their populations of Con A-activated cell cultures with Percoll before assaying for IL-2 activity. In addition, they used Ficoll-Hypaque to separate PBLs from the whole blood which has been shown to yield inconsistent degrees of lymphoproliferation with chicken lymphocytes (Maheswaran and Thies, 1975).

The responding cells used in this assay were obtained from birds of the same genotype (line 19-B15.1). The lymphocytes from these birds yielded the highest number of blast cells following Con A-activation which meant more tests could be performed (personal observation; Palmer et al., 1981). More importantly, by using responding cells from the same genotype, differences in levels of IL-2 production can not be attributed to genetic differences of the responding cells.

Preparation of crude supernatants

The primary difference between the avian and mammalian IL-2 assays is that the avain IL-2 assay includes chicken RBCs in its lectin-activated mononuclear cell cultures as a means of enhancing the production of IL-2. Kromer et al. (1984) showed that Con A bound to RBCs increased IL-2 production by ten-fold. Frederickson (T. L. Frederickson, USDA-ARS, East Lansing, Michigan, personal communication) has found that the mere addition of RBCs to the culture was equally as effective in enhancing IL-2 production. He also suggests that RBCs may play a role in enhancing IL-2 production by enhancing antigen presentation. Kromer et al. (1984) suggest that RBCs may augment antigen presentation by forming complexes with the mitogen which enhances the ability of Con A to combine with macrophages and T cells than would free mitogen.

An important consideration of working with chicken RBCs is the fact that they possess MHC determinants (Class I) that are shared by lymphocytes (Pink et al., 1977). It is therefore possible that antigen presentation may also be heightened via MHC recognition. Kromer et al. (1984), however, have reported that both MHC-compatible and -incompatible RBCs were equally as effective in the assay system. It should be noted that their study did not incorporate as many different MHC haplotypes as this study did. In addition, their bird populations are comparatively small so the genetic diversity would not be as great as it is in this study (S. J. Lamont, Department of Animal Science, Iowa State University, personal communication). Therefore, to avoid possible influences due to MHC-incompatibility, autologous RBCs are used.

Reference standard

Mammalian IL-2 studies traditionally incorporate a reference standard of IL-2 activity as a means of accounting for assay-to-assay differences and to provide other investigators with a comparative indication of the IL-2 levels observed in their study. This study has adopted this practice. But in order to utilize a reference standard an avian IL-2 reference standard had to be created because mammalian IL-2 does not augment chicken lymphoproliferation (Kromer et al., 1984).

The reference standard used for this study was a crude supernatant preparation obtained from a culture of one individual GH-B15.1 bird. The average log %CM giving 50% maximal proliferation of the reference standard is 1.12 ± 0.08. The standard deviation is unexpectedly low because lymphoproliferative assays traditionally have a high standard deviation. The reason for the low standard deviation may be due, in part, to the use of a log scale which tends to normalize values. Frederickson and Sharma (1985) have reported similar findings. They determined that a 6-fold change in the concentration of responder cells did not change the %CM that provides 50% maximal proliferation. The slopes of the response curves varied, but the log %CM yielding 50% maximal proliferation did not.

As stated previously, each laboratory investigating avian IL-2 expresses levels of IL-2 activity in a different manner. Schnetzler et al. (1983) expressed levels of IL-2 as the log %CM yielding 50% maximal proliferation without considering a reference standard. Kromer et al. (1984) presented their data in terms of gross cpm of ^{125}I-deoxyuridine

incorporation. Frederickson and Sharma (1985) were the first to use a reference standard in an avian IL-2 assay system. Recently, Vainio et al. (1986) defined 1 unit of IL-2 as 1/100 of the cpm from a culture containing a 1/20 dilution of the CM. Because of the dicotomy of approaches used to calculate avian IL-2 levels, a comparison was made to determine whether data expressed as raw cpm could be validly compared to data that has been adjusted based on a reference standard. In addition, a comparison was made between a method that uses the ratio of the cpm of the unknown CM to cpm of the reference standard CM and a method that uses the ratio of the log %CM of the unknown to the log %CM of the reference standard as a means of expressing IL-2 levels.

As shown in Table 15, there is a high correlation ($r = 0.94$) between calculations that utilize a reference standard. These data agree with the observations of Frederickson (personal communication) who now utilizes the ratio of the unknown cpm to the cpm of the reference standard as a means of expressing IL-2 activity. There was also a high positive correlation ($r = 0.89$, $r = 0.87$) between the IL-2 calculation methods that utilized a reference standard and the method that expressed IL-2 levels as raw cpm. It should be emphasized that a positive correlation, as seen with these data, indicates that there is a linear relationship between the values obtained using each calculation method. It does not imply that values obtained in one study can be quantitatively compared with values obtained from another study. In order to quantitatively compare results obtained from different studies a reference standard would have to be used. Based on these results,

however, a study utilizing any of the three methods described here, could validly compare relative relationships of levels of IL-2 production with another study that also used any other of these three methods.

Analysis of the Genetic Associations of IL-2 Production

The results of the inbred lines study and S1 line study present an enigma as to the source of genetic influence on levels of IL-2 production. The inbred lines study demonstrates that non-MHC influences are greater than MHC influences on levels of IL-2 production. The S1 line study, on the other hand, indicates that MHC influences are highly significant in affecting levels of IL-2 production.

The reason for the discrepancy of results between the two studies may be a result of the differing genetics of the birds used in each. The birds used from the inbred lines study are at least 81% inbred indicating that there is little within-line variability in their background (non-MHC) genes. The S1 line, a partially (approximately 40%) inbred line, has more variability in its background genes. In addition, the S1 sublines (genotypes) share a common pool of background genes so that each subline could posses all the allelic forms of the non-MHC gene(s) controlling levels of IL-2 production contained in the gene pool. It is conceivable, therefore, that a gene locus coding for a particular level of IL-2 production has been fixed within certain of the inbred lines. Conversely, there is a smaller chance that one allelic form of this gene has been fixed within the S1 line and the non-MHC genes are randomized throughout the sublines (genotypes) of the S1 line. The S1 line, therefore, is an appropriate genetic model system to investigate MHC-

associated control whereas the inbred lines are more appropriate for examination of non-MHC control.

Preliminary study

Before the different genetic stock were assayed to determine their relative levels of IL-2 production, a preliminary study was conducted to determine if there was evidence for a genetic association of levels of IL-2 production. In addition, the influence of sex and time of sampling on levels of IL-2 production were also investigated.

The preliminary study indicated that there are genetic differences of levels of IL-2 production between the nine different genotypes examined (Table 5). The finding that there appeared to be genetic differences was encouraging because it indicated that there may be a genetic association of IL-2 production, despite the paucity of reports supporting this notion.

The effect of sex differences was not significant on levels of IL-2 production (Table 5). The results of the inbred lines study and S1 line study also supported these data. Therefore, the gene(s) controlling levels of IL-2 production does not appear to be associated with sex.

Differences in levels of IL-2 production that occured as a result of obtaining samples in either the morning or afternoon were not significant. This indicates that IL-2 production is probably not a diurnal event. Since only two different sampling times were tested, it is impossible to be sure that there are not cyclic changes in levels of IL-2. Nevertheless, differences in levels of IL-2 production from

samples obtained in the morning and afternoon were not considered to be statistically significant in the inbred lines study and S1 line study.

The effect of week-to-week sampling diffences approached significance. As a result, in subsequent studies, equal numbers of samples from each of the genotypes were gathered during each week to insure that the weekly sampling effects would not favor one genotype over another. The fact that weekly sampling effects influenced levels of IL-2 production suggest that the ability to produce IL-2 is age-related. The association of age and levels of IL-2 production is well documented for mice, humans, and rats (Gillis et al., 1981; Thoman and Weigle, 1982; Gilman et al., 1982).

Genetic association of IL-2 production in the inbred lines

As stated previously, the inbred lines are highly inbred, and include some sets composed of sublines of birds that are congenic at the MHC region. Therefore, it is highly probable that a non-MHC gene locus mediating a certain level of IL-2 production has been fixed within each of these lines. The analysis of variance of the effects of non-MHC and MHC influences on levels of IL-2 production (Tables 7 and 8) strongly suggest non-MHC effects are greater than MHC effects. This notion is supported by the observation that two lines (Line 19 and Line GH) possessing the same MHC type (B13 and B15.1) differ significantly in their levels of IL-2 production whereas there is not a significant difference associated with MHC type differences within the same line (Table 9). These data agree with those of Schauenstein et al. (1985) who showed that there was not a difference in levels of IL-2 production

between Obese strain birds of three different MHC types.

It should be noted that three of the individual analyses of variance for the effects of MHC type (Table 7) show the influence of the MHC to be approaching significance. This indicates that MHC effects may also influence levels of IL-2 production in the ISU inbred lines, but to a lesser degree than non-MHC effects. The possibility of MHC influences on levels of IL-2 production is not unexpected since T cell immune response requires MHC recognition (Clark, 1983).

Genetic association of IL-2 production of the S1 line

Analysis of variance of the partially inbred S1 line indicate that the effects of the genes encoding Ea-B type and immune response to GAT but not immune response to RSV-induced tumors significantly influence levels of IL-2 production (Table 12). It should be emphasized at this point that the genes coding for Ea-B type, high or low immune response to GAT, and regression or progression of RSV-induced tumors have been shown to be associated or linked with the MHC (Schierman and Nordskog, 1961; Benedict et al., 1975; Schierman et al., 1977) It is therefore unexpected, based on a lack of association between MHC genes and IL-2 levels seen in earlier studies of murine and avian IL-2 and on the results of the inbred lines study, to see a significant association of the MHC and levels of IL-2 production (Farrar et al., 1982; Schauenstein et al., 1985). In addition, since the effects of the genes controlling Ea-B type and immune response to GAT are significant, the effect of the gene controlling the response to RSV-induced tumors would have also been expected to be significant because these genes have been shown to be

closely linked (Gebriel and Nordskog, 1983).

A possible explanation for the contrasting results between the inbred lines study and S1 line study is that there may be more than one gene locus controlling levels of IL-2 production. It appears that at least one gene locus is located outside the MHC and one gene locus may be encoded within the MHC. The results of the inbred lines study provide preliminary evidence of this possibility. Within the inbred lines, both MHC and non-MHC influences are observed although the effects of the MHC only approached significance at the 5% probability level. The results of the S1 line study, however, clearly support the possibility of MHC influences on levels of IL-2 production.

The heterogeneity of the S1 background genes may be one reason why non-MHC influences are not observed within the S1 line. As stated previously, it is probable that a non-MHC gene locus controlling a particular level of IL-2 production has not been fixed within the S1 line because the S1 line is not highly inbred. As a result of the variability of this gene locus in the non-MHC region, the effects of MHC influences on levels of IL-2 production are observable within the S1 line.

As stated previously, the ability to respond to RSV-induced tumors has been shown to be linked with the immune response to GAT and Ea-B type (Gebriel and Nordskog, 1983; Schierman et al., 1977). Therefore, an accordant effect on levels of IL-2 production would have been expected. This is not the case, however (Table 12). Unlike the association of Ea-B type and immune response to GAT on levels of IL-2 production, the effects of the ability to respond to RSV-induced tumors was not significantly

associated with IL-2 production. In addition, there was an inverse relationship between the effects of the ability to respond to RSV-induced tumors and the immune response to GAT and Ea-B type on levels of IL-2 production (Table 13).

The reason for the differences observed between the effects the ability to respond to RSV induced tumors and the effects of the gene loci controlling immune response to GAT and Ea-B type on levels of IL-2 production may be explained by the fact that the Ea-B and GAT were held constant in sublines when selecting for regression or progression of RSV-induced tumors. As a result, selection may have been primarily for non-MHC loci controlling RSV tumor response. Collins et al. (1980) have shown that non-MHC loci also influence the ability to regress or progress RSV-induced tumors. Because the first and second order interactions between RSV and MHC (immune response to GAT and Ea-B type) are significant, this lends credence to the notion that more than one gene locus controls levels of IL-2 production, and at least one locus is within and one is outside the MHC.

The possibility that at least two different loci may be controlling levels of IL-2 production, at least in this assay system, is supported by an earlier study by Morrow and Abplanalp (1981). They showed that proliferation in response to Con A is mediated by at least two different loci, one linked to the MHC (Con A2) and at least one located outside the MHC (Con A1).

It is conceivable, therefore, that differing levels of IL-2 production may be attributed to the same genetic control as that

influencing the immune response to Con A in IL-2 producing cells. Kromer et al. (1984) reported a high correlation between proliferation in response to Con A and levels of IL-2 production. Another report from their laboratory suggests that non-MHC control of the immune response to Con A accounts for differences in levels of IL-2 production (Schauenstein et al., 1985). Their results do not discount the possibility that levels of proliferation in response to Con A may be influenced by the ability to produce IL-2. The cells used in their studies contained a heterogeneous population of T lymphocyte subsets that included both cells that produce and cells that respond to IL-2. It is possible that levels of mitogen-induced T cell proliferation may be a consequence of both Con A-stimulation and response to the presence of IL-2. As a result, high levels of mitogen-induced T cell proliferation would be observed when high levels of IL-2 are measured.

In order to determine whether levels of IL-2 production are genetically controlled or whether levels of IL-2 production are a consequence of the genetic control of the immune response to Con A, an assay system that greatly reduces or eliminates non-IL-2-induced proliferation should be used. This could be accomplished by comparing the levels of proliferation of a homogeneous population of Con A induced T_H cells (i.e., IL-2 producing cells) with the levels of IL-2 produced by this population. The use of homogeneous populations of IL-2 producing cells would eliminate the effects of other cells and their soluble factors that also influence T cell proliferation. Because IL-2 producing cells will not respond to IL-2, their proliferation should be a result of

the activation by Con A. Moreover, to distinguish between IL-2 and other soluble factors that may also be present, antibody to avian IL-2 could be used to detect levels of IL-2 directly from the CM. Therefore, by comparing the levels of response due to Con A activation and levels of IL-2 measured directly from the CM, it may be possible to distinguish between the genetic control of response to Con A and levels of IL-2 production. The absence of a correlation would indicate that levels of IL-2 production are genetically controlled independent of Con A response. This experiment cannot be performed at this time because avian immunologists have not developed the reagents to identify and separate T lymphocyte subsets.

Associations of Levels of IL-2 Production and Disease

The association between abnormal levels of IL-2 production and a variey of disease states and immune disorders is well-documented (for a review, see Kromer et al., 1986; Welte and Mertelsmann, 1985). In this study, the inherent ability of each genotype to produce IL-2 is compared with their known ability to respond to different antigenic challenges. It should be emphasized that the birds were not assayed for levels of IL-2 production during antigenic challenge. The objective of this work is to examine the possibility of an association between differences of "normal" levels (unchallenged birds) of IL-2 production and immune response to different disease challenges.

As described previously, there is a significant association between the immune response to GAT and levels of IL-2 production. As illustrated in Table 13, high responders to GAT produced significantly higher levels

of IL-2 than low responders to GAT. There is not, however, an association between the ability to progress or regress RSV-induced tumors and levels of IL-2 production. Therefore, it appears the the humoral immune response to GAT but not to RSV-induced tumors may be associated with a genotype's inherent ability to produce IL-2.

Steadham et al. (1987) have demonstrated that B1B1 birds were significantly more resistant to Marek's disease than B19B19 birds of the S1 line. High responders to GAT, within each Ea-B type, were significantly more resistant to Marek's disease than low responders to GAT. In addition, they observed that there was not an association between resistance to Marek's disease and immune response to RSV-induced tumors, as demonstrated previously (Calnek et al., 1975). The results of this study correlate with the results of Steadham and his associates. Similar trends of significance of levels of IL-2 production are observed with the relative ability to resist Marek's disease. Genotypes with higher levels of IL-2 production also are more resistant to Marek's disease. This may suggest that resistance to Marek's disease (a lymphoproliferative disease) may be associated with the ability to produce IL-2.

Lamont et al. (1987) have demonstrated a highly significant association between resistance to P. multocida (etiological agent of fowl cholera) and Ea-B type of the S1 line. The association between response to GAT and resistance to P. multocida was less significant, and the RSV tumor response was not significant. A similar relationship of significance of IL-2 levels is observed within the S1 line. The

genotypes with higher IL-2 levels are more resistant to fowl cholera. It is possible that the inherent ability to produce IL-2 may influence the ability to resist P. multocida.

Comparisons of the trends of significant influences between immune response to an antigenic challenge and levels of IL-2 production are offered as possible avenues of further investigation. This study is designed to determine relative levels of IL-2 production in unchallenged birds. Additional studies that investigate the relationship between levels of IL-2 production and disease should be performed on challenged birds to confirm the role of IL-2 in avian disease resistance.

SUMMARY

Interleukin-2 is a soluble immunoenhancing factor that plays a central role in the augmentation of cell-mediated immune responses and may also influence T-dependent B cell responses. Because of the importance of IL-2 in the immune system, a great deal of work has been done in ascertaining its mechanism of action. The study of avian IL-2, however, was not possible until an effective and efficient avian IL-2 assay was recently developed. The avian IL-2 assay has been utilized in this study to examine relative levels of IL-2 production of the various genetic stock at the Iowa State Poultry Research Center.

The results of this study indicated that there is genetic control of levels of IL-2 production in the chicken. Because two different genetic model systems were used in this study, MHC as well as non-MHC influences on levels of IL-2 production could be examined. The study of the inbred lines indicated that the genetic control of IL-2 production was a result of non-MHC genes. The study of the partially-inbred S1 line, on the other hand, indicated that genetic control of IL-2 production was due to MHC genes. Therefore, the combined results of both genetic model systems indicate there may be at least two gene loci, one encoded outside and one encoded inside the MHC, controlling levels of IL-2 production.

This study does not discount the possibility that differing levels of IL-2 production may be a consequence of the genetic control of the immune response to Con A. Because Con A was used as the source of antigenic stimulation, it is possible that differences of levels of IL-2 production measured are a result of the genetic control of the immune

response to Con A. The responding cells in this study, however, were washed thoroughly and cultured in the presence of a Con A inhibitor to insure that the effect of any residual Con A would not influence the levels of IL-2 measured.

The possibility that the inherent ability to produce IL-2 affected disease resistance was also examined. Resistance to Marek's disease and fowl cholera but not to RSV-induced tumors was associated with the ability to produce IL-2.

The study of the role of IL-2 in the avian immune system is in its infancy. There is a great deal of work yet to be done in the examination of the role of IL-2 in the avian immune system. Because of the phylogenic difference between mammals and aves, the study of the avian immune system and the factors that govern its activity should prove useful in understanding the evolution of the immune system.

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