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Comparison of Immunologic Responses Between Mice Differing at the Agouti Locus : Immunologic Abnormalities in Lethal Yellow (Ay) Mice are Augmented by Obesity

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COMPARISON OF IMMUNOLOGIC RESPONSES BETWEEN MICE DIFFERING AT THE AGOUTI LOCUS: IMMUNOLOGIC ABNORMALITIES IN LETHAL YELLOW (A^y) MICE ARE AUGMENTED BY OBESITY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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D E D I C A T I O N

To my family, Tim, Nathan, and Amanda, for their patience and to my friends, Alethea F., Lynn W., and John B., who helped me keep a rational perspective during the course of this thesis.

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I. ABSTRACT

The agouti locus, located on chromosome 2 of the house mouse (mus musculus), functions in the complex regulation of pigment synthesis. The complexity of its regulatory role is revealed through the aberrations that become manifest when a mutation, specifically the lethal yellow (A^y) mutation, occurs at the agouti locus. The lethal yellow mutation is associated with an alteration in pigment synthesis, onset of obesity at approximately 120 days, and an increased susceptibility to cancer. Based on the putatvie relationship between cancer and immunity, this mutation may be correlated with altered immune mechanisms. This correlation was investigated by comparing a delayed-type hypersensitiivity (DTH) response to dinitrofluorobenzene (DNFB), in vivo humoral immunity to sheep red blood cell (SRBC), and in vitro lymphocyte reactivity of C57BL/6J A^y/a mice with congeneic a/a controls. Responses were compared between 42-day-old and 120-day-old mice to determine the correlation, if any, between altered immunity and age-onset obesity. Data indicate that the A^y mutation is directly liked to a supressed DTH response and, to some extent, a decreased reactivity to mitogen-induced lymphocyte proliferation. Obesity appears to play a role in the alteration in humoral immunity as demonstrated by an enhanced anti-SRBC IgM response and suppressed antibody-forming cell (AFC) response to SRBC. Finally, serum from obese yellow (A^y/a) mice was markedly suppressive in in vitro lymphocyte proliferation assays.

II. INTRODUCTION AND LITERATURE REVIEW

II.A. The Agouti Locus

The agouti locus, ubiquitous in most orders of mammalia, is located on chromosome 2 of Mus musculus (59). In the wildtype (agouti) mouse, this locus regulates the synthesis of two pigments, phaeomelanin and eumelanin which produce yellow and black hair color, respectively. The agouti genotype (A/A or +/+) is phenotypically expressed as a hair bearing a black shaft, a yellow band, and a black tip.

Eumelanin and phaeomelanin are synthesized in melanosomes which are small granules synthesized by the endoplasmic reticulum and Golgi complexes of melanocytes (85). The regulation of the synthesis of these two pigments is the product of many complex interactions. The switch in melanin synthesis from phaeomelanin to eumelanin appears to be correlated with an increase in tyrosinase activity (7). Tyrosinase functions in the oxidation of tyrosine to dopachrome, a melanin precursor. Three isozymes of tyrosinase have been identified which vary according to their attached carbohydrates (56). Glycosylation of tyrosinase may therefore determine the type of melanin synthesized (85). The switch to eumelanin synthesis may also be controlled by a pituitary hormone known as melanocyte stimulating hormone (MSH) (56,66). This hormone stimulates tyrosinase activity through cyclic AMP (cAMP). However, tyrosinase activity is not solely regulated by MSH and is believed to be

controlled by a number of other factors which can also operate through a cAMP messenger system (7). That cyclic AMP is central to this conversion is indicated by its ability to switch eumelanin synthesis to phaeomelanin (66).

II.A.1. Regulatory Role of the Agouti Locus

The mode of action imparted by the agouti locus in the regulation of pigment synthesis is unknown. However, at least five regulatory mechanisms, not necessarily distinct, have been proposed for its actions. First, the agouti locus is believed to indirectly influence pigment production by the melanocyte through a follicular regulatory factor. Melanocytes from black mice, producing only eumelanin, will begin to produce phaeomelanin when transplanted into the hair follicles of yellow mice. The reciprocal transplant, yellow to black, results in a shift in pigment synthesis from phaeomelanin to eumelanin (65). Thus the microenvironment of the melanocyte strongly influences its pigment production.

Secondly, the type of pigment produced appears to be dependent upon the ratio of an unidentified `regulatory parameter' putatively produced by the agouti locus and the bulb mass (19). A proposed model suggests that phaeomelanin is produced when the ratio of regulatory parameter to bulb mass exceeds a critical threshold. As a corollary to this, eumelanin is synthesized when this ratio falls below the critical threshold (19).

Third, a correlation between the structure of the melanosome and the type of pigment produced is noted. Melanosomes that contain eumelanin are rod-shaped and those that contain phaeomelanin are spherical (65). Thus, this suggests that the agouti locus plays a regulatory role in the determination of the ultrastructure of melanosomes (65).

Fourth, the agouti locus is postulated to be involved with the regulation of tyrosinase. A mutation at the agouti locus (A^y) which is marked by the synthesis of phaeomelanin only has been correlated with the absence of two of the three isozymic forms of tyrosinase (56).

Finally, Wolff (82) postulates that the regulatory factor(s) controlled by the agouti locus may focus its action on the endoplasmic reticulum, a major site of protein synthesis. He speculates that alterations in this region may affect a number of functions throughout the metabolic network (82).

II.A.2. Genetic Linkage of the Agouti with Immunoregulatory Genes

Chromosome 2 mapping reveals a close association between the agouti locus and several immunoregulatory gene sequences. This region is remarkably analogous to the Major Histocompatibility Complex (MHC) of chromosome 17 in the murine system (54). The MHC region is composed of a tightly linked series of genes, often referred to as H-2, which encodes for a number of traits of immunological significance including the histocompatibility antigens

(62), tumor virus susceptibility and immune responsiveness (50).

Three distinct classes of genes have been mapped in the MHC region of Chromosome 17. These are Class I (histocompatibility), Class II (immune response), and Class III (complement) genes. Briefly, antigens coded for by the Class I region were first recognized as the primary antigens involved in graft rejection, a measure of histocompatibility, and are thus often referred to as transplantation antigens. Three families of histocompatibility genes have been identified in this class and are referred to as K, D, and L (70). These histocompatibility loci code for histocompatibility antigens which are located on all nucleated cells and are found in the highest concentration on the membranes of B cell, T cells, and macrophages (70). These histocompatibility antigens allow for the differentiation of self from non-self antigens thus enabling the detection and subsequent elimination of aberrant cells by the immune system. Certain T-cell subsets only recognize antigen when presented in association with Class I antigens. This is referred to as MHC restriction.

Individual animals differ in their immunologic responsiveness to specific antigens. The ability to respond and/or the intensity of the response is inherited in a Mendelian fashion (6). This ability has been ascribed to immune response or Ir genes. These genes code for the strength of the serological response to T-dependent antigens (6,63). In other words, Ir genes regulate the interaction of antigen-presenting cells and the T-cell. The Ir gene

products, referred to as Ia antigens, are predominantly expressed on cell membranes of antigen presenting cells (B lymphocytes, macrophages, and dendritic cells) and are rarely expressed on T lymphocytes (63). Thus far, only a few structural Ia antigens have been identified. This presents a perplexing situation in that immunologists have been unable to explain how a relative paucity of immune response genes can control the magnitude of immune responses to an enormous variety of antigens (53).

The most commonly recognized Ir genes are linked to the histocompatibility antigens of the H-2 region on chromosome 17 and are commonly referred to as the Class II MHC genes (62). Similar to Class I MHC restriction, Class II MHC restriction also occurs. For example, interaction between helper T-cells and either macrophages or B cells occurs only if the helper T-cells possess specific receptors for the Class II antigens on the surface of antigen-presenting cells (70). As a result, antigen presenting cells and T-cells must have the same haplotype in order for T-cell activation to occur (53). Class II genes also control the proliferation, differentiation and effector functions of regulatory T-cells which ultimately control the B-cell response through the amplification or suppression of T-cell subsets (70).

Considerable attention to the MHC region has occurred as a result of the rapidity of graft rejection when the antigens coded by the H-2 region in donor and recipient mice are mismatched. However, even when these antigens were compatible, graft rejection continued

to occur, thus indicating that other loci on other chromosomes were also found to control graft survival. These loci are referred to as non-H-2 loci and code for non-H-2, or "minor histocompatibility" antigens. Disparity between non-H-2 loci may lead to graft rejection, albeit usually with less intensity than H-2 mismatched mice (51). Nevertheless, the potency of these non-H-2 loci should not be underestimated: the multiplicity of their effects can often result in graft rejection with magnitudes similar to that observed with H-2 disparity (51). These non-H-2 loci may also be as polymorphic as the MHC (51).

Significantly, several non-H-2 histocompatibility loci are located in the fifth linkage group of chromosome 2 near the agouti locus. Backcrossing of strain B10.LP indicates that the H-3 histocompatibility locus is linked to the agouti allele with a mapping distance of 10.3 centimorgans (cM) in males and 20.5 cM in females. A second weaker histocompatibility locus, H-13, is linked closer to the agouti locus than the H-3 locus. Additional histocompatibility loci are also believed to be linked to the H-3 and H-13 loci (54).

As observed with other histocompatibility loci (54), the histocompatibility genes of chromosome 2 are in close proximity with immune response genes, specifically the Ir-2 locus which has been shown to control the immune response to an erythrocyte antigen. This locus appears to be linked to the H-3/H-13 complex. Furthermore, the Ir-2 locus (denoted as such to differentiate it from the Ir-1 locus

of the MHC) has been determined to be linked to the agouti locus with a distance of 20 map units (20,54). A further similarity with the H-2 region of chromosome 17 is that chromosome 2 contains gene sequences which code for both lymphocyte surface markers, i.e. Ly-4 and Ly-m11, and a complement component (C5) (11,54). Chromosome 2 has additional immunologic importance in that the B-2-microglobulin gene is found in this linkage group (11). B-2-microglobulin is a small polypeptide of unknown function that is noncovalently linked to the Class I histocompatibility antigens (70) and has amino acid and structural homology with regions of the immunoglobulin molecules (60,67).

II.A.3. Mutations at the Agouti Locus

The complexity of the agouti locus is demonstrated by the wide array of effects that mutations at the agouti locus impart. One such mutation is the viable yellow (A^{VY}) mutation. This mutation is associated with an alteration in regulation of the hair color pattern. Additionally, it is associated with an increased susceptibility toward obesity and neoplasms (40,84). This mutation is a valuable model for the study of the possible correlation between the microenvironment and the expression of the agouti locus because it allows for the segregation of two phenotypes within a genotype. While homozygous viable yellow (A^{VY}/A^{VY}) are yellow, the heterozygotes (A^{VY}/a) may demonstrate two different phenotypes even though they are genetically identical. These two phenotypes are referred to

as mottled yellow and pseudoagouti. Only the mottled yellow become obese while the pseudoagouti remain lean. These phenotypic differences may result from the environmental modulation of the regulatory processes of the mutant gene product (85). Investigators have postulated that the environment of the reproductive tract of the dam plays a major role in the phenotypic differentiation of the A^{VY}/a embryos (85).

At least seventeen distinct alleles have been identified at the agouti locus (56). In addition to the viable yellow mutation which results in different pigmentation patterns, there is the lethal yellow mutation. This mutation, which is quite similar to but more severe than the viable yellow was first described by Cuenot in 1905 as referenced by Heston (34) and is designated A^{y}. A number of manifestations are associated with this allele, of which the most visible effect is the coat color. As a result of this mutation, hair bulb melanocytes of strain C57BL/6J mice produce only phaeomelanin resulting in a coat of yellow hair. This mutation does not seem to involve an impairment inherent with the melanocyte. When MSH or cyclic AMP is added to organ culture of skin from yellow mice, eumelanin synthesis is induced (66). This suggests that the agouti locus may impart its action on regulatory mechanisms involving the -MSH-cAMP system.

II.B. The Lethal Yellow (A^Y) Mutation

The A^Y mutation, unlike the viable yellow, is a recessive lethal mutation with homozygous blastocysts dying on the fifth day after conception (44). This is apparently due to a failure in implantation to the uterine wall or to intrinsic defects within A^Y/A^Y embryos (23). However, mice heterozygous (A^Y/a) for the lethal yellow gene are viable. For this reason, the A^Y mutation is maintained in a congeneic strain by inbreeding with the nonagouti (black) mouse designated as a/a. This strain is genetically identical to the A^Y/a at all loci within the genome except at the agouti locus. With the exception of the viable yellow, the A^Y gene is dominant to all other alleles identified at this locus (56). Like the viable yellow, A^Y is associated with obesity and an increased susceptibility towards cancer.

II.B.1. Molecular Studies of the Mutation

While the secondary physiological alterations imparted by the lethal yellow mutation are well known, the direct action of the gene on the gene product affected by this mutation has not been elucidated. The search for possible aberrations in protein function as a result of this yellow mutation has been complicated by the diverse effects of genomic background. The effects of the yellow mutation depend upon the residual background genome (82). For example, effects of background genome have been demonstrated in the study of embryonic survival in the A^Y/a mouse where decreased survival of

female A^Y/a embryos is noted in A^Y/a dams. This decreased survival of yellow embryos, however, was only observed in one of the strains studied (83) and, therefore, cannot be directly related to the mutation. Incidentally, selective loss of yellow heterozygotes in either yellow or black dams did not occur in strain C57BL/6J mice, the strain to be used in this research (24).

Since wide varieties of aberrations are associated with the A^Y allele, the lethal yellow mutation is speculated to interact with a regulatory mechanism (19) . In an effort to identify a polypeptide that may be under the direct influence of the agouti gene, a number of key metabolic enzymes were assayed for activity within yellow mice from different genomic backgrounds (84). These enzymes included those of glycogen and fat metabolism. Of the enzymes tested, cytoplasmic malic enzyme demonstrated the closest relation to the primary effect of the A^Y gene. This enzyme is intimately involved in fatty acid synthesis. Aberrations in activity of this enzyme may cause the excessive fatty acid deposition observed in the lethal yellow mouse (84). Other enzymes studied were different between yellow and non-yellow mice but these differences were restricted to specific strains (84).

II.B.2. Obesity and Hormonal Involvement

The lethal yellow mutation is associated with an age onset obesity linked by an increase in adipose tissue and an increase in muscular and skeletal growth (38). Onset of obesity is correlated

with an increased food intake and reduced energy requirements (38). Obesity is considered to be a physiological state associated with aberrations in hormone production and metabolism (47). The role of hormonal involvement and obesity (38) has led to the study of hormonal differences in the yellow mouse. However, data indicate that possible hormonal aberrations do not appear to be causally related to the onset of obesity. While adrenalectomy reduced the weight gain of the yellow mouse, the reduction was not enough to consider adrenal gland abnormalities to be the primary lesion in the induction of obesity (42). Furthermore, removal of the pituitary gland in the yellow mouse did not prevent obesity (85). Based on these data, the age-associated onset of obesity does not appear to result from hormonal imbalances but seems to be a result of some other metabolic aberration.

In related experiments, yellow and non-yellow mice were placed in parabiosis to detect possible metabolic aberrations that may alter growth characteristics of either member of the pair. While this did not influence weight gain nor growth of either the yellow or non-yellow mouse, results indicated that the A^{Y} allele exerted an effect on the amount of nonfat dry liver residue. Thus, researchers hypothesize that altered metabolic, and possibly hormonal imbalances, affect the liver metabolism (81). Other metabolic aberrations noted included a significant elevation in hexokinase activity in the adult yellow mouse (30). Additionally, glucose metabolism is impaired; yellow mice display more than twice the amount of blood sugar than

that of their lean littermates. This is also correlated with capillary dilatation and degranulation of beta cells in the pancreas of the yellow mouse (30).

II.B.2.a. Hormonal Implications in Reproductive Failure

In contrast to the lack of correlation noted previously between adrenalectomy, hypophysectomy and hormonal influences on obesity in the yellow mouse, evidence of possible hormonal lesions caused by this mutation has been presented. Data indicate an association between infertility and the A^{Y} mutation in the lethal yellow female mouse. These mice have fewer litters and stop reproducing at an earlier age than their congeneic black littermate (26). Depressed ovulation rates were partially restored by exogenous gonadotropins administered to these mice (26). Furthermore, A^{Y} ovaries grafted to a nonyellow host maintained their reproductive capacity longer than ovaries in the yellow host (25). These data suggest that reproductive failure is not due to defective ovaries and may suggest endocrine lesions within the hypothalomo-pituitary axis (25).

II.B.3. Susceptibility to Cancer

In addition to aberrant pigmentation and obesity, the lethal yellow mutation is also associated with an increased susceptibility to cancer. Heston (34) was the first to demonstrate that the A^{Y} gene, or some factor segregating with the gene, increased the

susceptibility to 20-methylcholanthrene induced tumorigenesis of pulmonary tumors in yellow mice. Subsequently, the lethal yellow gene was proven to be associated with an increased susceptibility to spontaneous pulmonary tumors (37), hepatomas, mammary tumors (39) and induced neoplasms of the skin (77). Deringer (15) also reported a susceptibility to reticular neoplasms. This increased susceptibility to mammary tumors, hepatomas and reticular neoplasms is demonstrated by an earlier onset in the yellow mouse as compared to the onset in agouti mice (15).

While the causative factor associated with the development of cancer is not known, investigators suggest that the factor involved with this increased tumor susceptibility is associated with an increase in body weight (39,55). By increasing the weight of nonyellow mice with gold-thioglucose injections and restricting the food intake of the lethal yellow mouse, the difference in tumor susceptibility between these mice was eliminated (38).

Hormonal changes are sometimes associated with an increased risk in selected neoplastic disorders (47). The effects of possible hormonal aberrations and cancer susceptibility in the yellow mouse has been studied to a limited extent. Male hypophysectomized A^{Y} mice demonstrate a significantly reduced incidence of spontaneous hepatomas (35). While the inhibitory effect of this condition is not known, it is speculated that inhibition may be due to a decreased function of the testes (35) suggesting a pituitary involvement of this mutation.

II.C. Immunologic Implications of the Lethal Yellow Mutation

II.C.1. Aberrations in immune function

The association of the lethal yellow gene with an increased susceptibility towards cancer suggests another manifestation among the broad milieu of effects of this mutation. A putative relationship between cancer and an impaired immune system is well documented. The close linkage association of the agouti locus with many immunoregulatory sequences and the increased susceptibility to cancer displayed by the lethal yellow mutation implicates a possible involvement of this mutation in immune dysfunction.

II.C.2. Cancer and Immunity

The correlation between cancer and aberrations in immunity is inconclusive. While the cause and effect pattern has not been firmly established, an association between cancer and immunosuppression has been observed. In many malignancies including leukemia, myeloma, Hodgkin's disease, and solid tumors, a suppression in cellular immunity has been demonstrated (29). With the exception of Hodgkin's disease, humoral immunity is also suppressed in these cases (29). The determination that a person with a primary immunodeficiency disease has a hundredfold increase in the incidence of cancer provides evidence for the causal effect of immunosuppression in cancer (1). Furthermore, the risk of cancer may be increased 10 to 100 times for patients receiving immunosuppressive drugs to prevent graft rejection (1). On the basis of these studies, a theory has

been proposed regarding the role of the immune system in eliminating malignant cells as a part of its immune surveillance function. This theory postulates that the immune system recognizes and responds to tumor specific antigens of transformed malignant cells and eliminates them (18).

Evidence against the aforementioned theory has also been presented. Detection of tumor-specific antigens and the subsequent use of immunotherapy in many cancers have been unsuccessful (58). If cancer is the result of immunosuppression, tissue specificity of cancer would not be expected. However, cancers observed in transplant recipients tend to be located within the lymphoid system or in the skin. Furthermore, cancers of the lung, breast, colon, and bladder occur in no greater frequency in immunosuppressed individuals than in the general population (46). These studies indicate that the association between cancer and immune dysfunction is more tenuous than previously believed.

The possibility of a genetic predisposition towards cancer has received considerable attention. A statistical correlation between histocompatibility antigens and susceptibility to neoplasia has been noted. This susceptibility may be influenced by genetic factors in the host. These may be regulated by the host's immunological responsiveness to specific tumor antigens and are closely associated with genes controlling the host's histocompatibility antigens (50). Certain tumors have responded to genetic selection and occur in high percentages in specific inbred

strains (36). For example, inherited susceptibility to virally-induced leukemias has been shown to be linked to the mouse H-2 locus (17). This susceptibility may involve a complex relationship between histocompatibility antigens and immune responsiveness (50,68).

To re-emphasize, the role of the immune system in response to neoplasia is uncertain. Cancer susceptibility appears to be under the control of genetics through the inability to respond to certain tumor antigens or susceptibility may be due to general immunosuppression, as observed in immunologically suppressed transplant patients. The lethal yellow mouse provides an exciting model for the elucidation of the role of immunology in neoplasia. The association of the A^{Y} gene with cancer susceptibility suggests a genetic tendency towards cancer. However, the mutation is associated with age-onset obesity. As will be described, obesity is associated with immunosuppression.

II.C.3. Obesity and Immunity

Obesity is postulated to play a role in immune dysfunction. Obese mice have been demonstrated to show an impaired cellular immunity as determined by aberrations in certain cell types and their functions (9,52). Genetically obese C57BL/6J ob/ob mice contain fewer mononuclear cells and Thy 1.2 positive lymphocytes than their lean controls (9). Cytotoxic activity of spleen cells and absolute spleen size were found to be significantly reduced in these obese animals (10). In contrast, natural killer (NK) activity and

antibody-dependent cellular cytotoxicity are increased (9). Other investigators observed that obese mice respond significantly less to the skin contactant, picryl chloride when compared to lean mice (52). This impairment of cellular immunity, however, is reversible (9,52). In vitro priming of lymphocytes eradicated any differences in activity between obese and lean mice which indicates that impaired immune function may be a consequence of an abnormal environment in obese animals (10).

There are conflicting reports on the effect of obesity on humoral, or B-cell, activity. Meade, et. al. (52) observed that obesity does not seem to depress antibody formation against sheep red blood cells (SRBC). In contrast, Chandra (9) observed a significant reduction in plaque forming cells (PFC) to SRBC (9). The marked decrease appears to be predominantly of the IgG isotype and thus may be due to a deficiency of helper T-cells (9).

The cause of the altered immunocompetency in obese mice is not known. However, environmental factors seem to be involved as indicated by the reversibility of the impairment in in vitro culture (52). In addition to metabolic aberration such as hyperphagia and hyperglycemia, endocrine alterations are also observed in genetically obese mice (9). For example, genetically obese mice display hyperinsulinemia, elevated levels of adrenocorticotrophic hormone (ACTH) and increased B-endorphins (9). Hormones have been shown to interfere with lymphocyte proliferation and function (45) where optimal levels of hormone seem to be essential in maintaining

immunologic homeostasis. For example, insulin enhances antibody synthesis by B-lymphocytes. However, high concentrations of insulin inhibit Interleukin-2 induced lymphocyte proliferation and suppresses hemolytic plaque-forming cells to sheep red blood cells (57). Likewise, ACTH increases mitogen-induced lymphocyte proliferation (45), but suppresses antibody production (57). Obesity is also believed to cause depressed growth hormone responses to various challenges and possible suppression of prolactin responses (47). The requirement of lymphocytes for growth hormone is readily observed during in vitro serum free culture (57). In vivo, the number of circulating B- and T-cells in adult mice have been found to be proportional to the level of growth hormone (45). Another hormone, prolactin, stimulates both antibody production by B-cells and delayed-type hypersensitivity to dinitrochlorobenzene by T-cells (45). Thus, the endocrine system and the immune system appear to be interrelated and proper functioning of immunocompetent cells appears to be under direct hormonal influence.

II.C.4. Possible Correlation Between Agouti Locus and Immunity

A limited number of investigations of possible aberrations involving immunity in the lethal yellow mouse have been conducted. Investigators propose that certain genes known to be associated with high tumor susceptibility may also be associated with low immunologic responsiveness. They demonstrated that the spleen cells of the nonagouti (a/a) genotype are more reactive in a graft versus host

reaction than are those of the congeneic A^Y or A^{VY} mice. This was demonstrated by splenic indices following allografts of A^Y, A^{VY}, or a/a splenocytes into YBR A^Y or C57BL/6J A^{VY} F_1 hybrids (YBR X C57Bl/10 or C57BL/6J X b10.BR F_1, respectively). Reciprocal skin grafts were also performed between A^Y or A^{VY} and a/a. Results indicate no apparent histocompatibility differences between A^Y or A^{VY} mice and their congeneic a/a mouse. Therefore, they suggest that the quantitative differences in the immune response is not associated with differences at a histocompatibility locus (21).

Studies of the effect of the physiologic environment of the immune response have been conducted using the viable yellow model. These investigations centered around the effects of phenotypic expression of the A^{VY} genotype. Their results indicate partitioning of the differences in immunological responsiveness according to phenotype. When compared to lean genetically identical pseudoagouti A^{VY}/a mice, obese A^{VY}/a mice exhibit decreased antibody response to a T-cell dependent immunogen, enhanced antibody responses to a T-cell independent immunogen (Type II pneumococcal polysaccharide) and increased levels of serum IgA. Pseudoagouti and nonagouti mice responded similarly (59). They concluded that alterations in immune function are the result of phenotypic expression of the A^{VY} genotype.

II.D. Objective

The purpose of this investigation was to determine if aberrations in immunity are part of the primary lesion of the lethal

yellow mutation and to subsequently ascertain if a correlation exists between immunoincompetency and age-onset obesity. Humoral and cell-mediated immunity were assessed in the lethal yellow mouse at six weeks and at 120 days, the age at which obesity becomes apparent (23,26). These data were compared with those of lean congeneic black age-matched littermates. The well-established tests of cellular immunity include *in vivo* assessment of delayed type hypersensitivity (DTH) and *in vitro* lymphocyte responses to T-cell mitogens (29). Humoral immunity was assessed *in vivo* by both enumerating antibody forming cells (AFC) and measuring antigen specific immunoglobulin levels and determined *in vitro* by LPS-induced lymphocyte proliferation of B-cells.

II.E. Rationale

The increased susceptibility towards cancer in the lethal yellow mouse strongly suggests an aberration in immune responsiveness. Evidence for this correlation has been presented through experiments demonstrating altered immunity in this tumor susceptible mouse line, as well as the viable yellow mouse. However, aberrations in the immune response were observed to separate with phenotype in the viable yellow mouse. This observation implicates environmental physiologic factors as the cause of these aberrations. This is further substantiated by data available on immunocompetency in the obese mouse model. However, studies on the immunocompetency in the lethal yellow mice, a more severe mutation, versus the effect

of their physiologic environment have not been conducted.

II.F. Measurement of Immunocompetency

The immune system involves all of the physiologic mechanisms that allow the body to defend itself from constituents that appear foreign including viruses, bacteria, and abnormal cells. To simplify discussion, the immune response is divided into two components, humoral and cell-mediated. However, these divisions are not absolute in that these two facets are intimately involved in the regulation of each other. The humoral arm of specific immunity is B-cell mediated in which the effector cells of the response are believed to be derived from the bone marrow (5). This facet of the immune response is commonly associated with immunoglobulins, or antibody. Immunity afforded by this arm of the immune response is transferrable through serum. Immunoglobulins are glycoproteins produced by the B-cell in response to stimulation by antigen. These immunoglobulins bind specifically to the antigenic conformation that induced its synthesis and subsequently allows the body to neutralize the possible effects of the antigen. Five classes of these glycoproteins differing in structural as well as biological properties have been identified and are denoted IgG, IgM, IgA, IgE, and IgD. IgG is found in highest concentration in the blood and is effective in opsonization and the eventual lysis of cellular antigen. IgM, the largest immunoglobulin, appears in the second highest concentration in the blood and is most efficient in the activation of a mechanism leading to cell lysis.

IgA is the most prevalent antibody in bodily secretions. IgE, found in very low concentrations, is involved in Type I hypersensitivity reactions. IgD is found primarily on the cell membrane on B-cells.

Cell-mediated immunity is typically associated with rejection of abnormal cells within the body. Such cells include tumor cells, virally-infected cells, and grafts. The effector cells in this process, derived from bone marrow stem-cells, are referred as T-cells and are differentiated within the thymus. Transfer of T-cell mediated immunity can only be accomplished by the transfer of T-lymphocytes.

II.F.1. Assessment of Cell-Mediated Immunity (CMI)

II.F.1.a. Delayed-type Hypersensitivity

Delayed-type hypersensitivity assays are utilized for the evaluation of the cellular or T-cell arm of the specific immune response. Hypersensitivity is an exaggerated reaction of immunologic specificity to an antigen or hapten. An initial, or sensitizing, dose of antigen or hapten is required before hypersensitivity reactions can be invoked (2). The exaggerated reaction is initiated, after a waiting period of 5 to 10 days (2), when a subsequent exposure to the sensitizing antigen is encountered. Subsequent exposure is referred to as the challenge. While immunologic in nature, hypersensitivities are generally detrimental to the host rather than protective.

Hypersensitivity reactions may be classified as immediate or delayed depending upon the time of onset after antigenic challenge and the requirement for antibody or T-cells (2). Immediate hypersensitivity develops within seconds or minutes after challenge and is antibody-mediated. Due to involvement of antibody, immediate hypersensitivity can be passively transferred through serum. In contrast, delayed-type hypersensitivity (DTH) is a hypersensitivity reaction that is characterized by a relatively slow onset when compared to immediate hypersensitivity. Additionally, DTH cannot be transferred from one animal to another by serum but is adoptively transferred through the transfer of T lymphocytes. A DTH reaction is initiated by T-cells which subsequently recruit a large population of monocytes and macrophages to the area where antigen is present. Hence, this reaction is often referred to as T-cell mediated hypersensitivity.

Immediate-type hypersensitivity is subdivided into three types on the basis of immunopathology (2). Type 1 hypersensitivity is IgE dependent and involves the participation of mast cells and basophils. IgE produced from a sensitizing dose of antigen binds to an Fc receptor for this immunoglobulin located on these two cell-types. Cross-linking of two bound IgE by antigen results in degranulation releasing histamine, heparin and other chemical mediators. These lead to vasodilation, smooth muscle contraction, hypotension, shortness of breath, edema, increased vascular permeability, and sometimes death. Type II, or cytotoxic,

hypersensitivity is IgG mediated and involves a complement-dependent cytolysis. This reaction is significant in mismatched blood transfusions and hemolytic diseases of newborns. Type III, or immune complex hypersensitivity, is the result of antigen-antibody complexes leading to the activation of the complement cascade. Subsequently, chemotactic factors for neutrophils are released. This neutrophil infiltration leads to local tissue destruction (71).

Four types of DTH reactions have been described (61). These include the Jones-Mote reaction, contact hypersensitivity, tuberculin-type hypersensitivity, and granulomatous hypersensitivity. Of these four, the first three occur within 72 hours after antigen challenge with the fourth type developing over a period of several weeks.

The Jones-Mote reaction is maximal at 24 hours. This manifestation is characterized by the infiltration of basophiles to the area immediately under the epidermis after an intradermal challenge to an antigen. This is known as cutaneous basophilic hypersensitivity (CBH). The Jones-Mote response is strongly regulated by cyclophosphamide-sensitive lymphocytes (61).

Contact hypersensitivity is maximal at 48 hours after antigen challenge. Predominantly an epidermal reaction, it is characterized by a mononuclear cell infiltrate accompanied by edema of the epidermis (61). Many of the antigens responsible for the initiation of this reaction are haptenes which covalently or non-covalently conjugate to self proteins. The antigen presenting cell in this

conjugate to self proteins. The antigen presenting cell in this reaction is the Langerhans cell (61).

The third type of DTH reaction is the tuberculin reaction. Like contact hypersensitivity, its maximal reactivity occurs 48-72 hours after antigen challenge. Histologically, it is characterized by an extensive infiltration of lymphocytes and macrophages.

The fourth, and perhaps the most deleterious DTH response is the granulatomas reaction. Granulatomas occur as a long-term effect of DTH. Macrophages migrating to the site of the DTH reaction become activated. These, in turn, release factors that cause lymphocyte activation. This process leads to giant cells such as those observed in tuberculosis. The net effect of this reaction is the release of lysosomal enzymes and cytotoxic factors that lead to further tissue damage (32).

II.F.1.b. Measurement of DTH

A number of methods have been described to detect a DTH response in the murine model. A common method is the footpad reaction. This is performed by injecting antigen into a metatarsal pad of the hindfoot (13) The visible reaction, as indicated by induration, will appear at 6 hours after challenge, peak at 18-24 hours, and recede after 48 hours (13). As with most types of DTH reactions, thickening of the skin occurs at the site of antigen injection. The thickness of the challenged foot is measured with a caliper and compared to the thickness of the control foot. Another

test of DTH reactivity is the skin test involving the intracutaneous injection of the antigen. The subsequent reaction parallels the footpad reaction. DTH begins 6 to 8 hours and peaks at 18-48 hours. Erythema is uncommon in DTH skin reactions in the mouse and cellular infiltration must be determined by touch (13). Problems arise in the murine model because mouse skin is thin and leakage into subcutaneous tissues occur (3). A third test is the percutaneous skin test reaction which is primarily used to determine contact hypersensitivity. A measured volume of chemical, or antigen, is applied to a confined, shaven area of the mouse. The subsequent response parallels that observed in the skin test reaction. A common test site is the ear. This is advantageous in that the ear does not need to be shaved and thus reduces irritation. Reactions have been traditionally measured in the same manner as the skin test reaction in which the ear lobes are measured with a caliper. Skin thickness is correlated with the intensity of the reaction (13). Unlike the previous two tests, this reaction is not complicated by an accompanying immediate type hypersensitivity (13).

Quantitation of DTH by measuring skin or footpad thickness is difficult to perform when responses are marginal. Since swelling or increased thickness may also be due to edema rather than from cellular infiltration (74) such methods may be incorrectly interpreted. Since DTH is primarily the result of the infiltration of a rapidly proliferating monocyte population, the use of radioisotopic DNA precursors provides a very sensitive assay for DTH responses.

This assay measures the amount of labeled monocytes that infiltrate at the site of antigen challenge (74). ^{125}I-UDR is typically used for labelling. Increased labelling efficiency can be achieved by increasing the time of availability of the label or by decreasing the size of the competing thymidine pool (41). The thymidine pool can be decreased by injecting small amounts of fluorodeoxyuridine (FDU). FDU blocks thymidine synthesis from deoxyuridine by binding and, thus, inhibiting the enzyme required for thymidine synthesis (41). Amplification of *in vivo* DTH reactions may also be potentiated by the use of cyclophosphamide. DTH is suppressed by blocking factors that occur when antibody complexes with antigen (48). Cyclophosphamide has long-lasting deleterious effects of the B-cell arm of the immune system thus blocking the modulating effect of B-cells on DTH (73).

II.F.2. Assessment of Humoral Immunity

Measurement of specific antibody is dependent upon the affinity of the antigen-antibody interaction. Amplification of this immunologic reaction can occur by the activation of the humoral component known as complement. Complement is a general term denoting a cascade of proteins activated in the presence of bound antibody that leads to the eventual lysis of cells. Hence, B-lymphocytes producing antibody to the cellular antigen in the presence of complement will lead to formation of plaques as a result of cellular lysis. This has been the foundation for a method of enumerating antibody-producing B-cells known as the Jerne Plaque Assay or the

hemolytic plaque assay. This is an important tool for the *in vitro* assessment of *in vivo* immunoreactivity. The host is sensitized with an antigen, i.e. SRBC, which invokes antibody production by the B-lymphocyte. Lymphocytes are subsequently isolated and cultured in the presence of the cellular antigen and complement. The antibody isotype IgM is the most efficient activator of the complement cascade. Thus most plaques that form in this assay are due to this isotype. Other lymphocytes producing different immunoglobulin isotypes can be quantified through the addition of species-specific anti-isotype serum.

Another assay used to assess humoral immunity involves evaluation of the level of antibody in the serum. Enzyme immuno-assays (EIA) are commonly utilized in the determination of antibody levels. EIA's incorporate the specificity of the antigen-antibody reaction with the efficiency of an enzyme. While many variations of EIA are performed, the basic component is an enzyme-labelled antibody. Specificity is provided by the antibody. Formation of the antigen-antibody complex is detected by the enzymatic conversion of a substrate by the antibody-bound enzyme. The enzyme utilized is chosen such that substrate conversion involves a colorimetric change. Thus, colorimetric conversion is an indirect measure of the amount of antibody bound to its specific antigen.

II.F.3. Mitogen Induced Lymphocyte Proliferation

In vitro mitogen-induced lymphocyte proliferation is used as

both a diagnostic and research tool to measure lymphocyte reactivity. Clinically, it has been used to detect and measure congenital and acquired immunologic deficiencies as well as to monitor the effects of various immunosuppressives and immunotherapeutic treatments (64). Mitogens are substances that activate lymphocytes without regard to antigenic specificity. In other words, mitogens added to cultures of nonsensitized lymphocytes will initiate blast transformation and proliferation. Cross-linking of the mitogen to surface molecules on the lymphocyte is believed to perturb the cell membrane and thus stimulate the lymphocyte to divide (33).

Certain lectins and bacterial endotoxins are known to have mitogenic effects on lymphocytes. These mitogens specifically bind to certain sugar groups or glycoproteins located on the lymphocyte membrane (33). For example, the plant lectin, Concanavalin A (Con A), binds to glycoproteins containing -mannosyl groups. Another plant lectin, phytohemmaglutinin, binds N-acetyl-galactosamine (4). These binding specificities have permitted the evaluation of specific subclasses of lymphocytes. It has been determined that murine lymphocytes bearing the ly 1 surface antigen respond to concanavalin A (51). This antigen is found both on undifferentiated T lymphocytes and on differentiated helper T cells (72). Phytohemmaglutinin (PHA) stimulates T-cells bearing the ly 1,2,3 surface antigen, or undifferentiated T-cells (51,72). While Con A and PHA demonstrate specificity toward T-cells, the

bacterial component, lipopolysaccharide (LPS) is a B-cell mitogen (33).

While these tests represent only a sample of the numerous tests available to measure immunocompetency, these will permit the detection of aberrations that may be prevalent within the broad spectrum of immune response mechanisms present in the body. In recapitulation, the intent of this research is to compare the cell-mediated immunity and humoral immunity between the lethal yellow (A^{Y}/a) mouse and its congeneic black (a/a) littermate at two points in their lifecycle. Cell-mediated immunity will by assessed through an *in vivo* radioisotopic DTH assay and through an *in vitro* proliferation response to the T-cell mitogens, PHA and Con A. Humoral immunity will be measured *in vivo* through a direct hemolytic plaque assay for the quantitation of specific B-cell activity to sheep red blood cells and through the determination of specific antibody levels in serum utilizing an indirect enzyme immunoassay. Furthermore, *in vitro* non-specific B-cell activity will be assayed by the proliferative activity of B-cells to LPS. Combined, these tests will allow the determination of immunologic alterations as a result of the lethal yellow mutation.

III. MATERIALS AND METHODS

III.A. Mice

Female C57BL/6J A^y/a (lethal yellow) and congeneic a/a (black) mice, obtained from Jackson Laboratories (Bay Harbor, Maine) were maintained in a colony at South Dakota State University, Brookings. Mice were sustained in 462 cm^2 cages at no more than 8 mice per cage. Food (Wayne Breeder Blox) and water were provided *ad libitum*.

All experiments were performed on the lethal yellow A^y/a mice and compared with congeneic black a/a mice. The effects of age-onset obesity was performed by comparing these mice at both 120 days (approximately 17 weeks) with 42 days (6 week) of age. Hence each series of experiments were performed on four groups of mice, 42-day-old A^y/a, 42-day-old a/a, 120-day-old A^y/a, and 120-day-old a/a female mice.

III.B. Delayed-Type Hypersensitivity

Two days prior to sensitization, all mice were injected subcutaneously with 150 mg/kg body weight of cyclophosphamide (Sigma, St. Louis, Missouri). The lower abdomen and both hind flanks on all mice were shaven on the following day. Forty-eight hours following injection of cyclophosphamide, six A^y/a and six a/a mice from each age group were sensitized with a topical application of a 10 mg/ml solution of 2,4-dinitrofluorobenzene (DNFB) (Sigma, St. Louis)

dissolved in a 1:1 mixture of olive oil:acetone. Twenty-five ul were applied to each of three shaven areas. Six control mice from each of the four groups were painted with an equivalent volume of the olive oil:acetone solvent. The concentration and volume of the sensitizing agent were determined by a preliminary pilot study. Five days following sensitization, all mice were challenged with a topical application of 5 ul DNFB (10 mg/ml) on the right ear lobe. An equivalent volume of the solvent was painted on the left ear of all mice. Ten hours following the challenge, all mice were injected intraperitoneally with 100 ul 5-fluoro-2-deoxyuridine (10^{-6} M in physiologic saline) (Sigma, St. Louis). Twenty minutes later, mice were injected intraperitoneally with 1.92 uCi of ^{125}I-deoxyuridine (689 uCi/mL) (Amersham, Arlington Heights, IL). After sixteen hours, mice were terminated via cervical dislocation. Ears were excised and placed in Wheaton omnivials (American Scientific Products,). Gamma emissions were counted and recorded on a Gamma 4000 gamma counter (Beckman, Irvine, California). Data were reported as a ratio of cpm in right ear/cpm in left ear.

III.C. In vivo Humoral Immunity

III.C.1. Immunization Procedure

III.C.1.a. Primary Response.

Forty-two-day-old and 120-day-old C57BL/6J A^y/a and a/a female mice were injected intraperitoneally with 0.25 ml washed SRBC (4 X 10^9 cells/ml) in sterile physiologic saline. Immune

responsiveness against SRBC was determined on days 4,5,6 and 7 following immunization.

III.C.1.b. Secondary Response.

Forty-two-day-old and 120-day-old C57BL/6J A^y/a and a/a female mice were injected three times at weekly intervals with 0.25 ml washed SRBC (4 X 10^9 SRBC/ml) in physiologic saline. Immune responsiveness against SRBC was assayed 4,5,6 and 7 days after the third immunization.

On the preselected assay days after both primary and secondary immunization, each mouse was weighed and exsanguinated via the subclavian artery. The spleen and thymus were excised and weighed. Serum was separated from the clotted blood via centrifugation at 500 X g on a Sorvall RT 6000 refrigerated centrifuge and stored at -20°C. The spleens were subsequently used for the enumeration of plaque-forming cells.

III.C.2. Assay of Humoral Immunity

III.C.2.a. Plaque-forming Cells

The spleens from immunized mice were removed as described and passed through a size 50 stainless steel mesh screen (Small Parts, Inc., Miami, Florida). Spleenocytes were washed in 10 ml RPMI 1640 (Sigma) and centrifuged at 200 X g in a Sorvall RT 6000 centrifuge using an H-1000 rotor. Cells were resuspended in one milliliter of RPMI 1640 supplemented with 10% SRBC adsorbed, heat inactivated fetal calf serum (FCS) (Gibco Laboratories, Grand Island, New York).

Forty microliters of each spleen suspension was placed in disposable blood dilution vials (American Scientific Products, McGraw Park, Illinois) containing 20 ml physiologic saline for quantitation of leukocytes. Red blood cells were lysed with five drops of Zap-Isotron II (Coulter Diagnostics, Inc.) and leukocyte counts were performed using a Model MHR Coulter Counter (Coulter Electronics, Hialeah, Florida).

Plaque-forming cells (PFC) were determined according to the Cunningham-Szenberg modification of the Jerne Plaque Assay (14,43). Seventy-five microliters of the suspended splenocytes were added to a mixture containing 125 ul SRBC (1 X 10^9 cells/ml in RPMI with 10% FCS), 25 ul SRBC adsorbed guinea pig complement (Gibco), and 25 ul RPMI. Forty microliters of this cell suspension was added to each of three coverslip chambers on a microscope slide. Evaporation was prevented by sealing the edges of the coverslip chamber with heat-liquified petroleum jelly. Slides were incubated thirty minutes at 37°C. Plaque-forming cells were enumerated on an Olympus microscope (Leeds, Minneapolis, Minnesota) using a 4X objective (40X final magnification). The number of plaque-forming colonies per 10^6 spleen cells were determined.

III.C.2.b. Enzyme Immunoassay (EIA)

III.C.2.b.i. Preparation of SRBC Membranes. Membranes from SRBC were prepared through osmotic lysis and release of hemoglobin according to the procedure outlined by Hanahan and Ekholm (28) using

hypotonic phosphate buffer prepared as described by Dodge, et. al. (16). Following the final wash, membranes were pooled and suspended in 20 mOsM (5.9 mM Na_2HPO_4, 1.2 mM NaH_2PO_4 phosphate buffer), pH 7.4, and stored at -20°C.

Protein concentrations were determined using Biorad Protein Assay (Biorad Laboratories, Richmond, California) and BCA Protein Assay (Pierce, Rockford, Illinois) against a standard curve of bovine serum albumin, Fraction V (Boehringer Mannheim Biochemicals, Indianapolis, IN). The average concentrations determined from the two separate assays was determined.

III.C.2.b.ii. EIA. Thawed SRBC membranes were solubilized in 0.1% sodium dodecyl sulfate (SDS) (Sigma).

Wells of a 96-well flat-bottomed Immulon 1 Removawell Strips (Dynatech Laboratories, Alexandria, VA) were coated with 100 ul of a 1:500 dilution of solubilized SRBC membranes (1.25 mg protein/ml) using 0.05 M carbonate buffer at pH 9.6. Control wells were coated with carbonate buffer, pH 9.6. Plates were incubated overnight in a humidity chamber at 4°C.

Plates were washed three times with 300 uL 20mM Tris (Tris hydroxymethyl aminomethane) (Sigma) buffer, pH 7.4 with 0.05% Tween 20 (Sigma). Unbound sites in the wells were blocked by the addition of two hundred microliters of 0.1% gelatin (Knox Gelatine, Inc., Englewood Cliffs, New Jersey) in Tris buffer without Tween 20. Following incubation at room temperature for one hour, the plates were washed three times with 300 uL Tween 20 supplemented Tris buffer

(Tris/tween). One hundred microliters of serum diluted in Tris/tween with 0.1% RIA (radioimmunoassay) grade bovine albumin (Sigma) were added in triplicate to both SRBC coated wells and control wells. Primary immune serum was diluted 1:25 for both IgG and IgM assays. Secondary immune serum was diluted 1:50 for IgM assays and 1:200 for IgG assays. Triplicate assays of a reference serum diluted 1:50 to assay IgM and 1:200 to assay for IgG were performed. Plates were incubated two hours at room temperature in a humidity chamber. After washing three times with 300 uL Tris/tween, 100 ul of 1:1000 alkaline phosphatase labelled goat anti-mouse IgG (Sigma) or anti-mouse IgM (Sigma) diluted with Tris/tween containing 0.1% bovine albumin were added to all wells. Plates were incubated for three hours at room temperature in a humidity chamber. Plates were washed three times with 300 uL Tris/Tween (pH 7.4) and 100 ul of 1 mg/ml p-nitrophenyl phosphate, disodium (Sigma) in 0.97% (v/v) diethanolamine buffer, pH 9.8, were added to all wells. Following thirty minutes of incubation at room temperature, the enzymatic reaction was stopped with 50 ul of 3N NaOH. Absorbance was determined at a wavelength of 410 nm using a Microelisa Minireader MR590 (Dynatech). The average absorbance of the control wells for each serum was subtracted from the average absorbance observed in the SRBC coated wells and this value was used to compare the responses. Absorbance was corrected according to the reference serum as described by Voller, et. al. (78).

III.D. Lymphocyte Proliferation

III.D.1. Collection of serum

Blood from A^y/a 42-day, A^y/a 120-day, a/a 42-day, and a/a 120-day C57BL/6J female mice was obtained from the subclavian artery during terminal exsanguination. Serum was removed following coagulation and centrifugation at 500 X g on a Sorvall RT 6000 refrigerated centrifuge (DuPont, Wilmington, Delaware) using an H-1000 rotor. Sera from each group were pooled, filter sterilized using 0.22 um Acrodisc (Gelman Sciences, Ann Arbor, MI) and stored at -20°C. until use.

III.D.2. Proliferation Assay

Mitogen induced lymphocyte proliferation assays were performed using a phenol extract of lipopolysaccharide (LPS) obtained from E. coli 055:B5, concanavalin A (Con A), and phytohemagglutinin from Phaeseoulis vulgaris (PHA). Preliminary assays demonstrated optimal mitogenic activity at the following concentrations: LPS (Sigma) - 20 ugm/ml; Con A, Type IV-S (Sigma) - 2 ugm/ml; and PHA (Sigma) - 4 ugm/ml.

Spleens were aseptically removed from each mouse, dispersed through a size 50 stainless steel mesh (Small Parts, Inc., Miami, Florida) and suspended in a minimal volume of RPMI 1640 (Sigma) with 50 mg/l gentamycin (Sigma). Due to the quantity of cells required, 3 spleens from the same genotype and age group were pooled. Clumps from the pooled spleens were allowed to settle for ten minutes. The

supernatant was removed and centrifuged at 200 X g for 10 minutes in a Sorvall RT 6000 refrigerated centrifuge using a H-1000 rotor. Spleen cells were resuspended in approximately 5 ml RPMI 1640 supplemented with 50mg/l gentamycin (Sigma), 1 mM sodium pyruvate (Sigma), and 2 mM L-glutamine (Flow Laboratories, McLean, VA). Viability counts were performed using trypan blue exclusion dye and an Improved Neubauer (C.A. Hausser & Son, Philadelphia) hemocytometer. Prior to incubation, the cell suspension was supplemented with serum to a final concentration of 10% (v/v). Serum supplements included sera obtained from the four groups of mice as well as CPSR-2 low-mitogen bovine serum (Sigma).

Two hundred microliters of each diluted cell suspension with the appropriate serum supplement were added in quadruplicate to sterile 96-well flat-bottomed tissue culture plates (Corning, New York). Control and treatment wells were seeded with 200 ul of cells at concentrations of 9 X 10^6 and 3 X 10^6 cells/ml for LPS and PHA/Con A controls, respectively . Fifty microliters mitogen at 100 ugm/ml, 10 ugm/ml, and 20 ugm/ml of LPS, Con A, and PHA, respectively, were added to appropriate wells. An equivalent volume of RPMI was added to control wells.

Cells were placed in a humidified Precision Automatic CO_2 incubator (GCA) and incubated for 48 hours (as determined by preliminary screens) at 37^0C. and 5% CO_2 prior to pulsing. Cells were pulsed with 1 ul 6-^{3}H-thymidine (2Ci/mmole, Amersham International). Twenty hours following addition of the label, cells were harvested

with a Brandel Cell Harvester (Gaithersburg, MD) on Brandel FP-201 filter discs and placed in Minivials (New England Nuclear). After air-drying, five milliliters of scintillation cocktail (1 liter Triton X-100, 2 liters scintillation grade toluene, 1.5 gm 2,5-diphenyloxazole (Fisher Scientific), and 0.5 gm p-bis-(2-(5-phenyloxazolyl))-benzene (New England Nuclear). Vials were stored at 4^{0}C. for at least 45 minutes prior to counting on a Beckman LS 1801 scintillation counter. Counts were performed for five minutes and recorded as number of counts per minute (cpm).

III.E. Statistical Analysis

Least squares analysis of variance using the F-test ($F_{0.05}$) was used to evaluate significance.

IV. RESULTS

IV.A. Weight Comparisons

IV.A.1. Total Body Weight

Forty-two-day-old and 120-day-old A^y/a and a/a mice were weighed to verify the putative correlation between age and the onset of obesity noted in 120-day-old A^y/a mice (Table 1). The average weights of 120-day-old A^y/a and a/a (LSMean = 25.75 g; where LSMean = Least squares mean) mice were significantly ($p<0.01$) greater than that of their 42-day-old counterparts (LSMean = 17.94 g). While 42-day-old A^y/a mice did not differ significantly in weight from 42-day-old a/a mice, the mean weight of 120-day-old A^y/a mice was significantly greater ($p<0.01$) than that of 120-day-old a/a mice.

IV.A.2. Organ Ratios

The size of the major lymphoid organs is often used as a general indicator of immunocompetence. Two of these organs, the spleen and the thymus, were removed from 120-day-old and 42-day-old A^y/a and a/a mice and weighed. The weights of these organs were corrected to the average body weight of mice within the age and genotype set (Table 1).

The corrected spleen weights did not differ significantly among the groups.

Thymus weights differed significantly ($p<0.01$) between the two age groups. Thymus glands from 120-day-old mice were significantly smaller (LSMean = 0.052 g) than were thymus glands

Table 1. Table of Total Body Weight and Relative Organ Weights[a] for 42-day-old and 120-day-old A^y/a and a/a mice.

	Total Body Weight (g)	Spleen Weight (g)	Thymus Weight (g)
42-day-old a/a	17.83[b]	0.096	0.071[e]
42-day-old A^y/a	18.05[b]	0.099	0.067[e]
120-day-old a/a	22.22[c]	0.094	0.055[f]
120-day-old A^y/a	29.28[c,d]	0.100	0.049[f]

[a]Weights corrected to the average body weight within the specified set.

[b,c,d]Those not sharing same superscripts have significantly different ($p<0.01$) body weights.

[e,f]Those not sharing same superscripts have signficantly different ($p<0.01$) relative thymus weights.

removed from 42-day-old mice (LSMean = 0.069 g). While nosignificant difference was observed between thymus weight and genotype, a trend was noted in which thymus glands from A^y/a mice were smaller (LSMean = 0.058 g) than those from a/a mice (LSMean = 0.063 g) with significance in this difference established at the 0.10 level.

IV.B. In vivo Cell-Mediated Immunity

Delayed-type hypersensitivity (DTH) against DNFB was used as an *in vivo* determination of cell-mediated immunity. One of the requirements for initiating a DTH reaction is exposure to an initial, or sensitizing, dose of antigen. The specific recruitment of cells does not occur after initial exposure to the antigen but requires a second exposure. Upon second exposure, or challenge, with the same antigen, a DTH response can be invoked. This is detected by the specific recruitment of effector cells to the site of antigen challenge. In this assay, cellular infiltration of *in vivo* labelled ^{125}I cells was used an indicator of DTH reactivity against the skin contactant, DNFB. Control mice did not receive a sensitizing dose of DNFB. Results (Figure 1) were recorded as a ratio of counts per minute (cpm) in sensitized ear/ cpm in control ear. The significant difference ($p<0.01$) in this ratio between sensitized mice (LSMean = 3.23) and control mice (LSMean = 1.09) is an indication of the specific recruitment of cells to the sight of antigen challenge, thus verifying this as a test of immunologic

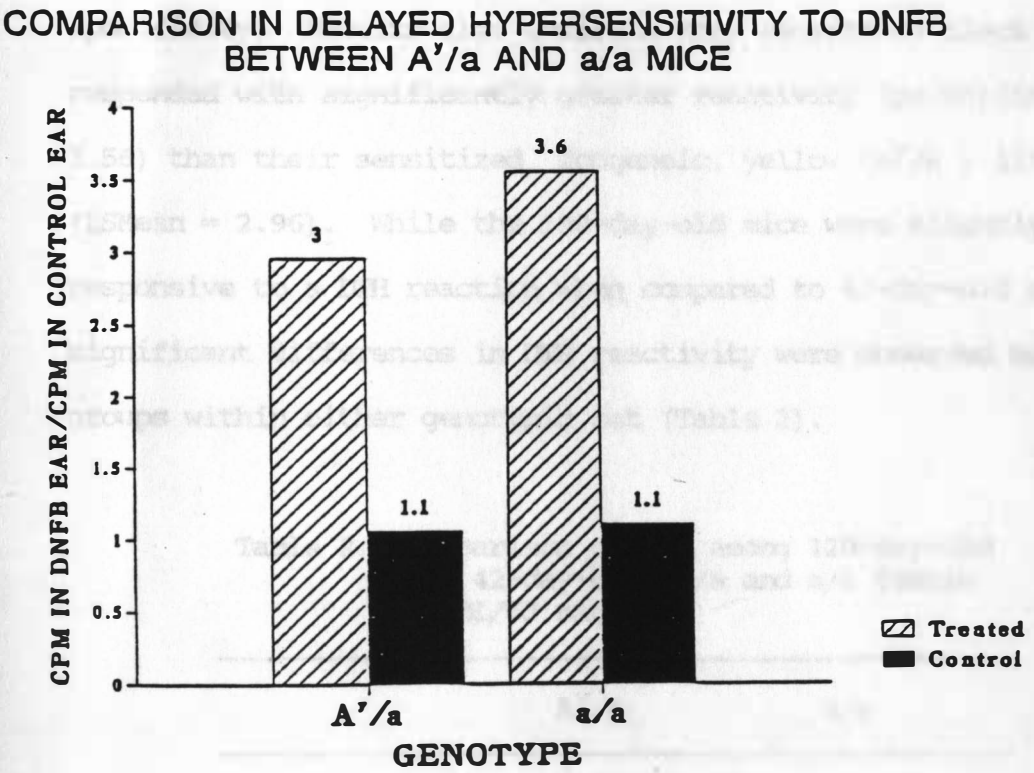

Figure 1. Graph of Delayed Type Hypersensitivity (DTH) between female C57BL/6J yellow (A^y/a) and black (a/a) mice as determined by the recruitment of ^{125}I-labelled cells to the site of the DNFB challenge, i.e. the right ear. DTH reactivity is measured as a ratio of cpm in challenged ear divided by cpm in control ear. Treatment groups received a topical application of DNFB five days prior to the challenge. Control mice did not receive an initial sensitizing dose of DNFB. Recruitment of effector cells to the DNFB challenged ear was significant. Control mice demonstrated no specific recruitment as indicated by a ratio of 1. A significant difference in DTH activity between A^y and a/a mice has been determined ($p<0.05$).

specificity. Results also indicate that sensitized black (a/a) mice responded with significantly greater reactivity ($p<.05$)(LSMean = 3.56) than their sensitized, congeneic, yellow (A^y/a) littermate (LSMean = 2.96). While the 120-day-old mice were slightly less responsive to a DTH reaction when compared to 42-day-old mice, no significant differences in DTH reactivity were observed between age groups within either genotypic set (Table 2).

Table 2. Comparison of DTH among 120-day-old and 42-day-old A^y/a and a/a female C57BL/6J mice.

	A^y/a	a/a
42-day-old	3.18[a,b]	3.65
120-day-old	2.74	3.48

[a]cpm in sensitized ear/cpm in control ear
[b]values represent means of the ratios.

IV.C. In vivo Humoral Immunity

IV.C.1. Antibody-forming Cells (AFC)

B-cell reactivity among the four sets of mice was compared using a direct hemolytic plaque assay to quantitate SRBC specific antibody forming cells. Figure 2 represents the number of antibody-forming cells per 10^6 spleen cells after either a primary or secondary exposure to SRBC. With the exception of the 120-day-old yellow (A^y/a) mice, the peak response following primary exposure to SRBC occurred on the fifth day post-immunization. A peak response

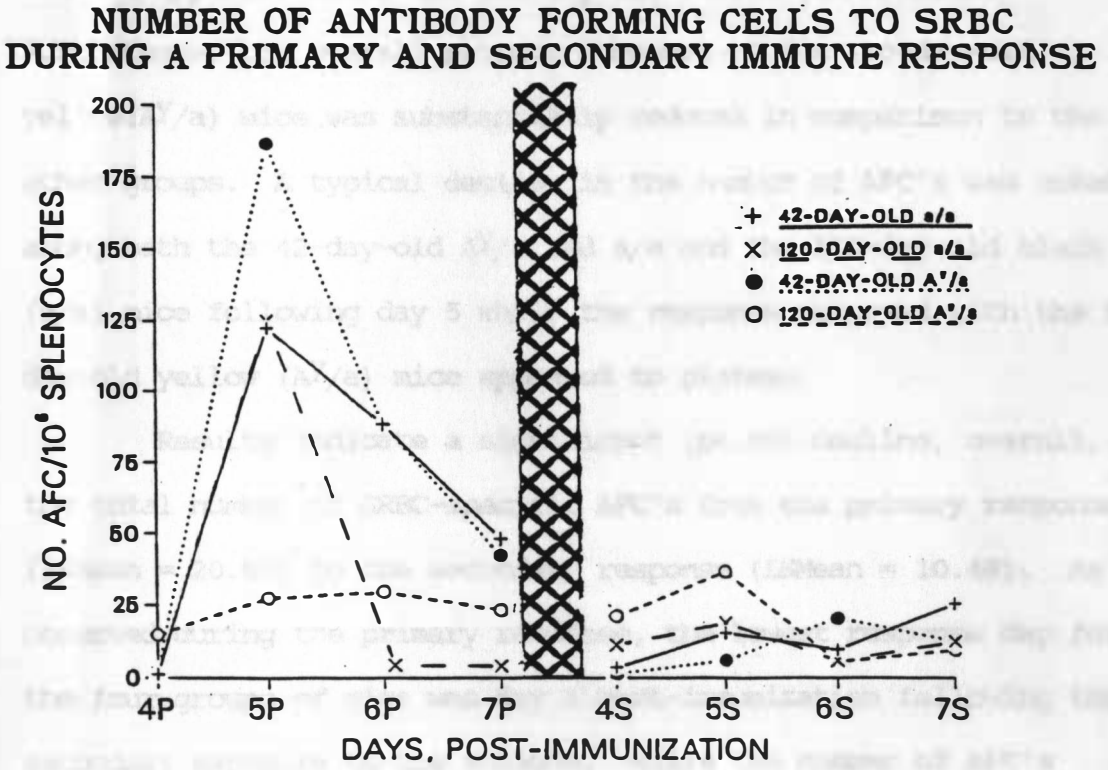

Figure 2. Number of antibody-forming cells to the T-dependent antigen, sheep red blood cell (SRBC) as determined by the direct hemolytic plaque assay. Values shown are the geometric means for six mice. Female C57BL/6J A^y/a and a/a mice were injected intraperitoneally with 1 X 10^9 SRBC. Assays were performed on days 4, 5, 6, and 7 (4p, 5p, 6p, and 7p) following primary exposure of the antigen. Mice were injected three times at weekly intervals to induce a secondary response to the antigen. Hemolytic plaque assays were performed on days 4, 5, 6, and 7 (4s, 5s, 6s, and 7s) after the third injection of the antigen.

was not apparent among the 120-day-old yellow (A^Y/a) mice. Furthermore, the overall primary response of the 120-day-old yellow(A^Y/a) mice was substantially reduced in comparison to the other groups. A typical decline in the number of AFC's was noted among both the 42-day-old A^Y/a and a/a and the 120-day-old black (a/a) mice following day 5 while the response observed with the 120-day-old yellow (A^Y/a) mice appeared to plateau.

Results indicate a significant ($p<.05$) decline, overall, in the total number of SRBC-specific AFC's from the primary response (LSMean = 20.08) to the secondary response (LSMean = 10.48). As was observed during the primary response, the lowest response day for the four groups of mice was day 4 post-immunization following the secondary exposure to the antigen. While the number of AFC's produced by the 120-day-old yellow (A^Y/a) mice was slightly higher than that produced by the mice in the three other groups on the fourth and fifth day post-secondary exposure, these results were not significantly meaningful.

IV.C.2. Enzyme Immunoassay (EIA)

IV.C.2.a. Primary response.

An EIA was performed to determine the relative levels of antigen-specific IgM and IgG in the sera from the four sets of mice following a primary exposure to the T-dependent antigen, SRBC. Figure 3 represents the relative concentrations of IgM on days 4, 5, 6, and 7 post-immunization. A significant increase in the overall

SERUM LEVELS OF ANTI-SRBC IgM DURING A PRIMARY IMMUNE RESPONSE

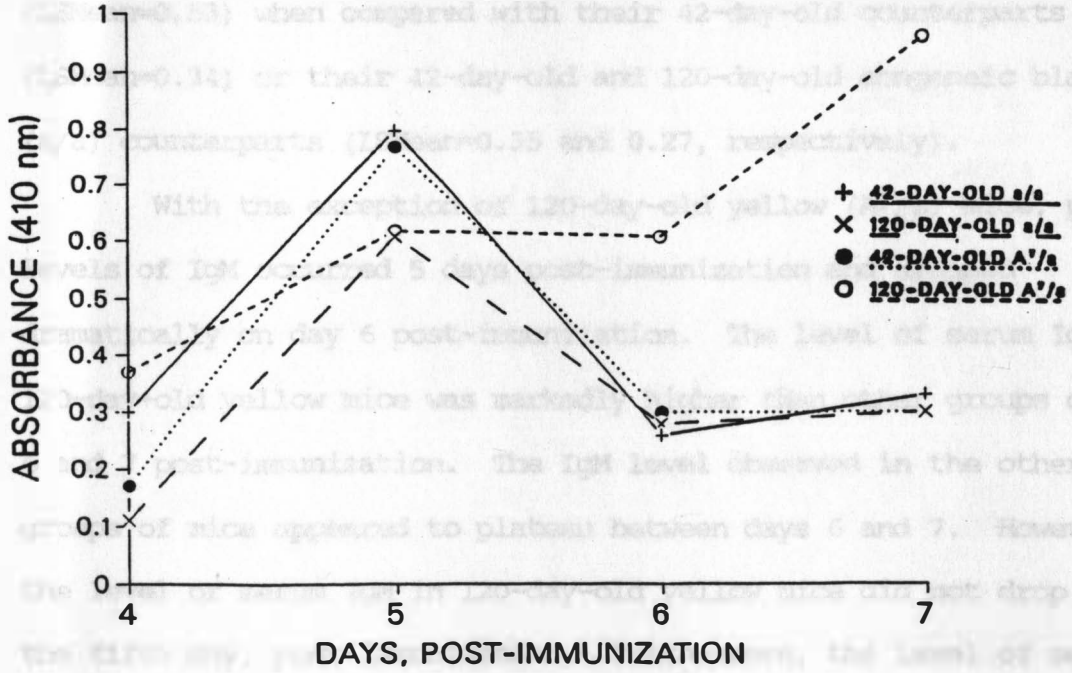

Figure 3. Relative levels of anti-SRBC IgM in serum of female C57BL/6J A^y/a and a/a mice on days 4, 5, 6, and 7 following intraperitoneal injection of 1 X 10^9 SRBC as determined by EIA using alkaline phosphatase labelled anti-mouse IgM. Serum was diluted 1:25.

IgM titer ($p<0.05$) was noted among 120-day-old yellow (A^Y/a) mice (LSMean=0.53) when compared with their 42-day-old counterparts (LSMean=0.34) or their 42-day-old and 120-day-old congeneic black (a/a) counterparts (LSMean=0.35 and 0.27, respectively).

With the exception of 120-day-old yellow (A^Y/a) mice, peak levels of IgM occurred 5 days post-immunization and dropped dramatically on day 6 post-immunization. The level of serum IgM in 120-day-old yellow mice was markedly higher than other groups on days 6 and 7 post-immunization. The IgM level observed in the other three groups of mice appeared to plateau between days 6 and 7. However, the level of serum IgM in 120-day-old yellow mice did not drop after the fifth day, post-immunization. Furthermore, the level of serum IgM was significantly higher ($p<0.05$) in 120-day-old yellow (A^Y/a) mice than that of the other three sets of mice. Levels of IgM in 42-day-old yellow (A^Y/a) mice did not differ from those observed in black (a/a) mice.

A comparison of the relative combined levels of IgG on days 4, 5, 6, and 7 after a primary exposure to SRBC revealed a significantly lower level of IgG in 120-day-old black (a/a) mice (LSMean = 0.16) when compared to its 42-day-old black counterpart (LSMean=0.40). As shown (Figure 4), levels of serum IgG in 120-day-old black (a/a) mice were below those observed in the other three sets of mice on days 4, 5, 6, and 7, post-immunization. Overall, IgG levels in young black mice were not significantly different from young yellow (LSMean = 0.39) nor old yellow (LSMean = 0.31) mice.

SERUM LEVELS OF ANTI-SRBC IgG DURING A PRIMARY IMMUNE RESPONSE

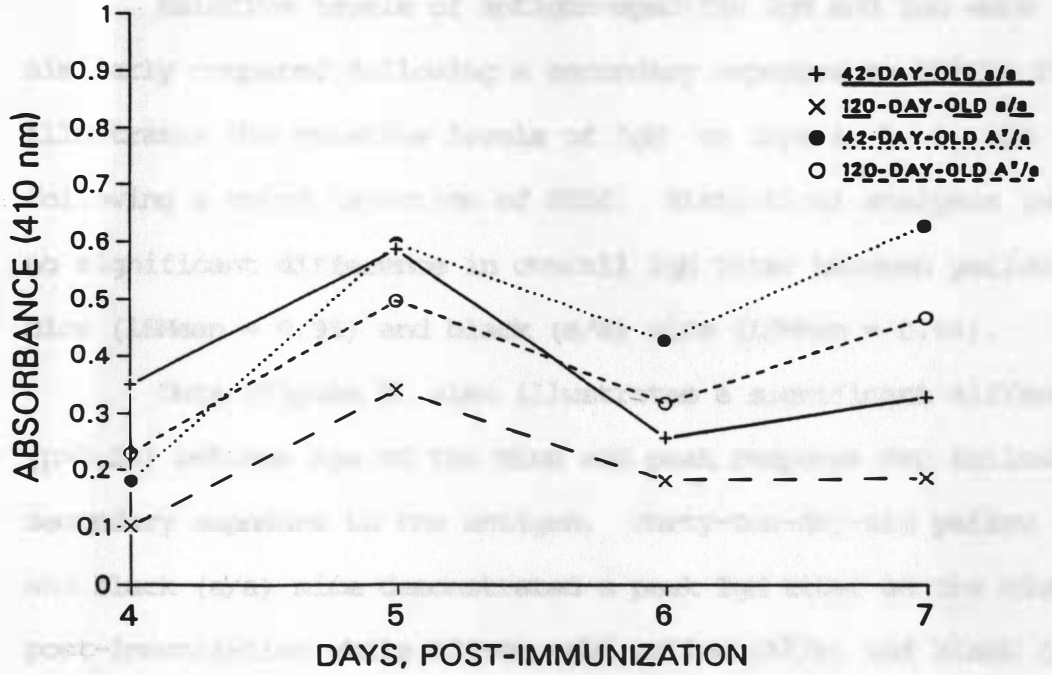

Figure 4. Relative levels of anti-SRBC IgG in serum of female C57BL/6J A^y/a and a/a mice on days 4, 5, 6, and 7 following intraperitoneal injection of 1 X 10^9 SRBC as determined by EIA using alkaline phosphatase labelled anti-mouse IgG. Serum was diluted 1:25.

IV.C.2.b. Secondary Response.

Relative levels of antigen-specific IgM and IgG were similarly compared following a secondary exposure to SRBC. Figure 5 illustrates the relative levels of IgM on days 4, 5, 6, and 7 following a third injection of SRBC. Statistical analysis indicates no significant difference in overall IgM titer between yellow (A^y/a) mice (LSMean = 0.91) and black (a/a) mice (LSMean = 0.64).

Data (Figure 5) also illustrates a significant difference ($p>0.05$) between age of the mice and peak response day following a secondary exposure to the antigen. Forty-two-day-old yellow (A^y/a) and black (a/a) mice demonstrated a peak IgM titer on the sixth day post-immunization while 120-day-old yellow (A^y/a) and black (a/a) mice peaked on the fifth day post-immunization.

Figure 6 illustrates the levels of IgG on days 4, 5, 6, and 7 following a third injection of SRBC. With the exception of young yellow mice, levels of IgG gradually rose from the fourth to the sixth day post-immunization and dropped from the sixth to the seventh day. No correlation between age and day of peak response was observed. Levels of IgG observed in the young yellow mice were erratic with a sharp peak observed on the sixth day, post-immunization. No significant differences in IgG levels during a secondary immune response were observed among the four sets of mice analyzed.

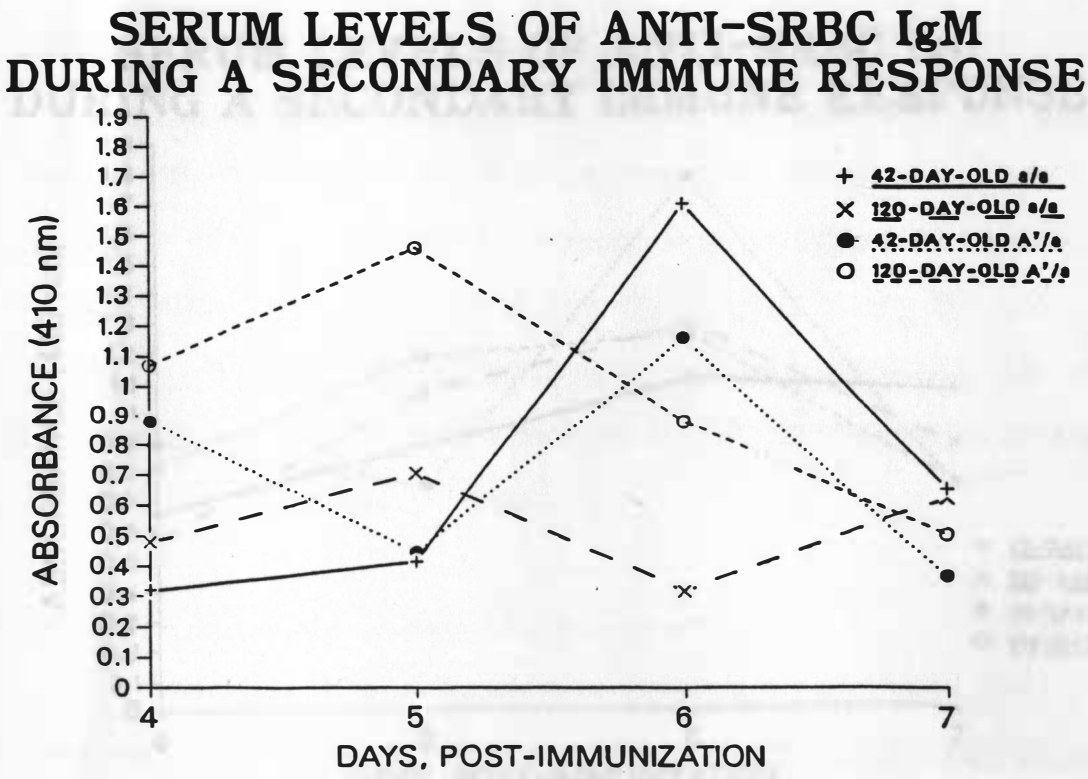

Figure 5. Relative levels of anti-SRBC IgM in serum of female C57BL/6J A^y/a and a/a mice on days 4, 5, 6, and 7 following a third intraperitoneal injection of 1 X 10^9 SRBC as determined by EIA using alkaline phosphatase labelled anti-mouse IgM. Serum was diluted 1:50.

SERUM LEVELS OF ANTI-SRBC IgG DURING A SECONDARY IMMUNE RESPONSE

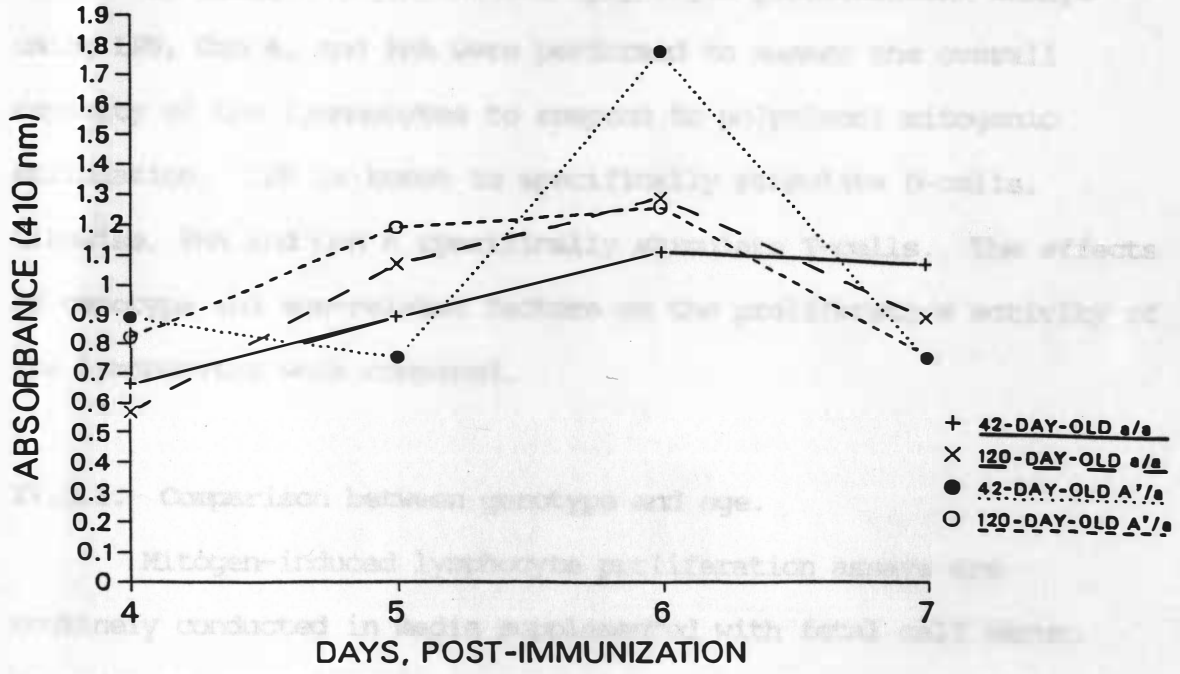

Figure 6. Relative levels of anti-SRBC IgG in serum of female C57BL/6J A^y/a and a/a mice on days 4, 5, 6, and 7 following a third intraperitoneal injection of 1 X 10^9 SRBC as determined by EIA using alkaline phosphatase labelled anti-mouse IgG. Serum was diluted 1:200.

IV.D. In vitro Mitogen-Induced Lymphocyte Proliferation

In vitro mitogen-induced lymphocyte proliferation assays using LPS, Con A, and PHA were performed to assess the overall capacity of the lymphocytes to respond to polyclonal mitogenic stimulation. LPS is known to specifically stimulate B-cells. Likewise, PHA and Con A specifically stimulate T-cells. The effects of genotype and age-related factors on the proliferative activity of the lymphocytes were compared.

IV.D.1. Comparison between genotype and age.

Mitogen-induced lymphocyte proliferation assays are routinely conducted in media supplemented with fetal calf serum. In order to investigate the possibility of environmental influences on lymphocyte reactivity, lymphocytes were cultured both in the presence of fetal calf serum, known to provide optimal growth conditions, and in media supplemented with serum from the four sets of mice.

A comparison of the overall proliferative activity between the two genotypes (Figure 7) during all culture conditions indicates that lymphocytes from genetically yellow (A^y/a) mice tended to be less reactive than that observed with their congeneic black (a/a) littermates. Although this difference was not significant for the LPS and Con A response, lymphoocytes from yellow (A^y/a) mice were

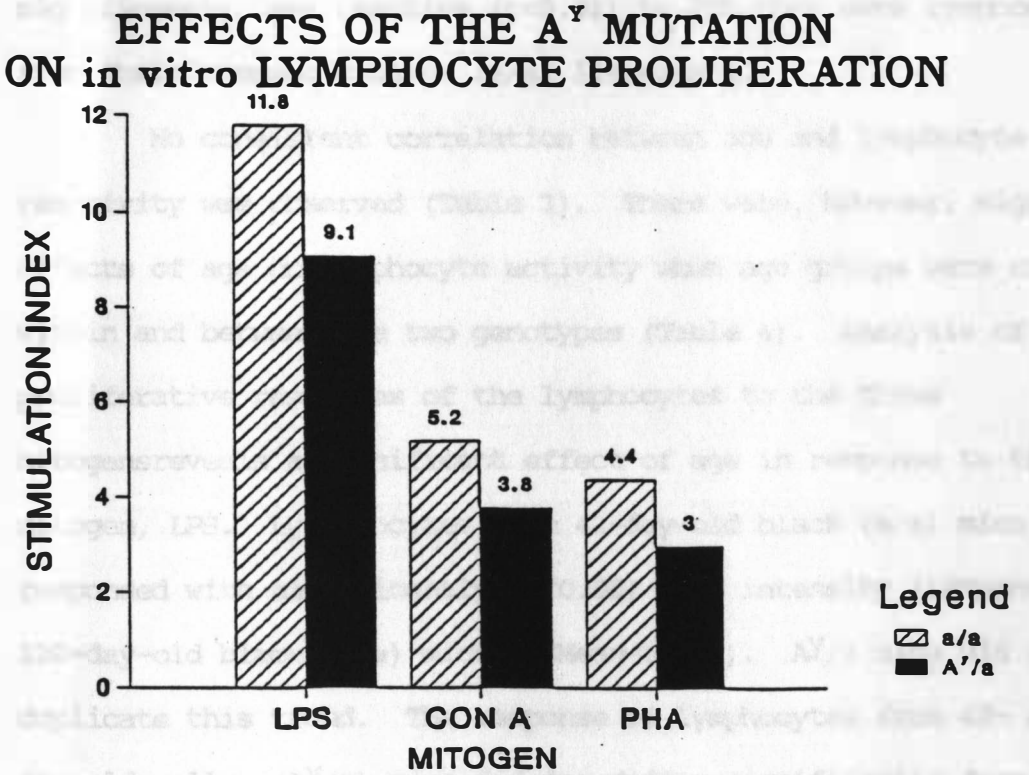

Figure 7. Stimulation indices (cpm of mitogen stimulated cells/cpm of control cells) of lymphocytes from female C57BL/6J A^Y/a and a/a mice cultured in the presence (stimulated) or absence (control) of LPS, Con A and PHA. Proliferation was determined by the the uptake of ^3H-thymidine. This figure represents the compilation of results of cells cultured separately in fetal bovine serum and serum from 42- and 120-day-old A^Y/a and a/a mice. The values shown for the mitogen, PHA, are statistically significant ($p<0.05$).

significantly less reactive ($p<0.01$) to PHA than were lymphoocytes from their congeneic black (a/a) littermate.

No consistent correlation between age and lymphocyte reactivity was observed (Table 3). There were, however, significant effects of age on lymphocyte activity when age groups were compared within and between the two genotypes (Table 4). Analysis of the proliferative responses of the lymphocytes to the three mitogensreveals a significant effect of age in response to the B-cell mitogen, LPS. Lymphoocytes from 42-day-old black (a/a) mice responded with significantly ($p<0.05$) less intensity (LSMean=9.06)than 120-day-old black (a/a) mice (LSMean=14.48). A^Y/a mice did not duplicate this trend. The response of lymphocytes from 42- and 120-day-old yellow (A^Y/a) mice did not differ significantly from each other. 120-day-old yellow (A^Y/a) mice responded with significantly less intensity (LSMean = 8.12) than 120-day-old black (a/a) mice (LSMean = 14.48).

Mitogen-stimulated proliferation assays in the presence of fetal calf serum revealed a decrease, albeit not significant, in lymphocyte reactivity towards LPS, Con A, and PHA among yellow (A^Y/a) mice when compared with their congeneic black (a/a) mice (Figure 8). Young 42-day-old yellow (A^Y/a) mice appeared to be less reactive to Con A and PHA than were the other groups of mice.

Table 3. Comparison between age and lymphocyte reactivity to B-cell and T-cell mitogens among A^y/a and a/a C57BL/6J mice.

	LPS[a]	Con A[b]	PHA[b]
42-day-old	9.5[c]	4.4	4.1
120-day-old	11.3	4.6	3.4

[a]B-cell mitogen
[b]T-cell mitogen
[c]$\frac{\text{cpm } (^3\text{H-Thymidine}) \text{ in mitogen stimulated wells}}{\text{cpm } (^3\text{H-Thymidine}) \text{ in control wells}}$

Table 4. Interaction between age groups within genotype in response to B-cell and T-cell mitogens.

	A^y/a		a/a	
Mitogen	42-day-old	120-day-old	42-day-old	120-day-old
LPS[b]	10.05[a]	8.12	9.06	14.48
Con A[c]	3.54	4.16	5.22	5.13
PHA[c]	2.85	3.19	3.88	5.02

[a]$\frac{\text{cpm } (^3\text{H-Thymidine}) \text{ in mitogen stimulated wells}}{\text{cpm } (^3\text{H-Thymidine}) \text{ in control wells}}$
[b]B-cell mitogen
[c]T-cell mitogen

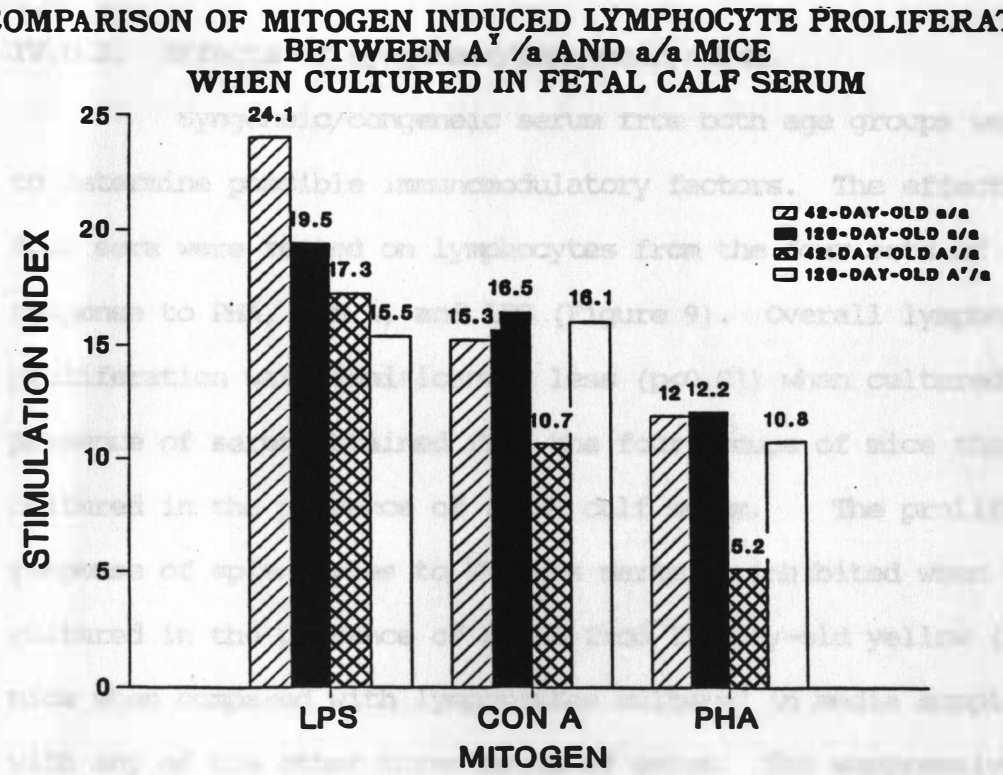

Figure 8. Stimulation indices (cpm of mitogen stimulated cells/cpm of control cells) of lymphocytes from 42- and 120-day-old female C57BL/6J A^{Y}/a and a/a mice in response to LPS, Con A, and PHA when cultured in the presence of fetal bovine serum. Proliferation was determined by the incorporation of exogenous ^{3}H-thymidine. Values shown represent no significant difference in responsiveness among the four groups of mice.

IV.D.2. Effects of syngeneic/congeneic serum.

Syngeneic/congeneic serum from both age groups were tested to determine possible immunomodulatory factors. The effects of these four sera were tested on lymphocytes from the four sets of mice in response to PHA, Con A, and LPS (Figure 9). Overall lymphocyte proliferation was significantly less ($p<0.01$) when cultured in the presence of serum obtained from the four groups of mice than when cultured in the presence of fetal calf serum. The proliferative response of splenocytes to PHA was markedly inhibited when they were cultured in the presence of serum from 120-day-old yellow (A^y/a) mice when compared with lymphocytes cultured in media supplemented with any of the other three pools of serum. The suppressive effect observed with serum from 120-day-old yellow (A^y/a) mice (LSMean = 1.28) was significantly greater ($p<.05$) than that observed with serum from 120-day-old black (a/a) mice (LSMean = 2.74) and serum from 42-day-old yellow (A^y/a) mice (LSMean = 2.74) but did not significantly differ from the response observed in the presence of serum from 42-day-old black (a/a) mice (LSMean = 1.9).

Analysis of lymphocyte reactivity in response to Con A (Figure 9) revealed the apparent suppressive effect of serum from 120-day-old yellow (A^y/a) mice when compared with the proliferative response of lymphocytes cultured in the presence of serum from the other three groups of mice. Lymphocytes cultured in sera obtained from 42-day-old yellow (A^y/a) and black (a/a), and 120-day-old black mice responded with similar intensity.

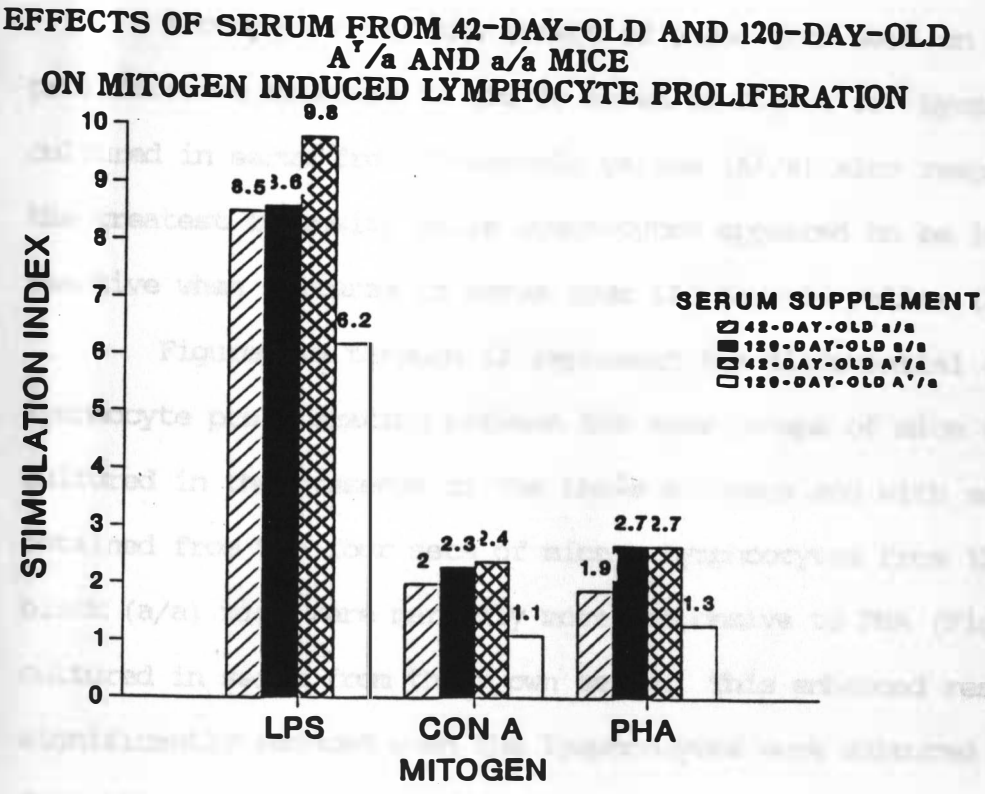

Figure 9. Lymphocytes from 42-day-old and 120-day-old a/a and A^Y/a mice were cultured in serum obtained from these mice. Proliferation was determined by ^3H-thymidine uptake, wherein the stimulation index = cpm of mitogen stimulated cells/cpm of control cells. Values shown for each serum supplement include the compilation of data of lymphocytes obtained from the four groups of mice.

A comparison of the effect of these four sera on the proliferative response to LPS is shown in Figure 9. Lymphocytes cultured in serum from 42-day-old yellow (A^y/a) mice responded with the greatest intensity while lymphocytes appeared to be least reactive when cultured in serum from 120-day-old yellow (A^y/a) mice.

Figures 10 through 12 represent the differential effects in lymphocyte proliferation between the four groups of mice when cultured in the presence of the three mitogens and with serum obtained from the four sets of mice. Lymphoocytes from 120-day-old black (a/a) mice were markedly more responsive to PHA (Figure 10)when cultured in serum from their own serum. This enhanced response was significantly reduced when the lymphoocytes were cultured in serum from 120-day-old yellow (A^y/a) mice. Furthermore, splenocytes from 120-day-old black (a/a) mice demonstrated a marked proliferative response to LPS when compared with the other three sets of mice (Figure 11). Again, this effect was obviated when cultured in the presence of serum from 120-day-old yellow (A^y/a) mice. Finally, the increased responsiveness observed in 120-day-old black (a/a) mice to PHA and LPS was not observed in response to Con A (Figure 12).

Lymphoocytes from 120-day-old yellow (A^y/a) mice tended to be least reactive to the three mitogens when cultured in serum obtained from 120-day-old yellow (A^y/a) mice (Figures 10-12). Enhanced reactivity of these lymphocytes to LPS appeared to occur when these cells were cultured in serum from the other three sets of mice

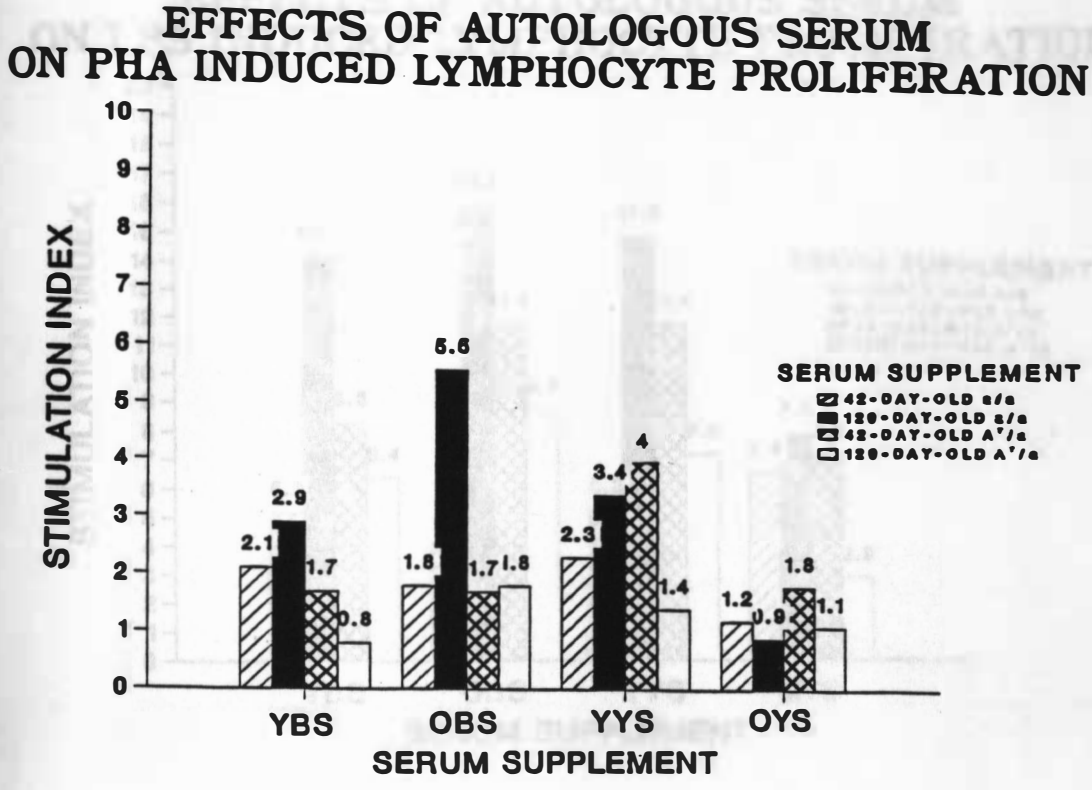

Figure 10. Lymphocytes from 42- and 120-day old female C57BL/6J A^y/a and a/a mice were cultured separately in the presence of serum from 42-day-old a/a mice (YBS), 120-day-old a/a mice (OBS), 42-day-old A^y/a mice (YYS), and 120-day-old A^y/a mice (OYS). The proliferative response of the lymphocytes to the T-cell mitogen, phytohemagglutinin (PHA), was determined through the incorporation of ^3H-thymidine. Values shown represent the stimulation index (cpm of mitogen stimulated cells/ cpm of control cells).

EFFECTS OF AUTOLOGOUS SERUM ON LPS INDUCED LYMPHOCYTE PROLIFERATION

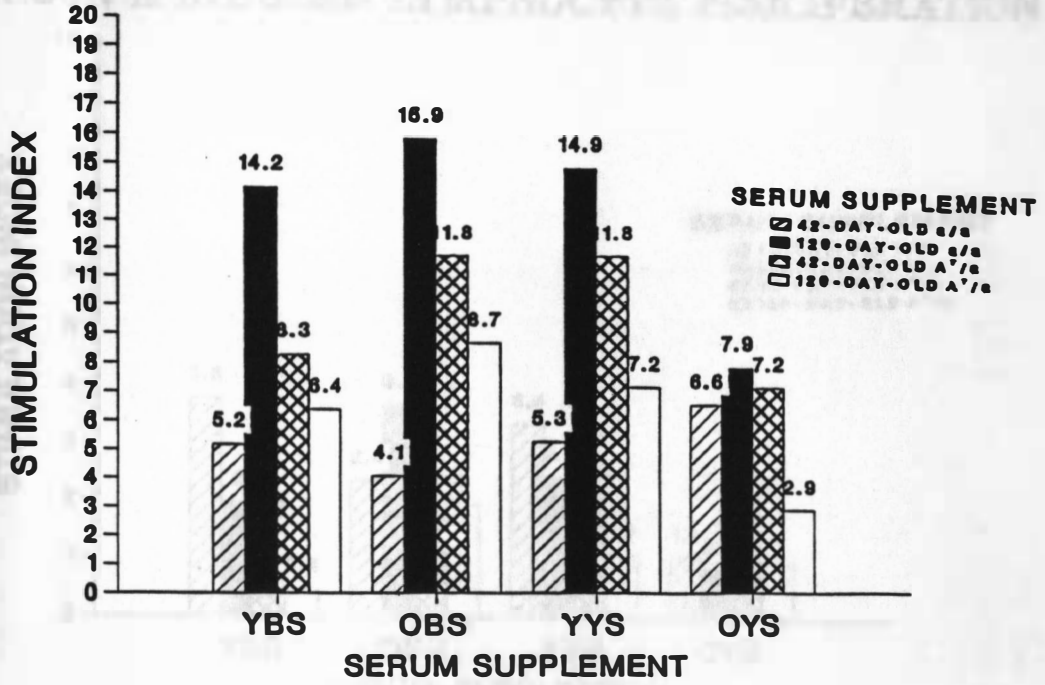

Figure 11. Lymphocytes from 42- and 120-day old female C57BL/6J A^Y/a and a/a mice were cultured separately in the presence of serum from 42-day-old a/a mice (YBS), 120-day-old a/a mice (OBS), 42-day-old A^Y/a mice (YYS), and 120-day-old A^Y/a mice (OYS). The proliferative response of the lymphocytes to the T-cell mitogen, Concanavilin A (Con A), was determined through the incorporation of ^3H-thymidine. Values shown represent the stimulation index (cpm of mitogen stimulated cells/cpm of control cells).

EFFECTS OF AUTOLOGOUS SERUM ON CON A INDUCED LYMPHOCYTE PROLIFERATION

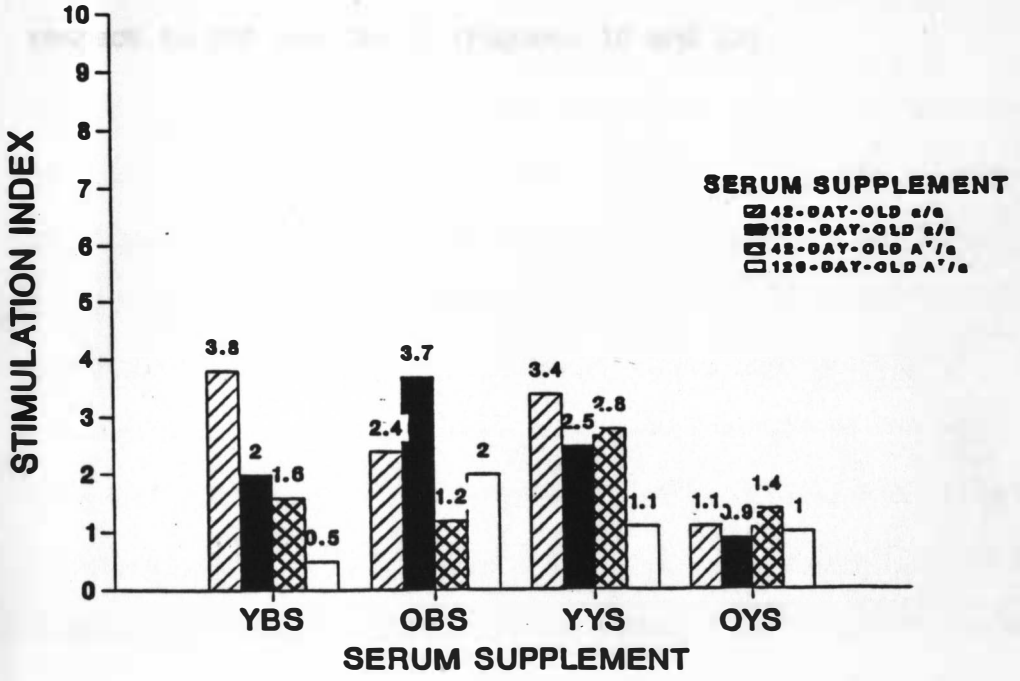

Figure 12. Lymphocytes from 42- and 120-day old female C57BL/6J A^Y/a and a/a mice were cultured separately in the presence of serum from 42-day-old a/a mice (YBS), 120-day-old a/a mice (OBS), 42-day-old A^Y/a mice (YYS), and 120-day-old A^Y/a mice (OYS). The proliferative response of the lymphocytes to the B-cell mitogen, LPS, was determined through the incorporation of ^3H-thymidine. Values shown represent the stimulation index (cpm of mitogen stimulated cells/cpm of control cells).

(Figure 11). However, this enhancement was not apparent with respect to PHA and Con A (Figures 10 and 12).

V. DISCUSSION

V. Weight Comparison

V.A.1. Total Body Weight.

Total body weights between 42-day-old and 120-day-old yellow (A^Y/a) and congeneic black (a/a) mice were compared to verify the correlation between age and the onset of obesity in lethal yellow (A^Y) mice. The mean weight of 120-day-old yellow (A^Y/a) mice differed significantly from 120-day-old black (a/a) mice. Moreover, the total body weight of 42-day-old yellow (A^Y/a) and black (a/a) mice did not differ. This indicates that an age-associated obesity has segregated with the A^Y/a mutation. Since obesity has been demonstrated to be associated with immune dysfunction, tests of immune function were performed on 42-day-old yellow (A^Y/a) while they were lean and compared with data obtained at the onset of obesity. Age-matched lean, black congeneic (a/a) littermates were used as controls.

V.A.2. Lymphoid Organ Weights

Evaluation of the immune system often includes a gross examination of the lymphoid organs from which immunologically competent immune cells arise (79). Among the most common points of examination include thymus and spleen mass.

The thymus is a primary lymphoid organ in which maturation and differentiation of T-cells occur. Results indicate a significant decrease in relative thymus weights with an increase in

age. This correlation was expected because thymic involution after puberty is typically observed (69). Unlike thymic depletion of neonates which leads to the impairment of T-cell-mediated immunity, adult thymic involution does not usually affect immune function. While not statistically significant ($p<.05$) the relative thymus weights of yellow (A^y/a) mice tended to be less than that of their congeneic black (a/a) littermates ($p<.10$). This may have an impact on cell-mediated immunity since the thymus is the primary organ of T-cell maturation. The thymus also acts as an endocrine gland secreting hormones that are important in T-cell maturation. These include thymosin, thymopoietin, and thymic humoral factor. Depletion of this primary organ would therefore lead to fewer circulating mature T-cells and, thus, an impairment in cell-mediated immunity.

The spleen is a secondary lymphoid organ containing both B- and T-cells. However, it is often considered to be a major B-cell organ (69). Relative spleen weights did not differ among the four sets of mice studied. Thus, no apparent immunological aberration is indicated on the basis of relative spleen size.

V.B. <u>In vivo Cell-mediated Immunity</u>

DTH was performed to compare <u>in vivo</u> cell-mediated immunity among the four sets of mice. Results indicated that the lethal yellow (A^y/a) mice were less responsive to DNFB than were their congeneic black (a/a) counterparts. Suppressed cell-mediated immunity as determined through DTH sensitivity seems to be directly

correlated with the A^{Y} mutation and does not appear to be causally related to obesity. This conclusion is based on the observation that age, correlating to the onset of obesity in yellow mice, did not appear to influence DTH reactivity. This observation differs from data obtained in relation to the viable yellow mutation (A^{VY}), which occurs at the agouti locus and displays similar manifestations to the lethal yellow mutation. Studies performed on the viable yellow mouse using the skin contactant, oxalazone, showed no significant difference in DTH reactivity from that of their genetically identical lean littermate nor from their congeneic black littermate (59). The differences noted between these studies on the A^{VY} mutation and our studies may illustrate a differential effect of the lethal yellow mutation in relation to the remarkably similar viable yellow mutation. However, this comparison must be used cautiously in that DNFB, instead of oxalozone, was used in our experiment. Furthermore, DTH responses of the viable yellow mouse were measured through ear swelling which is not as sensitive as the radioisotope labelling performed in our experiment (74).

Since DTH is a T-cell mediated immune mechanism, an impairment in T-cell activity is implicated in the lethal yellow (A^{Y}) mouse. Aberrations in T-cell activity have also been observed by other investigators. Gasser and Fischgrund (21) demonstrated decreased responsiveness in splenocytes from lethal yellow (A^{Y}) mice in a graft vs. host reaction when compared with splenocytes from their congeneic lean, black (a/a) littermates. Our data indicate an

alteration in the T_{DTH} subpopulation of T-cells and therefore complements data presented by Gasser and Fischgrund (21) which suggests alteration in the T_c subpopulation. Together these two studies demonstrate the severity of the T cell defect.

Alterations in T-cell function may result from a mutation at the A^y loci that may directly affect T-cell development or function. This would correlate with the observed decrease in thymus weights. Alternatively, suppressed DTH reactivity may represent an alteration not directly associated with the T-cell population. Many cellular interactions occur during the course of a T-cell mediated response. Processing of antigen prior to immunologic stimulation often requires presentation of the antigen to the T-cell by a macrophage. Antigen presentation requires proper cell to cell interaction which is believed to be regulated by the products of the immune response genes. Hence, an alteration in the expression of the immune response genes may lead to improper interaction and thus decreased immunoreactivity.

On a broader scale, suppressed DTH reactivity may be the result of altered responses to factors released by cells during an immunologic response. Antigen-stimulated T-cells release lymphokines such as macrophage chemotactic factor and migration inhibition factor that enable the specific recruitment of macrophages to the antigen-challenged site. These recruited cells also become activated through other lymphokines released by the antigen-stimulated T-cell. Activated macrophages, in turn, release factors

that further enhance T-cell activity. A decrease in synthesis or a structural change in any of these various factors could lead to suppressed DTH activity by an interruption of the cascade leading to the DTH reaction.

V.C. In vivo Humoral Immunity

In vivo humoral immunity was assayed through the enumeration of B-cells producing specific antibody to the T-dependent antigen, SRBC and through the measurement of serum SRBC specific IgM and IgG antibody levels. Humoral immunity was monitored through a predetermined period of time in order to detect possible differences in both the time and extent of peak responsiveness. Alterations in the peak response would indicate alterations in the regulation of the immune response.

V.C.1. Antibody-Forming Cells (AFC)

Enumeration of direct hemolytic plaques revealed a substantially reduced number of plaque-forming cells produced by the 120-day-old yellow (A^y/a) mice on days 5 and 6 following a primary exposure to SRBC. Whereas the number of direct plaques peaked for the lean, black controls and the 42-day-old yellow mice, a peak was not observed in the 120-day-old yellow (A^y) group. This may represent an impairment in the induction of antibody synthesis or may indicate a delay in the peak response to the antigen. This apparent suppression in antibody-forming B-cells may be a causally related to

obesity as 42-day-old yellow (A^y) mice responded similarly with lean, black (a/a) mice. This correlation between the immune response and obesity in the A^y mouse differs from observations reported in a separate strain of obese mice. A similar study performed on genetically obese (ob/ob) mice (10) demonstrated an enhancement, rather than a decrease, in the number of antibody-forming cells to SRBC in the obese mouse when compared to the lean mouse. This suggests physiologic differences in obesity between the genetically obese (ob/ob) mouse and the lethal yellow (A^y) mouse which may be a function of the mutation.

With the exception of 120-day-old yellow (A^y) mice, a decline in the number of direct plaques was observed following a secondary exposure to SRBC. This decrease in the number of direct plaques would be expected as a result of a switch in antibody synthesis from IgM to IgG. As mentioned previously, direct hemolytic plaque assays permit only the quantitation of B-cells producing antibody of the IgM isotype. Since this decrease was observed in these three sets of mice, it is interesting that the number of plaques did not decrease in the 120-day-old yellow mice. Unlike the three other sets of mice, the number of AFC's produced by 120-day-old yellow mice generally remained the same during the secondary response as compared to the number of AFC's produced in the primary response. Several possible defects may account for this observation. First, the plateau may indicate diminished T suppressor activity Secondly, it is possible that the obese yellow mice peaked at a later

time following the primary exposure to SRBC and then normalized to the levels observed in the other sets. Finally, this delayed peak of immunological reactivity might indicate alterations in the regulation of the overall response. The observed plateau may indicate a failure in isotype shift, a mechanism which is believed to be induced by the helper T-cell (33). Failure in isotype shift would lead to diminished IgG production, hence, indicating a defect in this T-cell population. An indirect plaque assay enabling the detection of AFC's producing IgG was attempted during our investigation. Unfortunately, an overall suppression of plaques was observed upon the addition of anti-mouse IgG to the lymphocyte culture, thus, invalidating the data. This curious phenomenon has been observed by other investigators as well (31).

V.C.2. Enzyme Immunoassay

Relative levels of serum IgM and IgG to SRBC were determined following both a primary and secondary exposure to SRBC. Inexplicably, the serum anti-SRBC IgM level in 120-day-old yellow (A^y) mice following a primary exposure to the antigen became significantly higher than the other three sets of mice. The increased IgM titer observed in this group of mice did not correlate with the decreased number of IgM plaques formed. These observations suggest that fewer B-cells are secreting higher concentrations of IgM antibodies, thus, implying an aberration in the regulation of antibody synthesis. Because of this apparent aberration in the

regulation of antibody synthesis, the lethal yellow model may serve useful for studies in hypersensitivity reactions where a dysfunction in immunologic regulation leads to a pathologic condition.

The regulatory mechanisms of the immune response have not been fully elucidated. However, helper T-cells are believed to regulate the shift in isotype synthesis from IgM to IgG. Without this shift, increased IgM levels without an increase in IgG would be expected. Suppressed function of the helper T-cell could be implicated in this investigation except that levels of serum anti-SRBC IgG in the old yellow mouse were equivalent to that observed in the other sets of mice. Furthermore, impairment of the helper T-cell function would also be associated with a failure to produce an anamnestic response since the helper T-cell is an integral part of this response. This was not the case as data indicates a secondary response in the 120-day-old yellow mouse statistically indistinguishable from the other mice.

Because altered humoral activity was observed only in 120-day-old mice, obesity, rather than genotype, seems to be intimately involved in the control of this facet of the immune system. Investigations using the viable yellow (A^{vy}) model have also indicated that altered immunological function is a secondary effect of obesity rather than genotype. Studies with the A^{vy} model revealed that anamnestic IgG responses to a T-dependent antigen were significantly reduced in obese, viable yellow mice when compared to their genetically identical, albeit phenotypically different, lean

littermates (59). Thus, this effect has been attributed to a secondary effect of obesity rather than the effect of the gene mutation. During our studies, a depressed anamnestic response was not detected in the lethal yellow mouse. Again, these contrasting observations suggest differences in the physiology associated with obesity observed among different types of obese mice.

A significant effect of age on the antibody response to SRBC was noted following a secondary exposure to the antigen. However, this effect was not associated with a genotype difference, and, therefore, is not pertinent to this discussion.

V.D. In vitro Assessment of the Immune Response

In vivo tests of the immune system are often complicated by the degree of interaction that occurs between the systems within the body. Therefore, in vitro tests are used to try to narrow the focus of possible aberrations. As pointed out, the lethal yellow mutation seems to be directly involved in suppressed cell-mediated immunity as demonstrated by the DTH test. However, through this in vivo assay, it was not possible to determine if the abnormality was specifically associated with T-cell function. Conversely, humoral immunity does not appear to be directly affected by this mutation in that aberrations are only observed in 120-day-old yellow mice. Since these mice weigh significantly more than the other three sets of mice, obesity likely plays a role in the altered immune response observed in response to SRBC. To help ascertain the source of the

aberration, *in vitro* lymphocyte stimulation assays to polyclonal mitogens were performed. Mitogens that specifically stimulate B-cell and T-cell populations were chosen. Lipopolysaccharide, a B-cell mitogen, and two T-cell mitogens, concanavalin A and phytohemagglutinin were utilized. This test served two purposes, one to determine if abnormal lymphocytes are a result of genotype and the other to determine if there is an effect of the physiologic environment on lymphocyte reactivity.

V.D.1. Lymphocyte Proliferation with Fetal Bovine Serum

Optimal growth conditions for mammalian cell culture requires the addition of serum to the growth medium. The most commonly recognized serum for most cell culture is fetal calf serum. During this study, lymphocyte proliferation assays were performed under such optimal growth conditions. While mice bearing the lethal yellow mutation tended to react with less magnitude, no significant differences were observed in the response. This suggests that the lethal yellow mutation is not directly associated with the ability of lymphocytes to respond to these polyclonal stimulants. From these observations, suppressed DTH reactivity as a result of the A^{y} appears to be due to an alteration that is not directly associated with T-cell function, but suggests defects in antigen presentation or regulation of T-cell activity.

V.D.2. Lymphocyte Proliferation with Congeneic/Syngeneic Serum

Cell-mediated immunity studies performed by Chandra and Au (10) using obese (ob/ob) mice indicate that impaired immunity is the result of an abnormal physiologic environment. They found the reactivity after in vitro stimulation of spleen cells from obese mice did not differ significantly from the reactivity of the cells obtained from lean controls. To ascertain the possible influence of serological factors in the response of obese yellow (A^y) mice in our study, lymphocytes were cultured in the presence of mitogens using media supplemented with 10% serum from the four sets of mice. Interestingly, stimulation indices were significantly less when lymphocytes were cultured with congeneic/syngeneic serum than when cultured with allogeneic fetal calf serum. Other investigators have observed a similar immunosuppressive effect of autologous serum (49,75). Veit and Michael (75) reported a suppression of antibody synthesis to SRBC when murine lymphocytes were cultures in the presence of normal mouse serum. Unfortunately, this immunosuppressive factor was not characterized.

Inexplicably, differences between genotype became apparent during the proliferative responses of splenocytes in the presence of congeneic/syngeneic serum to the various mitogens. When compared to their black (a/a) controls (Figure 7), lymphocytes isolated from yellow (A^y) mice revealed a significant difference in activity to the T-cell mitogen, PHA, when cultured in the five pools of serum. PHA is known to specifically stimulate cells bearing Ly 1,2,3 surface

markers (51) which are found on all undifferentiated T-cells. This observation suggests either fewer numbers of lymphocytes bearing the Ly 1,2,3 marker or decreased sensitivity of Ly 1,2,3+ cells to this mitogen on A^y/a lymphocytes. A significant difference in response to the other T-cell mitogen, Con A, known to stimulate Ly 1+ cells, was not observed. Since DTH T-cells bear this marker, one would have expected a decreased response to this mitogen. This indicates that the suppressed DTH response observed in this study is not intrinsically related to an aberrant T-cell population.

Lymphocytes cultured in the presence of serum from 120-day-old yellow (A^y) mice appeared to be less reactive than the other groups of mice. This effect was most significant ($p<.05$) in the PHA-induced blastogenesis assay. Since cells cultured under optimal conditions, in fetal calf serum, did not display this effect, this suggests the presence of some factor(s) in the serum of 120-day-old mice that may suppress the proliferative response of lymphocytes to this T-cell mitogen. Investigators have demonstrated a correlation between the presence of lipids in serum and suppressed lymphocyte reactivity (27). Cholesterol is an example of a lipid which has been demonstrated to exert immunosuppressive effects. Reportedly, increased cholesterol within macrophages inhibits the phagocytic process, thus affecting its function in killing or processing antigen (76). Failure to process antigen would likely lead to defective antigen-presentation to lymphocytes, inhibiting the immune response. The increased levels of cholesterol in the serum of obese yellow mice

(8) makes it tempting to speculate that this is at least partially responsible for this suppressive activity. In relation to this, the suppressed plaque-formation observed in response to SRBC in 120-day-old yellow (A^y/a) mice may also be related to hypercholestrolenemia.

While not statistically significant, cells cultured in the presence of LPS and cultured in serum from 120-day-old yellow mice also tended to be less responsive. Since LPS specifically stimulates B-lymphocytes, diminished reactivity of this population may also explain the decreased number of plaques observed in the 120-day-old yellow mice. As observed with the PHA response of T-cell populations, lipids in serum have been reported to affect lymphocyte reactivity to LPS (27).

The Con A-induced proliferative response of lymphocytes cultured in the presence of serum from 120-day-old (A^y/a) mice appeared to be similarly suppressed. Con A specifically stimulates the subset of T-lymphocytes bearing the ly 1 marker. This subpopulation includes both the helper T-cell and the T_{DTH} cell. If this population of T-cells were affected by factors in this serum, it would be logical to speculate that the intensity of a DTH reaction and the immunologic responsiveness to T-dependent antigens, such as SRBC, would be significantly suppressed in 120-day-old mice. This relationship, however, was not observed. Suppressed DTH activity in the lethal yellow mouse was not related to obesity.

V.E. Disparity Between In vitro and In vivo Results

Upon comparison of the effects of autologous serum among the four groups of mice (Figures 10-12), it was noted that lymphocytes from 120-day-old black (a/a) mice were more responsive to PHA and LPS than were lymphocytes from the other groups of mice, indicating heightened T-cell and B-cell reactivity. This was inconsistent with the significantly decreased anti-SRBC IgG titer observed in this group of mice (Figure 4). Speculatively, IgG levels produced by 120-day-old black (a/a) mice may have peaked earlier than the fourth day following immunization to SRBC. If antibody titer peaked earlier than day 4, the number of plaque-forming cells should also have peaked before the fourth day. However, heightened reactivity was not indicated by the results from the direct hemolytic plaque assay where peak responsiveness occurred on the fifth day post-immunization (Figure 2).

VI. CONCLUSION

Many complex physiological and immunological interactions are observed in the (A^y/a) mouse model. Data demonstrate the influences of this mutation on the immunoreactivity of the lethal yellow mouse. Body weight rather than genotype per se appears to exert an immunomodulatory effect. This was demonstrated in both humoral and CMI assays. Evidence for possibly depressed humoral immunity included decreased numbers of antibody-forming cells isolated from 120-day-old yellow mice and the reduced responsiveness of lymphocytes obtained from 120-day-old yellow mice to the B-cell mitogen, LPS, when compared with the other groups. CMI responses also appeared to be influenced by factors associated with obesity. Serum obtained from 120-day-old yellow (A^y) mice tended to suppress the proliferative response of lymphocytes to both T-cell mitogens.

Other irregularities in cell-mediated immunity were directly associated with the A^y mutation since the effects were apparent in both lean, as well as, obese yellow mice. This was demonstrated *in vivo* in the DTH reaction and, to a lesser extent, *in vitro* during lymphocyte proliferation utilizing T-cell mitogens. These observations further support the evidence of the influence of the A^y mutation on the immune system that has been reported in the literature (21).

Based on these data, one can only conjecture as to the direct or indirect role that the agouti locus plays in immunoregulation. The agouti locus is closely associated with many genes responsible

for immune regulation. These genes include the H-3 and H-13, as well as several other, minor histocompatibility loci (22). Additional gene sequences of immunological significance associated with the agouti locus include the Ir-2 genes which code for such lymphocyte surface antigens as Ly-4, Ly-m11 (which is believed to be the gene that codes for B-2-microglobulin), Ly-23, Qam9, and GM-3, and finally Pgp-2 (which controls a cell surface glycoprotein of macrophages) (22). Hence, this cluster of genes in the mouse may have a number of both biological and immunological implications comparable to the widely studied MHC of chromosome 17 (11). Since these genes are closely linked to the agouti locus, it thus seems plausible that a mutation at this locus may have an effect on these closely linked immunoregulatory gene sequences.

The lethal yellow mutation at the agouti locus has been associated with the absence of two of the three forms of tyrosinase. Investigators believe that this is a result of a glycosylation defect. On this assumption, one could speculate that the failure to glycosylate membrane proteins may also occur. This could result in an alteration of lymphocyte markers. This would explain the data obtained during Con A and PHA-induced lymphocyte proliferation. Since PHA binds to the N-acetyl-glucoseamine group of glycoproteins and Con A binds to -mannosyl, failure in the glycosylation of protein with N-acetylglucoseamine may account for the differences in binding and activation of the mitogens to the lymphocytes and, thus, explain the significantly suppressed response of the A^{y}/a lymphocytes

to PHA. This interpretation must be viewed cautiously, however. The observed increase in lymphocyte proliferation of 120-day-old black (a/a) mice to PHA may be the contributing factors to the overall difference in activity between the two genotypes. However, data on lymphocytes cultured in the presence of fetal calf serum does indicate a decrease in reactivity among A^{y}/a mice.

Speculative evidence for the alteration of one or more of the minor histocompatibility loci associated with the agouti locus is indicated in the literature. Of particular interest is an investigation performed by Wolff (81). His study involving the growth of A^{y}/a and a/a mice in parabiosis centered on the detection of possible hormonal differences between these mice. His data may be interpreted to be of immunological significance. In control groups where yellow mice were joined with yellow mice, or black mice joined with black mice, mortality ranged from 0-7%. On the other hand, when black mice were joined with yellow mice, mortality dramatically increased to 20-40% The majority of these deaths occurred three or more months after the union of the mice in parabiosis. It is therefore possible that this may have been a result of graft rejection. An incompatibility in minor histocompatibility antigens, such as the H-3, can be responsible for graft rejection. However, such rejection is usually much slower than that from incompatibility in the MHC and may take longer than 100 days to occur. Coincidentally, deaths of the heterogenous pairs of mice in Wolff's parabiosis experiment occurred within 100 days.

A final interesting aspect of the agouti locus is the presence of a retroviral sequence referred to as Emv-15, closely linked to the agouti locus (12). Retroviruses can be integrated into host chromosomes and act as mutagens either by two mechanisms. They can interrupt the normal gene expression of the host chromosome or activate the expression of cellular sequences flanking the integration sites (80). Thus, the possibility exists that the insertion of this retrovirus has altered the expression of one or more of these genes which play a role in immunoregulation. If an immune response gene, which codes for Ia antigens, is inactivated, the Ia antigen will not be expressed on the lymphocyte. This would impair cell-to-cell interaction and lead to suppressed DTH activity, as well as, other cell-mediated immune mechanism.

VII. SUMMARY

In summation, the lethal yellow mutation appears to be causally related to diminished immunoreactivity. Unfortunately, it is not a straight-forward mutational effect. It appears that both the physiological environment which either causes or results from obesity and the lymphocyte itself are altered as a result of this mutation. Whereas the obesity appears to be associated with the suppression of humoral immunity, direct effects of the mutation itself appear to be responsible for the reduced CMI response observed during the DTH reaction. Combined, these results provide another link between immunosuppression and cancer susceptibility.

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COMPARISON OF IMMUNOLOGIC RESPONSES
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IMMUNOLOGIC ABNORMALITIES IN LETHAL YELLOW (A^y) MICE
ARE AUGMENTED BY OBESITY

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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CERTIFICATION OF COMPLETION

Oral Examination and Thesis

This is to certify that NANCEE LOIS OIEN

in accordance with the requirements of South Dakota State University for the degree Master of Science has presented to this committee two unbound copies of an acceptable thesis done in the major field; and has satisfactorily passed the oral examination, the major field Microbiology and the Minor field CHEM.

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