Effects of Dietary Restriction on Immune Function in Rodent Model Systems

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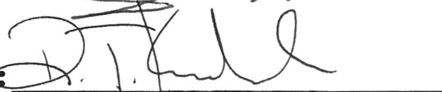

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INTRODUCTION

Humans and laboratory animals experience a decline in immune system function and DNA excision repair correlated with increased occurrence of cancer as a function of age. Dietary restriction is reported to be associated with increased lifespan and with increased DNA excision repair in laboratory animals. This report shows that dietary restriction is correlated with increased lifespan of male and female rats and mice, and with the capacity of cultured splenocytes from these animals to initiate blastogenesis in response to antigenic stimulation.

BACKGROUND INFORMATION

Aging

Cancer is a disease which is typically associated with old age. Two schools of thought have evolved to explain the age-related occurrence of cancer. One theory maintains that the high incidence of cancer in the elderly is a result of an accumulation of separate, low probability, carcinogenic events during the extended lifetime.[1-3] The other theory maintains that physiological changes normal to the process of aging cause decreases in immune system function and in deoxyribonucleic acid (DNA) repair mechanisms.[4-7] Data have been reported to support both of these theories, suggesting that increased age-related occurrence of independent initiation and promotion events, coupled with decreased age-associated capacity of the organism to deal with these events, may contribute singly or in concert to the onset of cancer associated with aging.[8]

DNA Damage and Repair

DNA Damage

DNA damage may be initiated in cells by exposure to ultraviolet (UV) light, ionizing radiation such as x-raysand cosmic radiation, endogenous and spontaneous oxidative events, and exposure to mutogenic xenobiotics. Humans are constantly exposed to radiation from the sun, other common light sources, and radioactive agents, and are increasingly exposed to genotoxic chemicals, allof which cause damage to DNA that must be repaired.

DNA Repair

Two primary forms of DNA excision repair are found in cells of vertebrates, including humans and rodent, short patch repair and long patch repair. It should be noted that when the DNA molecule forms the double helix conformation discovered by Watson and Crick, a major and a minor groove can be distinguished spiraling throughout the molecule.

Short Patch Repair. Short patch repair works on DNA alterations in the molecule's major groove which are generally caused by x-rays (ionizing radiation) or chemicals that mimic the x-rays. Ionizing radiation causes nicks in the phosphodiester backbone (chains of sugars connected by phosphate groups to which the nucleic acids are attached) of DNA activating an enzyme, exonuclease, to excise one to fifteen bases containing the damaged site. An enzyme known as DNA polymerase β then fills the gap by polymerizing the attachment of new base nucleotides to the helix using the other side of the DNA as a template. Finally, another enzyme, ligase, attaches the repaired DNA segment to the existing strand.

Long Patch Repair. In contrast, long patch DNA repair occurs in DNA damaged by UV light (non-ionizing radiation) or UV-like chemicals. UV light does not nick the phosphodiester backbone, so a specific endonuclease enzyme is required to open the sugar-phosphate bond in the backbone prior to exonuclease excision of approximately one hundred bases. Another enzyme, DNA polymerase α catalyzes the addition of new bases to the exposed 3'-hydroxy end of the excised DNA segment, again using the intact

DNA strand as the template. DNA polymerase β fills in the bases at the ends of the repaired segments, and ligase attaches the last phosphodiester bond to complete the repair. The aforementioned enzymes track up and down the helical grooves of DNA, repairing the strands as needed.

Immune System

The immune system is the body's mechanism for dealing with an invasion by either biological (bacteria, viruses, fungi, and protozoa) or abiotic agents (chemical and physical entities from trauma, irradiation, heat, cold, and toxins). The immune system recognizes the inflammatory response in tissue, and initiates a complex sequence of biochemical reactions that lead to functional and morphological changes.[9] The immune system has two different mechanisms for combating damage to cells: the vascular response and the cellular response.

Vascular Response

The vascular immune response is simply forcing the circulatory vessels to allow fluids to cross through the vessels, which are normally impermeable to these fluids, to the injured cells.[9] These fluids include the chemicals histamine, serotonin, kinins, plasmin, anaphylotoxins, and prostaglandins.[9] Their specific individual functions will not be discussed in the scope of this report.

Cellular Response

The response of the immune system to foreign material on the cellular level is centered on the activation and response of a group of hematopoietic cell lines derived from a common pluripotent stem cell. Pluripotent stem cells differentiate into either the committed stem cells, which give rise to hematopoietic cell lines such as red cells, granulocytes, macrophages, and megakaryocytes, or committed lymphoid precursors, which give rise B lymphocytes (B cells) or T lymphocytes (T cells).[10] The committed stem cell line progeny will be briefly discussed to be followed by an examination of the lymphatic system.

Granulocytes. The granulocytic series of cells consists of neutrophils, eosinophils, and basophils, with neutrophils being the most common. All three types of granulocytes commonly derived from the bone marrow function in a similar fashion. They aid the immune response by phagocytosis of foreign debris and utilization of lysosomal enzymes to lyse the cellular debris.[9]

The phagocytic process of granulocytes begins with the recognition of foreign particles that have been coated by an opsonic serum factor such as IgG. The phagocyte has receptors specific for the Fc region of the IgG on its membrane.[9] The particles are recognized and engulfed into the cell to form a vacuole called the phagosome. Phagosomes fuse with a lysosome to form a phagolysosome. The digestive enzymes in the phagolysosome partially digest the particle and release the fragments into the cellular space.[9]

Macrophages. Bone marrow-derived macrophages have many functions in the immune system, two of which include phagocytosis of foreign bodies and processing of antigen for the lymphocytes. In its function of phagocytosis, the macrophage closely resembles the granulocyte. The macrophage also processes antigens before presenting them to the lymphocytes to stimulate antibody formation. Finally, these cells possess the ability to divide to form other macrophages, epithelioid cells, or giant cells.[9]

Lymphatic System

The lymphatic system is another part of the body's defense against foreign intrusion. These cells are interrelated to produce chemicals which enhance their effectiveness against antigens.

Lymphocytes

Lymphocytes are immune cells derived from the bone marrow of most vertebrates. Burnet[11,12] postulated that the cells of the immune system, lymphocytes included, became committed to the production of a specific antibody molecule before exposure to an antigen. When antibodies to specific immunoglobulins are mixed with lymphocytes, lymphocytes are induced to blastogenic transformation and DNA synthesis.[13,14]

The immune system contains many variations of lymphocytes that are involved in different areas of the humoral immune response. These cells include T lymphocytes, T helper cells, T killer cells, T suppresser cells, and B lymphocytes.

T Lymphocytes. T lymphocytes are differentiated cells that have been programmed in the thymus to be different than the antibody producing B cells. When the body is invaded by foreign substances, T cells move to the site of infection. T cells that are specific to antigens at the infection site are activated by binding with the antigens by means of receptor proteins on the cell surface. T cells carry the antigen back to the lymphatic system and to lymph nodes. Once there, some T cells hold on to the antigen and become memory cells. Upon contact with the specific antigen, or mitogens used in research, T cells entes blastogenesis and proliferation begins. Active T cells produce lymphokines, low molecular weight proteins which facilitate in the removal and destruction of foreign particles by activating phagocytosis in macrophages and other leukocytes.[15]

T Helper Cells (T4 Cells). T helper cells are a form of T lymphocyte that is specific for its interaction with B cells. These cells react in the same manner as T cells upon contact with the antigen, but they specifically help the humoral response by stimulating macrophages and by presenting antigen to appropriate B cells in a form that the B cell can utilize. This process allows B cells to proliferate and form clones of antibody-producing cells.[10] T helper cells also differentiate the T killer cells from the other lymphotic cells.[15]

T Killer Cells. T killer cells are another form of basic T lymphocytes that has differentiated with specific characteristics. T killer cells are released from the thymus upon stimulation by T cells that have contacted antigens at the

sight of infection. These cells have direct cytotoxic activity against target cells such as those from tumors; they can produce interferon; and they maintain a general surveillance of tissue.[9]

T Suppresser Cells. T suppresser cells control the level of B cells in infected areas. These cells maintain B cell levels at a level that is optimal to the immune response. They are the control cells of the immune system.[15]

B Lymphocytes. B cells, like T cells, originate in the bone marrow but do not go through the thymus for programming. These cells are involved in the humoral response system on the antigen-antibody level. B cells are activated and induced to proliferate by the interaction with T cells. B cells receive antigen from T cells. Using this antigen, activated B cells produce antibodies on their cell surface and release antibodies into the surrounding fluid. Upon activation, these cells clone to form a large reserve of cells capable of antibody production.[16,17] Further down the line, B cells can also form plasma cells which store antibody. [9]

Interleukin 1 and Interleukin 2

In the humoral immune response, the interleukins serve as proteins responsible for the proliferation of lymphocytes. Interleukin 1 (IL-1) is produced by macrophages that have been stimulated by either antigens or mitogens. IL-1 binds to T cells and induces the production of interleukin 2 (IL-2). IL-2 stimulates the proliferation of T cells that bear specific

receptors for the IL-2. The production of IL-1 and IL-2 are important in stimulating T cells to clone and potentiate the immune system's response to foreign antigen.[18].

SIGNIFICANCE OF RESEARCH

DNA Repair System

Research into the effects of dietary restriction on DNA excision repair will be centered on the specific effects to the function of DNA polymerase α (deoxynucleotidyltransferase, E.C.2.7.7.7). DNA polymerase α is responsible for adding approximately 90% of the deoxynucleotide monophosphates to the growing end of the DNA primer during DNA synthesis necessary for both excision repair and mitosis. Prior studies have shown that the molecular weight of immunoaffinity purified enzyme is constant between related organisms. These studies have also shown that the primary structure of DNA polymerase α subunits is similar between species.

Polymerase α Isozymes

The study of DNA polymerase α is thought to be important to the understanding of aging because of the discovery that two different types of DNA polymerase α may be derived from fetal and adult cells. The two types of DNA polymerase α are called isozymes because they are different enzyme forms that perform the same function. Fetal cells show the presence of a DNA polymerase α (A_2) that is stable, highly active, and has a high affinity for binding to the DNA primer, while DNA polymerase α from the adult cells contains A_2 type and another form, A_1, which has charge differences and a lower binding affinity to the DNA primer stem. The adult-derived enzyme shows a significant increase in expression as the cell's age increases, with fetal-derived cells having 0% of A_1, and cells from a 66 year old male having 94% of

the total cellular pool in the A_1 form. It is important to note that both the ratio of A_1 to A_2 enzyme increases as age increases, and that the total cellular pool of enzyme decreases with age. This difference in enzyme expression may provide other researchers with a biomarker for aging. Furthermore, this fact suggests that age-related dysfunctions of DNA excision repair may be due to the gradual loss of expression of the A_2 enzyme and/or the increased expression of the A_1 enzyme over the A_2 enzyme. Aging could be significantly impacted by loss of the cells' ability to repair damaged DNA.

Dietary Restriction

The significance of using dietary restricted rodents comes from initial data that suggests that rodents with restricted diets live 25-40% longer. Testing rodents with and without dietary restriction for A_1 versus A_2 expression could reveal useful facts about human aging.

Immune System

The cells of the immune systems of animals undergo a vast amount of change which may explain the altered immune response associated with aging. One such change is the imbalance of distribution of specific T lymphocyte subsets[19,20] with a reduction of lectin-stimulated lymphocyte DNA synthesis which has been reported for older humans.[21,22,23] This phenomenon parallels the age-related decline in mitogen activation of T cells in the murine model system.[24] Akagawa et al[25] reported

that the 6-thioguanine-resistant stem cells were up to seven times as prevalent in the old mice as in the young ones. From the data, old mice contain a significantly greater population of stem cells that cannot respond to mitogen activation resulting in clonal differentiation into T cells, suggesting that ability to stimulate blastogenesis is decreased in the aged. This assumption is further correlated with findings showing that, although lymphocytes from young and old humans have similar numbers and binding affinities for mitogen receptors, circulating lymphocytes from older subjects are half as likely to enter into the first stage of cell division as are the circulating lymphocytes from young subjects.[26,27]

The immune responses in young and old humans is also different as seen by the number of cells that complete multiple divisions after stimulation.[26] This could be caused by the decreased ability of activated immune system cells to produce cytokines, such as the helper T cell growth factor IL-2 which is decreased in activated T cells from older humans.[23,28,29,30] Miller and Harrison[31] reported that mice in the last third of their lifespan exhibit three- to six-fold fewer biologically active helper and killer T lymphocytes compared to young mice. Data indicates that this decline in T cell function may be due to decrease in the viable cells that do not respond to antigenic stimulation while appearing normal. The decrease in regulatory T cell functions may be related to a decline in the individual cell's capacity to IL-2.[32,29,33,34,35]

Although the reasons for immune system decline are complex and not well correlated, the only known mechanism for increasing

lifespan, presumably by retarding the rate of immune system and DNA repair decline and the resultant increased expression of disease, is through dietary restriction.[36,37,38,39,40]

MATERIAL AND METHODS

Animal Preparation

C57Bl/6 (B6) mice and Fischer 344 (F-344) rats utilized in this study were reared at the National Center for Toxicological Research in the Specific Pathogen Free Barrier Facility, Jefferson, Arkansas.

The animals were prepared for research in the following methods:

1. Animals were held at 23° C on a 12/12 hour light/dark cycle with lights on form 6:00 am daily.
2. Nursing animals were maintained with females fed *ad libitum* (AL) on the NIH-31 rodent diet, and were continued to be fed AL until 14 weeks of age.
3. At 14 weeks the animals were divided into two groups and fed either NIH-31 AL or a diet identical to NIH-31 in vitamins and minerals but 60% restricted in calories (DR).
4. Both groups received AL water.
5. Animals were shipped to College Station, Texas, and were utilized immediately upon arrival.
6. Rats were sacrificed by decapitation and mice by cervical dislocation
7. Spleens, brains, livers, kidneys, lungs, peritoneal macrophages and blood were removed for various ongoing investigations.

Splenocyte Preparation

Splenocytes were removed and blastogenesis was examined in the following methods:

1. Spleens were aseptically excised from AL and DR animals and fascia was removed using scalpel, scissors and a syringe.
2. The spleens were minced and cells were expressed into RPMI 1640 medium.
3. Preparations of spleen cells were allowed to sediment for 5 minutes to remove debris, and the supernatants were centrifuged at 500 rpm for 10 min.
4. The pellets were resuspended in RPMI 1640 medium, counted, adjusted to contain 2.5×10^6 cells/ml, and dispensed into 6-well plates at 2 ml/well.

Blastogenesis Stimulation

Splenocyte suspensions were divided into two groups per dietary condition--the control group (no stimulation) and the experimental group (stimulation with Conconavalin A). Conconavalin A was added to a final concentration of 25 μg/ml.

^{3}H-Thymidine Incorporation and Assay

3H-Thymidine is incorporated into the splenocytes and activity is assayed in the following manner:

1. After 68 hrs., 1 μCi/ml 3H-thymidine (New England Nuclear, 24 Ci/mmol) was added to the medium and the cells were assayed for 3H-thymidine incorporation after 4 additional hours of culture.
2. Splenocytes to be assayed were dissolved by treatment with 0.1 Normal (N) NaOH containing 2% $NaCO_3$ for 15 min, 5 ml of cold 10% trichloroacetic acid (TCA) was added to each tube, and the tubes were vortex-mixed and held at 0° C for 30 min.
3. Each tube was poured across a glass fiber filter (Whatman AH34) held in a Gelman vacuum filter manifold.
4. The assay tubes were washed with a second 5 ml TCA aliquot followed by 5 ml of cold ethanol x 2, each of which was poured across the corresponding filter.
5. Filters were dried and counted using Beckman Scintiverse Scintillation cocktail.
6. Activation of splenocytes by Conconavalin A was reported as dpm of 3H-thymidine incorporated/10^6 cells.

RESULTS

F344 rats and B6 mice were SPF-housed and fed ad libitum or were dietary restricted (60%) to the end of their life span. The rate of survival of mice at the age of 28 months differed very little between the gender of the animals, but differed greatly between the AL and DR animals of either gender (Figure 1). Survival patterns for the aging F344 rats differed significantly from those of the B6 mice. If fed AL, no male rats survived to the age of 29 months, while female rats fed AL showed a survival rate of 22% at 31 months. The survival rate of the male F344 rats that were DR was 44%, while the DR females expressed a survival rate of 62% (Figure 2).

Both rats and mice showed a pattern of immune response similar to their survival rates. Six-week-old B6 mice exhibited ^{3}H-thymidine incorporation into DNA of mitogen-stimulated spleen cells at levels approximating 2.5 times the levels seen in cells from 24 month old AL animals (Figure 3). Even though levels of ^{3}H-thymidine incorporation did not vary between nonmitogen-stimulated AL or DR mice, DR animals showed approximately a 90% increase above AL animals for mitogen-stimulated cells. The F344 rats show a similar pattern in comparisons between ^{3}H-thymidine incorporation into unstimulated and mitogen-stimulated splenocytes from young of young rats and 29 month old rats that were AL or DR (Figure 4). These data show that DNA synthesis measured as ^{3}H-thymidine incorporation in unstimulated splenocytes did not differ between old vs young or between AL and

DR animals. However, mitogen-stimulated DNA synthesis in cells from old DR animals more closely resembled mitogen-stimulated DNA synthesis in splenocytes from young animals.

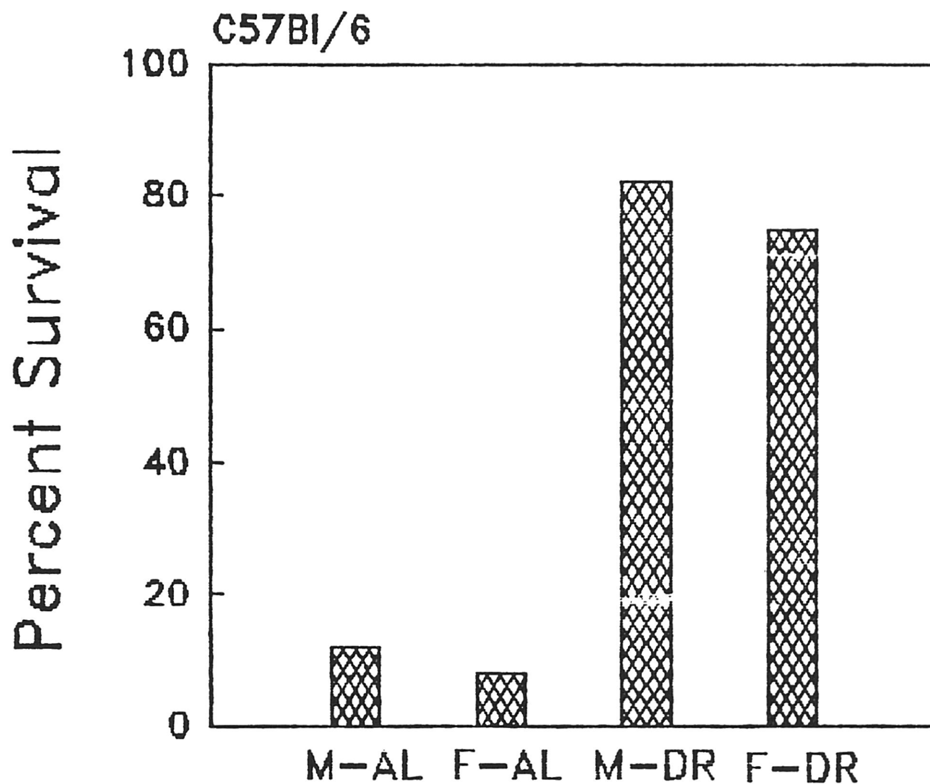

Figure 1: Percent survival of 28 month C57Bl/6 mice that were reared in the Specific Pathogen Free Barrier Facility at the National Center for Toxicological Research, Jefferson, Arkansas. Symbols: M-male; F-female; AL-ad libitum; DR-dietary restricted.

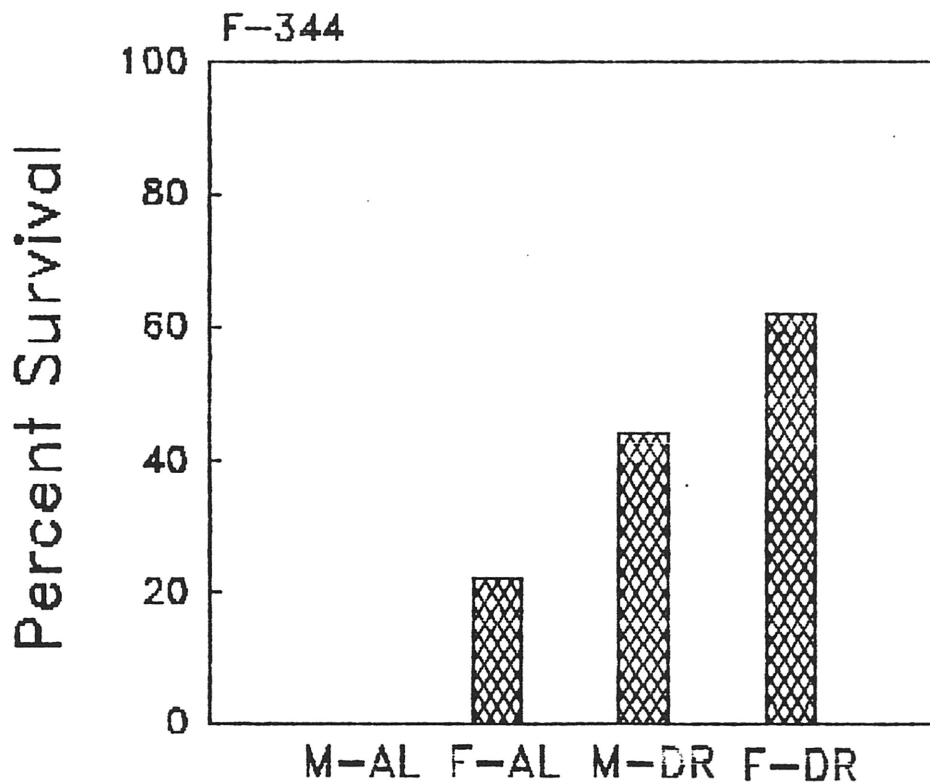

Figure 2: Percent survival of 29 month Fischer 344 rats that were reared in the Specific Pathogen Free Barrier Facility at the National Center for Toxicological Research, Jefferson, Arkansas. Symbols: M-male; F-female; AL-ad libitum; DR-dietary restricted.

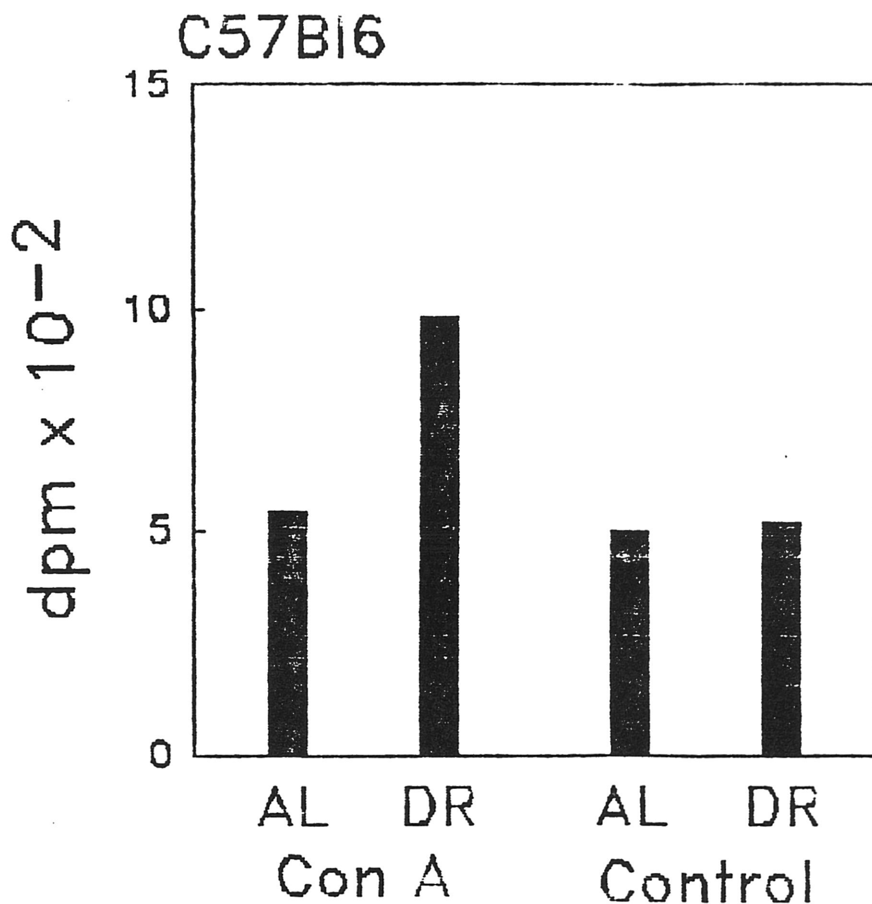

Figure 3: ^{3}H-Thymidine incorporation (measured in dpm) in the splenocytes of 24 month C57Bl/6 mice that were SPF-housed. The splenocytes were either mitogen-stimulated with conconavalin A (Con A) or non-stimulated (Control). Symbols: AL-ad libitum; DR-dietary restricted.

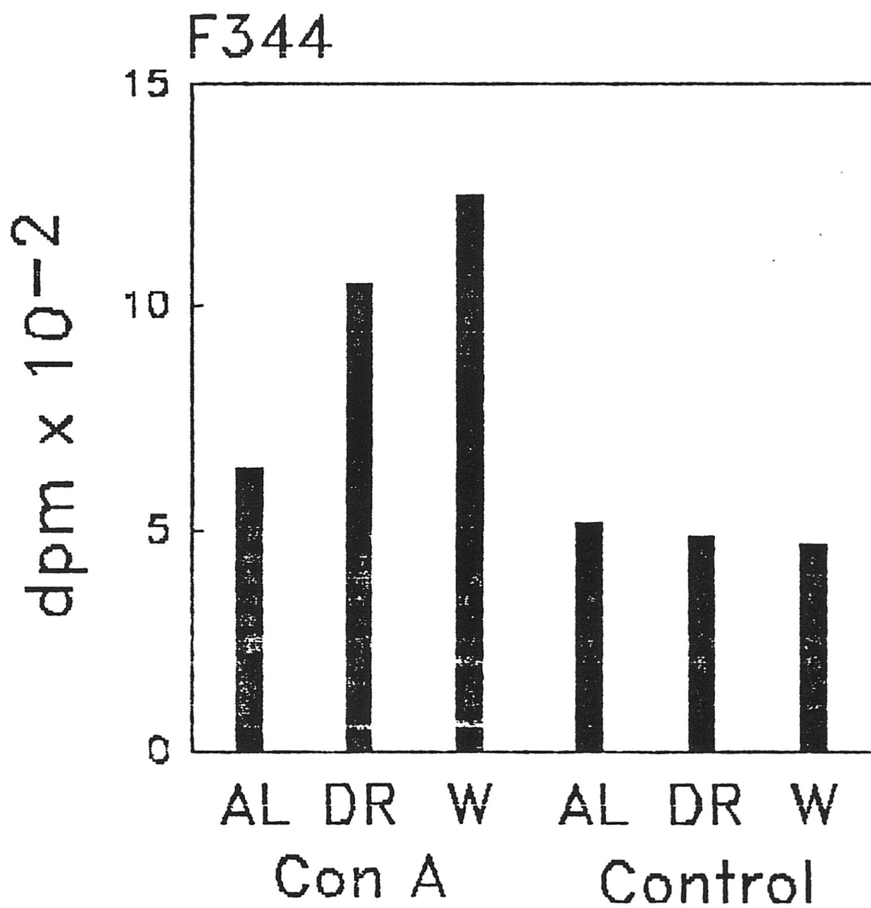

Figure 4: ^{3}H-Thymidine incorporation (measured in dpm) in the splenocytes of 29 month Fisher 344 rats that were SPF-housed. The splenocytes were either mitogen-stimulated with conconavalin A (Con A) or non-stimulated (Control). Symbols: AL-ad libitum; DR-dietary restricted; W-6 week old weanlings.

DISCUSSION

The literature shows that data exists which correlates a decrease in the functioning of the immune system in vertebrate animals with the increase of chronological age of those animals. Age-related alterations in the immune system are very complex, and as of yet, investigators have not correlated the two variables well. These studies include a well developed series of investigations into the impaired of B- and T-cells to undergo mitogen-stimulated proliferation in aged humans and mice and analyses of thymic hormone, growth factor, and cytokine alterations resulting in decreased immune function associated with aging. Cytokine secretion and cellular responses to those cytokines are clearly interrelated.

Although the total number of T-cells in circulation does not increase as a function of aging in humans[21,41,42], an apparent shift in the ratio of T4 and T8 lymphocytes exists, such that increased numbers of T4 cells compare with decreased numbers of T8 cells in the elderly.[19,20] Although T-cells from older humans continue to interact with mitogens like conconavalin A and phytohemagglutinin and do not exhibit a decline in peripheral blood T-cells, T-cells populations from aging humans contain fewer cells exhibiting ^{3}H-thymidine incorporation after mitogen stimulation than do T-cell populations from younger humans.[26,43] Up to 50% fewer of the mitogen-stimulated T-cells in peripheral blood of aged humans initiate DNA synthesis associated with blastogenesis compared with T-cells of young humans.[26] Mitogen-activated T-cells from older subjects exhibit the same cycle

time, but do not go through as many cycles of DNA synthesis and cell division as do the cells from younger subjects.[26]

Age-related decreases in B-cells or their functions are not as clearly delineated. Although older subjects' capacity to produce bone-marrow-derived B-cells does not seem to decline, the ability of the individual splenocyte precursor cells to respond to antigenic stimulation may decline by up to 50% with increased age. Zharhary and Klinman[26,43] reported a normal rate and extent of cell division and a normal amount of antibody secretion per cell in B-cells that responded to antigenic stimulation, but that the spleens from older mice produced a reduced frequency of cells responsive to antigens.

Cellular reception of a series of antigen- and lymphokine-delivered signals initiates virtually all cellular and humoral responses from the immune system. Apparently interleukin 1 is the initial mononuclear phagocyte lymphokine signal induced by lectin, antigen, microbial, and inflammatory agent exposure.[44] Even though stimulated peritoneal macrophage secretion of IL-1 does not appear to decrease with age in rats[45], macrophage secretion of IL-1 from mice does decrease as a function of age.

CONCLUSION

This investigation examined the mitogen-stimulated initiation of blastogenesis in splenocytes, measured by ^{3}H-thymidine incorporation, and has correlated the data with the rates of survival of ad libitum vs dietary restricted rats and mice. This preliminary data shows that male and female rats and mice exhibit and increased lifespan as a function of dietary restriction, and that the increased lifespan appears to be correlated with the capacity of the splenocytes from dietary restricted animals to respond to antigenic stimulation.

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