CHARACTERIZATION OF THE IMMUNE SYSTEM OF THE HISPID COTTON RAT (SIGMODON HISPIDUS) IN RESPONSE TO DIETARY PROTEIN, TEMPORAL CHANGES AND THE EFFICACY OF NYLON WOOL SEPARATION OF SPLENOCYTES

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SPLENOCYTE SUBPOPULATIONS OF WEANLING COTTON RATS (*SIGMODON HISPIDUS*) ARE INFLUENCED BY MODERATE PROTEIN INTAKE

ABSTRACT

Cotton rat (*Sigmodon hispidus*) populations frequently exhibit extreme numerical fluctuations in abundance, which have been attributed to changes in food quality and its possible affect on juvenile survival. Weanling cotton rats fed low protein diets have been shown to experience altered immune system function. We expanded these earlier studies to include investigations on the effects of moderate levels of dietary protein restriction on splenocyte subpopulations of weanling cotton rats and to determine the influences of onset of protein restriction at an earlier age. Weanling cotton rats (14 days old) were fed three isocaloric diets containing either 4, 8, or 16% protein for 24 days. We phenotyped splenocyte subpopulations into one of eight categories by dual-staining with a panel of surface markers for T-cells (peanut agglutinin, PNA; soybean agglutinin, SBA and *Helix pomatia* agglutinin, HP) and B-cells (rabbit-anti-rat immunoglobulin-G, BS). Total numbers of all splenocyte subpopulations examined were lower in weanling cotton rats subjected to moderate (8%) or severe (4%) protein restriction; B-cells were more sensitive to protein restriction than T-cells. Prevalence (% of total

splenocytes) of PNA^-/BS^+ splenocytes was reduced, while PNA^+/BS^- and SBA^-/HP^+ prevalence was elevated with decreased protein intake. Multivariate statistical analysis of immune parameters revealed that cellular yields (i.e., peripheral blood lymphocytes and splenocyte subpopulations) and subcellular components of the immune system (i.e., hemolytic complement activity and mean corpuscular volume) most accurately described the degree of protein restriction and suggested an overall change in immunocompetence as a result of either moderate or severe protein restriction.

INTRODUCTION

Numerous studies have documented a strong relationship between offspring survival and population density for several small-mammalian species (Lidicker, 1973; Boonstra, 1977; Burns, 1981). Alterations in juvenile survival in a changing environment may often be the single most important factor limiting reproductive success and population growth (Krebs and Meyers, 1974; Loudon, 1985). Unfortunately, our understanding of the physiological mechanisms for such environmentally-induced alterations is poor. Changes in immunocompetence have been implicated, either directly or indirectly, as one such mechanism causing altered survival in small mammal populations (McDonald et al., 1981; Mihok et al., 1985; Dobrowolska and Adamczewska-Andrzejewska, 1991; Lochmiller et al., 1994).

Among the many environmental factors capable of influencing juvenile survival in herbivore populations, White (1978) has postulated that the quality of protein food resources in the habitat is one of the most influential. Protein malnutrition has been shown to impair development of immune system function in laboratory rodents (Kenney et al., 1968; Bises et al., 1987; Woodward and Miller, 1991). Recently, elevated delayed-type hypersensitivity (DTH) responses and reduced hemolytic complement activity have been documented in weanling cotton rats (Sigmodon hispidus) subjected to severe, protein restriction (Vestey et al., 1993). Additionally, Lochmiller et al. (1993) showed that lymphoproliferative responses of moderately, protein-calorie restricted cotton rats were normal or elevated, but the reverse was observed for severely, protein-calorie restricted animals. These previous investigations have suggested that protein-induced immune alterations in cotton rats may be the result of numerical changes in specific splenocyte subpopulations. If true, in situ monitoring of immunocompetence in wild individuals could be greatly simplified. Our primary objective in this study was to examine if protein intake during early weanling development results in qualitative and quantitative changes in splenocyte subpopulations of the cotton rat. Unlike previous studies, we were particularly interested in documenting responses to both moderate and severe levels of protein restriction in the diet, and determining the

influences of onset of protein restriction at an earlier age.

MATERIALS AND METHODS

Experimental design.--Cotton rats used in this study were obtained from the National Institutes of Health (28-30 generations in captivity). Adult females were bred and maintained on a nutritionally complete ration for laboratory rodents (Purina, St. Louis, MO) through gestation and lactation. Offspring (n = 39) from 9 litters were weaned (14 days old) and randomly assigned to one of three isocaloric diets containing either 4, 8, or 16% protein (United States Biochemical, Cleveland, OH), which were provided ad libitum over a 24-day feeding trial. Weanling cotton rats were paired by gender and housed in polypropylene boxes containing an elevated wire-mesh floor underlayed with wood shavings. Following 3 mortalities in the 4% protein group, 1 in each of the 8 and 16% protein groups, sample sizes were 10, 12, and 12, respectively. Causes of mortalities were unknown; a Chi-squared contingency test (Koopmans, 1981) indicated no difference ($P < 0.05$) in mortality rates among diet groups. Body mass was recorded initially and at 6-day intervals. Daily food intake (g/g body mass) was determined every three days by dividing total intake per cage by the total average body mass during the respective interval.

Morphology and blood analysis.--Upon termination of the trial, animals were anesthetized with an intramuscular injection of ketamine hydrochloride (Aveco Co. Inc., Fort Dodge, IA, 50 mg/kg body mass). Morphological characteristics of animals were recorded, including body mass (g), lean body mass (measured by total body electrical conductivity; Model SA-2, EM Scan Inc., Springfield, IL), and total body length (mm). Blood was collected via the retro-orbital plexus for hematology and serum chemistry. Anesthetized animals were euthanized by cervical dislocation, and mass of the spleen, thymus, popliteal lymph node (paired), Peyer's patches (see Lochmiller et al., 1992), and liver determined to the nearest 0.1 mg. Counts of leukocytes (WBC, $x10^3/mm^3$), erythrocytes (RBC, $x10^6/mm^3$), and platelets (PLT, $x10^6/mm^3$), yields ($x10^6/mm^3$) of mononuclear cells from spleen, thymus, and popliteal lymph node, and values for hemoglobin (HGB, g/dl), hematocrit (HCT, %), mean corpuscular volume (MCV, μm^3), mean corpuscular hemoglobin (MCH, pg), and mean corpuscular hemoglobin concentration (MCHC, %) were determined using a Serono 9000 cell counter (Serono-Baker Diagnostics, Allentown, PA). Differential counts of white blood cells were performed by classifying 100 cells under oil immersion, light microscopy.

Serum was obtained by centrifugation and stored at -70°C for later analysis of hemolytic complement activity and total serum protein concentration (Biuret method using a

human serum standard; Kingsley, 1942). Hemolytic complement activity was determined using a slight modification of the assay described by DeWaal et al. (1988). Briefly, two-fold serial dilutions of cotton rat serum (40 µl) were made in a 96-well, round-bottom microtiter plate. Twenty-five µl of washed sheep red blood cells (0.6% SRBC in veronal buffer, Colorado Serum Co., Denver, CO) and 25 µl of a 1/80 dilution (in veronal buffer) of rabbit-anti-SRBC antibody (Rash-Nordic Immunological Laboratories, Capistrano Beach, CA) were added to each sample well. Plates were lightly vortexed, incubated for 1.5 h at 37°C, centrifuged for 5 min at 275 x g, and absorbance (414 nm) of supernatant measured on a Titertek Multiscan II (ICN Biomedicals Inc., Costa Mesa, CA). Controls were used to generate a standard curve and hemolytic complement activity was expressed as CH_{50} units/ml serum, where 1 CH_{50} unit equaled the amount of complement giving lysis of 50% of the SRBC (Kabat and Mayer, 1961).

Phenotyping splenocytes.--Due to a lack of commercially available monoclonal antibodies recognizing lymphocyte surface antigens of cotton rats, we selected a suite of non-specific lectins with an affinity for T-lymphocytes and an antibody specific for immunoglobulin (Ig-G; heavy and light chain) in laboratory rats. Splenocytes were dual-stained with combinations of these surface markers as described below.

The spleen was aseptically removed, gently disintegrated into a single cell suspension using a glass-on-glass tissue homogenizer containing 5 ml of Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma, St. Louis, MO) and placed on ice. Cells were decanted and washed once in RPMI-1640 and resulting pellet resuspended in 10 ml of tris-buffered ammonium chloride (0.83%; pH 7.2) to lyse erythrocytes. Following two additional washes in RPMI-1640, cells were resuspended in 2.5 ml of RPMI-1640 and counted (Serono 9000). Splenocytes were then adjusted to $10x10^6$ cells/ml in phosphate buffered saline (pH 7.4). An aliquot of $6x10^6$ cells was treated with 10 µl of neuraminidase type VI (0.01 U, Sigma, cat # N-3001) to expose surface receptors, and 2 ml of tris-buffered Hank's balanced salt solution (TH; Sigma) supplemented with 0.5% bovine serum albumin and 0.02% sodium azide (TH-S; pH 7.45) and incubated for 45 min at 37°C. After cells were washed twice in TH-S, an aliquot of $2x10^6$ cells was treated with 100 µl of tetramethylrhodamine isothiocyanate (TRITC) conjugated-*Helix pomatia* agglutinin (HP) (TRITC-HP, 100 µg/ml, Sigma, cat # L-1261), incubated for 30 min at 4°C, and washed twice in TH-S. Cells were then treated with 100 µl of fluorescein isothiocyanate (FITC) conjugated-soybean agglutinin (SBA) (FITC-SBA; 100 µg/ml, Sigma, cat # L-1020) and washed twice in TH-S.

To a separate aliquot of $2x10^6$ splenocytes, 100 µl of TRITC-peanut agglutinin (PNA) (TRITC-PNA, 250 µg/ml, Sigma,

cat # L-3766) and 40 µl of FITC-rabbit-anti-rat immunoglobulin-G (BS) (FITC-BS, 1:10 in distilled water, Cappel Research Products, Durham, NC, cat # 55746) were added simultaneously, and the mixture incubated for 30 min at 4°C followed by two washes in TH-S.

Dual-stained cells were mounted on a microscope slide in polyvinyl alcohol and phenotyped using epifluorescent microscopy (1000X magnification, Olympus BH-2). Each cell was first located and enumerated under bright field illumination then examined with the FITC and TRITC exciter filters in position. The first 100 cells were characterized as SBA^+/HP^-, SBA^+/HP^+, SBA^-/HP^+ or SBA^-/HP^-; or as PNA^+/BS^+, PNA^+/BS^-, PNA^-/BS^+ or PNA^-/BS^- (+ = positive for the stain). For each subpopulation, results were expressed as prevalence (% of total splenocytes), total yield (splenocytes $x10^6$), relative splenic yield (splenocytes $x10^5$/mg spleen), and relative total yield (splenocytes $x10^6$/g body mass).

Statistical analysis.--Weanling cotton rats were separated into 13 "litter blocks" consisting of three siblings of the same gender which were randomly assigned to either a 4, 8, or 16% protein diet. Any particular litter (n = 9) may have contributed 1 or 2 "litter blocks" to the experiment depending on litter size. A randomized block design (PROC ANOVA; SAS Institute Inc., 1982) was then used to examine differences in measures of immunocompetence and morphology among protein diet groups, with "litter block" (block effect) and diet as main effects. When data on an

animal was missing, the analysis was conducted using the analysis of covariance procedure (Steel and Torrie, 1980, p. 426). The test for least significant difference (LSD) was used to indicate a significant ($P < 0.05$) difference in means (Steel and Torrie, 1980); gender of offspring had no significant influence on immune parameters. Levene's test was used to test for homogeneity of variance (Snedecor and Cochran, 1980); all significant ($\underline{P} < 0.05$) variables were transformed prior to further statistical analysis.

Given the complex, interrelated nature of the immune system, the impact of protein intake on overall immune system development was examined using multivariate statistical analysis. Stepwise discriminant analysis (PROC STEPDISC; SAS Institute Inc., 1982) was used to produce a reduced set of discriminator parameters that would provide the best separation of individuals among the three diet groups. Discriminant function analysis (PROC DISCRIM; SAS Institute Inc., 1982) using a jackknife procedure was used to determine classification accuracy among diets. Canonical discriminant analysis (PROC CANDISC; SAS, Institute Inc., 1982) was used to assess relationships between immune parameters and the three diet groups in reduced dimensions.

RESULTS

Intake and morphology.--Initial body mass did not differ ($\underline{P} = 0.228$) among diet groups, with an overall mean of 18.6 ± 0.5 (SE) g, however, dietary protein did affect ($\underline{P}$

< 0.001) body mass on days 6, 12, 18, and 24 (Fig. 1). Body mass only increased by 0.08% from days 6 - 24 for weanlings fed 4% protein, whereas during the same time period, body mass increased by 33 and 46%, respectively, for weanlings fed 8% and 16% protein diets (Fig. 1). Dietary effects on development were also reflected ($P < 0.001$) in lean body mass and absolute liver mass, which increased with increased protein intake, however, relative liver mass was similar among diet groups, with an overall mean of 64.6 ± 0.8 mg/g body mass (Table 1).

Average daily food intake was similar among diet groups for days 1 - 6 and 6 - 12 ($P \geq 0.134$), with overall means of 0.176 ± 0.003 and 0.177 ± 0.003 g/g body mass, respectively, but there were attempts by weanlings fed 4% protein to compensate for protein restriction with increased ($P < 0.008$) food intake by days 12 - 18 and 18 - 24. Relative food intake remained similar between the 8 and 16% protein groups for days 12 - 18 and 18 - 24, with overall means of 0.163 ± 0.004 and 0.151 ± 0.004 g/g body mass, respectively (Fig. 2).

Hematology.--Red blood cell counts, MCH, and MCHC did not differ ($P \geq 0.075$) among diet groups, with overall means of 6.1 ± 0.06 $x10^6/mm^3$, 18.1 ± 0.6 pg, and 31.6 ± 0.4%, respectively (Table 1). Hemoglobin and HCT values were affected ($P \leq 0.033$) by dietary protein with weanlings fed 4% protein averaging 13-15% less HGB and HCT than those fed 8 or 16% protein (Table 1). Mean corpuscular volume

differed ($P = 0.025$) among all diet groups with values increasing as protein quality increased in the diet (Table 1). The opposite trend was observed for concentrations of platelets ($P = 0.024$). Relative abundances of lymphocytes, neutrophils, eosinophils, and monocytes in blood were similar ($P \geq 0.123$) among diet groups (Table 1), with overall means of 72.5 ± 1.6, 25.1 ± 1.2, 0.2 ± 0.2, and 2.1 ± 0.7%, respectively. However, WBC levels were sensitive ($P = 0.009$) to dietary protein; WBC values for weanlings fed 4% protein were 48 and 36% lower respectively, compared to those fed 8 and 16% protein (Table 1). These differences were largely attributed to changes ($P = 0.001$) in absolute lymphocyte numbers (Table 1). Absolute numbers of neutrophils, eosinophils, and monocytes were unaffected by dietary protein ($P \geq 0.353$), with overall means of 2.4 ± 0.3, 0.02 ± 0.02, and 0.2 ± 0.06 $x10^3/mm^3$, respectively.

Lymphoid organs.--Development of all lymphoid organs examined was suppressed in weanlings fed 4 and 8% protein diets. Spleen, thymus, popliteal node, and Peyer's patch masses were all influenced ($P < 0.001$) by dietary protein and were half to one-fourth the size in weanlings fed 4 or 8% protein compared to those fed 16% protein (Figs. 3, 4, 5, and Table 1). Splenic index (mg/g body mass), and absolute and relative (cells/mg spleen) splenocyte yields were all sensitive ($P < 0.001$) to protein restriction; values were smaller with decreased protein intake (Fig. 3). Protein restriction influenced ($P \leq 0.005$) thymic and Peyer's patch

indices (mg/g body mass), and relative thymocyte yield (cells/mg thymus), with 29 - 55% lower values for weanlings fed 4% protein compared to those fed 8 or 16% protein (Fig. 4 and Table 1). We also observed significant ($P < 0.002$) changes in both absolute thymocyte yield and mononuclear cell yield from the popliteal nodes, with yields 54 - 88% lower for weanlings fed 4 or 8% compared to 16% protein (Figs. 4 and 5). Relative values indicated that differences in popliteal lymph node size were merely a reflection of proportional declines in body mass.

Serum chemistry.--Both total serum protein concentration ($P = 0.01$), and hemolytic complement activity ($P = 0.001$) were significantly influenced by protein content of the diets. Total serum protein concentrations were at least 1.2-fold greater in weanlings fed 16% protein and hemolytic complement activity was about 3-fold greater in the 16% compared to the 4% protein group (Figs. 6 and 7).

Splenocyte phenotypes.--Dietary protein influenced prevalence of only two splenocyte subpopulations examined. Prevalence of SBA^-/HP^+ splenocytes in weanlings fed 4% protein averaged 23% higher ($P = 0.039$) than those fed 16% protein (Fig. 8). Protein restriction influenced ($P < 0.005$) prevalence of PNA^-/BS^+ splenocytes, with values 54 and 60% lower, respectively, in weanlings fed either 4 or 8% protein compared to those fed 16% protein (Fig. 9). However, prevalence of SBA^+/HP^-, SBA^+/HP^+, SBA^-/HP^-, PNA^+/BS^+, PNA^-/BS^-, and PNA^+/BS^- splenocytes among diet

groups was unaffected ($P \geq 0.061$) by dietary protein, with overall means of 2.6 ± 0.4, 14.9 ± 0.7, 42.7 ± 1.2, 6.0 ± 0.6, 41.6 ± 0.1, and 46.8 ± 2.6%, respectively (Figs. 8 and 9).

Total yields of all eight splenocyte subpopulations examined were influenced ($P \leq 0.002$) by protein restriction, with yields averaging 61 - 97% lower in weanlings fed either 4 or 8% protein compared to those fed 16% protein (Figs. 8 and 9). Subpopulations also differed ($P \leq 0.026$) in response to dietary protein when expressed on a relative basis to spleen and body mass. Relative splenic yield of PNA^-/BS^+ splenocytes, and relative total yields of PNA^-/BS^+, PNA^-/BS^-, PNA^+/BS^+, SBA^-/HP^+, and SBA^+/HP^- splenocytes averaged 43 - 90% lower in weanlings fed 4 or 8% compared to 16% protein (Figs. 8 and 9). Relative splenic yields of PNA^-/BS^-, PNA^+/BS^-, and SBA^+/HP^+ splenocytes averaged 47 - 67% lower in weanlings fed 4% than those fed 8 or 16% protein (Figs. 8 and 9). Relative splenic yields of PNA^+/BS^+, SBA^+/HP^- splenocytes, and relative total yield of SBA^+/HP^+ splenocytes were remarkably sensitive to dietary protein, with yields 70 - 83% lower in the 4% compared to the 16% diet group (Figs. 8 and 9). Protein restriction strongly influenced the relative splenic yield of SBA^-/HP^- splenocytes, and relative total yield of PNA^+/BS^- splenocytes, indicated by 22 - 73% lower yields in the 4 or 8% groups compared to the 16% protein group (Figs. 8 and 9).

Relative total yields of SBA^-/HP^- splenocytes were higher with decreased protein intake (Fig. 8).

Multivariate analysis.--The immune system is highly complex and composed of multiple interacting components. Therefore, to adequately document how dietary protein influences the system as a whole we used multivariate statistical analysis of hematology, hemolytic complement activity, lymphoid morphology and abundance of splenocyte subpopulations as measures of immune development. Stepwise discriminant analysis selected hemolytic complement activity (rank transformed), relative thymocyte yield, blood lymphocyte count (log transformed), and MCV (rank transformed) as variables that contributed in the separation of diet groups. Multivariate analysis of variance indicated significant differences among all diet group means (Wilks' λ =0.044; df = 2, 21; $P < 0.001$). Discriminant function analysis classified animals 100% (29/29) correctly by diet. Within covariance matrices were heterogeneous, therefore, canonical variate analysis was not optimal at discriminating, but could be used with discretion, as suggested by Neff and Marcus (1980). Along the first canonical variate, 94% of the variation among diet groups was accounted for by a combined influence of hemolytic complement activity (59%), MCV (17%), relative thymocyte yield (11%), and blood lymphocyte count (6%). Along the second canonical variate, blood lymphocyte count (2.9%), MCV (1.7%), relative thymocyte yield (0.9%), and hemolytic

complement activity (0.8%) accounted for 6% of the variation among diet groups (Fig. 11).

We were also interested whether the influence of protein restriction on immunocompetence could be detected simply by analyzing quantitative and qualitative changes in splenocyte subpopulations. Stepwise discriminant analysis, considering only prevalence of splenocyte subpopulations, selected PNA^-/BS^+ and SBA^+/HP^- splenocytes as parameters that contributed in the separation of diet groups (Wilks' $\lambda = 0.593$; df = 2, 29; $\underline{P} = 0.004$). Discriminant function analysis correctly classified 75% (9/12), 42% (5/12) and 56% (5/9) of the animals to the 16, 8, and 4% protein diet groups, respectively. Along the first canonical variate, 98% of the variation among diet groups was accounted for by a combined influence of the prevalence of PNA^-/BS^+ (60%) and SBA^+/HP^- (38%) splenocytes; whereas, the prevalence of SBA^+/HP^- (1.3%) and PNA^-/BS^+ (0.6%) splenocytes accounted for 2% of the variation among diet groups along the second canonical variate (Fig. 11).

Stepwise discriminant analysis was also used to examine overall changes in relative splenic yields of splenocyte subpopulations. The procedure selected SBA^-/HP^-, PNA^-/BS^+ (rank transformed) and SBA^+/HP^+ (rank transformed) as variables that contributed to the separation of diet groups (Wilks' $\lambda = 0.353$, df = 2, 28, $\underline{P} < 0.001$). Discriminant function analysis correctly classified 75% (9/12), 50% (6/12), and 89% (8/9) of the animals into 16, 8, and 4%

protein groups, respectively. Along the first canonical variate, 92% of the variability among diet groups was accounted for by a combined effect of SBA^-/HP^- (39%), PNA^-/BS^+ (27%), and SBA^+/HP^+ (26%) splenocytes; along the second canonical variate, 8% of the variation among diet groups was accounted for by a combined influence of PNA^-/BS^+ (4.5%), SBA^-/HP^- (1.9%), and SBA^+/HP^+ (1.7%) splenocytes (Fig. 11).

DISCUSSION

The moderate-restricted diet (8% protein) was formulated to provide recommended maintenance (8% protein) requirements for laboratory voles (National Research Council, 1978), which have feeding habits similar to cotton rats. Our high quality (16% protein) diet was formulated to provide protein in excess of weanling growth requirements of laboratory voles (National Research Council, 1978). Weanling cotton rats from all dietary groups increased in body mass to some degree and no severe mass loss occurred on any of the diets. Total serum protein reflects the nutritive state of an animal (Coles, 1967), and in weanling cotton rats indicated that those fed a moderate (8% protein) or low (4% protein) quality diet were protein restricted.

Both moderate and low quality protein diets adversely influenced immune organ development of weanling cotton rats. A moderate quality protein diet resulted in impaired development of the spleen, thymus, Peyer's patches, and popliteal lymph nodes. For the spleen, impairment was

greater than that due to suppressed body growth, similar to our previous study (Vestey et al., 1993). Vestey et al. (1993) found that weanling (18 days old) cotton rats that were severely protein restricted had suppressed development of the thymus, which was attributed to lower body mass. In contrast, we observed that thymic suppression was greater than that attributed to suppressed body growth. Differences between studies in sensitivity of the thymus to protein restriction probably reflect differences in age of weaning and when diets were administered. Offspring in our study were subjected to protein restriction when 14 days old compared to 18 days old in Vestey et al. (1993). Spleen and thymus gland development appear to be remarkably more sensitive to protein restriction at 14 days old compared to 18 days old indicating an important period when splenic and thymic development are extremely sensitive to dietary protein. Protein restriction also affected mononuclear cellularity in the spleen, thymus, and popliteal lymph nodes, but the spleen was most sensitive. Cellularity of the spleen was suppressed by even moderate levels of protein restriction, and suppression was greater than that attributed to organ size.

Hemolytic complement activity is a nonspecific antibody-induced cytolytic defense mechanism against foreign cells. Hemolytic complement activity in weanling cotton rats was extremely sensitive to dietary protein, indicated by suppressed activity in both moderately and severely

protein restricted weanlings. Conversely, Vestey et al. (1993) found severe protein restriction did not influence hemolytic complement activity in weanling cotton rats, but did reduce activity in juveniles. Weanlings in our study were subjected to dietary protein restriction at an earlier age than those of Vestey et al. (1993) indicating hemolytic complement activity is extremely sensitive to early onset of protein restriction. This sensitivity may be because individual components of the hemolytic complement system need adequate protein for optimal development; possibly during early weanling development these components are not priority sites of maintenance. Additionally, Chandra and Newberne (1977) suggested protein malnutrition during early stages of development can frequently lead to the damage of complement synthesizing tissues, such as the liver and spleen (Ruddy et al., 1972), development of which, was severely impaired in weanling cotton rats fed either a moderately and severely protein-restricted diet.

A review of the literature revealed that sensitivity of specific lymphocyte subpopulations to dietary restriction is highly variable, depending in part upon the animal species and nature of nutritional insult. For example, protein malnutrition in laboratory rats has been shown to effect T-cells more profoundly than B-cells (Slobodianik et al., 1984; Bises et al., 1987), but Woodward and Miller (1991) reported greater reductions in B-cells than T-cells in laboratory mice. As a result, we were not completely sure

just how weanling cotton rat lymphocyte subpopulations would respond to protein restriction. The surface markers we employed to identify unique subpopulations of splenocytes should not be used as unequivocal markers of T- and B-cells. However, given their general binding characteristics for selected cell types in other rodent species, we felt confident that a general idea of how T- and B-cells were responding to varying levels of dietary protein could be gleaned.

The PNA label has been demonstrated to bind peripheral lymphocytes (usually immature) that are either T-cells or "non-T, non-B" cells (Reisner et al., 1976; Uni and Heller, 1991). The BS label binds specifically to Ig-G on the surface of B-cells, however, BS may bind to a small number of T-cells through Fc surface receptors (Reynolds and Ortaldo, 1990). Based on these criteria, we suggest that splenocytes possessing PNA^+/BS^- and PNA^+/BS^+ phenotypes are subpopulations of T-cells, PNA^-/BS^+ represent primarily B-cells, and PNA^-/BS^- phenotypes represent a subpopulation of non-T non-B cells. However, 40% of the splenocytes in weanling cotton rats were PNA^-/BS^-, indicating a substantial number of splenic T- and B-cells are not accounted for with our panel of surface markers. We suspect a considerable number of them are B-cells due to the low percentage (10%) of PNA^-/BS^+ splenocytes. The HP and SBA labels bind predominantly to T-cells in a variety of mammalian species (Hellstrom et al., 1976, Swanborg et al., 1977, Haller et

al., 1978, Morein et al., 1979). Assuming similar binding characteristics in cotton rats, we suggest that splenocytes possessing SBA^+/HP^-, SBA^-/HP^+ and SBA^+/HP^+ phenotypes are subpopulations of T-cells and SBA^-/HP^- phenotypes are predominately B-cells.

Numerically, all splenocyte subpopulations (including T- and B-cells) of weanling cotton rats were affected by protein restriction, even at moderate levels. Although they were not a large proportion of the total splenocyte pool, B-cells appeared to be remarkably sensitive to protein restriction. We observed a 21-fold reduction in PNA^-/BS^+ splenocytes compared to a 9-fold reduction in PNA^+/BS^- splenocytes in cotton rats fed 4% compared to 16% protein. Multivariate analysis of splenocyte subpopulations provided further evidence that in weanling cotton rats, B-cells PNA^-/BS^+, SBA^-/HP^-) are influenced the greatest by protein restriction.

The exact mechanisms whereby protein restriction influences lymphocyte numbers in weanling cotton rats is unknown. However, protein restriction in laboratory rats has been reported to decrease secretion of lymphokines necessary for optimal T- and B-cell proliferation (Coffman et al., 1990; Ruscetti, 1990; Mengheri et al., 1992). Decreased secretion of lymphokines may reflect limited protein necessary for production of lymphokines. Protein restriction has also been reported to result in elevated levels of immunoregulatory hormones, resulting in decreased

T- and B-cell proliferation (Betz and Fox, 1991; Redmond et al., 1991). Observed numerical alterations in splenocyte subpopulations may be related to altered (increased or decreased for a particular subpopulation) production of glycoprotein receptors, hence alter binding of surface markers.

We have demonstrated the importance of analyzing immunocompetence as a multivariate system when assessing how protein restriction influences development. Our observations indicate that lymphoid organs of weanling cotton rats are notably affected by dietary protein. However, cellular yields (i.e., peripheral blood lymphocytes and splenocyte subpopulations) and subcellular components of the immune system (i.e., hemolytic complement activity and MCV) most accurately describe the degree of protein restriction and the effect of protein restriction on overall immunocompetence of weanling cotton rats. Several investigators (i.e., Dobrowolska and Adamczewska-Andrzejewska, 1991) have suggested a relationship between altered immunocompetence and population dynamics. Evidence in weanling cotton rats of quantitative and qualitative changes in splenocyte subpopulations, especially B-cells, and remarkable alterations in nonspecific immunity in response to both moderate and severe protein restriction may lend insight to the physiological mechanism by which environment influences survival and the dynamics of small mammal populations.

LITERATURE CITED

Betz, M., and B. S. Fox. 1991. Prostaglandin E_2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. The Journal of Immunology, 146:108-113.

Bises, G., E. Mengheri, and S. Gaetani. 1987. T-lymphocyte subsets in zinc deficient and protein malnourished rats. Nutrition Reports International, 36:1371-1378.

Boonstra, R. 1977. Predation on Microtus townsendii populations: impact and vulnerability. Canadian Journal of Zoology, 55:1631-1643.

Burns, G. R. 1981. Population dynamics of island populations of subarctic Clethrionomys rutilus. Canadian Journal of Zoology, 59:2115-2122.

Chandra, R. K., and P. M. Newberne. 1977. Nutrition, immunity, and infection. Plenum Press, New York, 246 pp.

Coffman, R. L., A. O'Garra, and D. J. Volkman. 1990. Interleukins and interferons acting on B-lymphocytes. Pp. 88-103, in Immunophysiology (J. J. Oppenheim and E. M. Shevach, eds.). Oxford University Press, New York, 424 pp.

Coles, E. H. 1967. Veterinary clinical pathology. W. B. Saunders, Philadelphia, Pennsylvania, 455 pp.

DeWaal, R. M., G. Schrijver, M. J. Bogman, K. J. Assmann, and R. A. Koene. 1988. An improved sensitive simple microassay of mouse complement. Journal of Immunological Methods, 108:213-221.

Dobrowolska, A., and K. A. Adamczewska-Andrzejewska. 1991. Seasonal and long-term changes in serum gamma-globulin levels in comparing the physiology and population density of the common vole, Microtus arvalis Pall. 1779. Journal of Interdisciplinary Cycle Research, 22:1-19.

Haller, O., M. Gildlund, U. Hellstrom, S. Hammarstrom, and H. Wigzell. 1978. A new surface marker on mice natural killer cells: receptors for Helix pomatia A hemagglutinin. European Journal of Immunology, 8:765-771.

Hellstrom, U., M. L. Dillner, S. Hammarstrom, and P. Perlmann. 1976. The interaction of nonmitogenic and mitogenic lectins with T lymphocytes: association of cellular receptor sites. Scandinavian Journal of Immunology, 5:45-54.

Kabat, E. A., and M. M. Mayer. 1961. Experimental immunochemistry. Second edition. Charles C. Thomas, Springfield, Illinois, 567 pp.

Kenney, M. A., C. E. Roderick, L. Arnich, and F. Piedad. 1968. Effect of protein deficiency on the spleen and antibody formation in rats. Journal of Nutrition, 95:173-178.

Kingsley, G. R. 1942. The direct biuret method for determination of serum proteins as applied to photoelectric and visual colorimetry. Journal of Laboratory and Clinical Medicine, 27:840-845.

Koopmans, L. H. 1981. An introduction to contemporary statistics. Duxbury Press, Boston, Massachusetts, 599 pp.

Krebs, C. J., and J. Meyers. 1974. Population cycles in small mammals. Advances in Ecological Research, 8:267-399.

Lidicker, W. Z. 1973. Regulation of numbers in an island population of California vole, a problem in community dynamics. Ecological Monographs, 43:271-302.

Lochmiller, R. L., M. R. Vestey, and D. Nash. 1992. Gut associated lymphoid tissue in the cotton rat (Sigmodon hispidus) and its response to protein restriction. Journal of Wildlife Diseases, 28:1-9.

Lochmiller, R. L., M. R. Vestey, and S. T. McMurry. 1993. Selected immune responses of adult cotton rats (Sigmodon hispidus) to dietary restriction. Comparative Biochemistry and Physiology, 104:593-599.

_____. 1994. Temporal variation in humoral and cell-mediated immune response in a Sigmodon hispidus population. Ecology, 75:236-245.

Loudon, A. S. I. 1985. Lactation and neonatal survival of mammals. Symposium of the Zoological Society of London, 54:183-207.

McDonald, I. R., A. K. Lee, A. J. Bradley, and K. A. Than. 1981. Endocrine changes in dasyurid marsupials with differing mortality patterns. General and Comparative Endocrinology, 44:292-301.

Mengheri, E., F. Nobili, G. Crocchioni, and J. A. Lewis. 1992. Protein starvation impairs the ability of activated lymphocytes to produce interferon-γ. Journal of Interferon Research, 12:17-21.

Mihok, S., B. N. Turner, and S. L. Iverson. 1985. The characterization of vole population dynamics. Ecological Monographs, 55:399-420.

Morein, B., U. Hellstrom, L. G. Axelsson, C. Johansson, and S. Hammarstrom. 1979. Helix pomatia A hemagglutinin, A surface marker for bovine T-lymphocytes. Veterinary Immunology and Immunopathology, 1:27-36.

National Research Council. 1978. Nutrient requirements of domestic animals, number 10: Nutrient requirements of laboratory animals. Third edition. National Academy of Sciences, Washington D.C., 96 pp.

Neff, N. A., and L. F. Marcus. 1980. A survey of multivariate methods for systematics. American Museum of Natural History, New York, 243 pp.

Redmond, H. P., J. Shou, C. J. Kelly, S. Schreiber, E. Miller, P. Leon, and J. M. Daly. 1991. Immunosuppressive mechanisms in protein-calorie malnutrition. Surgery, 110:311-317.

Reisner, Y., M. Linker-Israeli, and N. Sharon. 1976. Separation of mouse thymocytes into two subpopulations by use of peanut agglutinin. Cellular Immunology, 25:124-129.

Reynolds, C. W., and J. R. Ortaldo. 1990. Nature and physiology of natural effector cells. Pp. 336-349, in Immunophysiology (J. J. Oppenheim and E. M. Shevach, eds.). Oxford University Press, New York, 424 pp.

Ruddy, S., I. Gigli, and K. F. Austen. 1972. The complement system of man. New England Journal of Medicine, 287:545-549.

Ruscetti, F. W. 1990. Interleukin-2. Pp. 46-66, in Immunophysiology (J. J. Oppenheim and E. M. Shevach, eds.). Oxford University Press, New York, 424 pp.

SAS Institute, Inc. 1982. SAS user's guide: Statistics, 1982 edition. SAS Institute Inc., Cary, North Carolina, 584 pp.

Slobodianik, N. H., R. C. Cosarinsky, S. H. Langini, M. E. Roux, M. E. Rio, and J. C. Sanhuja. 1984. Effect of severe protein deficiency on surface and intracellular markers of growing rats' lymphoid organs. Nutrition Reports International, 29:957-964.

Snedecor, G. W., and W. G. Cochran. 1980. Statistical methods. Seventh edition. Iowa State University Press, Ames, Iowa, 507 pp.

Steel, R. G. D., and J. H. Torrie. 1980. Principles and procedures of statistics: a biomedical approach. Second edition. McGraw-Hill Publishing Company, New York, 633 pp.

Swanborg, R. H., U. Hellstrom, H. Perlmann, S. Hammarstrom, and P. Perlmann. 1977. A new rat lymphocyte surface

marker: characterization and separation of cells with receptors for Helix pomatia hemagglutinin. Scandinavian Journal of Immunology, 6:235-239.

Uni, Z., and E. D. Heller. 1991. Peanut agglutinin as a marker of maturation and activation of chicken thymic-derived lymphocytes. Poultry Science, 70:1516-1520.

Vestey, M. R., S. T. McMurry, and R. L. Lochmiller. 1993. Influence of dietary protein on selected measures of humoral and cellular immunity in the cotton rat Sigmodon hispidus. Canadian Journal of Zoology, 71:579-586.

White, T. C. R. 1978. The importance of a relative shortage of food in animal ecology. Oecologia, 33:71-86.

Woodward, B. D., and R. G. Miller. 1991. Depression of the thymus-dependent immunity in wasting protein-energy malnutrition does not depend on an altered ratio of helper ($CD4^+$) to suppressor ($CD8^+$) T-cells or on a disproportionately large atrophy of the T-cell relative to the B-cell pool. American Journal of Clinical Nutrition, 53:1329-1335.

Table 1. Selected morphological and hematological values of weanling cotton rats fed isocaloric diets containing 4, 8, or 16% protein.

		4% (n = 10)		8% (n = 12)		16% (n = 12)	
Variable	Units	Mean	SE	Mean	SE	Mean	SE
Lean body tissue	g	26.7^{a}	3.5	38.3^{ab}	3.1	62.8^{b}	6.9
Liver	g	1.4^{a}	0.1	2.1^{b}	0.1	3.1^{c}	0.2
	mg/g body mass	64.0^{a}	6.5	66.4^{a}	2.4	63.5^{a}	1.6
Peyer's patches	mg	15.4^{a}	2.3	32.9^{b}	2.8	49.1^{c*}	5.3
	mg/g body mass	0.7^{a}	0.1	1.0^{b}	0.1	1.0^{b*}	0.1
WBC	$x10^{3}/mm^{3}$	6.2^{a}	0.7	11.9^{b**}	1.5	9.7^{b}	0.9
RBC	$x10^{6}/mm^{3}$	6.0^{a}	0.1	6.2^{a}	0.2	6.2^{a}	0.2
HGB	g/dL	10.0^{a}	0.5	11.5^{b}	0.2	11.7^{b}	0.5
HCT	%	32.5^{a}	0.6	35.7^{b}	0.8	36.9^{b}	1.5
MCV	μm^{3}	55.5^{a}	0.5	58.0^{b}	1.6	59.6^{c}	0.6
MCH	pg	16.7^{a}	0.8	18.7^{a}	0.7	19.0^{a}	0.2
MCHC	%	30.7^{a}	1.5	32.2^{a}	0.4	31.9^{a}	0.2
PLT	$x10^{3}/mm^{3}$	846.7^{a}	56.4	731.1^{a}	39.6	669.5^{b}	39.5
Lymphocytes	%	68.8^{a}	4.3	74.3^{a}	2.9	74.3^{a}	3.7
	$x10^{3}/mm^{3}$	4.1^{a}	0.4	8.7^{b}	0.9	7.1^{b}	0.7
Neutrophils	%	27.7^{a}	4.1	23.2^{a}	3.3	24.3^{a}	3.7
	$x10^{3}/mm^{3}$	1.9^{a}	0.3	2.9^{a}	0.7	2.5^{a}	0.6
Eosinophils	%	0.1^{a}	0.1	0^{a}	0	0.6^{a}	0.4
	$x10^{3}/mm^{3}$	0.01^{a}	0	0^{a}	0	0.06^{a}	0.04
Monocytes	%	3.5^{a}	1.5	2.0^{a}	0.7	0.8^{a}	0.2
	$x10^{3}/mm^{3}$	0.3^{a}	0.1	0.3^{b}	0.1	0.1^{b}	0.03

^{abc} Values with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

* n = 11; ** n = 9

Fig. 1.--Body mass for weanling cotton rats (n = 12) fed 4, 8, or 16% protein diets. Values represent means (± SE); bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

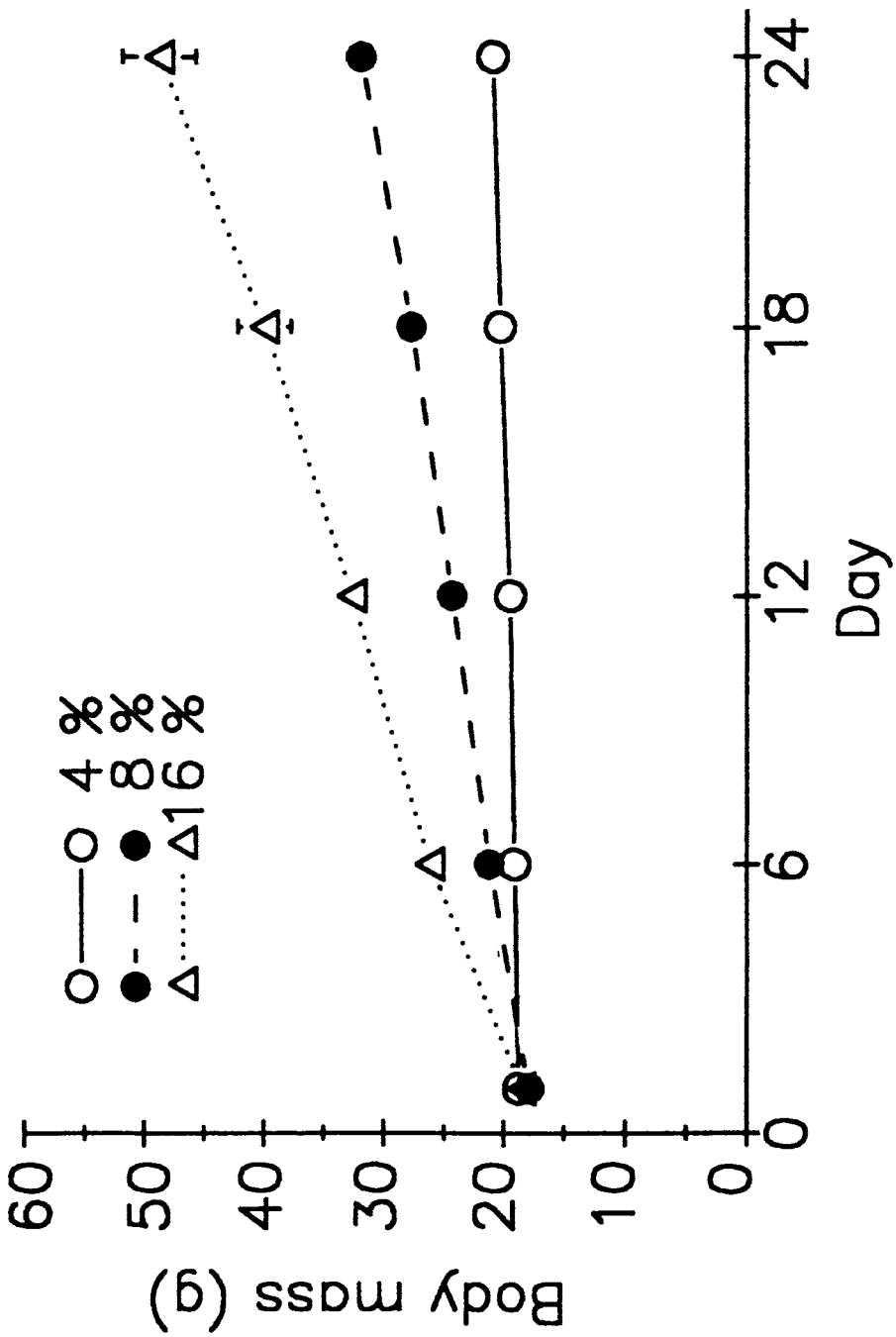

Body mass (g)
60
50
40
30
20
10
0
0
6
12
18
24
Day
4%
8%
16%

Fig. 2.--Daily food intake (g/g body mass) of weanling cotton rats fed 4 (n = 11), 8 (n = 12), or 16% (n = 12) protein diets. Values represent means (± SE); bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

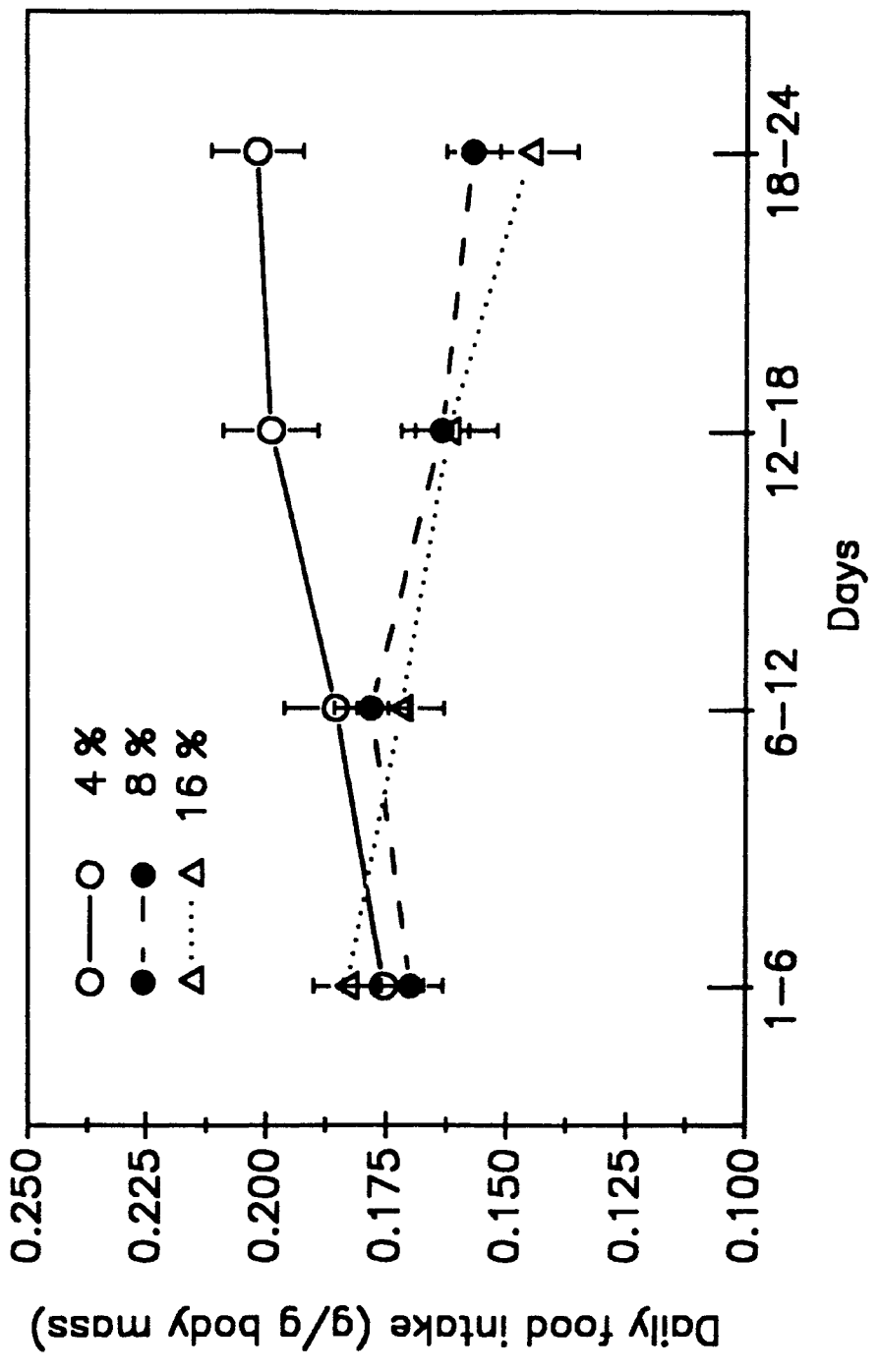

Daily food intake (g/g body mass)
0.250
0.225
0.200
0.175
0.150
0.125
0.100
1–6
6–12
12–18
18–24
Days
4 %
8 %
16 %

Fig. 3.--Absolute spleen mass (a), splenic index (b), total splenocyte yield (c), and relative splenocyte yield (d) for weanling cotton rats fed 4 (n = 10), 8 (n = 12), or 16% (n = 12) protein diets. Values represent means (± SE); bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

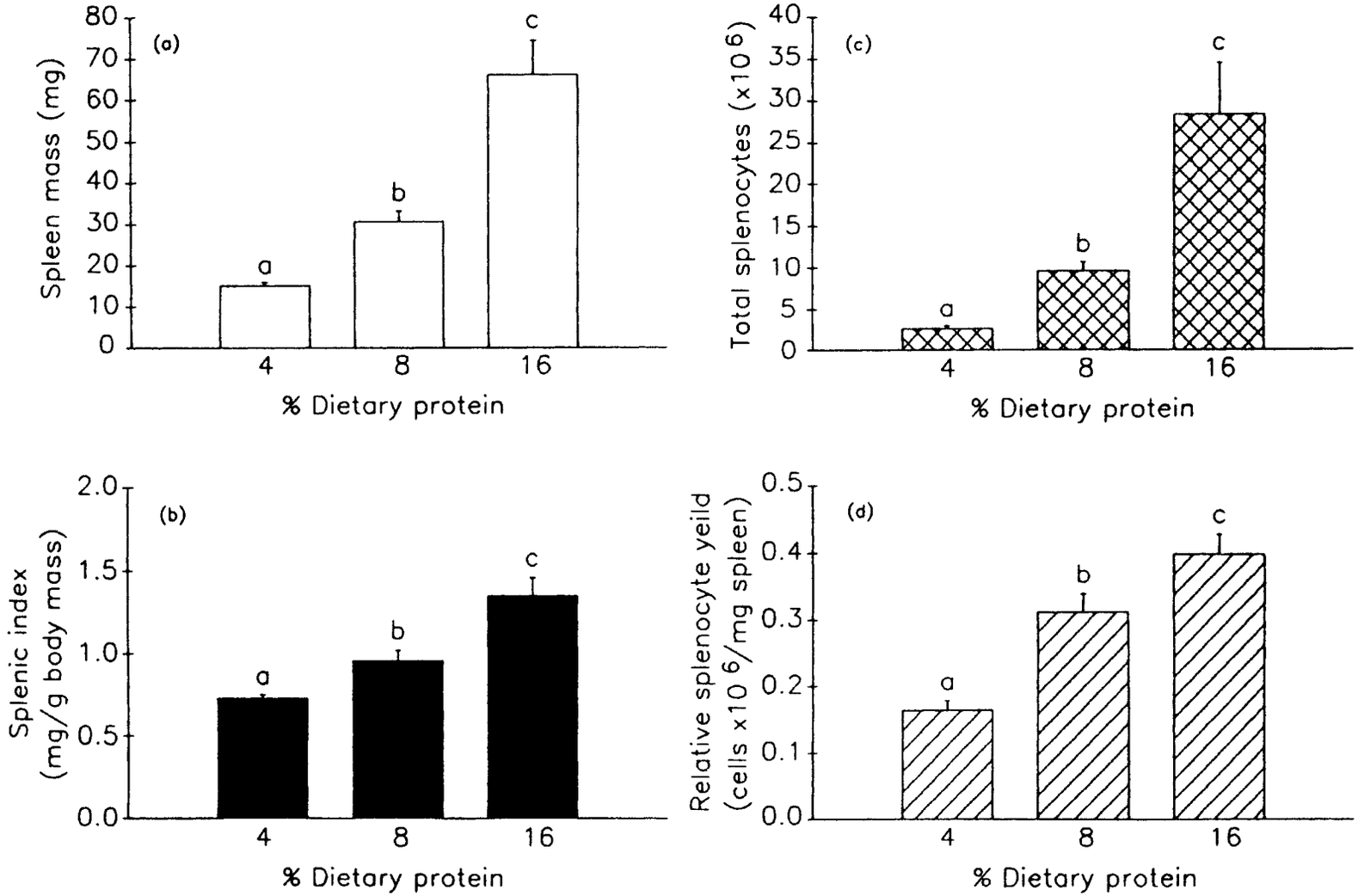

(a)
Spleen mass (mg)
80
70
60
50
40
30
20
10
0
a
b
c
4
8
16
% Dietary protein
(c)
Total splenocytes (x10 6)
40
35
30
25
20
15
10
5
0
a
b
c
4
8
16
% Dietary protein
(b)
Splenic index
(mg/g body mass)
2.0
1.5
1.0
0.5
0.0
a
b
c
4
8
16
% Dietary protein
(d)
Relative splenocyte yeild
(cells x10 6/mg spleen)
0.5
0.4
0.3
0.2
0.1
0.0
a
b
c
4
8
16
% Dietary protein

Fig. 4.--Absolute thymus mass (a), thymic index (b), total thymocyte yield (c), and relative thymocyte yield (d) for weanling cotton rats fed 4 (n = 10), 8 (n = 12), or 16% (n = 12) protein diets. Values represent means (± SE); bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

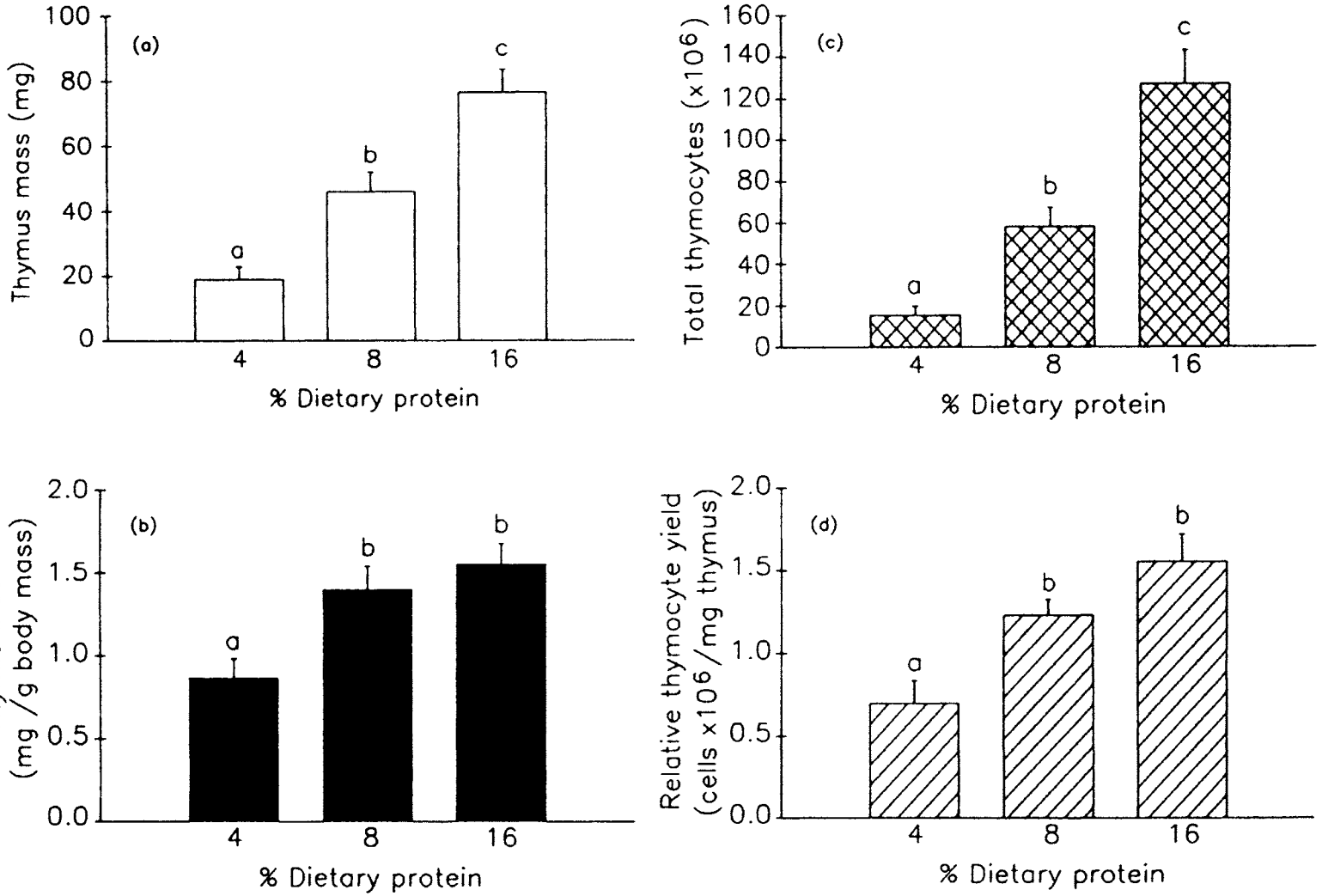

(a)
Thymus mass (mg)
100
80
60
40
20
0
a
b
c
4
8
16
% Dietary protein
(c)
Total thymocytes (x10⁶)
160
140
120
100
80
60
40
20
0
a
b
c
4
8
16
% Dietary protein
(b)
Thymic index
(mg /g body mass)
2.0
1.5
1.0
0.5
0.0
a
b
b
4
8
16
% Dietary protein
(d)
Relative thymocyte yield
(cells x10⁶/mg thymus)
2.0
1.5
1.0
0.5
0.0
a
b
b
4
8
16
% Dietary protein

Fig. 5.--Absolute paired popliteal node (PPN) mass (a), relative paired popliteal node mass (b), total mononuclear cell yield (c), and relative mononuclear cell yield (d) for weanling cotton rats fed 4 (n = 10), 8 (n = 12), or 16% (n = 12) protein diets. Values represent means (± SE); bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

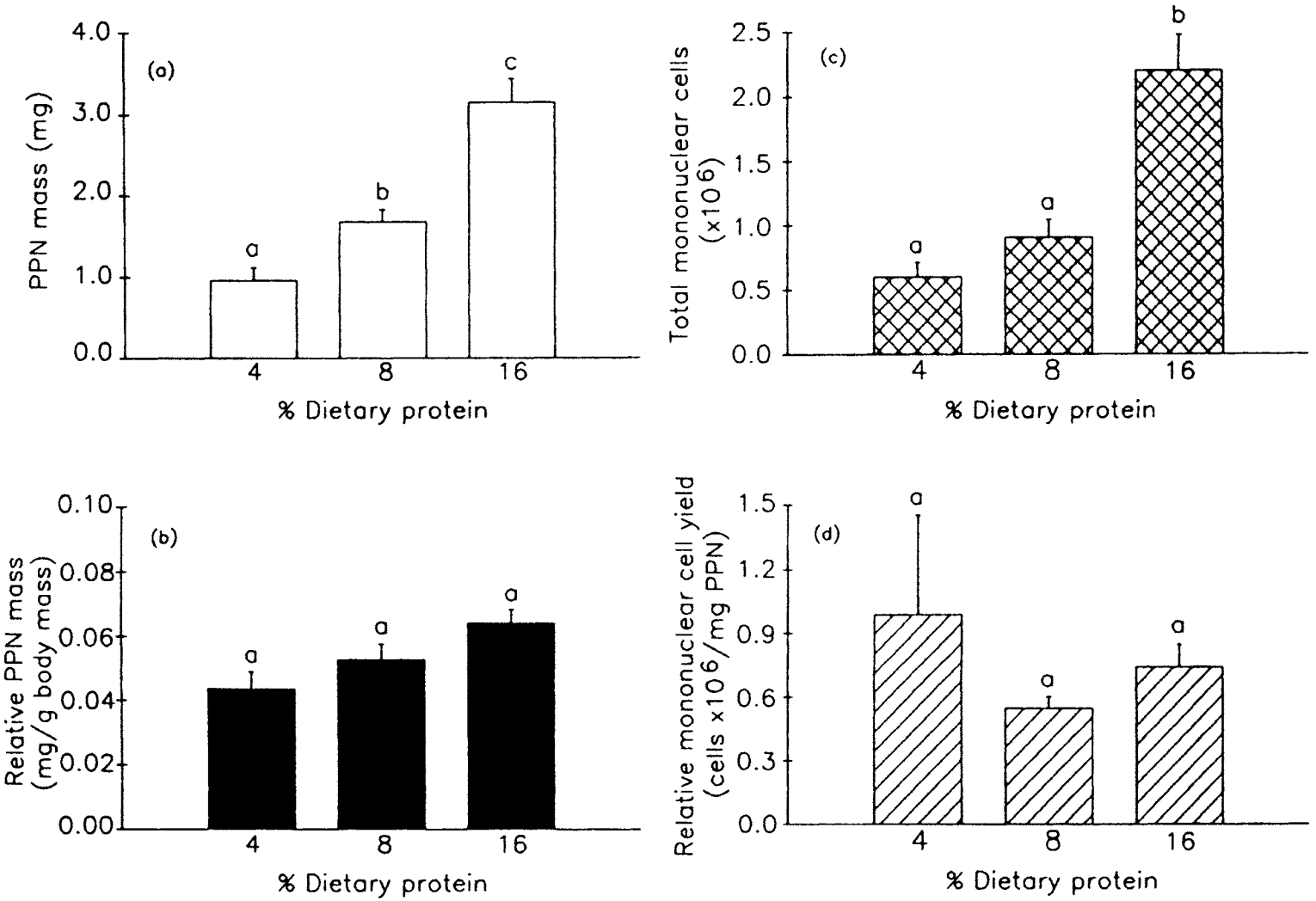

(a)
PPN mass (mg)
4.0
3.0
2.0
1.0
0.0
a
b
c
4
8
16
% Dietary protein
(c)
Total mononuclear cells (x10 6)
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
4
8
16
% Dietary protein
(b)
Relative PPN mass (mg/g body mass)
0.10
0.08
0.06
0.04
0.02
0.00
a
a
a
4
8
16
% Dietary protein
(d)
Relative mononuclear cell yield (cells x10 6/mg PPN)
1.5
1.2
0.9
0.6
0.3
0.0
a
a
a
4
8
16
% Dietary protein

Fig. 6.--Total serum protein for weanling cotton rats fed 4 (n = 10), 8 (n = 12), or 16% (n = 12) protein diets. Values represent means (± SE); bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

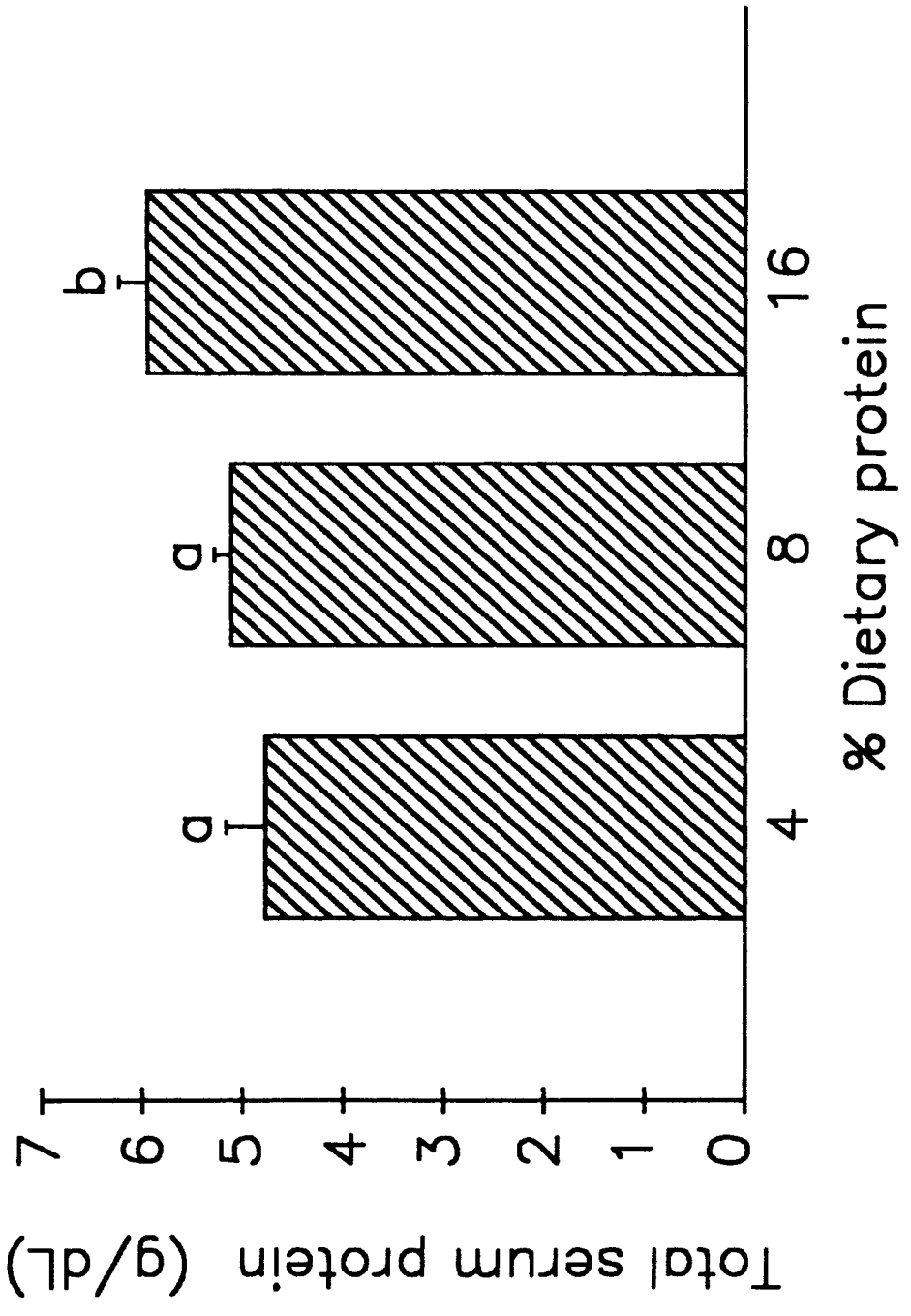

Total serum protein (g/dL)
0
1
2
3
4
5
6
7
a
a
b
4
8
16
% Dietary protein

Fig. 7.--Hemolytic complement activity (CH_{50} units/ml serum) for weanling cotton rats fed 4 (n = 10), 8 (n = 12), or 16% (n = 12) protein diets. Values represent means (± SE); bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

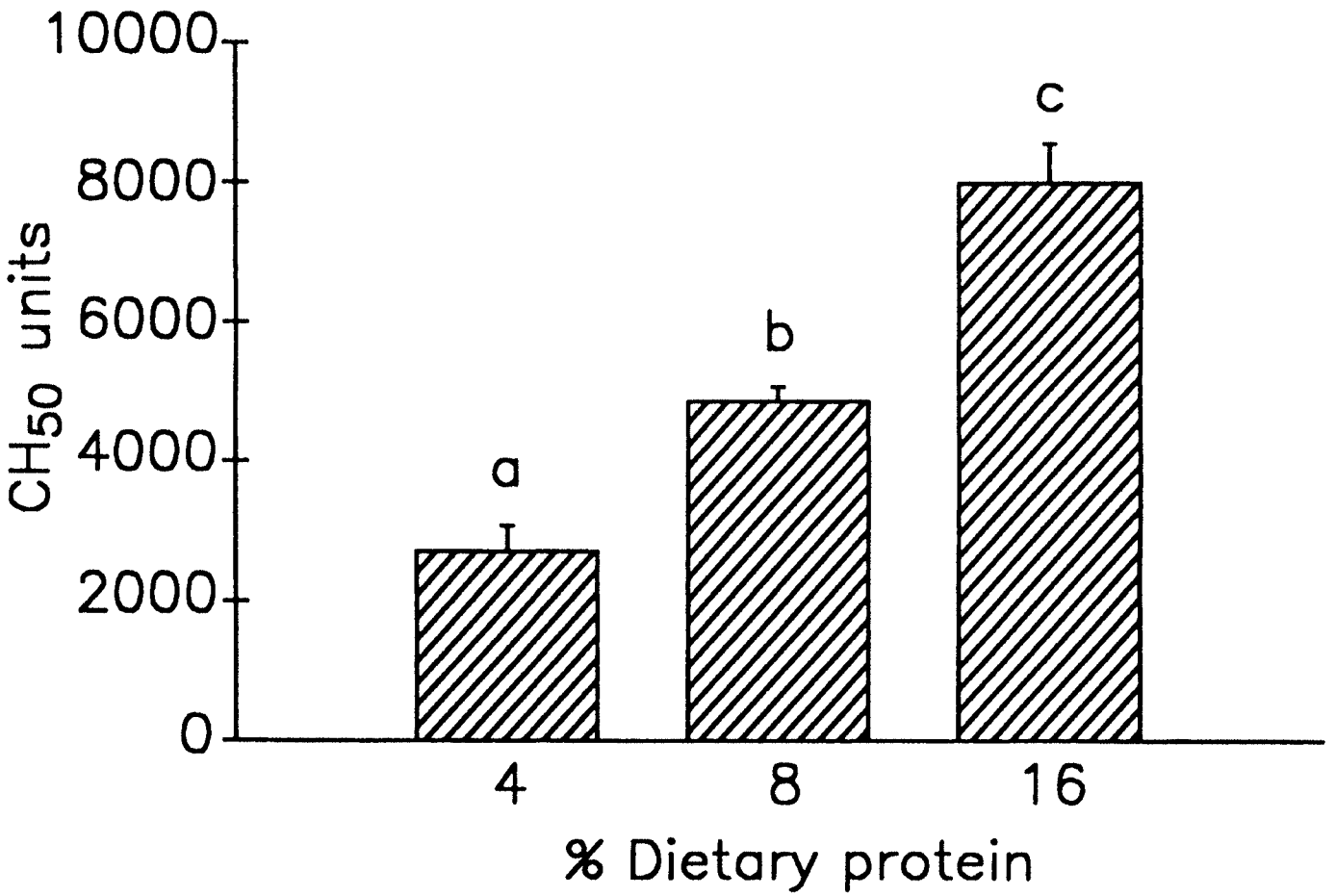

10000
8000
6000
4000
2000
0
CH_{50} units
a
b
c
4
8
16
% Dietary protein

Fig. 8.--Splenocyte subpopulations (mean ± SE) of weanling cotton rats fed 4 (n = 9), 8 (n = 12), or 16% (n = 12) protein diets as characterized by dual-labeling with FITC-soybean agglutinin (SBA) and TRITC-*Helix pomatia* agglutinin (HP) and represented as (a) prevalence, (b) total yield, (c) relative splenic yield, and (d) relative total yield. Bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

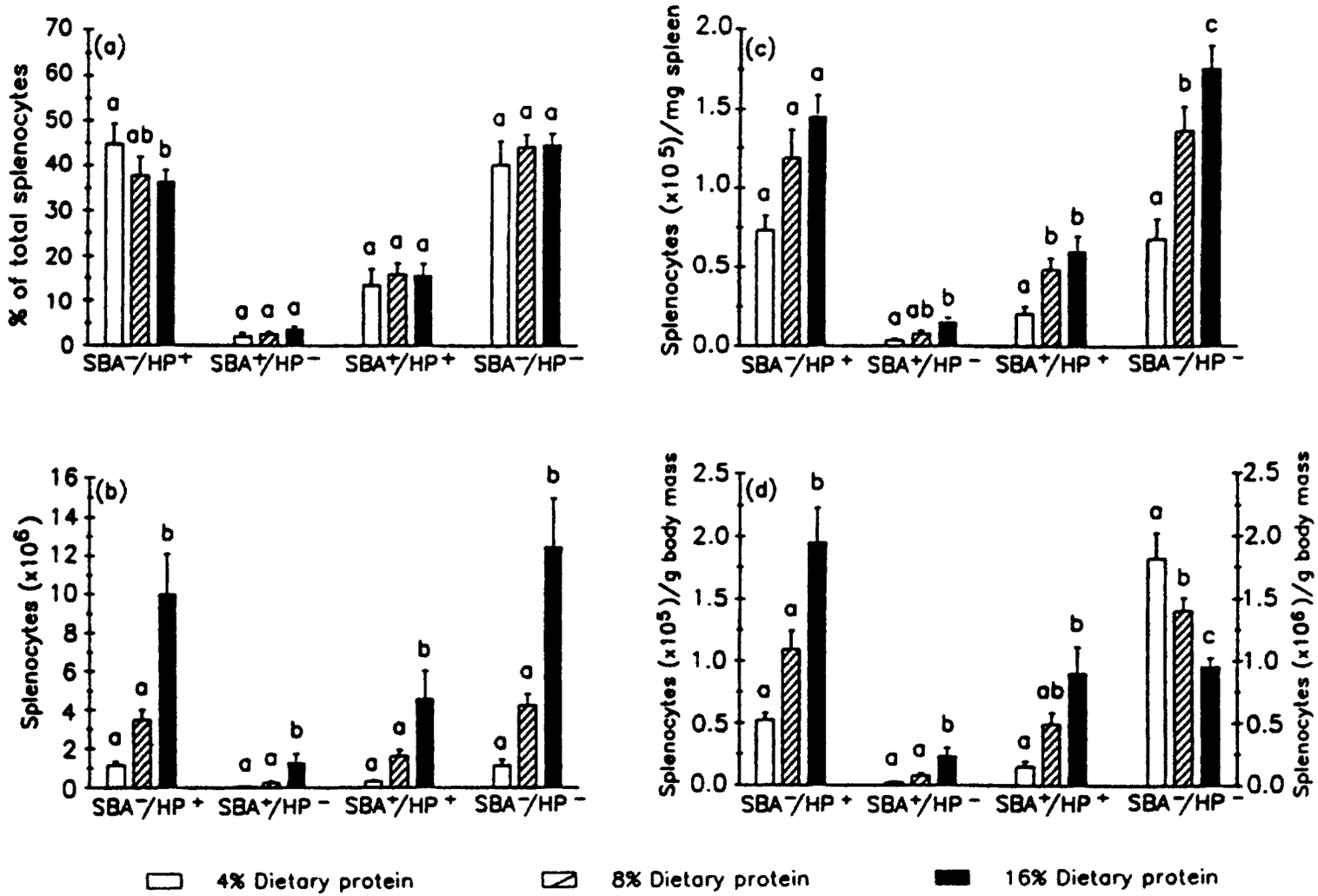

(a)
% of total splenocytes
70
60
50
40
30
20
10
0
a
ab
b
a a a
a a a
a a a
SBA⁻/HP⁺
SBA⁺/HP⁻
SBA⁺/HP⁺
SBA⁻/HP⁻
(c)
Splenocytes (x10 5)/mg spleen
2.0
1.5
1.0
0.5
0.0
a
a
a
a ab b
a
b
b
a
b
c
SBA⁻/HP⁺
SBA⁺/HP⁻
SBA⁺/HP⁺
SBA⁻/HP⁻
(b)
Splenocytes (x10⁶)
16
14
12
10
8
6
4
2
0
a
a
b
a a b
a
b
a
a
b
SBA⁻/HP⁺
SBA⁺/HP⁻
SBA⁺/HP⁺
SBA⁻/HP⁻
(d)
Splenocytes (x10⁵)/g body mass
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
a a b
a
ab
b
a
b
c
Splenocytes (x10⁶)/g body mass
SBA⁻/HP⁺
SBA⁺/HP⁻
SBA⁺/HP⁺
SBA⁻/HP⁻
4% Dietary protein
8% Dietary protein
16% Dietary protein

Fig. 9.--Splenocyte subpopulations (mean ± SE) weanling cotton rats fed 4 (n = 10), 8 (n = 12), or 16% (n = 12) protein diets as characterized by dual-labeling with FITC-rabbit-anti-rat immunoglobulin-G (BS) and TRITC-peanut agglutinin (PNA) and represented as (a) prevalence, (b) total yield, (c) relative splenic yield, and (d) relative total yield. Bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

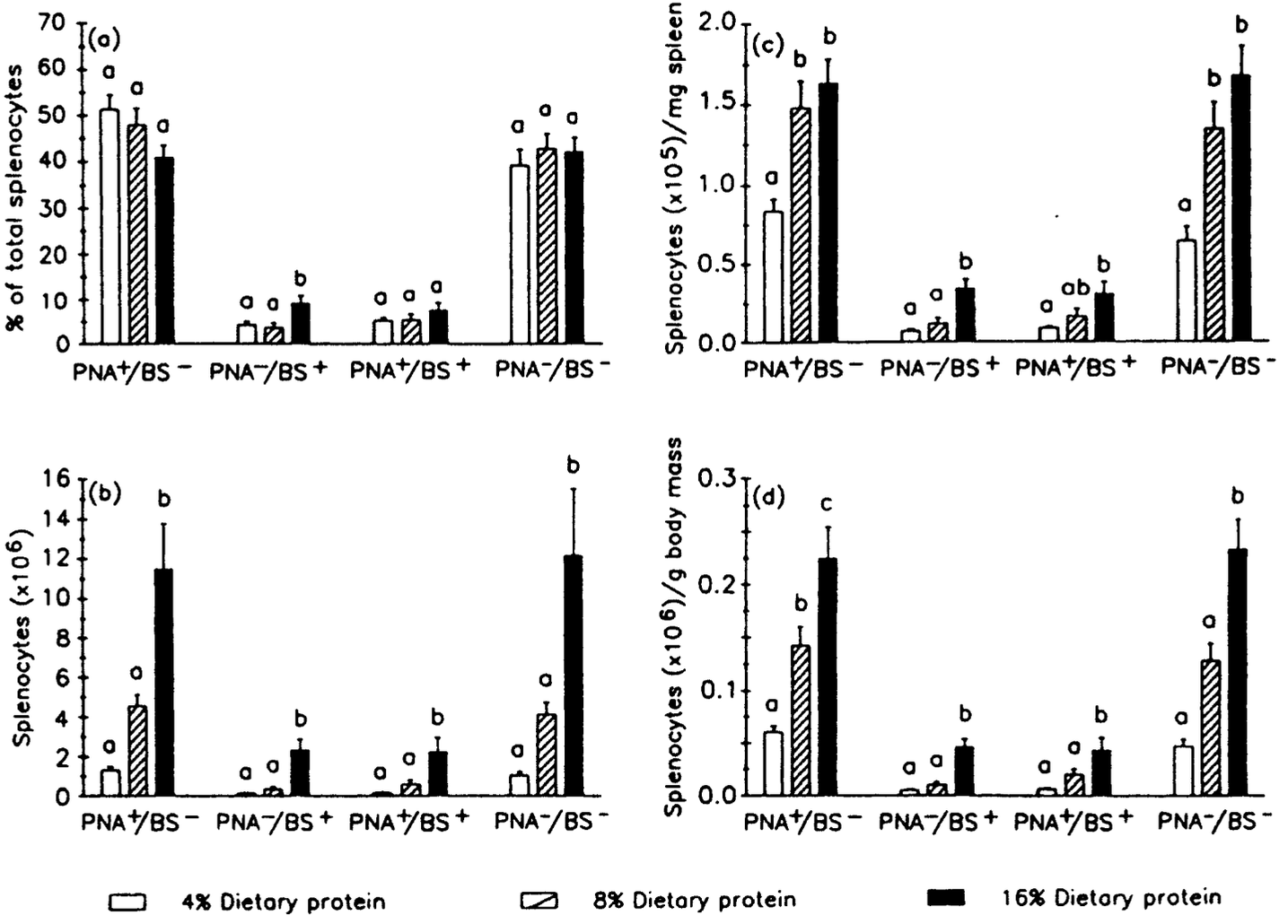

(a)
% of total splenocytes
70
60
50
40
30
20
10
0
(b)
Splenocytes (x10⁶)
16
14
12
10
8
6
4
2
0
(c)
Splenocytes (x10⁵)/mg spleen
2.0
1.5
1.0
0.5
0.0
(d)
Splenocytes (x10⁶)/g body mass
0.3
0.2
0.1
0.0
PNA⁺/BS⁻
PNA⁻/BS⁺
PNA⁺/BS⁺
PNA⁻/BS⁻
4% Dietary protein
8% Dietary protein
16% Dietary protein

Fig. 10.--Splenic index (mg spleen/g body mass) and splenocyte yield ($x10^6$/g body mass) for weanling cotton rats fed 4 ($\underline{n}$ = 11; * $\underline{n}$ = 10), 8 ($\underline{n}$ = 12), or 16% ($\underline{n}$ = 12) protein diets. The relative ratio of these two indices are represented as x-fold values. Indices represent means (± SE); bars with no superscripts in common indicate significantly different means ($\underline{P} < 0.05$) using the test for least significant difference.

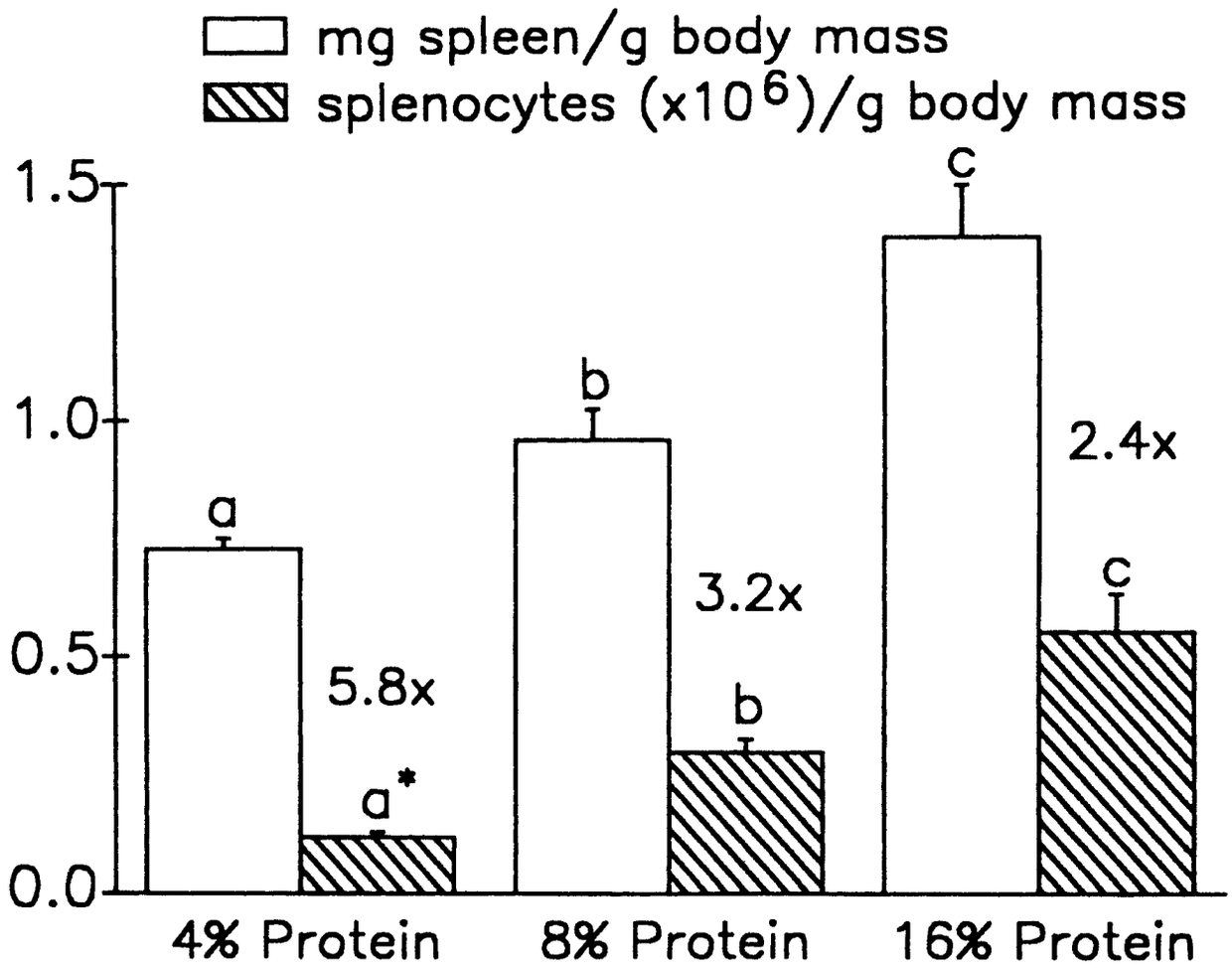
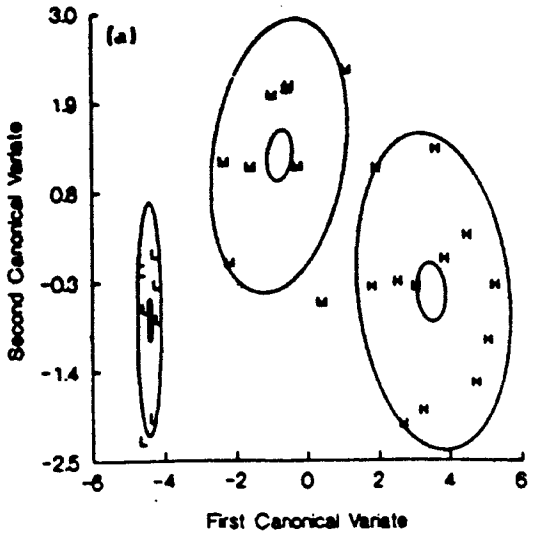
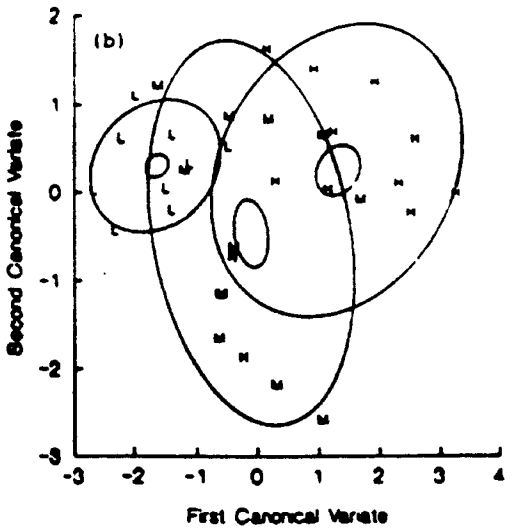
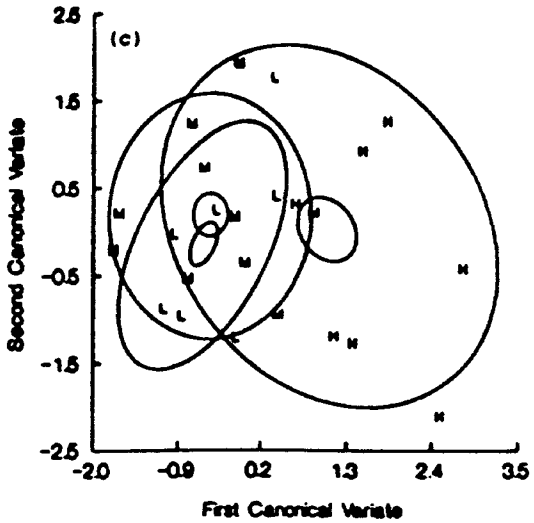

mg spleen/g body mass
splenocytes (x10^6)/g body mass
1.5
1.0
0.5
0.0
a
5.8x
a*
b
3.2x
b
c
2.4x
c
4% Protein
8% Protein
16% Protein

Fig. 11.--Plot of canonical scores derived from linear combinations of immunological parameters selected by stepwise discriminant analysis to discriminate weanling cotton rats according to dietary protein (4, L; 8, M; or 16%, H). Immunological parameters selected include (a) hemolytic complement activity, relative thymocyte yield, blood lymphocyte count, and mean corpuscular volume, (b) prevalence (% of total splenocyte) of PNA^-/BS^+ and SBA^+/HP^- splenocytes, and (c) relative splenic yields (splenocytes/mg spleen) of SBA^-/HP^-, PNA^-/BS^+, and SBA^+/HP^+ splenocytes. Confidence ellipses (95%) were constructed about both the means and samples for each diet group.


(a)
Second Canonical Variate
First Canonical Variate
3.0
1.9
0.8
-0.3
-1.4
-2.5
-6
-4
-2
0
2
4
6


(b)
Second Canonical Variate
First Canonical Variate
2
1
0
-1
-2
-3
-3
-2
-1
0
1
2
3
4


(c)
Second Canonical Variate
First Canonical Variate
2.5
1.5
0.5
-0.5
-1.5
-2.5
-2.0
-0.9
0.2
1.3
2.4
3.5

CHAPTER II

QUANTITATIVE AND QUALITATIVE NUMERICAL ALTERATIONS IN SPLENOCYTE SUBPOPULATIONS OF THE COTTON RAT (SIGMODON HISPIDUS) ACROSS SEASONS

ABSTRACT

Previous research in our laboratory has documented seasonal alterations in humoral and cell-mediated immunity in cotton rat (Sigmodon hispidus) populations. Based on these observations, we hypothesized that these seasonal differences in immune function were attributable in part to qualitative and quantitative numerical changes in specific splenocyte subpopulations. Lymphocytes were harvested from spleens of 139 cotton rats collected from a tallgrass prairie in central Oklahoma from December 1991 to September 1992. Unique splenocyte subpopulations were identified using fluorescein conjugated cell surface markers (concanavalin-A, peanut agglutinin, soybean agglutinin, Helix pomatia agglutinin, pokeweed mitogen, and rabbit-anti-rat immunoglobulin-G. All subpopulations examined were more abundant in fall and winter than spring and summer. Several plausible explanations for seasonal variation in abundance of splenocyte subpopulations are discussed.

INTRODUCTION

Environmentally induced changes in immunocompetence have been implicated in the regulation of small mammal populations (Dobrowolska and Adamczewska-Andrzejewska, 1991). A multitude of factors including physiological stress (Kelley, 1985), behavioral interactions (Vessey, 1964; Kelley, 1985), and nutritional deficiencies (Gershwin et al., 1985) have been shown to decrease immunocompetence in laboratory rodents. Similar mechanisms in wild populations may predispose individuals to enhanced predation risks due to subclinical infections or result in direct mortality (e.g., infectious disease). Evidence in support of immune involvement in population regulation can be found for Microtus montanus (Seed et al., 1978), Microtus arvalis (Dobrowolska and Adamczewska-Andrzejewska, 1991), and dasyurid marsupials (Bradley et al., 1980).

Previous work in our laboratory (Lochmiller et al., 1993) indicated that cotton rats (Sigmodon hispidus) collected from wild populations respond differently than captive-raised animals (defined in Lochmiller et al., 1993) in lymphoproliferative response assays. Furthermore, Lochmiller et al. (1994) observed temporal variation in humoral and cell-mediated immune responsiveness within a cotton rat population monitored over a 2-year period. These findings prompted us to explore the hypothesis that quantitative and qualitative changes in specific subpopulations of splenocytes govern seasonal variation in

immunocompetence of cotton rats. We used selected lectins that recognize glycoprotein receptors exhibited on the surface of certain T- and B-cells, as well as antibodies to membrane-bound immunoglobulin on B-cells, to document temporal changes in splenocyte subpopulations of the cotton rat throughout the year.

MATERIAL AND METHODS

Animals and morphology.--Cotton rats (n = 139) were captured from a population located 5 - 8 km west of Stillwater in central Oklahoma (36°04' N, 97°09' W) in fall (December 1991), winter (February 1992), spring (May 1992), and summer (September 1992) using Sherman live traps baited with rolled oats. Ewing et al. (1984) previously described the vegetation in this habitat type. Animals were returned to the laboratory, housed in polypropylene boxes containing wood shavings, and provided rodent chow (Purina 5001, St. Louis, MO, 24% crude protein) and water ad libitum. The animal room was maintained on a 14L:10D photoperiod with an ambient temperature of 25°C. After a 1-week acclimation to captivity, cotton rats were anesthetized with an intramuscular injection of ketamine hydrochloride (Aveco Co. Inc., Fort Dodge, IA; 50 mg/kg body mass) and euthanized by cervical dislocation. Spleen and thymus were aseptically removed and mass determined to the nearest 0.1 mg. The splenic and thymic index (Mittal and Woodward, 1991) was calculated as mg/g body mass.

Preparation of splenocytes.--The spleen was rinsed in 70% isopropyl alcohol, gently disrupted in 5 ml of Roswell Park Memorial Institute (RPMI) supplemented medium (RPMI-S, pH 7.2) containing RPMI-1640 (Sigma) with L-glutamine (2.05 mM), sodium pyruvate (1.0 mM), non-essential amino acids (1.0 mM, Sigma, cat. # M-7145), 2-mercaptoethanol ($2x10^{-5}$ M), penicillin (100,000 U/L), streptomycin (100 mg/L), and normal horse serum (10%), and placed on ice. Cells were washed via centrifugation (4°C, 8 min, 275 x g) in RPMI-S and erythrocytes removed by lysis with tris-buffered ammonium chloride (0.83%; pH 7.2). Following two washes in RPMI-S, cells were resuspended in 2.5 ml of RPMI-S and counted using a Serono 9000 series cell counter (Serono-Baker Diagnostics, Allentown, PA).

Phenotyping of splenocytes.--Due to a lack of commercially available monoclonal antibodies to lymphocyte surface antigens of cotton rats, we selected a suite of lectins with an affinity for T-cell glycoprotein receptors and an antibody specific for membrane-bound immunoglobulin on B-cells to phenotype cotton rat splenocytes. Splenocytes were washed in 10 ml of phosphate buffered saline (pH 7.4) and adjusted to $10x10^6$ cells/ml in phosphate buffered saline. An aliquot of $9x10^6$ cells was treated with 10 µl (0.01 U) of neuraminidase type VI (Sigma), to expose surface receptors, and 2 ml of tris-buffered Hank's balanced salt solution supplemented with 0.5% bovine serum albumin and 0.02% sodium azide (TH-S; pH 7.45) and incubated for 45 min

at 37°C. Neuraminidase-treated cells were washed twice in TH-S and aliquots of $3x10^6$ cells were placed in microcentrifuge tubes, centrifuged, and resuspended in the residual media (approximately 100 µl) then treated with 100 µl of FITC-lectin (soybean agglutinin (SBA); 100 µg/ml, peanut agglutinin (PNA); 250 µg/ml, or Helix pomatia agglutinin (HP); 100 µg/ml, Sigma). Aliquots of $3x10^6$ cells (not treated with neuraminidase) were placed in microcentrifuge tubes, centrifuged, resuspended in the residual media and treated with 100 µl of FITC-lectin (Con-A; 50 µg/ml, or PWM; 50 µg/ml, Sigma), or 40 µl of FITC-BS (rabbit-anti-rat immunoglobulin-G, heavy and light chain; 1:10 in distilled water, Cappel Research Products, Durham, NC); these cells were washed once in phosphate buffered saline, supplemented with bovine serum albumin (1.0%) prior to addition of FITC-BS. Cells were incubated for 30 min at 4°C (SBA, PNA, HP, Con-A, and PWM-treated) or 37°C (BS-treated). Cells were washed three times in phosphate buffered saline (Con-A, PWM, and BS-treated) or TH-S (SBA, PNA, and HP-treated), resuspended in the residual media (approximately 100 µl), and mounted on microscope slides in polyvinyl alcohol.

Cells were phenotyped via epifluorescent microscopy under oil immersion (1000X magnification, Olympus BH-2). Cells were first detected under bright field illumination, counted, then with the FITC-exciter filter (Jacobs Instrument Co., Shawnee Mission, KS) in position,

characterized as positive (stain speckled or capped on cell surface) or negative for the appropriate marker. Differential counts of splenocyte subpopulations were expressed as prevalence (% of total splenocyte yield), relative splenic yield (splenocytes/mg spleen), and total yield (splenocytes/spleen).

Statistical analysis.--One-way analysis of variance for unequal sample sizes (PROC GLM; SAS Institute, 1982) was used to test for seasonal differences in gross lymphoid organ morphology and measures of abundance for specific splenocyte subpopulations. Levene's test of homogeneity of variance (Snedecor and Cochran, 1980) was performed; relative splenic yields for all subpopulations were rank transformed and other variables were either rank (prevalence of PWM^+, BS^+, and PNA^+ splenocytes, thymic index) or log (total yield of Con-A^+, PWM^+, and SBA^+ splenocytes, splenic index) transformed as necessary prior to further statistical analysis. Means were separated using the test of least significant difference (LSD; SAS Institute, 1982). Means are reported with corresponding standard error.

RESULTS

There was significant ($P = 0.001$) seasonal variation in body mass, with animals averaging 125.9 ± 6.4 g in summer compared to an overall average body mass of 96.7 ± 3.3 g for other seasons. Spleen masses were significantly ($P = 0.005$) different among seasons, with heavier spleens in spring (251

± 31 mg) and summer (278 ± 18 mg) than fall (190 ± 14 mg) or winter (197 ± 17 mg); however, differences were simply a reflection of greater body mass, as the splenic index was similar among seasons, with an overall mean of 2.2 ± 0.1 mg/g body mass. There was also seasonal variation ($\underline{P}$ = 0.012) in total splenocyte yields, with greater yields in summer (87 ± 7 $x10^6$) than either fall (57 ± 5 $x10^6$) or spring (64 ± 6 $x10^6$); yields in winter (70 ± 9 $x10^6$) were similar to all other months. Splenocyte yields were proportional to spleen masses. Trends in thymus mass and the thymic index were the opposite of spleen trends, with significant ($\underline{P}$ < 0.001) seasonal changes that were characterized by greater values in fall than other seasons. Thymus mass averaged 57 ± 4 mg in the fall compared to an overall average of 29 ± 3 mg in other seasons, whereas, the thymic index was characterized by an average value of 0.65 ± 0.05 mg/g body mass in fall to an overall average of 0.36 ± 0.03 mg/g body mass in other seasons.

In general, the majority of subpopulations we examined were more abundant in fall and winter compared to spring and summer (Figs. 1, 2, and 3). Average annual prevalence of cells positive for the T-cell labels Con-A^+ (53 ± 7%), PNA^+ (59 ± 8%), SBA^+ (32 ± 10%), HP^+ (42 ± 5%) and the B-cell labels BS^+ (25 ± 2%) and PWM^+ (7 ± 3%) indicated that T-cells were about twice as abundant as B-cells in the cotton rat. Prevalence of PNA^+, Con-A^+, and PWM^+ splenocytes, total yield of SBA^+ splenocytes, and relative splenic yields

of Con-A$^+$, PNA$^+$, and SBA$^+$ splenocytes were significantly ($P < 0.05$) influenced by season, with values 1.3 - 4.9 times greater in either fall or winter than either spring or summer. Similar trends were observed for total yields of PNA$^+$ (1.2 - 1.4 times greater) and PWM$^+$ (3.7 - 7.5 times greater) splenocytes. Significant seasonal differences ($P < 0.001$) were also apparent for prevalence of HP$^+$ and BS$^+$ splenocytes and relative splenic yield of HP$^+$ splenocytes, with 29 - 64% greater values in fall than any other season. Seasonal differences ($P < 0.001$) in relative splenic yield of PWM$^+$ splenocytes were also apparent, with yields 11% higher in winter than fall, but yields in fall were 6-fold higher than spring and 4-fold higher than summer. Total yield of Con-A$^+$ splenocytes changed seasonally ($P = 0.009$), with 22 - 41% lower yields in spring than any other season. Seasonal differences ($P < 0.001$) in the relative splenic yield of BS$^+$ cells was also observed, with yields in fall 62% greater than spring, and yields in winter 50% greater than spring.

DISCUSSION

Seasonal changes in immune organs such as the spleen and thymus gland have been widely documented for a variety of species. However, seasonal variation in these immune organs appears to be very species-dependent as indicated by the multitude of trends recorded for various small mammal species (Newson, 1962; Sealander and Bickerstaff, 1967;

Kendall and Twigg, 1981; Lochmiller et al., 1994). Our observations in the cotton rat did not reveal any changes in spleen size across seasons that could not be explained by body mass. This was not the case for the thymus gland, which was largest in fall, similar to our previous studies with the cotton rat (Lochmiller et al., 1994). Thymus mass changes may be a reflection of altered nutritional status (Peitz et al., 1993) or just as likely may represent a normal physiological change due to some external stimuli such as photoperiod (Ozoga and Verme, 1978). It is well known that involution of the thymus occurs with age, however, age did not explain differences in thymus mass in our study.

Assessment of variation in splenocyte subpopulations across seasons was accomplished using a suite of FITC-conjugated surface markers with well-described binding characteristics for other species. Concanavalin-A and SBA have been shown to bind primarily to T-cells (Hellstrom et al., 1976); HP has been used as a T-cell and natural killer cell marker in laboratory mice (Haller et al., 1978; Mattes and Holden, 1981) and rats (Swanborg et al., 1977); and PNA preferentially binds T-cells (predominately immature T-cells) in mice (London et al., 1978), and chickens (Uni and Heller, 1991). Pokeweed mitogen and BS preferentially bind B-cells, but PWM has some specificity for T-cells (Greaves and Janossy, 1972) as does BS due to Fc receptors on some T-cells (Reynolds and Ortaldo, 1990). Although we can not be

absolutely certain these cell-surface markers bind the same cells in cotton rats as they do in other species, they do provide a means of identifying and monitoring changes in specific splenocyte subpopulations. Given their general binding characteristics for selected cell types, we may obtain general ideas about seasonal variation of T- and B-cells in the cotton rat.

All phenotypes examined were more abundant, on a relative and absolute basis, in fall or winter compared to spring and summer. Abundance of both PWM^+ and BS^+ splenocytes was lowest during summer, however the effect on PWM^+ splenocytes was much more pronounced, suggesting only a fraction of the B-cells changed across seasons. Dobrowolska and Adamczewska-Andrzejewska (1991) have observed seasonal fluctuations in serum gamma-globulin levels, with lowest concentrations in early summer in wild populations of M. arvalis, which could be attributed to changes in B-cell abundance.

Pronounced seasonal variation in subpopulations of T-cells was very apparent in our study. Among the three T-cell markers used (SBA^+, $Con\text{-}A^+$, and PNA^+), SBA^+ splenocytes changed most profoundly across seasons. There are numerous factors that may have contributed to variations in abundance of splenocyte subpopulations of cotton rats across seasons. Seasonal variation in nutrient availability (Cameron and McClure, 1988) may be one of the most influential extrinsic factors influencing splenocyte subpopulations, as nutrition

is widely known to have numerous effects on immunocompetence (Klurfeld, 1993) and disease resistance (Hart et al., 1985). Restricted protein intake has been shown to result in a reduction in T-cell (Slobodianik et al., 1984), and B-cell (Woodward and Miller, 1991) abundance. Vestey et al. (1993) suggested that elevated delayed-type hypersensitivity responses of protein-restricted cotton rats may be the result of quantitative alterations in T-suppressor cells or T-helper:T-suppressor cell ratios. Changes in humoral immunity in cotton rats subjected to protein restriction (Vestey et al., 1993) could be due in part to reductions in B-cell abundance. These reports of altered immune function in response to protein restriction are interesting, because splenocyte subpopulations of cotton rats were lowest during summer, a season when nutritional quality of herbivore diets reach their lowest in central Oklahoma (Peitz et al., 1993).

Other possible contributing factors to the observed seasonal changes in splenocyte subpopulations across seasons include physiological stress, reproduction and genetic shifts in the population. Elevated blood-glucocorticoid levels, such as cortisol in conjunction with the stress response can have powerful immunosuppressive consequences on T-cell function and humoral immunity (Griffin, 1989; Plotnikoff et al., 1991). McLean (1982) observed that following glucocorticoid administration, cotton rats experienced increased susceptibility to Keystone virus infection and lower neutralizing antibody titers, which may

be secondary to alterations in T- and B-cell numbers. Cotton rats were reproductively active during spring and summer, seasons when splenocyte subpopulations were lowest. Low numbers of splenocyte subpopulations may be due to the influence of sex steroid hormones, which can suppress both humoral and cell-mediated immune responses (Grossman, 1985). Polymorphism of the major histocompatibility complex and immune response (Ir) genes, is considerable within a species (Klein, 1986; Banacerraf and McDevitt, 1972) and may contribute to observed changes in subpopulations. Lochmiller et al. (1994) have documented significant seasonal changes in immunological phenotypes within wild cotton rat populations. Lastly, as some cells have both T- and B-cell markers (Reynolds and Ortaldo, 1990), it is possible that the proportion of cells bearing both receptors increased during fall and winter, accounting for the greater abundance of all splenocyte subpopulations in these two seasons.

We have demonstrated that variation in quantitative and qualitative characteristics of subpopulations of both T- and B-cells occur across seasons in the wild cotton rat. These observations support our hypothesis that temporal changes in functional immunoresponsiveness in wild cotton rat populations (Lochmiller et al., 1994) could be due in part to numerical alterations in specific T- and B-cell subpopulations. These observations warrant further, more detailed, investigations to determine if predictable

Gershwin, M. E., R. S. Beach, and L. S. Hurley. 1985. Nutrition and immunity. Academic Press, New York, 417 pp.

Greaves, M. F., and G. Janossy. 1972. Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. Transplantation Reviews, 11:87-130.

Griffin, J. F. T. 1989. Stress and Immunity: a unifying concept. Veterinary Immunology and Immunopathology, 20:263-312.

Grossman, C. J. 1985. Interactions between the gonadal steroids and the immune system. Science, 227:257-261.

Haller, O., M. Gildlund, U. Hellstrom, S. Hammarstrom, and H. Wigzell. 1978. A new surface marker on mice natural killer cells: receptors for Helix pomatia A hemagglutinin. European Journal of Immunology, 8:765-771.

Hart, R. P., S. D. Bradshaw, and J. B. Iveson. 1985. Salmonella infections in a marsupial, the quokka (Setonix brachyurus), in relation to seasonal changes in condition and environmental stress. Applied and Environmental Microbiology, 49:1276-1281.

Hellstrom, U., M. L. Dillner, S. Hammarstrom, and P. Perlmann. 1976. The interaction of nonmitogenic and mitogenic lectins with T lymphocytes: association of cellular receptor sites. Scandinavian Journal of Immunology, 5:45-54.

Kelley, K. W. 1985. Immunological consequences of changing environmental stimuli. Pp. 193-223, in Animal stress (G. P. Moberg, ed.). American Physiological Society, Bethesda, Maryland, 324 pp.

Kendall, M. D., and G. I. Twigg. 1981. The weight of the thymus gland in a population of wild bank voles, Clethrionomys glareolus from Wicken Fen, Cambridgeshire. Journal of Zoology (London), 194:323-329.

Klein, J. 1986. Natural history of the major histocompatibility complex. John Wily and Sons, New York, 775 pp.

Klurfeld, D. M. 1993. Nutrition and immunology. Plenum Press, New York, 358 pp.

Lochmiller, R. L., M. R. Vestey, and S. T. McMurry. 1993. Phenotypic variation in lymphoproliferative responsiveness to mitogenic stimulation in cotton rats. Journal of Mammalogy, 74:189-197.

_____. 1994. Temporal variation in humoral and cell-mediated immune response in a Sigmodon hispidus population. Ecology, 75:236-245.

London, J., S. Berrih, and J. F. Bach. 1978. Peanut agglutinin I. A new tool for studying lymphocyte subpopulations. The Journal of Immunology, 121:438-443.

Mattes, M. J., and H. T. Holden. 1981. The distribution of Helix pomatia lectin receptors on mouse lymphoid cells

and other tissues. European Journal of Immunology, 11:358-365.

McLean, R. G. 1982. Potentiation of Keystone virus infection in cotton rats by glucocorticoid-induced stress. Journal of Wildlife Diseases, 18:141-148.

Mittal, A., and B. Woodward. 1991. Development of ultrastructural abnormalities in the thymic stellate epithelial cells of weanling mice subjected to severe food intake restriction. Nutrition Research, 11:491-500.

Newson, J. 1962. Seasonal differences in reticulocyte count, haemoglobin level and spleen weight in wild voles. British Journal of Haematology, 8:296-302.

Ozoga, J. J., and L. J. Verme. 1978. The thymus gland as a nutritional status indicator in deer. The Journal of Wildlife Management, 42:791-798.

Peitz, D. C. 1993. Essential amino acid nutritional ecology of cottontail rabbits (Sylvilagus floridanus). Masters thesis, Oklahoma State University, Stillwater, 142 pp.

Plotnikoff, N., A. Murgo, R. Faith, and J. Wybran. 1991. Stress and immunity. CRC Press, Boca Raton, Florida, 558 pp.

Reynolds, C. W., and J. R. Ortaldo. 1990. Nature and physiology of natural effector cells. Pp. 336-349, in Immunophysiology (J. J. Oppenheim and E. M. Shevach, eds.). Oxford University Press, New York, 424 pp.

SAS Institute, Inc. 1982. SAS user's guide: Statistics, 1982 edition. SAS Institute Inc., Cary, North Carolina, 584 pp.

Sealander, J. A., and L. K. Bickerstaff. 1967. Seasonal changes in reticulocyte number and relative weights of the spleen, thymus, and kidneys in the northern red-backed mouse. Canadian Journal of Zoology, 45:253-260.

Seed, J. R., A. J. Pinter, P. U. Ashman, S. T. Ackerman, and L. King. 1978. Composition of organ weights of field and laboratory Microtus montanus infected with Trypanosoma brucei gambiense. American Midland Naturalist, 100:126-134.

Slobodianik, N. H., R. C. Cosarinsky, S. H. Langini, M. E. Roux, M. E. Rio, and J. C. Sanhuja. 1984. Effect of severe protein deficiency on surface and intracellular markers of growing rats' lymphoid organs. Nutrition Reports International, 29:957-964.

Snedecor, G. W., and W. G. Cochran. 1980. Statistical methods. Seventh edition. Iowa State University Press, Iowa. 507 pp.

Swanborg, R. H., U. Hellstrom, H. Perlmann, S. Hammarstrom, and P. Perlmann. 1977. A new rat lymphocyte surface marker. Characterization and separation of cells with receptors for Helix pomatia hemagglutinin. Scandinavian Journal of Immunology, 6:235-239.

Uni, Z., and E. D. Heller. 1991. Peanut agglutinin as a marker of maturation and activation of chicken thymic-derived lymphocytes. Poultry Science, 70:1516-1520.

Vessey, S. H. 1964. Effects of grouping on levels of circulating antibodies in mice. Proceedings of the Society for Experimental Biology and Medicine, 115:252-255.

Vestey, M. R., S. T. McMurry, and R. L. Lochmiller. 1993. Influence of dietary protein on selected measures of humoral and cellular immunity in the cotton rat Sigmodon hispidus. Canadian Journal of Zoology, 71:579-586.

Woodward, B. D., and R. G. Miller. 1991. Depression of the thymus-dependent immunity in wasting protein-energy malnutrition does not depend on an altered ratio of helper ($CD4^+$) to suppressor ($CD8^+$) T-cells or on a disproportionately large atrophy of the T-cell relative to the B-cell pool. American Journal of Clinical Immunology, 53:1329-1335.

Fig. 1.--Seasonal variation in abundance (% of total splenocytes) of splenocyte subpopulations in cotton rats trapped from a population in central Oklahoma from December 1991 to September 1992. Splenocyte subpopulations were identified using fluorescein conjugated (a) concanavalin-A (Con-A, 50 µg/ml), (b) peanut agglutinin (PNA, 250 µg/ml), (c) soybean agglutinin (SBA, 100 µg/ml), (d) Helix pomatia agglutinin (HP, 100 µg/ml), (e) pokeweed mitogen (PWM, 50 µg/ml), and (f) rabbit-anti-rat immunoglobulin-G (1:10). Data expressed as mean (± SE); bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

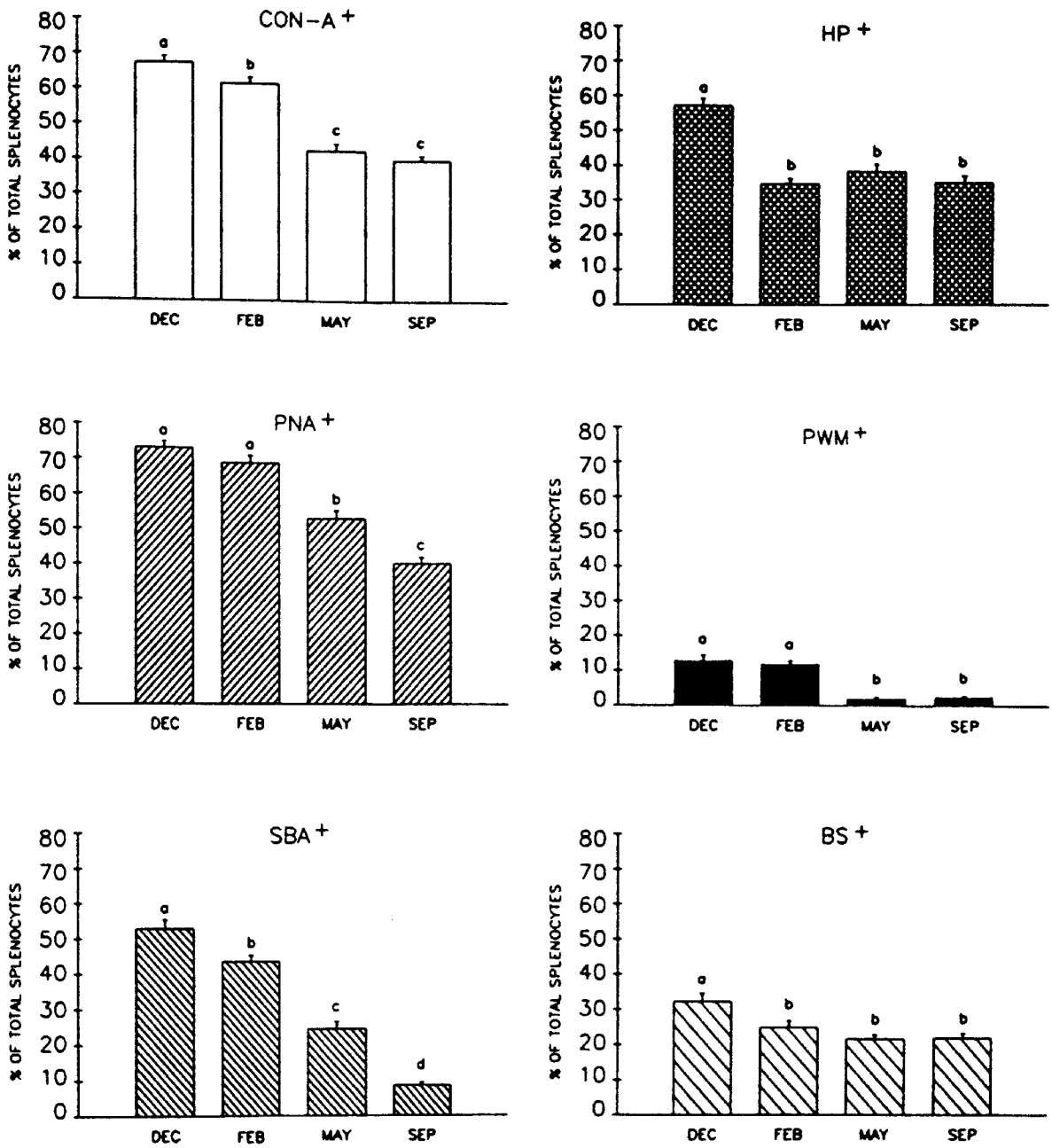

CON-A +
HP +
PNA +
PWM +
SBA +
BS +
% OF TOTAL SPLENOCYTES
0
10
20
30
40
50
60
70
80
DEC
FEB
MAY
SEP
a
b
c
d

Fig. 2.--Seasonal variation in abundance (splenocytes/mg spleen) of splenocyte subpopulations in cotton rats trapped from a population in central Oklahoma from December 1991 to September 1992. Splenocyte subpopulations were identified using fluorescein conjugated (a) concanavalin-A (Con-A, 50 µg/ml), (b) peanut agglutinin (PNA, 250 µg/ml), (c) soybean agglutinin (SBA, 100 µg/ml), (d) Helix pomatia agglutinin (HP, 100 µg/ml), (e) pokeweed mitogen (PWM, 50 µg/ml), and (f) rabbit-anti-rat immunoglobulin-G (1:10). Data expressed as mean (± SE); bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

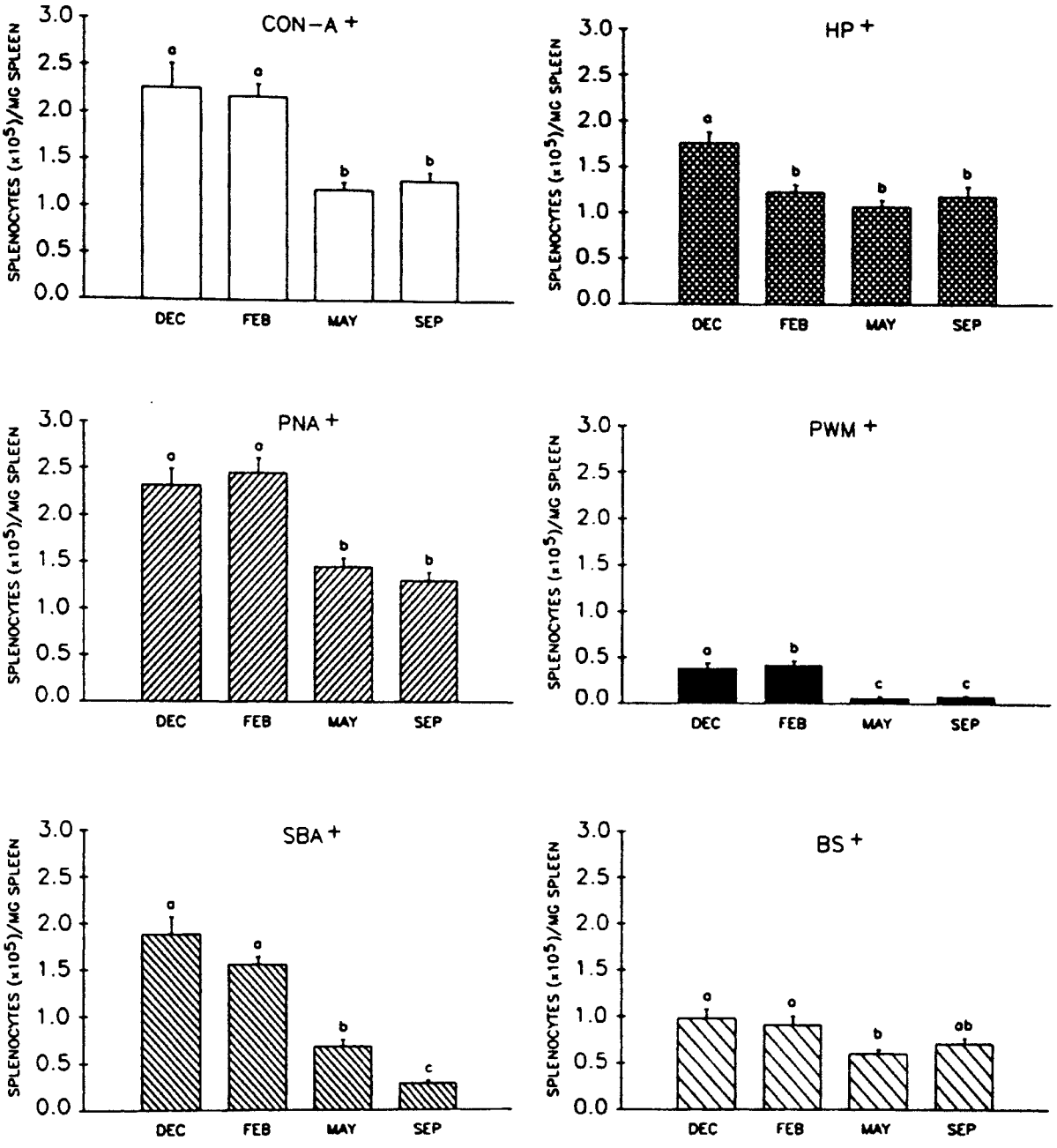
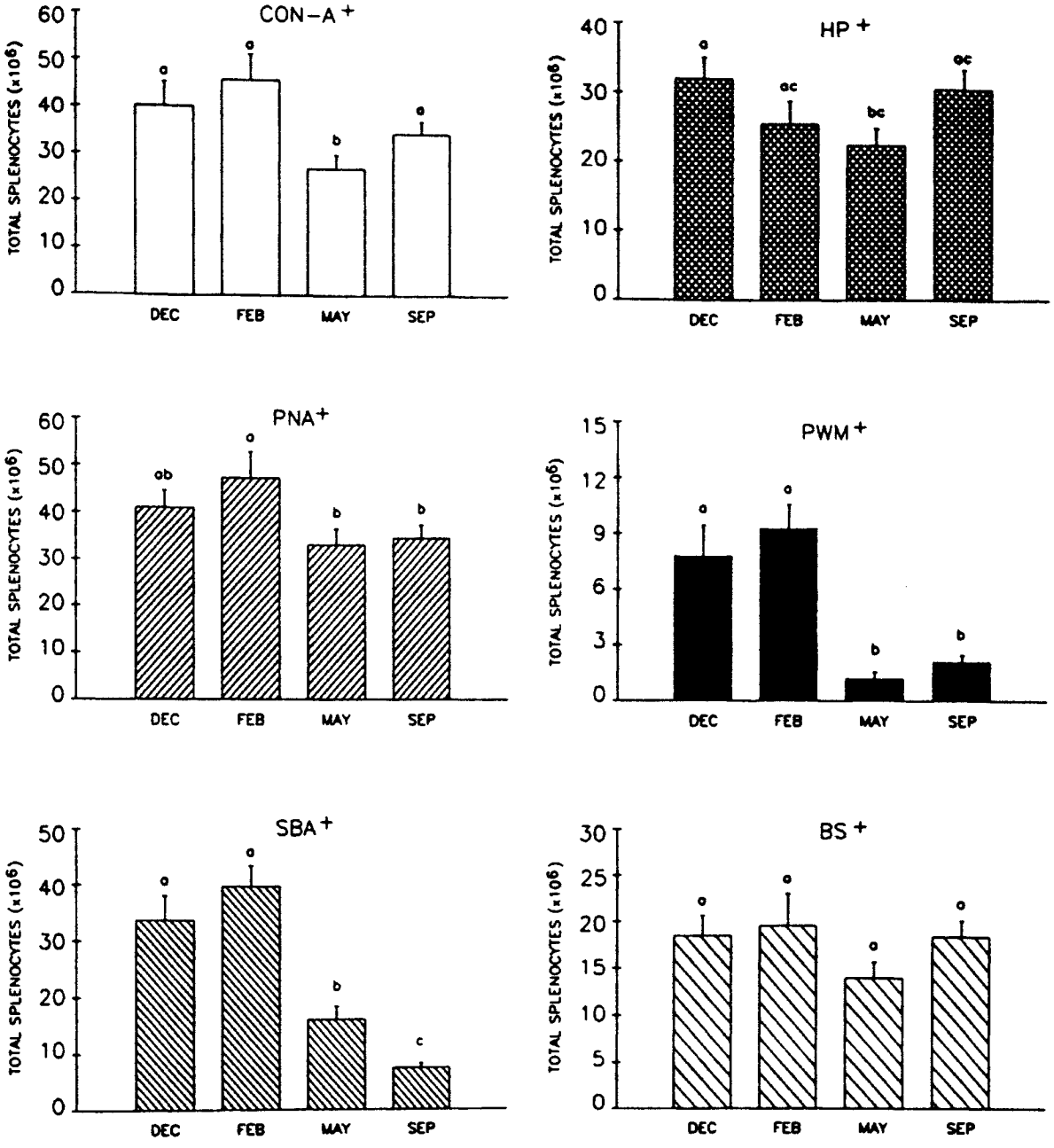

CON-A +
HP +
PNA +
PWM +
SBA +
BS +
SPLENOCYTES (x10⁵)/MG SPLEEN
3.0
2.5
2.0
1.5
1.0
0.5
0.0
DEC
FEB
MAY
SEP
a
b
c
ab

Fig. 3.--Seasonal variation in absolute number of splenocyte subpopulations in cotton rats trapped from a population in central Oklahoma from December 1991 to September 1992. Splenocyte subpopulations were identified using fluorescein conjugated (a) concanavalin-A (Con-A, 50 µg/ml), (b) peanut agglutinin (PNA, 250 µg/ml), (c) soybean agglutinin (SBA, 100 µg/ml), (d) Helix pomatia agglutinin (HP, 100 µg/ml), (e) pokeweed mitogen (PWM, 50 µg/ml), and (f) rabbit-anti-rat immunoglobulin-G (1:10). Data expressed as mean (± SE); bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.


CON-A +
HP +
PNA+
PWM +
SBA+
BS +
TOTAL SPLENOCYTES (x10⁶)
DEC
FEB
MAY
SEP

CHAPTER III

PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF NYLON WOOL-SEPARATED SPLENOCYTES OF COTTON RATS (SIGMODON HISPIDUS)

ABSTRACT

We investigated the efficacy of nylon wool columns for the partial purification of splenocyte subpopulations in the cotton rat (Sigmodon hispidus). Lymphocytes within the pre-separated cell population and the non-adherent and adherent subpopulations were phenotyped by labeling with fluorescein isothiocyanate (FITC) conjugated markers, peanut agglutinin (PNA), Helix pomatia agglutinin (HP) (predominately T-cell markers) and rabbit-anti-rat immunoglobulin-G (BS) (predominately a B-cell marker). Subpopulations were subjected to polyclonal activation with the mitogens concanavalin-A (Con-A), pokeweed mitogen (PWM), interleukin-2 (IL-2) and Salmonella typhimurium (STM) to characterize functional responses. PNA^+ cells were non-adherent, BS^+ cells were adherent and HP^+ cells were not uniquely characterized as adherent or non-adherent. The PNA^+:BS^+ ratio was 4 times greater in non-adherent than in adherent subpopulations. Lymphoproliferation responses of cells cultured with PWM and IL-2 were 2-3 times greater in non-adherent than adherent subpopulations. Separation of cotton rat splenocytes by nylon wool adherence provided enriched splenocyte subpopulations.

INTRODUCTION

The cotton rat (Sigmodon hispidus) has served as a model for various pulmonary diseases (Ambrose and Wyde, 1993; Prince et al., 1993) and recently as a small-animal model for immunotoxicity studies (McMurry et al., 1994). Additionally, there have been increased desires among physiological ecologists to understand interrelationships among immunocompetence, environment, and demography in wild populations (Dobrowolska and Adamczewska-Andrzejewska, 1991). Use of the cotton rat in such models necessitates the development of baseline immunological techniques, which often require the enrichment of primary lymphocyte preparations. Of the many procedures available, adherence to nylon wool columns has been used most frequently, with varying degrees of success, to separate unpurified lymphocyte preparations into T-cell (non-adherent) and B-cell (adherent) subpopulations in laboratory mice (Julius et al., 1973) and rats (Havenith et al., 1992), chickens (Lamont and Van Alten, 1981), sheep (Gorrell et al., 1992), and humans (Van Schooten et al., 1992).

Information on the applicability of this technique for the cotton rat is lacking. The primary objective of this study was to examine the efficacy of nylon wool columns as a means of enriching T-cells through selective removal of B-cell and macrophage populations in the cotton rat. Pre-separated cells, and resulting cell fractions (non-adherent and adherent subpopulations) were characterized

phenotypically and functionally by subtyping with cell-surface markers and measuring lymphoproliferative responses to a suite of mitogens.

MATERIAL AND METHODS

Animals

Adult male and female inbred (28-30 generations) cotton rats originating from the National Institutes of Health (NIH) were housed in polypropylene boxes with wood shavings and fed commercially available rodent chow (Purina 5001, St. Louis, MO) and water ad libitum. The animal room was maintained on a 14L:10D photoperiod with an ambient temperature of 25 ± 1(SE)°C.

Preparation of cell suspensions

Cotton rats (n = 12) were anesthetized with an intramuscular injection of ketamine hydrochloride (Aveco Co. Inc., Fort Dodge, IA) at 50 mg/kg body mass and euthanized via cervical dislocation. The spleen was removed and mass determined to the nearest 0.1 mg. Spleens were gently disrupted in 5 ml of Roswell Park Memorial Institute-1640 (RPMI-1640, Sigma, St. Louis, MO) supplemented medium (RPMI-S, pH 7.2) containing RPMI-1640, L-glutamine (2.05 mM), sodium pyruvate (1.0 mM), non-essential amino acids (1.0 mM), 2-mercaptoethanol ($2x10^{-5}$ M), penicillin (100,000 U/L), streptomycin (100 mg/L) and normal horse serum (10%) (Sigma). Cell suspensions were washed and erythrocytes removed by lysing with tris-buffered (0.83%) ammonium chloride. Cell preparations designated for

lymphocyte subtyping (n = 6) were washed twice in 5 ml of phosphate buffered saline (PBS) supplemented with 5% fetal calf serum (FCS) (PBS-F) and cells counted. Cell preparations designated for lymphoproliferation response assays (n = 6) were washed twice in 5 ml of RPMI-S and cells counted. All cell counts were performed using a Serono 9000 cell counter (Serono-Baker Diagnostics, Allentown, PA).

Separation of cell populations using nylon wool

Splenic cell preparations were separated on combed, scrubbed, nylon wool using a modification of the procedure originally described by Julius et al. (1973). Briefly, 10-ml syringes fitted with leurlock stopcocks were packed to the 5 ml mark with 0.6 g of nylon wool (Robbins Scientific, Sunnyvale, CA) and columns rinsed once with 30 ml of PBS and twice with 30 ml of PBS-F prior to a 1 h incubation at 37°C. After incubation, columns were rinsed with 30 ml of warm (37°C) PBS-F. Cell suspensions were concentrated to a volume of 1.0 ml (7.45×10^7 - 1.73×10^8 total cells) and added to the column dropwise; the stopcock was opened slightly to allow the mixture to enter the nylon wool column. Cells were packed into the wool by addition of warm PBS-F (3 ml) to the top of each column; columns were incubated for 45 min at 37°C. Non-adherent cells were flushed from the column by adding PBS-F dropwise and collecting the first 20 ml of effluent in a 30 ml centrifuge tube. Previous analysis, following the procedure described above, indicated 20 ml of effluent contained 95% of all cells eluted from a nylon wool

column (Fig. 1), similar to laboratory mice (Coligan et al., 1992). To remove adherent cells, 3 ml of PBS was plunged vigorously through the nylon wool column three times and cells pooled. Both non-adherent and adherent subpopulations were washed in PBS, and cells adjusted to either $1.0x10^7$ cells/ml of PBS-F (lymphocyte subtyping) or $5.56x10^6$ cells/ml of RPMI-S (lymphoproliferative response assays). Viable cell counts were performed using the trypan blue exclusion technique.

Lymphocyte subtyping

Aliquots of cells were removed from the pre-separated population, non-adherent subpopulation, and adherent cell subpopulation for counting and phenotyping using three FITC-conjugated surface markers: peanut agglutinin (PNA, 250 µg/ml, Sigma), Helix pomatia agglutinin (HP, 100 µg/ml, Sigma) and rabbit-anti-rat immunoglobulin-G (BS, 1:10 in distilled water, Cappel Research Products, Durham, NC). We used a slight modification of the procedure described by Mattes and Holden (Mattes and Holden, 1981) to label lymphocytes from all three populations. Briefly, cells for PNA and HP labeling were washed in 5 ml Tris-buffered Hank's balanced salt solution (TH, Sigma) supplemented with 0.5% bovine serum albumin (BSA) and 0.02% sodium azide (TH-S, pH 7.45). Cells for BS labeling were washed in 5 ml of PBS supplemented with 5% BSA (PBS-BSA) and placed on ice. Cells for PNA and HP labeling were resuspended in 2 ml TH-S with 10 µl of neuraminidase type VI (1000 U/ml, Sigma) and

incubated for 45 min at 37°C (tubes were mixed by inversion mid-way through incubation). After incubation, cells were washed twice in 2 ml of TH-S and resuspended in 200 µl TH-S ($4.0x10^6$ cells). To label the splenocytes, aliquots of 100 µl ($2.0x10^6$ cells) were added to microcentrifuge tubes and centrifuged; resulting cell pellets were resuspended in the residual media, to which either 100 µl PNA, 100 µl HP or 40 µl BS was added followed by a 30 min incubation at either 4°C (PNA and HP) or 37°C (BS). Following incubation, cells were washed three times in 1 ml TH-S (PNA and HP) or PBS-BSA (BS) and resuspended in the residual media. Labeled cells were mounted on microscope slides in polyvinyl alcohol. Cells were phenotyped via epifluorescent microscopy under oil immersion (1000X magnification, Olympus BH-2). Cells were first detected under bright field illumination and then characterized as positive (stain speckled or capped on cell surface) or negative for the appropriate marker with the FITC-exciter filter (Jacobs Instrument Co., Shawnee Mission, KS) in position; relative abundance (%) of each phenotype was recorded.

Lymphoproliferation response assays

Functional characteristics of cells from all three populations were described using a lymphoproliferation response assay measuring cellular uptake of ^{3}H-thymidine in stimulated cultures. Aliquots of $5.0x10^5$ cells/90 µl were cultured in triplicate wells of a 96-well, polystyrene, flat-bottom tissue culture plate (Corning Glass Works,

Corning, NY) in the presence of concanavalin-A (Con-A, 5.0 µg/ml of culture, Sigma), pokeweed mitogen (PWM, 0.625 µg/ml of culture, Sigma), interleukin-2 (IL-2, 40.0 U/ml of culture, Boehringer Mannheim Corp., Indianapolis, IN) and protein extract from cell walls of Salmonella typhimurium (STM, 10 µg/ml of culture, RIBI ImmunoChem Research, Hamilton, MT). Controls consisted of aliquots of cells in the absence of mitogenic stimulation. After 54 h of incubation at 37°C, ^{3}H-thymidine (1.0 µCi/well) was added to each well and incubated an additional 18 h. Cells were harvested onto glass-fiber filters using a PHD Cell Harvester (Cambridge Technology Inc., Watertown, MA). Filters were air-dried prior to addition of 2.5 ml of Betamax ES scintillant (ICN Biochemicals Inc., Irvine, CA). Activity (counts per minute, CPM) of ^{3}H-thymidine was determined with a liquid scintillation counter (Beckman Instruments, Fullerton, CA) and stimulation indices calculated as the ratio of CPM for stimulated cultures to unstimulated control cultures.

Statistical methods

Homogeneity of variances among the three cell populations was examined using Levene's test (Snedecor and Cochran, 1980). A randomized block design (PROC ANOVA, SAS Institute, 1982), with animal (block effect) and subpopulation as main effects, was used to examine differences in the relative abundance of selected lymphocyte

subtypes and stimulation indices between non-adherent and adherent subpopulations.

RESULTS

Of the cells added to the nylon wool column, 37% were recovered in the non-adherent subpopulation and 18% recovered in the adherent subpopulation, yielding a total recovery of 55%. Cell viability ranged from 96% in the pre-separated cell population to 94% in the adherent subpopulation, indicating the plunging of adherent cells from the nylon wool did not affect their viability.

Variances were homogeneous ($\underline{P} \geq 0.109$) about the mean among the three cell populations for relative abundance of PNA^+, HP^+ and BS^+ splenocytes. Variances about the mean for stimulation indices of PWM, IL-2 and Con-A were heterogeneous ($\underline{P} = 0.004$, $\underline{P} = 0.020$, $\underline{P} = 0.042$ respectively) among the three subpopulations. Stimulation indices for PWM and IL-2 were more variable in the pre-separated subpopulation than other subpopulations; stimulation indices for Con-A showed similar variability between the pre-separated population and the non-adherent subpopulations, and decreased variability in the adherent subpopulations (Fig. 3-4). Variances were homogeneous ($\underline{P} = 0.091$) about the mean among the three cell populations for stimulation indices of cells in response to STM.

There was a decrease ($\underline{P} = 0.027$) in the relative abundance of PNA^+ cells in the adherent fraction compared to those in the non-adherent subpopulation; relative abundance

of HP^+ cells remained similar in both subpopulations (Fig. 2). Relative abundance of BS^+ cells was lower ($P = 0.004$) in the non-adherent than adherent subpopulation (Fig. 2); the PNA^+:BS^+ ratio was 4 times greater ($P = 0.021$) in the non-adherent than adherent subpopulation (Fig. 2).

Lymphoproliferative responses of cells cultured with PWM and IL-2 were greater ($P = 0.029$, $P = 0.028$, respectively) in the non-adherent compared to adherent subpopulations (Fig. 3). Lymphoproliferative responses to Con-A and STM stimulation were similar ($P \geq 0.341$) among all three cell subpopulations (Fig. 3).

DISCUSSION

Nylon wool has been reported to deplete lymphoid cell preparations of immunoglobulin (Ig)-bearing cells (B-cells) and macrophages due to their adherence to nylon wool (Julius et al., 1973), resulting in an enriched T-cell fraction. The efficacy of this procedure varies among animal species. B-cells in splenocyte preparations were reduced from 45% to 2-5% in the non-adherent subpopulations of laboratory mice (Julius et al., 1973) and only reduced to 24% in the non-adherent subpopulations of chickens (Lamont and Van Alten, 1981) following nylon wool-separation. The BS^+ cells of cotton rats were reduced from 26% (Fig. 4) in the pre-separated population to 11% (Fig. 2) in the non-adherent subpopulation, indicating this subtype is adhering to the nylon wool.

Observed reductions in the relative abundance of PNA^+ cells in the adherent subpopulation of cotton rats was in agreement with previous studies indicating that PNA binds preferentially to T-cells (typically immature T-cells, with some binding of non-T-cells) in mice (London et al., 1978; London, 1980), cows (Nagi and Babiuk, 1989) and chickens (Uni and Heller, 1991). Additionally, the increased PNA^+:BS^+ ratio in the non-adherent subpopulation, compared to adherent subpopulation (Fig. 2), indicates the non-adherent subpopulation is enriched in PNA^+ splenocytes. Although HP is primarily a T-cell and natural killer cell (non-T, non-B cells) surface marker in cows (Morein et al., 1979) laboratory mice (Haller et al., 1978; Mattes and Holden, 1981) and laboratory rats (Swanborg et al., 1977), we observed no difference in relative abundance of HP^+ cells among cell subpopulations in the cotton rat. This suggested that some subfraction(s) of HP^+ cells in the cotton rat adhered to nylon wool. Some of the HP^+ cells in the adherent subpopulation of cotton rats may be B-cells, as a portion of B-cells in laboratory rats has been documented to remain bound to Helix lectin-agarose columns during fractionation techniques (Mattes and Holden, 1981). Approximately 50% of the non-adherent cells were PNA^+, and with the HP^+ cells, comprised about 89% of the non-adherent subpopulation in cotton rats.

Responses of pre-separated cells of cotton rats to PWM stimulation, primarily a T- and B-cell mitogen (Greaves and

Janossy, 1972), were seven times greater than responses of adherent and non-adherent subpopulations (Fig. 4). The higher stimulation index of pre-separated cells is primarily attributed to the presence of all lymphocyte subpopulations (T- and B-cells, accessory cells). The dependence of B-cell proliferation on the presence of both T-cells and macrophages (Tizard, 1987) could have contributed to lower responses to PWM in the non-adherent subpopulation. Lower responses to PWM in the adherent subpopulation may be due in part to the relative absence of T-cells, hence, decreased T-cell-dependent B-cell proliferation. A similar explanation may be used for the decreased response to IL-2 stimulation in adherent subpopulations, as IL-2 costimulates proliferation and differentiation of B-cells and stimulates proliferation of T-cells, NK-cells, and monocytes (Coffman et al., 1990; Delves and Roitt, 1992). Furthermore, the decreased variability (see standard error bars, Fig. 3-4) of PWM and IL-2 stimulation indices in non-adherent and adherent subpopulations indicates nylon wool is effectively enriching cell populations.

The lack of any differences in stimulation indices between the non-adherent and adherent subpopulations for Con-A and STM were unexpected. Because Con-A predominantly stimulates T-cells (Greaves and Janossy, 1972) and STM stimulates B-cells (Severinson and Larsson, 1986), we expected Con-A stimulation indices to be greatest in the non-adherent subpopulation and lowest in the adherent

subpopulation. Similarly, STM stimulation indices were expected to increase in the adherent subpopulation and decrease in the non-adherent subpopulation. Similar responses between the non-adherent and adherent cell subpopulations following Con-A and STM stimulation suggest that contaminating T-cell subsets were present in the adherent subpopulation and B-cells were present in the non-adherent subpopulation. Interestingly, stimulation indices for Con-A were less variable in the adherent subpopulations, whereas, stimulation indices for STM showed no change in variability about the mean.

Phenotypic characterization of splenic preparations in the cotton rat using cell surface markers indicates that BS^+ cells are predominately adherent to nylon wool, PNA^+ cells are predominately non-adherent to nylon wool, and HP^+ cells apparently consist of both adherent and non-adherent subtypes. Additionally, lymphoproliferative responses to PWM and IL-2 indicate nylon wool columns are efficient at partially enriching splenic preparations for T-cells. The decreased variability in functional assays among cotton rats following nylon wool separation, as indicated by PWM, IL-2 and Con-A stimulation indices, further indicate partial enrichment of splenic preparations. However, responses to mitogens and BS labeling indicated that non-adherent subpopulations possess contaminating B-cell subpopulations.

REFERENCES

Ambrose, M.W. and Wyde, P.R., 1993. Parainfluenza virus type 3 (PIV3)-specific and non-virus-specific delayed type hypersensitivity responses in cotton rats given different PIV3 antigen preparations. Vaccine, 11:336-342.

Bradley, A.J., McDonald, I.R., Lee, A.K., 1980. Stress and mortality in a small marsupial (Antechinus stuartii Macleay). Gen. Comp. Endocrinol., 40:188-200.

Coffman, R.L., O'Garra, A., Volkman, D.J., 1990. Interleukins and interferons acting on B lymphocytes. In: J.J. Oppenheim, and E.M. Shevach (Editors), Immunophysiology: The role of cells and cytokines in immunity and inflammation. Oxford University Press, New York, pp. 88-103.

Coligan, J.E., Kruisbeek, A.M., Marguiles, D.H., 1992. In vitro assays for mouse lymphocyte function. In: E.M. Shevach, and W. Strober (Editors), Current protocols in immunology. John Wiley and Sons, New York, pp. 3.0.1-3.5.6.

Delves, P.J. and Roitt, I.M., 1992. Interleukin 2. In: I.M. Roitt, and P.J. Delves (Editors), Encyclopedia of immunology. Academic Press, San Diego, pp. 903-906.

Dobrowolska, A. and Adamczewska-Andrzejewska, K.A., 1991. Seasonal and long-term changes in serum gamma-globulin levels in comparing the physiology and population density of the common vole, Microtus arvalis Pall. 1779. J. Interdiscipl. Cycl. Res., 22:1-19.

Gorrell, M.D., Brandon, M.R., Sheffer, D., Adams, R.J., Narayan, O., 1992. Ovine lentivirus is macrophagetropic and does not replicate productively in T lymphocytes. J. Virol., 66:2679-2688.

Greaves, M.F. and Janossy, G., 1972. Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. Transplant. Rev., 11:87-130.

Haller, O., Gildlund, M., Hellstrom, U., Hammarstrom, S., Wigzell, H., 1978. A new surface marker on mouse natural killer cells: receptors for Helix pomatia A hemagglutinin. Eur. J. Immunol., 8:765-771.

Havenith, C.E.G., Breedijk, A.J., Verdaasdonk, M.A.M., Kamperijk, E.W.A., Beelen, R.H.J., 1992. An improved and rapid method for the isolation of rat lymph node or spleen T lymphocytes for T cell proliferation assays. J. Immunol., 153:73-79.

Julius, M.H., Simpson, E., Herzenberg, L.A., 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol., 3:645-649.

Lamont, S.J. and Van Alten, P.J., 1981. Characteristics of nylon fiber adherence-separated chicken splenocytes. J. Immunol., 40:181-191.

London, J., 1980. Peanut agglutinin VI. Identification of murine T lymphocyte subsets during ontogenesis by use of peanut agglutinin and Ly-6.2 antiserum. J. Immunol., 125:1702-1707.

London, J., Berrih, S., Bach, J.F., 1978. Peanut agglutinin I. A new tool for studying lymphocyte subpopulations. J. Immunol., 121:438-443.

Mattes, M.J. and Holden, H.T., 1981. The distribution of Helix pomatia lectin receptors on mouse lymphoid cells and other tissues. Eur. J. Immunol., 11:358-365.

Morein, B., Hellstrom, U., Axelsson, L.G., Johansson, C., Hammarstrom, S., 1979. Helix pomatia A hemagglutinin, A surface marker for bovine T-lymphocytes. Vet. Immunol. Immunopathol., 1:27-36.

Nagi, A.M. and Babiuk, L.A., 1989. Peanut agglutinin (PNA): binding and stimulation of bovine intestinal and peripheral blood leukocytes. Vet. Immunol. Immunopathol., 22:67-78.

Prince, G.A, Porter, D.D., Jenson, A.B., Horswood, R.L., Chanock, R.M, and Ginsberg, H.S. 1993. Pathogenesis of adenovirus type 5 pneumonia in cotton rats (Sigmodon hispidus). J. Virol., 67:101-111.

SAS Institute, Inc., 1982. SAS user's guide; Statistics. SAS Institute, Inc. Cary, NC., 584 pp.

Severinson, E. and Larsson, E.L., 1986. Lymphocyte responses to polyclonal B and T cell activators. In: D.M. Weir (Editor), Cellular Immunology. Blackwell Scientific Publishers, Oxford, pp. 63.1-63.18.

Snedecor, G.W. and Cochran, W.G., 1980. Statistical Methods, 7. Iowa State University Press, Ames, IA, 507 pp.

Swanborg, R.H., Hellstrom, U., Perlmann, H., Hammarstrom, S. Perlmann, P., 1977. A new rat lymphocyte surface marker: Characterization and separation of cells with receptors for Helix pomatia hemagglutinin. Scand. J. Immunol., 6:235-239.

Tizard, I., 1987. Veterinary immunology: An Instruction, 3. W.B. Saunders; Philadelphia, PA., 401 pp.

Uni, Z. and Heller, E.D., 1991. Peanut agglutinin as a marker of maturation and activation of chicken thymic-derived lymphocytes. Poult. Sci., 70:1516-1520.

Van Schooten, W.C.A., Maiore, T., Jones, K.R., Paborsky, L., 1992. Purified HLA class II peptide complexes can induce adherence and activation of peptide-specific human T cell clones. J. Immunol., 148:1043-1048.

Figure 1. Recovery of cotton rat splenocytes (mean ± SE) per cumulative ml of effluent passed through a nylon wool column; recovery also presented as percent of the total splenocytes (mean ± SE) eluted.

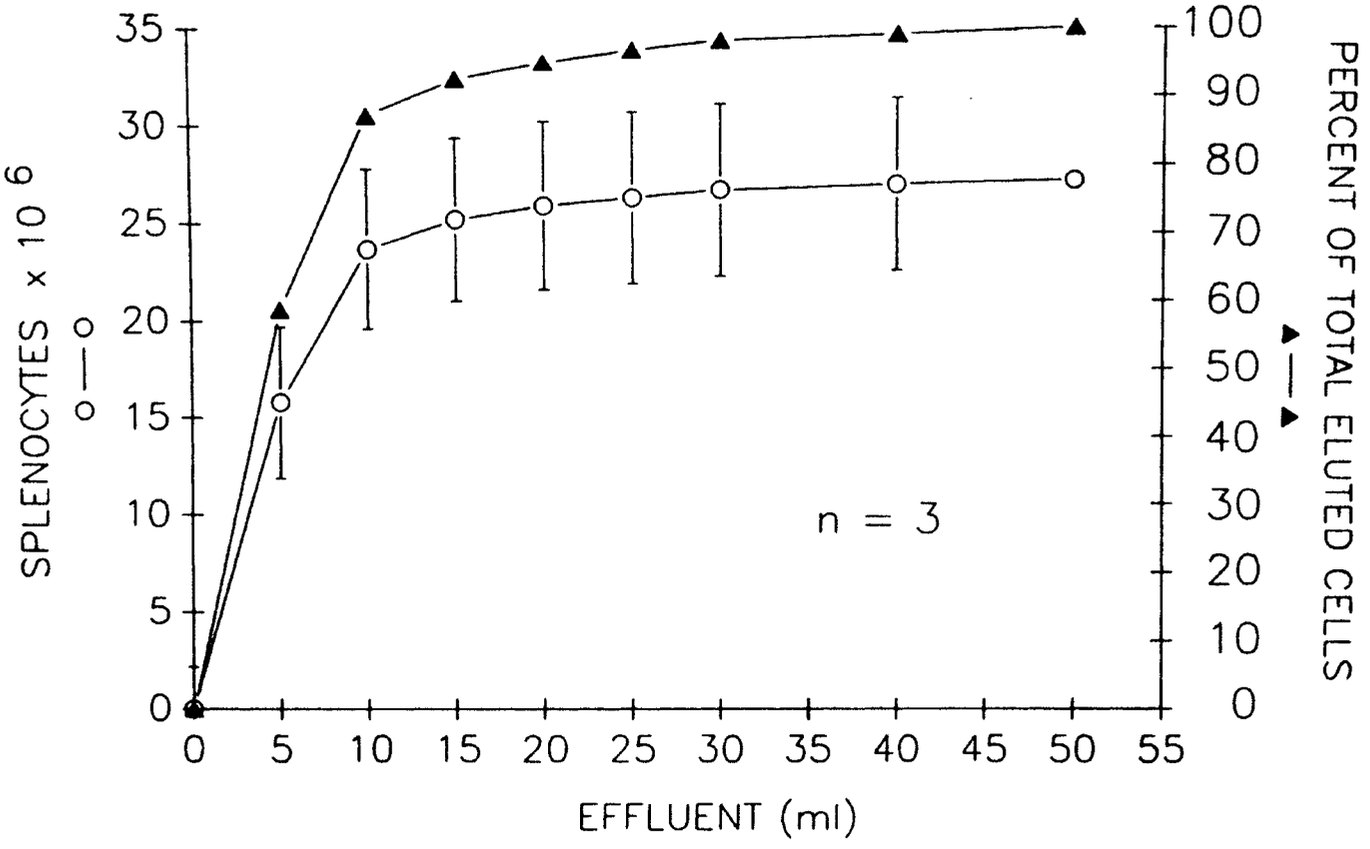

SPLENOCYTES x 10 6
o—o
35
30
25
20
15
10
5
0
0 5 10 15 20 25 30 35 40 45 50 55
EFFLUENT (ml)
n = 3
100
90
80
70
60
50
40
30
20
10
0
PERCENT OF TOTAL ELUTED CELLS
▲—▲

Figure 2. Relative abundance (%) of cotton rat splenocyte subtypes (mean ± SE) in non-adherent (NAC) and adherent (AC) subpopulations following nylon wool separation. Splenocyte subtypes were determined by cell surface staining with fluorescein isothiocyanate-conjugated (a) peanut agglutinin (PNA, 250 μg/ml), (b) Helix pomatia agglutinin (HP, 100 μg/ml), (c) rabbit-anti-rat immunoglobulin-G (BS, 1:10) and (d) the ratio of PNA^+ splenocytes to BS^+ splenocytes. Bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

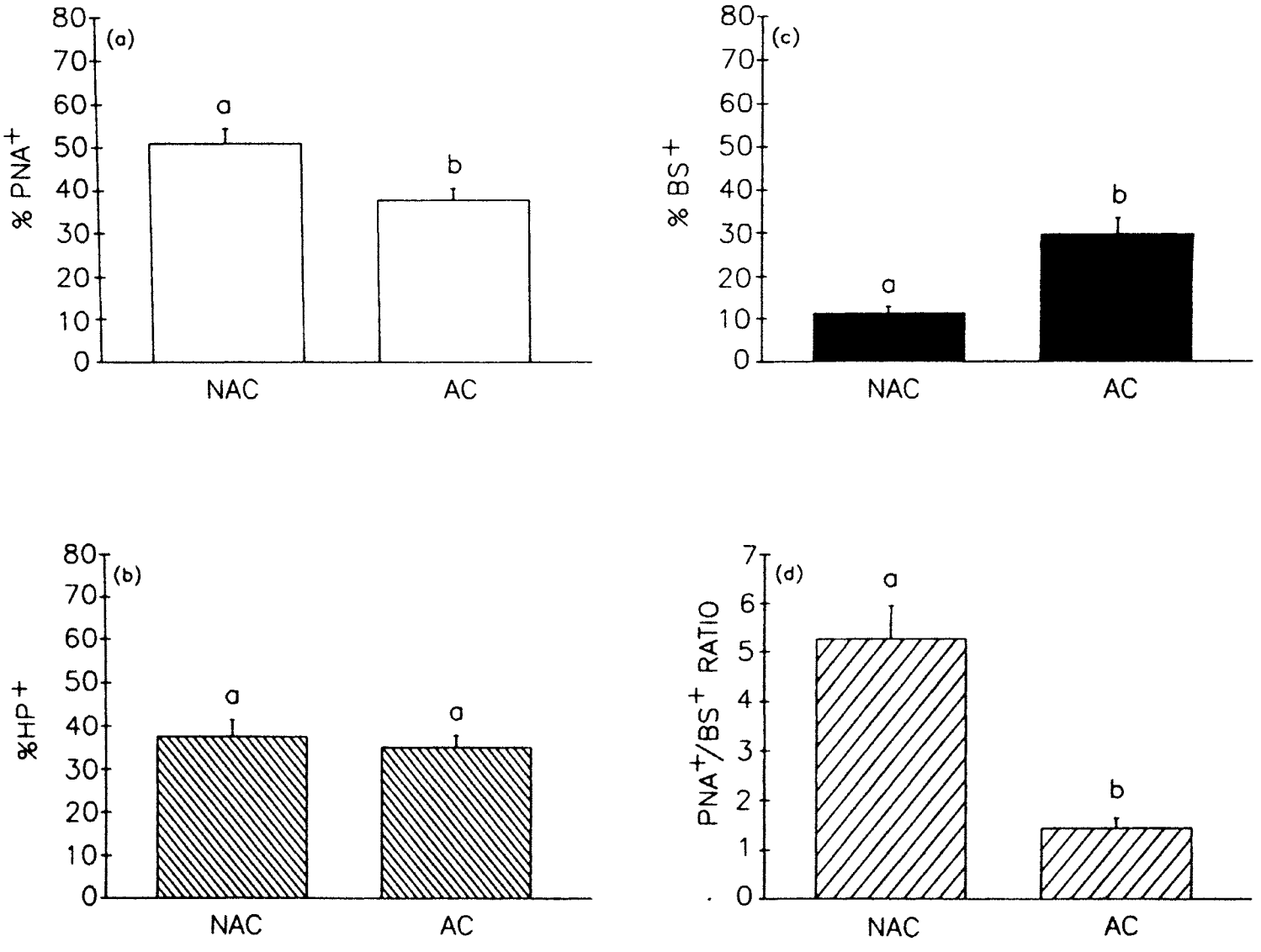

(a)
% PNA+
80
70
60
50
40
30
20
10
0
a
b
NAC
AC
(c)
% BS+
a
b
NAC
AC
(b)
%HP+
a
a
NAC
AC
(d)
PNA+/BS+ RATIO
7
6
5
4
3
2
1
0
a
b
NAC
AC

Figure 3. Stimulation indices represented as the ratio of ^{3}H-thymidine (1.0 μCi/well) uptake by stimulated cells to that of unstimulated control cells for cotton rat splenocytes in non-adherent (NAC) and adherent (AC) subpopulations following nylon wool separation. Responses to (a) concanavalin-A (Con-A, 5.0 μg/ml culture), (b) pokeweed mitogen (PWM, 0.625 μg/ml culture), (c) interleukin-2 (IL-2, 40.0 U/ml), and (d) protein extract from cell walls of Salmonella typhimurium (STM, 10 μg/ml culture) were measured. Bars with no superscripts in common indicate significantly different means ($P < 0.05$) using the test for least significant difference.

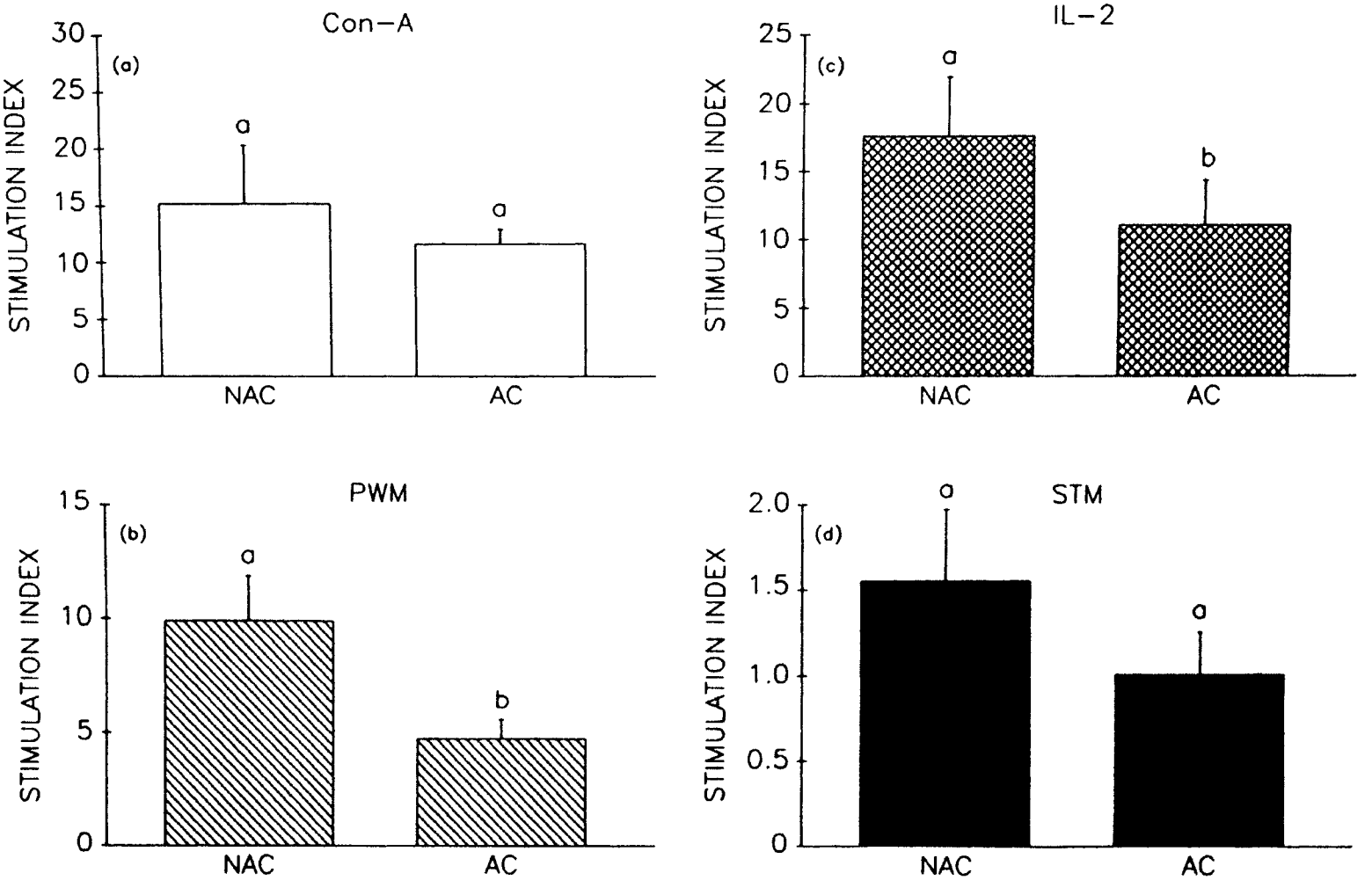
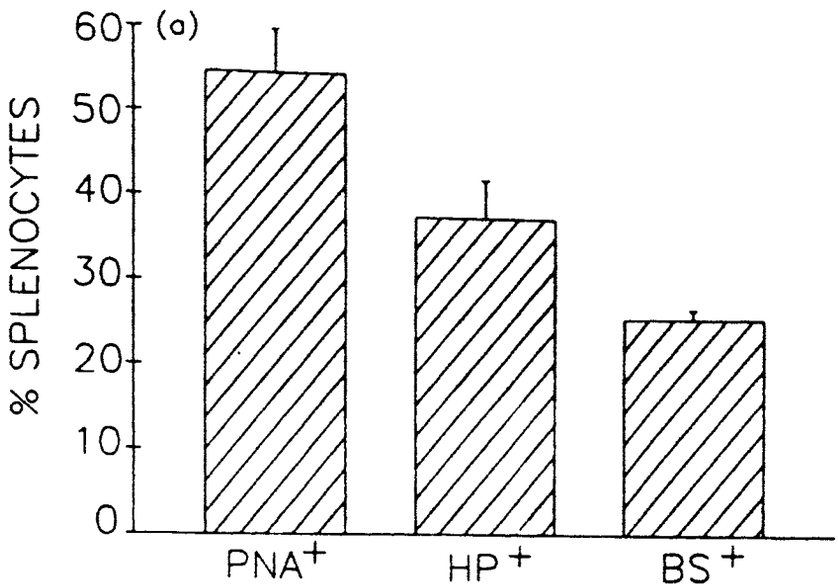
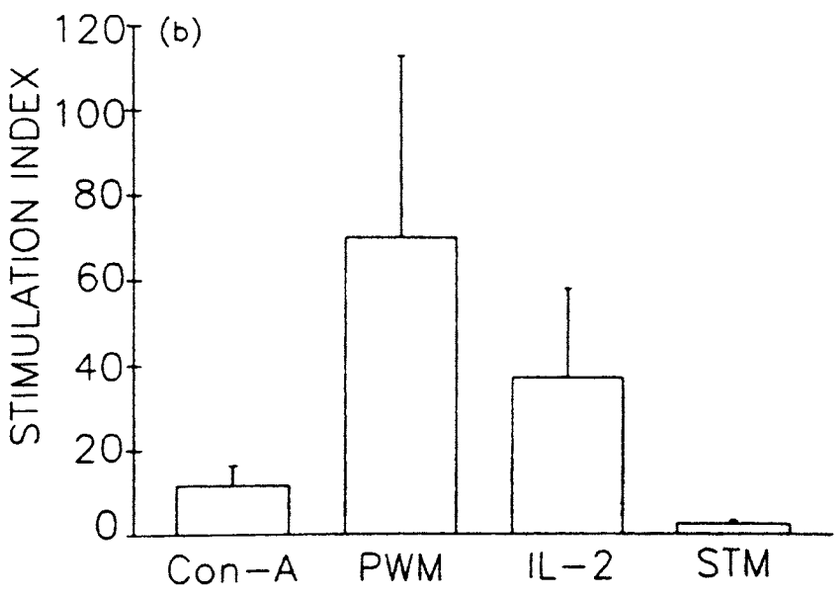

Con-A
(a)
STIMULATION INDEX
30
25
20
15
10
5
0
a
a
NAC
AC
IL-2
(c)
25
20
15
10
5
0
a
b
PWM
(b)
15
10
5
0
a
b
STM
(d)
2.0
1.5
1.0
0.5
0

Figure 4. Phenotypic and functional characteristics of pre-nylon wool-separated suspensions of cotton rat splenocytes. (a) Percent of splenocytes (mean ± SE) positive for fluorescein isothiocyanate conjugated peanut agglutinin (PNA, 250 µg/ml), Helix pomatia agglutinin (HP, 100 µg/ml) and rabbit-anti-rat immunoglobulin-G (BS, 1:10). (b) Stimulation indices (counts per minute (CPM); mean ± SE) represented as the ratio of ^{3}H-thymidine (1.0 µCi/well) uptake by stimulated cells to that of unstimulated control cells in response to concanavalin-A (Con-A, 5.0 µg/ml culture), interleukin-2 (IL-2, 40.0 U/ml culture), pokeweed mitogen (PWM, 0.625 µg/ml culture), and Salmonella typhimurium (STM, 10 µg/ml culture).


(a)
% SPLENOCYTES
60
50
40
30
20
10
0
PNA+
HP+
BS+


(b)
STIMULATION INDEX
120
100
80
60
40
20
0
Con-A
PWM
IL-2
STM

VITA

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Candidate for the Degree of

Master of Science

Thesis: CHARACTERIZATION OF THE IMMUNE SYSTEM OF THE HISPID COTTON RAT (SIGMODON HISPIDUS) IN RESPONSE TO DIETARY PROTEIN, TEMPORAL CHANGES AND THE EFFICACY OF NYLON-WOOL SEPARATION OF SPLENOCYTES

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