Phenotypic and Functional Characterization of Cotton Rat (*Sigmodon hispidus*) Splenocytes Separated on Nylon Wool

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We investigated the efficacy of nylon wool columns for the partial purification of splenocyte subpopulations in the cotton rat (*Sigmodon hispidus*). Lymphocytes within the unseparated cell population and the nonadherent 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 nonadherent, BS+ cells were adherent and HP+ cells were not uniquely characterized as adherent or nonadherent. The PNA+:BS+ ratio in nonadherent subpopulations was 4 times that in adherent subpopulations. In nonadherent subpopulations lymphoproliferation responses of cells cultured with PWM and IL-2 were 2-3 times that in adherent subpopulations. Separation of cotton rat splenocytes by nylon wool adherence provided enriched splenocyte subpopulations.

INTRODUCTION

Increased desires among physiological ecologists to understand interrelationships among immunocompetence, environment, and demography in wild populations (*1*) has necessitated the development of baseline immunological techniques applicable to wild animal species. Such techniques 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 (nonadherent) and B-cell (adherent) subpopulations in laboratory mice (*2*) and rats (*3*), chickens (*4*), sheep (*5*), and humans (*6*).

Information on the applicability of this technique for wild rodent species such as the hispid cotton rat (*Sigmodon hispidus*) 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. Unseparated cells and resulting cell fractions (nonadherent 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 its 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), nonessential amino acids (1.0 mM), 2-mercaptoethanol (2×10^{-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. The cell preparations

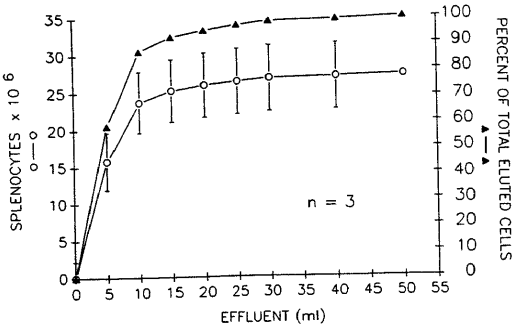

Figure 1. Recovery of cotton rat splenocytes (mean±SE) per cumulative ml of effluent passed through a nylon wool column; recovery also presented as percent of the total splenocytes (mean±SE) eluted.

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 (Robbins Scientific, Sunnyvale, CA) using a modification of the procedure originally described by Julius et al. (*2*). Briefly, 10-ml syringes fitted with leurlock stopcocks were packed to the 5-ml mark with 0.6 g of nylon wool 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. Nonadherent 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 results with laboratory mice (*7*). To remove adherent cells, 3 ml of PBS was vigorously forced (by using the syringe plunger) through the nylon wool column three times and cells pooled. Both nonadherent and adherent subpopulations were washed in PBS, and cells adjusted to either 1.0×10^7 cells/ml of PBS-F (lymphocyte subtyping) or 5.56×10^6 cells/ml of RPMI-S (lymphoproliferative response assays). Viable cell counts were performed by using the Trypan Blue exclusion technique.

Lymphocyte Subtyping. Aliquots of cells were removed from the unseparated population, nonadherent subpopulation, and adherent cell subpopulation for counting and phenotyping by 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 (*8*) 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 (4.0×10^6 cells). To label the splenocytes, aliquots of 100 μl (2.0×10^6 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 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 poly(vinyl alcohol). Cells were phenotyped via epifluorescent microscopy under oil immersion (1000X magnification, Olympus BH-2). Cells were first detected under bright field

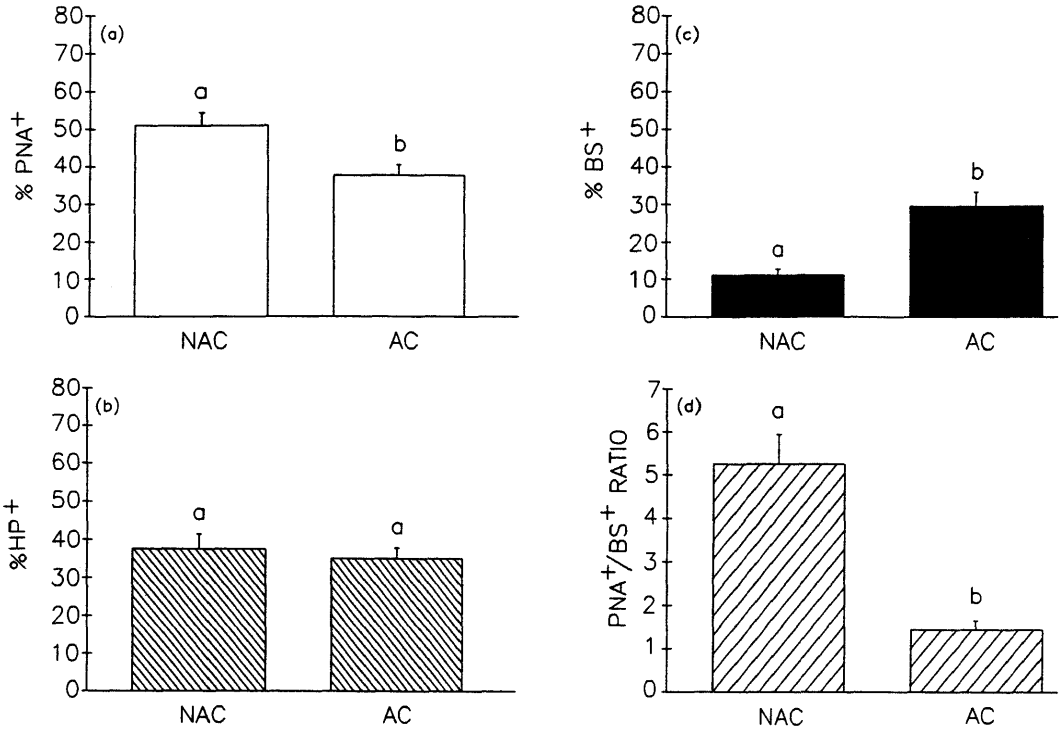

Figure 2. Relative abundance (%) of cotton rat splenocyte subtypes (mean±SE) in nonadherent (NAC) and adherent (AC) subpopulations following nylon wool separation. Splenocyte subtypes were determined by cell surface staining with fluorescein isothiocyanate-conjugated (a) peanut agglutinin (PNA, 250 μg/ml), (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 ($P<0.05$) according to the test for least significant difference.

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 5.0×10^5 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) or protein extract from cell walls of *Salmonella typhimurium* (STM, 10 μg/ml of culture, RIBI Immuno-Chem 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) was added to each well and incubated an additional 18 h. Cells were harvested onto glass-fiber filters with 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 (*9*). A randomized block design (PROC ANOVA, *10*), with animal (block effect) and subpopu-

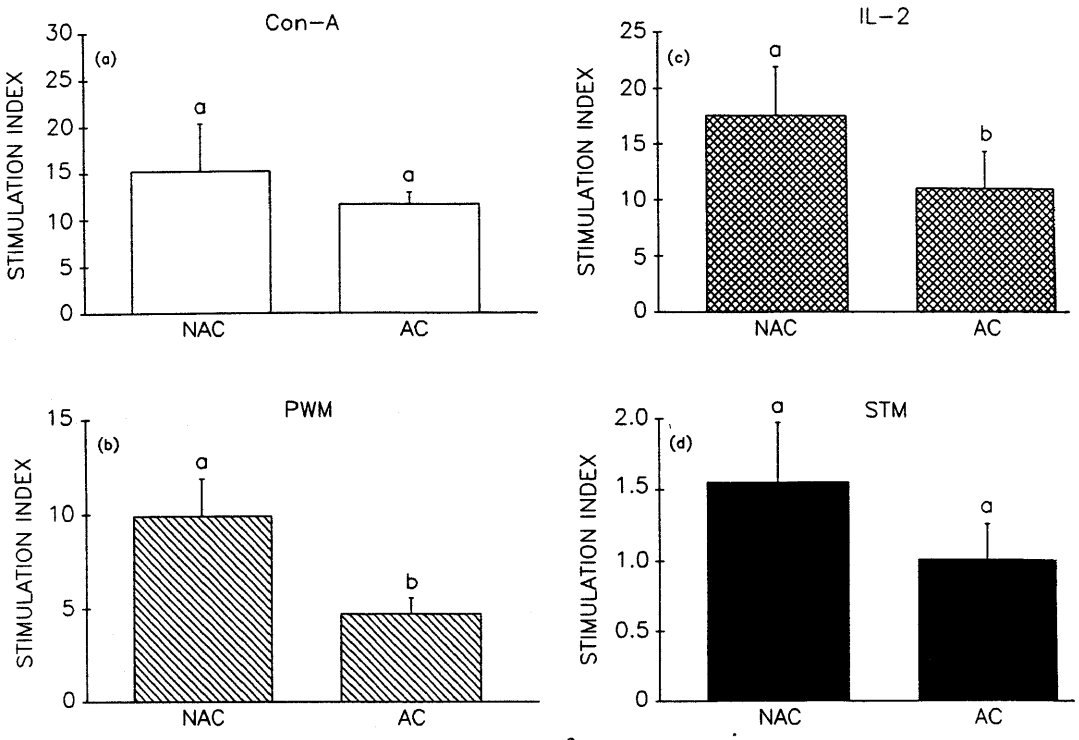

Figure 3. Stimulation indices represented as the ratio of [^{3}H]-thymidine (1.0 μCi/well) uptake by stimulated cells to that of unstimulated control cells for cotton rat splenocytes in nonadherent (NAC) and adherent (AC) subpopulations following nylon wool separation. Responses to (a) concanavalin-A (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 ($P<0.05$) according to the test for least significant difference.

lation as main effects, was used to examine differences in the relative abundance of selected lymphocyte subtypes and stimulation indices between nonadherent and adherent subpopulations.

RESULTS

Of the cells added to the nylon wool column, 37% were recovered in the nonadherent subpopulation and 18% recovered in the adherent subpopulation, yielding a total recovery of 55%. Cell viability ranged from 96% in the unseparated cell population to 94% in the adherent subpopulation, indicating that the method of separating adherent cells from the nylon wool did not affect their viability.

Variances were homogeneous (P = 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 (P = 0.004, 0.020, 0.042 respectively) among the three subpopulations. Stimulation indices for PWM and IL-2 were more variable in the unseparated population than subpopulations; stimulation indices for Con-A showed similar variability between the unseparated population and the nonadherent subpopulations, and decreased variability in the adherent subpopulations (Figs. 3, 4). Variances were homogeneous (P = 0.091) about the mean among the three cell populations for stimulation indices of cells in response to STM.

The relative abundance of PNA+ cells is greater (P = 0.027) in the nonadherent fraction than in the adherent subpopulation; relative abundance of HP+ cells is similar in both subpopulations (Fig. 2). Relative abundance of BS+ cells was lower (P = 0.004) in the nonadherent than adherent subpopulation (Fig. 2); the PNA+:BS+ ratio was 4 times as great (P = 0.021) in the

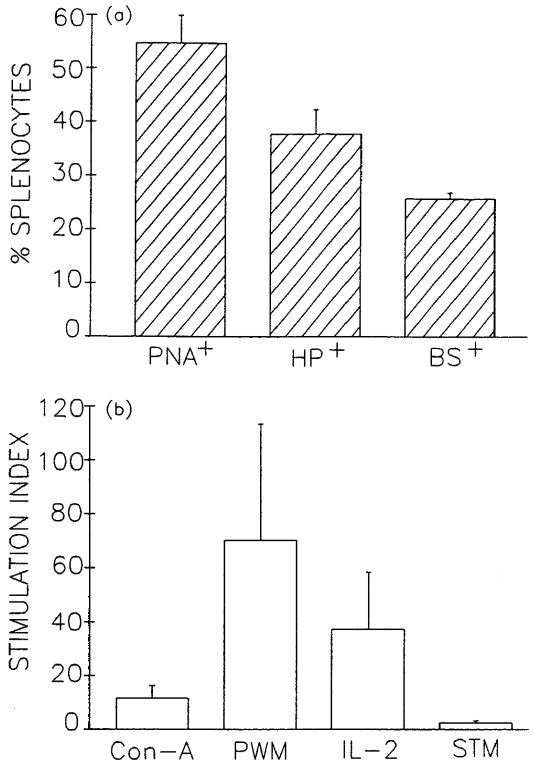

Figure 4. Phenotypic and functional characteristics of pre-nylon wool-separated suspensions of cotton rat splenocytes. (a) Percent of splenocytes (mean±SE) positive for fluorescein isothiocyanate-conjugated peanut agglutinin (PNA, 250 μg/ml), *Helix pomatia* agglutinin (HP, 100 μg/ml) and rabbit-anti-rat 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).

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

DISCUSSION

Nylon wool depletes lymphoid cell preparations of immunoglobulin (Ig)-bearing cells (B-cells) and macrophages owing to their adherence to nylon wool (*2*), 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 nonadherent subpopulations of laboratory mice (*2*) but only reduced to 24% in the nonadherent subpopulations of chickens (*4*) following separation on nylon wool. The BS+ cells of cotton rats were reduced from 26% (Fig. 4) in the unseparated population to 11% (Fig. 2) in the nonadherent 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 to non-T-cells) in mice (*11*, *12*), cows (*13*), and chickens (*14*). Additionally, the increased PNA+:BS+ ratio in the nonadherent subpopulation, compared to the adherent subpopulation (Fig. 2), indicates that the nonadherent 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 (*15*), laboratory mice (*8*, *16*), and laboratory rats (*17*), we observed no difference in relative abundance of HP+ cells among cell subpopulations in the cotton rat. This suggests that some subfraction(s) of HP+ cells in the cotton rat adhere 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 remain bound to *Helix* lectin-agarose columns during fractionation techniques (*8*). Approximately 50% of the nonadherent cells were PNA+, and with the HP+ cells, comprised ~89% of the nonadherent subpopulation in cotton rats.

Responses of unseparated cells of cotton rats to PWM stimulation, primarily a T-and B-cell mitogen (*18*), were seven times those of adherent and nonadherent subpopulations (Fig. 4). The higher stimulation index of unseparated cells is primarily attributed to the presence of all lymphocyte subpopulations (T- and B-cells, accessory cells). The reduced numbers of B-cells (*19*) could have contributed to lower responses to PWM in the nonadherent 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 co-stimulates proliferation and differentiation of B-cells and stimulates proliferation of T-cells, NK-cells, and monocytes (*20, 21*). Furthermore, the decreased variability (see standard error bars, Figs. 3, 4) of PWM and IL-2 stimulation indices in nonadherent and adherent subpopulations compared with the unseparated population indicates nylon wool is effectively enriching cell populations.

The lack of any difference in stimulation indices between the nonadherent and adherent subpopulations for Con-A and STM was unexpected. Because Con-A predominantly stimulates T-cells (*18*) and STM stimulates B-cells (*22*), we expected Con-A stimulation indices to be greatest in the nonadherent subpopulation and lowest in the adherent subpopulation. Similarly, STM stimulation indices were expected to increase in the adherent subpopulation and decrease in the nonadherent subpopulation. Lack of different responses between the nonadherent and adherent cell subpopulations following Con-A and STM stimulation suggests that contaminating T-cell subsets were present in the adherent subpopulation and B-cells were present in the nonadherent subpopulation. 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 nonadherent to nylon wool, and HP+ cells apparently consist of both adherent and nonadherent subtypes. Additionally, lympho-proliferative 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 nonadherent subpopulations contain contaminating B-cell subpopulations.

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