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Nonspecific Lymphocyte Responses in F344 and LEW Rats: Susceptibility to Murine Respiratory Mycoplasmosis and Examination of Cellular Basis for Strain Differences

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Mycoplasma pulmonis **produces a mitogen which may play a role in the pathogenesis of murine respiratory mycoplasmosis in rats. Since LEW rats are more susceptible to this disease than F344 rats are, these two strains were used to examine a possible association between disease severity and the level of nonspecific lymphocyte stimulation by mitogens, including *M. pulmonis* membrane preparations. F344 and LEW spleen, lung, blood, and lymph node lymphocytes were exposed to various mitogens. LEW lymphocytes gave a significantly higher response to mitogenic stimulation, regardless of their anatomical source. These differences in lymphocyte responsiveness were primarily due to differences within the nonadherent cell population. Significantly higher numbers of W3/25$^+$ (T helper) cells were found in LEW lymphoid populations, whereas no difference was found in MRC OX-8$^+$ (T suppressor/cytotoxic) cells. These data suggest an association between disease severity and host responsiveness to nonspecific stimuli.**

Murine respiratory mycoplasmosis (MRM) due to *Mycoplasma pulmonis* infection is a chronic, naturally occurring respiratory disease of laboratory rats (10). We have shown that LEW rats develop more severe pulmonary and upper respiratory tract lesions after intranasal inoculation of *M. pulmonis* than do F344 rats, even when matched for age, sex, and microbial and environmental factors (15). One of the principal histological differences between the two strains is the striking increase in the number of lymphocytes, including an increase in the amount of bronchial-associated lymphoid tissue, in the lungs of infected LEW rats compared with that in the lungs of infected F344 rats (15). All B-lymphocyte and plasma cell populations as well as total T-lymphocyte numbers dramatically increase in the LEW strain for at least 120 days after infection. In contrast, only T cells and immunoglobulin A (IgA)-bearing B lymphocytes and plasma cells (IgM, IgG, and IgA) show increased numbers in F344 animals. The increase is less than in LEW animals and reaches a maximum at 21 days postinfection (17).

Both viable *M. pulmonis* and crude membrane (MPM) preparations of this organism can nonspecifically activate lymphocytes (37). This mitogenic activity is associated with an external membrane protein of the mycoplasma and primarily stimulates B lymphocytes, although some T lymphocytes may also be activated (36). One possible explanation for the differences in MRM between LEW and F344 rats is that the variation in lesions may be due in part to differences in the responsiveness of LEW and F344 lymphocytes to the mitogenic activity of *M. pulmonis*. The present in vitro studies were undertaken to examine this hypothesis. MPM and other mitogens were used to compare the responsiveness of LEW and F344 lymphocytes, the effects of antigen-primed cells and immune serum on the responses to MPM were examined, and the cellular parameters responsible for the differences were investigated. LEW lymphocytes had a significantly higher nonspecific response than F344 lymphocytes to MPM, phytohemagglutinin (PHA; mitogenic for T lymphocytes [41]), pokeweed mitogen (PWM; a T-dependent, B-lymphocyte mitogen [24]), and *Salmonella typhimurium* mitogen (STM; a B-cell mitogen). These differences in lymphocyte responsiveness were shown to be primarily due to differences in the nonadherent cell population and appear to be related to a relatively higher proportion of W3/25$^+$ (T helper) cells in LEW lymphoid populations.

MATERIALS AND METHODS

Animals. Male and female pathogen-free LEW and F344/Ld rats, 8 to 12 weeks old, reared and maintained in Trexler-type plastic film isolators, were used in all experiments (8, 30). Within any single experiment, either male rats exclusively or equal numbers of males and females of each strain were used. The pathogen-free status of animal colonies was monitored by serological (including enzyme-linked immunosorbent assay [ELISA] for serum antimycoplasmal antibodies) and cultural techniques as described previously for mycoplasmal, viral, fungal, and other bacterial pathogens (9, 15). The colony was consistently negative for all pathogens, and the animals used were negative for IgG and IgM antimycoplasmal antibodies.

Mycoplasmas. *M. pulmonis* UAB 5782, used for these studies, was isolated from the lungs of a rat with natural MRM. The initial isolate was cloned three times in the medium described by Hayflick (21) and identified as a pure culture of *M. pulmonis* by immunofluorescence (18).

Mitogens. (i) MPM. *M. pulmonis* UAB 5782 was grown at 37°C in medium containing 20 g of mycoplasma broth base per liter without crystal violet (GIBCO Laboratories, Grand Island, N.Y.), supplemented with 20% gamma-globulin-free horse serum (GIBCO), 0.5% glucose, and 0.5% CVAR enrichment (GIBCO) at pH 7.8. Cultures were harvested in the late-log phase of growth by centrifugation for 20 min at 4,000 × *g* at 4°C. The pellets were then used in the preparation of a crude membrane fraction by osmotic lysis in

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distilled water after preloading the organisms with 2 M glycerol as described by Razin (40) and Rottem et al. (43). Unlysed organisms were pelleted by centrifugation at 4,000 × *g* at 4°C for 20 min, and membranes were harvested from the supernatants by centrifugation at 30,000 × *g* at 4°C for 1 h. Protein determinations were made on the membrane preparation by the micromethod of Bio-Rad Laboratories, Richmond, Calif. (4), and the concentration of MPM was adjusted to 200 μg of protein per ml. The absence of viable organisms was verified by culture, and the MPM was stored in aliquots at −70°C.

(ii) Other mitogens. PHA-M (Difco Laboratories, Inc., Detroit, Mich.), PWM (GIBCO), and STM (Ribi Immunochemical Research, Inc., Hamilton, Mont.) were diluted as specified by the manufacturers.

The final dilutions of all mitogens were prepared on the day of use in RPMI 1640 (GIBCO) containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Research Organics, Cleveland, Ohio), 0.2% $NaHCO_3$, and 2 mM L-glutamine (GIBCO).

Cell collection and preparation of lymphoid suspensions. Rats were anesthetized with a combination of ketamine hydrochloride (Bristol Laboratories, Syracuse, N.Y.) and pentobarbital sodium (Butler Co., Columbus, Ohio). Blood was collected by cardiac puncture for analysis by ELISA.

In preliminary experiments, both the starting cell suspension and the mononuclear cell suspension from each source were characterized for viability (by trypan blue exclusion), phagocytic ability (phagocytosis of fluorescent carboxylated microspheres [Polysciences Corp., Warrington, Pa.] [46] or yeasts [47] or both), pinocytic ability (neutral red [11]), the presence of nonspecific esterase (22) (without fixation to increase specificity for murine macrophages [C. C. Stewart, 1982 RES Workshop on Isolation and Characterization of Macrophages]), and morphological appearance (with Wright-Giemsa stain).

Lung mononuclear cells were collected as previously described (16, 17). Initial lung lymphoid suspension contained over 50% macrophages. Routinely, the proportion of macrophages in lung suspensions was reduced to 10 to 15% by repeated incubation with iron powder (10 mg/ml) in plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.) for 30 min at 37°C followed by magnetic sedimentaion. This step does not cause selective loss of B lymphocytes (17). A considerable proportion of nonlymphoid cells (up to 15%), consisting mainly of ciliated epithelial cells, remained in lung mononuclear cell populations.

Because of the small number of lymphocytes that can be recovered from uninfected rat lungs, most of the studies were performed with lymphocytes from other tissues. Spleens or mesenteric lymph nodes were aseptically removed, minced, and pressed through a sterile nylon screen (mesh size, 250 μm) into cold RPMI 1640 (pH 7.2) containing 10 mM HEPES, 2% fetal calf serum (GIBCO), and 20 μg of gentamicin (Elkins-Sinn, Inc., Cherry Hill, N.J.) per ml. Mononuclear cell populations from lymph nodes were used after being washed twice with cold medium. Splenic cells were washed in cold medium and loaded onto Ficoll-Hypaque lymphocyte separation medium (Pharmacia Fine Chemicals, Piscataway, N.J.). Gradients were spun at room temperature for 25 min at 400 × *g*, and the mononuclear cell layers were removed, washed twice in cold medium, and suspended in culture medium containing rat serum at either 5 or 10%, depending on the assay to be performed. After Ficoll-Hypaque purification, spleen mononuclear cell populations contained 15 to 18% macrophages as determined by all methods. After two depletions as described above, spleen populations contained less than 6% phagocytic cells. Lymph node populations contained 1 to 3% macrophages and were not depleted of adherent cells.

Peripheral blood lymphocytes were obtained by collecting 3 ml of blood by cardiac puncture into a syringe containing 2 ml of RPMI 1640 with 5 U of sodium heparin per ml. The cells were washed, and the mononuclear cells were isolated on Ficoll-Hypaque gradients and suspended at 4×10^6 cells per ml in culture medium containing 5% rat serum.

Lymphocyte transformation assays. Two lymphocyte transformation assays were used to ensure that any strain differences were not artifacts of the methodology. In the macroassay, the concentration of lymphoid cells was adjusted to 2×10^6 in RPMI 1640 containing 10% fresh autologous serum. Aliquots of cells (0.5 ml) and mitogen (0.5 ml), each at twice the final concentration, were added to polystyrene tubes (12 by 75 mm) (no. 2058; Becton Dickinson Labware, Oxnard, Calif.). The optimal time of culture for both LEW and F344 lymphocytes was 96 h for all mitogens (data not shown). After incubation for 72 h at 37°C, 1 μCi of 5-[^{125}I]iodo-2'-deoxyuridine (Amersham Corp., Arlington Heights, Ill.) in 0.025 ml of medium was added to each tube. After an additional 24 h of incubation, the cells were spun down and the medium was removed. The cells were then suspended in cold 10% trichloroacetic acid, the precipitate was washed twice in cold 10% trichloroacetic acid, and the radiolabel incorporated was measured in a gamma counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

In the microassay, 4×10^5 cells per well were cultured in a humidified atmosphere containing 95% air and 5% CO_2 for a total of 48 h in 96-well flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) with mitogen in a final volume of 0.2 ml per well. The culture medium contained 2.5% pooled frozen rat serum unless otherwise indicated. At 24 h before harvesting, 0.5 μCi of [*methyl*-^{3}H]thymidine (Amersham) was added to each culture well. The cells were collected onto glass fiber strips, lysed, and washed with distilled water by a cell harvester (Bellco Glass, Inc., Vineland, N.J.). The strips were allowed to dry and were then counted in Econofluor (New England Nuclear Corp., Boston, Mass.) with a Betatrac 6895 scintillation counter (TM Analytic, Inc., Elk Grove, Ill.).

All experimental treatments were tested in duplicate or triplicate, and each experiment was repeated at least once. The amount of radiolabel incorporation was expressed as the geometric mean of the counts per minute (×/÷ standard deviation [SD]).

Immune serum and *M. pulmonis* antigen-primed cells. To obtain antigen-primed cells and serum containing antibodies to *M. pulmonis*, six LEW and six F344 rats were injected intraperitoneally with 0.25 ml of a suspension of MPM in saline containing 500 μg of protein per ml. Control rats received an equal volume of sterile saline. At 14 days after injection, all rats were bled by cardiac puncture and spleen cells were prepared as described above. The sera from each group were pooled, and the presence of antibody to *M. pulmonis* was confirmed by ELISA (9). Sera pooled from immunized animals were strongly positive in the ELISA for both IgM and IgG antibodies directed against *M. pulmonis*; sera from control rats were negative.

Mitogen-pulsed cultures. The mitogenic response to PHA was shown to require the presence of macrophages (48). To investigate whether macrophages were responsible for the differences in the response of LEW and F344 cells to this mitogen, we exposed lymphocytes to PHA bound to macro-

phages. After splenocytes were collected from both strains, erythrocytes were lysed by Tris-buffered ammonium chloride (33). Samples (0.1 ml) of suspensions containing 4×10^6 cells per ml from each strain were placed in wells in flat-bottomed microtiter plates. After 1.5 h of incubation at 37°C, these wells were washed twice and the nonadherent cells were discarded. The adherent cells were pulsed with 0, 1, 1.5, or 2.0 mg of PHA per ml and incubated again for 1 h, after which the medium was removed and the wells were washed three times with culture medium. No cells were added to control wells, so the only source of mitogen was that bound to the plastic. No additional mitogen was added to the cultures.

Cells from each strain were depleted to contain less than 6% macrophages by adherence to plastic or by iron phagocytosis and magnetic sedimentation or both. Nonadherent cells were then added to PHA-pulsed adherent cells in various combinations. Cultures were incubated for 48 h and treated as described above.

Immunofluorescence characterization. Initially, the numbers of B and T lymphocytes in lymphoid populations from the spleen and lung were determined by using dichlorotriazinylaminofluorescein-labeled rabbit antisera to rat $F(ab)_2$ and rat T cells (16, 17). These two antisera do not cross-react in double-labeling experiments, and neither reacts with phagocytic cells (16, 17).

More detailed studies of the T-cell subpopulations present in lymphoid tissues were performed with monoclonal antibodies to rat T-cell markers and analysis by a fluorescence-activated cell sorter (FACS). Supernatants from W3/25 and MRC OX-8 hybridomas were kindly provided by Neil Barclay and Alan Williams (Medical Research Council Cellular Immunology Unit, University of Oxford, Oxford, England). W3/25 antibody labels T helper cells and some macrophages and dendritic cells (2, 32). MRC OX-8 antibody labels T suppressor/cytotoxic cells and NK cells (5, 6, 14, 31). Fluorescein-conjugated, affinity-purified $F(ab)_2$ fragments of rabbit anti-mouse IgG (FITC-RAM; Pel-Freez Biologicals, Rodgers, Ark.) were passed through a column of rat immunoglobulin linked to cyanogen-bromide-activated Sepharose 4B (Pharmacia) (52) to remove cross-reactive antibodies which would bind to rat B cells and were used as the secondary reagent in labeling studies.

Lymphoid cells (10^6) were pelleted in small, siliconized conical tubes. The cells were suspended in 0.1 ml of monoclonal antibody (diluted 1:2) with phosphate-buffered saline supplemented with 0.2% sodium azide and 1.3 mM magnesium sulfate (PBS-azide) and incubated on ice for 30 min. The cells were pelleted through a fetal calf serum cushion and washed once with PBS-azide. The above steps were repeated with FITC-RAM antibody. After the last wash, the cells were pelleted and suspended in PBS-azide with 4% Formalin. The addition of 4% Formalin does not affect the amount of fluorescent labeling on cells and minimizes the loss of label due to capping or shedding of bound antibody or both (25). All experiments included cells labeled with FITC-RAM antibody alone.

A total of 10^4 cells were analyzed with a Becton Dickinson FACS IV. By appropriate setting of the light scattering gate, contaminating erythrocytes and cellular debris were excluded from analysis; fluorescence was measured on a logarithmic scale. The percent fluorescence-positive cells was determined by setting the fluorescent gate at approximately 5% positive, by using cells stained with FITC-RAM alone (background fluorescence), and by measuring the number of fluorescence-positive cells in the sample stained with monoclonal antibody and FITC-RAM with this same gate setting. The percent positive cells was calculated by the following formula: 100 × (number of fluorescence-positive cells − number of background fluorescent cells)/(10,000 − number of background fluorescent cells). The experiment was repeated four times, with three animals of each strain per experiment. Data from these experiments are expressed as the arithmetic mean ± SD of the percent positive cells.

TABLE 1. LEW and F344 splenic lymphocyte responses to stimulation by mitogens

Mitogen	Assay	Dose[a]	Response[b] of:	
			LEW cells	F344 cells
MPM	Micro	5 μg/ml	30,381 (1.4)[c]	12,123 (1.4)
	Macro	50 μg/ml	10,204 (1.1)[c]	3,471 (1.2)
PHA	Micro	0.2 mg/ml	65,607 (1.5)[c]	33,970 (2.0)
	Macro	0.2 mg/ml	31,709 (1.0)[c]	7,843 (1.1)
PWM	Micro	ND[d]	ND	ND
	Macro	2 μl/ml	32,942 (1.1)[c]	16,145 (2.0)
STM	Micro	20 μg/ml	7,916 (1.2)[c]	5,640 (1.4)
	Macro	ND	ND	ND
None	Micro		444 (1.8)	328 (1.9)
	Macro		447 (2.1)	418 (1.4)

[a] Dose is optimum for each strain in corresponding assay based on dose-response curve.
[b] Geometric mean in counts per minute (×/÷ SD) of [^{3}H]thymidine incorporation in triplicate cultures in three experiments with lymphocytes from three animals per strain for each experiment.
[c] LEW lymphocytes gave a significantly higher response ($P < 0.05$) to mitogen stimulation than did F344 lymphocytes.
[d] ND, Not done.

Statistics. Factorial design experiments were analyzed by the analysis of variance (13, 20). When analysis of variance indicated that a significant difference occurred among a group of means, Duncan's mean ratio test, Student's range test, or the least squares means test was used to determine the nature of the difference (13, 20). All other data were analyzed by Student's independent or paired t test (13, 20). A probability (P) of <0.05 was accepted as significant.

RESULTS

Activation of splenic lymphocytes by different mitogens. Splenic lymphoid cells from both LEW and F344 rats were nonspecifically activated to proliferate by MPM, although lymphocytes from LEW rats were significantly more responsive in both micro- and macroassays (Table 1). Increased responsiveness of LEW rat lymphocytes was not limited to MPM, as shown by their greater response to PHA, PWM, and STM (Table 1). Dose-response studies (data not shown) indicated that the differences in responsiveness of the strains were not dependent on the mitogen dose or the total time of culture. Note that with spleen lymphocytes, there was no significant difference in the spontaneous level of radiolabel incorporation.

Comparison of lymphocyte source. LEW and F344 lymphocytes collected from the lungs, mesenteric lymph nodes, peripheral lymph nodes, and peripheral blood were compared for their responsiveness to PHA and MPM. LEW lymphocytes gave higher responses, regardless of the source of cells, with both MPM and PHA (Table 2). In addition, there was significantly higher spontaneous incorporation of

TABLE 2. Mitogen responses of lymphocytes from various lymphoid tissues[a]

Mitogen	Dose	Lymphocytes	Response[b] of:	
			LEW cells	F344 cells
PHA	0.2 mg/ml	Lung	26,535 (1.1)[c]	9,443 (1.1)
	0.2 mg/ml	PLN[d]	22,652 (1.1)[c]	3,102 (1.1)
	0.2 mg/ml	MLN[d]	105,175 (1.0)[c]	1,170 (1.1)
	0.2 mg/ml	Blood	60,983 (1.1)[c]	15,009 (1.1)
MPM	50 μg/ml	Lung	34,394 (1.1)[c]	4,063 (1.0)
	5 μg/ml	MLN	15,030 (1.0)[c]	309 (1.3)
	5 μg/ml	Blood	7,230 (1.1)[c]	101 (1.2)
None		Lung	1,111 (1.3)[c]	575 (1.4)
		PLN	644 (1.2)	915 (1.3)
		MLN	1,392 (1.6)	745 (1.8)
		Blood	1,868 (1.4)[c]	176 (1.1)

[a] Data from lung tissue were obtained with the macroassay; data from other tissues were obtained with the microassay. For each assay, values obtained with the optimum dose for both strains for each assay is presented.

[b] Geometric mean in counts per minute (×/÷ SD) of [^{3}H]thymidine incorporation in triplicate cultures for three different experiments with three animals per strain per experiment.

[c] LEW lymphocytes gave a significantly higher response ($P < 0.05$) to mitogen stimulation than did F344 lymphocytes.

[d] PLN, Peripheral lymph node lymphocytes (pooled cervical and popliteal); MLN, mesenteric lymph node lymphocytes.

TABLE 3. Effects of antibody and primed cells on MPM response of spleen lymphocytes

Cells	Presence of antibody[a]	Response[b] of:	
		LEW cells	F344 cells
Normal[c]	−	16,815 (1.7)	3,984 (1.9)
Primed[c]	−	16,155 (1.4)	5,432 (1.4)
Normal	+	4,188 (1.6)	1,339 (1.9)
Primed	+	6,701 (1.6)	2,751 (1.6)
Control normal[d]	−	323 (1.8)	236 (1.2)
Control primed[d]	−	394 (1.9)	149 (1.3)

[a] LEW and F344 rats immunized with only saline were found to have no antibody (−) to MPM, whereas the immunized group produced antibody (+) to MPM, as determined by ELISA.

[b] Geometric mean in counts per minute (×/÷ SD) of [^{3}H]thymidine incorporation in triplicate cultures for two experiments with four animals per strain per experiment. Each experiment was performed with the microassay, and an MPM dose of 5 μg/ml was used. There was no difference between normal and primed cells from the same strain of rat; however, antibody to *M. pulmonis* significantly inhibited ($P < 0.001$) the response to MPM in both strains.

[c] Cells from LEW and F344 rats immunized with MPM were considered primed cells, whereas those from rats immunized with saline only were considered normal cells.

[d] Control cultures were incubated without mitogen. There was no significant difference in background between normal and primed cells for either strain or any significant difference between LEW and F344 background levels.

radiolabel by LEW lung and blood lymphocytes than by F344 lymphocytes from these sites.

Effects of serum and primed cells. To determine whether the difference in responsiveness between LEW and F344 lymphocytes was due to factors in normal serum, the responses of lymphocytes from both strains to MPM and PHA were compared following supplementation with either heterologous and homologous ELISA-negative rat serum. The source of the serum (LEW and F344) did not affect the responses of F344 cells to either mitogen. In contrast, when using LEW cells, F344 serum was slightly inhibitory for MPM responses (10,441 cpm [1.0 SD] for LEW cells with LEW serum compared with 9,456 cpm [1.0 SD] for LEW cells with F344 serum; $P < 0.05$) and slightly stimulatory for PHA responses (26,918 cpm [1.2 SD] for LEW cells with LEW serum compared with 36,136 cpm [1.1 SD] for LEW cells with F344 serum). However, LEW cells were still more responsive than F344 cells ($P < 0.01$) in all cases.

To determine whether previous exposure to *M. pulmonis* affects responsiveness, spleen cells and pooled sera from immunized and control rats of both strains were used in a factorial design experiment. The different factors were (i) strain: LEW and F344; (ii) mitogen: none; MPM, 5 μg/ml; PHA, 0.2 mg/ml; (iii) source of cells: immune or normal animals; and (iv) source of serum: immune or normal. The normal and immune serum from each strain were used with the corresponding cells only.

Immunization with *M. pulmonis* membranes did not affect MPM or PHA responses in either strain when normal or immune cells were compared in the presence of normal serum (Table 3); however, antibody to *M. pulmonis* significantly inhibited ($P < 0.001$) the response to MPM in both strains, regardless of whether the cells were from normal or immunized animals. LEW responses were higher than F344 responses for both MPM ($P < 0.001$) and PHA ($P = 0.003$).

Admixture studies. The effect of using an admixture of LEW and F344 cells (1:1 ratio; cell density the same as in the macroculture) was examined to determine whether the difference in responsiveness was due only to a difference in the number of proliferating cells or to a difference in some control mechanism (Table 4). The expected intermediate response with the admixture of cells occurred only with PWM. In all other cases, the cell mixtures gave results higher than or equal to those seen in cultures containing only LEW cells. These last results cannot be explained by costimulation between lymphocytes of the two strains, as there was no pronounced increase in radiolabel incorporation in the mixture of cells without mitogen.

Role of adherent cells in lymphocyte activation. To examine the possibility that the differences in the responses of LEW and F344 lymphocytes are caused by differential processing of mitogen by macrophages, the following experiments were performed. Preliminary studies showed that plastic adherent cells alone did not respond to MPM, PWM, or PHA. Depletion of adherent cells to less than 6% macrophages

TABLE 4. Effects of spleen cell mixtures on responsiveness to mitogens[a]

Mitogen	Response[b] of:		
	F344 cells	LEW cells	LEW-F344 (1:1) cell mixture
None	525 (1.2)[c]	433 (1.2)[c,d]	336 (1.2)[d]
MPM	4,727 (1.1)[c]	9,936 (1.1)[d]	11.481 (1.2)[e]
PHA	6,237 (1.2)[c]	31,188 (1.2)[d]	50,367 (1.1)[e]
PWM	24,981 (1.1)[c]	36,686 (1.2)[d]	31,108 (1.2)[d]

[a] All animals used in these experiments were males.

[b] Geometric mean in counts per minute (×/÷ SD) of [^{125}I]iododeoxyuridine incorporated in triplicate cultures in two experiments with four animals per strain per experiment. The macroassay was used for each experiment. MPM was used at a dose of 50 μg/ml, PHA was used at a dose of 0.2 mg/ml, and PWM was used at a dose of 2 μl/ml.

[c–e] Letter superscripts are used to indicate significant difference ($P < 0.05$) between cell cultures exposed to a given mitogen. Means for the same mitogen with the same letter superscript are not different. No statistical comparison was made between responses to different mitogens.

significantly decreased the responses of LEW and F344 splenic lymphocytes to both MPM and PHA to approximately one-third or less of the response seen with cultures containing 18 to 20% macrophages. However, lymphocytes from LEW rats still responded to a greater degree than did lymphocytes from F344 rats (3,500 cpm for LEW lymphocytes versus 1,525 cpm for F344 lymphocytes and 35,46 cpm for LEW lymphocytes versus 8,520 for F344 lymphocytes with macrophage-depleted cultures for MPM and PHA, respectively; $P < 0.01$ for both mitogens).

Because responses to PHA are macrohage dependent in rats (48), PHA-pulsed adherent LEW and F344 cells were cultured with untreated homologous and heterologous nonadherent cells. Nonadherent cells were added to pulsed adherent cells in various combinations: (i) F344 adherent cells with F344 nonadherent cells, (ii) F344 adherent cells with LEW nonadherent cells, (iii) F344 adherent cells with a 1:1 mixture of LEW and F344 nonadherent cells, (iv) the same combinations as above with LEW adherent cells, (v) F344 nonadherent cells without adherent cells, and (vi) LEW nonadherent cells without adherent cells.

The results (Table 5) indicate that the presence of adherent cells during PHA pulse enhanced the lymphocyte response; however, the difference in the responses of the LEW and the F344 cells depended on the source of nonadherent cells, not on the source of adherent cells. Also, the admixture of LEW and F344 nonadherent cells gave responses that were higher than expected, regardless of the souce of adherent cells.

Immunofluorescence characterization of lymphocyte populations. LEW and F344 lymphoid populations from lungs, spleen, or lymph nodes showed no differences in the number of cells with surface or cytoplasmic immunoglobulin or in the number of cells with surface T-cell markers identified by dichlorotriazinylaminofluorscein-labeled anti-rat T-cell serum (data not shown). Lung cultures contained an appreciable proportion (40 to 50%) of cells with the morphological appearance of lymphocytes that did not carry T- or B-cell markers; fewer than 20% of these cells were found in cell populations from other tissue. There was no significant difference in the number of these "null" lymphocytes in the LEW and F344 suspensions.

Although analysis with rabbit antisera to rat T cells showed no difference between LEW and F344 lymphoid populations in regard to the total number of T cells, the admixture and mitogen pulse experiments suggested a difference between the two rat strains in regard to regulatory influences residing within the nonadherent cell population. In addition, it is known that all mature T cells in peripheral lymphoid organs of rats do not stain with anti-T-cell serum (7). To determine whether there was a difference in T-cell subpopulations, splenic lymphocytes and mesenteric node lymphocytes from the two strains were analyzed with a FACS after immunofluorescent staining with monoclonal antibodies (Table 6). More W3/25$^+$ cells were found in LEW rats ($P < 0.007$), but there was little difference in the number of MRC OX-8$^+$ cells between the two strains. Thus, the helper/suppressor cell ratio was found to be significantly higher in LEW rats than in F344 rats.

TABLE 5. Role of adherent cells in F344 and LEW spleen lymphocyte responses to PHA

Adherent cells	Nonadherent cells	Response[a]
	F344	3,126 (1.4)[b]
	LEW	15,246 (1.1)[c]
F344	F344	22,066 (1.0)[c,d]
LEW	F344	24,547 (1.0)[d]
F344	F/L[f]	40,146 (1.1)[e]
F344	LEW	48,045 (1.2)[e]
LEW	F/L	49,181 (1.2)[e]
LEW	LEW	52,373 (1.2)[e]

[a] Geometric mean in counts per minute (×/÷ SD) of [^{3}H]thymidine incorporation in triplicate cultures in two experiments with four animals per strain per experiment. Experiments were performed by the microassay. Data presented are with adherent cells pulsed with 2.0 mg of PHA per ml, which gave optimum responses. Cultures without PHA did not respond.

[b–e] Letter superscripts are used to indicate significant differences ($P < 0.05$) between cell combinations as determined by Duncan's means ratio test. Means for groups with any letter superscript in common are not significantly different from each other. Means that share no superscript are significantly different.

[f] F/L, 1:1 mixture of F344 and LEW nonadherent cells.

TABLE 6. T-cell subpopulations in lymphoid populations from F344 and LEW rats

Lymphocytes	Strain	% positive cells[a]		
		W3/25[b]	OX-8[c]	W3/25 to OX-8[d]
Spleen	F344	25.9 ± 2.3	25.8 ± 4.5	1.02 ± 0.1
	LEW	40.5 ± 4.5	22.7 ± 4.0	1.83 ± 0.5
MLN[e]	F344	33.8 ± 2.7	17.8 ± 0.5	1.90 ± 0.2
	LEW	49.4 ± 2.5	18.1 ± 3.2	2.76 ± 0.4

[a] Numbers represent mean and average deviation of percent positive cells for four experiments for spleen lymphocytes and two experiments for MLN lymphocytes, with four animals per experiment.

[b] LEW lymphocytes contained a greater proportion of W3/25 positive cells than F344 lymphocytes ($P = 0.007$ by paired t test).

[c] LEW and F344 lymphocytes did not differ in their proportions of OX-8 positive cells ($P = 0.385$ by paired t test).

[d] Ratio between the proportion of W3/25 positive cells and the proportion of MRC OX-8 positive cells.

[e] MLN, Lymphocytes from mesenteric lymph nodes.

DISCUSSION

Our data show that LEW lymphocytes have higher nonspecific responses to MPM, PHA, PWM, and STM than do F344 lymphocytes. These differences (MPM, PHA, and PWM) can be demonstrated with lymphocytes from different lymphoid tissues and are not due to differences in optimal times of culture or optimal concentrations of mitogen, since the responses of the LEW lymphocytes are higher than those of F344 lymphocytes under all conditions tested. Since initial studies were performed with autologous serum, the possibility that the differences were due to serum factors was investigated. Although normal serum slightly affected the lymphocyte responses to both MPM and PHA, serum factors could not completely explain the differences between the two strains, as LEW lymphocytes gave consistently higher responses regardless of the source of serum. In agreement with the results of Naot (34), immune sera inhibited MPM responses (Table 3). However, antiserum from either strain was capable of inhibiting MPM-induced lymphocyte proliferation at a concentration of 2.5%. This suggests that the in vivo differences in lymphoid responses between the two strains are not due to differences in the ability to produce inhibiting antibody. Furthermore, the differences found in in vitro responses cannot be due to undetected mycoplasma infection in LEW rats. All the cultural and serological evidence suggests that neither the LEW rats nor the F344 rats had been exposed to

mycoplasmal antigen. After immunization with MPM, no difference could be detected in antigen-primed versus naive lymphocytes for either strain. This is not unexpected, as the length of culture used in these studies was shorter than is required to demonstrate antigen-mediated transformation in vitro (12).

The difference in the responsiveness of LEW and F344 lymphocytes also does not seem to be due solely to differences in the number of cells capable of proliferating in response to mitogen. MPM primarily affects B cells (36), and there is no significant difference in B-cell numbers in LEW and F344 lymphoid populations. Furthermore, in the admixture studies with both MPM and PHA (Table 3), the admixtures had responses equal to or greater than that of LEW cells alone. If the difference was due to the number of responding cells, one would expect that the admixture of cells would give intermediate responses. The data instead suggest that the differences in the responsiveness of LEW and F344 lymphocytes are due to differences in regulation.

The involvement of adherent cells in the MPM and PHA lymphocyte responses of both rat strains is clearly indicated in our study as well as in those of others (1, 23, 42). However, adherent cells do not appear to be responsible for the difference in responsiveness of LEW and F344 lymphocytes for either mitogen. After depletion of adherent cells, LEW lymphocyte responses to both MPM and PHA were still higher than those of F344 lymphocytes. Furthermore, in the experiments with PHA-pulsed adherent cells, the response of nonadherent LEW cells was greater than that of nonadherent F344 cells regardless of the source of adherent cells.

The proposal that regulatory lymphocytes are important in the differences in response of LEW and F344 cultures is supported by the FACS data (Table 6), which show that LEW rats possess significantly more cells with the T helper cell phenotype ($W3/25^+$) than do F344 rats. Although W3/25 antibody stains some macrophage populations in addition to T helper cells (2, 32), it is unlikely that $W3/25^+$ macrophages account for the strain differences found in the FACS data, as no difference was found in the number of macrophages in LEW and F344 mononuclear suspensions on the basis of functional markers and cell morphology.

Other investigators have found a higher incidence (46 to 52%) of W3/25-positive cells in F344 spleen cell populations (44). The reason for this discrepancy with our results (26% $W3/25^+$ cells) is unclear but may include the use of different sublines of F344 rats or differences in environmental factors such as antigen exposure or diet. Differences in methodology may also contribute to this discrepancy; we were careful to minimize binding of FITC-labeled anti-mouse IgG antibody via Fc receptors and cross-reactions with rat immunoglobulin.

A reasonable hypothesis to explain the difference in responsiveness of LEW and F344 lymphocytes to MPM, PHA, and PWM is the difference in the numbers of $W3/25^+$ cells, presumably T helper cells. This hypothesis is strengthened by the fact that $W3/25^+$ cells are known to be preferentially stimulated by PHA (50). LEW rats give high responses to experimental allergic encephalomyelitis (51), peptidoglycan-induced experimental arthritis (49), and other experimentally induced autoimmune diseases (27–29, 45), as well as to MRM; they also give high responses to certain antigens and other mitogens (38). F334 rats generally give low responses in such experimental systems, although they have been compared directly with LEW rats in only one study, involving arthritis experimentally induced with peptidoglycan (49). It seems likely that a single basic genetic mechanism is responsible for the high response of LEW animals in most, if not all, of these systems. Differences in numbers of $W3/25^+$ T helper cells could also explain these differences in susceptibility to disease.

In regard to MRM, it should be remembered that the role of nonspecific mitogenicity in disease pathogenesis is still uncertain. Even though an interstitial pneumonia was produced with intranasal instillation of MPM (35), other membrane components such as proteases (39) and cytotoxic substances (19), which are known to be present in membrane preparations of other mycoplasmas, were not excluded. These could produce similar changes through cell death and direct tissue injury. Furthermore, hypersensitivity and antigen-specific reactions in the lungs could also produce similar pathological changes (3, 26). Of course, $W3/25^+$ cells could also affect these host responses. Even if mitogenicity is not a major pathogenic mechanism, the difference in numbers of $W3/25^+$ cells may be at least partly responsible for the differences in lesion development.

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LITERATURE CITED

1. **Arala-Chaves, M. P., L. Hope, S. H. Korn, and H. Fudenberg.** 1978. Role of adherent cells in immune responses to phytohemagglutinin and concanavalin A. Eur. J. Immunol. **8**:77–81.
2. **Barclay, A. N.** 1981. The localization of populations of lymphocytes defined by monoclonal antibodies in rat lymphoid tissues. Immunology **42**:593–600.
3. **Bice, D. E., M. A. Degen, D. L. Harris, and B. A. Muggenburg.** 1982. Recruitment of antibody-forming cells in the lung after local immunization is nonspecific. Am. Rev. Respir. Dis. **126**:635–639.
4. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **Brideau, R. J., P. B. Carter, W. B. McMaster, D. W. Mason, and A. F. Williams.** 1980. Two subsets of rat T lymphocytes defined with monoclonal antibodies. Eur. J. Immunol. **10**:609–615.
6. **Cantrell, D. A., R. A. Robins, C. G. Brooks, and R. W. Baldwin.** 1982. Phenotype of rat natural killer cells defined by monoclonal antibodies marking rat lymphocyte subsets. Immunology **45**:97–103.
7. **Carter, P. B., and H. Bazin.** 1980. Immunology, p. 181. *In* H. J. Baker, J. R. Lindsey, and S. H. Weisbroth (ed.), The laboratory rat, vol. 2. Research applications. Academic Press, Inc., New York.
8. **Cassell, G. H., and J. K. Davis.** 1978. Protective effect of vaccination against *Mycoplasma pulmonis* respiratory diseease in rats. Infect. Immun. **21**:69–75.
9. **Cassell, G. H., J. R. Lindsey, M. K. Davidson, J. K. Davis, M. B. Brown, and K. Mayo.** 1981. Detection of natural *Mycoplasma pulmonis* infection in rats and mice by an enzyme-linked immunosorbant assay (ELISA). Lab. Anim. Sci. **31**:676–682.
10. **Cassell, G. H., J. R. Lindsey, R. G. Overcash, and H. J. Baker.** 1973. Murine mycoplasma respiratory disease. Ann. N.Y. Acad. Sci. **225**:395–413.
11. **Cohn, Z. A., and E. Wiener.** 1963. Particulate hydrolases of macrophages. II. Biochemical and morphological responses to particle ingestion. J. Exp. Med. **118**:1009–1020.

12. **Corradin, G., H. M. Etlinger, and J. M. Chiller.** 1977. Lymphocyte specificity to protein antigens. I. Characterization of the antigen-induced *in vitro* T cell-dependent proliferative response with lymph node cells from primed mice. J. Immunol. **119**:1048–1053.
13. **Cox, D. R.** 1970. The analysis of binary data, p. 14. Methuen and Co., Ltd., London.
14. **Dallman, M. J., D. W. Mason, and M. Webb.** 1982. The roles of host and donor cells in the rejection of skin allografts by T cell-deprived rats injected with syngeneic T cells. Eur. J. Immunol. **12**:511–518.
15. **Davis, J. K., and G. H. Cassell.** 1982. Murine respiratory mycoplasmosis in F344 and LEW rats. Strain differences in lesion severity. Vet. Pathol. **19**:280–293.
16. **Davis, J. K., P. A. Maddox, R. B. Thorp, and G. H. Cassell.** 1980. Immunofluorescent characterization of lymphocytes in lungs of rats infected with *Mycoplasma pulmonis*. Infect. Immun. **27**:255–259.
17. **Davis, J. K., R. B. Thorp, P. A. Maddox, M. B. Brown, and G. H. Cassell.** 1982. Murine respiratory mycoplasmosis in F344 and LEW rats: evolution of lesions and lung lymphoid cell populations. Infect. Immun. **36**:720–729.
18. **Del Guidice, R. A., and M. F. Barile.** 1974. Immunofluorescent procedures for mycoplasma identification. Dev. Biol. Stand. **23**:134–137.
19. **Gabridge, M. G., C. K. Johnson, and A. M. Cameron.** 1974. Cytotoxicity of *Mycoplasma pneumoniae* membranes. Infect. Immun. **10**:1127–1134.
20. **Goldstein, A.** 1964. Biostatistics: an introductory text. Macmillan Publishing Co., Inc., New York.
21. **Hayflick, L.** 1965. Tissue cultures and mycoplasmas. Tex. Rep. Biol. Med. **23**(Suppl 1):285–303.
22. **Kaplow, L. S.** 1981. Identification of mononuclear macrophages, p. 200. *In* H. B. Herscowitz, H. T. Holden, J. A. Bellanti, and A. Ghaffer (ed.), Manual of macrophage methodology, collection, characterization, and function. Marcel Dekker, Inc., New York.
23. **Katz, R., R. Simon-Tov, and Y. Naot.** 1983. Comparison of mitogens from *Mycoplasma pulmonis* and *Mycoplasma neurolyticum*. Yale J. Biol. Med. **56**:613–621.
24. **Keightley, R. G., M. D. Cooper, and A. R. Lawton.** 1976. The T cell dependence of B cell differentiation induced by pokeweed mitogen. J. Immunol. **117**:1538–1544.
25. **Lanier, L. L., and N. L. Warner.** 1981. Paraformaldehyde fixation of hematopoietic cells for quantitative flow cytometry (FACS) analysis. J. Immunol. Methods **47**:25–30.
26. **Larsen, G. L., D. A. Parrish, and P. M. Hensen.** 1983. Lung defense: the paradox of inflammation. Chest **83**(Suppl):1S–5S.
27. **Lennon, V. A., J. M. Lindstrom, and M. E. Seybold.** 1975. Experimental autoimmune myasthenia: a model of myasthenia gravis in rats and guinea pigs. J. Exp. Med. **141**:1365–1375.
28. **Levine, S., and R. Sowinski.** 1970. Allergic inflammation, infarction and induced localization in the testes. Am. J. Pathol. **59**:437–451.
29. **Levine, S., and E. J. Wenk.** 1968. The production and transfer of allergic adrenalitis. Am. J. Pathol. **52**:41–53.
30. **Lindsey, J. R., H. J. Baker, R. G. Overcash, G. H. Cassell, and C. E. Hunt.** 1971. Murine chronic respiratory disease. Significance as a research complication and experimental production with *M. pulmonis*. Am. J. Pathol. **64**:675–707.
31. **Lyscom, N., and M. J. Brueton.** 1982. Intraepithelial, lamina propria and Peyer's patch lymphocytes of the rat small intestine: isolation and characterization in terms of immunoglobulin markers and receptors for monoclonal antibodies. Immunology **45**:775–783.
32. **Mayrhofer, G., C. W. Pugh, and A. N. Barclay.** 1983. Distribution, ontogeny and origin in the rat of Ia-positive cells with dendritic morphology and of Ia antigen in epithelia, with special reference to the intestines. Eur. J. Immunol. **13**:112–122.
33. **Mishell, B. B., and S. M. Shiigi (ed.).** 1980. Selected methods in cellular immunology, p. 3. W. H. Freeman and Co., San Francisco.
34. **Naot, Y.** 1982. *In vitro* studies on the mitogenic activity of mycoplasmal species towards lymphocytes. Rev. Infect. Dis. **4**(Suppl):S205–S209.
35. **Naot, Y., S. Davidson, and E. S. Lindenbaum.** 1981. Mitogenicity and pathogenicity of *Mycoplasma pulmonis* in rats. Atypical interstitial pneumonia induced by mitogenic mycoplasmal membranes. J. Infect. Dis. **143**:55–62.
36. **Naot, Y., S. Merchan, E. Ben-David, and H. Ginsburg.** 1979. Mitogenic activity of *Mycoplasma pulmonis* in rats. I. Stimulation of rat B and T lymphocytes. Immunology **86**:399–406.
37. **Naot, Y., J. G. Tully, and H. Ginsburg.** 1977. Lymphocyte activation by various mycoplasma strains and species. Infect. Immun. **18**:310–317.
38. **Raff, H. V., and D. J. Hinrichs.** 1977. Suppressor cell influence in selected strains in inbred rats. II. Strain-dependent mitogen- and antigen-related low responsiveness. Cell. Immunol. **29**:96–108.
39. **Razin, S.** 1978. The mycoplasmas. Microbiol. Rev. **42**:414–470.
40. **Razin, S.** 1983. Cell lysis and isolation of membranes, p. 225. *In* S. Razin and J. G. Tully (ed.), Methods in mycoplasmology. Academic Press, Inc., New York.
41. **Rodey, G. E., and R. A. Good.** 1969. The *in vitro* response to phytohemagglutin of lymphoid cells from normal and neonatally thymectomized mice. Int. Arch. Allergy Appl. Immunol. **36**:399–407.
42. **Rosenstreich, D. L., J. L. Farrar, and S. Dougherty.** 1976. Absolute macrophage dependency of T lymphocyte activation by mitogens. J. Immunol. **116**:131–139.
43. **Rottem, S., O. Stein, and S. Razin.** 1968. Reassembly of mycoplasma membranes disaggregated by detergents. Arch. Biochem. Biophys. **125**:46–56.
44. **Shonnard, J. W., B. K. Davis, and D. V. Cramer.** 1981. *In vitro* responses of BN strain inbred rats. Transplant. Proc. **13**:1390–1391.
45. **Silverman, D. A., and N. R. Rose.** 1975. Spontaneous and methylcholanthrene-enhanced thyroiditis in BUF rats. II. Induction of experimental autoimmune thyroiditis without complete Freund's adjuvant. J. Immunol. **114**:148–150.
46. **Steinkamp, J. A., J. S. Wilson, G. C. Saunders, and C. C. Stewart.** 1982. Phagocytosis: flow cytometric quantitation with fluorescent microspheres. Science **215**:64–66.
47. **Stewart, C. C., H. Lin, and C. Ades.** 1975. Proliferation and colony-forming ability of peritoneal exudate cells in liquid culture. J. Exp. Med. **1**:1114–1132.
48. **Stoecker, C. A., B. M. Rickard, and C. A. Abel.** 1978. Effect of T- and B-lymphocyte mitogens on interactions between lymphocytes and macrophages. Cell. Immunol. **35**:362–377.
49. **Wilder, R. L., G. B. Calandra, A. J. Garvin, K. D. Wright, and C. T. Hansen.** 1982. Strain and sex variation in the susceptibility to streptococcal cell wall-induced polyarthritis in the rat. Arthritis Rheum. **25**:1064–1072.
50. **Williams, J. M., H. M. Shapiro, E. L. Milford, and T. B. Strom.** 1982. Multiparameter flow cytometric analysis of lymphocyte subpopulation activation in lectin-stimulated cultures. J. Immunol. **128**:2676–2681.
51. **Williams, R. M., and M. J. Moore.** 1973. Linkage of susceptibility to experimental allergic encephalomyelitis to major histocompatibility locus in rats. J. Exp. Med. **138**:775–783.
52. **Wolfsy, L., and B. Burr.** 1969. The use of affinity chromatography for specific purification of antibodies and antigens. J. Immunol. **103**:380–382.