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Predisposing Factors in *Pneumocystis carinii* Pneumonia: Effects of Tetracycline, Protein Malnutrition, and Corticosteroids on Hosts

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Components of the immunosuppressive regimen used to reactivate latent *Pneumocystis carinii* infection were analyzed for their effects on the growth, nutrition, and lymphoid system of hosts. Rats that were administered either tetracycline or a low-protein (8%) diet alone for 7 weeks developed few abnormalities, but animals on the combined regimen developed lower body and lymphoid organ weights, lower serum albumin levels, and fewer circulating lymphocytes. Rats that were administered corticosteroids and tetracycline experienced severe wasting, debilitation, and generalized lymphocyte depletion; the low-protein diet increased the magnitude of these changes. Alterations in the frequency of occurrence of specific lymphocyte subsets occurred only in rats given corticosteroids and consisted mainly of a greater decline in peripheral blood T helper cells than in T suppressor cells. The data suggest that long-term tetracycline administration and a low-protein diet have a variety of adverse effects on the host which enhance the immunosuppressive properties of corticosteroids.

Pneumocystis carinii is a major opportunistic pulmonary pathogen, but little is known about the predisposing factors important in the development of infection with this organism. Serum antibodies, although prevalent in the normal population, do not offer protection against *P. carinii* pneumonia (12, 19, 31); rather, antibodies may possibly function as opsonins (18, 32). Support for the importance of cellular immune factors comes from the occurrence of *P. carinii* infections in patients receiving corticosteroids and cytotoxic drugs (29), in athymic (nude) mice (33), and in disease states, such as protein malnutrition and acquired immunodeficiency syndrome, that are characterized by impaired T-cell function (5, 13).

Animal models have been helpful in formulating concepts about *P. carinii*. Rats that were administered corticosteroids, a low-protein diet, and tetracycline spontaneously developed *P. carinii* pneumonia within ca. 8 weeks through a mechanism of reactivation of latent infection (7, 13). We have used this model to study the pathogenesis, humoral immune responses, and changes in lymphocyte subsets in *P. carinii* pneumonia (28a, 30, 32, 34). These studies focused mainly on corticosteroids, yet little is known about the contributions of the other components of this regimen to the impairment of host defenses. Such issues have important clinical applications, because opportunistic infections tend to occur more frequently when combinations of potentially immunosuppressive agents are used. In the present study, we compared the effects of tetracycline and a low-protein diet with and without corticosteroids on the growth, nutrition, and lymphoid system of rats.

MATERIALS AND METHODS

Rats. Adult male Lewis rats obtained from Harlan Sprague-Dawley, Indianapolis, Ind., were used instead of the customary outbred Sprague-Dawley rats because we wanted to develop an inbred rat strain for future studies of lymphocyte function. In our previous study of *P. carinii* infection and lymphocyte subsets, we used Lewis rats weighing 300 g (28a). In the present study, we chose younger rats weighing 200 g to allow for the greater effects of tetracycline and protein malnutrition on the host.

The rats were housed in a conventional colony room, ate a regular diet, and drank tap water for 1 to 2 weeks before the study began. The rats were then randomly divided into six groups by the protocol shown in Table 1. The major variables were tetracycline (1 mg/ml) in the drinking water, a low-protein (8%) diet with vitamins, and corticosteroids (cortisone acetate [25 mg] injected subcutaneously twice weekly). At 1- to 2-week intervals, three to eight rats from each group were weighed and then exsanguinated by cardiac puncture under halothane anesthesia. The rats were sacrificed 48 h after the last corticosteroid injection. Samples of blood from each rat were sent to the University of Cincinnati Medical Center Clinical Laboratories for the following tests: total and differential leukocyte (WBC) count, total protein level, and serum albumin level. The thymus, spleen, and bone marrow were removed for analysis.

Reagents. The following monoclonal antibodies (obtained from Accurate Chemical Co., Westbury, N.Y.) were used: W3/13, a pan-T-cell marker; W3/25, which identifies helper (inducer) T cells; and OX8, which identifies suppressor (cytotoxic) cells. Fluorescein-conjugated $F(ab')_2$ fragment rabbit anti-mouse immunoglobulin G (IgG) (Cappel Laboratories, Cochranville, Pa.) was used as the second label for T cells in the indirect fluorescent antibody technique; this antibody was absorbed with rat IgG on a Sepharose 4B column. Fluorescein-conjugated $F(ab')_2$ fragment rabbit anti-rat IgG (heavy and light chain) (Cappel Laboratories) was used to identify rat B cells.

Lymphocyte studies. These studies were described in detail previously (28a). Briefly, single-cell suspensions were prepared in RPMI 1640 medium from the thymus, spleen, and bone marrow; peripheral blood lymphocytes were obtained by Ficoll-Hypaque density gradient centrifugation. Cell counts were performed in a hemacytometer, and viability as determined by trypan blue exclusion was >90%.

For T-cell analysis, a 0.1-ml suspension of 10^6 cells was incubated with 0.1 ml of the monoclonal antibody at 4°C for 45 min, washed, and incubated with 0.1 ml of the fluorescein-conjugated goat anti-mouse IgG; for B-cell analysis, 10^6

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TABLE 1. Experimental protocol for rat groups

Group	Drinking water	Diet	Corticosteroids
1	Plain	Regular	No
2	Tetracycline	Regular	No
3	Plain	Low protein	No
4	Tetracycline	Low protein	No
5	Tetracycline	Regular	Yes
6	Tetracycline	Low Protein	Yes

cells were incubated with 0.1 ml of fluorescein-conjugated rabbit anti-rat IgG. The cells were analyzed by flow microfluorometry in a fluorescent-activated cell sorter (FACS III; Becton-Dickinson, Mountain View, Calif.) (11). The pulse height analyzer was set to terminate after passage of 20,000 viable lymphocytes, and the number of cells staining with a particular anitserum was expressed as the frequency within or percentage of the total cell population. Cell viability and size were determined by staining the populations with ethidium bromide and propidium iodide, respectively. Controls included standardization of the system by use of Covaspheres (Covalent Technology Corp., Ann Arbor, Mich.) and the lack of staining when the cell population was analyzed without anitbody (autofluorescence) or when phosphate-buffered saline was substituted for the monoclonal antibody. There was a strong correlation between the results obtained with the FACS and those obtained with conventional fluorescence microscopy in selected studies.

The frequency of occurrence of lymphocyte subsets for each group of rats was determined from a specimen pooled from group members. This was necessary because of the profound lymphocyte depletion which resulted in the corticosteroid-treated rats (groups 5 and 6). In preliminary studies, we found that the frequency of occurrence of the lymphocyte subsets in the pooled specimen correlated well with the mean values for the individual members. The frequency of occurrence of each lymphocyte subset at different body sites in groups 2 through 6 on the experimental regimens was compared with the corresponding subset frequency in simultaneously examined rats in group 1 (the control group), and expressed as Δ% by the following formula: Δ% = subset frequency (percent) in the experimental group − subset frequency (percent) in the control group. In selected studies the absolute numbers of T helper (Th) and T suppressor (Ts) cells within each group were also calculated and expressed as the Th/Ts ratio.

RESULTS

General changes. Group 1 control rats remained healthy, and their mean body weight had increased by 62% after 7 weeks, when the study was terminated (Fig. 1). Group 2 rats on the tetracycline regimen first gained and then lost weight. The weight of group 3 rats on the low-protein diet remained relatively constant throughout the study. Group 4 rats, which received both tetracycline and the low-protein diet, weighed consistently less than did rats in group 2 or 3.

Group 5 corticosteroid-treated rats became chronically ill and wasted and had lost 40% of their original weight after 5 weeks. Group 6 rats, which received corticosteroids and the low-protein diet, lost weight even faster. Rats in groups 5 and 6 began dying after 5 weeks and had such severe involution of lymphoid tissues that they were not included in the final phase of the study.

The mean spleen weight in group 1 rats remained relatively constant, whereas the thymus weight declined by 64% after 7 weeks (Fig. 1). The ratio of the weight of each of these organs to total body weight declined over time (Fig. 2). The spleen and thymus weights of group 2 rats were similar to those of group 1 rats except at the end of the study, when

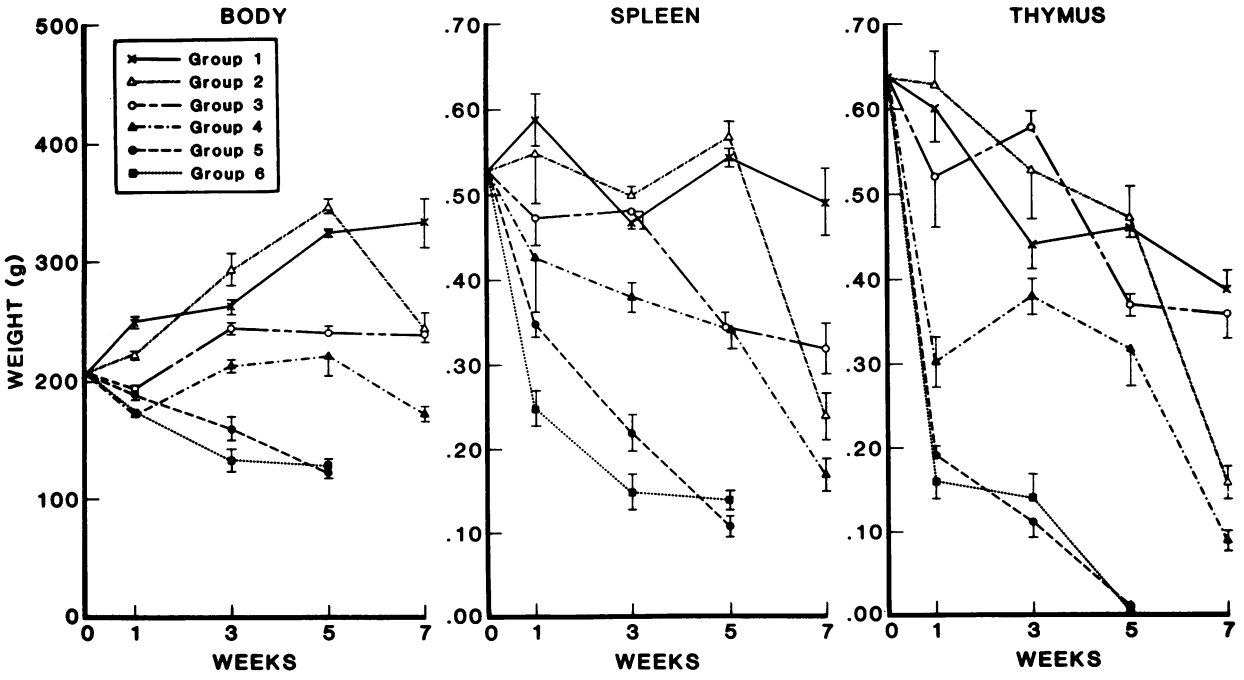

FIG. 1. Body, spleen, and thymus weights of the different rat groups over time. Each point represents the mean (± standard error of the mean) weight of three to eight rats.

they declined; a similar pattern occurred with the ratio of these organ weights to total body weight. Group 3 rats had lower spleen weights than did group 1 rats, but the thymus weights were similar. Group 4 rats exhibited lower spleen and thymus weights than did rats in groups 2 and 3, but the ratio of the organ weights to total body weight was similar.

Rats in groups 5 and 6 exhibited massive depletion of all lymphoid tissues. After 5 weeks the thymus in both groups had decreased to an undetectable size, and spleen weights had fallen by 75 to 80%. The ratio of thymus and spleen weights to total body weight showed a steep decline, which indicated that corticosteroids had a greater effect on the lymphoid system than on the rest of the body.

Changes in lymphocytes. Mean WBC counts in peripheral blood among group 1 rats remained relatively constant throughout the study and showed a preponderance (80 to 85%) of lymphocytes (Fig. 3). Rats in groups 2 and 3 had wide fluctuations in WBC and lymphocyte counts at different times during the study. Group 4 rats had consistently lower WBC and lymphocyte counts than did rats in group 1, 2, or 3.

Mean WBC counts among group 5 rats fell by 68%, to 2,300/mm^3, by week 5 of the study. At this point, lymphocytes constituted only 13% of the WBC, and the quantitative lymphocyte count had fallen by 95%, to 300/mm^3. The decline in lymphocytes among rats in group 6 was even steeper during the early part of the study; by week 5 the lymphocyte count was 260/mm^3.

Lymphocyte subsets. The frequency of occurrence of the lymphocyte subsets was measured in the thymus, spleen, peripheral blood, and bone marrow of the rats in each experimental group and compared with the corresponding frequencies among group 1 control rats. Group 2 rats had lymphocyte subset frequencies which were very similar to those in group 1 rats throughout the study, and hence the Δ%'s were small (Fig. 4). The lymphocyte subset frequencies varied somewhat more widely at the different body sites among group 3 rats, without any consistent pattern; the major change was the decline in OX8 (Ts) cells in the thymus. Lymphocyte subset frequencies also varied among the group 4 rats, with the major changes being an increase in OX8 cells in the thymus and a decrease in W3/13 (pan-T) cells in peripheral blood. The changes in lymphocyte subset frequencies in group 4 rats appeared to be unrelated to the changes in group 2 and 3 rats.

Rats in groups 5 and 6 exhibited a large decline in the frequency of occurrence of lymphocyte subsets in the thymus during week 1 of the study; these values returned to the base line by week 2 in group 5 rats but could not be measured accurately in group 6 rats because of an insufficient amount of tissue (Fig. 4). The changes in lymphocyte subset frequencies in the spleen were less marked and less consistent. The most noticeable change in the peripheral blood of both rat groups was the fall in the frequency of W3/25 (Th) cells. OX8 and B cells declined by lesser amounts, whereas the changes in W3/13 cells were inconsistent. The major change in the bone marrow was an increase in the frequency of W3/13 cells, although wide fluctuations occurred at different points in the study. Small increases were found in the frequency of W3/25 and OX8 cells, but the changes in B cells were inconsistent among the groups.

The effects of corticosteroids on Th cells in peripheral blood were more clearly seen when the Th/Ts ratio was calculated (Fig. 3). The Th/Ts ratio remained ≥1.80 in groups 1 through 4 throughout the study. By contrast, the Th/Ts ratio fell to 1.29 after 5 weeks in group 5 rats and to 1.05 after 3 weeks and 1.00 after 5 weeks in group 6 rats.

Serum proteins. Serum albumin and total protein levels among rats in groups 1, 2, and 3 were similar except for the increased levels in the group 3 rats at the end of the study (Fig. 5). Total serum albumin and total protein levels in group 4 rats were lower than in the other groups at most points in the study. Serum albumin and total protein levels in group 5 and 6 rats rose early in the study, and the serum albumin levels remained elevated in group 6 rats for 5 weeks.

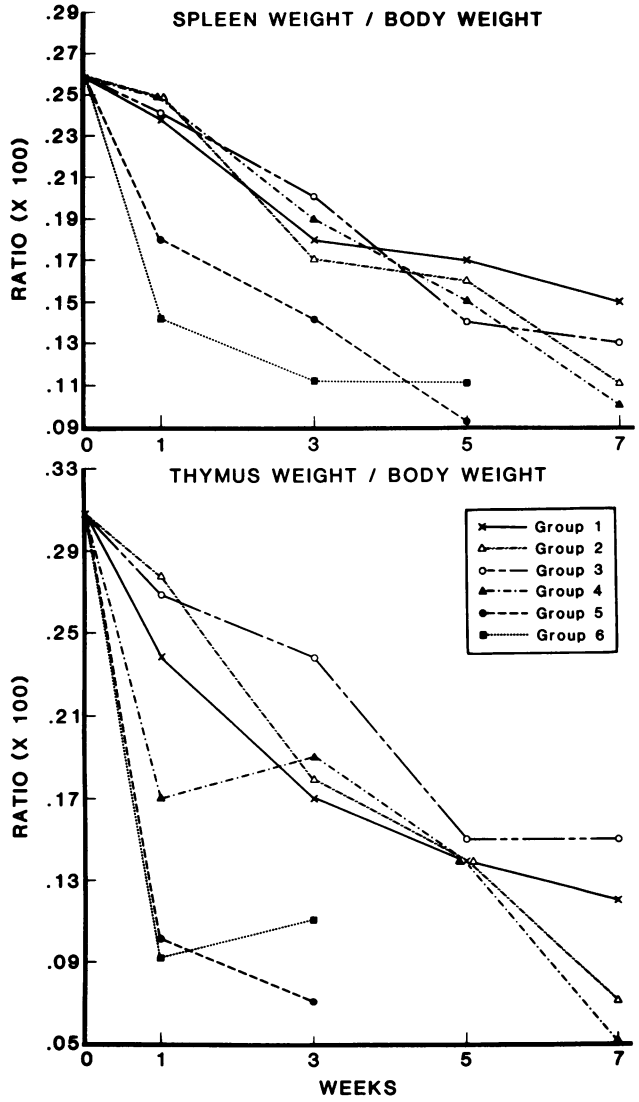

FIG. 2. Ratios of spleen and thymus weights to body weight of the different rat groups over time. Each point represents the mean ratio for three to eight rats.

DISCUSSION

P. carinii is a slow-growing organism of low virulence. In early animal model studies, the administration of corticosteroids in immunosuppressive doses sufficient to foster the

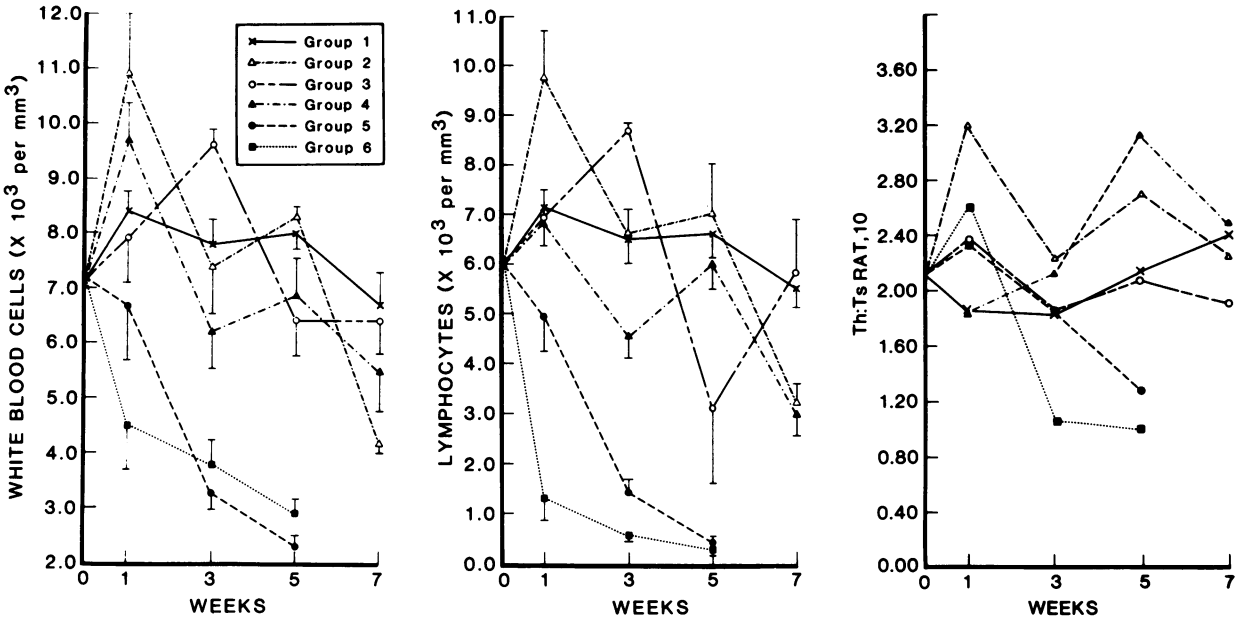

FIG. 3. Peripheral blood total WBC counts, lymphocyte counts, and Th-Ts cell ratios in the different rat groups over time. Each point in the left and middle graphs represents the mean (± standard error of the mean) counts for three to eight rats. The Th/Ts ratios were calculated from pooled specimens.

propagation of *P. carinii* frequently resulted in early death from overwhelming systemic *Corynebacterium kutscheri* infection; tetracyclines were effective in preventing this complication and became part of the standard regimen (7). Long-term tetracycline administration itself does not enhance the development of *P. carinii* infection (30), but by altering the natural microbial flora of hosts, it may enhance the pathogenicity of other parasites and the growth of resistant bacteria (16, 20). Other antibiotics have occasionally been used in animal models of *P. carinii* infection (22), but it is unknown whether these agents offer advantages over the tetracyclines.

In the present study, rats on the tetracycline regimen differed little from the control rats except at the end of the protocol, when they experienced weight loss and involution of the lymphoid organs. The lack of changes in the frequency of lymphocyte subsets after tetracycline administration does not rule out other, subtler effects of this drug that could not be detected by analysis of cell surface markers. Tetracyclines have a variety of effects on lymphocyte function, including inhibition of delayed hypersensitivity, response to mitogenic stimulation, and T-cell growth (9, 25–27). Studies of specific cellular immune responses to *P. carinii* are needed to examine more directly the effects of tetracyclines on host defenses against this organism. These studies have only rarely been successful because of a lack of organism preparations free from contaminating host tissues (10); however, recent advances in the cultivation of *P. carinii* suggest that such studies may now be more feasible (3). Tetracyclines also affect a variety of other functions of host cells, including leukocyte chemotaxis and phagocytosis (1, 6, 17), as well as inducing changes in alveolar epithelial cells (8). The significance of these effects is unknown, but since other cell types have been postulated as having a role in host defenses against *P. carinii* (18, 21, 28), the subject is worthy of further investigation.

Severe protein malnutrition itself can lead to clinical and experimental *P. carinii* pneumonia (13). The low-protein diet has become part of our standard animal regimen because it enhances the magnitude and uniformity of *P. carinii* infection achieved through the use of corticosteroids (29). Rats on the low-protein diet in the present study gained less weight than the control rats but exhibited few other consistent abnormalities in their lymphoid tissues or serum proteins. Clinical protein malnutrition is associated with a reduction in the number of circulating T cells and impaired cellular immunity (2). It is possible that the use of a diet more severely restricted in protein content or institution of such a diet at an earlier age might have resulted in some lymphocyte abnormalities in the present study.

The combination of tetracycline and low-protein diet had additive effects on the host. Rats on this regimen had consistently lower body weights, lower lymphoid organ weights, fewer circulating lymphocytes, and lower serum albumin and total protein levels than did rats receiving either agent alone. Although few definite changes in lymphocyte subsets were detected, the abnormalities produced by the tetracycline–low-protein diet combination on the host suggest that there is an increased potential for immunosuppression. The antianabolic properties of tetracycline (14) may have augmented the effects of the dietary protein deprivation, but the mechanism of this interaction and its precise relationship to host immune function are unclear.

The most pronounced immunosuppressive effects in this study were obtained with the corticosteroid component of the regimen. Rats that were administered corticosteroids and tetracycline experienced severe wasting, debilitation, and lymphoid tissue depletion. The addition of the low-protein diet to this regimen increased the magnitude of these changes. The lymphocytopenia in the thymus and spleen involved all subsets, whereas in peripheral blood there was a greater decline in Th than in Ts cells, with a concomitant decline in the Th/Ts ratio. The greater effect of corticosteroids on circulating Th cells has also been found in humans and appears to be an important property of these drugs (23, 24). The mechanism of corticosteroid-induced peripheral blood

lymphocytopenia is thought to be due to redistribution of the lymphocytes to other body compartments, particularly the bone marrow (4). Although the corticosteroid-treated rats in the present study had an increase in W3/13 (pan-T)-positive cells in their bone marrow, cell-sorting analysis revealed a high frequency of granulocytes that also stained with this antibody (28a).

Rats on the corticosteroid–tetracycline–low-protein diet regimen had higher levels of serum albumin than did the rats in other groups during the early phases of the study. Previous studies have shown that the rise in serum proteins with corticosteroid administration in the presence of protein malnutrition is at the expense of muscle protein (15). The relationship of corticosteroids to protein metabolism and immune function has long been a subject of interest because malnourished children have elevated serum cortisol levels.

In conclusion, this study examined the effects of components of the long-term immunosuppressive regimen used to

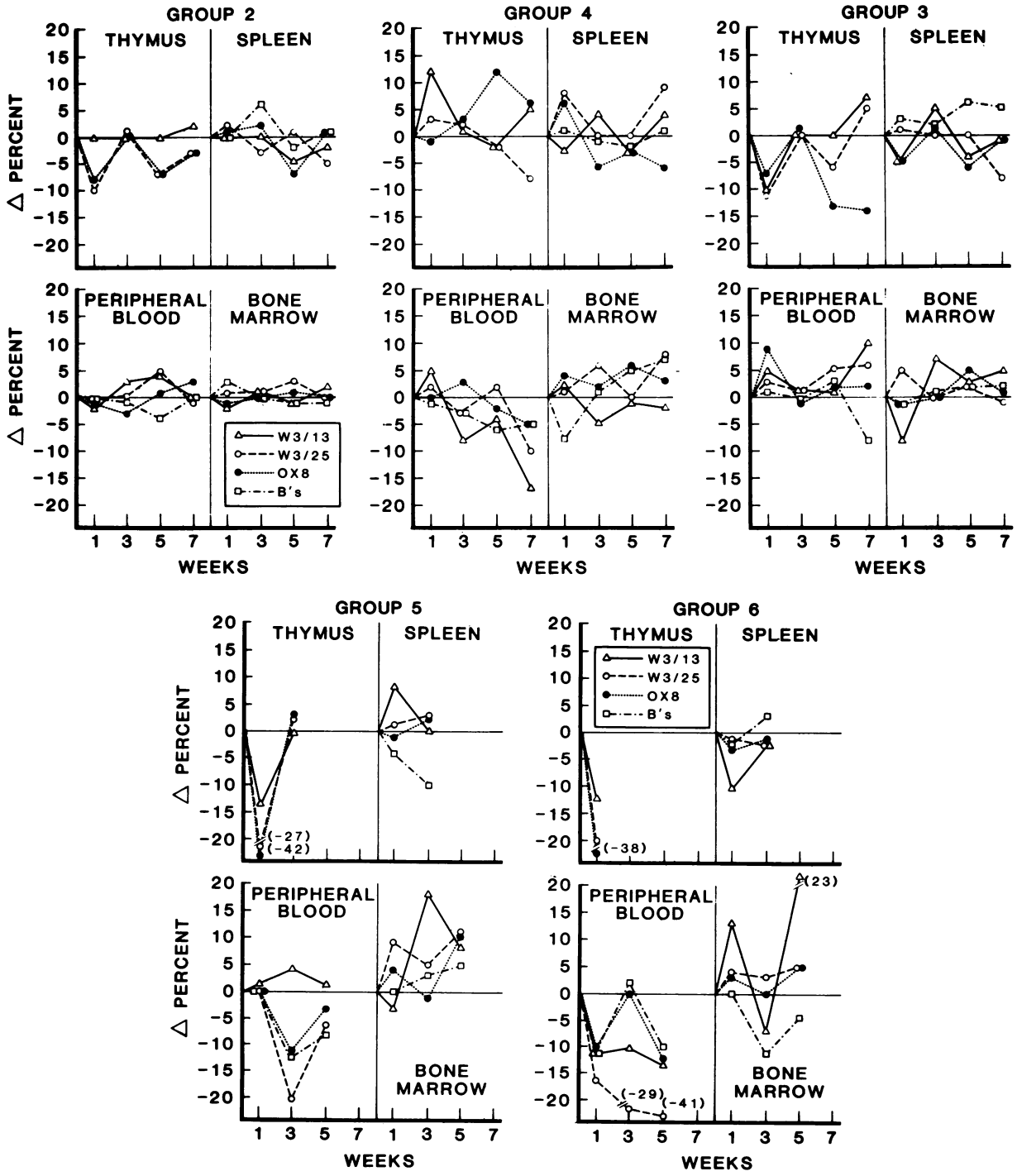

FIG. 4. Relative change in frequency of occurrence of the lymphocyte subsets in the experimental groups compared with group 1 (control) rats at different body sites, expressed as Δ%.

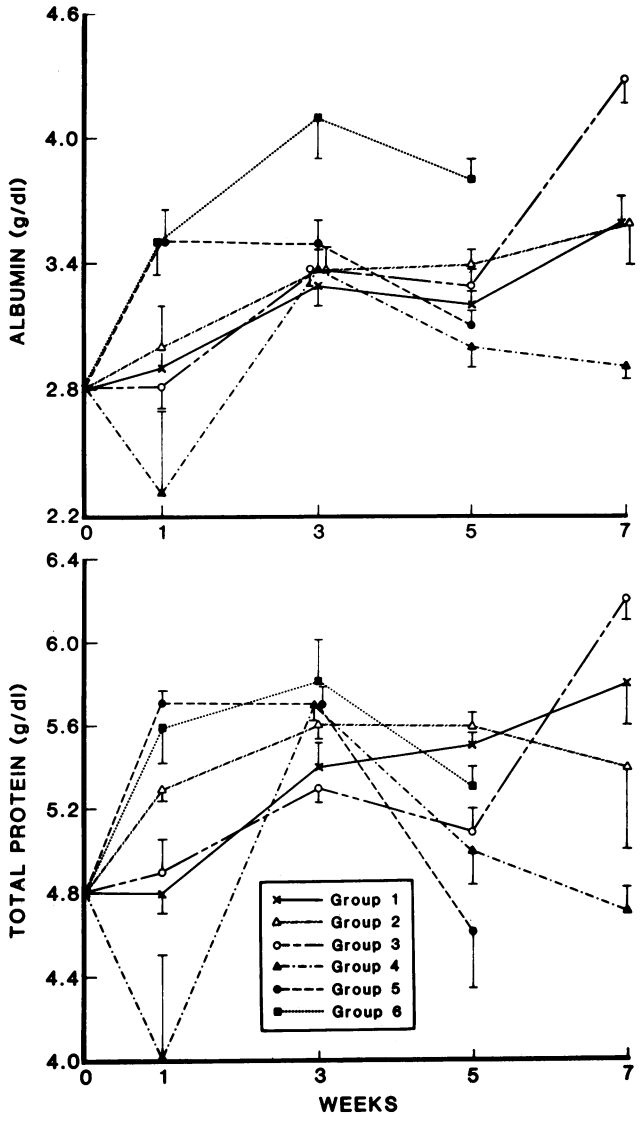

FIG. 5. Serum albumin and total protein concentrations in the different rat groups. Each point represents the mean (± standard error of the mean) concentration measured for three to eight rats.

reactivate latent *P. carinii* infection in rats. Tetracycline administration and low-protein diet, particularly in combination, result in measurable changes in the growth, nutrition, and lymphoid system of the host which appear to enhance the effects of the corticosteroids.

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