PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 756-761 (1976)

Changes in the Composition of Canine Respiratory Cells Obtained by Bronchial Lavage following Irradiation or Drug Immunosuppression (39301)

HERBERT Y. REYNOLDS, JOHN A. KAZMIEROWSKI, AND DAVID C. DALE[1]
(Introduced by Sheldon M. Wolff)

Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Pulmonary infections occur frequently in patients with neoplastic diseases (1) or as a consequence of their cytotoxic antineoplastic chemotherapy. The resulting granulocytopenia is often an important factor predisposing these subjects to infections. However, relatively little is known about the milieu of the lower respiratory tract of immunodeficient hosts, despite the frequent involvement of this organ with infection.

Pulmonary immunity to infectious agents is maintained in part by a combination of cellular elements in the lower respiratory tract (2), which includes alveolar macrophages that are probably derived from circulating blood monocytes (3, 4), lymphocytes, and polymorphonuclear cells. The present study in dogs attempts to assess the impact of acute and chronic bone marrow suppression to alter the integrity of the lymphoreticular system of the lungs by recording cellular changes in the respiratory tract and peripheral blood.

Materials and methods. Beagles, 6 months old and weighing 10–12 kg, were individually caged in a temperature-controlled room and maintained as in previous studies (5). These dogs were chosen because they could be easily handled and considerable experience was available regarding their response to total body irradiation (6).

Two means of immunosuppression were used:

(i) *Radiation.* A group of four dogs received 350 rad (midline tissue dose) of total body gamma irradiation from bilateral opposing cobalt-60 sources at 46 rad/min. This radiation dose produced bone marrow failure with leukopenia and thrombocytopenia but without diarrhea or other serious toxicity (5, 6). In these animals, the total white blood counts were less than 1000/mm^3 by 6 days after irradiation and counts were less than 100 cells/mm^3 by 10–12 days after irradiation.

Because the irradiation induced anorexia in some animals, dogs received 500 ml of Ringer's lactate solution (Abbott Laboratories, North Chicago, Ill.) daily by subcutaneous clysis, beginning the day after irradiation. In addition, these dogs were given daily transfusions of platelets as previously described (5) to keep platelet counts above 25,000/mm^3. They did not receive antibiotics.

(ii) *Drug regimens.* Dogs were given one of the following drug schedules: (1) no treatment, (2) daily intramuscular (im) injection of 1 mg/kg methylprednisolone sodium succinate (Upjohn Co., Kalamazoo, Mich.), (3) 2 mg/kg methylprednisolone im on alternate days, (4) daily cyclophosphamide 2 mg/kg im (200 mg Cytoxan reconstituted with 10 ml sterile water, Mead Johnson Laboratories, Evansville, Ind.), (5) daily cyclophosphamide (2 mg/kg) and methylprednisolone (1 mg/kg), and (6) on alternate days cyclophosphamide (2 mg/kg) and methylprednisolone (2 mg/kg). These doses of methylprednisolone and cyclophosphamide are equivalent to those humans might receive for various anti-inflammatory diseases. The cyclophosphamide treatment either daily or alternate day administration reduced total peripheral blood counts of the dogs to about 1000–1500 cells/mm^3 after approximately 2 weeks. At this point the cyclophosphamide dose was reduced to approximately 1.5 mg/kg to maintain white blood counts in the range of 2000–4000 cells/mm^3. All drug therapies were continued for 6 weeks.

Pulmonary lavage and recovery of respiratory cells. Dogs were lightly anesthetized

[1] Present address: Department of Medicine, University of Washington School of Medicine, Seattle, Washington.

with intravenous sodium thiamylal (Surital, Parke, Davis and Co.). In the group of irradiated dogs, a lower lobe bronchus was intubated with a cuffed endobronchial tube with a radiopaque tip (Metras bronchographic catheter, 19F, Rusch, Inc., New York). The other dogs were lavaged through a fiberoptic bronchoscope (model F0 8800, American Cystoscope Makers, New York) positioned in a lower lobe orifice. A lower lobe was washed with two 50-ml aliquots of 0.9% sterile saline. Sequential lavages at 1- or 2-week intervals were done in alternate lower lobes of each dog studied.

The recoverable lung lavage fluid was immediately strained through a layer of very loose cotton gauze to remove mucus and then centrifuged at 500*g* for 5 min at 25°. The supernatant fluid was decanted and the respiratory cell pellet was resuspended in modified Hank's balanced salt solution (prepared without Ca^{2+} and Mg^{2+} ions and phenol red by the NIH Media Unit). Cells were washed and pelleted twice before a final suspension of cells was made in McCoy's 5A Medium (Grand Island Biological, New York).

Aerobic bacterial cultures of bronchial lavage fluid were made on nutrient blood agar and MacConkey agar plates.

Size and functional differentiation of respiratory cells. Respiratory cells were suspended at a density of approximately 1–2 × 10^7 cells/ml of medium. A total cell count (Coulter) and cell viability by eosin dye exclusion (7) were done. Differential cell counts of 500 cells were made from an unstained wet mount and from a cytocentrifuge prepared Wright–Giemsa stain. Macrophages were distinguished from lymphocytes by positive staining with neutral red (8) and by ingestion of polystyrene latex balls (mean diameter 1.1 μm, Dow Chemical Co., Midland, Mich.). Size and morphology under phase-contrast microscopy and in stained smears were used as well.

Observations. Body weight was determined at weekly intervals. Rectal temperatures of the dogs were measured each morning during the study; a temperature less than 39° was considered within normal limits. Complete blood cell counts were made every 2 days during the study periods. Total leukocyte counts were measured with an electronic particle counter (model Fn, Coulter Electronics, Inc., Hialeah, Fla.); counts of less then 1500–2000 cells/mm^3 were done by direct visual counting with a hemocytometer. Differential counts were made on air-dried Wright's stained smears.

Results. Reproducibility of pulmonary lavage samples. Each dog served as its own pretreatment lavage control and then had at least three lung lavages subsequently. The recovery of lavage fluid was similar whether a Metras catheter or the bronchofiberscope were used; therefore, results from both lavage procedures have been combined. Sixty-two lower lobe lavages were performed. The mean recovery of lavage fluid was 68.7 ± 1.4 (SE) ml (range measured, 53–87 ml). Aspirated lavage fluid was foamy and white and free of visible blood, but respiratory cell pellets usually contained 2–8% erythrocytes. Respiratory cell viability was 92.6 ± 0.5% (range observed, 84–99%) from all lavages. Neither radiation nor the immunosuppressive drugs affected the viability of the recoverable respiratory cells. Dogs seemingly tolerated the procedure well and recovered quickly after the anesthesia abated.

Effects of radiation. In Fig. 1, the total number of respiratory cells and lymphocytes were compared with peripheral blood leukocyte values for a group of four dogs prior to irradiation and at intervals following.

Bronchial lavages were done 1 day after irradiation to document immediate cellular effects in the lungs. Mean respiratory cell counts did not differ from the control values; viability of the respiratory cells was similar also (Table I). Six days after irradiation, the number of respiratory cells and lymphoyctes were unchanged and the ratio between the percentage of macrophages and lymphocytes in the respiratory cell recovery was maintained. In contrast, blood leukocyte counts had decreased to less than 1000 cells/mm^3. All peripheral elements were depressed (granulocytes, monocytes, and lymphocytes) as illustrated for this dog model previously (5). On the twelfth day, when peripheral leukocyte counts were quite low (Fig. 1), there was a slight increase in the number of recoverable respiratory cells

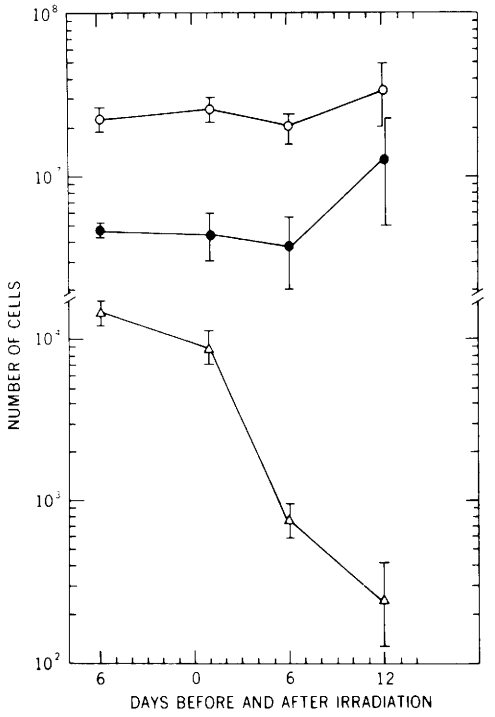

FIG. 1. Mean counts ± SE for respiratory cells and total peripheral blood leukocytes are given for a group of four dogs before irradiation and at intervals following irradiation. Total respiratory cells (○) and respiratory lymphocytes (●) obtained by bronchial lavage are contrasted with blood leukocyte counts/mm³ (△).

TABLE I. VIABILITY AND COMPOSITION OF CANINE RESPIRATORY CELLS OBTAINED BY PULMONARY LAVAGE BEFORE IRRADIATION AND AT INTERVALS FOLLOWING

Days before and after irradiation	Cell viability (%)	Macrophages (%)	Lymphocytes (%)
−6	96.0 ± 1.0[a]	73.0 ± 2.0	22.0 ± 2.0
+1	93.0 ± 1.8	84.8 ± 2.3	16.3 ± 1.9
+6	93.3 ± 2.7	83.8 ± 2.2	18.0 ± 2.5
+12	96.3 ± 1.8	66.3 ± 12.7	35.6 ± 10.0

[a] Mean ± SE for results from four dogs.

(from 2.1 × 10^7 to 3.4 × 10^7 mean cell count) reflecting principally an increase in respiratory lymphocytes. Lymphocytes accounted for approximately one-third of the respiratory cells in these lavages (Table I). Dogs did not survive longer than 15–18 days without antibiotic support, so further pulmonary lavages in this group of animals were not possible. Thus, an abrupt cessation of bone marrow function and subsequent peripheral blood leukopenia did not effect the recovery of lung macrophages for at least 12 days after irradiation. The increase in the number of respiratory lymphocytes found by 12 days is unexplained but may represent some regenerative response of lung associated lymphoid tissue to the initial irradiation effect.

An assessment of alveolar macrophage function with such parameters as viability, adherence to glass surfaces, and phagocytic ingestion of latex particles was not different from control values when macrophages were examined one, 6 and 12 days after irradiation.

Effects of drug suppression. All dogs had a preliminary lung lavage to establish control cell values; 2 weeks later a pair of dogs was assigned to a drug regimen for the next 6 weeks. Lung lavages were done at 2-week intervals after drug therapy was begun. Cell counts from each dog of a pair were combined and average values are given for total respiratory cells and lymphocytes (Fig. 2).

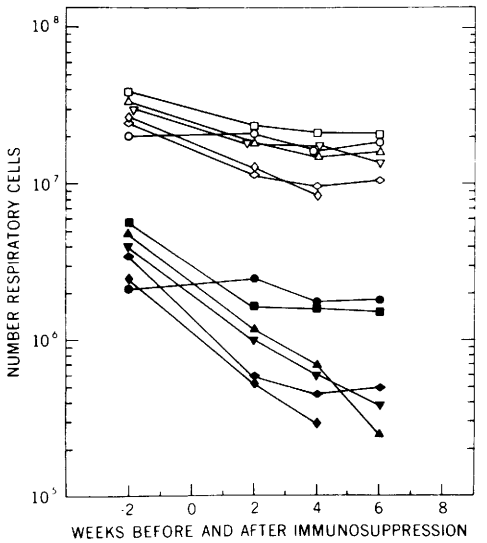

FIG. 2. Average respiratory cells counts obtained by bronchial lavage from a pair of dogs are shown before treatment and at 2-week intervals after various regimens of drug immunosuppression. Open symbols denote total respiratory cells; closed symbols give absolute lymphocyte counts. The following drugs were used: (△) daily methylprednisolone; (□) alternate day methylprednisolone; (◇) daily cyclophosphamide; (◇) daily cyclophosphamide and methylprednisolone; (▽) alternate day cyclophosphamide and methylprednisolone; and (○) no treatment controls.

Control dogs given no drugs had minimal cell fluctuations during the 8 weeks of observation. A typical respiratory cell differential count for these controls was similar to preirradiation values given in Table I. Alveolar macrophages comprised 75 to 85% of the total respiratory cell population. The proportions of macrophages and lymphocytes obtained are similar to values reported from normal dog lung washings (9–11).

Respiratory cells from dogs receiving daily or alternate day methylprednisolone decreased about 25% (Fig. 2) after 2 weeks of treatment compared with presteroid control values; thereafter, total cell counts were stable. Respiratory lymphocytes also decreased after 2 weeks of steroids and then remained stable in the alternate day treated pair (■); in contrast, lymphocyte counts continued to decrease in the daily steroid treated dogs (▲). Alveolar macrophages were unaffected, quantitatively, in steroid treated dogs.

Low dose daily methylprednisolone (1 mg/kg) or high dose (2 mg/kg) every other day had the same effect of decreasing peripheral blood lymphocytes and monocytes. In all instances, the degree of monocytopenia was somewhat greater than the lymphopenia. The absolute numbers of lymphocytes decreased by approximately 50% of control values after 2 to 4 weeks of steroid treatment. Likewise, blood monocyte counts were depressed by about 75% of control values.

It should be emphasized that although peripheral blood lymphocyte and monocyte counts eventually stabilized after several weeks of steroid treatment, there was a progressive decrease in the number of recoverable respiratory lymphocytes (▲) from the daily methylprednisolone treated dogs.

All steroid-treated dogs remained active and apparently healthy. There was no evidence of infection and daily morning rectal temperatures remained within the normal range. However, the weight loss of these dogs was profound during the 6 weeks of treatment. Dogs receiving daily steroids lost about 60% of their body weight; alternate day treated dogs lost 50%.

Daily cyclophosphamide reduced total respiratory cells and lymphocytes after two weeks by greater than 50%, whereupon all cell counts stabilized (Fig. 2). However, the daily combination of cyclophosphamide and methylprednisolone had a marked effect on both total respiratory cell recovery (◇) and lymphocyte counts (◆). These dogs could be maintained for only 4 weeks of therapy. The alternate day cyclophosphamide and methylprednisolone therapy permitted survival of the dogs for 6 weeks of therapy and had less effect on the total respiratory cell counts (no appreciable change in number of alveolar macrophages), but respiratory lymphocytes (▼) decreased progressively throughout treatment. Blood leukocyte counts were more easily controlled in the 2000–4000/mm^3 range by reducing the cyclophosphamide dosage and lymphocyte and monocyte counts stabilized at approximately 50% of pretreatment values. However, it was apparent that a progressive decrease of respiratory lymphocytes occurred with the combination treatment. Weight loss was considerable in these dogs and approximated about 50% of original body weight at the end of 6 weeks of treatment. Rectal temperatures remained normal and evidence of intercurrent infection was lacking.

Discussion. The effect of immunosuppressive therapy to alter specific cellular components of pulmonary immunity as sampled by serial bronchial lavages was the object of this study. First, a single dose of irradiation was used to cause acute bone marrow depression and leukopenia. The abrupt monocytopenia which was present by 6 days after irradiation and which persisted until the death of the animals had no effect on the number of recoverable alveolar macrophages. Although circulating monocytes are thought to be the precursor cells for lymphoreticular macrophages in the lungs (3, 4), long periods of monocytopenia in humans with hematologic malignancies (12) have not affected the recovery of alveolar macrophages. Undoubtedly, this attests to the longevity of tissue macrophages, including alveolar ones, which might survive for periods of several months. Moreover, these human studies have shown that alveolar macrophages incorporate tridiated thymidine into scheduled DNA synthesis and hence may replicate to some degree *in situ* in the lungs (13). Thus, under periods of

monocyte deprivation, the pulmonary macrophage population may be capable of sustaining itself in part.

With acute suppression of bone marrow and blood granulocytes, it has been shown that dogs are very susceptible to pneumonia (5, 6); however, restoring granulocytes by transfusion can cure an established Pseudomonas pneumonia in many instances (5). Thus susceptibility to pulmonary infection is in part a function of available circulating granulocytes. Whereas acute immunosuppression may not affect the number of recoverable alveolar macrophages (Fig. 1, Table I), the ability to mobilize granulocytes to the lung and other sites of acute infection is basic to host's immunity against many bacterial agents.

Second, drug therapy with methylprednisolone and with cyclophosphamide, singly and in various combinations, was used to create a chronic form of bone marrow suppression. All forms of therapy had some initial effect within 2 weeks of decreasing the number of recoverable respiratory cells (Fig. 2), then total counts stabilized (reflecting principally the number of alveolar macrophages), except in the daily cyclophosphamide-methylprednisolone treated dogs.

There was an appreciable difference in the recovery of respiratory lymphocytes for the two steroid regimens. Daily methylprednisolone caused a progressive decrease in respiratory lymphocytes, whereas the alternate day schedule, using twice the steroid dose, had less effect. The less dramatic changes in respiratory lymphocyte counts produced by alternate day steroid treatment may relate to other observations in humans that this regimen reduces susceptibility to infection (14). In peripheral blood, canine monocytes seemed to be more susceptible to steroid administration than lymphocytes, as was found in human studies (14, 15), but both cell types were depressed. Although the dogs remained frisky and healthy, weight loss was profound. These dogs appeared to be very susceptible to the cytolytic effects of steroids, particularly lymphocytes recoverable from the lower respiratory tract. Steroids probably cause a dissolution of lymphoid tissue in these animals. In this respect, dogs may be similar to other steroid sensitive species, such as the mouse, rat, rabbit, and hamster (16).

Summary. Canine respiratory cells, obtained by bronchial lavage, and blood leukocytes were monitored to observe cellular changes following acute and chronic immunosuppression. Irradiation (350 R) produced bone marrow suppression and prompt peripheral blood leukopenia, but did not affect recovery of pulmonary alveolar macrophages or lymphocytes for 12 days after. Treatment for 6 weeks with daily methylprednisolone (1 mg/kg) caused a progressive decrease in the number of recoverable respiratory lymphocytes, whereas alternate day methylprednisolone (2 mg/kg) had less effect. Cyclophosphamide in combination with steroids generally augmented the progressive loss of blood and respiratory lymphocytes. Recovery of alveolar macrophages was not changed appreciably. Thus, the population of lung macrophages, sampled by pulmonary lavage, withstood acute and chronic forms of immunosuppression very well. In contrast, canine lymphocytes seem more susceptible to injury, especially to drug regimens containing steroids.

The authors appreciate the assistance of Mr. Edward W. Harvey and Miss Patricia L. Johnson in these studies.

1. Levine, A. S., Schimpff, S. C., Graw, R. G., Jr., and Young, R. C., Sem. Hematol. **11,** 141 (1974).
2. Reynolds, H. Y., J. Infect. Dis. **130,** Suppl., 134 (1974).
3. Van Furth, R., Sem. Hematol. **7,** 125 (1970).
4. Godleski, J. J., and Brain, J. D., J. Exp. Med. **136,** 630 (1972).
5. Dale, D. C., Reynolds, H. Y., Pennington, J. E., Elin, R. J., Pitts, T. W., and Graw, R. G., Jr., J. Clin. Invest. **54,** 664 (1974).
6. Nutter, J. E., Graw, R. G., Jr., and Baum, S. J., Publication AD-763352, SR 73-5, Armed Forces Radiobiology Institute, Bethesda, Md.
7. Hanks, J. H., and Wallace, J. H., Proc. Soc. Exp. Biol. Med. **98,** 188 (1958).
8. Cohn, Z. A., and Weiner, E., J. Exp. Med. **118,** 1009 (1963).
9. Reynolds, H. Y., and Thompson, R. E., Fed. Proc. **31,** 936 (Abstract) (1972).
10. Kaltrieder, H. B., and Salmon, S. E., J. Clin. Invest. **52,** 2211 (1973).
11. Kaltrieder, H. B., Turner, F. N., and Salmon, S. E., Amer. Rev. Resp. Dis. **111,** 257 (1975).

12. Golde, D. W., Finley, T. N., and Cline, M. J., N. Engl. J. Med. **290,** 875 (1974).
13. Golde, D. W., Byers, L. A., and Finley, T. N., Nature (London) **247,** 373 (1974).
14. Dale, D. C., Fauci, A. S., and Wolff, S. M., N. Engl. J. Med. **291,** 1154 (1974).
15. Fauci, A. S., and Dale, D. C., J. Clin. Invest. **53,** 240 (1974).
16. Claman, H. N., N. Engl. J. Med. **287,** 388 (1972).

Received September 23, 1975. P.S.E.B.M., 1976, Vol. 151.