PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 156, 406-410 (1977)

Effects of Chronic Methotrexate Therapy on Numbers and Function of Rabbit Alveolar Macrophages[1] (39946)

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Introduction. The use of increasingly aggressive cancer chemotherapy to treat leukemias and lymphomas has been accompanied by a marked increase in infectious complications, particularly pneumonia (1-5). Furthermore, interstitial pneumonias and fibrosis, without definitive identification of causative organisms, have been reported to occur in patients receiving various chemotherapeutic regimens, particularly those including methotrexate (6-9). The pathological findings of both the interstitial pneumonias caused by infectious agents (e.g., pneumocystis carinii, cytomegalic virus) and those attributed to drug effects can be strikingly similar (4, 7, 9, 10) and suggest that some common pathogenesis may underlie all of these pneumonias despite the nonisolation of causative organisms in the "drug-associated" pneumonias.

The number and functions of peripheral phagocytic cells have been studied extensively in patients with leukemia receiving irradiation and/or chemotherapy (11-13), but to our knowledge no one has studied the numbers and functions of alveolar macrophages quantitatively as affected by long-term chemotherapy. Reynolds *et al.* (14) showed that canine alveolar macrophages were more resistant to acute and subacute doses of steroids and cyclophosphamide than canine lymphocytes with respect to absolute numbers, but did no functional studies. Lockard *et al.* (15) demonstrated abnormalities of bacterial ingestion by alveolar macrophages from rats treated with cyclophosphamide. Their experiments were very short term and no mention was made of changes in peripheral blood counts of the animals. For these reasons, we have studied the alveolar macrophages isolated from normal rabbits treated with saline or methotrexate to determine whether the numbers and *in vitro* phagocytic and bactericidal activities of these cells are altered by methotrexate therapy.

Methods. Healthy male New Zealand albino rabbits, weighing 2.5 to 3.0 kg and approximately 4 months of age at the time of onset of treatment, were used in all experiments. The dose of methotrexate (MTX) was chosen in order to approximate nontoxic doses that have been used therapeutically in man (6). The dose given was 2.3 mg/kg twice weekly, a dose estimated to be analogous to 1.0 mg/kg twice weekly in humans (16), and which we noted not to cause neutropenia in the rabbit. As controls New Zealand albino rabbits of the same weight and age characteristics were used. These animals were housed in the same animal quarters as the drug-treated rabbits and received saline injections twice weekly with a volume equal to that of methotrexate. Complete blood counts were performed weekly on all animals and at the time of sacrifice. The rabbits were sacrificed with an air embolus into a marginal ear vein and the lungs and tracheobronchial trees were dissected free. The lungs were lavaged four times with 50 ml of sterile saline solution. After lavage, the lung tissue was dissected free and weighed. Touch preparations on glass slides were made of the cut surfaces of the lavaged lungs. The wash-outs were collected in sterile plastic containers (Falcon), centrifuged at 1500*g* for 5 min, the supernatants removed, the cell pellets washed twice with Hank's buffered saline solution (HBSS) with 1% gelatin (Difco), and the cells finally resuspended in HBSS to give a final concentration of 1×10^7 cells/ml. Cell counts were made on both the saline and HBSS-suspended cells. The viability of the cells contained in the washings was evalu-

[1] Supported in part by Grant PDT-39 from the American Cancer Society, Inc.

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ISSN 0037-9727

ated microscopically by exclusion of trypan blue. Smears of the washings were made and stained with either Wrights stain or α-naphthyl acetate esterase stain by the technique of Yam *et al.* (17). The latter stain distinguishes mononuclear phagocytes (monocytes and macrophages) from lymphocytes and neutrophils.

Bactericidal capacities of the alveolar macrophages (AM) were determined by a modification of the method of Quie *et al.* (18). The bacterial species used in these studies were the 502A strain of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Bacteria were grown in Penassay broth (Difco) at 37° for 18 hr. The bacteria were washed with saline twice, then resuspended in saline to give an optical density of 3.12 at 620 nm in a Coleman Junior II spectrophotometer, Model 6/20, and then resuspended in HBSS with 1% gelatin at a dilution of 1:100.

Appropriate dilutions of cells and bacteria were mixed together in duplicate with 10% pooled normal rabbit sera to give a cell/bacterium ratio of approximately 2.5:1 in HBSS with 1% gelatin. Each tube contained 5×10^6 cells and a total volume of 1 ml. Additional mixtures were made for all experiments without cells as bacterial controls. After 0.1-ml samples of the mixtures were taken for zero-time viable colony counts, the tubes were tumbled gently for 1 hr at 37°, with the additional samples for viable bacterial colony counts removed at 30 and 60 min. The 0.1-ml samples were mixed with 9.9 ml of sterile water, agitated to lyse the cells, and then mixed with agar and poured into petri dishes. After 60 min of incubation, a 10-fold excess of bacteria was added to the tubes, the mixtures were incubated at 37° for an additional 15 min with gentle tumbling, and samples were taken and smeared on glass slides. These smears were examined microscopically after Wright's staining to determine the presence of intracellular bacteria. The numbers of viable colonies were determined after 24 hr of incubation of the petri dishes at 37°.

Experiments using the same bacterial strains were evaluated for the numbers of viable intracellular bacteria by a similar method with the following modification: gentamicin sulfate (Schering), at a final concentration of 10 μg/ml (this exceeded the minimal bactericidal concentration of the two strains for gentamicin by a factor of >2), was added after 30 min of incubation of cells and bacteria in HBSS with 10% normal rabbit serum and samples were subsequently taken 30 min later. These 0.1-ml samples were diluted 1:100 in sterile water as outlined above and the colony counts were made in identical fashion as from the tubes without gentamicin. The addition of gentamicin sulfate, an aminoglycoside antibiotic, allows one to distinguish intra- and extracellular bacteria, as it does not penetrate into leukocytes and therefore kills only extracellular bacteria (19, 20).

Results. The 22 rabbits included in Table I were similar with respect to appearance and weight gain throughout the study. Table I summarizes the mean peripheral blood counts and the numbers of AM at the time of sacrifice for the rabbits receiving methotrexate in the control groups. For this purpose, the control animals were combined into one group, although two received saline for 2 months and three for 9 months. There were no differences between these subgroups for either white blood counts or numbers of AM per gram wet lung weight. Greater than 95% of the cells in the lung washings from all the rabbits were esterase positive, and >99% of the cells excluded trypan blue. The AM from all of the animals appeared morphologically similarly after Wrights staining. Methotrexate treatment was associated with a statistically significant decrease in numbers of AM in all groups (by Student's *t* test) and a particularly severe depression was observed after 9 months. As shown, the absolute number of peripheral leukocytes and PMNs remained normal in each treatment group, with no significant variations seen as compared to the control group. Table II describes the individual complete blood counts at the time of sacrifice of the rabbits who showed profound decreases in the number of months of methotrexate therapy. There was no fall in any of the white cell series including the monocyte.

Wrights-stained and esterase-stained touch preparations from the cut surfaces of all

TABLE I. NUMBERS OF AVEOLAR MACROPHAGES AND PERIPHERAL BLOOD COUNTS OF RABBITS RECEIVING METHOTREXATE, 2.3 mg/kg, TWICE EACH WEEK.

Treatment (months)	Number	AM × 10^4/g wet lung weight ± 1 SEM	WBC × 10^9/liter ± 1 SEM	PMN × 10^9/liter ± 1 SEM
Saline (2)	3	674.2 ± 161.2	10.1 ± 3.1	4.7 ± 1.9
Saline (9)	2			
Methotrexate (1)	4	193.3 ± 41.7*	10.2 ± 1.3	3.1 ± 0.3
Methotrexate (2)	3	235.3 ± 68.0*	9.6 ± 0.8	4.8 ± 1.0
Methotrexate (6)	6	119.3 ± 24.0**	8.0 ± 0.9	3.2 ± 0.7
Methotrexate (9)	4	8.4 ± 1.1***	10.7 ± 1.4	5.4 ± 1.8

* $P < 0.05$ compared to controls.
** $P < 0.01$ compared to controls.
*** $P < 0.001$ compared to controls.

TABLE II. INDIVIDUAL BLOOD COUNTS OF RABBITS RECEIVING MTX FOR 9 MONTHS.

HCT (%)	WBC × 10^9/liter	PMN × 10^9/liter	Monocytes × 10^9/liter
37	8.73	2.27	0.61
39	9.04	6.05	0.36
42	14.62	10.09	0.88
38	10.44	3.03	0.63
Mean ± 1 SEM 39 ± 1.1	10.71 ± 1.36	5.36 ± 1.78	0.62 ± 0.11

rabbits sacrificed revealed no obvious differences between residual numbers of esterase-positive cells after lavage of the lungs between the saline control animals and the methotrexate-treated animals.

Figures 1 and 2 show the *in vitro* bactericidal activities of alveolar macrophages of the saline-treated rabbits as compared to those of rabbits treated with methotrexate for 1 or 6 months against *S. aureus* 502A and *P. aeruginosa*. The killing of *P. aeruginosa* is significantly deficient by the AM of rabbits who received MTX for 6 months after 60 min ($P < 0.01$ at 60 min using Student's t test), but not after 30 min ($P = 0.07$). These same AM are significantly deficient in the killing of *S. aureus* 502A after 30 min ($P < 0.01$), but not after 60 min ($P < 0.9$). AM of rabbits receiving methotrexate for only 1 month show normal killing against *S. aureus* 502A and *P. aeruginosa* after both 30 and 60 min. Insufficient numbers of AM were obtained from rabbits who received methotrexate for 9 months to conduct the bactericidal experiments.

When a 10-fold excess of bacteria was added after 60 min to the cells in basic medium with 10% serum and incubated for 15 min at 37°, there appeared to be no differences microscopically between the number of intracellular bacteria from the AM of the methotrexate-treated animals as

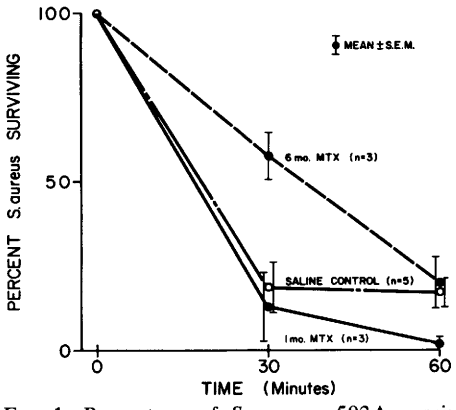

FIG. 1. Percentage of *S. aureus* 502A surviving after incubations with alveolar macrophages of saline- and methotrexate-treated rabbits.

compared to that from the AM of the saline-treated controls. When gentamicin sulfate was added to the cell bacteria mixtures after 30 min of incubation, less than 5% bacteria survived in the tubes containing the antibiotic, bacteria, and cells from animals treated with methotrexate for 6 months, suggesting that the *Pseudomonas* surviving in the non-gentamicin-containing mixtures after 60 min were predominantly extracellular. Thus, the diminished killing of *Pseudomonas* by AM of animals receiving long-term treatment with methotrexate was presumably a result of defective phagocytosis.

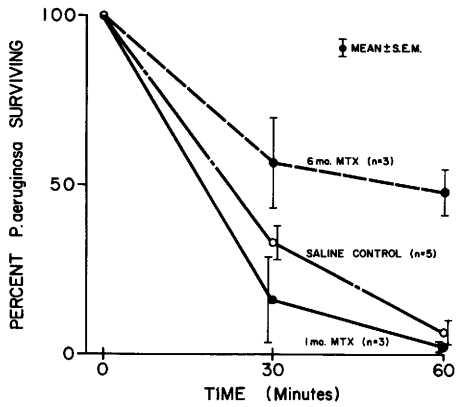

FIG. 2. Percentage of *P. aeruginosa* surviving after incubations with alveolar macrophages of saline- and methotrexate-treated rabbits.

Our methods cannot adequately evaluate killing of bacteria when phagocytosis is subnormal, so an additional defect in intracellular killing cannot be entirely ruled out.

Discussion. These results show an orderly and clear-cut decrease in the population of AM expressed as numbers of cells per gram of wet lung weight after even 1 month of treatment with low-dose methotrexate. This is particularly surprising in view of the entirely normal numbers of peripheral blood polymorphonuclear leukocytes and monocytes of all rabbits studied.

On the other hand, abnormalities of killing and/or phagocytosis are only significant for the AM of rabbits receiving the drug for at least 6 months. The differences are demonstrated against both a gram-positive and a gram-negative organism representative of the organism that causes pneumonia in patients with leukemia receiving chemotherapy (5).

The alveolar macrophage may be more susceptible to cancer chemotherapy because it is a hematopoietic cell of bone marrow origin (21, 22), but its susceptibility to these agents may be distinctive from that of other white blood cells because it is turned over at a much slower rate than polymorphonuclear leukocytes (22). Golde *et al.* (23) have shown in three leukemic patients with long-term sustained peripheral blood monocytopenias (40 to 60 days) that morphologically and functionally normal AM were present in their lung washings. This study could not absolutely quantify AM and it is possible that the numbers of AM were decreased in each of these patients from those of healthy patients. Furthermore, it is unclear in the normal steady state in animals whether tissue macrophages are self-renewing or whether peripheral blood monocytes migrate to tissues and replenish the tissue macrophage pool (24).

It is entirely possible that methotrexate-associated pneumonias may have an infectious etiology, and that abnormalities of AM numbers and functions demonstrated in this study may be two factors which predispose patients receiving low-dose and long-term methotrexate to pneumonias. There is only scant evidence that methotrexate itself is directly toxic to lung parenchymal tissue (25), and the matter is even more confusing because many of the patients who have been diagnosed to have methotrexate-associated pneumonia tolerated retreatment without experiencing subsequent toxicity (9). Despite the fact that infectious agents have not been isolated in the reported cases of methotrexate-associated pneumonias, it is possible that bacterial pneumonias of persons receiving methotrexate are far more common than reported, as those pneumonias with definite infectious etiologies would not normally be documented in the literature.

If adverse effects demonstrated by this study in rabbits occur in humans, it is possible that all patients receiving methotrexate may be particularly susceptible to the development of pneumonia, even when their peripheral white blood counts are normal.

Summary. Rabbits were treated with biweekly intramuscular methotrexate, 2.3 mg/kg, for 1, 2, 6, and 9 months. During these periods, none manifested leukopenia, neutropenia, or monocytopenia of the peripheral blood. After sacrifice, saline lavages of the lungs revealed progressively decreasing numbers of alveolar macrophages with time. Bactericidal function tests of the macrophages against *S. aureus* 502A and *P. aeruginosa* revealed defective phagocytosis after 6 months of treatment.

I am indebted to Ms. Carol Wass and Ms. Esperanza Virtusio for expert technical assistance and to Ms. Diana McKenzie and Ms. Diane Reck for expert secretarial assistance.

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Received April 21, 1977. P.S.E.B.M. 1977, Vol. 156.