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"Treatment of Mycobacterium intracellulare Infected Mice with Walter Reed Compound H"

Final Comprehensive Report

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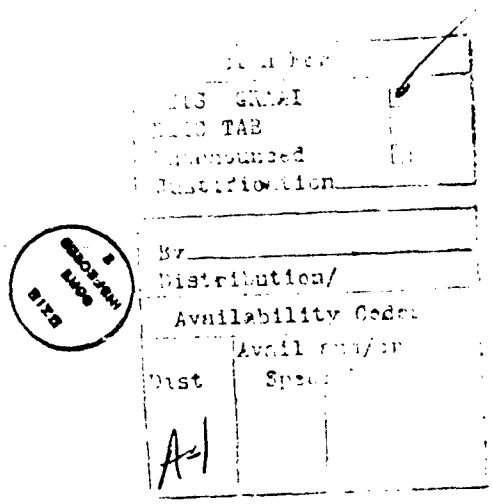
Summary

The antimycobacterial activity of 21 thiosemicarbazone compounds on growth of strains of M. intracellulare was determined. The minimal inhibitory concentration for 5 of the compounds against strains growing in 7H9 broth was 1 mcg/ml or less. One of these compounds, compound H, was chosen for further study and its effectiveness for treating M. intracellulare infected Swiss-Webster mice was determined. The drug was remarkably effective in eliminating organisms from the spleens and lungs of mice that had been infected by either intravenous or intraperitoneal injections of M. intracellulare serotype 12 (Trudeau D-673).



Forward

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committees on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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"Treatment of Mycobacterium intracellulare Infected Mice with Walter Reed Compound H"

Introduction

Certain thiosemicarbazones are inhibitory to growth of strains of bacteria including Mycobacterium tuberculosis, Mycobacterium leprae, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, enterococci, Neisseria gonorrhoeae, and Neiserria meningitidis (1). This study was initiated in order to examine the potential usefulness of a group of thiosemicarbazones synthesized at the Walter Reed Institute of Research, Walter Reed Army Medical Center, Washington, D.C. for inhibiting growth of Mycobacterium avium-intracellulare both in vitro and in vivo. This organism produces a progressive infection in man that is refractory to treatment with the usual antituberculosis drugs and additional chemotherapeutic agents are needed for treating individuals infected with M. avium-intracellulare.

Materials and Methods

Organisms

Most of the strains used in the initial phases of this study were obtained from patients with pulmonary infections at the National Jewish Hospital and Research Center/National Asthma Center, Denver, CO. These strains were characterized in the usual biochemical manner (2) and were serologically classified using the methods of Schaefer (3). For the in vivo studies M. intracellulare serotype 12 was obtained from Dr. Frank Collins at the Trudeau Institute, Saranac Lake, N.Y. (Trudeau designation is #D-673) (4). Strains were grown in 7H9 liquid medium and stored frozen at -70°. Prior to use in experiments, cultures were thawed, transferred to fresh 7H9 broth and incubated at 37° with vigorous shaking for aeration until early log phase growth was obtained. Dilutions of these cultures were used for inoculation of liquid or solid media and injection of animals. Colony counts were determined by plating dilutions of cultures and tissue homogenates on 7H11 agar. Plates were incubated in a 5% CO_2 atmosphere at 37° for 21 days.

Compounds

Twenty-one thiosemicarbazone compounds were received from the Walter Reed Institute of Research. The chemical structures of the compounds were and remain unknown to these investigators. All compounds were dissolved in dimethylsulfoxide (DMSO) at a 10,000 mcg/ml concentration before use in the in vitro studies.

In-vitro Studies

For preliminary studies the thiosemicarbazones in DMSO were added to 5 ml of 7H9 broth in standard screw cap test tubes to final thiosemicarbazone concentrations of 1, 10, and 100 mcg/ml. Control tubes contained 7H9 broth only and 7H9 broth + DMSO at an equivalent concentration to that of the drug tubes. The tubes were inoculated with 0.1 ml of a log phase culture (O.D. approximately 0.1 at 525 nm) of M. intracellulare serotype 9 or M. intracellulare strain Allen. Tubes were incubated for 7 days at 37° on a roller-tube apparatus and visually read for turbidity. Growth in the presence of the thiosemicarbazone compounds was compared to that in the control tubes. Compounds labeled as A (lot BH 58586), B, (lot BH 49578), D (lot BH 58451), F (lot BH 58540), G (lot BH 67316), H (lot BH 48062), J (lot BH 67718), L (lot BH 47912), N (lot BH 67325), O (lot BH 58559), R (lot BH 67478), S (lot BH 67450), U (lot BH 50286), V (lot BH 58577), Y (lot BH 58568), Z (lot BH 64717), AA (lot BH 67469), BB (lot BH 67334), CC (lot BH 70073), DD (lot BH 67487), and EE (lot BH 67307) were screened in this manner. In addition to the turbidity readings, 0.01 ml samples were taken from some of the tubes, diluted in fresh 7H9 broth and inoculated on the surface of 7H11 agar plates for colony count determinations. These were made in order to attempt to determine whether the drugs were bacteriostatic or bactericidal.

On the basis of the preliminary screening of all 21 compounds, five were chosen for study of their effectiveness for inhibiting growth of an additional five strains of M. intracellulare (designated strains I-V). The experimental methodology was the same as used in the previous experiment except that a 0.1 mcg/ml concentration was included.

Later it was decided to test the effectiveness of thiosemicarbazone compound H against species of other mycobacteria and additional strains of M. avium-intracellulare. To do this thiosemicarbazone compound H at final concentrations of 0.1, 1, and 10 mcg/ml was added to 7H11 agar and plates prepared. Plates containing the various concentrations of compound H were then inoculated with 10 strains of M. tuberculosis (resistant to ethionamide), 20 strains of M. scrofulaceum, and 20 strains of M. intracellulare. The plates were inoculated with two dilutions of each strain with a goal of achieving at least one plate with an inoculum ranging from 100-500 colonies. After inoculation plates were incubated in a 5% CO_2 atmosphere at 37° for 3 weeks. Strains were considered to be susceptible if at least 90% growth inhibition was observed.

In-vivo Studies

For these studies four to five week old male Swiss-Webster mice (strain CD-1; Charles River Breeding Laboratories, Wilmington, MA) were given intravenous, intraperitoneal, or subcutaneous injections with 10^6 to 10^7 M. intracellulare serotype 12. Mice infected by the various routes were then divided into treatment and control groups. Compound H was blended into powdered mouse food (Lab Blox in meal form; Wayne Feeds, Allied Mills Inc., Specialty Feeds, Chicago, IL) at a concentration of 0.1312 gm per 175 gm of food and the mice were given the food ad lib. The amount of compound H was based on the assumption that a 0.1% amount of thiosemicarbazone in the diet is safe. Blood levels of compound H were not determined. Control groups were comprised of uninfected mice receiving drug, infected mice that received no treatment and uninfected mice with no treatment. Body weights of the mice were determined throughout the course of the experiments. Mice were sacrificed at one day, one week, two weeks, three weeks, one month, two months, and three months after infection. Treatment with thiosemicarbazone compound H started two-three weeks after infection. On the sacrifice dates, spleens, lungs, and lymph nodes from mice were removed for cultures. The organs were weighed, placed in Teflon-to-glass tissue grinders containing a small amount of diluent, and homogenized. Dilutions of the homogenates were then plated on 7H11 agar. Bacterial growth in the various tissues was calculated on the basis of bacteria per gm of tissue. Data points shown in Figures 1-7 are the mean counts from the tissues of 4 to 6 mice.

Results

In vitro Studies

The results of screening the effectiveness of the twenty-one thiosemicarbazone compounds on growth of two strains of M. intracellulare growing in 7H9 broth are shown in Table 1. It can be seen that compounds H, L, S, Z, and AA inhibited growth of at least one of the two strains at a 1 mcg/ml or less concentration. Compounds B, D, N, O, R, U, Y, DD, and EE inhibited at least one of the strains at a concentration between 1 and 10 mcg/ml and all other compounds inhibited at higher concentrations. It can also be seen in Table 1 that certain discrepant results were obtained. For example, compound D appeared to inhibit growth at 10 mcg for both strains and yet the 100 mcg/ml concentration tube showed turbidity. To explain these results samples were taken from the discrepant tubes and plated on 7H11 agar to obtain colony counts. These data are shown in Table 2. It is apparent that the turbidity seen in tube D-100 for both strains type 9 and Allen was not due to bacterial growth. Some idea as to whether the compounds are bactericidal or bacteriostatic was also obtained from the data shown in Table 2. Samples taken from tube H-1 with no turbidity indicate that the drug is apparently bactericidal against serotype 9 and bacteriostatic against strain Allen. Similar results were obtained with compound L and N. Compounds S and Z seem to be bacteriostatic for both strains. Compound AA appeared to be bactericidal against strain Allen and bacteriostatic against serotype 9.

On the basis of the above data indicating that certain drugs inhibited growth of one or more of the M. intracellulare strains tested at a 1 mcg/ml concentration or less, five additional strains were tested in the same manner including a 0.1 mcg/ml concentration. Compound H inhibited all strains at a concentration of 0.1 to 1 mcg/ml. Compound L inhibited strain III at a concentration of less that 0.1 mcg and the other four strains at a concentration between 1 and 10 mcg. Compounds S, Z, and A all had similar effects in that strains I, IV, and V were inhibited by 1 to 10 mcg, and strains II and III by 0.1 to 1 mcg/ml.

Because of the effectiveness of compound H on the M. intracellulare strains, an additional 20 strains were tested at various concentrations of compound H. However, this experiment was carried out with the strains growing on an agar based medium, 7H11 agar. These data are shown in Table 4 along with the results of the inoculation of 10 ethionamide-resistant strains of M. tuberculosis and 20 strains of M. scrofulaceum . The results indicate that 7 of the 20 strains of M. intracellulare were inhibited by a concentration of 1 to 10 mcg of compound H. Similar results were obtained with the other strains tested. In fact, 9 of the 10 M. intracellulare strains were inhibited by 1 to 10 mcg concentrations. The 10-fold difference of susceptibility of the strains of M. intracellulare growing on agar vs. the liquid broth medium is unexplained. However, the phenomenon of variable results between susceptibility test results performed in a liquid medium and in a solid medium is a recognized one in clinical microbiology (5).

In vivo Studies

The effect of M. intracellulare infection and/or treatment with compound H on mouse body weights is shown in Figure 1. Infected mice did not change their weights appreciably during the course of the experiment. However, the two groups of mice which received the drug lost a significant amount of body weight during the course of the experiment. The toxicity of the treatment dose of compound H is further illustrated by the fact that of 25 mice receiving compound H only, 10 died within a 2 month period, 20 died within a 3 month period, and none were alive after a 3 month period. Some of these mice were necropsied by Lynn Jankovsky, DVM, (Veterinary Pathologist, University of Colorado Health Sciences Center). Dr. Jankovsky reported that these mice had died of what appeared to be an "interstitial pneumonia."

The multiplication of M. intracellulare in various tissues of mice after receiving injections of bacteria by various routes is shown in Figures 2-4. Data shown in these figures indicate that subcutaneous injections did not lead to bacterial multiplication in either spleens,

popliteal lymph nodes, or lungs in the test mice. Intravenous injection of M. intracellulare led to a 1-2 log increase of bacteria in spleens. Lung counts (intravenous injection) were difficult to interpret since there seemed to be a gradual decrease in number of bacteria seen in the lungs through 8 weeks with a marked increase between the 8th and 12th week time period. Lymph node infection data following intravenous injection was also difficult to interpret. The varying counts are probably due to difficulty in culturing and obtaining popliteal lymph node material. The intraperitoneal route of infection also led to a progressive (10-fold) increase in number of bacteria seen in spleens and lungs. In fact, the intraperitoneal route of injection apppeared to offeran excellent model for evaluation of drug effectiveness for treating pulmonary disease due to M. intracellulare. Lymph node data following intraperitoneal injection was difficult to interpret.

Results of treating mice with compound H that had been infected with M. intracellulare by the various routes are shown in Figures 5-7. Figure 5 indicates that intravenous infected and treated mice had almost a 1 log decrease in number of bacteria following the 12 week treatment period when bacteria in spleens are follwed. Even more impressive is the effect of the drug on bacteria in spleens of intraperitoneally infected mice. The number of bacteria in the spleens was four log units lower than in the untreated controls. Figure 6 shows lymph node data for intraperitoneally infected mice. It is apparent that treatment with compound H decreased the number of bacteria by 2 log units after the 12 week period. Lung counts for intraperitoneally infected mice are shown in Figure 7. At the end of the 12 week treatment period, the number of bacteria was decreased by approximately 4 log units.

Discussion

Although the experiments described in this report should be considered to be preliminary in nature, we feel that two important observations have been made. First, infection of Swiss-Webster mice with Mycobacterium intracellulare serotype 12 (Trudeau D-673) leads to the development of an apparently progressive pulmonary infection. Lack of such a model has hampered research into new antimycobacterial agents effective against M. intracellulare for several years. It is apparent that one can readily follow bacterial multiplication in either spleens or lungs after injection of M. intracellulare by either the intravnenous or intraperitoneal route. Subcutaneous injection of this strain did not appear to be a satisfactory arrangement. Secondly, and most importantly, several of the thiosemicarbazones studied had substantial antimycobacterial activity in vitro and one, compound H, was shown to be markedly effective in preventing multiplication of M. intracellulare serotype 12 in both spleens and lungs of infected mice. Unfortunately the experiments were carried out using a drug dosage which was apparently relatively close to the toxic level.

Future Plans

The data described above leads us to suggest the following additional work:

1. Intravenous and intraperitoneal injection of various amounts of M. intracellulare serotype 12 should be carried out to determine the infectious dose with this organism and to determine whether this infection will eventually lead to a progressive disease in mice or whether the infection eventually will become a chronic situation. The pathology of the infection in mice is also of interest. It is planned to follow the infected mice for a much longer period than the 12 weeks initially used.

2. The in vitro studies should be expanded to include additional species of nontuberculous mycobacteria and drug resistant strains of M. tuberculosis that are involved in infections in man. Of special interest would be the susceptibility of strains of Mycobacterium fortuitum, Mycobacterium chelonei, and Mycobacterium marinum. All of these species are being increasingly implicated in diseases in man and are generally refractory to treatment with many of the commonly used antituberculosis drugs.

3. Employing the mouse model described above the treatment experiments with compound H (and possibly other thiosemicarbazones) should be repeated employing various dosages of compound H which are generally lower than those used in the initial experiments. It is unfortunate that the level of compound H employed generally led to toxicity in the mice. In other words, we propose to repeat the experiments and determine the dose-response relationships for compound H and M. intracellulare in Swiss-Webster mice.

We are prepared to pursue the above using our own resources.

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Table 1

Effect of Thiosemicarbazones on Growth of M. intracellulare in 7H9 Broth

Compound-Conc(mcg/ml)	Turbidity Type 9	Turbidity Allen
Control	4+	4+
Control + DMSO	4+	4+
A-1	4+	4+
-10	4+	2+
-100	0	0
B-1	4+	4+
-10	0	0
-100	0	0
D-1	4+	4+
-10	0	0
-100	3+	3+
F-1	4+	4+
-10	4+	4+
-100	1+	1+
G-1	4+	4+
-10	4+	1+
-100	1+	1+
H-1	0	0
-10	0	1+
-100	0	1+
J-1	4+	4+
-10	4+	4+
-100	0	0
L-1	0	4+
-10	0	0
-100	0	0
N-1	4+	4+
-10	0	4+
-100	1+	3+
O-1	4+	4+
-10	0	+
-100	0	0

Compound-Conc(mcg/ml)	Turbidity Type 9	Turbidity Allen
R-1	4+	4+
-10	0	0
-100	0	0
S-1	0	4+
-10	0	0
-100	0	0
U-1	4+	4+
-10	0	4+
-100	0	0
V-1	4+	4+
-10	2+	3+
-100	0	0
Y-1	+	4+
-10	0	0
-100	0	0
Z-1	0	4+
-10	0	0
-100	0	0
AA-1	0	4+
-10	0	0
-100	0	0
BB-1	4+	4+
-10	4+	4+
-100	0	0
CC-1	4+	4+
-10	4+	4+
-100	0	0
DD-1	4+	4+
-10	0	+
-100	0	0
EE-1	+	4+
-10	0	2+
-100	0	0

Table 2

Subculture Results from 7H9 Broth cultures

Tube	No of Colonies/0.01 ml Sample	
	Serotype 9	Allen
Control	TNTC*	TNTC
Control + DMSO	TNTC	TNTC
D-100	0	1
H-1	18	TNTC
H-100	0	34
L-1	0	TNTC
N-100	20	ND**
S-1	TNTC	TNTC
Z-1	TNTC	TNTC
AA-1	TNTC	47

*TNTC = To numerous to count
**ND = Not done

Table 3

Effect of Thiosemicarbazones on Growth of *M. intracellulare* Strains

	Turbidity / Strain				
Compound-Conc(mcg/ml)	I	II	III	IV	V
Control	3+	4+	4+	3+	4+
Control + DMSO	3+	4+	4+	3+	4+
H-0.1	3+	4+	3+	2+	+
-1	0	0	0	0	0
-10	0	0	0	0	0
-100	0	0	0	0	0
L-0.1	3+	4+	0	3+	2+
-1	2+	0	0	0	+
-10	0	0	0	0	0
-100	0	0	0	0	0
S-0.1	4+	3+	3+	4+	4+
-1	2+	0	0	2+	2+
-10	0	0	0	0	0
-100	0	0	0	0	0
Z-0.1	3+	4+	+	4+	4+
-1	2+	0	0	2+	2+
-10	0	0	0	0	0
-100	0	0	0	0	0
AA-0.1	3+	4+	+	+	3+
-1	2+	0	0	+	2+
-10	0	0	0	0	0
-100	0	0	0	0	0

Table 4

Inhibition of Mycobacteria Growing on 7H11 Agar by Compound H

Species	Number tested	Number inhibited Concentration mcg/ml 0.1	1.0	10.0
M. tuberculosis*	10	0	1	8
M. scrofulaceum	20	0	0	9
M. intracellulare	20	0	0	7

*Ethionamide-resistant strains

FIG. 1. EFFECT OF INFECTION AND TREATMENT ON MOUSE WEIGHTS

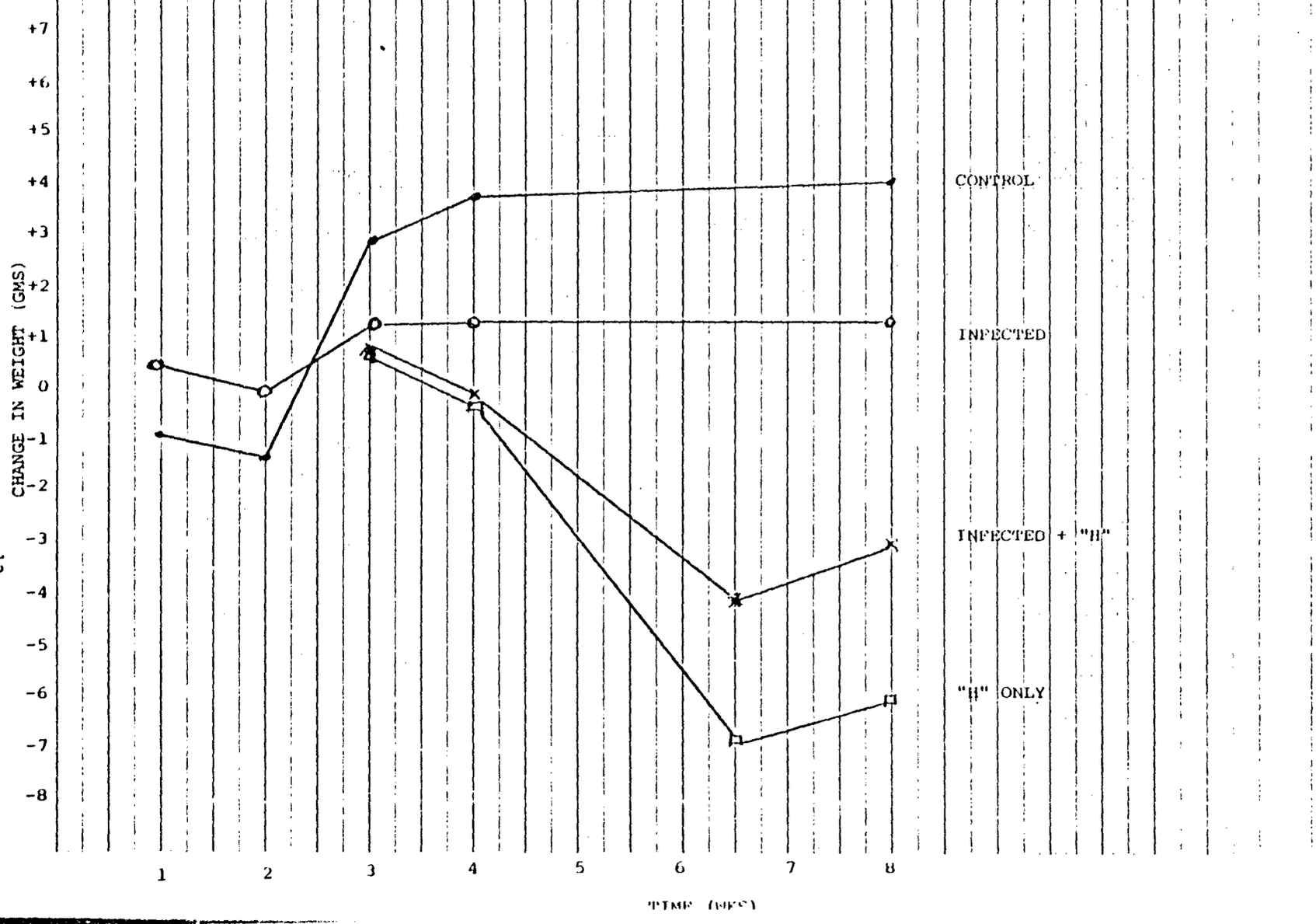

FIG. 2. BACTERIAL COUNTS IN SPLEENS OF MICE INFECTED BY VARIOUS ROUTE OF INFECTION

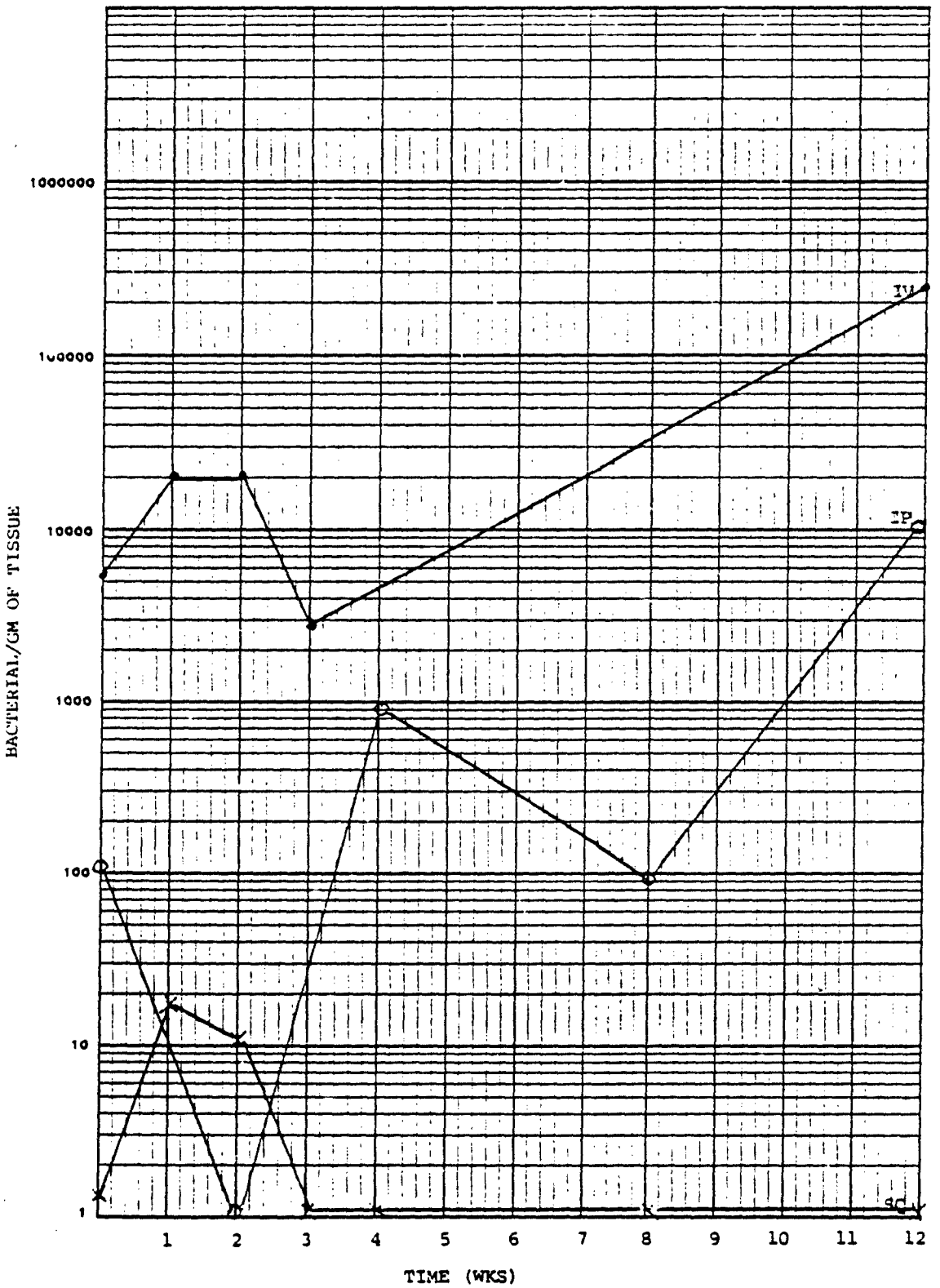

Fig. 3. BACTERIAL COUNTS IN POPLITEAL LYMPH NODES OF MICE INFECTED BY VARIOUS ROUTES OF INJECTION

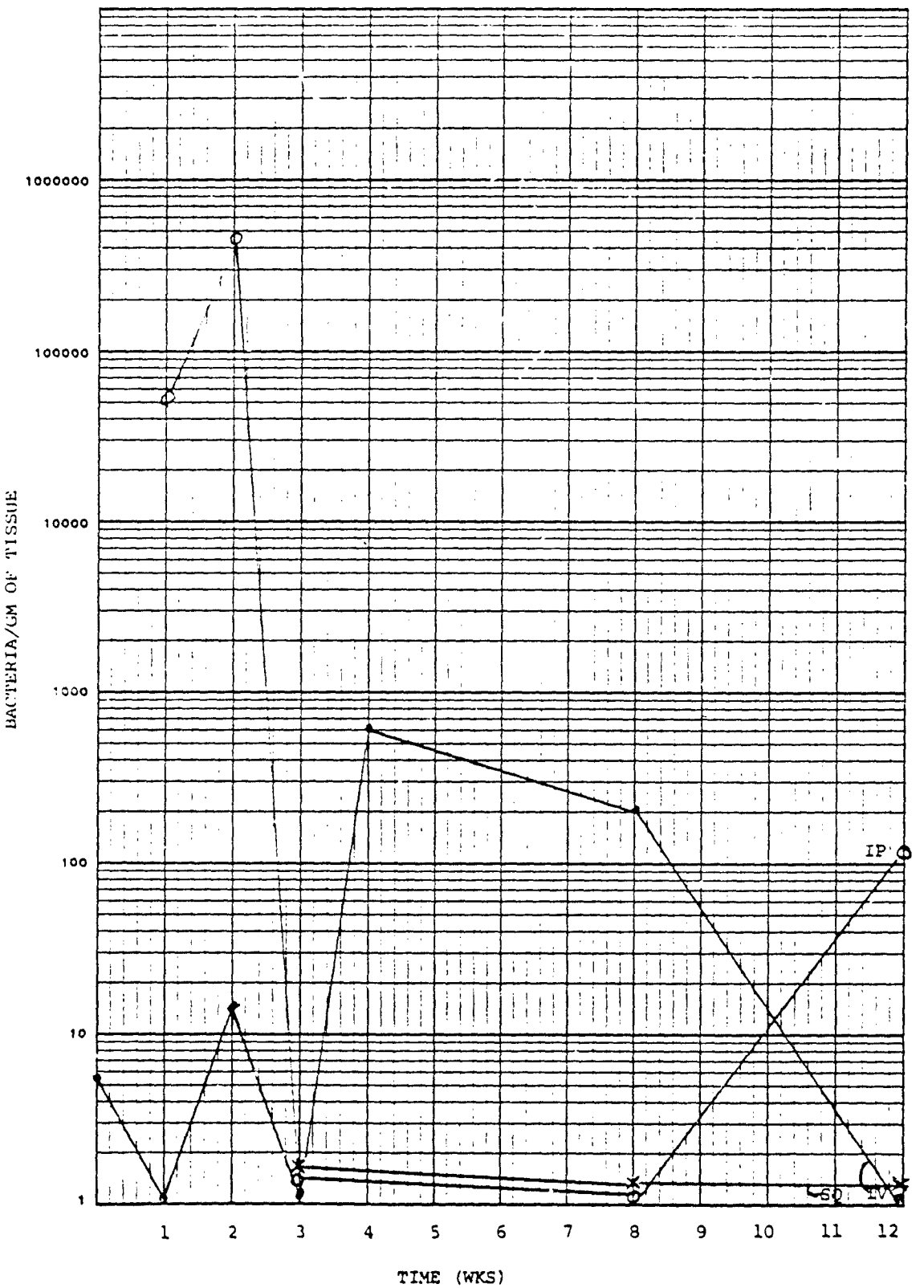

FIG. 4. BACTERIAL COUNTS IN LUNGS OF MICE

INFECTED BY VARIOUS ROUTES OF INJECTION

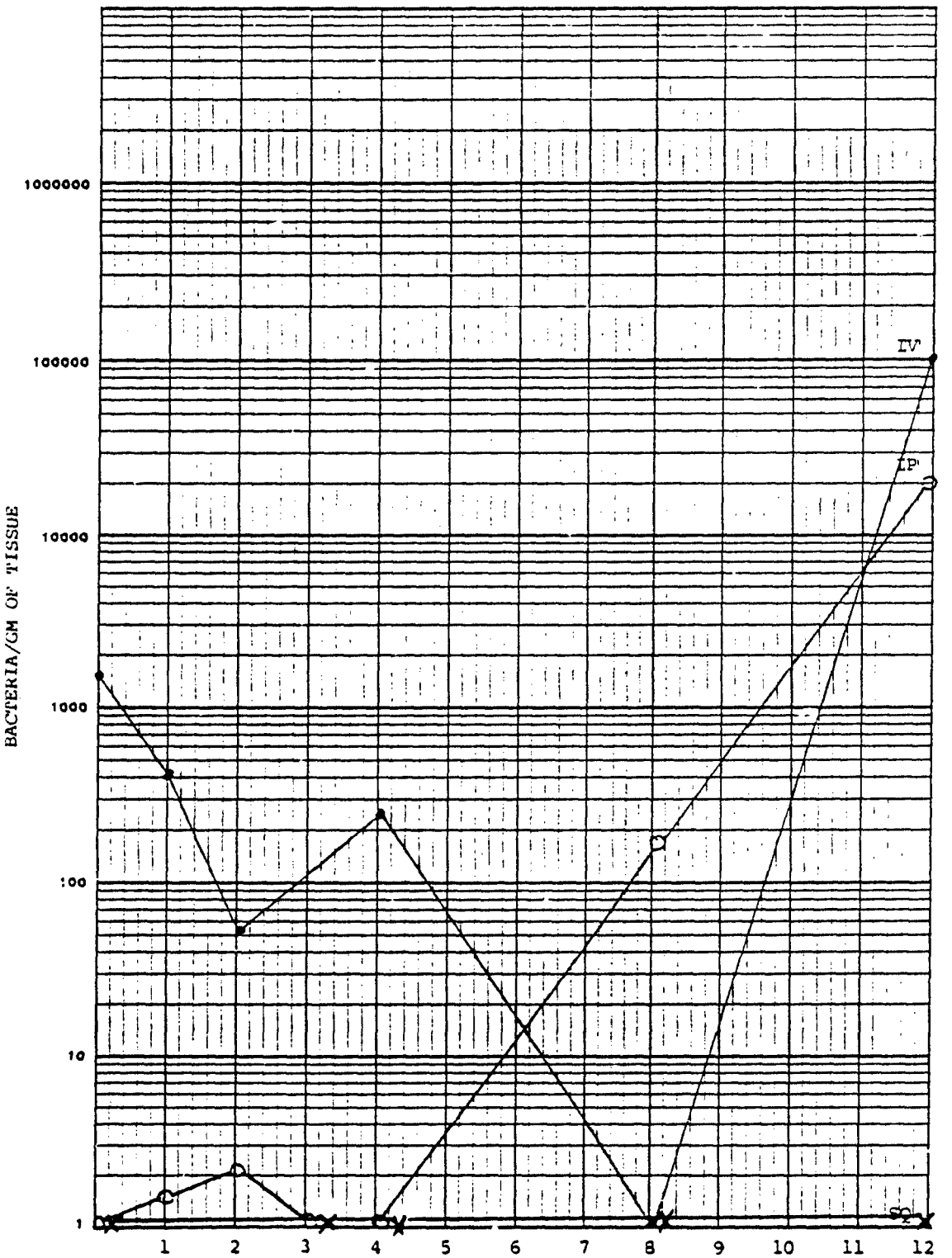

FIG. 5. EFFECT OF TREATMENT ON BACTERIAL COUNTS IN SPLEENS

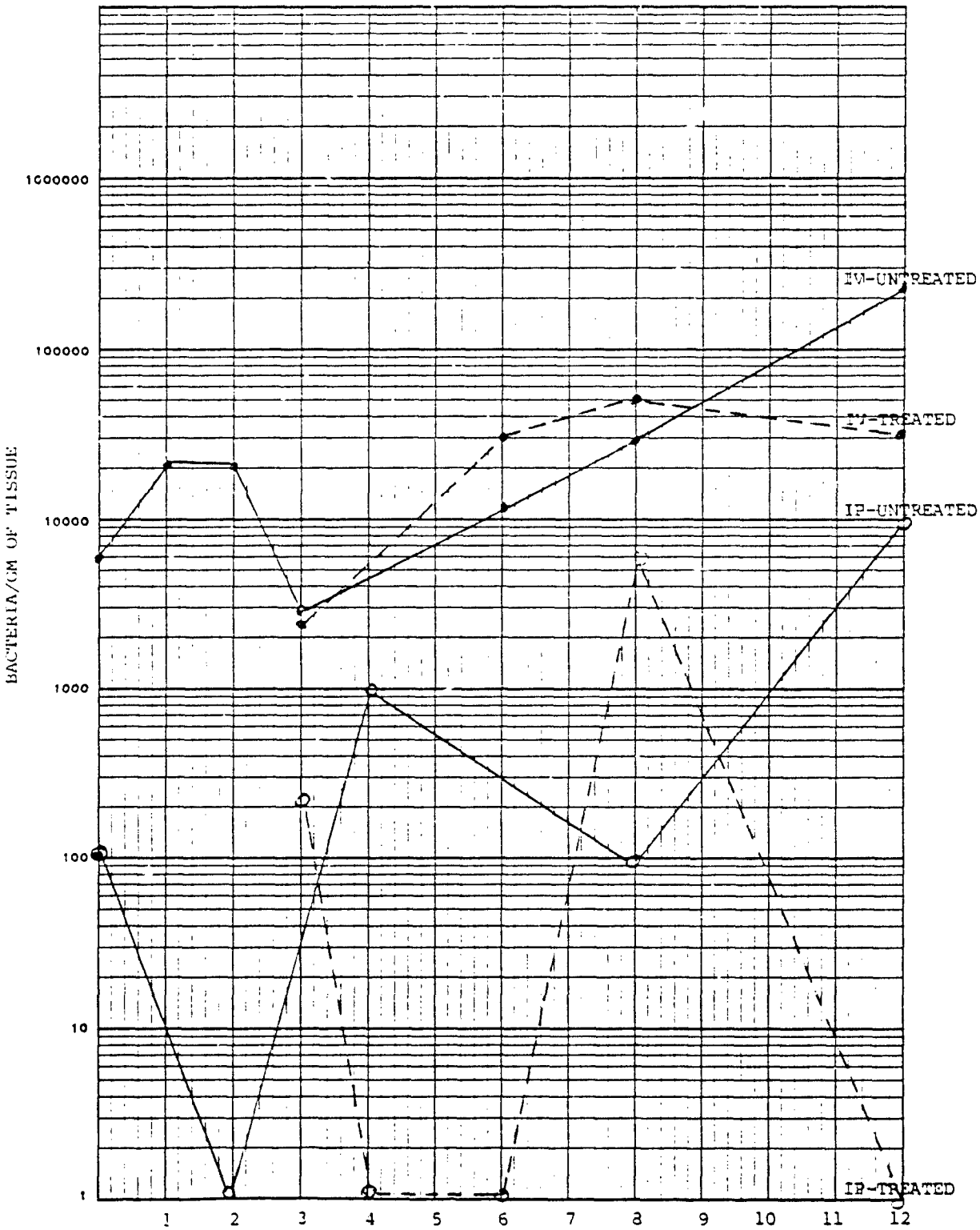

FIG. 6. EFFECT OF TREATMENT ON BACTERIAL COUNTS IN POPLITEAL LYMPH NODES

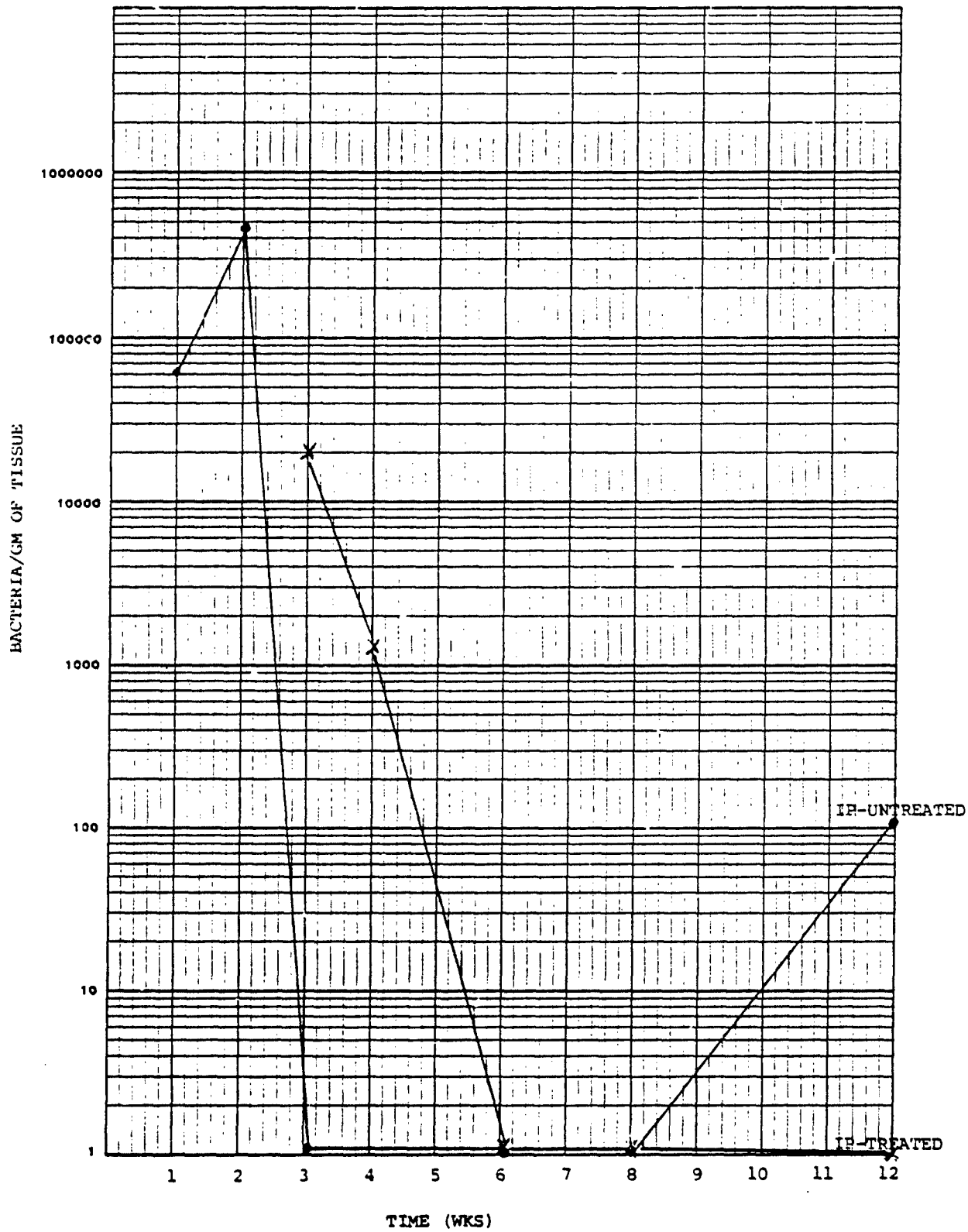

FIG. 7. EFFECT OF TREATMENT ON BACTERIAL COUNTS IN LUNGS