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GRADUATE COLLEGE

THE EFFECT OF CARBON MONOXIDE EXPOSURE ON SUSCEPTIBILITY OF MICE TO RESPIRATORY INFECTION WITH *LISTERIA MONOCYTOGENES*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

RALPH FRANK UNGAR

Norman, Oklahoma

1972

THE EFFECT OF CARBON MONOXIDE EXPOSURE ON SUSCEPTIBILITY OF MICE TO RESPIRATORY INFECTION WITH *LISTERIA MONOCYTOGENES*

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THE EFFECT OF CARBON MONOXIDE EXPOSURE ON SUSCEPTIBILITY OF MICE TO RESPIRATORY INFECTION WITH *LISTERIA MONOCYTOGENES*

CHAPTER I

INTRODUCTION

Carbon monoxide (CO), a colorless nonirritating gas, is the most abundant and widely distributed air pollutant found in the lower atmosphere (15). Emission of CO generally exceeds that of all other gaseous pollutants combined, particularly in urban atmospheres. This gas constitutes an important, world wide environmental hazard. More persons in the United States succumb each year to acute CO poisoning (>1000) than to any other single toxic agent except alcohol (25).

The acute toxicity associated with tissue hypoxia during CO exposure is well understood and involves the preferential binding of CO instead of oxygen to hemoglobin forming carboxyhemoglobin (COHb) (1). Because the affinity of CO for hemoglobin is 200 to 250 times that of oxygen, a small quantity of CO can reversibly inactivate a substantial

percentage of the oxygen-carrying capacity of the blood (1). It is possible, therefore, that exposure to concentrations of CO above 0.1 per cent (1000 ppm) can be fatal to both lower animals and humans (35). Another major effect of CO may be attributed to alteration of oxyhemoglobin (O_2Hb) dissociation characteristics produced by COHb, with a resultant impaired unloading of O_2 at the tissues.

The results of animal exposure to concentrations of CO below 1000 ppm present a picture which is far less clear. However, differences of exposure response occur among animals of the same species while repeated sub-lethal exposures may result in acclimatization to concentrations of CO which are lethal to unacclimatized animals.

The more obvious adverse effect of CO on human health is generally associated with its ability to impair oxygen transport in blood by mechanisms already mentioned. Therefore, attention should be given to the fact that CO does under certain circumstances, produce profound effects on mechanisms other than the oxygen-hemoglobin system. Several specific alterations in a number of endocrine organs have been attributed to CO. Lilienthal (25) in his review on carbon monoxide, made reference to several cases of acute hyperthyroidism following exposure to CO and increased activity of the thyroid and bioassay of hypophyseal depletion of thyrotropic hormone as evidenced by anatomical changes. Patterson and coworkers found that food intake and gastro-intestinal

motility were inhibited by repeated exposure to CO (30). Among the most subtle alterations occurring in response to CO exposure are those which involve shifts in compartmental concentrations of trace metals. New techniques now allow for detection of minute amounts of these important subcellular constituents, and their alterations during the course of various disease states, including chronic CO exposure (28, 32). The activity of several enzymes has been found to be altered during exposure to CO (12, 36). The significance of these and other findings (7, 18) and the extrapolation to the whole organism must, of necessity, await further investigations relative to the effect of CO on biological processes.

The biologic and toxic effects of atmospheric gases such as sulfur dioxide (SO_2), nitrogen dioxide (NO_2), ozone (O_3), and carbon monoxide (CO) in urban populations continue to attract much attention; particularly with regard to the role these pollutants play in altering man's susceptibility to infection. In the past, the effect of air pollutants on resistance to infection has been studied from two viewpoints, namely, epidemiology and animal experimentation. This investigation will be limited primarily to the effect of low exposures of the air pollutant, CO, on resistance to infection by respiratory challenge with Listeria monocytogenes.

It is well established now, that certain inhaled gases alter the susceptibility of experimental animals to bacterial infection. Increased incidence of spontaneous

pneumonia has been noted in guinea pigs undergoing ozone exposure (39) and chronic exposure to light irradiated auto dust (22). Purvis, et al. (33) studied the effect of atmospheric pollutants on the susceptibility of animals to respiratory infection. He reported a decrease in the resistance of mice to respiratory infection with Klebsiella pneumoniae when exposed for 3 hours to 4 ppm of ozone. Ehrlich (10) reporting on the effect of NO_2 on resistance of mice to Klebsiella pneumoniae noted that a 2 hour pre-exposure to 3.5 ppm of the gas enhanced the mortality rate. Coffin (5) has shown enhanced mortality of mice to group 'C' Streptococci by exposure to 25 ppm of CO and 0.15 ppm total oxidant. The literature contains significant scientific information on pollutant gases SO_2, O_3, and NO_2 relative to their effect on the pathogenesis of disease. The reports as a whole reflect a consistant pattern, namely, that these particular air pollutants impart profound alterations in the hosts' resistance to respiratory infection. However, the experimental evidence relative to CO and its effect on susceptibility is not as prevalent. One such study was undertaken by Stupfel, et al. (40) who reported in a monograph devoted to the biological effects of CO, that the LD_{50} dose of Serratia marcescens was the same for two groups of mice; one exposed to 50 ppm of CO (5 days/week for 56 days) and the other unexposed. Nevertheless, contrary to Stupel's findings, it was noted during this study that mice exposed to 100 ppm of CO for a 5 day

period demonstrated increased resistance to a listeria infection. Search of the literature failed to reveal any other pertinent studies relevant to this specific area.

As mentioned earlier, CO is considered one of the most important and widely distributed urban atmospheric pollutants and reportedly constitutes a health hazard. The fact that so few investigations have been concerned with this gas and its effect on host resistance is somewhat difficult to comprehend; particularly in view of the detailed studies devoted to the other air pollutants. The experimental and epidemiological data pertaining to CO and its effect in man and animals are particularly sparse in regard to the low concentration level found in community air pollution. In case of acute poisoning with high concentrations of the gas the biologic effects on man's health and activities is well understood. However, meaningful effects of exposure to low concentrations of CO have not as yet been clearly identified.

One wonders at this point if the present-day ambient air exposure levels of CO are harmful only to the extent of its reaction with hemoproteins or can CO per se also exert an effect quite apart from this, which in turn, could alter the course of an infection. If it could be demonstrated that ambient-air concentrations of CO can in fact mediate cellular or sub-cellular responses, be they deleterious or not, perhaps the role of CO in the pathogenesis of microbial disease

might be somewhat clarified. Some knowledge of the complete spectrum of biological actions of CO under ambient air concentrations might furnish an alternative basis for analyzing the effects of this gas on man.

In an effort to study the underlying causes of altered resistance to bacterial infection imparted by gaseous pollutants, consideration must be given to bacterial clearance rates governed by a summation of the mechanisms that constantly remove bacteria from the system and render them non viable in situ. Mechanisms possibly involved are:

a) Removal by means of ciliary activity and mucus flow.

b) Removal by means of lymphatic drainage.

c) Bacterial inactivation or death in situ (phagocytosis and destruction by intracellular enzymes, antibodies and other antibacterial substances).

Stimulation or suppression of any one of these mechanisms would of necessity serve in altering the hosts' resistance to a given bacterial infection.

This study was undertaken to determine the effect of low CO exposure levels on respiratory infection with Listeria monocytogenes and the part this gas plays in altering the normal functions associated with the latter mechanism (c above). Other aspects were designed for measuring the parameters which generally are considered indices of acquired (cellular) resistance.

Listeria monocytogenes is a member of the family Cornybacteriacea, order Eubacteriales. The smooth, pathogenic form is a small gram-positive, nonsporeforming, non-acid fast, diphtheroid-like rod with rounded ends measuring 1.0 to 2.0 μ by 0.5 μ.

Listeriosis is one of the most recently recognized and least understood of all bacterial infections of man, domesticated animals and wild life. This bacterium is often relegated to the class of laboratory curiosities--a micro-organism that can produce a high circulating monocytosis of the peripheral blood or a marked purulent conjunctivitis in a susceptible laboratory animal. Investigators of this disease see it as a potential menace, an indiscriminate killer of young and old alike, and, until the advent of antibiotics, individuals known to survive attacks were usually left with permanent physical or mental defects.

Listeria monocytogenes is a facultative, intracellular parasite with a high pathogenicity for many animals species including mice (16). The disease in man was believed to be limited largely to meningitis. However, Seeliger (38) has reported that it frequently causes abortions and stillbirths in women. The mode of infection appears to be hematogenous and/or upper respiratory (16).

Listeria monocytogenes provides an excellent model involving an acute infection in laboratory animals in which acquired resistance depends upon a cell-mediated form of

immunity which is associated with mononuclear cells. It has been well established that the principle agent of resistance to a listeria infection in mice is the macrophage (phagocytic cells) and that cellular immunity plays a vital role in this protection (26, 29, 31).

The major points to be considered in this study follow:

a) Determination of what effect a low concentration of CO has on resistance of mice infected via upper respiratory tract.

b) Influence of CO on the phagocytic activity of alveolar and peritoneal macrophages.

c) Investigation of the intracellular fate of L. monocytogenes within normal and CO influenced macrophages.

d) To determine if the resistance provided by listeria immunization is altered by CO exposure.

CHAPTER II

MATERIALS AND METHODS

Respiratory Exposure of Mice to Listeria monocytogenes

The Henderson Apparatus, a modification of that described by Henderson (19), was used to expose the mice to aerosols generated by a Collison nebulizer. The total, non-circulating air flow through the apparatus was 28 liters/min of which the nebulizer contributed 8 liters/min of droplet particles of 5 microns or less. The cloud was assayed by sampling with porton, all glass, short stem impingers (12 liters/min) which ontained 10 ml of 1% Difco tryptose.

The aerosol was sampled for 1 min periods. The mice were exposed for 1 min during test runs and exposures varied from 1-9 min in MLD determinations followed by a 1 min sterile air wash. Inoculum for test runs and MLD determinations was 1.5×10^8 cells/ml nebulizer fluid.

The ambient air temperature in the exposure chamber was 76-80 F. and the relative humidity was 70%. Prior to returning the mice to their cages, their noses were swabbed with 5% formaldehyde. The formulae for determination of

spray factor and infective dose were those elaborated by Elberg and Henderson (11). Serial ten-fold dilutions were made of impingers which were used to sample the aerosol following animal exposure. A sample was withdrawn from the nebulizer suspension prior to animal exposure. Appropriate dilutions of the impinging liquid were made in a 0.1% tryptose--0.5% sodium chloride solution, and 0.1 portions were spread on Trypticase soy agar (TSA) with glass rods. The plates were incubated overnight at 37 C.

Carbon Monoxide (CO)
Exposure System

The exposure system consisted of a 12-cu ft plexiglass chamber (2-ft x 3-ft x 2-ft) into which carbon monoxide was introduced after having been diluted with oil-free compressed air in a mixing chamber just prior to entry. The diluted CO entered the chamber through four 0.25-inch ports located at the corners of one end of the chamber. The rate of flow through the chamber was determined to be 0.5 cfm (23). Exit ports at the other end of the chamber were identical to the entry ports in size and location. Each entry port was connected to the mixing chamber by a 0.25-inch polyvinyl tube.

The mixing chamber was a polyethylene bottle which had been sealed and modified such that the polyvinyl tubing, after being inserted into a bored hole, formed an airtight seal with the bottle. An identical arrangement following the exit ports allowed the chamber's gaseous environment to be

homogenized prior to exit via an exhaust tunnel. Connected to the exit mixing chamber was a 15-ft 0.25-inch polyvinyl gas sampling line which tied the chamber into a Beckman Infrared Analyzer Model 315A(L) (41-inch cell length). Both mixing chambers and the exposure chamber itself were located in a large 90-cu ft hood which was maintained under slight negative pressure utilizing an exhaust fan. This allowed any escaping gas to be rapidly evacuated from the hood. At the gas entry end of the exposure chamber was a 1-ft by 1.5-ft access port which could be sealed by a overlapping plexiglass port cover.

The CO source tank was located outside the hood and connected to the gas mixing chamber inside the hood by a line from a Mine Safety Appliance single stage regulator on the tank which entered the hood through a small aperture. The compressed air was similarly connected from the compressed air source to the gas mixing chamber through a separate aperture while another aperture permitted the gas sampling line to exit from the hood for quantitative analysis.

The CO infrared analyzer could be adjusted to monitor the exposure chamber either continuously or, by manual adjustment, intermittently. During the evening and early morning hours the exposure chamber was monitored on a continuous basis. Prior to exposure, the analyzer was calibrated with 100 percent nitrogen and then with CO of known concentration. Following calibration of the analyzer, the mixture of CO and

compressed air within the exposure chamber could be adjusted for proper proportions to meet the desired CO concentration. Once the calibration was completed, the desired CO concentration could be maintained within ± 10 ppm using this exposure arrangement. All experimental animal exposures were conducted at CO concentrations of 100 ± 10 ppm. The exposure times varied from 1 to 6 days and will be discussed with the associated experiment.

Experimental Animals

Male inbred BALB/cJ mice, 6- to 8-weeks old, were used in all experiments. They were housed in metal cages and given water and mouse chow ad libitum.

Listeria monocytogenes

Three different strains were used throughout the study.

a) Virulent, streptomycin-resistant 4-52 strain. The culture was received from Guy P. Youmans, Department of Microbiology, Northwestern University Medical School, Chicago, Illinois. The Minimum Inhibatory Concentration (MIC) to streptomycin was 500 µg/ml.

b) Virulent A 4413 (TL-2) strain. Culture was received from Sidney Silverman, U.S. Army Biological Laboratories, Fort Detrick, Md. The MIC to streptomycin was 15 µg/ml.

c) Attenuated AT16(Ry) strain. This culture was also received from Sidney Silverman. The MIC to streptomycin was 15 µg/ml.

Cultures were kept at 4 C on TSA plates with 5% human blood. They were sub-cultured monthly to fresh TSA plates and passed through mice at 6 month intervals.

Tissue Culture

Tissue culture medium consisted of Eagle's Minimun Essential Medium, (MEM) containing inactivated fetal calf serum in a final concentration of 20%. The pH was adjusted to 7.0 with 1% sodium bicarbonate. All glassware was washed in 7x cleanser, acid-washed, and thoroughly rinsed in double-distilled water.

Macrophage Disruption

The procedure for quantitative recovery of viable intracellular listeria was dependent upon an effective method for disintegration of the phagocytic cells. The procedure as outlined by Hsu (21) was investigated and as reported by the author, immediate and complete disintegration of macrophages was observed using a 0.5% solution of sodium desoxycholate. However, the viability of listeria was found to be markedly reduced. A combination of procedures reported by Maxwell et al. (27) and Armstrong (2) and slightly modified in this laboratory was employed. Macrophages were allowed to attach to the inner surface of 16 x 85 mm rubber-stoppered Leighton tubes which contained 1 ml of MEM. Medium was decanted and replaced with 1 ml of an appropriately diluted suspension of listeria in MEM. After the macrophages had been infected

for the desired time, the tubes were washed several times with HBSS. To each tube was added 1 ml of MEM and several drops of sterile glass beads (0.1 mm in diameter). All tubes were subjected to 10 seconds of sonication with the Bronwill-Blackstone sonicator (20,000 cps). The Leighton tubes were held horizontally over the probe and the flat window area exposed. The contents of each tube were mixed thoroughly on a vortex mixer immediately and at frequent intervals thereafter for approximately 30 minutes. Samples were removed, diluted, and plate counts carried out on TSA with 5% blood.

Enumeration and Isolation of Endogenous Microorganisms in Mice

Mice were examined before and after carbon monoxide exposure (100 ppm/5 days) to determine the effect on quantitative distribution of endogenous microorganisms. Mice were ether killed and placed in isopropyl alcohol to reduce the number of external contaminants. The tissues of each animal were removed aseptically and processed separately.

To determine the number of recoverable coliform bacteria from the intestinal tract, a 10 mg portion of the large colon was removed. Tissues were placed in an homogenizer tube containing 5 ml of sterile 0.85% saline and emulsified with Teflon grinder. Serial tenfold dilutions of fecal emulsions were made and cultured to nutrient agar plates for coliform counts. Undiluted samples were cultured to Eosin Methylene Blue (EMB), Salmonella - Shigella Agar (S-S) and

Sabouraud Dextrose Agar (SDA) with 30 units of penicillin and streptomycin.

The lungs, spleen, and blood were examined for the presence of bacteria and fungi. Tissues were placed in homogenizer tubes and emulsified. Undiluted emulsions (0.1 ml) were cultured to EMB, S-S, SDA and Brain Heart Infusion (BHI) with 10% human blood. Sabouraud plates were held at room temperature. All other inoculated media were incubated at 37 C. Conventional procedures for identification were followed.

Macrophage Isolation

a) Lung. Alveolar macrophages were collected according to a method modified from that of Bennett (3). The animals were sacrificed by cervical dislocation. No exudate-inducing agent was used. Lungs were aseptically removed and disected free of extraneous tissue. They were then processed through two rinses in saline (50 units of penicillin and streptomycin/ml), two rinses in Hanks balanced salt solution (HBSS) with penicillin and streptomycin and one rinse in Minimal Essential Medium with streptomycin. The lungs were then placed in sterile petri dishes (60 x 15 mm) with 5 ml of MEM (6 units/ml of heparin). Using a 26 gauge needle the lungs were carefully inflated with the medium and then minced with small fine tissue scissors. A sterile stainless steel fine mesh sieve was placed in the petri dish. The tissue

suspension was pipetted with care using a 10 ml plastic syringe prepared by cutting the needle attachment base and enlarging the opening. The supernate was collected in silicon coated tubes, care being taken to avoid clumps and fragments of tissue. The cells were centrifuged at 200 x g for 10 minutes. Cell pellets were resuspended in 5 ml of HBSS and again centrifuged. The supernatant fluid was discarded and cells adjusted with MEM to an appropriate concentration. Differential counts were performed on the cell suspensions. Viability was assessed by trypan blue exclusion test.

b) Peritoneal. Peritoneal cells were obtained from mice without the use of an exudate-inducing agent. The animals were sacrificed by cervical dislocation. Exudate cells were collected by asceptic technique. A small ventral midline incision was made in the peritoneal wall and the viscera and peritoneal cavity were washed with approximately 3 ml of HBSS containing 6 units of heparin/ml. The peritoneal washings which consisted of a pool of 5 mice/experimental run were centrifuged at 200 x g for 10 minutes and the supernatant discarded. The packed cells were washed twice with HBSS without heparin and adjusted with MEM to an appropriate concentration. Differential cell counts were done on cell suspensions. Viability was assessed by trypan blue exclusion test.

Determination of MLD for Aerosol Infection

To determine what effect, if any, carbon monoxide would have on an upper respiratory listeria infection in

mice, an MLD (defined herein as the minimal number of organisms providing 100% mortality between the 5th and 7th day of infection) was established.

Mice were divided into two major groups. The first group was further divided into 6 subgroups (8 mice/group) with each receiving a 1, 2, 3, 5, 6, or 9 minute listeria aerosol exposure from nebulizer fluid containing 1.5×10^8 organisms/ml of strain A4413 (TL-2). The second group served as the non-infected controls. Mice were observed for a ten day period and the number of deaths occurring per day recorded. Autopsies were frequently performed to confirm the cause of death.

The infecting bacterium was grown on Tryptic Soy Agar (TSA) for two days at 37 C and subsequently transferred to tryptic soy broth in a flat surfaced flask with an electromagnetic stirrer. Cultures were incubated at 37 C for 18 hours with constant stirring. Preparatory procedures for aerosol suspensions in collison nebulizer, diluents for the impinging liquid and serial dilution for plate counts were essentially those as outlined by Roessler and Kautter (34). The number of organisms used for the aerosol infection was standardized turbidimetrically at 530 nm on a Coleman Junior spectrophotometer. Per cent transmittance was correlated with the number of organisms as determined by plate counts and McFarland's bacterial standards.

CO Exposed-Aerosol Infection of Mice with Listeria Monocytogenes

To determine the effect carbon monoxide would have on animals infected via the respiratory route with listeria (str TL-2) 120 mice were divided into three major groups. Forty mice comprised Group 1 which was subdivided into 5 smaller regiments having 8 mice per subgroup. Group 1 animals were exposed to 100 ppm of carbon monoxide and thereafter at 2, 3, 4, 5, and 6 days, 8 mice per day were removed and subjected to aerosol infection with listeria. Group 2- CO control mice (8 for each exposure day) were identically exposed to the gas. However, this group was not infected. Group 3 consisted of 40 mice which did not receive any exposure to carbon monoxide but were infected.

Eight mice from Group 1 and 8 mice from Group 3 were placed in the exposure tube side-by-side and simultaneously exposed to a passing aerosol generated from the nebulizer fluid containing 1.5×10^8 listeria/ml.

Mice were observed daily and deaths recorded. Autopsies were frequently performed to confirm cause of death. Severity of infection was determined by higher mortality rate and earlier time of death.

In Vitro Phagocytosis of L. monocytogenes by Peritoneal and Alveolar Macrophages

In order to assess the effect of CO on the phagocytic function of macrophages under minimal influence from the

cellular immune mechanism, macrophages were obtained from animals pre-exposed to the gas. Cells were then infected with listeria in-vitro.

One hundred fifteen mice were divided into 3 major groups. Group 1 comprised 50 mice which were exposed to 100 ppm of CO for 5 days. The animals were then divided into 2 subgroups of 25 mice each from which peritoneal and alveolar macrophages were obtained for phagocytic studies. Group 2 consisted of 50 mice which were not exposed to CO and served as the normal controls. The mice were divided into 2 subgroups of 25 mice each. Peritoneal and alveolar macrophages were obtained for phagocytic studies. Group 3 animals, designed as CO shelf controls, were used to determine whether there was a stress connected simply to the exposure protocol per se. Other than not having received any CO, the mice were treated identically to group 1.

One experimental run per day for 5 days was performed on group 1 and group 2 mice and two runs per day for 2 days were done with group 3. Each experimental run comprised a pool of either peritoneal macrophages from 4 to 6 mice or alveolar macrophages from 5 to 6 mice.

Experimental approach to phagocytic study

Peritoneal macrophages.--The cell pellets from the peritoneal macrophage pools were resuspended with MEM to a concentration of approximately 1 x 10^6 cell/ml. One ml of

macrophage suspension was pipetted into a series of 8 Leighton tubes (15 x 85 mm) containing 10 x 50 mm coverslips. The tubes were incubated at 37 C for 3 hours during which time cells attached to the coverslips. The medium was removed following incubation and replaced with 1 ml of MEM containing sufficient listeria to give an organism to macrophage ratio of 12:1. Controls received only MEM. Phagocytosis was allowed to take place for 30, 60, and 90 min at 37 C following which 3 Leighton tubes were removed per time interval. The coverslips were removed from 1 control tube and 2 infected tubes, rinsed in saline and stained with Wrights. Slides were examined for the number and proportion of infected cells as compared to controls and the number of infected cells containing 1 to 5, 6 to 10, and greater than 10 listeria was recorded.

Alveolar macrophages.--The cell pellets from the alveolar macrophage pools were resuspended with MEM to a concentration of approximately 8 x 10^5 cells/ml. One ml of macrophage suspension was pipetted into a series of 9 Leighton tubes containing coverslips. The tubes were incubated at 37 C for 5 hours during which time cells attached to the coverslips. The medium was removed following incubation and replaced with 1 ml of MEM containing a listeria to macrophage ratio of 30:1. Controls received only MEM. Macrophages were maintained at 37 C during which time infection occurred. Three Leighton tubes were removed at 2, 3, and 4

hour intervals, coverslips removed, rinsed in saline and stained with Wrights. Slides were examined as in the peritoneal study.

Bacterial inocula were prepared from an overnight growth of listeria, str.TL-2, in TSB. The cultures were standardized by determining the per cent transmittance of a saline suspension at 530 nm on the Coleman Jr. spectrophotometer. The per cent transmittance was correlated with the number of organisms as determined by plate counts and the McFarland's bacterial standards.

Experimental data represent the average of 5 separate experimental runs (4-6 mice/run) in which peritoneal macrophages from 20 oil immersion fields and 100 alveolar macrophages were counted in duplicate.

Quantitative Assay of Viable Intracellular Listeria

An attempt was made to examine the effect of CO on an in vitro infection of macrophages. The infected cells were maintained in tissue cultures and the bacillary multiplication followed for various time intervals.

One hundred twenty-five mice were divided into 3 major groups: Group 1 comprised the CO exposed mice (100 ppm for 5 days), group 2 served as the non-exposed (normal) controls; group 3 were the shelf controls. Each major group was subdivided in the same manner as in the study on phagocytosis. Thereafter, the peritoneal and alveolar macrophages were obtained for assay studies.

One experimental run per day for 6 days was performed on groups 1 and 2 mice. One experimental run was done with group 3. Each experimental run comprised a pool of either peritoneal or alveolar macrophages obtained from 5 mice/cell population.

Experimental approach to assay study

Peritoneal macrophages.--The cell pellets from the peritoneal macrophage pools were resuspended with MEM to a concentration of approximately 1 x 10^6 cells/ml. One ml of macrophage suspension was pipetted into a series of 10 Leighton tubes without coverslips and incubated for 3 hours at 37 C during which time cells attached to the inner surface of the tubes. The MEM was removed and replaced with 1 ml of MEM containing sufficient listeria to give an organism to macrophage ratio of 6:1. Controls received only MEM. The mixtures were incubated for 90 min at 37 C during which time the cells became infected. Following this period, the infecting medium was removed and the tubes were washed thoroughly with HBSS and MEM. All tubes received 1 ml of 15 µg of streptomycin per ml of MEM (MEM-S). The antibiotic was added at this time to suppress the extracellular multiplication of listeria (2). Immediately, and at 12, 24, and 36 hours after the addition of streptomycin, Leighton tubes were removed and intracellular organisms quantitated. Controls were examined at the 36 hour interval. MEM with streptomycin

was replaced with 1 ml of MEM without streptomycin. The macrophages were disrupted according to the procedure outlined earlier. Samples of lysate were diluted, plated on TSA and incubated at 37 C for 48 hours. Intracellular count was calculated as the number of listeria/ml of lysate.

Alveolar macrophages.--The cell pellets from the alveolar macrophage pools were resuspended with MEM to a concentration of approximately 8 x 10^5 cells/ml. One ml of the macrophage suspension was pipetted into a series of 10 Leighton tubes without coverslips and incubated 5 hours at 37 C for cell attachment. The MEM was removed and replaced with 1 ml of MEM containing sufficient listeria to give an organism to macrophage ratio of 10:1. Controls received only MEM. The mixtures were incubated for 3 hours at 37 C while cells became infected. As with the peritoneal study, tubes were washed in MEM with 15 µg/ml of streptomycin. Immediately, and at 8, 16, and 24 hours after the addition of streptomycin, Leighton tubes were removed and intracellular organisms quantitated. Controls were examined at the 24 hour interval. Procedure for colony counts was identical to that described above. Bacterial inocula were standardized as outlined earlier in the study on phagocytosis.

Experimental data for each cell population (peritoneal and alveolar) represent the average of 6 separate experimental runs (5 mice/run). Two Leighton tubes were used for each

quantitation interval per experimental run and colony counts were done in duplicate for each Leighton tube.

Blood Cell Determination

Blood was obtained by means of cardiac puncture from groups 1 and 2 mice used in the phagocytic activity study. Duplication blood smears were made and stained with Wright's stain. Differential cell counts were made from these preparations. The "white" leucocyte pipettes with 1.0% acetic acid were used for total WBC counts.

Spleen Assay for Viable Infecting Listeria

The enumeration of viable infecting listeria in the spleens of mice has been used successfully by Mackaness (26) and Coppel (6) as a means for measuring the cellular immune response. Mackaness found that 90% of an infecting suspension of Listeria monocytogenes given intravenously (I.V.) could be found in the liver and spleen. Also, the growth of listeria in these two organs was exactly the same; therefore, in subsequent experiments, he used only the spleen as the assay organ for growth of listeria in vivo.

To determine the effect CO would have on cellular immunity, 150 mice were divided into two major groups; group 1 was composed of CO exposed and group 2 non-CO exposed mice. Group 1 comprised 70 mice which were divided into 3 smaller subgroups designated as A_1, A_2, and A_4. The 3 subgroups were then exposed to 100 ppm of CO for 5 days. Immediately

following exposure, A_1 mice were infected (I.V.) with 2 x 10^5 of a streptomycin-resistant listeria 4-52. The streptomycin-resistant mutant was used because a marker was needed to distinguish the infecting from the vaccinating organisms with the A_2 and A_4 mice. The infecting dose was one which permitted survival of all the mice, since under these conditions there was found to be less variation in spleen counts between individual animals (6). On the 1st, 2nd, 3rd, and 4th days following infection, 5 mice per experimental run were sacrificed by cervical dislocation. Spleens were removed aseptically, dissected free of extraneous tissue and placed with 5 ml of a sterile 0.01 M phosphate buffer (pH 7.0) in sterile tissue grinder tubes. The organs were homogenized in a Teflon grinder and appropriate dilutions made for bacterial counts. Plating was accomplished on TSA containing 200 ug/ml of streptomycin. Listeria counts from each spleen homogenate were done in duplicate. Cultures were incubated and colonies counted after 48 hours incubation at 37 C. Spleen counts were calculated as the number of listeria per milliter of spleen homogenate. CO-exposed, non-infected mice were included as controls.

Immediately following CO exposure, mice in the A_2 and A_4 subgroups were immunized intraperitoneally with 1.2 x 10^8 of the attenuated AT16 (Ry) strain of listeria. The A_2 and A_4 groups, after 2 and 4 days respectively, were infected intravenously with 2 x 10^5 of the streptomycin-

resistant listeria (4-52). Spleens were removed, processed and cultured in the same manner as that used with the A_1 group above.

Group 2, non-CO exposed mice were processed exactly as those in group 1. The schedule for infecting and immunizing animals was performed as with the CO-exposed group 1 mice. Table 1 shows details of this study.

Statistical Analysis

CO-exposed vs non-exposed mice infected with a listeria aerosol: The mean survival times for respective exposure days were compared by the one-way analysis of variance.

Studies relating to the effect of CO on phagocytic activity in vitro and spleen assay were assessed by the Student's "T" Test. Means of exposed groups were compared to those of unexposed controls.

TABLE 1

SCHEDULE FOR SPLEEN ASSAY, CHALLENGE AND IMMUNIZATION OF MICE EXPOSED AND NON EXPOSED TO CO

	Mice Subgroup		
Day	A_1	A_2	A_4
1	challenge*	immunize**	immunize
2	SA***		
3	SA	challenge	
4	SA	SA	
5	SA	SA	challenge
6		SA	SA
7		SA	SA
8			SA
9			SA

* Challenged intravenously with 2×10^5 cells of 4-52 Listeria.

** Immunized intraperitoneally with 1.2×10^8 cells of At 16(Ry) Listeria.

*** Spleen assay (SA). Five mice were sacrificed/assay day.

CHAPTER III

RESULTS

Macrophage Disruption

A short period of sonication (10 sec) followed by vortex mixing of attached infected macrophages during a 30 min period, proved to be an effective method for disruption of the phagocytic cells. Microscopic examination revealed macrophages to be completely disintegrated. The effect of sonication on viability of listeria suspended in saline (0.85% NaCl) and distilled water is shown in Figure 1. The viability is unaffected by periods of sonication up to 90 seconds, at which time there were 2.9×10^6 organisms in the sonicated samples and 2.7×10^6 organisms is the non-sonicated controls.

Enumeration and Isolation of Endogenous Microorganisms in Mice

The effect of CO (100 ppm/5 days) on the quantitative evaluation of coliform bacteria in the colon of mice is shown in Table 2. No significant change in the number of coliform organisms was observed. The coliform count in the CO-exposed

Figure 1.--Effect of sonication on viability of Listeria monocytogenes suspended in sodium chloride and distilled water.

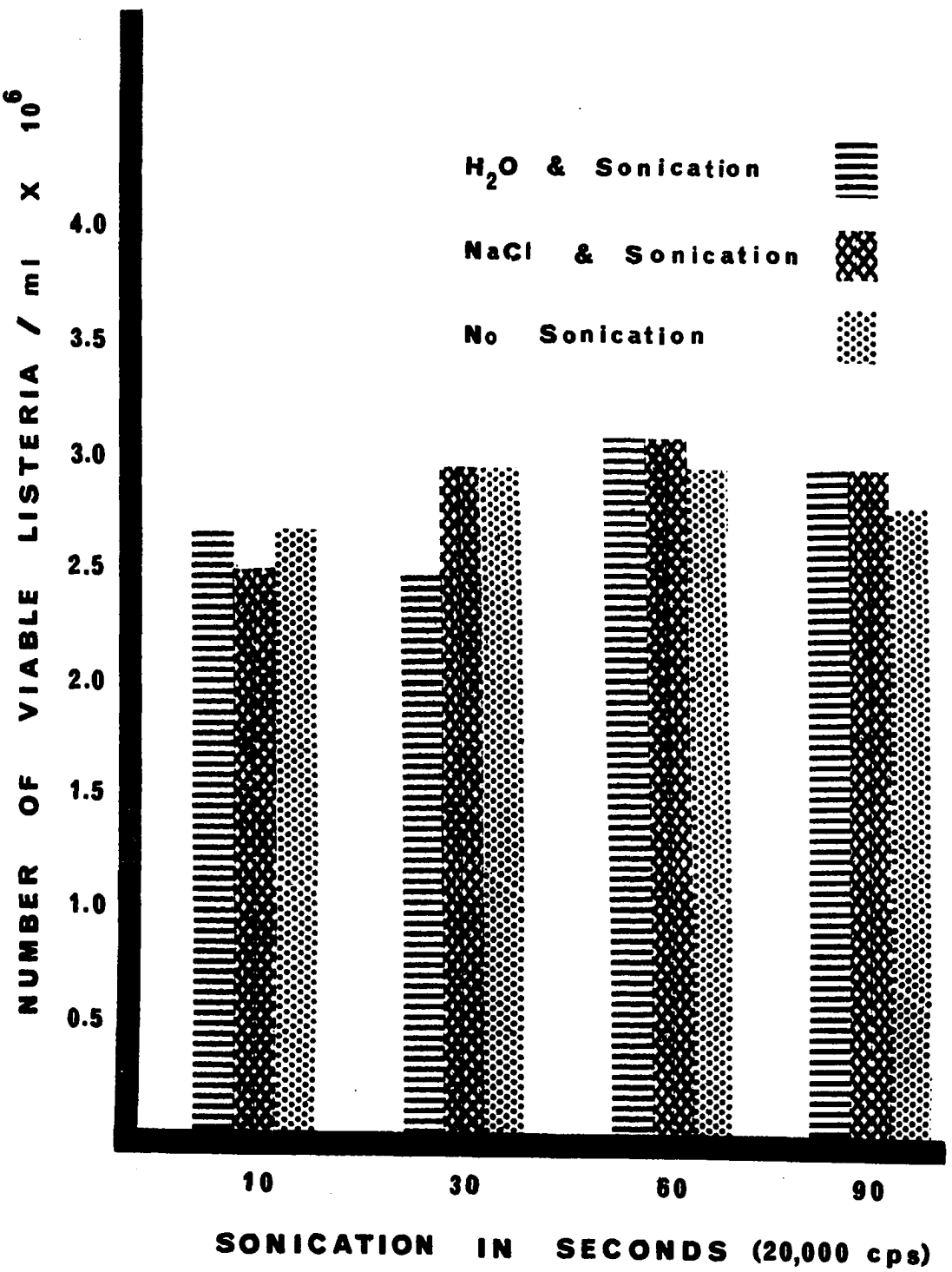

NUMBER OF VIABLE LISTERIA / ml X 10^6
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
H_2O & Sonication
NaCl & Sonication
No Sonication
10
30
60
90
SONICATION IN SECONDS (20,000 cps)

TABLE 2

A COMPARATIVE QUANTITATION OF COLIFORM BACTERIA IN 10 MG OF FECES FROM CARBON MONOXIDE EXPOSED AND NON-EXPOSED MICE

Experimental Group*	Number of coliform bacteria/sample	
	Carbon Monoxide Exposed	Non-Exposed
1	3×10^7	3×10^7
2	7×10^6	5.5×10^6
3	1.2×10^7	1.7×10^7
4	1×10^7	5×10^6
5	2×10^6	1.6×10^7
6	3×10^6	5×10^6
7	1×10^6	2×10^6
8	6×10^6	7×10^6
9		1×10^6
10		1.5×10^6
TOTAL MEAN:	8.8×10^6	9×10^6

* Averages of 5 mice/experimental group.

mice ranged from 1 x 10^6 to 1.2 x 10^7 while in the unexposed mice the range was 1 x 10^6 to 1.7 x 10^7. The bacterial flora remained essentially unchanged. Figure 2 compares the frequency of recoverable organisms from the exposed and non-exposed animals. Escherichia and yeast cells were the predominating organisms in both groups of animals, CO-exposed and unexposed. Escherichia was isolated from all mice in both groups, while the yeast cells were recovered from 79% of the CO-exposed and 81% of the unexposed animals.

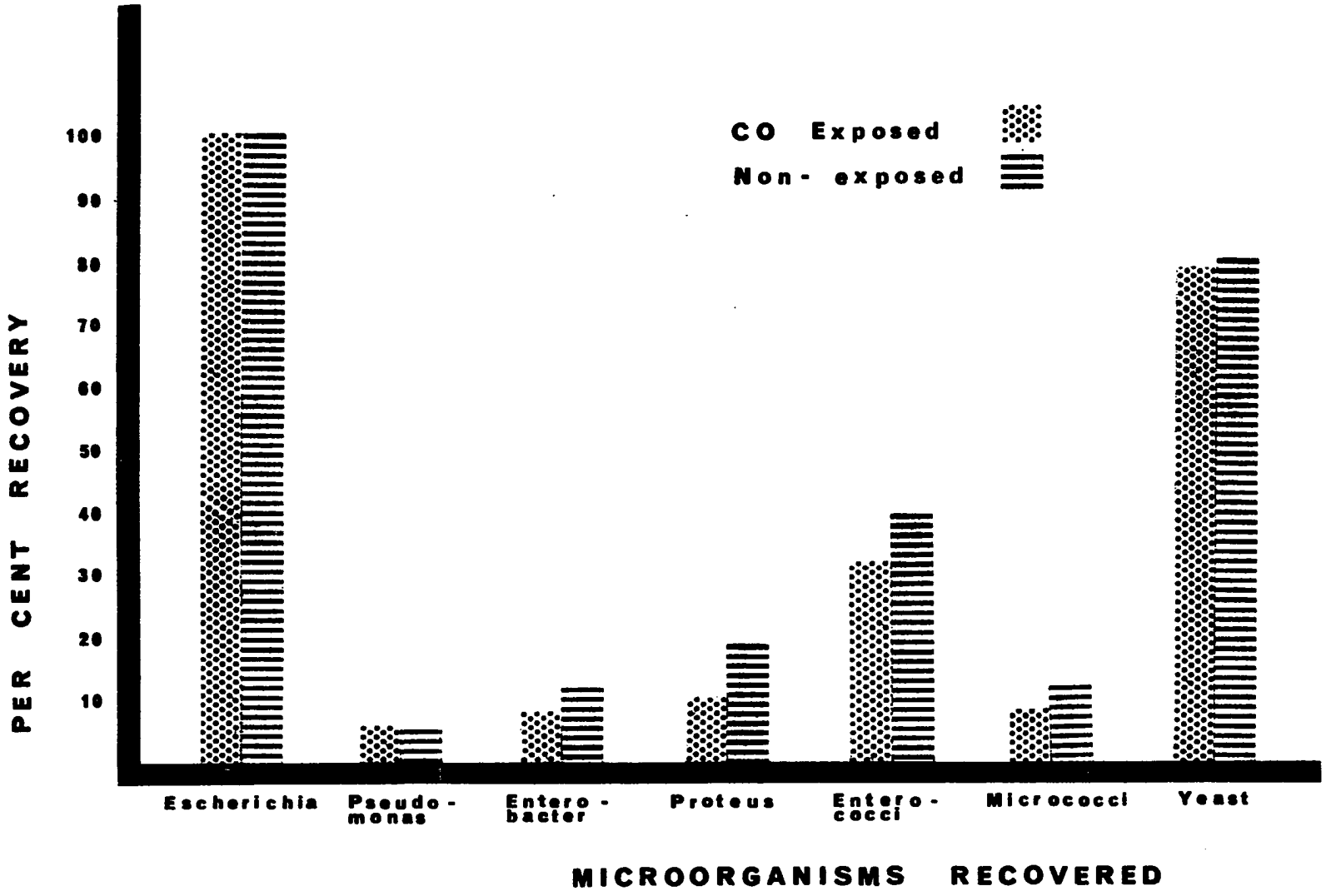
The yeast cell isolated from both groups of mice was identified as Candida rugosa. No fungal organisms were recovered from tissues or blood following 2 weeks incubation. There were no salmonella or shigella recovered from the spleen, lung, or feces. Occasionally several colonies of micrococcus from spleen and neisseriae from lung tissues were observed in both CO and non-CO exposed mice.

Macrophage Isolation

The isolation techniques provided an adequate yield of both peritoneal and alveolar macrophages. Although the number of erythrocytes from lung preparations exceeded the number of nucleated cells, lysis of the former was not found to be necessary in that they agglutinated in subsequent tissue culture work and were readily removed by several washes.

Microscopic examination of Wright's stained smears from the cell preparations showed that approximately 85-90%

Figure 2.--Effect of CO on the intestinal flora (aerobes) in non-infected mice.


CO Exposed
Non- exposed
PER CENT RECOVERY
100
90
80
70
60
50
40
30
20
10
Escherichia
Pseudo-monas
Entero-bacter
Proteus
Entero-cocci
Micrococci
Yeast
MICROORGANISMS RECOVERED

of the cells were mononuclear (macrophages), 8-10% lymphocytes and 3% polymorphonuclear cells.

Trypan blue exclusion test for macrophages from both CO and non-CO animals revealed a viability of 90-95%.

Determination of MLD for Aerosol Infection

Minimal lethal dose (MLD) for purpose of this study has been defined as the minimal number of organisms providing 100% mortality of mice within 5 to 7 days. Results of MLD determination are shown in Table 3. The MLD for mice infected with Listeria (TL-2) was determined to be 5000 organisms/animal. The dose was achieved by subjecting mice to a 1 minute aerosol exposure which served as the inoculum for challenge runs in those experiments associated with upper respiratory listeria infections.

There were no deaths in the non-infected control mice at the end of 10 days.

CO Exposed Aerosol Infection of Mice with Listeria monocytogenes

The data in Figure 3A indicate that a 2 day exposure of mice to 100 ppm of CO confers a marked degree of protection to a listeria aerosol challenge. However, it should be noted that this group received 3500 organisms/animal whereas mice in all other subgroups received 5000 organisms/animal. The increase in resistance is demonstrated by a lower mortality rate and increased survival time in the CO-exposed-infected

TABLE 3

DETERMINATION OF MLD CHALLENGE DOSE FOR CARBON MONOXIDE EXPOSED AEROSOL INFECTED MICE*

Aerosol exposure (min)	Per cent mortality of mice aerosolized with Listeria monocytogenes (T-2 str.)** Days post infection								
	1	2	3	4	5	6	7	8	9
1	12.5	12.5	25	62	87.5	100	-		
2	0	12.5	25	50	100	-			
3	0	50	75	100	-				
5	25	50	62	100	-				
6	0	25	50	100	-				
9		50	100	-					

* Minimal number of organisms providing 100% mortality within 5 to 7 days.

** Infecting aerosol for all mice was derived from nebulizer fluid containing 1.5×10^8 organisms/ml.

mice when compared to the non-CO-infected mice. The difference, in terms of average survival time, between these two groups was significant at $P<0.01$. Figures 3 (B, C, D, and E) present data which indicate that a 3, 4, 5, and 6 day exposure of mice to 100 ppm of CO provides them with a high degree of protection to a listeria aerosol challenge containing 1.5×10^8 organisms/ml of nebulizer fluid (5000 organisms/animal). The enhanced resistance in CO exposed mice is evidenced in not only the lower death rates but also in the increased length of survival times. The differences relative to the average survival times among the CO exposed and unexposed mice were significant at $P<0.05$ with the exception of the 4 day exposure in which case the level of significance was $P<0.25$. When the average survival times of the mice exposed for days 3, 4, 5, and 6 were combined and compared to the combined unexposed animals, a statistically significant difference was observed ($P<0.01$).

No deaths were observed in the CO exposed, non-infected control mice. Listeria monocytogenes was recovered from spleens of randomly selected mice at the time of death.

In-Vitro Phagocytosis of Listeria monocytogenes

The extent of phagocytic activity of Listeria monocytogenes by alveolar and peritoneal macrophages obtained from pre-exposed mice was assessed in vitro.

Figure 3.--Per cent mortality of CO exposed (100 ppm) and non-exposed mice infected with Listeria (TL-2).

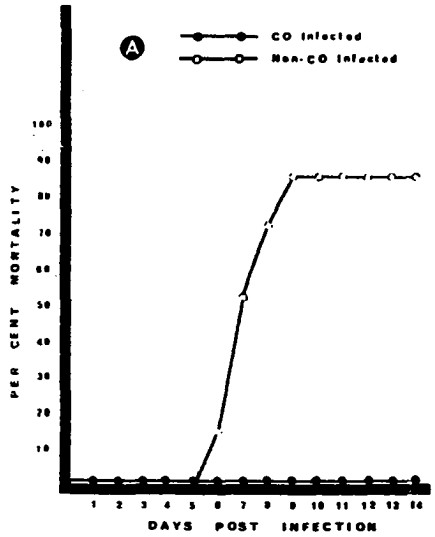
A) Two days exposure.
(Dose of 3,500 organisms/animal)

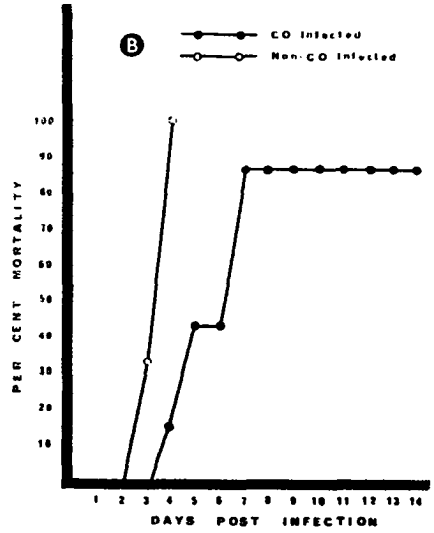
B) Three days exposure.
(MLD of 5,000 organisms/animal)

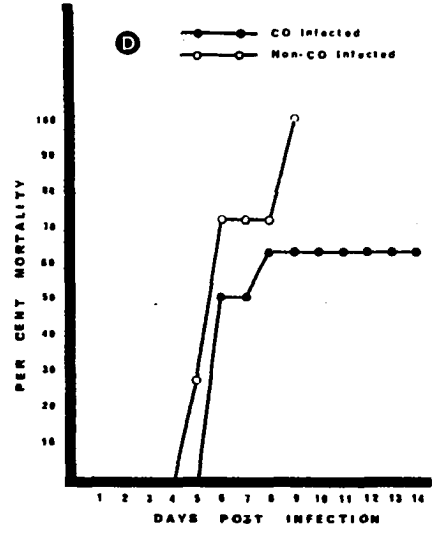
C) Four days of exposure.
(MLD of 5,000 organisms/animal)

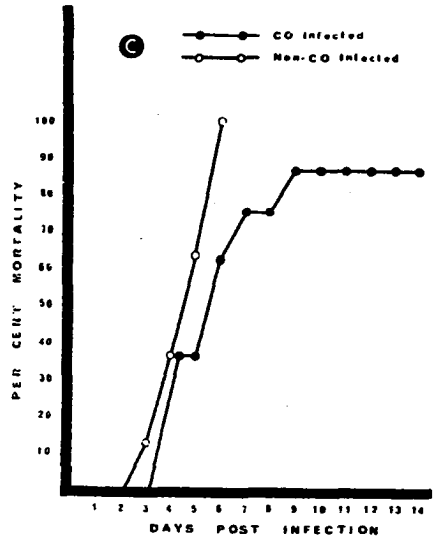
D) Five days of exposure.
(MLD of 5,000 organisms/animal)

E) Six days of exposure.
(MLD of 5,000 organisms/animal)


A
CO infected
Non-CO infected
PER CENT MORTALITY
DAYS POST INFECTION


B
CO infected
Non-CO infected
PER CENT MORTALITY
DAYS POST INFECTION


D
CO infected
Non-CO infected
PER CENT MORTALITY
DAYS POST INFECTION


C
CO infected
Non-CO infected
PER CENT MORTALITY
DAYS POST INFECTION

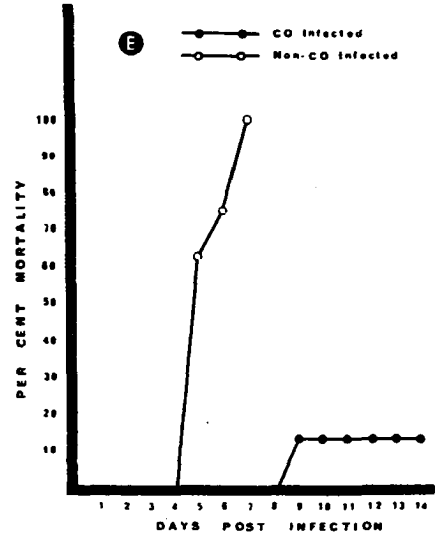

E
CO infected
Non-CO infected
PER CENT MORTALITY
DAYS POST INFECTION

Table 4 summarizes the results of the phagocytic activity displayed by peritoneal macrophages. Phagocytosis by peritoneal macrophages derived from CO exposed animals progressed at a significantly higher rate when compared to the macrophages from unexposed animals ($P<0.01$). The enhanced phagocytic activity was evident during the 30, 60, and 90 min sampling times. A demonstrable difference is also apparent in the CO infected macrophages containing 1-5 organisms at the 30 min sample time with a marked shift to increased numbers in the >10 as compared to the control macrophages.

Macrophages obtained from CO (shelf) controls phagocytized listeria at a rate similar to the normal, non-exposed mice. Per cent of infected cells was 32, 43, and 72 at 30, 60, and 90 min respectively.

Table 5 summarizes the results of the phagocytic activity displayed by alveolar macrophages. As with the peritoneal macrophages, the per cent of infected macrophages from CO exposed mice showed an enhanced capacity to phagocytize listeria when compared to the non-exposed, infected controls. A statistically significant difference was noted between the two groups at the 2 and 3 hour sampling times ($P<0.01$). The 4 hour sample, although not revealing a marked difference between the two groups, was significant at $P<0.05$.

There was no demonstrable difference in phagocytic activity between macrophages of CO (shelf) controls and

TABLE 4

PHAGOCYTOSIS OF L. MONOCYTOGENES (TL-2) BY PERITONEAL MACROPHAGES DERIVED FROM MICE EXPOSED TO 100 PPM OF CARBON MONOXIDE FOR 5 DAYS, NON-CARBON MONOXIDE EXPOSED, AND SHELF CONTROL[a]

Sample time (min.)	Carbon Monoxide Exposed				No Carbon Monoxide				Shelf Control
	% macrophages infected	% macrophages containing			% macrophages infected	% macrophages containing			
		1-5	6-10	>10		1-5	6-10	>10	
30	65[b]	45	18	2	31	28	3	0	--[c]
60	87[b]	11	36	40	58	21	31	6	--
90	94[b]	2	15	77	73	8	32	33	--

[a]Mice housed in non-carbon monoxide environment adjacent to carbon monoxide exposed mice.

[b]Significant at $P < 0.01$.

[c]No demonstrable difference in phagocytic activity between macrophages from shelf control and non-carbon monoxide exposed mice.

TABLE 5

PHAGOCYTOSIS OF L. MONOCYTOGENES (TL-2) BY ALVEOLAR MACROPHAGES DERIVED FROM MICE EXPOSED TO 100 PPM OF CARBON MONOXIDE FOR 5 DAYS, NON-CARBON MONOXIDE EXPOSED, AND SHELF CONTROL MICE[a]

Sample time (hrs)	Carbon Monoxide Exposed				No Carbon Monoxide				Shelf Control
	% macrophages infected	% macrophages containing			% macrophages infected	% macrophages containing			
		1-5	6-10	>10		1-5	6-10	>10	
2	42[b]	25	10	7	24	16	6	2	--[d]
3	45[b]	13	13	19	35	14	13	8	--
4	48[c]	14	13	21	40	15	13	12	--

[a]Mice housed in non-carbon monoxide environment adjacent to carbon monoxide exposed mice.

[b]Significant at $P < 0.01$.

[c]Significant at $P < 0.05$.

[d]No demonstrable difference in phagocytic activity between macrophages from shelf control and non-carbon monoxide exposed mice.

non-CO exposed mice. Per cent of infected macrophages was 25, 36, and 43 at 2, 3, and 4 hours, respectively.

Quantitative Assay of Viable Intracellular Listeria

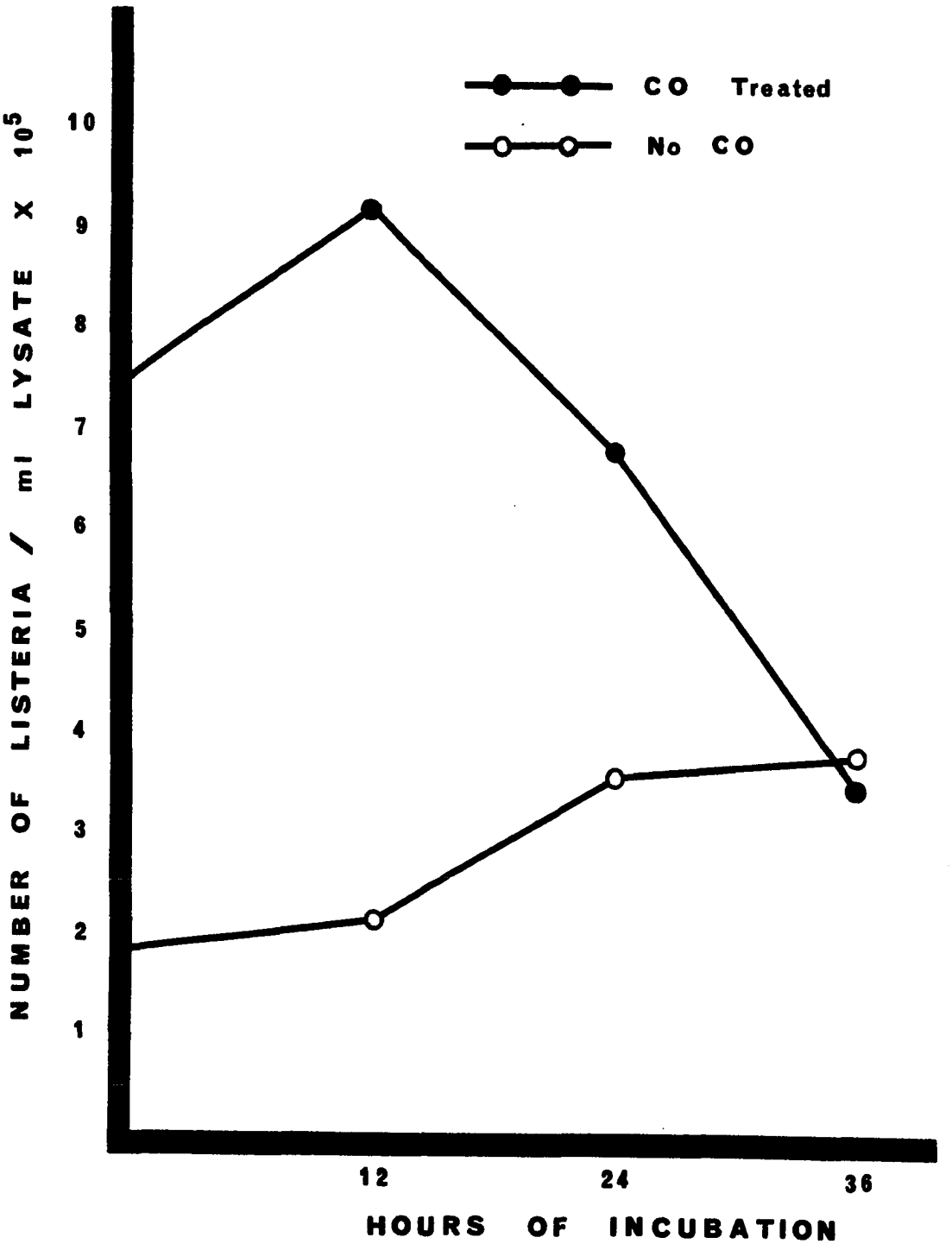
Macrophages derived from CO exposed (100 ppm/5 days) and non-exposed animals were evaluated with regard to their ability to cope with a listeria infection in vitro. For purpose of this experiment, it was assumed that the bacterial plate counts on cell lysates taken at the various time intervals after initial infection indicated either the extent of listericidal activity or intracellular multiplication.

Peritoneal macrophages

Figure 4 illustrates the results of this experiment. Macrophages obtained from CO exposed mice and infected in vitro, showed an initial 21.6% increase in the number of intracellular organisms during the first 12 hours of infection. Thereafter, a precipitous drop (62.2%) was observed up to the 36 hour sample at which time the experiment was concluded. In contrast the non-CO treated macrophages showed an initial 9% increase in the number of intracellular organisms during the first 12 hours of infection. Thereafter, the intracellular replication steadily progressed throughout the 36 hour infection during which time we see a 77.8% increase in the number of organisms.

The number of intracellular organisms at the base line level, '0' hr sample, (7.5×10^5) from the CO treated cells

Figure 4.--Quantitative assay of viable L. monocytogenes (TL-2) within peritoneal macrophages derived from CO exposed and non-exposed mice. Cells were infected in vitro.


CO Treated
No CO
NUMBER OF LISTERIA / ml LYSATE X 10^5
10
9
8
7
6
5
4
3
2
1
12
24
36
HOURS OF INCUBATION

as compared to the number of organisms from the non-CO treated cells (1.9×10^5) confirms the observation made earlier regarding enhanced phagocytic activity of the CO-treated macrophages (see Table 4).

Alveolar macrophages

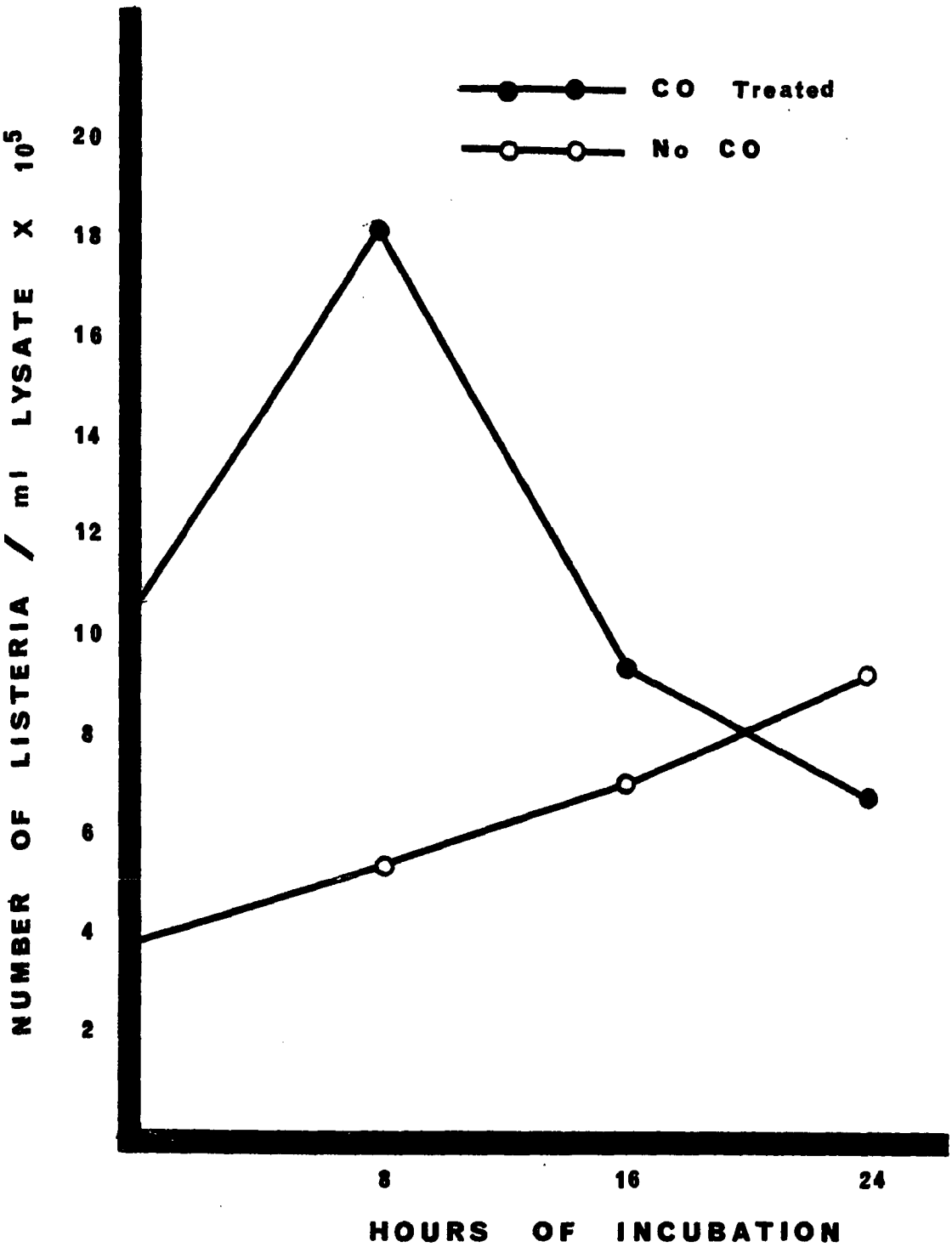
Figure 5 illustrates the results of this experiment. Alveolar macrophages derived from CO exposed animals and infected in vitro, showed an initial 71.4% increase in the number of intracellular organisms during the first 8 hours of infection. Thereafter, a marked drop in the number of organisms (63.3%) was observed in the final 24 hour sample. In contrast the non-CO treated macrophages showed an initial 34.5% increase in the number of intracellular organisms during the first 8 hours of infection. Thereafter, the intracellular replication progressed steadily throughout the 24 hr infection during which time a 70.9% increase is noted.

As with the peritoneal macrophages, the number of intracellular organisms at the base line level, 0 hr sample, (10.5×10^5) from the CO-treated cells as compared to the number of organisms from the non-CO treated cells (3.9×10^5) is in agreement with the earlier observations relative to enhanced phagocytic activity of the CO-treated macrophages (see Table 5).

Blood Cell Determination

Normal white blood counts (WBC) for mice used in this study were in the range of 4,000 to 8,000 mm^3. The mean

Figure 5.--Quantitative assay of viable L. monocytogenes (TL-2) within alveolar macrophages derived from CO exposed and non-exposed mice. Cells were infected in vitro.


CO Treated
No CO
NUMBER OF LISTERIA / ml LYSATE X 10^5
20
18
16
14
12
10
8
6
4
2
8
16
24
HOURS OF INCUBATION

count in the normal controls was 5,987. The counts for mice exposed to CO (100 ppm/5 days) varied from 3,000 to 8,800/mm^3. The mean count was 4,779. There was no statistically significant difference in the WBC counts between the normal and CO exposed mice.

Per cent cell types representing the normal and CO exposed animals show similar differential counts. Results are shown in Table 6.

Spleen Assay for Viable Infecting Listeria

Prior to examining the effects of CO on the immune response, as assessed by enumeration of infecting listeria in the spleens of mice, two preliminary studies were undertaken:

1. Determination of the highest immunizing dose of the attenuated listeria AT 16(Ry) tolerated when administered intraperitoneally. For this study, 5 groups of mice with 8/group were injected I.P. with 1 of 5 doses of the attenuated listeria and observed for 10 days. Mice showing signs of ill-effect and those dying were recorded. The highest immunizing dose tolerated by mice was approximately 1.2×10^8 organisms. Coppel (6), using approximately 1.3×10^8 organisms as the tolerated immunizing dose determined the time of onset of the immune response afforded by listeria AT 16(Ry) to be evident at 2 days and reaching maximum protection on the 5th day.

2. Establish a challenge dose with virulent 4-52 listeria which would permit survival of all the mice for

TABLE 6

PERIPHERAL WHITE BLOOD CELL COUNT (WBC) AND DIFFERENTIAL ON MICE EXPOSED TO 100 PPM OF CARBON MONOXIDE AND NON-EXPOSED CONTROLS

	Number pf WBC (per mm^3)*	Per cent cell types			
		lymphocyte	monocyte	neutrophile	eosinophil
Carbon monoxide exposed	4,779	67.0	9.0	24.0	0
Non-carbon monoxide exposed	5,987	72.4	7.5	20.1	0

*Mean of 5 separate groups of mice with 4 mice/group.

10 days following intravenous injection. Five groups of mice with 8/group were infected intravenously with 1 of 5 doses of virulent 4-52 listeria and observed for 10 days. The infecting dose which permitted survival of all mice when injected I.V. was found to be 2×10^5 organisms.

Figure 6 shows the results of the A_1 group of mice infected immediately following 5 days of exposure to CO and the non-exposed controls. In the normal controls, the organisms replicated at a rather vigorous rate for the first 2 days. Thereafter, a slow but steady decline was evident. In the CO treated mice early growth of the infecting organisms was slow. The maximum number of organisms was recovered on the 2nd day after which time a rapid decline was observed. When comparing the spleen counts of the two groups, a significant difference is noted between each subgroup respective to the day of post infection. For days 1, 2, and 4 the differences are significant at $P < 0.025$ and for day 3 $P < 0.10$.

Figure 7 shows the results of the A_2 groups of mice which were immunized immediately after CO exposure and infected 2 days later. Replication of organisms during the first 24 hours in both groups of mice proceeded slowly. This replication was less apparent in the CO treated group. By the 3rd day after infection, no organisms were found in the undiluted spleen homogenates of either group. When comparing the spleen counts of the two groups, a significant difference was noted between each subgroup at days 1 and 2. ($P < 0.025$).

Figure 6.--The effect of pre-exposure to CO on spleen assay for Listeria monocytogenes from non-immunized mice infected I.V. with 2 x 10^5 organisms.

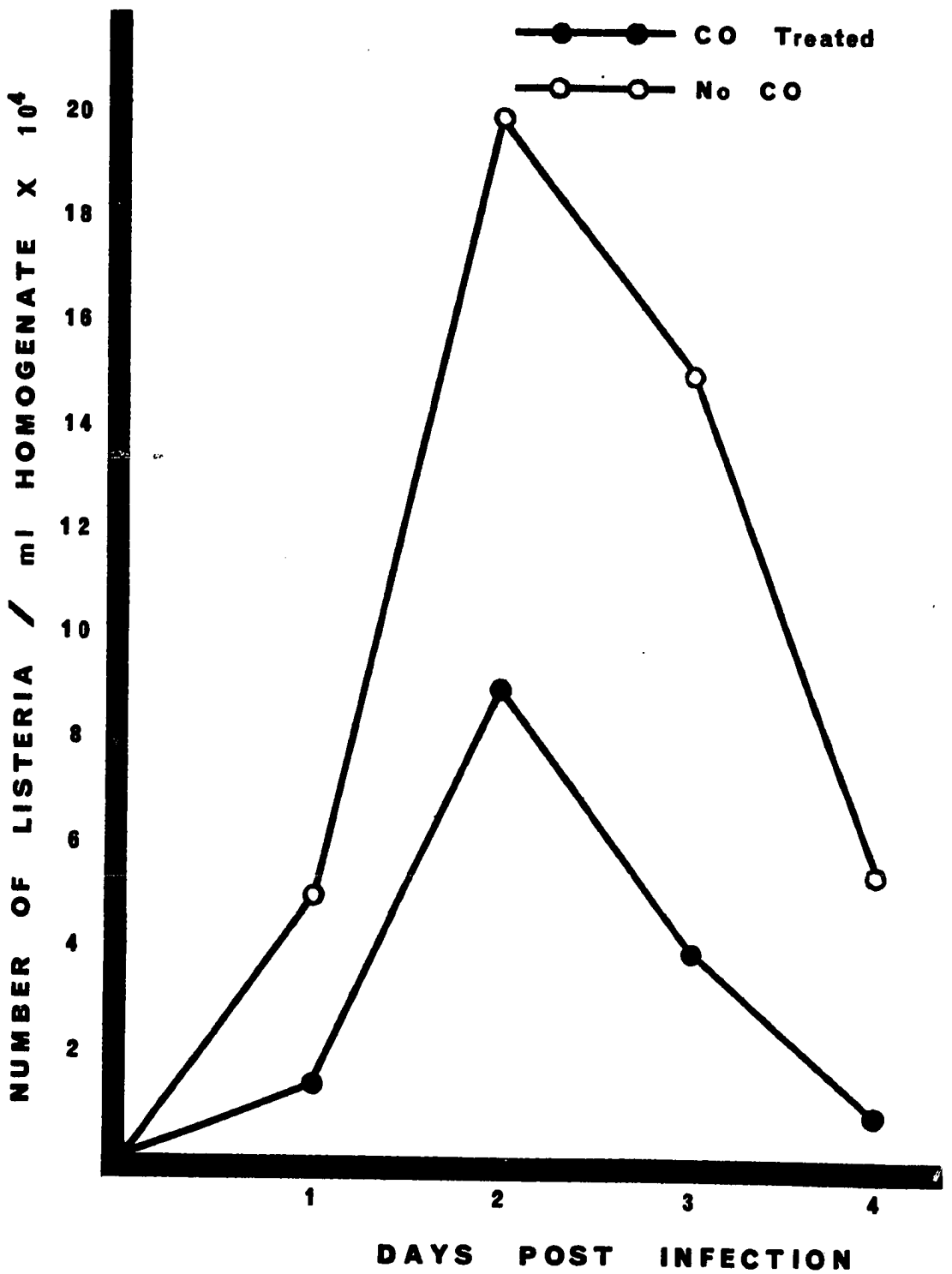
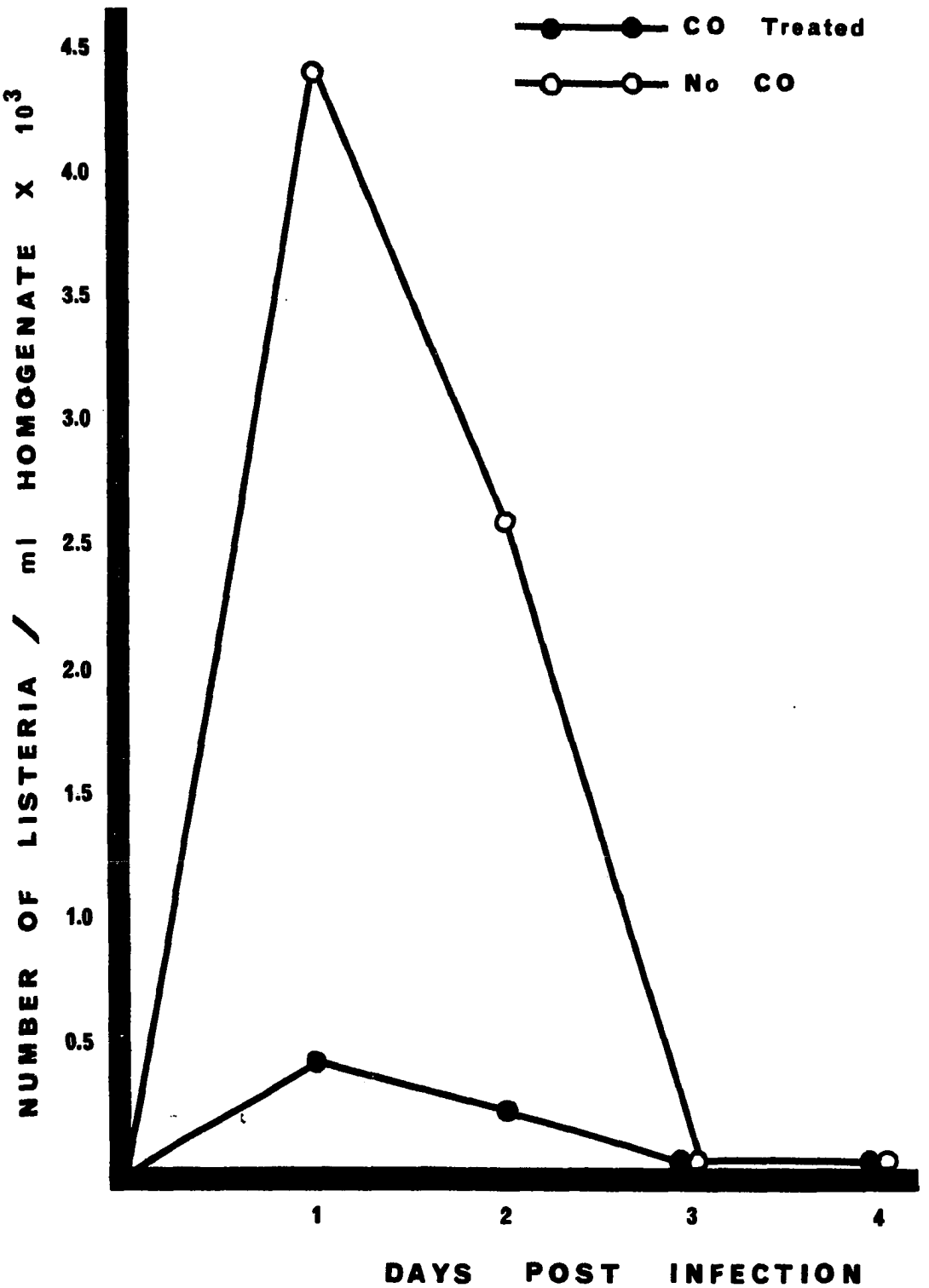

CO Treated
No CO
NUMBER OF LISTERIA / ml HOMOGENATE X 10^4
20
18
16
14
12
10
8
6
4
2
1
2
3
4
DAYS POST INFECTION

Figure 7.--The effect of pre-exposure to CO on spleen assay for L. monocytogenes (4-52 strain) from mice immunized I. P. with attenuated listeria and challenged I.V. with 2 x 10^5 organisms.


CO Treated
No CO
NUMBER OF LISTERIA / ml HOMOGENATE X 10^3
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
1
2
3
4
DAYS POST INFECTION

No organisms were recovered from the spleens of the A_4 group of mice which were infected 4 days following immunization.

Spleen homogenates from non-infected control mice were negative for Listeria monocytogenes.

CHAPTER IV

DISCUSSION

Experiments were performed to demonstrate the effect of CO on susceptibility of mice to respiratory infection. Mice exposed to 100 ppm of CO for 2, 3, 4, 5 and 6 days and infected with Listeria monocytogenes by the respiratory route exhibited increased resistance to the infection. This was demonstrated by a decrease in mortality and an increase in survival time. Traditional studies of CO toxicology have emphasized the dramatic effects of heavy exposures; remarkably little information is available about the consequences of exposure to the low concentrations found in the polluted community air. The concentration of CO chosen for this study was a level to which animals could be exposed without manifesting an acute CO toxicity and also a level below that normally encountered by man in his environment (e.g. 400-500 ppm of CO inhaled in cigarette smoke) and present for brief periods in confined areas of automobile congestion (14, 15, 24).

No attempt was made to correlate the days of exposure with the degree of resistance. A study for this purpose would involve exposing mice to various concentrations of CO for

differing time periods and serve to determine if a dose-effect relationship existed.

A consistent pattern of acquired resistance was observed in the CO exposed animals for the various time intervals, each of which tend to confirm the other. There is little experimental evidence available in the literature to support the claims incriminating CO as a distinct health hazard at the lower levels of exposure. Results of this portion of the study suggest that low levels of CO may play an important part in respiratory disease. The effects of CO exposure as reported herein, hopefully, will serve to stimulate further investigation in this area.

The possibility that CO exposure might influence the endogenous microbial flora in mice was investigated. The coliform bacteria constitute a large part of the normal intestinal flora of man and animals. Within the intestine they normally do not cause disease and may even contribute to normal function and nutrition. In this habitat there is an extremely delicate host-parasite relationship. However, factors serving to create an imbalance could conceivably lead to proliferation with dissemination and disease. Schaedle (37) observed a rapid and profound change in the composition of bacterial flora of mice by a variety of unrelated disturbances. Particularly noted were the changes associated with environmental temperatures. Should any one of the conditions (proliferation, dissemination, disease) be incurred, it is

entirely possible that cellular immunity would be invoked through the effect of bacterial endotoxic 'O' antigens; thus permitting macrophages to exhibit non-specificity in phagocytic activity. Under the conditions of this experiment CO did not alter the numbers or distribution of coliform bacilli. Thus it is unlikely that non-specific activation of macrophages was involved in or contributed to the enhanced phagocytic and bactericidal activity observed in the study against listeria infection.

Salmonella (31) and certain fungi (20) have been shown to activate macrophages, and while in the activated state these cells demonstrated increased phagocytic capacity. The possibility that mice used in this study harbored enteric pathogens (salmonella, shigella) and/or fungi was considered, as well as the influence CO may have exerted in potentiation of these organisms. The observation that no enteric pathogens or fungi were recovered from either group of mice (CO exposed and unexposed) implies that enhanced phagocytic activity noted earlier in the study was in all probability, not associated with the specific sequences for activation of macrophages which in turn could inactivate a variety of intracellular organisms.

The mononuclear phagocytes, which reportedly originate in the bone marrow, are transported by the peripheral blood as monocytes and eventually become tissue macrophages (13). An increase in blood monocytes observed in mice exposed to

CO might well indicate enhanced hematopoietic activity in the monocyte-macrophage cell system. Should this occur, substantial numbers of potential macrophages (blood monocytes) would be readily available and upon stimulation by an inflammatory process, migrate in larger numbers to contain the invading agent(s) more effectively. Examination of the peripheral white blood cells from CO exposed and unexposed mice did not reveal any noticeable change in numbers nor types of cells. Thus it is highly improbable that the increased resistance of the CO exposed-listeria infected mice was associated with a pre-conditioning of the monocyte-macrophage system.

The observation that mice exposed to low levels of CO were endowed with a greater capacity to resist a listeria infection via the respiratory tract was of great interest. In view of this, the next obvious avenue of investigation was directed at an in situ mechanism which resides primarily with the pulmonary macrophages and serves to clear bacteria in the lung (4, 17, 26). These cells make up a significant total of the lung cell population and represent the major cellular host defense apparatus responsible for early resistance to bacterial infection.

The results of the studies associated with phagocytosis indicated that CO, under the experimental conditions employed, served to enhance the phagocytic and bactericidal activity of not only alveolar but also peritoneal macrophages. Of interest was the observation that the peritoneal

macrophages demonstrated a much greater degree of efficiency in the up-take of listeria than did the alveolar cells under identical experimental conditions. This is in agreement with the finding of Degre (9), who made a similar observation with macrophages from normal mice. It would appear that the protection afforded to the mice by CO exposure, in all probability, was related to the direct or indirect influence this gas exerted on the macrophage phagocytic activities; particularly the pulmonary phagocytes which constitute the first line of cellular defense against aerosol infections. Not only were the CO exposed phagocytes empowered to ingest listeria more effectively but they also exhibited a marked degree of efficiency relative to suppressing the replication of this facultative intracellular bacterium. This is a very essential function, if listeria is to be contained and the host spared of any serious consequences.

Supplementing this host defense against infection are the macrophages located elsewhere within the host and which no doubt possess the accentuated ability for phagocytosis. This is illustrated by the enhanced antibacterial activity of the macrophages from the intraperitoneal cavity and phagocytic cells in the spleens of the CO exposed mice.

Cell-mediated immunity (CMI) is invested in the "activated" macrophages which in turn represent the final step through which this form of resistance is expressed. The major effector arm in resistance against listeria is the

"activated" macrophage. Thus an attempt was made to examine the specific antibacterial mechanism known to be effective against listeria, namely, cellular immunity.

The effect of immunization on the non-CO-exposed mice is reflected by a sharp decrease in numbers of listeria within the spleen tissue during the 1st and 2nd days of infection and no recoverable organisms thereafter. The CO-exposed, non-immunized mice, demonstrated a marked capability for suppressing replication of listeria as evidenced by a 70 and 55 per cent decrease for the 1st and 2nd days, respectively. The immune response, in the absence of CO accounted for a 91 and 98 per cent decrease in listeria replication for the 1st, and 2nd days respectively. However, the decrease in replication was more apparent in the CO immunized animals (99.2 and 99.9 per cent for the 1st and 2nd days respectively). These results suggest that CO may serve to influence factors associated with cell-mediated immunity. Perhaps a more meaningful approach for a study of this nature might be directed at a few of the major assays developed for the study of CMI in vitro (e.g., lymphotoxin activity, lymphocyte transformation, migration inhibitor factor). Also some consideration could be given to allograft immunity using the mouse skin allograft as a model for studying cellular immunity.

Little is known regarding the determinants for the well documented phagocytic and bactericidal activities of the

macrophages. The actual kinetics by which macrophages destroy bacteria are unequivocal; nevertheless, much is known about the bactericidal mechanisms of the polymorphonuclear leucocytes. Any attempt to present a working hypothesis as related to an intracellular microbicidal mechanism(s) under the influence of CO becomes extremely difficult.

In view of the alterations in macrophage function as induced by CO, it would be most desirable to continue studies along the following lines:

(1) Many of the functions of macrophages are intimately related to the cell membrane. Cross, et al. (8) have reported that alveolar macrophage plasma membranes possess a Na^{+}-K^{+} stimulated Mg^{++}dependent ATPase system which may be directly related to the phagocytic and bactericidal activities of the cell. This enzyme system would serve as a convenient biochemical marker to quantitate the interaction of lung cells and CO.

(2) The effect of CO may be indirect or secondary. The gas may influence specific physiological factors which in turn modify the phagocytic properties (i.e., assess the effect of CO on the endocrine function).

(3) As suggested earlier, a study designed for measuring the effect of CO on those parameters generally considered indices of cellular immune response with particular emphasis on in vitro assays and allograft immunity.

(4) Assess the effect of CO on intracellular soluble enzymes or a look at the antibacterial activities of extracts from macrophages of CO exposed animals.

CHAPTER V

SUMMARY AND CONCLUSIONS

Male inbred BALB/cj mice were exposed to 100 ppm of carbon monoxide (CO) for varying lengths of time (2, 3, 4, 5, and 6 days) and subsequently infected via the respiratory route with Listeria monocytogenes. Mice so treated evidenced increased resistance to listeria infection as manifested by a lower death rate and an increased length of survival. No dose-effect relationship could be established under the experimental conditions of this study. The finding in this portion of the study suggests that low levels of CO can exert a beneficial effect on the host's defensive mechanism.

Carbon monoxide did not alter the numbers or distribution of coliform bacteria in the colon of mice. The bacterial flora per se remained essentially unchanged.

Exploratory studies conducted to define the mechanism responsible for increased resistance to infection were directed at the cellular capacities for phagocytosis and bacterial inactivation. In vitro phagocytic properties of pulmonary and peritoneal macrophages derived from mice exposed to CO demonstrated enhanced phagocytic properties. Likewise the

macrophages exhibited a marked ability to suppress the intracellular proliferation of listeria.

The suppression of listeria replication was further reflected in the spleen assay studies of the CO exposed, non-immunized and immunized mice. Both experimental groups evidenced enhanced antibacterial activity.

It would appear that CO served as a non-specific activator for macrophage phagocytic activity which in turn played a dominant role in affording the animals with a marked degree of protection against listeria infection.

The action of CO, in all probability, is related to alterations associated with biochemical or immunologic activities of the phagocytic cells in some as yet unexplained manner which endows the cells with a greater capacity to phagocytize and kill microorganisms.

Suggestions for future studies are included in the discussion.

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