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Effect of Ammonia on In Vivo and In Vitro Immune Responses

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The effects of exposure of animals to ammonia on their delayed type of dermal response, the mitogenic and antigenic responses of their lymphocytes, and the bactericidal and phagocytic activities of their alveolar macrophages were examined. Experimental guinea pigs vaccinated with *Mycobacterium bovis* BCG were exposed to 3.75 μg of ammonia per dl of air (50 ppm) or 6.75 μg of ammonia per dl of air (90 ppm), whereas control animals also vaccinated with BCG were maintained in the normal environment. The dalayed type of dermal response to tuberculin injected 3 weeks later was significantly ($P < 0.05$) less in experimental animals exposed to 6.75 μg of ammonia per dl than in control animals. In vitro, the response of blood lymphocytes and bronchial lymphocytes to phytohemagglutinin, concanavalin A, and tuberculin stimulation was significantly ($P < 0.01$) less than the response of lymphocytes from control animals. The response of normal blood lymphocytes to phytohemagglutinin incubated in medium containing 1 or 10 mg of ammonia per dl was significantly ($P < 0.01$) reduced as compared with the response of lymphocytes incubated without ammonia. The viability of lymphocytes incubated with these concentrations of ammonia was significantly ($P < 0.01$) affected. There was no significant difference in the bactericidal or phagocytic activities of alveolar macrophages collected from animals exposed to ammonia and control animals. However, ammonia added to the culture of alveolar macrophages from normal animals significantly inhibited their bactericidal activity.

Animals are frequently exposed to a high concentration of ammonia (a common pollutant of air) in their environment. Ammonia at a concentration of 1.9 μg/dl of air (25 ppm) caused a greater incidence of mycoplasmal pulmonary lesions in the infected animals than in control animals maintained in the control environment (2). The susceptibility of birds to viral respiratory infections also increased after their exposure to 1.5 μg of ammonia per dl (20 ppm) (1). Interestingly, ammonia usually does not penetrate to the distal airways (3, 4). This observation suggests that ammonia might affect the defense system of the host by means other than local suppression of the immunity.

In this study, we determined the effects of exposing guinea pigs to ammonia in air on their immune system. We studied the delayed type of dermal response, antigenic and mitogenic responses of blood and bronchial lymphocytes, and the bactericidal and phagocytic activities of alveolar macrophages. The effect of addition of ammonia to the tissue culture medium on the viability and response of normal lymphocytes to a mitogen and on the bactericidal and phagocytic activities of alveolar macrophages was also investigated. The term ammonia was used to express the total concentration of NH_3 and NH_4^+ in an aqueous solution. The concentration of nonionic ammonia (NH_3) in the blood plasma is highly pH dependent; at a pH of 7.4, only 2.5% of total ammonia ($NH_3 + NH_4^+$) is in solution. Each 0.3-U change in pH near the physiological pH range of plasma contributes to a doubling of nonionic ammonia (13).

MATERIALS AND METHODS

Animals and ammonia exposure. Twenty-four guinea pigs of Hartley strain (The Ohio State University, Columbus, Ohio) were vaccinated subcutaneously with a single dose of 10^4 live *Mycobacterium bovis* BCG (lot no. D257, Glaxo Inc., Fort Lauderdale, Fla.). After 3 weeks, these animals were challenged intradermally with 2.5 μg of purified protein derivative of tuberculin (PPD) in 50 μl of commercial diluent (Tubersol; Connaught Laboratories, Willowdale, Ontario, Canada). Eight PPD-positive animals served as controls and were maintained in the chamber in the normal environment with the ammonia concentration below 1.12 μg/dl (15 ppm). Two other groups of eight PPD-positive guinea pigs each were placed separately into two chambers. Ammonia concentration in one chamber was maintained at 3.75 μg/dl (50 ppm) and in another at 6.75 μg/dl (90 ppm). A steady concentration of ammonia in the chambers was maintained by its continuous flow from a gas tank, and the concentration was monitored daily with detector tubes for ammonia SC type from 0.37 to 9.75 μg/dl (5 to 130 ppm) (Arthur H. Thomas Co., Philadelphia, Pa.). After 3 weeks, control and experimental animals were again challenged intradermally with PPD, and the diameters of the erythema were measured with a caliper.

Clinical and hematological examinations. Water and food consumption by guinea pigs were monitored daily. Animals were also observed for any sign of distress, conjunctivitis, or respiratory diseases. All guinea pigs were weighed at the beginning and end of the experiments. Blood samples were collected by cardiac puncture from each guinea pig at the beginning and end of the experiments. Erythrocytes and leukocytes (including differential cell counts) were counted. Ammonia concentration in the blood was determined by an enzymatic method that uses glutamate dehydrogenase and NADH (Gilford Diagnostic Ammonia Kit, Gilford Instrument Laboratories, Inc., Cleveland, Ohio). Glutamate dehydrogenase catalyzes the reductive amination of 2-oxoglutarate and concomitantly oxidizes NADH. The NAD formation, measured by a decrease in the absorbance at 340 nm in a UV/VIS spectrophotometer (Perkin-Elmer Lambda

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3), was proportional to the ammonium ion concentration in the blood. The commercial standards and our standards, prepared by dilution of ammonium hydroxide, were always checked before blood samples were tested.

Separation of lymphocytes. Blood was collected by cardiac puncture into syringes containing anticoagulant from ammonia-exposed, control, and normal guinea pigs. The blood samples were subsequently diluted with 3% gelatin (2:1 vol/vol) and allowed to stand at 37°C for 25 to 30 min. The cloudy supernatant fluid was removed every 5 min during sedimentation of the erythrocytes. Lymphocytes from the supernatant fluid were finally separated by a Ficoll-Hypaque gradient and washed in Eagle minimal essential medium supplemented with 10% inactivated cavian serum, 0.5% gentamicin sulfate (Schering Corp., Kenilworth, N.J.), and 1% L-glutamine (KC Biologicals, Lenexa, Kans.). The concentration of lymphocytes was then adjusted to 4×10^6/ml. The efficacy of separation of this method was 70.2 ± 6.4%, and it yielded more than 96% lymphocytes (including monocytes) with viability of more than 96 ± 0.31% as determined by trypan blue dye exclusion. Bronchial leukocytes were obtained by a tracheobronchial lavage (9) with minimal essential medium from ammonia-exposed, control, and normal guinea pigs. Approximately 15% of the lavaged leukocytes were lymphocytes (see Results). A suspension of the unfractionated bronchial leukocytes (4×10^6/ml) was prepared for the lymphoblastogenesis assay and the bactericidal (10^7/ml) and the phagocytosis (10^6/ml) tests.

Blastogenesis of lymphocytes from ammonia-exposed and control animals. Quadruplicate (four wells) cultures of 2×10^5 blood lymphocytes or bronchial leukocytes in 50 μl of medium were established in the wells of microculture plates (Linbro Scientific, Hamden, Conn.). Medium (150 μl) was added to the control wells. In the experimental wells, 50 μl of medium and then 100 μl of a 1.0-μg/ml solution of phytohemagglutinin (PHA) (Sigma Chemical Co., St. Louis, Mo.), 100 μl of a 1.0-μg/ml solution of concanavalin A (Sigma), or 100 μl of a 24-μg/ml solution of PPD were added so that the concentration of the mitogen or antigen was optimal in the well.

Cultures were incubated for 3 days at 37°C in a 5% CO_2 humidified atmosphere incubator. Eighteen hours before termination, 0.5 μCi of [*methyl*-^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) was added to each well. After 72 h, cultures were terminated and harvested for scintillation counting. The [^{3}H]thymidine incorporated into the lymphocytes was measured in total counts per minute on a Beckman LS 7000 liquid scintillation spectrophotometer.

Viability and blastogenesis of ammonia-exposed lymphocytes in vitro. The percentage of unstained lymphocytes (live) was determined by trypan blue exclusion test at 0, 2, 4, 24, 48, and 72 h of the incubation in medium alone or medium containing different concentrations of ammonia (as below).

Eighty wells filled with 2×10^5 lymphocytes from normal animals per 50 μl of blood per well were divided into five sets (A, B, C, D, and E) with four quadruplicates (16 wells per set). The wells of the first quadruplicate of set A received 100 μl of medium, whereas wells of the remaining three quadruplicates received 100 μl of medium containing 1.0, 5.0, or 10.0 μg of PHA per ml. An additional 50 μl of medium was placed into the wells of this set. The wells of set B were filled with medium or medium containing various concentrations of the mitogen, as above. However, the last addition of 50 μl of medium was replaced by 50 μl of medium containing ammonium hydroxide (NH_4OH; Mallinckrodt, Inc., Paris, Ky.) which brought the ammonia concentration to 0.01 mg/dl. The wells of sets C, D, and E were filled as in set B, with the exception that the final concentration of ammonium in the medium was adjusted to 0.1 mg/dl (set C), 1.0 mg/dl (set D), and 10.0 mg/dl (set E).

Bactericidal and phagocytic activities of alveolar macrophages from ammonia-exposed and control animals. Bactericidal and phagocytic capacities of the alveolar macrophages (alveolar and bronchial) from control and experimental guinea pigs were measured by ingestion of *Staphylococcus aureus*. An 18-h broth culture of *S. aureus* 305 was washed in minimal essential medium and suspended at a concentration of 10^8/ml (determined by Petroff-Hausser counting chamber and confirmed by plate counts of staphylococci). Staphylococcal suspension (100 μl) and 100 μl of bronchial leukocytes (10^7/ml) were mixed 10:1 (bacteria/cells). Triplicate samples in glass tubes were incubated at 37°C for 1 h in a 5% CO_2 incubator. Lysostaphin (to kill extracellular staphylococci) at a concentration of 32 IU (Sigma) was added to these samples, and the mixture was incubated at 37°C for 1 h. Subsequently, trypsin was added to break down lysostaphin. After 1 h of incubation, the mixture was sonified at a power setting of 35 for 15 s (Branson Sonifier; Branson Sonic Power Co., Danbury, Conn.) and plated on brain heart infusion agar (Difco Laboratories, Detroit, Mich.). Plate counts of viable staphylococci were done by the drop method, and results were expressed as the number of CFU. Controls for this experiment included staphylococci, bronchial leukocytes, lysostaphin-treated staphylococci, trypsin-treated staphylococci, and trypsin-treated lysostaphin subsequently incubated with staphylococci.

Phagocytosis by alveolar macrophages was measured in Linbro chambers. The suspension of bronchial leukocytes (50 μl) (10^6/ml) was distributed into each chamber, and cells were allowed to adhere to glass. After 2 h of incubation, nonadherent cells were removed by gentle washing with minimal essential medium, and 50 μl of staphylococcal suspension (10^8 heat-killed *S. aureus* cells per ml) was added. The cultures were then centrifuged for 5 min and incubated for 30 min. Subsequently, 32 IU of lysostaphin was added per chamber. The cultures were then washed, fixed in 10% Formalin, and stained with Wright stain. The percentage of macrophages with phagocytized bacteria for each preparation was calculated from observations of 100 of these cells.

Bactericidal and phagocytic activities of ammonia-exposed alveolar macrophages in vitro. Bactericidal and phagocytic tests (see above) were modified by preincubation of the macrophages with various concentrations of ammonia (0.01, 0.1, 1.0, or 5.0 mg/dl) at 37°C for 1 h before staphylococci were added. Thereafter, these tests were performed in the presence of ammonia. Controls were incubated without ammonia.

Statistical analysis. Experimental results were expressed as the mean ± standard deviation. The significance was evaluated with the Student *t* test.

RESULTS

Clinical and hematological observations. Guinea pigs maintained at 3.75 or 6.75 μg of ammonia per dl of air did not lose weight or show any signs of distress, conjunctivitis, or respiratory diseases. There were no significant differences in the number of erythrocytes and leukocytes in the blood between control (erythrocyte 5.4 ± 0.3 × 10^6/mm^3; leukocyte 6.7 ± 0.69 × 10^3/mm^3) and experimental (erythrocyte 4.8 ± 0.5 × 10^6/mm^3; leukocyte 5.6 ± 0.64 × 10^3/mm^3)

animals. The difference between numbers of bronchial leukocytes and their types from animals of both groups was insignificant. The yield of bronchial leukocytes was ca. 30×10^6 viable cells per animal. Differential counts established that ca. 70% of the lavaged leukocytes were macrophages, 15% were lymphocytes, and 15% were eosinophils. The difference in concentration of ammonia in the blood of animals from the experimental group (199.5 ± 52.9 μg/dl) and from the control group (151.1 ± 77.8 μg/dl) was insignificant.

Delayed type of dermal response of ammonia-exposed animals. A significantly ($P < 0.05$) smaller dermal reaction to injection of PPD at 24, 72, or 96 h was observed in guinea pigs maintained at 6.75 μg of ammonia per dl than was observed in animals maintained at 3.75 μg of ammonia per dl or in the normal environment (Table 1). Approximately 2 mm of diameter of dermal lesion was observed infrequently at 3 h postinjection of PPD, that usually disappeared at 6 h.

Blastogenesis of lymphocytes from ammonia-exposed animals. Blood lymphocytes from guinea pigs exposed to 3.75 μg of ammonia per dl for 3 weeks responded to PHA (18,007 ± 3,466 cpm), concanavalin A (19,522 ± 5,764 cpm), and PPD (11,678 ± 2,542 cpm) as did lymphocytes from control animals (21,824 ± 1,892; 25,289 ± 1,710; and 14,858 ± 2,688, respectively) maintained in the normal environment. The responsiveness of bronchial lymphocytes was similar for experimental and control animals. In contrast, the response to mitogens of blood and bronchial lymphocytes from animals exposed to 6.75 μg/dl for 3 weeks was significantly ($P < 0.01$) lower than the response of lymphocytes from control animals (Fig. 1 and 2). The responsiveness to PPD of blood (4,776 ± 512 cpm) and bronchial (907 ± 40 cpm) lymphocytes from experimental guinea pigs also was significantly ($P < 0.01$) reduced (Fig. 1 and 2) as compared with the response of blood (7,291 ± 1,272 cpm) and bronchial (1,199 ± 27 cpm) lymphocytes from control guinea pigs.

Viability and blastogenesis of ammonia-exposed lymphocytes in vitro. Ammonia added in various concentrations to the tissue culture affected viability of blood lymphocytes from normal animals (Fig. 3). Addition of ammonia at concentrations of 1.0 or 10.0 mg/dl significantly ($P < 0.01$) decreased the viability of lymphocytes, whereas addition of 0.1 mg/dl and lower concentrations of ammonia did not affect viability of the cavian lymphocytes. Addition of 1.0 or 10.0 mg of ammonia per dl to the lymphocyte culture significantly ($P < 0.01$) reduced response to PHA stimulation (Fig. 4). In contrast, lower concentrations of ammonia in vitro did not significantly suppress the responsiveness of the lymphocytes.

Bactericidal and phagocytic activities of alveolar macrophages from ammonia-exposed and control animals. There was no significant difference in the bactericidal activity of alveolar macrophages from experimental ($1.4 \pm 1.0 \times 10^7$ CFU) and control animals ($2.0 \pm 0.9 \times 10^7$ CFU). The difference in the percentage of the phagocytizing cells from experimental guinea pigs (43.9 ± 2.8%) and from control animals (46.4 ± 4.5%) was insignificant.

TABLE 1. Dermal response to the injection of PPD in animals exposed to various concentrations of ammonia

Concn of NH_3	Mean diam of redness (mm) at:			
	24 h	48 h	72 h	96 h
0.0[a]	17.6 ± 1.7[b]	15.5 ± 1.9	12.0 ± 1.1	10.4 ± 1.5
3.8 mg/ml	15.2 ± 2.23	13.8 ± 1.6	12.6 ± 1.0	12.6 ± 0.7
6.08 mg/dl	14.4 ± 2.2[c]	14.7 ± 1.8	8.7 ± 1.4[c]	0.0[c]

[a] Concentration of ammonia was below 1.12 μg/dl.
[b] Values are means of eight guinea pigs per group ± standard deviation.
[c] Significant ($P < 0.05$) when compared with control group.

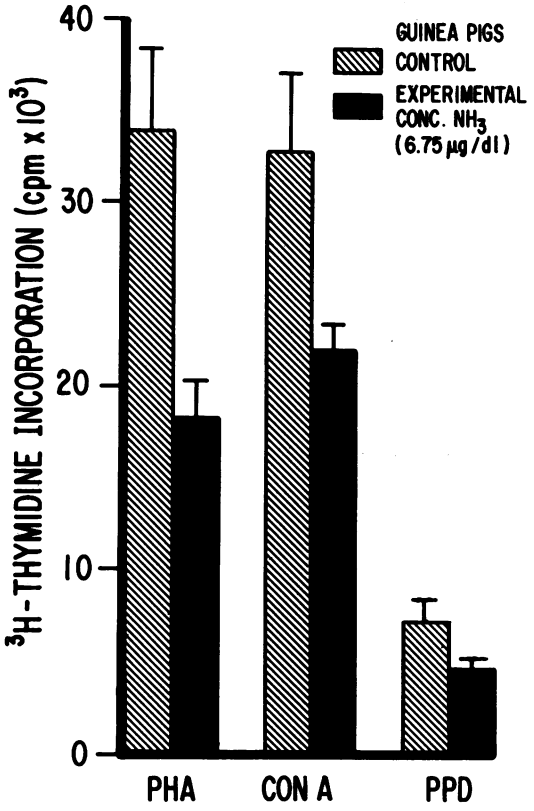

FIG. 1. Response of blood lymphocytes from ammonia-exposed animals and control animals after incubation with PHA, concanavalin A, or PPD. Bars, Mean [^{3}H]thymidine incorporation (cpm × 10^3) ± standard deviation for lymphocytes from eight animals.

Bactericidal and phagocytic activities of ammonia-exposed alveolar macrophages in vitro. The exposure of alveolar macrophages to ammonia at concentrations of 1.0 and 5.0 mg/dl significantly ($P < 0.01$) inhibited their bactericidal activity (Fig. 5). Other concentrations of ammonia (0.1 and 0.01 mg/dl) did not inhibit bactericidal activity of alveolar macrophages. In contrast, the phagocytic activity was not affected even by high concentrations of ammonia.

DISCUSSION

Lymphocytes from animals exposed to 6.75 μg of ammonia per dl showed significantly lower responses to mitogens and PPD than did those from control animals, suggesting that ammonia absorbed into the circulation does affect blood lymphocytes. The nonionic form of ammonia (NH_3) is more toxic and more readily absorbed by cells than is the ionic form (NH_4^+), and it is also pH dependent (13). Unfortunately, nonionic ammonia in solution cannot be determined by direct measurement (12). In this study, only a small increase of the concentration of ammonia in the blood (ionic form) was observed in animals exposed to 6.75 μg of NH_3 per dl. Schaerdel et al. (10) reported that the concentration of ammonia (NH_4^+) in the blood increased significantly in a linear fashion with increasing ammonia concentration in the

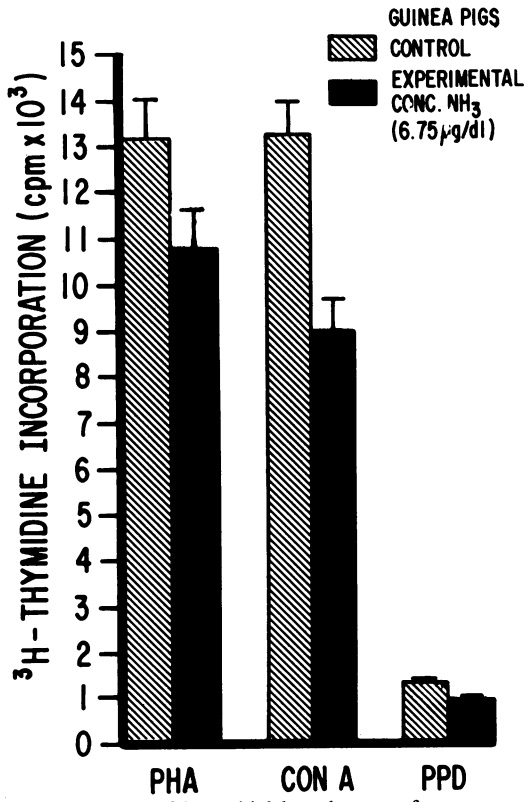

FIG. 2. Response of bronchial lymphocytes from ammonia-exposed animals and control animals after incubation with PHA, concanavalin A, or PPD. Bars, Mean [^{3}H]thymidine incorporation (cpm $\times$ 10^3) $\pm$ standard deviation for lymphocytes from eight animals.

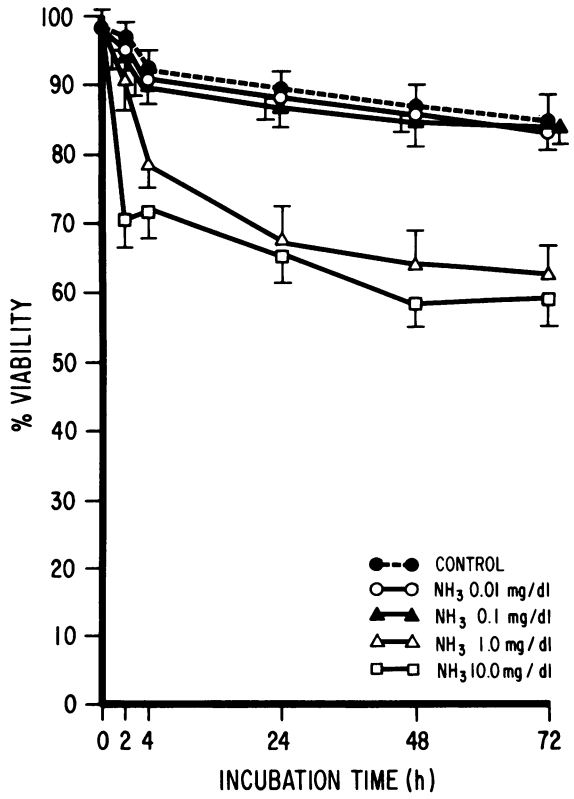

FIG. 3. Percentage of viable blood lymphocytes from unexposed animals after incubation in control medium and in media containing various concentrations of ammonia. Points, Mean percentage $\pm$ standard deviation of viable lymphocytes from five animals.

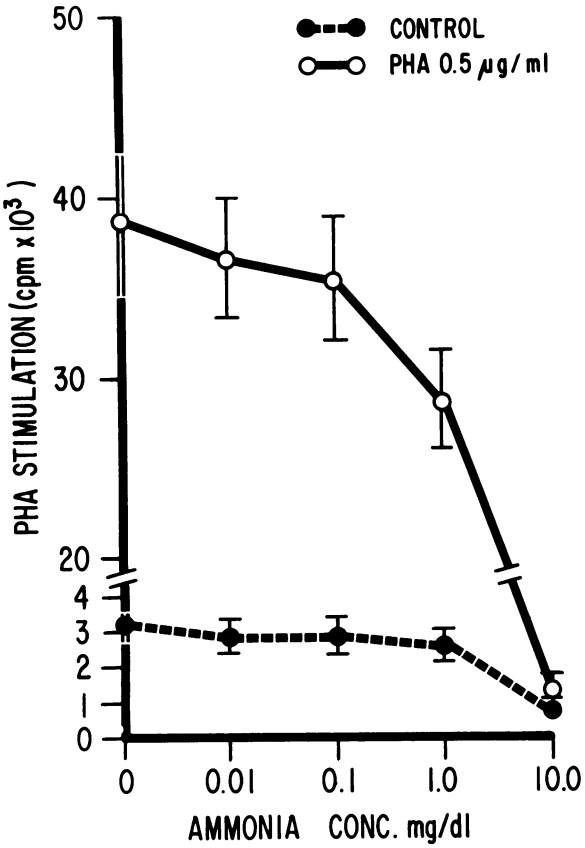

FIG. 4. Response of blood lymphocytes from unexposed animals stimulated with PHA or those from animals unstimulated with PHA and incubated in medium containing various concentrations of ammonia. Points, Mean [^{3}H]thymidine incorporation (cpm $\times$ 10^3) $\pm$ standard deviation for lymphocytes from five animals.

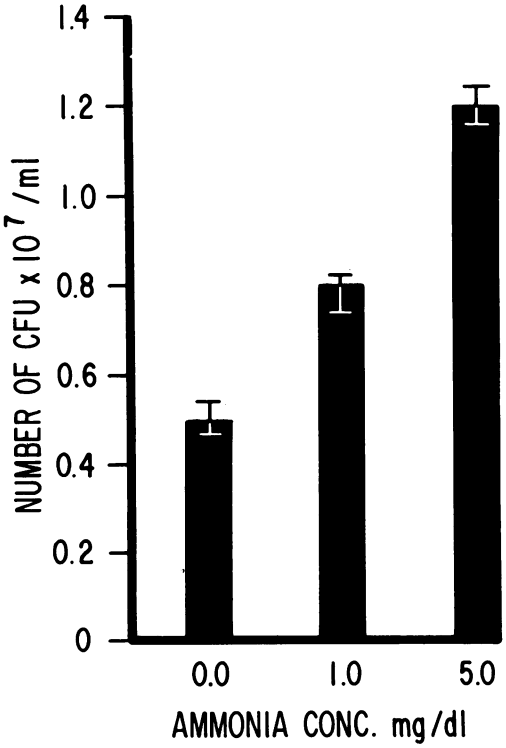

FIG. 5. Bactericidal capacity of normal alveolar macrophages cultured in medium containing from 0 to 5.0 mg of ammonia per dl. Bars, Mean CFU value $\pm$ standard deviation for alveolar macrophages from five animals.

environment, indicating that there was absorption of ammonia from the respiratory tract. They suggested that the body compensated for a high environmental ammonia concentration by increasing ammonia metabolism after 8 h of exposure. Therefore, concentration of ammonia in the respiratory tract circulation of exposed guinea pigs could be greater than that in the peripheral blood, which could consequently affect circulating lymphocytes. In rabbits, the increase of ammonia in the blood (3.1 mg/dl), by injection of urease or ammonium salts, was correlated with a decrease of the number of lymphocytes (5). Based on these observations, it was not likely that stress caused the reduction in responses of lymphocytes from ammonia-exposed guinea pigs. In addition, the ammonia-exposed animals did not show any signs of being under stress during the experimental period.

Addition of ammonia up to the concentration of 1.0 or 10.0 mg/dl of medium adversely affected the viability and mitogenic responsiveness of blood lymphocytes in vitro. These concentrations of ammonia in the blood are considered to be toxic. It has been demonstrated that a concentration of ammonia as low as 5 mg/dl inhibited production of interferon (7), mitochondrial respiration (15), or action of diphtheria toxin (8). The inhibition of these various activities by ammonia is probably due to alterations at the cell membrane level. The concentration of 0.09 mg of ammonia per dl was found in the blood of normal guinea pigs (14), and this concentration or lower did not affect the viability and mitogenic responsiveness of blood lymphocytes in vitro (Fig. 3 and 4).

Bactericidal and phagocytic activities of alveolar macrophages from experimental and control animals indicated that exposing guinea pigs to ammonia did not reduce these activities. In rats exposed to 7.5 μg of ammonia per dl (100 ppm), the intrapulmonary killing of staphylococci was also not reduced as compared with control animals (11). In that study and others (3, 4), it was concluded that NH_3 did not penetrate to the distal airways. In contrast, exposure of normal alveolar macrophages to ammonia at concentrations of 1.0 mg/dl or higher in vitro significantly inhibited their bactericidal activity. According to Gordon et al. (6), ammonia contributes to the blocking phagosome-lysosome fusion, thus reducing the killing of bacteria.

Exposure of animals to ammonia significantly reduced the cell-mediated response (i.e., dermal lesion). Stimulation of sensitized lymphocytes with PPD causes them to release lymphokines which subsequently attract inflammatory cells to the site of intradermal injection. The results of this study indicate that the responsiveness of blood or bronchial lymphocytes to PPD was reduced in ammonia-exposed animals. Therefore, it can be suggested that in vivo ammonia inhibits the release of lymphokines and the mediation of the specific inflammatory reaction, consequently compromising the ability of the host to eliminate infection.

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