Effects of Nitrogen Dioxide on Pulmonary Cell Population[1]

DONALD E. GARDNER, ROBERT S. HOLZMAN, AND DAVID L. COFFIN[2]

National Air Pollution Control Administration, Health Effects Research Program, Cincinnati, Ohio 45237

Studies from this laboratory have shown that ozone produces changes in the number and function of cells obtained by pulmonary lavage. In similar experiments, rabbits exposed to levels of NO_2 from ambient to 60 ppm demonstrated increased numbers of polymorphonuclear leukocytes in the lung washings. This phenomenon persisted for more than 72 hr after a single 3-hr exposure. When streptococci were instilled in the lungs of NO_2-exposed anesthetized rabbits 30 min before lavage, a pronounced inhibition of phagocytic activity was observed. With these criteria, NO_2 appeared less effective than ozone as a pulmonary irritant.

The principal identifiable components in oxidant-polluted atmospheres are ozone (O_3), peroxyacetyl nitrates, and oxides of nitrogen, mainly nitrogen dioxide (NO_2). In estimating the relative biological importance of these gases when they are mixed in polluted atmospheres, one should study the biological effect of each independently as well as in combination.

Coffin et al. (1, 2) have shown that the addition of ozone to the air supply of experimental animals enhances mortality from experimentally induced infections in the lung, delays the removal of bacteria from the lung, depresses the phagocytosis of bacteria by pulmonary alveolar macrophages, and causes a marked leukocytic pulmonary infiltration.

Nitrogen dioxide also enhances mortality from experimentally induced infections (3). Because of this similarity, other parameters previously used in ozone studies are being applied to studies of the effects of NO_2.

MATERIALS AND METHODS

New Zealand rabbits of both sexes, weighing between 1 and 2 kg, were exposed to NO_2 for 3 hr in a stainless-steel chamber with a volume of 11.4 ft³. Nitrogen dioxide (from commercially obtained cylinders of 1% NO_2 in nitrogen) was mixed with filtered ambient air and delivered to the chamber at a total flow rate of 11.4 ft³/min.

The NO_2 concentrations were monitored by the Saltzman (8) method and were expressed as parts per million (ppm) by volume. Pulmonary alveolar

macrophages were harvested by the techniques previously described by Coffin et al. (2). The isolated cells were sedimented by centrifugation at 365 × g for 15 min and were resuspended in buffered saline solution (pH 7.2) to a volume of 1.0 ml for every 0.1 ml of packed cells. Smears prepared from this suspension were stained by Giemsa's method. Total and differential counts were made. Eosin Y stain demonstrated that more than 99% of the cells were viable.

The in vivo phagocytic activity of pulmonary macrophages was studied. Forty rabbits were randomly subjected to various levels of NO_2. The animals were exposed for 3 hr and then were anesthetized by an intravenous injection of sodium pentobarbital. The trachea was exposed, and 1.0 ml of concentrated (10^8 organisms) *Streptococcus pyogenes*, Lancefield group C, was injected intratracheally. After 30 min, the rabbits were killed with pentobarbital. Their lungs were lavaged, and smears of the sedimented cells were prepared for staining. Slides were coded and read by a technician who was not aware of the code. One-hundred consecutive macrophages were counted on each slide and classified as containing 0, 1 to 3, 4 to 6, 7 to 9, or 10 or more bacteria. A cumulative frequency distribution was plotted, and the median number of bacteria per cell was determined for each animal. Group means were compared by analysis of variance.

Ten rabbits were exposed at each of five levels of NO_2 (8, 16, 20, 36, and 60 ppm), whereas 20 were reserved for controls. Differential counts were performed on the cells concentrated from washout fluid after staining by Giemsa's method.

RESULTS

Ninety-eight per cent of the cells obtained from healthy, unexposed rabbits were mononuclear macrophages. Small lymphocytes comprised 1%, and the remaining 1% represented an occasional polymorphonuclear leukocyte (heterophile) or eosinophile.

Nitrogen dioxide, at levels as low as 8 ppm, caused a significant (0.05) increase in the percentage of intra-alveolar heterophiles obtainable by pulmonary lavage. The dose reponse curve shown in Fig. 1 is similar in shape to that previously described for ozone; however, NO_2 is less potent than ozone, as considerably higher concentrations of NO_2 were necessary to produce equivalent changes. For example, 25.0 ppm of NO_2 (0.98 mmoles/m^3) increases the heterophiles to 20%. Ozone produces an equal response at a level of 2 ppm (0.08 mmoles/m^3).

To study the influx of heterophiles, rabbits were exposed to 40 ppm of NO_2 for 3 hr and were sacrificed at 0, 3, 6, and 9 hr after removal from the chamber. A level of 40 ppm of NO_2 was chosen because it represents approximately the same percentage of heterophiles as 5 ppm of O_3, which was used in the previous study. The peak infiltration of heterophiles was found to occur between 6 and 9 hr after exposure (Fig. 2).

In a second experiment, 35 rabbits were exposed to 40 ppm of NO_2 to determine the duration of the response. An abnormally high proportion of heterophiles persisted for as long as 72 hr (Fig. 2). Figure 2 compares the deviation of response of heterophiles after O_3 and NO_2 exposure.

There was a marked depression of phagocytic activity by NO_2. Ten ppm of NO_2 for 3 hr reduced the median number of streptococci per cell from 4.1 to 2.1 ($\alpha < 0.05$). In addition, although 83% of the macrophages from unexposed animals contained at least one bacterium, only 66% of the macrophages from exposed

animals showed phagocytic activity ($\alpha < 0.05$). Figure 3 is a comparison of the reduction in phagocytosis elicited by NO_2 with the O_3-induced depression previously reported by Coffin et al. (2).

DISCUSSION

Quantitation of pulmonary inflammation induced by toxic agents is readily accomplished by assessing the dose-response relationship between agent and heterophilic infiltration. This method is simple, reproducible, and can be performed by any technician familiar with blood-counting procedures.

In evaluating the relative toxicity of gases, several factors must be borne in mind. The con-
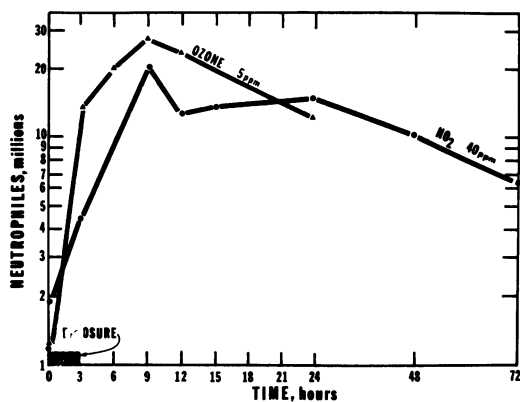

FIG. 2. Comparison of the effect of O_3 and NO_2 on pulmonary heterophiles (neutrophiles).
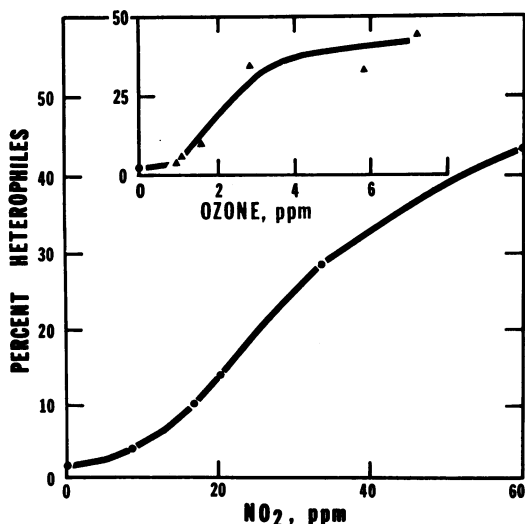

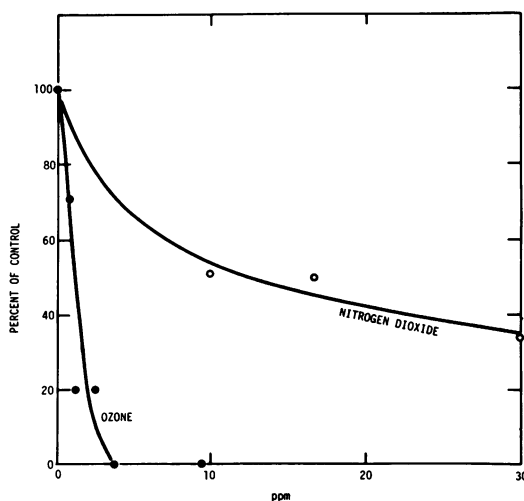FIG. 1. Effect of air pollutants on pulmonary heterophiles.

FIG. 3. Depression of phagocytosis by ozone and nitrogen dioxide (100% = median number of bacteria per cell in controls).

centration in the chamber atmosphere does not reflect the actual dose delivered to the alveoli, because some of the gas is either physically dissolved or chemically degraded as it passes through the respiratory system. The fraction of gas removed in this manner would be expected to vary from species to species because of anatomical differences and from gas to gas because of their physical and chemical properties. For purposes of environmental toxicology, it is difficult to determine the actual amount delivered to the lungs, and it is more practical to express dosage in terms of levels in the chamber atmosphere.

In long-term studies employing intermittent exposure at 1 ppm of O_3, chronic bronchiolitis and fibrosis were produced in a number of small laboratory rodents (10). In contrast to these findings, animals exposed chronically to 25 ppm of NO_2 showed no such demonstrable histological effect (12). In view of similarities in the acute biological effects of these two gases, i.e., on enhancement of mortality from experimental infection, on rate of bacterial clearance from the lung, on the infiltration of heterophiles, and on the depression of phagocytosis, it is reasonable to believe that their modes of action are closely related (1–3).

Either extracellular or cellular mechanisms may be responsible for the observed decrease in phagocytosis. Among the former, one possibility is that the air pollutant, either O_3 or NO_2, inactivates an opsonogenic factor in the extracellular milieu. Ozone has been shown to react with at least one extracellular pulmonary protein, lysozyme, both in vivo and in vitro (4). Nitrogen dioxide, however, does not share this ability. Pollutant-induced destruction of surfactant, by altering the surface tension of the alveolar lining, may affect macrophage motility. Exposure of rats to 1 ppm of NO_2 is followed by the peroxidation of lung lipids (11). Whether the oxidized lipids are in fact components of surfactant remains to be established.

Direct attack upon the cell is also possible. A change in charge, surface tension, or "adhesiveness" of the cell membrane would affect the rate of engulfment. Inhibition of intracellular metabolic processes necessary for phagocytosis is yet another possibility. Nitrogen dioxide depresses the in vitro phagocytosis of BCG and

also prevents the activation of the hexose monophosphate shunt which normally accompanies ingestion (7). Some studies of neutrophil phagocytosis suggest, however, that this activation occurs after the event rather than being a necessary prerequisite (5, 6).

These possibilities are not mutually exclusive, and several of them may operate simultaneously. Studies currently in progress may help assess the relative importance of each.

The variability in the number of heterophiles seemed to be influenced by increasing the NO_2 level; this invalidated the usual analysis of variance, and thus the Kruskal-Wallis "analysis of variance by ranks" (9) was substituted.

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