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Effects of Inhaled Endotoxin-Containing Bacteria

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Results of a study of pulmonary response-producing mechanisms resulting from the inhalation of endotoxin are reported. A number of experimental variations in challenging unimmunized rabbits with *Escherichia coli* without envelope antigens was attempted. Changes in arterial blood gases, leukocytes, platelets, and peripheral complement were monitored as indices of inhalation response. When either purified endotoxin or heat-treated *E. coli* cells were used for a single challenge, consistent elevations in P_{aO_2} with concurrent depressions in P_{aCO_2} were noted 4 hr after challenge. Experimental animals also exhibited a leukocytosis 10 min post challenge, which persisted up to 4 hr, but no evidence of fever could be documented. Since occupational exposure to endotoxin-contaminated dusts would involve exposures of varying concentration and duration, a comparison was made between single and dual challenges with the same dose given 24 hr apart, in an effort to see if something analogous to a pulmonary Shwartzman reaction might take place in unimmunized animals. When an aerosol sensitizing dose of *E. coli* was followed 24 hr later by an intravenous provoking dose of the same cell suspension, marked decreases in P_{aO_2} occurred within several hours after challenge and continued for up to 8 hr. Moreover, a decline in the number of circulating platelets coincided with the observed depressions of P_{aO_2}. A portion of the animals died with hemorrhagic lung injury, but in those surviving, P_{aO_2} values returned to normal within 24 hr. Indomethacin was shown to be effective in inhibiting these changes observed following dual challenge, thus pointing to the role of prostaglandins in the response. These experiments demonstrate that common endotoxin-containing microorganisms may cause pulmonary reactions in at least two ways. The ubiquity of gram-negative organisms allows for possible pulmonary injury in individuals associated with a wide variety of occupational exposures.

INTRODUCTION

The recognition of pulmonary injury resulting from the inhalation of microbially contaminated dusts is growing in importance. Microbial enzymes and certain cell wall components have been implicated in a variety of injury-producing mechanisms (Marx and Flaherty, 1976). Foremost among the microbial contaminants of organic dusts associated with occupational lung diseases are gram-negative bacteria or their endotoxins. Reports have appeared recently which associate the presence of gram-negative bacteria or their endotoxins in dust or aerosols of occupational origin with the nonspecific symptoms of fever, shivering, and malaise (Rylander *et al.*, 1977). Dust from cotton mills (Cavagna *et al.*, 1969; Rylander *et al.*, 1975a), grain elevators (Dutkiewicz, 1978), animal processing plants (Dutkiewicz, 1978), and numerous other sources have all been shown to be significantly contaminated with predominantly gram-negative microorganisms.

As a general class of biologically active material, endotoxins have been well studied (Morrison and Ulevitch, 1978). However, not as much information exists

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regarding the potential of this surface component to induce pulmonary damage after inhalation. Most of the pertinent information available centers on the systemic effects of single aerosol challenge with endotoxin rather than on an evaluation of direct lung toxicity. In the work reported here, we have examined the potential of inhaled endotoxin to induce pulmonary injury following sequential aerosol exposure. Our findings suggest that inhaled gram-negative bacteria or their endotoxins can lead to altered lung function in at least two ways, and that the potential exists for the initiation of a pulmonary Shwartzman reaction after repeated exposures.

MATERIALS AND METHODS

Animals. Outbred New Zealand rabbits of either sex, weighing 2.0–2.5 kg, were obtained from Hilltop Laboratory Animals, Inc. (Scottdale, Pa.).

Escherichia coli. Serotype 0111 B4 was grown overnight on a rotary shaker in Todd–Hewitt broth (Difco, Detroit, Mich.) at 37°C. The cultures were harvested and centrifuged at 800*g*, and the sedimented organisms washed three times with sterile, pyrogen-free saline (PFS). Aliquots of the washed organisms were then resuspended to the original volume with Todd–Hewitt broth and stored at −70°C until needed. Prior to aerosolization, the bacteria suspensions were routinely washed and resuspended to volume with PFS. An aliquot was removed to quantitate the organisms in the inoculum by standard pour-plate count. A second inoculum was prepared in the above manner except the washed cultures were heated to 100°C for 45 min, prior to rewashing and storage in −70°C. This procedure was used to remove the outer, B envelope antigen. The viable and heat-treated *E. coli* cells served as particulate endotoxin sources.

Endotoxin. Homologous endotoxin, prepared and purified from *E. coli* 0111 B4 by the method of Westphal and Jann (1965), was used for aerosol challenge.

Aerosol challenge. Aerosols were administered into a 1.5×10^4-cm^3 Lucite chamber capable of accommodating the heads of four rabbits simultaneously. Collars made of rubber dental dams were fitted over the ports of the chamber through which the animals' heads were placed, thereby ensuring a closed system. Five milliliters of approximately 10^9 viable or heat-treated *E. coli* cells were aerosolized over a 30-min period by means of a No. 40 DeVilbiss nebulizer attached to a pressure pump operating at 2 psi, and delivering a flow rate of 13 liters/min through the chamber. Such conditions result in an aerosol of particles with a mean mass diameter of 2.3 μm (Larson *et al.*, 1976). Sterile PFS was used as the vehicle for aerosol challenge throughout. Standard pour-plate counts of serial tenfold dilutions of lung homogenates from such animals revealed that this kind of exposure resulted in the deposition of approximately 10^6 viable *E. coli* per gram of lung parenchyma. When rabbits were challenged with purified endotoxin, 5 ml of homologous endotoxin (1 mg/ml) in PFS was aerosolized over a 30-min period.

Blood gas analysis. The procedure and rationale of utilizing blood gas analysis as an index of pulmonary damage has been reported previously (Olenchock and Burrell, 1976). All blood gas analyses were performed within 30 min on a Corning Model 161 blood gas system (Corning Scientific Instruments, Corning, N.Y.). Calibration of the blood gas analyzing system was performed between each read-

ing using humidified compressed gas mixtures containing 5.14% CO_2/N_2 balance and 10.8% CO_2/O_2 balance (Matheson Gas Products, East Rutherford, N.J.) and room air. Calculations of gas tensions were corrected daily for barometric pressure and the vapor pressure of water. Samples were obtained from the medial artery of the ear immediately before challenge and at various intervals afterward. The whole blood remaining after each analysis was clarified by centrifugation, and the plasma removed and stored at −70°C for determination of systemic complement values.

Complement assays. Assays of hemolytic complement activity, designated as 50% hemolytic complement (CH_{50}) units per milliliter, were performed by the method of Mayer (1961). The rabbit plasma samples were diluted 1/10 or 1/15 with Veronal-buffered saline before each determination.

Platelet and leukocyte counts. The platelet values were determined by the method of Rees and Ecker (Miale, 1967). Total circulating leukocyte values were determined by standard techniques using Turck's diluting fluid, (Miale, 1967).

Body temperature measurements. The rectal temperatures of rabbits were measured with a Tele-Thermometer (YSI Model 47, Yellow Springs Instrument Co., Yellow Springs, Ohio) equipped with a YSI No. 401 probe. Rabbits were conditioned to tolerate the restraining boxes and rectal probes without temperature elevations. The thermister probes were inserted 1 hr before aerosol challenge to ensure stable prechallenge temperatures below 40°C. Temperatures were recorded, with a scanning speed of 1 min, every 15 min during, and for up to, 6 to 8 hr following aerosol challenge.

Indomethacin. Indomethacin sodium was prepared from indomethacin powder (Sigma Chemical Co., St. Louis, Mo.). An aqueous solution was prepared fresh daily by adding 0.012 to 0.023 g Na_2CO_3 (anhydrous) to 5.0 ml of water containing indomethacin (100 mg/ml). Rabbits were injected (ip) with 60 mg/kg of indomethacin 1 hr prior to challenge.

RESULTS

Single Aerosol Challenge

A single 30-min aerosol challenge with 5.0 ml of viable *E. coli* cells, in a concentration of approximately 10^9 organisms/ml, failed to elicit consistent changes in the arterial blood gases of experimental animals. However, when the experiment was repeated using heat-treated cells for aerosol challenge, an average increase in P_{aO_2} of 12 mm Hg was noted by 4 hr postchallenge (Fig. 1). P_{aCO_2} values declined concurrently. This elevation in P_{aO_2} persisted for up to 6 hr. The same type of response was noted in animals receiving an aerosol of purified endotoxin (1 mg/ml). Control animals receiving an aerosol of only PFS did not demonstrate this increase in P_{aO_2} (Fig. 1). The P_{aO_2} and P_{aCO_2} values of the experimental animals returned to near-prechallenge values by 24 hr after challenge.

In an effort to ascertain if the increases in P_{aO_2} were the result of an endotoxin-induced pyrexia, the experiment was repeated and the rectal temperature of the rabbits monitored during, and for up to, 8 hr after challenge. No statistically significant increases in body temperature were demonstrable in rabbits given a single aerosol of heat-treated *E. coli* cells.

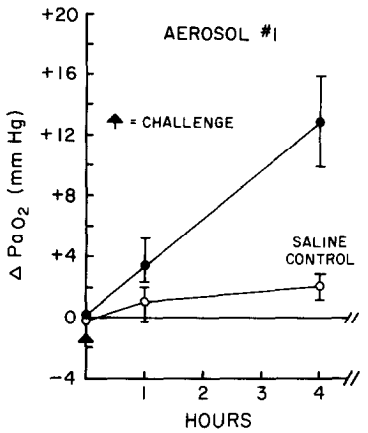

FIG. 1. Changes in arterial oxygen tensions (P_{aO_2}) of rabbits receiving a 30-min aerosol of heat-treated *E. coli* cells (●) or control animals challenged with an aerosol of pyrogen-free saline (○). Each value represents the mean change in P_{aO_2} from prechallenge values. A total of 12 rabbits was used for each determination. Vertical bars indicate the SEM.

Additionally, animals receiving a single aerosol of *E. coli* cells exhibited a leukocytosis (Fig. 2). This leukocytosis was evident 10 min after termination of the aerosol and persisted for up to 4 hr. An average 35% increase in total circulating WBC was noted when compared to control animals challenged with an aerosol of PFS.

Total circulating platelet and complement values (CH_{50}/ml) remained unchanged after a single aerosol challenge with heat-treated *E. coli* cells.

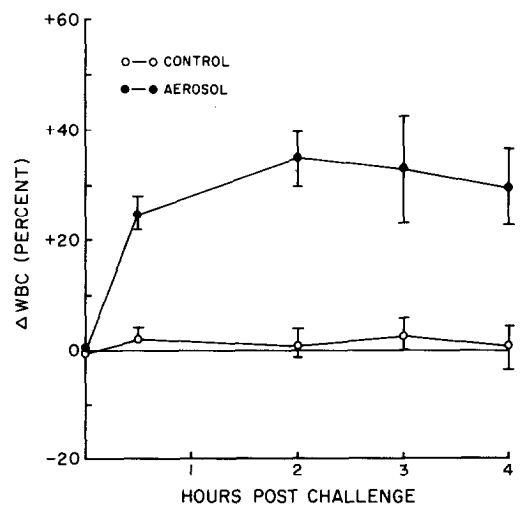

FIG. 2. Changes in total circulating leukocytes of rabbits exposed to aerosols of heat-treated *E. coli* cells (●) or of pyrogen-free saline (○). Values shown represent the mean percentage change in total circulating leukocytes (per mm^3) from prechallenge values of 12 rabbits. Vertical bars indicate the SEM.

Dual Aerosol Challenge

Since an occupational exposure to gram-negative bacteria and their endotoxins would involve repeated exposure of varying concentration and duration, a comparison was made between single and dual challenges, with the same dose, given 24 hr apart. The purpose of the second challenge was to see if a response analogous to a pulmonary Shwartzman reaction might take place in unimmunized animals. This reaction is maximally induced in rabbits within 18–24 hr of the first dose. Several double-challenge variations were attempted. Rabbits were challenged with heat-treated *E. coli* cells, delivered by aerosol or intravenous injection (0.5 of a suspension containing 10^9 cells/ml). The same animals were then challenged again, 24 hr later, with the same dose by either method by delivery. From these double-challenge variations, two consistent response patterns emerged.

With double aerosol challenges, rabbits responded with a mean increase in P_{aO_2} after the first aerosol (Fig. 3). After the second aerosol challenge, given 24 hr later, the increase in P_{aO_2} above prechallenge values was still present and maximal at 4 hr. However, the standard errors were reduced, and the animals tended to show less of an increase in P_{aO_2} (Fig. 3). The P_{aCO_2} values declined concurrently with the elevation of P_{aO_2} values. A leukocytosis was present after each aerosol challenge, but the total circulating platelet and complement values (CH_{50}/ml) remained unchanged.

A markedly different type of response was observed when an aerosol challenge with heat-treated *E. coli* was followed 24 hr later by an intravenous administration of the same dose. An elevation in P_{aO_2} was observed after the aerosol challenge, as before. Twenty-four hours later, when these same animals were challenged intravenously with a standard dose (0.5 ml of 10^9 heat-treated cells/ml), a marked and rapid decline in P_{aO_2} of approximately 20 mm Hg was observed (Fig. 4). The P_{aCO_2} values remained unchanged. The response was maximal by 1 hr and represented an average decline of approximately 50% below prechallenge values. A portion of the animals died, but in the survivors, P_{aO_2} values returned to normal by 4–6 hr.

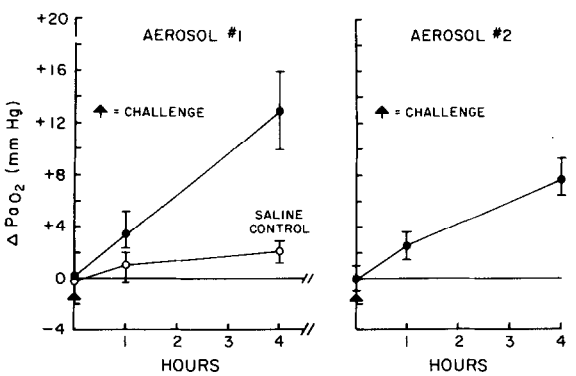

FIG. 3. Changes in arterial oxygen tension (P_{aO_2}) of rabbits receiving two 30-min aerosols of heat-treated *E. coli* cells. Values represent the mean change in P_{aO_2} from prechallenge values, determined prior to each aerosol challenge. Aerosol 2 was administered 24 hr after aerosol 1. A total of 12 rabbits was used for each determination.

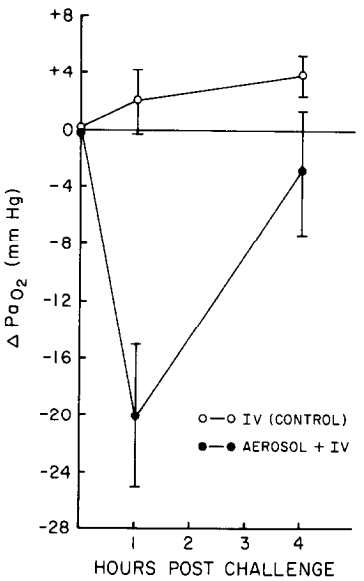

FIG. 4. Changes in arterial oxygen tension (P_{aO_2}) of rabbits after an intravenous challenge with 0.5 ml of heat-treated *E. coli* cells (10^9/ml). The experimental animals received a 30-min aerosol challenge 24 hr previously. Values represent the mean change in P_{aO_2} of 12 animals from prechallenge values determined prior to the iv challenge. Control animals were challenged iv without a prior aerosol. Vertical bars indicate the SEM.

Control animals receiving PFS or an intravenous challenge of heat-treated *E. coli* cells, without prior aerosol challenge, failed to exhibit this rapid decline in P_{aO_2} values.

Necropsy of the animals that died revealed grossly hemorrhaged lungs. There was no evidence of gastrointestinal hemorrhages, renal cortical necrosis, or joint involvement which suggests a focal lung reaction was produced, rather than a generalized response.

Histological sections of the lung tissues revealed an extensive interstitial pneumonitis, hemorrhage, and focal accumulations of leukocytes and lymphocytes in the interstitial spaces. Sections of lung, liver, adrenal, and renal tissues appeared normal.

Moreover, rabbits challenged intravenously after aerosol challenge exhibited a pronounced thrombocytopenia (Fig. 5). A mean decline of approximately 50% was observed, and like the decline in P_{aO_2} values, the thrombocytopenia was also maximal by 1 hr. Platelet values in experimental animals returned to approximately baseline values by 24 hr after the intravenous challenge. Control animals given PFS or a single dose of heat-treated *E. coli* cells intravenously failed to exhibit this decline in circulating platelets.

Although the intravenous administration of homologous endotoxin (0.5 μg/kg) evoked the same type of response as the heat-treated cells, the extent and duration of both the decline in P_{aO_2} and the thrombocytopenia were not as intense (data not presented).

Pretreatment of the rabbits with indomethacin abrogated both the decline in

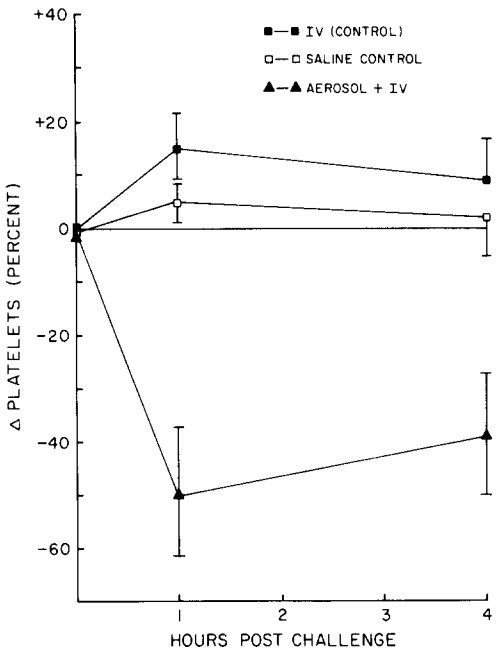

FIG. 5. Changes in circulating platelets (per mm^3) of rabbits after an intravenous challenge with 0.5 ml of heat-treated *E. coli* cells (10^9/ml). The experimental animals received a 30-min aerosol challenge 24 hr prior to the iv challenge. Control animals were inoculated with heat-treated *E. coli* cells or pyrogen-free saline iv, without receiving a previous aerosol challenge. A total of 12 rabbits was used for each determination. Vertical bars indicate the SEM.

P_{aO_2} and the thrombocytopenia (Fig. 6). Our preliminary experiments indicate that the intraperitoneal administration of indomethacin (60 mg/kg) is equally effective in eliminating the drop in P_{aO_2} values and thrombocytopenia when administered either 1 hr prior to the aerosol (preparative) challenge or the intravenous (provoking) challenge (although only the latter data are presented in Fig. 6). Indomethacin pretreatment did not affect the blood gases or platelet and leukocyte counts of control animals receiving saline aerosols.

DISCUSSION

Monitoring arterial blood gases after aerosol challenge with heat-treated *E. coli* cells adds a new dimension to our understanding of the adverse effects of inhaled endotoxin. Previous reports have indicated changes in body temperature (Snell, 1966), hematologic challenges (Snell, 1966; Snella and Rylander, 1977), and increased numbers of leukocytes in lung lavages after aerosol challenge (Rylander *et al.*, 1975b) with gram-negative bacteria or their endotoxins, but no assessment of changes in lung function have been reported. Monitoring arterial blood gases affords the opportunity of observing changes in pulmonary function in a manner analogous to the human situation (Lopez Marino *et al.*, 1973; Palmer and Kelman, 1973). This established technique has been utilized previously in the development

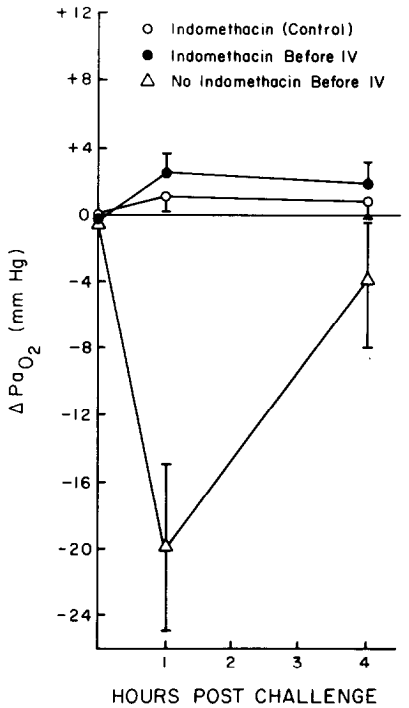

FIG. 6. The inhibition of the decline in arterial oxygen tension (P_{aO_2}) by indomethacin. Rabbits were given indomethacin (60 mg/kg ip) 1 hr prior to an intravenous challenge with 0.5 ml of heat-treated *E. coli* cells (10^9/ml). These same animals had received a 30-min aerosol challenge 24 hr previously. A second group of rabbits was challenged without pretreatment with indomethacin. Values represent the mean change in P_{aO_2} of four animals from prechallenge values determined prior to the iv challenge. Control animals were sham treated with saline.

of an experimental model of allergic lung disease and can be applied to the rabbit with confidence (Olenchock and Burrell, 1976; Ulevitch and Cochran, 1978).

The elevations in P_{aO_2} after a single or double aerosol challenge with heat-treated *E. coli* or homologous endotoxin are comparable to increases reported by Ulevitch and Cochran (1978) after a single intravenous challenge of rabbits with 5 mg of endotoxin prepared from *Serratia marcescens*. It is noteworthy that our data indicate that aerosols of smaller doses of endotoxin, compatible with dosage levels occurring spontaneously in the environment, can cause the same pulmonary dysfunction as large lethal doses of endotoxin given intravenously. Cinkotai *et al.* (1977) have demonstrated endotoxin levels of 0.2–1.6 g/m^3 of air in certain work environments.

The failure of viable *E. coli* cells to initiate the same type of response as the heat-treated organisms suggests that the endotoxin on the cell surface is responsible for the changes in arterial blood gases. It is generally accepted that the capsular B antigen of *E. coli*, and the capsular antigens of other enteric organisms, can mask or cover over the O or somatic antigens, which are an integral part of the lipopolysaccharide complex of endotoxin (Morrison and Ulevitch, 1978). Removing the B antigen by heating *E. coli* cultures to 100°C for 45 min is routinely

employed to permit the serological reaction of *E. coli* O antigens with typing antisera (Ewing and Martin, 1974).

Our inability to demonstrate a significant rise in temperature in experimental animals receiving single or double aerosols of heat-treated *E. coli* cells suggests that the increase in P_{aO_2} is not due to an endotoxin-induced pyrexia. Snell *et al.* (1966) observed a sustained 6-hr fever in rabbits exposed for 45 min to an aerosol containing a high concentration of endotoxin (3 mg/ml) and suggested that endotoxin is absorbed very quickly from the lung surface in a biologically active form. This hypothesis is compatible with the finding that the lung has little, if any, ability to detoxify endotoxin after an intravenous administration (Mori *et al.*, 1973). Endotoxin is sequestered in the lung, in an active form, but can be cleared and detoxified by the liver if the challenge dose is shunted there first. Since our experiments utilized a lower dose of endotoxin or whole heat-treated cells as an endotoxin source, the inability to demonstrate fever may be related to dosage differences and sequestration of the challenge doses by the pulmonary tissues.

However, the leukocytosis present concurrently with the elevations in P_{aO_2} indicates that an aerosol exposure to gram-negative bacteria or their endotoxins does result in systemic effects. Our findings are contrary to those of Snell *et al.* (1966) who observed a sustained leukopoenia in rabbits after an aerosol of more concentrated *E. coli* endotoxin, comparable to that observed by others after an intravenous challenge with endotoxin.

The results from the double-exposure experiments indicate that inhaled gram-negative bacteria or their endotoxins can possibly lead to pulmonary response in two ways. Double aerosol challenge results in a gradual increase in P_{aO_2}, and a leukocytosis. The diminished elevation in P_{aO_2} after the second challenge is suggestive of the well-known tolerance phenomenon associated with repeated endotoxin challenge (Chedid and Parant, 1971). To date, preliminary experiments indicate the four successive aerosol challenges, administered 24 hr apart, do not render the experimental animals totally unresponsive at the time of the fourth challenge. Further experiments are warranted to establish whether tolerance can be produced after repeated aerosol challenge.

The dramatic decline in P_{aO_2} that occurs when an aerosol challenge is followed by an intravenous challenge suggests that the lung can be the biological target of another type of endotoxin-mediated adverse reaction. In contrast to double aerosol challenge, this response is rapid, affects circulating platelets, and can be lethal. The initiation of this response appears limited to the particular combination of an aerosol challenge with heat-treated *E. coli* cells preceding an intravenous challenge with the same dose. The failure of each challenge to separately evoke this response, its rapid induction by the intravenous challenge, and the histopathologic picture produced suggest that a pulmonary Shwartzman reaction was produced in the lung. Our ability to inhibit these responses with indomethacin strongly supports this concept. Indomethacin is a potent inhibitor of prostaglandin synthesis, and a report indicates its effectiveness in preventing the generalized Shwartzman reaction (Howes *et al.*, 1978). Prostaglandins are produced by macrophages and such production is intensified by endotoxin exposure (Kurland and Bockman, 1978).

Throughout the course of this study we were unable to demonstrate changes in systemic complement levels (CH_{50}/ml) after aerosol or intravenous challenge. Endotoxin can activate complement via both classical and alternative pathways (Morrison and Kline, 1977) and the role of endotoxin-induced complement activation in lethal bacterial endotoxin hypotension and coagulative changes has recently been reported by Ulevitch and Cochran (1978). Our inability to demonstrate complement activation may be due to the limited sensitivity of the assay system or to the threshold level of the response. Further experiments, utilizing animals deficient in complement components or treated with cobra venom factor, are required to definitively establish whether complement is involved in these responses.

These findings suggest that inhalation of gram-negative bacteria or their endotoxin can result in altered lung function and additionally, in systemic effects. Whether these results reflect direct toxin action or structural damage or are secondary to other biological activities of endotoxin remains to be determined. Since the lung is known to sequester, but not actively detoxify, endotoxin, the possibility of damage from repeated contacts, rather than a single exposure, could be very important in explaining idiopathic disease. The inhalation or large doses, capable of entering the systemic circulation, could possibly have very adverse effects in individuals recently exposed to smaller amounts of endotoxin in the work environment. Since the levels of endotoxin contamination in cotton mills and other occupational settings fluctuate markedly, the potential for repeated aerosol exposure of varying intensities exists. Moreover, a gram-negative bacteremia, from a totally unrelated source, could conceivably result in a very adverse reaction in individuals subject to chronic aerosol exposure of gram-negative bacteria or their endotoxin.

As a direct consequence of its ability to cause inflammatory changes in the lung, endotoxin might play a pivotal role in the initiation of occupational lung diseases with an immunological etiology. Recent reports (Olenchock and Burrell, 1976; Pinto *et al.*, 1979) indicate the necessity of inflammatory changes in the lung to allow for the escape of certain antigens from the lung compartment to the systemic circulation for effective sensitization. In view of the widespread contamination of certain dusts of occupational origin with endotoxin, the role of endotoxin in initiating or amplifying these diseases warrants further investigation.

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