progressively inhibited (Fig 4) and the animals developed both precipitating serum antibodies and delayed skin reactivity. Skin biopsies at 48 hours revealed perivascular infiltration by lymphocytes and histiocytes characteristic of cell-mediated hypersensitivity, superimposed upon a nonspecific acute inflammatory reaction.

**DISCUSSION**

The granulomatous pneumonitis which we observed is similar to that seen in most cases of chronic farmer's lung and other human hypersensitivity pneumonitides. The early bronchiolar lesions, occurring as early as four days after inoculation of previously unsensitized animals, are considered to represent direct irritation of the tissue by the relatively large (about 1 micron) and antigenically complex *M. faeni* spores and hyphae. This interpretation is consistent with the fact that skin tests with *M. faeni* antigen are notoriously unreliable clinically because of irritative properties. The bronchiolar localization of lesions is probably also related to the particle size, and it would be of interest to perform similar experiments with smaller particles—perhaps purified antigenic components adsorbed onto inert particles of known size and shape. The later granulomatous lesions strongly suggest cell-mediated hypersensitivity to the antigen, which is supported by the MIF and skin test data. That the skin tests read at 48 hours reflect at least in part CMI is illustrated in Figure 5 which shows a rough linear correlation between the skin test size and percent inhibition of alveolar macrophages obtained at subsequent sacrifice. An additional hypothesis, that *M. faeni* particles may act as an immunologic adjuvant, is discussed in detail elsewhere, but remains speculative at present. We found no evidence for a type III (immune complex) reaction in our animals, but recognize that this and perhaps other mechanisms in addition to CMI may be important in the pathogenesis of hypersensitivity pneumonitis in some instances.

**REFERENCES**


**Immunologic Mechanisms in Experimental Interstitial Pneumonitis**

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**Session 9: Hypersensitivity Pneumonitis (B)**

The lung, by virtue of its unique exposure to both the circulation and the external environment, is particularly vulnerable to immunologic insult. Modes of well-documented immune pulmonary injury include alveolar capillary injury following either sequestration of circulating immune complexes, or fixation of antivascular basement membrane antibody. In addition, inhaled antigens are known to provoke asthmatic-type reactions in atopic individuals, but their role in inducing interstitial disease is less clear.

We have employed an animal model to assess pulmo-
nary reactions to inhaled organic substances, and in particular to discriminate between humoral and cellular patterns of immune injury. Antibody-mediated injury was studied by administering a protein antigen, bovine serum albumin (BSA), in aerosol form to BSA-sensitized rabbits possessing high titers of complement-fixing anti-BSA antibody. Cell-mediated or "delayed type" hypersensitivity injury, on the other hand, was simulated through a nonspecific stimulation of thymus-dependent lymphocytes (T-cells) present within the lung. This was achieved by the administration of a T-cell mitogen, concanavalin A (Con A), in aerosol form to nonsensitized rabbits. Lastly, the effects of humoral and cellular injury were studied in BSA-sensitized rabbits by challenge with aerosol mixtures containing both BSA and Con A.

**Materials and Methods**

Female New Zealand white rabbits weighing 4-5 lb were employed. Crystallized BSA, obtained commercially, and Con A, prepared by affinity chromatography on Sephadex columns, were dissolved in phosphate-buffered saline (PBS). For immunization, BSA was incorporated in alum and injected intramuscularly (gluteal) and subcutaneously (dorsal) on day 0 (10 mg BSA), day 7 (20 mg) and day 14 (20 mg). Serum antibody was measured by immunodiffusion in gel and by quantitative precipitin analysis. Arthus reactivity was tested immediately prior to aerosol challenge by direct or reverse-passive techniques. Aerosols were generated with a DeVilbiss ultrasonic nebulizer from solutions containing 5 mg protein/ml PBS, or 10 mg/ml in the case of combined challenge. Immunodiffusion, quantitative precipitin and sedimentation profiles were carried out on samples of 125I-BSA obtained before and after nebulization. These

### Table 1—Summary of Pulmonary Lesions Observed Following Aerosol-Challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization</th>
<th>Aerosol Challenge</th>
<th>MeL*</th>
<th>RUL</th>
<th>RML</th>
<th>RLLh</th>
<th>LUL</th>
<th>LLLh</th>
<th>LLLp</th>
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<td>1</td>
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<tr>
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<tr>
<td>3</td>
<td>BSA</td>
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<tr>
<td>4</td>
<td>None</td>
<td>Con-A</td>
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*MeL = mediastinal lobe; RUL = right upper lobe; RML = right middle lobe; RLLh = hilar portion of right lower lobe; RLLp = peripheral portion, right lower lobe; LUL = left upper lobe, LLLh = hilar portion of left lower lobe; LLLp = peripheral portion of left lower lobe.

**Lesions graded from 0 (no injury) to ++++ (severe injury).

"-" indicates tissue not examined.
studies revealed no measurable effect of nebulization upon the BSA molecule. Approximately 10-15 percent of Con A was found to be aggregated following nebulization; after centrifugation, the remaining nonaggregated Con A retained full mitogenic activity for human lymphocytes, as measured by stimulation of 3H-thymidine incorporation. For challenge, aerosols were introduced into a plexiglass chamber into which the rabbit's head had been inserted and sealed. In a separate study, aerosol challenge was administered to five lightly anesthetized animals via a plastic endotracheal tube, inserted 2.5 cm into the trachea with the aid of a pediatric laryngoscope. All challenge procedures consisted of administering 20 ml of aerosolized protein solution per day, given repeatedly on day 28, 29 and 30, since preliminary studies had shown this to yield maximal injury in BSA-sensitized rabbits receiving BSA aerosol challenge. All animals were sacrificed on day 31 by sodium pentobarbital overdose, and the lungs inflated with buffered isotonic formalin or frozen section embedding medium. Fluorescence microscopy was performed as previously described. Fluorescein-conjugated antibodies employed included rabbit antiCon A, rabbit antiBSA, goat antirabbit Ig, goat antirabbit C3, normal rabbit Ig and normal goat Ig. Goat antirabbit T-cell antibody (ATG) was the gift of Dr. A. Stavitsky, Cleveland, Ohio. Ultrastructural studies were carried out with an AEI transmission electron microscope.

Results

An outline of the experimental and control groups employed in this study, together with a summary of histopathologic changes, is given in Table 1.

All animals immunized with BSA developed precipitating antibodies to BSA, as demonstrated by immunodiffusion in agarose; the mean antibody titer by quantitative precipitin analysis was $0.706 \pm 0.463$ mg antibody protein/ml. All BSA-sensitized animals gave positive Arthus skin test reactions; their sera mediated reverse passive reactions in normal animals. Immunofluorescence microscopy revealed deposition of BSA, rabbit immunoglobulin and C3 along venules in skin test sites. Despite good Arthus reactivity, none of seven sensitized animals challenged with BSA aerosol (group 1) developed grossly evident pulmonary lesions, and microscopic examination of lung tissues revealed only scattered foci of mononuclear perivascular inflammation (Fig 1). Fibrinoid necrosis was not observed, and immunofluorescence staining for BSA, immunoglobulin and C3 were negative. Direct immunofluorescence staining with ATG revealed an occasional positive cell; the majority (>99 percent) of inflammatory cells failed to stain with any of the conjugates employed. Unsensitized controls challenged with BSA, (group 2) as well as sensitized controls receiving no challenge (group 3) were grossly and microscopically normal.

Cell-mediated injury was simulated by exposing four unsensitized (group 4) and three BSA-sensitized rabbits (group 5) to Con A aerosols. Following sacrifice, none showed gross evidence of injury, but microscopically six of seven were found to have patchy, predominantly interstitial areas of inflammation (Fig 2). Electron microscopy revealed a spectrum of inflammatory cells, including neutrophils, eosinophils, lymphocytes and histiocytes within extracapillary spaces of alveolar septae and, to a lesser extent, within alveolar spaces. Mitotic figures were rare, although occasional "blast" forms were noted. Immunofluorescence staining did not reveal deposits of immunoglobulin, C3 or Con A, although occasional cells were seen whose cytoplasm stained with ATG (Fig 3). Granulocytes and mast cells stained nonspecifically in a granular pattern with all conjugates employed.

The nature and extent of injury resulting from combined humoral and cellular mechanisms was studied in nine animals sensitized to BSA, then challenged with a mixture of BSA plus Con A (group 6). Following sacri-

Figure 1. Perivascular inflammation in a BSA-sensitized rabbit challenged with BSA aerosol (H & E stain, x 140).

Figure 2. Interstitial inflammation in a nonsensitized animal exposed to Con A aerosol (H & E stain, x 140).

Figure 3. Intralveolar T-cells (arrows) visualized by immunofluorescence after staining with FITC-ATG (x 400).
revealed marked interstitial edema and inflammation comprising neutrophils, eosinophils, lymphocytes, histocytes and immature plasmablasts. Neither endothelial damage nor type II epithelial proliferation was noted. Immunofluorescence staining revealed focal accumulations of IgC in a granular pattern within alveolar spaces (Fig 5), but no deposits of C3 or BSA were noted. Membrane-bound Con A was occasionally detected on alveolar cells. An occasional alveolar cell stained homogeneously with ATG; similar staining was not observed with reagents specific for immunoglobulin or C3, or with normal globulin controls.

The importance of systemic immunity to BSA in this model of combined injury was assessed by administering BSA-Con A aerosols to six nonsensitized animals (group 6). At necropsy, these animals displayed only those moderate, interstitial changes seen in groups 4 and 5 following exposure to Con A alone, thus arguing for an immunologic enhancement of injury in group 6.

In a separate study, BSA-Con A aerosol was administered via an endotracheal tube to three BSA-sensitized rabbits (not shown in Table 1). Two unsensitized controls received PBS and FITC-BSA, respectively. Neither control showed evidence of injury, whereas each of the three sensitized animals showed extensive, grossly visible areas of granulomatous, pseudomembranous tracheitis (Fig 6). This was in contrast to all other animals observed in this study, where large airway injury had been conspicuously absent.

**Discussion**

The concept of hypersensitivity pneumonitis rests on two fundamental observations: first, the clear association between chronic inhalation of certain environmental dusts and the onset of respiratory symptoms and dysfunction, and, second, the high incidence of systemic immunity in exposed individuals to one or more antigenic moieties present in these dusts. Initially, emphasis was placed upon the role of humoral antibody, thought to form immune complexes with inhaled antigens at or near alveolar spaces. Subsequent demonstrations of such antibody in asymptomatic cohorts, however, as well as demonstrations of cellular immunity against inhaled environmental antigens in exposed individuals, suggested a more complex pathogenesis.

The present study undertook an assessment of pulmonary injury mediated by inhaled organic dusts, under conditions designed to distinguish between antibody-mediated, immune-complex injury and cell-mediated injury. In studying the former, BSA challenge to BSA-sensitized rabbits was employed, since this combination has served as the archetype for studies of experimental immune complex injury to kidney and blood vessels. In developing a model for cell-mediated injury, on the other hand, we elected to simulate antigenic challenge by administering T-cell mitogens for several reasons; active sensitization of recipient animals would likely lead to formations of humoral antibody, as well as sensitized T-cells, while use of the classic approach, involving adoptive sensitization in rabbits by cell-transfer, results in...
pulmonary vascular lesions in the absence of antigenic challenge, due probably to histocompatibility differences. Mitogenic stimulation, on the other hand, with Con A should efficiently stimulate most T-cells present, thereby amplifying cell-mediated reactions, in contrast to specific antigen which stimulates only the small percentage of T-cells recognizing that antigen.

Animals sensitized, then challenged with BSA developed only minimal injury, despite the presence of both circulating precipitins and cutaneous Arthus reactivity to intradermal antigen challenge. Moreover, our ability to demonstrate antigen, immunoglobulin and C3 in venules at Arthus skin test sites, but not in pulmonary vascular lesions, argues against any significant immune-complex damage in the lung. Conversely, inhalation of Con A resulted in injury resembling that seen in the Hamman-Rich syndrome, and suggests that plant lectins and other mitogenic substances which occur naturally may, when inhaled, provoke similar interstitial injury in man. The present demonstration of Con A-induced injury in unsensitized recipients further suggests that T-cells within the lung can be stimulated by inhaled materials, and that such T-cell stimulation, if sufficient in degree, may play an important role in naturally-occurring environmental lung disease. Conceivably, complex antigen mixtures might prove as pathogenic as mitogens, by virtue of their ability to stimulate larger numbers of T-cells in toto.

Injury produced following combined challenge to BSA-sensitized animals clearly differs both in extent and pattern from that expected had changes induced by Con A alone been simply superimposed upon that seen in BSA-sensitized, BSA challenged animals. The observed association of granulomatous interstitial inflammation, vasculitis and necrosis, together with granulomatous airway injury seen in intubated animals, bears a close resemblance to the changes in Wegener's granulomatosis. The presence of immunoglobulin aggregates, demonstrated within diseased alveoli by immunofluorescence, suggests that Con A-induced injury is enhancing the effectiveness of humoral mechanisms, perhaps by facilitating contact between circulating antibody and intraalveolar antigen. Similarly, demonstrations of T-cells at the sites of injury argues for concomitant cell-mediated reactions.

This study indicates that cellular mechanisms may be particularly important in initiating immunologic inhalation injury, but that humoral mechanisms may serve to severely augment such cell-mediated injury. In addition, an association between inhalation injury and the clinical entities of Wegener's granulomatosis and Hamman-Rich syndrome is suggested.

References

Humidifier Lung: Hypersensitivity Pneumonitis Related to Thermotolerant Bacterial Aerosols*

Peter F. Kohler, M.D.; Gary Gross, M.D.; John Salvaggio, M.D.; and June Hawkins, M.D.

Antigens from thermophilic Actinomyces species growing in air conditioners and humidifiers have been implicated in causing hypersensitivity pneumonitis. A similar role for thermotolerant bacteria has not been documented despite the fact that these organisms are uniformly present in contaminated humidifiers and air conditioners, exceeding Actinomyces colonies by approximately 15-fold, and often are recovered when cultures are negative for thermophilic Actinomyces. In fact, to prevent overgrowth by thermophilic bacteria, inhibitors are frequently included in media used for culturing Actinomyces.

Limited information is available on the taxonomy of these bacterial species. They are classified as thermotolerant because growth is enhanced at 56°C compared to 37°C. The rod-shaped bacilli, resembling B. sereus, are both gram-negative and positive.

Our interest in the pathogenic potential of thermotolerant bacterial aerosols arose when they were cultured from the home humidifiers of two housewives with hypersensitivity pneumonitis.

CASE REPORTS

CASE 1

The patient, a 31-year-old white housewife with atopic rhinitis and asthma, developed increasing cough, dyspnea, chills, fever, and intense myalgia in late September, 1972. Chest x-ray film demonstrated bilateral basilar infiltrates; she was hospitalized for ten days and received antibiotics for presumptive bacterial pneumonia. Within six hours of returning home, symptoms recurred and she was readmitted for another ten days. Once again following discharge, fever, cough, dyspnea and myalgias developed after six hours in her home and she was referred to the University of Colorado Medical Center. On admission, her temperature was 39.2°C, bilateral crackling rales were present at the lung bases, chest x-ray film showed basilar infiltrates, WBC was 24,900/mm³ with 90 percent neutrophils, and PO₂ was 52

*From the Division of Clinical Immunology, Department of Medicine, University of Colorado Medical Center, Denver. Supported by grant RR-51 from the General Clinical Research Centers Program, Division of Research Resources, National Institutes of Health.