EXPERIMENTAL IMMUNE COMPLEX DISEASE OF THE LUNG

THE PATHOGENESIS OF A LABORATORY MODEL RESEMBLING CERTAIN HUMAN INTERSTITIAL LUNG DISEASES*

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There are increasing clinical (1-6), functional (7), radiological (4, 5, 6, 8), and histological (3, 9-14) evidences that interstitial pneumonitis with or without pulmonary fibrosis may develop in patients with underlying diseases which appear to be associated with circulating antigen-antibody complexes. The pathogenesis of these human lesions, however, has not been studied in experimental animal models. Rabbits with acute or chronic serum sickness have not shown pulmonary changes (15, 16). On the other hand, acute anaphylactic shock, a condition characterized by preferential precipitation of circulating antigen-antibody complexes within the lumina of pulmonary capillaries (17-19), is caused by a mechanism which has no relationship with chronic pulmonary diseases in man.

These considerations have suggested the possibility that antigen-antibody complexes present in large amount and for prolonged periods of time in the circulation of rabbits may localize in their lungs, thereby producing inflammatory reactions comparable to those commonly seen in the kidneys of the same animals.

The results of the studies described here show that rabbits making a hyperactive antibody response to injections of bovine serum albumin (BSA), when maintained in a state of relative antigen-antibody equivalence by high, multiple, daily doses of BSA, develop membranous and/or proliferative lesions of the lung which are associated with deposition of antigen, host immunoglobulin, complement, and fibrinogen in pulmonary structures. The inflammatory changes, which are presumably produced by antigen-antibody complexes, cover the morphologic spectrum of certain human interstitial pneumonitis (20, 21).

Materials and Methods

Induction of Serum Sickness.—58 female albino rabbits weighing 2-3 kg were purchased from a local breeder, and fed Purina rabbit pellets and water ad libitum. 51 rabbits were used

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1 Abbreviations used in this paper: BSA, bovine serum albumin; H & E, hemotoxylin and eosin; PAS, periodic acid-Schiff; Rh, rabbit.
for immunization and seven as untreated controls. Crystallized BSA (Armor Pharmaceutical Company, Kankakee, Ill.), diluted in physiologic saline to 2.5 mg/ml, was injected intravenously daily. The doses of antigen given to different rabbits were varied according to the procedure described by Dixon, Feldman, and Vazquez (15) in an attempt to match the antibody response and thereby to keep the ratio of antigen to antibody in the circulation near equivalence or in slight antigen excess. The initial dose was 12.5 mg/day. In order to neutralize all the antibody elaborated in animals with best response, daily doses up to 150 mg of antigen were required. Since a single injection of such amount of BSA frequently provoked anaphylactic symptoms or fatal anaphylaxis, a daily dose higher than 50 mg was divided into two equal portions slowly injected at intervals of 2–8 hours. Furthermore this procedure of multiple injections was adopted also because it might allow the formation of larger amounts and longer persistence of immune complexes in the circulation than in rabbits receiving a single daily injection (15, 22). With such type of immunization anaphylactic symptoms were prevented and the best responders could be included in this study. All rabbits were bled weekly 18–24 h after a BSA injection. The quantities of antigen and/or antibody in the circulation were determined by capillary precipitin test. Two or three times a week, the 24-h urine output was collected from each rabbit and the presence of proteinuria and hematuria determined. Urinary protein was measured according to the method of Shevky and Stafford (23) and hematuria was detected by Labstix (Ames Company, Elkhart, Ind.). Blood urea nitrogen concentration was measured every 2 wk or more frequently if animals developed oliguria and renal failure. Rabbits were sacrificed by exsanguination under sodium pentobarbital anesthesia at different intervals of time, and lungs and kidneys were examined by morphological and immunohistochemical techniques. In some rabbits, which showed a progressive decrease of antibody titer, the injection of antigen was discontinued.

Preparation of Tissues for Histological Studies.—Lungs and kidneys were removed and weighed. Part of each specimen was fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with hemotoxylin and eosin (H & E), periodic acid-Schiff (PAS) reagent, silver-methenamine (Jones), and Masson trichrome. Another portion of the specimen was fixed in a mixture of paraformaldehyde and glutaraldehyde (24) postfixed in osmium tetroxide and embedded in Epon 812. Sections cut at 1 μ were stained with toluidine blue and examined by light microscopy. Sections for electron microscopy were cut with an LKB Ultratome (LKB Instruments, Inc., Rockville, Md.) and examined with Siemens 101 Electron Microscope (Siemens Corp., Medical Industrial Div., Iselin, N. J.).

Immunohistological Techniques.—The globulin fractions of antisera to BSA, pneumococcus Type II, rat globulin, rabbit IgG (Hyland Div., Travenol Laboratories, Costa Mesa, Calif.), rabbit fibrinogen, and rabbit albumin (Cappel Laboratories, Inc., Downington, Pa.) were separated and conjugated with fluorescein or ferritin as described elsewhere (25, 26). The antiserum to rabbit C3, which was used for immunoelectron microscopy, was a gift of Dr. Charles G. Corchin. The antiserum to rabbit C3, used for immunofluorescence, was purchased from Cappel Laboratories. The staining of specimens and the control experiments were performed as described in a previous publication (27). The sections were viewed with a Leitz Ortholux microscope (E. Leitz, Inc., Rockleigh, N. J.) equipped with a Fluoem fluorescence illuminator. The intensity and extent of fluorescence was arbitrarily graded as 0, negative; ±, minimal; 1 +, slight; 2 +, moderate; and 3 +, marked.

RESULTS

The findings described here were obtained from 58 rabbits studied for a period of 2–33 wk. On the basis of immunologic response the animals injected with BSA for prolonged period of time were divided into three groups.

In the first group (Group I), consisting of 18 rabbits, there was a progressive
rise of antibody titer after 2–3 wk of immunization. This required an increase in the amount of BSA injected in order to maintain a state of relative equivalence. In 14 of these rabbits a plateau of 100–150 mg of BSA/day was reached and in most of them there was little variation in the amount of antigen required. Antibody production remained high for variable periods of time but in some rabbits eventually declined (Rb 48, 45, 20, 21). 13 of these rabbits (Rb 7, 15, 52, 65, 23, 28, 35, 37, 73, 17, 24, 81, 91) developed membranous and/or proliferative pulmonary lesions, together with glomerulonephritis and marked proteinuria. The maximum dose of BSA during immunization, the dose at time of sacrifice, the duration of immunization and that of proteinuria, the level of BUN at time of sacrifice, and the immunofluorescence and morphologic findings of the lung are given in Table I. Fig. 1 shows the course of experiment in a rabbit (Rb 15) with high antibody response.

In the majority of these rabbits the gross feature of the lung was normal. In Rb 15, 35, and 73 the lungs were enlarged, with small or large hemorrhages beneath the pleura (Fig. 2). The cut surface was edematous or congested and areas of hepatization were present.

Morphologically the pulmonary changes developing in the rabbits of this group were membranous and/or proliferative. In two rabbits (Rb 15 and 73) thickening of alveolar capillary walls was seen without marked proliferation (Fig. 3). This lesion resembled the changes of idiopathic membranous nephrop-

### TABLE I

<table>
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Abbreviations: 0, negative; --, not tested; ±, minimal amount; +, a slight amount; ++, moderate amount; ++++, marked amount.
Fig. 1. Rb 15, injected with BSA for 70 days. In order to match antibody production, the antigen dose was increased up to 150 mg/day. Proteinuria started 43 days and renal failure 58 days after beginning of immunization. At sacrifice the rabbit showed membranous pneumonia.

athy or those of membranous systemic lupus erythematosus glomerulonephritis. More commonly, however, the thickening of capillary walls was associated with proliferation of septal cells, increase of PAS-positive material in the interstitium, accumulation of polymorphonuclear leukocytes in capillaries with partial or complete obliteration of their lumina (Figs. 4, 5, 6), and alveolar hemorrhages. These changes produced a marked thickening and distortion of alveolar septa.

By electron microscopy the most characteristic finding was represented by deposits of electron-opaque material in the walls of alveolar capillaries and in the interstitium. In the capillaries the deposits were mainly subendothelial. The membranous thickening of alveolar capillary wall, seen by light microscopy, was produced by extensive accumulation of deposits between the endothelium and the alveolar basement membrane (Figs. 7, 8). Only rare focal subepithelial deposits, comparable to the glomerular "humps", were seen in rabbits with
marked proliferative lesions (Fig. 9). Likewise, interstitial deposits were observed mainly in animals with proliferative pneumonitis.

The alveolar capillary walls, the interstitium, and the walls of the terminal bronchioles contained deposits of BSA (Figs. 10, 12) and rabbit IgG (Fig. 11). The staining pattern obtained with respective specific fluorescein-conjugated antisera was similar, i.e., uniformly distributed along capillary walls and interstitium in form of discrete granular deposits, corresponding to the position of the electron-opaque aggregates seen by electron microscopy. Demonstration of deposits of C3 in the lung was not readily achieved by immunofluorescence. Staining of lung tissues with fluorescein-conjugated antibody to rabbit albumin was consistently negative.

The experiments of immunoelectron microscopy confirmed that the electron-opaque deposits indeed contained BSA and rabbit IgG. However, specific binding of ferritin-conjugated antibody to C3 was also observed in the deposits. Fig. 13 shows a section through a lung capillary wall of Rb 35. The basement membrane and the subendothelial deposits are tagged by ferritin-conjugated antibody to BSA. Fig. 14 illustrates the localization of ferritin-conjugated antibody to rabbit C3 in the subendothelial electron-opaque deposits and in the basement membrane. Controls for the foregoing experiments were carried out on fragments of lung from normal rabbits and rabbits with lung disease as follows: (a) Fragments of lung from normal rabbits were treated with ferritin-conjugated antibody to BSA or to rabbit globulin. There was no specific localization of ferritin-conjugated antibody. (b) Fragments of lung from rabbits with chronic serum sickness and pneumonitis were treated with ferritin-conjugated...
Figs. 3–18 (with the exception of the inset of Fig. 3) illustrate pathologic aspects of pulmonary tissue from rabbits of Group I.

Fig. 3. Rb 15. The lumina of alveolar capillaries (c) are patent. The arrowheads indicate membranous thickenings of alveolar capillary walls. (a), alveolar space. Silver-methenamine staining. × 1,000. The inset shows a normal alveolar septum of Rb 30. Silver-methenamine staining × 1,000.

Fig. 4. Rb 7. Proliferative and exudative pneumonitis. H & E stain. × 400.
FIGS. 5 and 6. Rb 7. The lumina of alveolar capillaries are obliterated by proliferated cells and polymorphonuclear leukocytes. The arrows indicate some focal thickening of the capillary walls. (a), alveolar space; (m), alveolar macrophage. Toluidine Blue. × 1,600.

FIG. 7. This picture shows the boxed area of Fig. 8 at higher magnification. Some subendothelial electron-opaque deposits are indicated by arrows. (c), lumen of the alveolar capillary; (EN) endothelial cell; (a), alveolar space; (m), part of an alveolar macrophage; (rbc), red blood cell. × 10,000.

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Fig. 8. Rb 15. Membranous pneumonitis. Areas of increased electron-opacity (arrows) are visible in the alveolar capillary walls. The lumina of the capillaries contain erythrocytes (rbc), a polymorphonuclear leukocyte (Pmn) and two mononuclear cells (N), probably lymphocytes; (S), septal cells; (E), epithelial cell type 2; (m), parts of alveolar macrophages; (a), alveolar space. The boxed area is shown at higher magnification in Fig. 7. × 4,000.
Fig. 9. Rb 7. The picture shows some aspects of proliferative and exudative pneumonitis. The lumina of alveolar capillaries are obliterated by proliferated cells (N) and by a polymorphonuclear leukocyte (Pmn). The thickness of the alveolar septum (its vertical axis runs from a to a) is greatly increased. The boxed area is seen, at higher magnification, in the lower part of the plate. × 6,000. The inset shows three focal subepithelial electron-opaque deposits (arrows). × 14,000.
Fig. 10. Rb 15. Sections of lung stained with fluorescein-conjugated antibody to BSA. Granular deposits are seen along the alveolar capillary walls and in the interstitium. (c) lumina of alveolar capillaries; (s), lumen of an alveolar sac. × 1,000.

Fig. 11. Rb 15. Section of lung stained with fluorescein-conjugated antibody to rabbit IgG. Granular deposits are present in the alveolar capillary walls and in the interstitium. (a) alveolar space; (c) lumina of alveolar capillaries; (s) alveolar sac. × 1,000.
antibody to pneumococcus Type II or to rat globulin. Fig. 15 illustrates the result of one of these experiments in which lung of Rb 35 was treated with ferritin-conjugated antibody to rat globulin. The electron-opaque deposits and the alveolar basement membrane do not contain ferritin granules.

Fig. 16 shows that the interstitium of some rabbits with severe lesions contained precipitates of fibrinogen products. Fluorescein-conjugated antibody to rabbit fibrinogen stained the interstitium and some alveolar capillary walls (Fig. 17). The fluorescence pattern was coarse and ribbon-like, therefore different from that of BSA and rabbit IgG. In these rabbits, fibrils with the periodicity of fibrin, progressive increase of interstitial collagen, interstitial sclerosis, and thickening of the basement membrane of alveolar capillaries (Fig. 18) were seen by electron microscopy.

The study of the lungs of rabbits which received high doses of BSA (Rb 48, 45, 20, 21) but in which antibody production progressively declined and BSA injections were discontinued, showed an increased amount of collagen in alveolar septa.

The second group (Group II) consisted of seven rabbits which developed only a slight or moderate antibody response throughout the course of the experiment. This antigen-antibody equivalence was maintained with low doses of BSA. Fig. 19 illustrates the course of one of these experiments. In four rabbits there was a progressive decline of the antibody response and eventually BSA administration was stopped. Absence of pulmonary lesions or slight changes only were observed. Nevertheless all these animals developed proteinuria and a variable degree of glomerular changes.

Ten rabbits (Group III) failed to produce demonstrable precipitating antibody. The animals were immunized for periods ranging from 5 to 15 wk and although the daily injection of BSA was maintained at the minimum level of 12.5 mg/day, or less, there was permanent excess of antigen in the circulation. The lungs and the kidneys of these rabbits were consistently normal.

Group IV included 16 rabbits which were sacrificed 10–19 days after beginning of immunization during acute serum sickness. Acute serum sickness was characterized by the appearance of proteinuria, increase of BUN, and demonstration of glomerular granular deposits of IgG, C3, and BSA by immunofluorescence microscopy with corresponding focal subepithelial deposits in glomerular capillary walls seen by electron microscopy. In some of these rabbits focal and granular deposits of BSA and host IgG were observed in the interstitium and capillary walls of the lung. The deposits were seen mainly within the cytoplasm of polymorphonuclear leukocytes. Areas of slight to moderate cellular infiltration were observed by light microscopy. The studies performed by electron microscopy showed infiltration of polymorphonuclear leukocytes. They did not demonstrate, however, electron-opaque deposits in pulmonary structures. Kidneys and lungs of noninjected rabbits (Group V) were consistently normal.
DISCUSSION

The results of this study show that rabbits which make an hyperactive antibody response to daily injections of BSA, when maintained in relative antigen-antibody equivalence by elevated doses of antigen, develop membranous and/or proliferative pneumonitis which is associated with deposition of BSA, host IgG, and fibrinogen in pulmonary structures. The granular pattern of fluorescence observed in the lung is comparable to that seen in renal glomeruli in immune complex disease and remarkably different from the linear staining characteristic of Goodpasture's syndrome (28, 29) or experimental pneumonitis produced in rats by antilung serum (30, 31). Immunoelectron microscopy confirms that antigen, immunoglobulin, presumably containing antibody to BSA, and complement are present in the electron-opaque deposits of the capillary walls and the interstitium. It is therefore conceivable that these immunologic reactants form antigen-antibody complexes. Then, the lesions of the lung would result, like those of the renal glomeruli, from deposition of circulating immune complexes which have no immunologic relationship with the lung. The pulmonary structure, the physicochemical characteristic of the complexes as well as the release of mediators should have importance in the process of deposition in tissues (32).

In previous studies of rabbits with chronic serum sickness extraglomerular lesions were not observed (15, 16, 22). The only extra-renal deposits of antigen found were in the splenic arterioles although there was no morphologic abnormality associated with this deposition (15). Therefore, the most probable explanation for the development of pulmonary lesions in rabbits of Group I is that the different procedure of immunization used in these studies may elicit the formation of larger amount of immune complex and/or a longer persistence of critical levels of complexes in the circulation. This interpretation is supported by the observation that deposition of BSA, rabbit IgG and C9, and lung changes comparable to those seen in Group I were not present in rabbits of Groups II, III, IV and V, and in rabbits studied in previous experiments (15, 22).

Immunization of rabbits with fractionated doses of antigen was adopted in order to avoid anaphylaxis and following the assumption that in patients with

Fig. 12. Rb 52. Section of lung stained with fluorescein-conjugated antibody to BSA. Granular deposits are seen in the wall of a terminal bronchiole. (l) lumen of the bronchiole; (EP), epithelium; (E), elastic tissue. × 1,000. The inset shows granular deposits of BSA along the alveolar capillary walls of Rb 15. (c) lumina of alveolar capillaries. × 1,500.

Fig. 13. Rb 35. Alveolar capillary walls from tissue treated with ferritin-conjugated antibody to BSA. Several electron-opaque deposits (arrows) are seen in the capillary walls. (c) lumina of capillaries; (a) alveolar space. × 4,000. The inset shows an oblique section of the alveolar wall enclosed in the boxed area of Fig. 13, at higher magnification. Ferritin is localized in the electron-opaque deposits (d) and in the basement membrane (b). Some ferritin granules are present also in the epithelial (EP) and endothelial (EN) cytoplasm. × 35,000.
systemic lupus erythematosus the release of DNA into the circulation can be envisioned as a slow and continuous process hardly reproducible by single daily intravenous injection of large amount of antigen in an experimental animal. With multiple injections of antigen the best antibody responders were included in this study and thus the percent of rabbits developing chronic serum sickness with high antigen intake was much greater than that previously reported by Dixon et al. (15) and Andres et al. (22).

Dixon, Feldman, and Vazquez (15) have shown that in experimental immune complex glomerulonephritis the critical factor determining the development of lesions is the quantity of antibody formed by rabbit. Furthermore, the same etiologic and pathogenetic mechanisms may produce a variety of histologic lesions resembling the spectrum of human glomerular diseases. Likewise, in experimental immune complex disease of the lung the antibody response seems to be the critical factor since only rabbits with hyperactive responses develop pulmonary changes. Moreover the same etiologic and pathogenetic mechanism is responsible for a variety of membranous, membranoproliferative, or proliferative pulmonary lesions that appear to cover a large spectrum of human pathology.

Much remains yet to be learned about human lung diseases produced by antigen-antibody complexes because the opportunity to view the development of pulmonary lesions during the early stages by biopsy is rare. The present knowledge is based mainly on studies of tissues obtained at autopsy. The most comparable lesion is the "interstitial pneumonitis" which seems to be produced by an inflammatory process involving the supporting structures and the capillaries rather than the alveoli. Proliferation of interstitial cells, interstitial edema, accumulation of polymorphonuclear leukocytes and mononuclear cells with progressive increase of reticulin fibrils and of interstitial fibrosis are considered the basic elements of this disease (20, 21).

It has been proposed that in rare instances (sensitization to Aspergillus fumigatus, coffee bean dust, inhalation of pituitary snuff, bird breeder's disease etc.) lung injury in man may be mediated by antigen-antibody complexes which are formed when the inhaled antigen reacts with antibody at alveolar level, thereby producing an Arthus-
Fig. 16. The picture shows an alveolar septum of Rb 23. The thickness of the septum is increased (vertical axis from a to a). The lumina of the alveolar capillaries and the interstitium contain polymorphonuclear leukocytes (Pmn) and proliferated cells (N). Deposits of fibrin (F) are visible in the interstitium. × 10,000.
Fig. 17. Rb 23. Section of lung stained with fluorescein-conjugated antibody to rabbit fibrinogen. Fluorescent deposits are seen in the interstitium (arrows) and along alveolar capillary walls. (c), lumina of alveolar capillaries; (a), alveolar space. X 1,000.

Fig. 18. The picture shows part of an alveolar capillary wall and of the interstitium in Rb 23. The thickness of the capillary wall is increased because organized material is present in the subendothelial space (b). The lumen of the capillary (c) contains the remains of a degenerated polymorphonuclear leukocyte. Collagen is visible in the interstitium (f). (s), septal cell; (a), alveolar space. X 14,000.
type reaction tentatively defined as "extrinsic allergic alveolitis" (33-35). The hypothesis that pulmonary lesions may also be elicited by circulating antigen-antibody complexes has not been considered.

Recent clinical, histological, radiological, and functional studies, however, suggest that lung disease may be associated with circulating antigen-antibody complexes. Hemoptysis and radiologic signs of lung infiltrates have been observed in patients with glomerulonephritis which appeared produced by circulating immune complexes (5, 6). Gamma globulin complexes with sedimentation rates ranging between 9 to 17 S, presumably antigen-antibody complexes, have been shown in the circulation of patients with idiopathic pulmonary fibrosis (4). In systemic lupus erythematosus, a disease in which the pathogenetic role of circulating complexes has been firmly established (36), clinical (1, 2) as well as roentgenographic (8) evidence of pulmonary changes has been reported. Furthermore, in patients with lupus the measurement of pulmonary function has shown an impairment of diffusing capacity of the lung seemingly due to an increase of membrane resistance (7). Finally, in occasional patients with systemic lupus erythematosus pulmonary interstitial fibrosis (2, 3), proliferative alveolitis (12, 13), interstitial pneumonitis (37), pulmonary hemosiderosis (9, 10), classic "wire loop" changes (11), or subendothelial electron-opaque deposits in alveolar capillaries (14) have been observed. It is likely that the routine use of lung biopsy in patients with underlying immune complex disease may contribute to define the pathogenetic role of circulating antigen-antibody complexes in pulmonary lesions in man.
SUMMARY

Membranous and/or proliferative pneumonitis, similar in certain features to human interstitial pneumonitis, developed in rabbits making hyperactive antibody response to daily injections of bovine serum albumin (BSA) administered in multiple large doses sufficient to maintain the state of relative antigen-antibody equivalence. The pulmonary lesions were associated with deposition in alveolar capillary walls and interstitium of antigen, host globulin and complement, presumably in immune complexes. In some rabbits chronic interstitial pneumonitis, characterized by thickening of alveolar capillary walls, interstitial fibrosis and deposition of fibrinogen, was observed.

The production of immune complex pneumonitis seems to depend on the degree of the antibody response because rabbits developing chronic serum sickness with low doses of BSA, rabbits with acute serum sickness as well as nonresponders showed no pulmonary alterations. This observation is comparable to that described by Dixon in his studies on experimental immune complex glomerulonephritis. It is conceivable that the pulmonary pathology shown here is produced by formation of larger amounts of complexes which may persist longer at critical levels in the circulation than in rabbits immunized with a single daily injection of BSA.

In conclusion this study suggests: first, that experimental chronic serum sickness can be used as a model, not only for glomerulonephritis, but also for experimental systemic disease, comparable to human systemic diseases produced by circulating antigen-antibody complexes; and second, that the pathogenesis proposed here offers an alternative to using antilung basement membrane pneumonitis for the experimental approach to the study of human lung immunopathology.

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BIBLIOGRAPHY


