THE RESPONSE OF THE RABBIT'S LUNG TO INTRATRACHEALLY INJECTED PLASMA OR SERUM LIPOGLOBULINS†

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who, unbound by tradition or fashion, followed his nose.

Lipoproteins of the blood have been shown to be retained in the arterial wall selectively at sites of increased permeability, and they also remain in the living corneal stroma following injection in native plasma or serum. In these sites they elicit a lipophagocytic, mononuclear cell, and fibroblastic reaction that simulates morphologic features of the early atherosclerotic plaque. These observations have led to interest in the lesion-producing potential of blood lipoproteins in other extravascular sites, first to learn more of the biology of the lipoproteins that may relate to the mechanisms of arteriosclerosis, and secondly to investigate the possibility that the blood lipoproteins may participate in the histogenesis of other etiologically and pathogenetically obscure disease processes.

The present report describes the sequential morphological reactions of the rabbit's lung to certain lipoprotein-rich fractions of human or rabbit plasma or serum and to hypercholesterolemic, hyperlipidproteinemic rabbit serum introduced intratracheally. The lungs of the experimental animals have revealed striking interstitial and alveolar infiltrative and proliferative phenomena.

MATERIALS AND METHODS

The study has involved a total of 49 New Zealand white rabbits. These were of either sex and weighed 2.5 to 4.0 kg. each.

Fifteen rabbits were given intratracheally 3 ml. each of a lipoprotein-rich globulin fraction from recently out-dated, pooled human ACD plasma. Five fractions of similar composition were used. These fractions were precipitated with the aid of polymers of ethylene glycol. After removal of the glycols, they were concentrated by re-solution in 0.15 M NaCl, at one tenth the volume of the original plasma. The material, by cellulose acetate electrophoresis, was a mixture of alpha, beta and gamma globulins with a dense band in the β globulin region staining for lipid. The lipid and protein composition of the individual lipid-rich globulin fractions ranged from: total cholesterol 780.0-945.0 mg.%, free cholesterol 187.5-245.7 mg.%, fatty acids 33.6-45.2 mEq./L., lipid phosphorus 13.5-21.6 mg.%, total protein 2.0-4.5 gm.%. The pH of the fractions in 0.15 M NaCl was between 6.5-6.7.

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† This investigation was carried out under a grant-in-aid from the U. S. Public Health Service (H-1794).

Received for publication 24 November 1970.
Fourteen rabbits were given intratracheally 3 ml. each of hypercholesterolemic, hyperlipoproteinemic rabbit serum from four different egg-yolk fed rabbits. Gofman originally showed that the hyperlipidemia of cholesterol-fed rabbits is chiefly due to the presence of low-density plasma lipoproteins. The opalescent sera were freed of chylomicrons by centrifugation at 20,000 x g for one hour. The lipid and protein contents of these sera ranged from: total cholesterol 745.0-887.8 mg.%; free cholesterol 186.0-272.5 mg.%; fatty acids 33.0-42.0 mEq./L.; lipid phosphorus 15.2-26.2 mg.%; total proteins 4.75-6.39 gm.%. Three rabbits in this group were egg-fed and were given intratracheal injections of their own hypercholesterolemic serum.

Five rabbits received intratracheally 3 ml. of a lipid-rich serum globulin fraction in physiological saline prepared by polyethylene glycol precipitation from 60 ml. of normal rabbit serum. This lipid-rich, homologous serum globulin fraction was concentrated by dissolving it in one tenth its original volume of 0.9 percent saline and contained: total cholesterol 531.2 mg.%; free cholesterol 157.6 mg.%; fatty acids 32.0 mEq./L.; phospholipids 16.8 mg.%; total protein 2.1 gm.%.

Four rabbits were each given 3 ml. of purified human serum β lipoprotein, intratracheally. This fraction was prepared by precipitation of the globulins of pooled normal human serum with polyethylene glycol. They were then redissolved in saline, density 1.065. The fraction was centrifuged in a Spinco preparative ultracentrifuge (No. 40 rotor) at 105,000 x g for 18 hours. The top clear lipid layer was collected. This was brought to a salt content of 0.9 percent by dilution, and finally was sterilized by passage through a Swinnex-13-filter unit. The lipids and proteins of the fraction as injected were: total cholesterol 381.8 mg.%; free cholesterol 89.9 mg.%; fatty acids 24.0 mEq./L.; phospholipids 9.0 mg.%; total proteins 0.36 gm.%. Its pH was 6.5. On cellulose acetate electrophoresis the fraction gave a single protein band which stained (osmic acid) strongly for lipids as well. This band had the mobility of a beta lipoglobulin.

Eleven rabbits, as controls, were each given 3 ml. of pooled, chylomicron-free, normal rabbit serum intratracheally. The lipid and protein content of the control serum was: total cholesterol 34.4 mg.%; free cholesterol 7.5 mg.%; fatty acids 10.4 mEq./L.; phospholipids 4.7 mg.%; total proteins 5.6 gm.%.

All materials were sterile and were injected into the trachea with sterile technique. A small cut-down over the trachea was made with the rabbits under Nembutal® anesthesia.

The animals in the experimental group receiving lipoglobulin-rich plasma fractions, hypercholesterolemic, hyperlipoproteinemic serum, or purified serum lipoglobulin, were sacrificed at intervals of from 6 hours to 127 days following injection. Three animals died, one, 30 minutes after the injection, another after two days, and a third after 20 days. The 35 rabbits remaining in this group were examined as follows: 9 at 6 to 72 hours, 9 at 5 to 8 days, 6 at 11 to 12 days, 6 at 17 days, and 5 at intervals thereafter. The 11 animals receiving normal rabbit serum as controls were sacrificed from 2 to 17 days after injection. The lungs and major organs of all animals were examined grossly and histologically. Frozen sections were stained for lipids by Sudan IV with or without counterstain. All paraffin sections were stained with hemotoxylin and eosin for routine survey and with PAS and iron stains where appropriate.

Determinations of lipids and of proteins in the fractions and sera to be injected were carried out by standard methods in use in this laboratory. These include: for cholesterol and cholesterol esters, a modified Schoenheimer-Sperry method; for lipid phosphorus, a modified Youngsburg procedure; for fatty acids, a modified Stoddard and Drury technique. Total proteins were determined by the usual micro-Kjeldahl procedure.
Electrophoretic analysis of the lipoprotein-rich serum fractions was carried out in Veronal buffer, pH 8.6, on cellulose acetate strips using a Beckman Model R-101 microzone cell. Protein staining was accomplished with ponceau S and lipids with one percent osmic acid by a method under development in this laboratory.

RESULTS

The lungs of all animals in the experimental groups, namely those receiving concentrated lipoprotein-rich fractions of plasma or serum or homologous or autologous lipoproteins as hypercholesterolemic rabbit serum, or purified serum lipoproteins exhibited qualitatively the same morphologic changes and therefore will be described together.

In an animal dying 30 minutes after injection of a lipoprotein-rich globulin fraction from human plasma an occasional focal hemorrhage was seen grossly, and the lungs appeared mottled. Microscopically, sudanophilic fluid was present in scattered zones of alveoli. Focal alveolar hemorrhages were observed.

At six hours after intratracheal injection of any of the lipid-rich fractions described or of hypercholesterolemic serum there were many polymorphonuclear leucocytes in the alveoli. The injected sudanophilic material was distributed mostly in alveoli in the central portions of the lung, but there was also fluid exudate in scattered subpleural regions. At six hours there was also congestion of the alveolar capillaries and beginning evidence of increase of mononuclear cells in the alveolar walls. No necrosis was present and there was no evidence that the reaction was due to infection. Lung cultures consistently proved negative. No definite involvement of pulmonary vessels or bronchi was seen at this time interval. In the fat stains phagocytosis of lipid by an occasional intra-alveolar cell was observed but most of the sudanophilic material in the alveoli was still extracellular and in a diffuse form.

In lungs from animals sacrificed 12 hours after injection of any of the lipoprotein-rich preparations or of hypercholesterolemic serum there were grossly darker patches of consolidation alternating with lighter over-distended zones. In the sudan stains, marked phagocytosis of lipid by mononuclear cells was now present in the pulmonary alveoli and in the alveolar walls. Numerous polymorphonuclear leucocytes were present in the alveoli and in the bronchioles but only a few in the alveolar walls. Masses of lymphocytes and of small mononuclear cells were now appearing in the interstitial tissues of the lung, especially around blood vessels. Larger mononuclear cells in increasing numbers were seen in the alveolar walls and in the alveoli themselves. Many of these were phagocytic, containing sudanophilic granules in their cytoplasms. They appeared vacuolated often in hematoxylin and eosin sections.
The walls of the smaller pulmonary veins and arteries were the sites of interesting changes. The endothelium of many of these vessels was raised and beneath it there were accumulations of lymphocytes and mononuclear cells forming nodular masses that impinged upon the lumen. An occasional polymorphonuclear leucocyte was present in the wall of a vessel but no red blood cells. The medias of the small pulmonary arterioles, at this stage, except in an occasional instance, were free of infiltrating cells. As already indicated, the perivascular lung tissue was now heavily infiltrated with mononuclear cells. There was no necrosis or abscess formation and no evidence morphologically or by culture of bacterial infection.

At 24 hours the lipoprotein-injected rabbit lungs revealed a markedly changed microscopic appearance. Over large areas, not only were the perivascular spaces crowded with lymphocytes and mononuclear cells, but now also the alveolar walls were thickened and broadened by the presence of large numbers of mononuclear cells with irregular oval nuclei and prominent nucleoli. In the fat stains almost all sudanophilic material was now within macrophages either in the alveoli or in the alveolar walls. The groups of polymorphonuclear leucocytes described earlier were now confined to the centers of small alveolar spaces or had disappeared altogether. Beneath the pleura focally there was a definite accumulation of large mononuclear cells in the alveoli and in the alveolar walls. Throughout the lung the most striking change was the broadening of the alveolar walls and perivascular zones by massive accumulations of mononuclear cells of a histiocytic type. These cells completely overshadowed the polymorphonuclear exudate that was so prominent at the 6 or 12 hour stage. The wall of nearly every thin-walled pulmonary vessel was infiltrated by rows and groups of lymphocytes and larger mononuclear forms. The intimas of many of these vessels again contained nodular, subendothelial groups of the same cells. There were no thrombi. The overall picture at this stage was no longer that of an acute intra-alveolar pyogenic process, but suggested rather a combined infiltrative-proliferative interstitial lesion. An occasional mitotic figure was seen at 24 hours among the masses of mononuclear cells in the alveolar walls or in the alveoli. Foamy phagocytic cells were present, but many of these were unstained by sudan, indicating accumulation in them of materials other than lipid.

At time intervals from 24 hours to about three weeks following injection of lipoprotein-rich serum fractions or of hyperlipoproteinemic homologous or autologous rabbit serum the lungs of rabbits exhibited a progression of the changes already described. Over wide areas the alveolar walls were massively thickened by crowding of large mononuclear cells. The alveoli were correspondingly reduced in size. Polymorphonuclear cells disappeared
Fig. 1. Rabbit 971. Lung, 6 hours after intratracheal injection of lipoglobulin-rich fraction of human serum. The alveoli contain protein-rich fluid. Cells are accumulating in the alveoli and alveolar walls. H & E. ×100.

Fig. 2. Rabbit 1113. Lung, 72 hours after intratracheal injection of clear, hyperlipoproteinemic, hypercholesterolemic rabbit serum. Prominent interstitial accumulations of mononuclear cells. H & E. ×100.
Fig. 3. Rabbit 1114. Lung, 72 hours after intratracheal injection of clear, hyperlipoproteinemic, hypercholesterolemic serum. Lipid-filled macrophages in alveoli and in alveolar walls. Sudan IV (without counter stain). ×450.

Fig. 4. Rabbit 1113. Lung, 72 hours after intratracheal injection of clear, hyperlipoproteinemic, hypercholesterolemic serum. Lipophages in alveoli and alveolar walls. H & E. ×1000.
Fig. 5. Rabbit 1186. Lung, 7 days after intratracheal injection of electrophoretically pure human serum beta lipoprotein in saline. Thickening and increased cellularity of alveolar walls. H & E. ×450.

Fig. 6. Rabbit 1114. Lung, 72 hours after intratracheal injection of clear hyperlipoproteinemic, hypercholesterolemic, rabbit serum. Detail of cells in alveolar walls, with a mitotic figure near center. H & E. ×1000.
Fig. 7. Rabbit 904. Lung, 6 days after intratracheal injection of lipoprotein-rich human plasma fraction. Massive sub endothelial accumulation of lymphocyte-like cells in a vein. Interstitial and intra-alveolar cellular accumulations. H & E. ×100.

Fig. 8. Rabbit 972. Lung, 12 hours after intratracheal injection of lipoprotein-rich fraction of human serum. Sub endothelial accumulation of lymphocyte-like cells in a pulmonary artery. Massive alveolar and interstitial cellular accumulations. H & E. ×100.
completely from the lesions after 48—72 hours and sudanophilic material, either free or in cells, likewise disappeared during the first week. Mitoses in the cells of the alveolar walls and in large mononuclear cells in the alveoli were so frequent during the first week of the development of the lesion and crowding of cells became so intense as almost to suggest tumor. Solid masses of tissue-like mononuclear cells were observed protruding in tongue-like fashion into small bronchioles. Small veins were often obliterated completely by accumulations of lymphocytes and mononuclear cells in their walls and lumina. The walls of pulmonary arteries were similarly involved but to a lesser extent. Within alveoli, decreasing numbers of macrophages were observed as the time interval following injection grew longer. These large intra-alveolar cells were frequently seen passing through pores in the alveolar walls. Some exhibited foamy cytoplasms which after the first few days did not stain with sudan dyes. Many of the mononuclear cells, both in the alveolar walls and in the alveoli contained PAS positive granules in their cytoplasms. In zones of prior hemorrhage Turnbull blue stains revealed iron positive macrophages in alveolar spaces. Extending from terminal bronchioles into the surrounding involved areas of lung, many alveoli were lined by rows of atypical bronchial epithelial cells. In several instances the pleurae were involved in the infiltrative-proliferative process described. Raised, patch-like areas were observed grossly on the pleural surface. These consisted histologically of subpleural masses of mononuclear cells in the subpleural connective tissue extending in some places to proliferating plaque-like masses at the surface. It should be emphasized that in all of the changes described necrosis was not seen nor was there evidence of a foreign-body type granulomatous reaction.

After an interval of about three weeks the pulmonary lesions described began to regress. The massive cell accumulations in the alveolar walls became less prominent, but the alveolar walls remained thickened as compared to normal controls or to those of rabbits given injections of normal rabbit serum. Interstitial scarring was not a prominent feature of the regression of the lesions, although in one animal a few alveoli obliterated by dense fibrous connective tissue were observed. Focal accumulations of lymphocytes and mononuclear cells persisted around blood vessels and bronchioles for many weeks. These were in excess of the same cells normally observed in these locations. In summary, over many weeks the interstitial lesions became less cellular, without prominent organization.

The lungs of control rabbits, given equal quantities of normal rabbit serum of low lipid content revealed focal pulmonary edema in the early stages with an occasional focal hemorrhage. The cellular exudative, infiltrative and proliferative phenomena observed in the experimental groups were
not found in the lungs of the control animals. Examination of organs other than the lungs of control and experimental animals revealed nothing of note.

DISCUSSION

The present report describes striking interstitial and intraalveolar cellular accumulations in the rabbit's lung following intratracheal injection of concentrated blood lipoproteins either in prepared fractions or in hypercholesterolemic serum. This reaction is etiologically nonspecific as virtually identical changes have been observed following the introduction by various routes of many foreign substances, including several classes of lipids. The reaction of the rabbit's lung to Freund's adjuvant, for example, has been extensively studied and the lesions described closely simulate those reported here.²

It is less well known that substances of endogenous origin, such as plasma lipoproteins, can give rise to this response in the pulmonary parenchyma. Soluble plasma lipoproteins are not retained in most tissue sites and no exudative or proliferative reactions in normally vascularized connective tissue occur following their injection locally.² Such sites are the subcutaneous connective tissues, or the sclera of the eye. However, in the vessel wall and in the cornea lipoproteins are retained following injection and elicit lipo-phagic mononuclear cell reactions.¹ It would appear from the present observations that in the lung also blood lipoproteins in concentrated form are at least partially removed by phagocytosis during the course of a massive infiltrative-proliferative cellular reaction involving alveolar cells (alveolar cells in the multipotential sense of Bertalanffy).¹ This suggests, as do the parallel observations in the artery wall and in the cornea, that in the conditions of the experiment the lipoproteins may be denatured as to their solubility in the pulmonary alveoli. In this state of particulate aggregation they may elicit the cellular responses described. The accumulation of cells, beginning at about 6 hours and becoming massive after 24-48 hours would seem to be too rapid to represent a type of hypersensitivity reaction to foreign protein. Also, the same changes were elicited in animals given autologous hyperlipidemic serum.

In the conditions of the present experiments the cellular response continues long after stainable lipids are no longer demonstrable. This observation suggests that a term such as "non-lipid" reticuloendotheliosis may have restricted chronological meaning and no etiological significance.

Blood lipoproteins are readily denatured by alterations of their physical environment, for example, by dehydration of their solutions,¹⁰ and also they are known to form insoluble complexes in vitro with mucopolysaccharides or with gelatin.¹¹,¹² They also may participate in antigen-antibody reactions.¹²
These properties and the fact that they can be rapidly and completely removed from local sites where they have elicited tissue reactions should render them suspect in considerations of the pathogenesis of infiltrative-proliferative histiocytic lesions in the lung or in other tissues. If concentrated and denatured in an extravascular tissue site, they may constitute in effect a kind of endogenous Freund's adjuvant.

Whether or not the pulmonary changes described in the experimental animal find any counterpart in the various interstitial pneumonias and reticuloendothelioses of man remains a matter for investigation. Also of interest might be a study of the reaction of pulmonary tissues to an excess of its own complex lipoprotein surfactant, particularly if the physical properties of the complex were altered.

**SUMMARY**

Concentrated human or rabbit serum lipoproteins introduced intratracheally into rabbits elicit in the lung a striking infiltrative-proliferative interstitial and alveolar cellular reaction that persists after stainable lipid is no longer present. It is suggested that endogenously denatured serum lipoglobulins may play a role in the pathogenesis of certain types of histiocytic tissue reactions.

**ACKNOWLEDGMENT**

The technical assistance of Edward Iannucci, Peter Integlia and Helen Cavallaro is gratefully acknowledged.

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