LUNG INJURY MEDIATED BY ANTIBODIES TO ENDOTHELIUM

I. In the Rabbit a Repeated Interaction of Heterologous Anti-angiotensin–converting Enzyme Antibodies with Alveolar Endothelium Results in Resistance to Immune Injury Through Antigenic Modulation*

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In the kidney, lesions mediated by antibodies against glomerular basement membrane antigens or by circulating or in situ–formed immune complexes have been extensively studied. Much less is known about similar pathogenic mechanisms in the lung. In laboratory animals it appears difficult to induce antibody-mediated pneumonitis. The endothelium of the alveolar capillary wall is, in contrast to that of the glomerular capillary wall, nonfenestrated. It has been proposed that this type of endothelium prevents easy access of macromolecules such as IgG or immune complexes to the alveolar basement membrane (ABM)[1] (1). Support for this view has come from experiments in rabbits (1) and in rats (2) where it was found that under normal physiological conditions heterologous antibodies against ABM do not bind to this structure. If, however, the endothelium is damaged by exposure of animals to 100% oxygen, both heterologous antibodies (1, 2) and autologous IgG (1) localize along the ABM and induce pneumonitis.

In patients with Goodpasture's disease there is evidence that autoantibodies reacting with the ABM cause hemorrhagic pneumonitis. However, the sequence of events resulting in this injury is not clearly defined (3). Patients with high concentrations of serum antibodies strongly reacting with ABM in vitro may not develop signs of lung injury. In contrast, patients with low levels of antibodies

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[1] *Abbreviations used in this paper:* ABM, alveolar basement membrane; ACE, angiotensin-converting enzyme; Gt, goat; HRP, horseradish peroxidase; IF, immunofluorescence; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline; Rb, rabbit.

reacting weakly with ABM in vitro occasionally develop fulminating lung disease (3–5). The pulmonary component of Goodpasture's disease is often precipitated by secondary events such as infections (5). These observations in man are consistent with the hypothesis that factors increasing the permeability of the alveolar capillary wall have an important role in the induction of antibody-mediated lung disease.

The aim of the present study was to explore the effects of the interaction of antibodies with the endothelium of the alveolar capillary wall. To this purpose rabbits were repeatedly injected with heterologous antibodies specific for angiotensin-converting enzyme (ACE), a well-defined antigen expressed on the plasma membrane of lung endothelial cells (6, 7). It was found that on the first day of injection, anti-ACE antibodies induce fatal pulmonary edema in the majority of rabbits and cause redistribution of ACE on the plasma membrane of alveolar capillary wall endothelial cells and local fixation of complement. Continued administration of antibodies to surviving rabbits resulted in disapperance of ACE from the surface of lung endothelial cells. This "immunological enzymectomy" appeared to render the rabbits refractory to the damaging effects of anti-ACE antibodies. The results indicate that IgG antibody reacting in vivo with an antigen expressed on the plasma membrane of lung endothelial cells causes an antigenic modulation similar to that observed in other systems characterized by interaction of plasma membrane receptors with soluble ligands. The clinical and immunopathological events appear relevant for the understanding of the pathogenesis of antibody-mediated injury in the lung and, probably, in other organs.

Materials and Methods

Animals. Female New Zealand white rabbits (Beckens Farms, Sanborn, NY), each weighing 2.5–3 kg, were used. They were allowed free access to water and Purina rabbit pellets.

Goat Anti-rabbit ACE (Gt Anti-RbACE) Antibody Preparations. Rabbit pulmonary ACE was purified by a modification of earlier published methods (8, 9). Frozen rabbit lungs (Pel-Freeze Biologicals, Rogers, AR) were homogenized in a buffer containing 20 mM Tris-HCl, pH 7.8, 30 mM KCl, 5 mM magnesium acetate, and 0.025 M sucrose and centrifuged at 16,000 *g* for 1 h. The pellet was dispersed in 10 mM Tris-HCl, pH 7.8, and 0.5% Nonidet-P40, stirred vigorously for 3 h, and centrifuged at 16,000 *g* for 1 h. The resulting supernatant containing solubilized enzyme was mixed with a slurry of DEAE cellulose equilibrated in 10 mM potassium phosphate, pH 6.5, 0.5% Nonidet-P40 and filtered. The filter cake was resuspended in potassium phosphate buffer containing 0.025% Nonidet-P40, mixed, and refiltered. The enzyme was eluted with 0.2 M and 0.35 M KCl containing 0.025% Nonidet-P40, concentrated, dialyzed extensively in 1 mM potassium phosphate, pH 7.5, 0.025% Nonidet-P40, and sequentially fractionated in a series of negative adsorption steps with calcium phosphate gel. The concentrated eluate was diluted with 10 mM Tris-HCl, pH 7.8, reconcentrated six times to eliminate residual detergent, and then applied to a Sephadex G-200 column under vacuum. Two peaks of activity in the eluate were combined and the final purification step was performed with a glycerol gradient (5–20% in 10mM Tris-HCl) run at 38,000 rpm, with a Beckman SW 40 Ti rotor, for 18 h at 4°C in a Beckman L2-65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The activity was resolved in a single peak that was assayed according to the method of Cushman and Cheung (10). The protocol resulted in a 1,000-fold purification with a final specific activity consistent with previous experience (8, 9, 11). The purified enzyme showed a single band on polyacrylamide gel electrophoresis under both native and reducing conditions.

Antibodies to RbACE were raised in goats by immunization with the purified enzyme in complete Freund's adjuvant (11). In all experiments the same pool of Gt anti-RbACE γ-globulin isolated by ammonium sulfate precipitation was used. Isolation of IgG from γ-globulin was achieved by fractionation by DEAE cellulose chromatography (12). The pooled IgG fractions were dialyzed in 0.1 M sodium acetate, pH 4.5, and treated with pepsin (Sigma Chemical Co., St. Louis, MO) at a ratio of 1 mg pepsin/100 mg protein at 37°C for 18 h (13). The digest was centrifuged and divided into two aliquots. The first aliquot was dialyzed in 0.1 M Tris-HCl, 0.2 M NaCl, 2 mM EDTA, pH 7.8, and applied to a 4-cm × 100-cm column of Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ) at a flow rate of 11 ml/h. The $F(ab')_2$ was recovered in the second protein peak, concentrated, dialyzed in phosphate-buffered saline (PBS), and then applied to a column of Protein A Sepharose CL4B (Pharmacia) in 0.1 M sodium phosphate, pH 7.6. The second aliquot of pepsin digest was dialyzed in 0.1 M sodium phosphate, pH 7.5, and treated with papain (Sigma), at a ratio of 1:100 (wt/wt) in the presence of 1 mM EDTA and 10 mM L-cysteine at 37°C for 18 h (14). The digestion was terminated with iodacetic acid at a final concentration of 15 mM. The digest was dialyzed in 0.1 M Tris-HCl, 0.2 M NaCl, 2 mM EDTA, pH 7.8, and applied to the Sephacryl S-200 column as described. The eluted Fab was recovered from the third protein peak, dialyzed in PBS, and applied to a column of Protein A Sepharose as described above. There was no detectable IgG in either the $F(ab')_2$ or the Fab preparations as determined by polyacrylamide gel electrophoresis with a loading of 100 μg protein. Both $F(ab')_2$ and Fab gave single arcs against Rb anti-GtIgG by immunoelectrophoresis in agarose. IgG and $F(ab')_2$ formed precipitin bands against purified converting enzyme by the Ouchterlony immunodiffusion technique, whereas Fab did not.

Preparation of Radiolabeled Antibodies to Rabbit ABM and to Keyhole Limpet Hemocyanin (KLH). Rabbit ABM was prepared according to a modification (1) of a method described by Meezan et al. (15). Rabbit lung pairs were purchased from Pel-Freeze Biologicals. Purified KLH was obtained from Calbiochem-Behring Corp., San Diego, CA. Antisera to rabbit ABM and KLH were raised in rats by immunization with the antigenic preparations in complete Freund's adjuvant. The γ-globulin fraction of the immune sera was isolated by ammonium sulfate precipitation. The rat anti-RbABM and anti-KLH antibodies were purified from the γ-globulin preparations by affinity column chromatography with the antigens coupled to cyanogen bromide-activated Sepharose 4 B (Pharmacia Fine Chemicals). The purified antibody preparations were iodinated by the chloramine-T method (16).

Design of Experiments. A. Rabbits treated with intravenous injections of Gt anti-RbACE γ-globulin: (*a*) 49 normal rabbits were started on a daily schedule of 3 doses of 7 mg of Gt anti-RbACE γ-globulin in 3 ml of PBS, injected in a lateral ear vein for a maximum of 4 d. 15 normal rabbits were similarly injected with normal Gt γ-globulin. At autopsy of rabbits that died or were sacrificed, fragments of lung, kidney, liver, spleen, adrenal, and choroid plexus were removed for immunopathologic studies. Before processing, lungs were inflated with a solution of 0.5% bovine serum albumin in PBS delivered via a cannula inserted in the trachea (1). (*b*) 15 rabbits were depleted of polymorphonuclear leukocytes by the administration of 1.75 mg/kg body weight of NH_2 (Mustargen HCl, Merck, Sharp and Dohme, West Point, PA) (17). These rabbits were then also started on a schedule of 3 intravenous doses of 7 mg of Gt anti-RbACE γ-globulin per day.

B. Rabbits injected with horse radish peroxidase (HRP)-labeled Gt anti-RbACE γ-globulin: Immunoelectron microscopic studies were performed according to a methodology proposed by Vogt et al. (18) and modified by Abrahamson and Caulfield (19). One normal and one polymorphonuclear leukocyte–depleted rabbit were intravenously injected, over a 5-h period, with 20 mg of HRP-labeled Gt anti-RbACE γ-globulin. As controls, one polymorphonuclear leukocyte-depleted rabbit was injected with 20 mg of HRP-labeled normal Gt γ-globulin and another polymorphonuclear leukocyte–depleted rabbit was injected simultaneously with 20 mg of unlabeled Gt anti-RbACE γ-globulin and 20 mg of HRP-labeled normal Gt γ-globulin. After completion of the injections, the animals were sacrificed and their lung tissue processed for immunoelectron microscopy

(see below). The γ-globulin preparations were labeled with HRP as described by Nakane and Kawaoi (20).

C. Rabbits injected with Gt anti-RbACE IgG, or $F(ab')_2$ or Fab fragments of Gt anti-RbACE IgG: One rabbit received 52 mg of Gt anti-RbACE IgG, two rabbits 18 mg each of Gt anti-RbACE $F(ab')_2$, and three rabbits 25 mg each of Gt anti-RbACE Fab. The injections were given intravenously over a period of 5 h. After termination of the injection, the animals were sacrificed and various organs were immediately removed for immunopathologic studies. In two additional rabbits the injection in one of 25 mg of Gt anti-RbACE Fab and in the other of 25 mg of Fab fragments of normal GtIgG was followed by the intravenous administration over a 15-min period of 3 ml of a Rb anti-GtIgG antiserum (Cappel Laboratories, Cochranville, PA). These latter two animals were sacrificed 10 min later.

D. Rabbits to which, following a single injection of Gt anti-RbACE γ-globulin, radiolabeled-rat anti-RbABM antibodies were administered: Immediately following an intravenous injection of 7 mg of Gt anti-RbACE γ-globulin, seven rabbits received a mixture of ^{125}I-labeled, purified rat anti-RbABM antibodies and ^{131}I-labeled, purified rat anti-KLH antibodies. The animals were sacrificed half an hour later and their lungs, livers, and kidneys prepared for measurement of the specific uptake of the anti-ABM antibodies (1). Seven other rabbits were similarly treated, except that instead of Gt anti-RbACE γ-globulin they were injected with 7 mg of normal Gt γ-globulin. The specific uptake of the anti-ABM antibodies in various organs was calculated as described in an earlier publication (1). For statistical analysis, the Student's *t*-test was used.

Determination of ACE Activity in Serum, and in Lung and Kidney Tissue Homogenates. After removal of standardized sized specimens from lung (one specimen from each lobe) and kidney for immunopathologic studies, the remaining tissue was homogenized in PBS and assayed for ACE activity according to the method of Cushman and Cheung (10). ACE activity was also determined in serum samples obtained before sacrifice of the rabbits.

Immunofluorescence (IF) Microscopy. Direct IF tests on tissues obtained at autopsy of all rabbits were performed according to methods described in an earlier publication (21). FITC-conjugated antisera to GtIgG, RbIgG, RbC3, and Rbfibrin were purchased from Cappel Laboratories. They were found to be monospecific by immunoelectrophoresis as well as by immunodiffusion. Gt anti-RbACE IgG was conjugated with FITC by a standard method (21). To determine whether polymorphonuclear leukocytes were present in sections of frozen lung tissue from NH_2-treated rabbits that died on the first day of administration of Gt anti-RbACE γ-globulin, an FITC-conjugated antiserum to rabbit polymorphonuclear leukocyte cationic proteins was used. This antiserum was prepared as described in an earlier publication (22). In indirect IF tests frozen sections of normal rabbit tissues were incubated with serial dilutions of Gt anti-RbACE and normal Gt γ-globulin preparations, followed by incubation with FITC-labeled antiserum to GtIgG. Sections for IF microscopy were examined with Leitz Ortholux microscope equipped with epifluorescence optics and appropriate filters for FITC.

Light Microscopy. Tissue specimens removed at autopsy were fixed in 10% buffered formalin and embedded in paraffin. 3-μm thick sections were stained wtih hematoxylin-eosin and periodic acid Schiff.

Electron Microscopy. Fragments of lung and kidney tissue were fixed by submersion in Karnovsky's fixative (23), postfixed in 1% osmium tetroxide, and embedded in Epon 812-Araldite. Perfusion-fixation of the lungs was not performed to avoid removal of inflammatory cells from vascular lumina. Thin sections stained with uranyl acetate and lead citrate were examined with a JEOL 100S electron microscope.

Immunoelectron Microscopy. Fragments of lung of rabbits injected with HRP-labeled γ-globulin preparations were fixed by submersion in Karnovsky's fixative. ~40 micron-thick sections of lung were obtained using a Smith-Farquhar tissue chopper. HRP was visualized by incubation of the sections in diaminobenzidine hydrochloride (Sigma) followed by addition of 0.003% H_2O_2 (24). The sections were "flat embedded" in Epon 812-Araldite and thin sectioned for electron microscopy (25).

Results

In Vitro Reactivity of the GT Anti-RbACE Preparations. Testing of the Gt anti-RbACE preparations in vitro by indirect IF microscopy showed the following reactivity: linear binding of GtIgG, $F(ab')_2$, and Fab along the endothelium of alveolar capillaries of the lung (Fig. 1*A*), of capillaries of the choroid plexus (Fig. 2*A*), of periportal vessels of the liver, and, though weakly, of the sinusoidal wall of the adrenal; in addition, binding was observed to the brush border of proximal tubules of the kidney.

Rabbits Treated with Intravenous Injections of Gt Anti-RbACE γ-Globulin. 1) Normal rabbits: On the first day of the antibody injections the majority of the rabbits showed signs of respiratory distress and 69% died from pulmonary edema. On the following days the mortality fell dramatically (Table I) and the rabbits tolerated the injections of the anti-ACE γ-globulin without overt symptoms.

The results of IF microscopy studies of the lung are summarized in Table I. The deposits of GtIgG and RbC3 found along the alveolar capillary walls on the first 3 d of antibody administration were granular in appearance (Figs. 1*B* and 3). The granular pattern of binding of anti-ACE antibodies in vivo contrasted with the linear pattern observed in vitro (Fig. 1*A*). Furthermore, in direct IF microscopy the distribution of ACE in the lungs of rabbits injected with Gt anti-Rb ACE γ-globulin was also granular (Fig. 1*C*). In Table I it should be noted that on day 4, the last day of antibody administration, immune deposits were no longer detectable in the lungs (Fig. 4). In addition, when these lungs were studied by indirect IF microscopy it was found that ACE was absent or present in only minimal amounts. In lung tissue of rabbits that were sacrificed, fibrin appeared localized within the lumen of alveolar capillaries and, in animals that died on the first day of injection with pulmonary edema, also in the alveolar space. The results of direct IF tests for the presence of GtIgG in selected organs other than the lung are presented in Table II. In the kidneys of some of the rabbits sacrificed on day 4 or 5, weak and ill-defined localization of GtIgG was found along the glomerular capillary walls. GtIgG was never detected on the brush border of proximal tubules. In the spleen granular localization of GtIgG was observed in perifollicular zones. Whenever GtIgG was seen in the choroid plexus (Fig. 2*B*) or the adrenal, it was present in a granular pattern along the capillary or sinusoidal walls, respectively.

Quantitative studies (Fig. 5) showed a precipitous drop in ACE activity in the lungs of rabbits injected with anti-ACE γ-globulin. Discontinuation of the γ-globulin administration resulted, within 24 h, in a significant increase in the amount of ACE activity present in the lung; normal values were found on day 21. The values of ACE activity determinations in serum paralleled those in lung. No change in kidney ACE activity was observed.

In animals that died with pulmonary edema the findings by light and electron microscopy were similar to those described in an earlier publication (11), and included areas of alveolar capillary wall degeneration and necrosis, and accumulation in capillary lumina of occasionally disintegrated polymorphonuclear leukocytes, platelets, and fibrin. The polymorphonuclear leukocytes contained an increased amount of glycogen (26) and glycogen granules were found free in vascular lumina. Areas of frank necrosis were absent in lungs of rabbits that

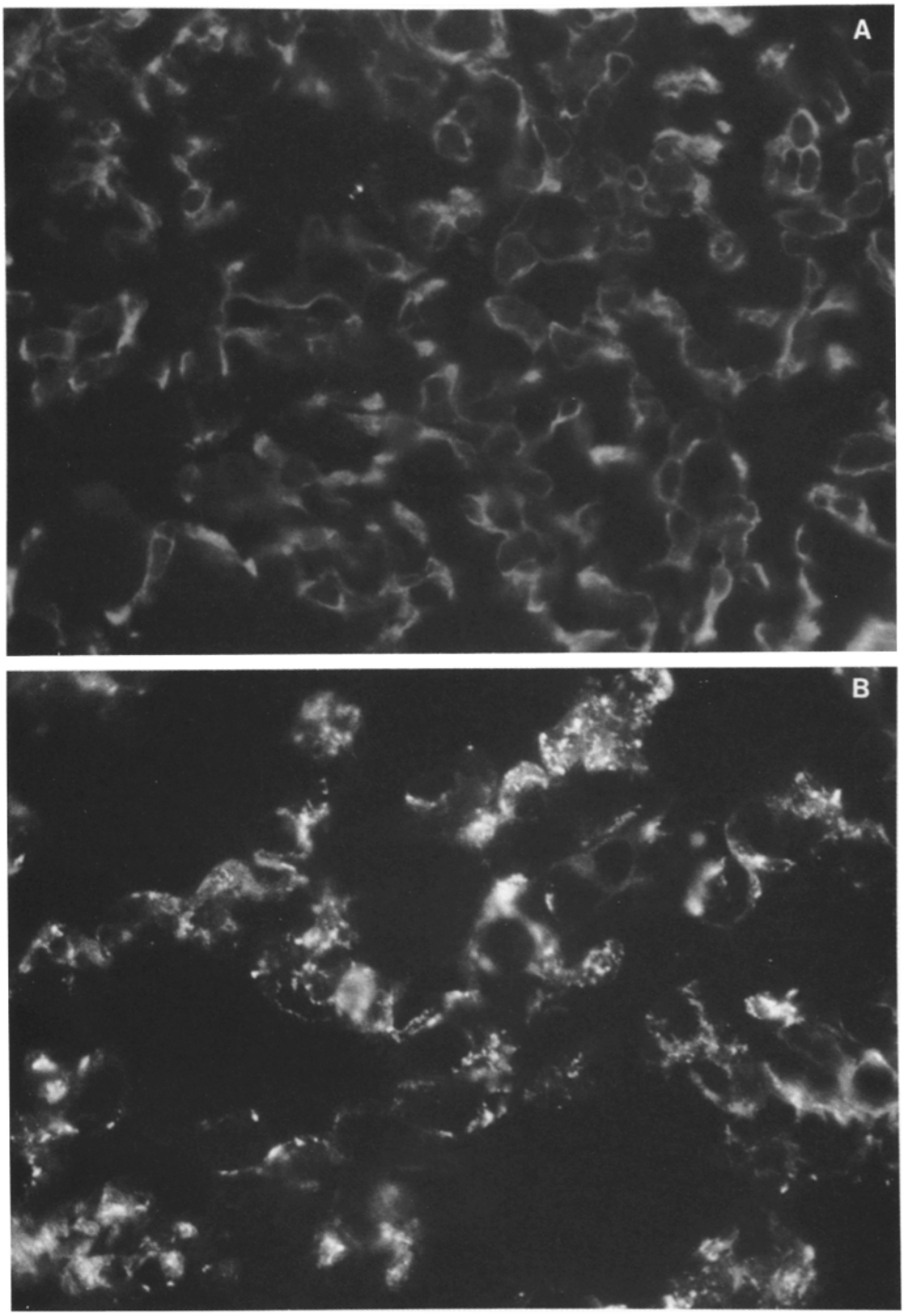

FIGURE 1. (*A*) Immunofluorescence micrograph showing in vitro binding of Gt anti-RbACE IgG to alveolar capillary walls of normal lung. The staining pattern is linear. × 600. (*B*) Immunofluorescence micrograph showing in vivo binding of Gt anti-RbACE IgG along alveolar capillary walls. The staining pattern is granular. × 600. (*C*) Immunofluorescence micrograph showing a granular distribution of ACE after in vivo binding of Gt anti-RbACE IgG along alveolar capillary walls. × 600.

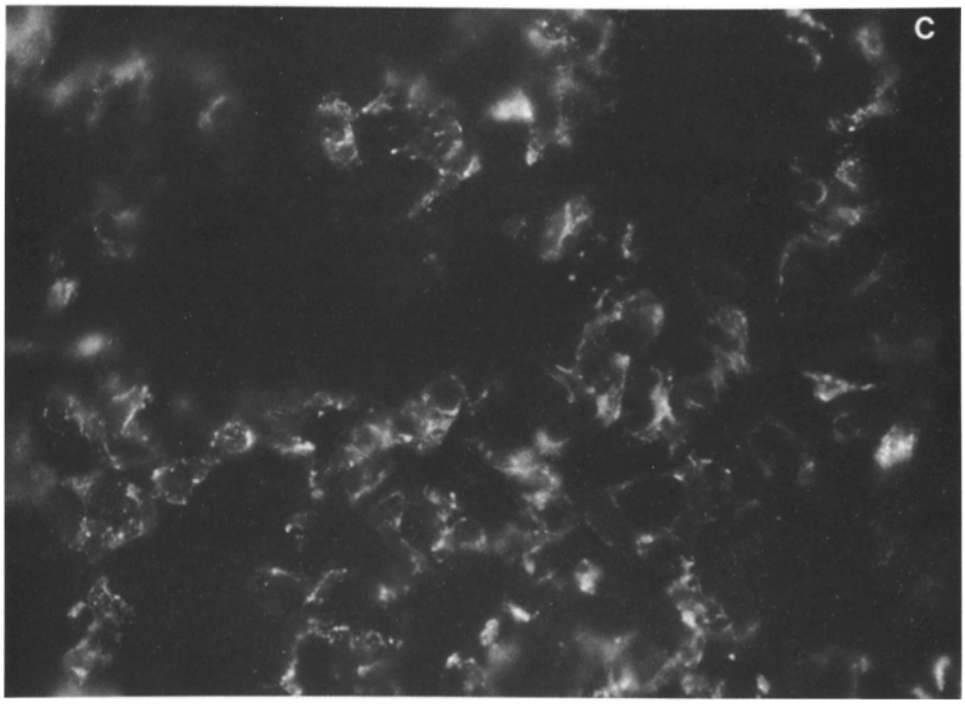

FIGURE 1C.

survived the first three injections of Gt anti-ACE γ-globulin and were sacrificed on day 1. In these animals the alveolar septa were not thickened; the alveolar endothelium, however, was markedly swollen and displayed an increased number of caveolae and vacuoles of variable size. In rabbits sacrificed on day 2 through 5 the alveolar septa were thickened. This thickening was caused by influx of mononuclear and polymorphonuclear leukocytes, proliferation of septal cells, and the presence of many type II pneumocytes. Over days 2 through 5 the polymorphonculear leukocytes gradually regained their normal appearance. Many macrophages were present in the alveolar space. On day 5 the endothelium was still swollen and showed an abundant rough endoplasmic reticulum and many free ribosomes. On day 21 the lung morphology had returned to normal and scarring was not found.

Rabbits injected with normal Gt γ-globulin were sacrificed on day 1 ($n = 7$), 4 ($n = 5$), 5 ($n = 1$), and 21 ($n = 2$). In these rabbits the results of all studies, including quantitation of ACE in lungs, kidneys, and sera, were normal.

2) Polymorphonuclear leukocyte–depleted rabbits: On the first day of Gt anti-RbACE γ-globulin injections, no polymorphonuclear leukocytes could be found in the circulation of HN_2-treated rabbits. The mortality (40%) on the first day in this group of animals was not statistically significant different from that observed in non-polymorphonuclear leukocyte–depleted rabbits.

In this group of rabbits the results obtained by IF microscopy were similar to those obtained in rabbits nondepleted of polymorphonuclear leukocytes and injected with anti-ACE antibody: initially the lung immune deposits were gran-

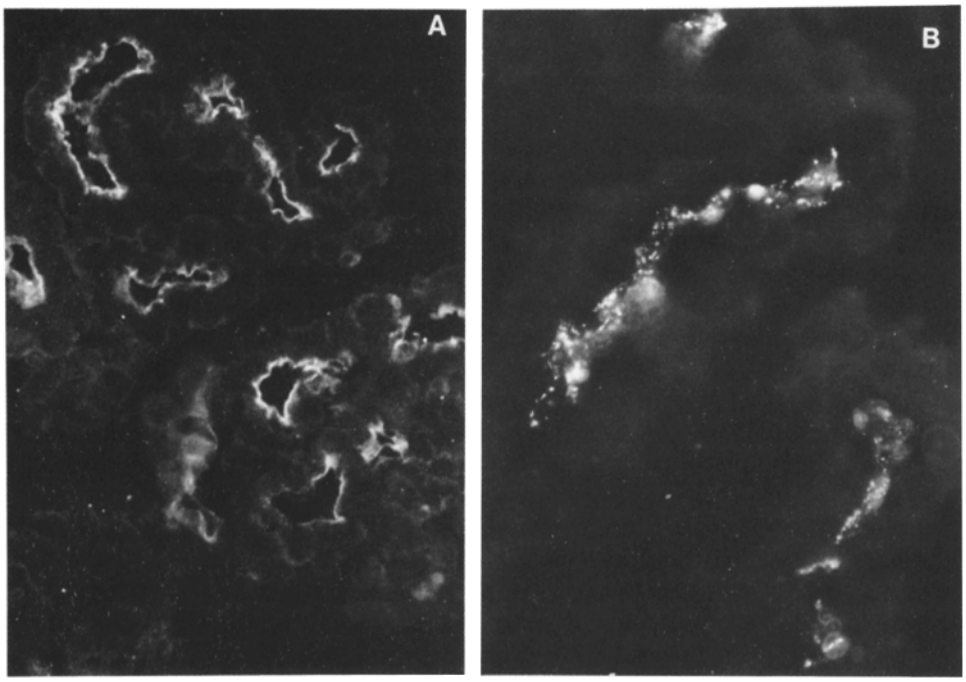

FIGURE 2. (*A*) Immunofluorescence micrograph showing in vitro binding of Gt anti-RbACE IgG to the endothelium of capillaries of the choroid plexus. The staining pattern is linear. × 250. (*B*) Immunofluorescence micrograph showing in vivo binding of Gt anti-RbACE IgG to the endothelium of capillaries of the choroid plexus. The staining pattern is granular. × 400.

TABLE I

Mortality and Immunofluorescence Findings in Lungs of Rabbits Injected on Day 1 through 4 with 21 mg of Goat Anti-rabbit Angiotensin-converting Enzyme γ-Globulin per Day

Day	Mortality in percent	No. of rabbits studied by DIF microscopy	Deposits along alveolar capillary wall: percentage of rabbits positive			
			GtIgG	RbIgG	RbC3	Rb Fibrin
1	69	22	100	0	81	81
2	13	7	57	0	29	71
3	0	3	33	0	ND	ND
4	0	5	0	0	0	20
5	0	7	0	0	0	14
21	0	5	0	0	0	0

Abbreviations: DIF, direct immunofluorescence; Gt, goat; Rb, rabbit; ND, not done.

ular and, on day 4 (n = 2), were no longer detectable. No, or only an occasional polymorphonuclear leukocyte was seen after staining sections of frozen lung tissue of rabbits that died on day 1 with FITC-conjugated antiserum to cationic proteins of rabbit polymorphonuclear leukocyte.

With the exception of absence of polymorphonuclear leukocytes on day 1, light and electron microscopy revealed lung lesions similar to those seen in rabbits nondepleted of polymorphonuclear leukocytes and injected with anti-

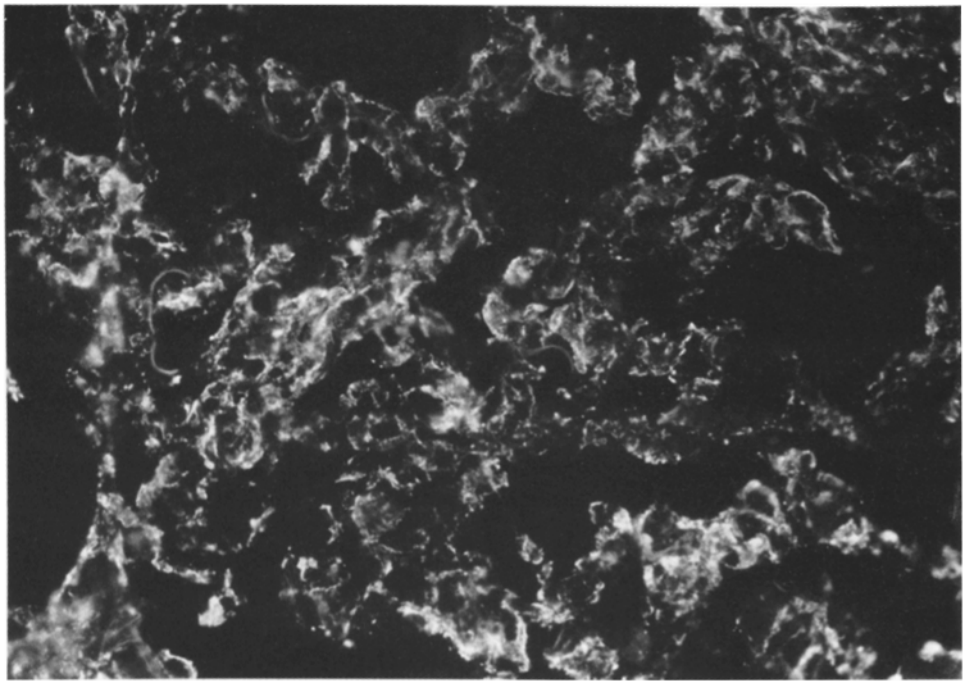

FIGURE 3. Immunofluorescence micrograph showing the binding of RbC3 along alveolar capillary walls of a rabbit injected with Gt anti-RbACE γ-globulin. The staining pattern is granular. × 250.

ACE antibody.

Immunoelectron Microscopy. Injected HRP-labeled Gt anti-ACE γ-globulin was found to be irregularly distributed along the surface of the endothelium of alveolar capillaries; it was also found in endothelial caveolae and had accumulated in vacuoles of variable size within the endothelial cytoplasm (Fig. 6). HRP could not be demonstrated in lung tissue of the control rabbits.

Rabbits Injected with Gt Anti-RbACE IgG, or F(ab')$_2$ or Fab Fragments of Gt Anti-RbACE IgG. GtIgG or F(ab')$_2$ fragments injected into rabbits were found, together with RbC3, in a granular pattern along alveolar capillary walls. In contrast, Fab fragments of Gt anti-RbACE IgG localized in a linear pattern along alveolar capillary walls (Fig. 7*A*). In direct IF microscopy the distribution of ACE was also linear (Fig. 7*B*). Complement fixation could not be detected in the lungs of rabbits injected with Fab fragments.

When the injection of Fab fragments of Gt anti-RbACE IgG was followed by an injection of Rb anti-GtIgG serum, fine granular deposits of Gt Fab fragments (Fig. 7*A*, inset), RbIgG and RbC3 were seen along alveolar capillary walls. Injection of Fab fragments of normal GtIgG followed by Rb anti-GtIgG serum, did not result in formation of granular immune deposits along alveolar capillary walls. However, precipitates composed of Gt Fab fragments, RbIgG, and RbC3 were occasionally present in the lumen of alveolar capillaries.

Rabbits to Which, Following a Single Injection of Gt Anti-RbACE γ-Globulin, Radiolabeled Rat Anti-RbABM Antibodies Were Administered. In the rabbits injected with

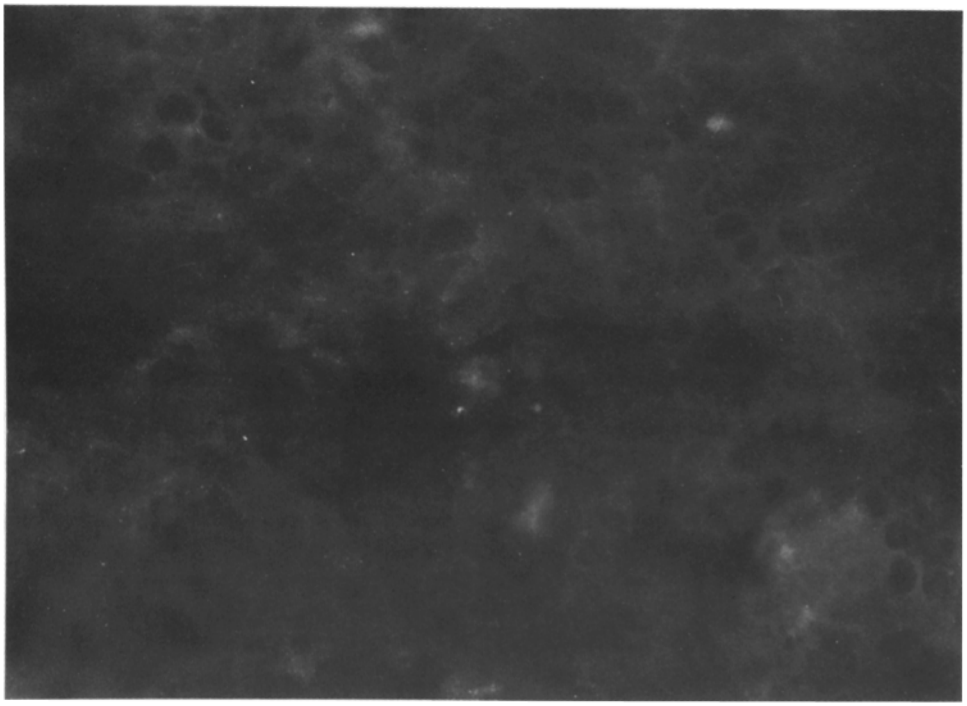

FIGURE 4. Immunofluorescence micrograph showing absence of binding of GtIgG to lung tissue in a rabbit sacrificed on the fourth day of administration of Gt anti-RbACE γ-globulin. × 600.

TABLE II

Localization of Goat IgG in Selected Organs of Rabbits Injected on Day 1 through 4 with 21 mg of Goat Anti-rabbit Angiotensin-converting Enzyme γ-Globulin per Day

Day	Kidney*	Spleen	Liver	Adrenal	Choroid plexus
1	0/22	8/14	0/14	3/13	3/9
2	0/7	3/8	0/8	0/8	2/8
3	ND	ND	ND	ND	ND
4	3/5	1/5	0/5	0/5	3/5
5	3/7	2/7	0/7	0/7	2/4
21	0/5	0/5	0/5	0/5	0/2

* Number of organs with deposits of goat IgG over total number of organs studied.

anti-ACE γ-globulin the specific uptake of ^{125}I-rat anti-RbABM antibodies (as corrected percent uptake of total injected dose of ^{125}I) was 2.4 ± 0.8 (mean ± SD) in the lungs, 11.3 ± 1.1 in the kidneys, and 31.6 ± 3.7 in the liver. In rabbits injected with normal Gt γ-globulin these values were: lungs 0.5 ± 0.1, kidneys 10.7 ± 0.7, and liver 36.2 ± 1.5. The only statistically significant difference was the increased binding of anti-ABM antibodies to the lungs in the group of rabbits treated with anti-ACE γ-globulin.

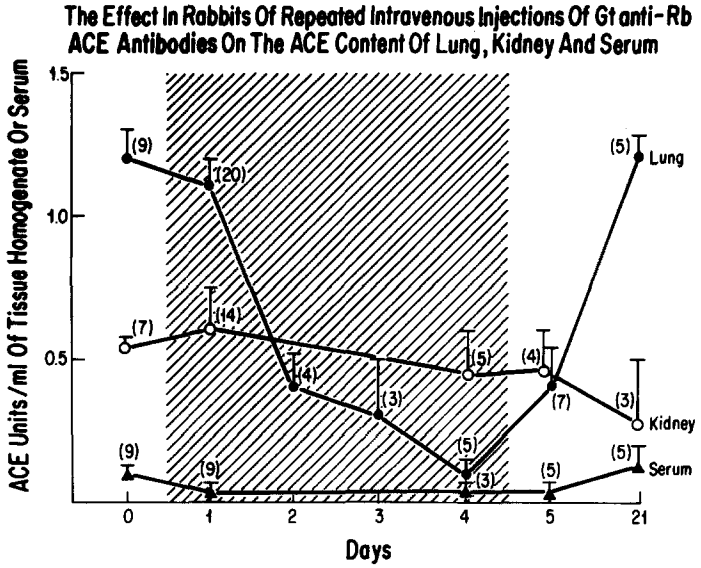

FIGURE 5. Quantitation of ACE activity in lung, kidney, and serum. Indicated are means ± 2 SE. Between parentheses, the number of rabbits studied. Gt anti-RbACE γ-globulin was administered during the time period represented as a shaded area. The values of lung and kidney ACE activity on day 0 were obtained from rabbits injected with normal Gt γ-globulin.

Discussion

ACE catalyzes the conversion of angiotensin I into the potent vasoactive peptide angiotensin II, and the degradation of the hypotensive and edematogenic peptide bradykinin (27–29). Although ACE is found on the surface of most endothelial cells, a major site of ACE activity appears to be the lung (6, 7, 9, 29–31). Results of immunohistochemical studies show ACE on the plasma membrane and in caveolae of lung endothelial cells (7). Previous studies have shown that rabbits (11) and rats (31) injected with large doses of heterologous anti-ACE antibodies develop increased vascular permeability in the lung (31) and frequently die of pulmonary edema.

The aim of the present study was to investigate the effects of a relatively long-term interaction of Gt anti-RbACE antibodies with lung ACE. To this purpose, small doses of antibody were slowly injected for a maximum of 4 d. Despite these precautions a high mortality, due to pulmonary edema, was recorded on the first day of injection. Increased alveolar capillary wall permeability, as reflected in the uptake of anti-RbABM antibodies, was also found in rabbits that did not die from pulmonary edema, confirming an earlier observation in rats (31). The role of polymorphonuclear leukocytes in the induction of increased alveolar capillary wall permeability does not appear essential because of mortality rate of rabbits depleted or not depleted of polymorphonuclear leukocytes was not significantly different.

Rabbits surviving the first day of anti-ACE antibody injection, became resistant to subsequent administration. The suppression of immune injury appears to be related to an "immunologic enzymectomy" caused by ligand-surface antigen

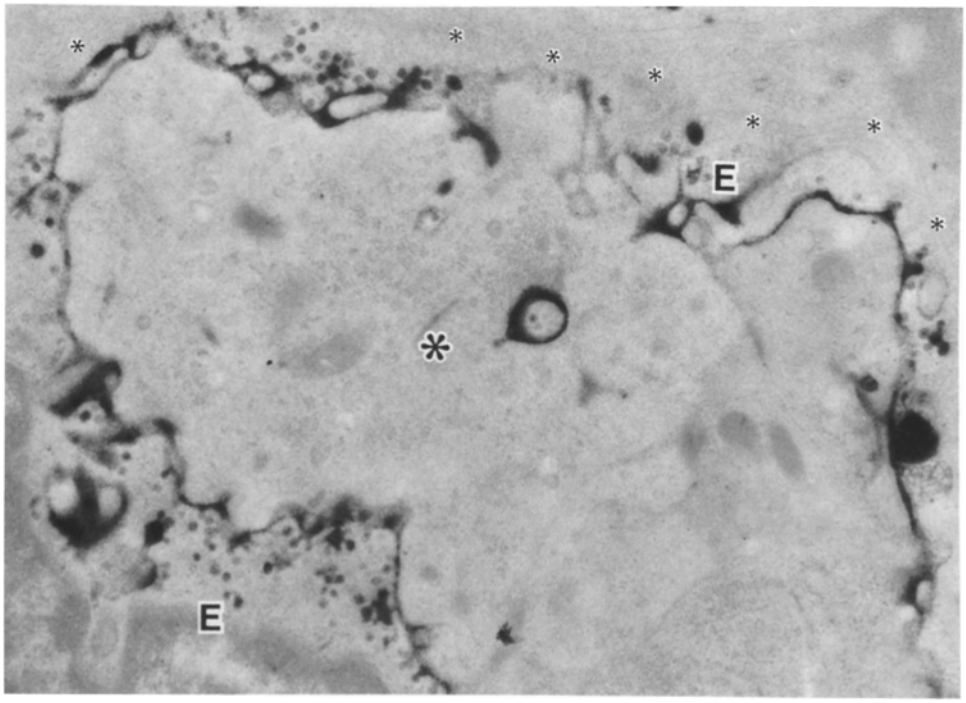

FIGURE 6. Immunoelectron micrograph of lung of a rabbit injected with HRP-labeled Gt anti-RbACE γ-globulin. Black HRP reaction product is seen in an irregular distribution along the surface of the alveolar capillary endothelium (E), in endothelial caveolae, and in vacuoles in the endothelial cytoplasm. The lumen of the alveolar capillary is obliterated by cytoplasm of an unidentified cell (large asterisk). Small asterisks: alveolar basement membrane. Unstained section. × 6,000.

interaction at the level of the plasma membrane of endothelial cells. Gt anti-RbACE antibodies bound in vitro to the endothelium of the lung with a distinctly linear immunofluorescence pattern, corresponding to the even distribution of ACE along the plasmamembrane of lung endothelial cells observed by immunoelectron microscopy (7). In contrast, after in vivo injection of the antibody, diffuse granular deposits of ACE, GtIgG, and RbC3 were observed along alveolar capillary walls and also, for example, along capillary walls of the choroid plexus. This indicates that in vivo cross-linking of ACE molecules by divalent antibodies induces a migration of the enzyme in the plane of the plasma membrane, resulting in the formation of microaggregates (32). This interpretation is supported by the observation of linear deposits along alveolar capillary walls of Gt Fab antibody fragments, contracting with the granular pattern observed after injection of Gt $F(ab')_2$ antibody fragments. The monovalent Fab antibody fragments, although binding to the plasma membrane of the alveolar endothelium, did not induce redistribution of ACE. Addition of a "piggyback" antibody (Rb anti-GtIgG) to cross-link the Fab-ACE complex, resulted in antigen redistribution. Similar studies have been performed on cultures of skeletal muscle using antibodies to acetylcholine receptors (33–34). It was found that, despite binding, the monovalent Fab fragments of anti-acetylcholine receptor antibodies did not enhance

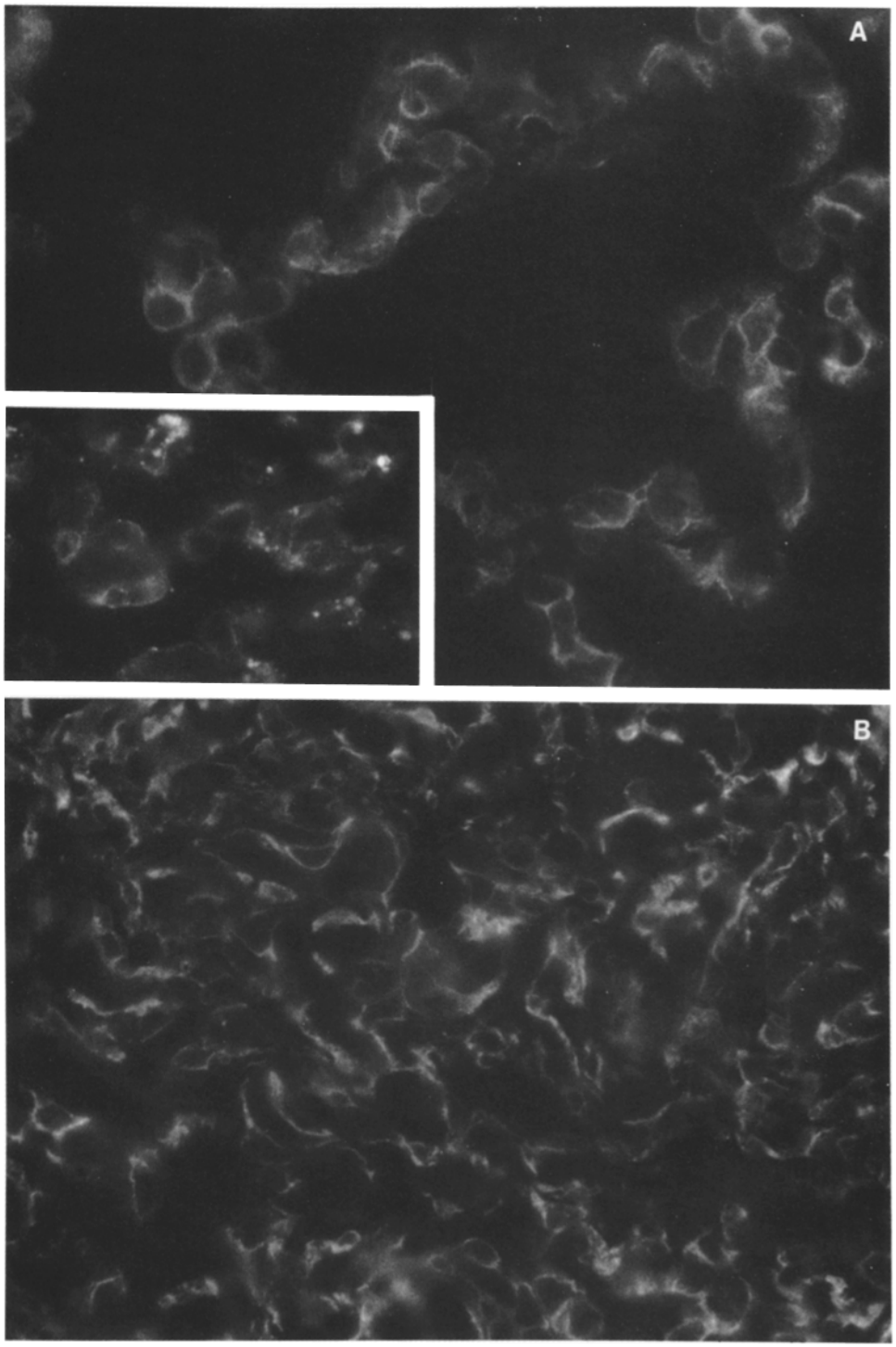

FIGURE 7. (*A*) Immunofluorescence micrograph showing in vivo linear binding of Fab fragments of Gt anti-RbACE IgG along the endothelium of alveolar capillaries. × 600. Inset: Immunofluorescence micrograph showing in vivo granular binding of Fab fragments of Gt anti-RbACE IgG along the alveolar capillary endothelium in a rabbit that, following the administration of the antibody fragments, was injected with Rb anti-GtIgG antiserum. × 600. (*B*) Immunofluorescence micrograph showing a linear distribution of ACE after in vivo binding of Fab fragments of Gt anti-RbACE IgG along the endothelium of alveolar capillaries. × 600.

degradation of the receptors. However, when "piggyback" antibodies were added to the system, the degradation rate increased.

In vivo cross-linking of ACE molecules by divalent antibodies led to disappearance of ACE from the surface of lung endothelial cells. Whether ACE was internalized (35), shed into the circulation (36), or both, is not clear from our study. The evidence for disappearance of ACE is based on the following observations: first, despite continued injections of Gt anti-RbACE antibodies, deposits of GtIgG or RbC3 were not present in the lungs of the rabbits sacrificed at day 4; second, ACE was no longer demonstrable by indirect IF microscopy in the lungs of rabbits sacrificed at day 4; third, quantitative measurement of ACE activity in lung homogenates showed, in agreement with the IF data, a striking decrease in activity of the enzyme on day 4. 24 h after discontinuation of antibody administration, there was a reappearance of ACE activity in the lung and normal levels were recorded 17 d after the last antibody injection. The endothelial cells denuded of ACE were no longer susceptible to immune injury, as evidence by the sharp drop in mortality during days 2, 3, and 4 of antibody injection. The morphological abnormalities found in the lungs of rabbits sacrificed on those days may reflect repair processes.

The in vivo disappearance of ACE from the surface of lung endothelial cells under the influence of specific antibody is an example of "antigenic modulation" (32). Antigenic modulation was first described by Boyse et al. (37, 38) who showed that surface isoantigens on mouse leukemic cells disappear when the cells are exposed to antibodies. Such phenotypic alteration occurs in vitro as well (39), and is reversed by eliminating the antibodies. It has been demonstrated that antigenic modulation is an active process requiring metabolically intact cells and does not result from long-term masking of isoantigens by IgG (39). It was later shown that anti-Ig antibody can redistribute surface Ig on murine lymphocytes into polar aggregates ("caps") (40, 41). After incubation of lymphocytes under capping conditions, surface Ig totally disappears through endocytosis and/or external stripping. Surface Ig reappears shortly after reculturing lymphocytes in a medium devoid of anti-Ig antibody. Antibody-induced antigenic modulation has also been demonstrated for histocompatibility antigens (42–45), Burkitt lymphoma cell surface antigens (46) and Gross leukemia cell surface antigens (47). In persistent viral infections, such as measles, infected cells express viral glycoprotein antigens on their surface. When exposed to anti-measles antibodies, however, the viral antigens rapidly coalesce into a pole of the cell and then disappear from the cell surface, shed into the medium in form of immune complexes (48, 49). The infected cells, denuded of viral surface antigens, are no longer susceptible to both humoral and cell-mediated immune responses (50). Such a mechanism of antibody-induced insensitivity to immunologic injury appears to have a central role in the pathogenesis of subacute sclerosing panencephalitis and other persistent viral infections associated with high antibody responses (36). The results of the present study show that antigenic modulation occurs in the lungs of rabbits injected with anti-ACE antibodies reacting with the plasma membrane of endothelial cells. The reappearance of ACE after suspension of antibody injections indicates that antigenic modulation, as in cell cultures (50), is at least partially reversible in vivo. It is conceivable that the expression of other

antigens on the plasma membrane of endothelial cells may be modulated by antibodies. In this regard it is attractive to consider the possibility that antigenic modulation may contribute, together with other factors, to the resistance to rejection occurring in grafts exposed to transplantation antibodies (51). Furthermore, it does not escape our notice that patching and shedding of plasma membrane antigens, such as those expressed on the plasma membrane of glomerular visceral epithelial cells (52), might result in local accumulation of immune complexes (Heymann glomerulonephritis). This hypothesis is now being tested in our laboratory.

To avoid anaphylactic complication, the administration of Gt anti-ACE γ-globulin had to be limited to 4 d. To study the chronic effects of anti-ACE antibodies on lung and other organs it will be necessary to render rabbits immunologically tolerant to GtIgG.

Summary

To study the effects of relatively long-term interaction of antibodies with surface antigens of lung endothelium, rabbits were intravenously injected for a maximum of 4 d with goat anti-rabbit lung angiotensin-converting enzyme (Gt anti-RbACE) antibodies. On day 1 69%, on day 2 13%, and on days 3 and 4 of injection none of the rabbits developed lethal pulmonary edema. By immunofluorescence microscopy, deposits of GtIgG, frequently in association with RbC3, were found along the endothelium of alveolar capillary walls in all rabbits studied on day 1, in 57% on day 2, in 33% on day 3, and in none of them on day 4. While in vitro anti-ACE antibodies bound in a linear pattern to the lung endothelium, the binding pattern in vivo was distinctly granular. The in vivo interaction of antibodies with ACE also redistributed ACE in a granular pattern along capillary walls. In contrast to the granular deposition of injected anti-ACE IgG and $F(ab')_2$ fragments of anti-ACE IgG, Fab fragments of anti-ACE IgG localized, without fixing C3, in a linear pattern along the endothelium of lung capillaries and did not modify the normal distribution of ACE. However, when the injection of Fab fragments of Gt anti-RbACE IgG was followed by an injection of Rb anti-GtIgG serum, granular deposits of Gt Fab fragments, RbIgG and RbC3 were seen along alveolar capillary walls. Biochemical measurement of ACE activity in lung homogenates provided data in agreement with those obtained by immunofluorescence microscopy, showing diminished activity to none on day 4, with some return of ACE activity on day 5, 24 h after the last injection of antibody, and normal values on day 21. The results obtained indicate that divalent antibodies to an antigen expressed on the plasma membrane of rabbit lung endothelial cells promotes a rapid redistribution of antigenic receptors, fixation of complement and, in surviving rabbits, disappearance of the antigen from the endothelial cells that are no longer susceptible to immune injury. In vivo "immunologic enzymectomy" induced by a ligand-surface antigen interaction is an example of antigenic modulation. These events may have an important role in the pathogenesis of inflammatory lesions induced by antibodies reacting with antigens expressed on the plasma membrane of cells in the lung and in other organs.

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