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Interaction of Antibody with Forssman Antigen in Guinea Pigs

A Mechanism of Adaptation to Antibody- and Complement-Mediated Injury

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Forssman antigen is a glycosphingolipid with antigenic specificity determined by extra-membrane haptenic sugars similar to blood group antigens and antigens that are the main barrier to xenogeneic organ transplantation. Herein, we describe the localization of Forssman antigen in guinea pig lungs and kidneys and the consequences of its interaction with antibodies* in vitro *and* in vivo *(Forssman reaction). Exposure of cultured guinea pig aortic endothelial cells to Forssman antibodies induced rapid redistribution of antigen-antibody complexes at the cell surface, followed by shedding that occurred by blebbing of plasma membrane as vesicles or fragments, and was associated with disappearance of antigen from the cell surface (antigenic modulation). Guinea pigs surviving frequent intravenous injections of increasing amounts of antibodies, for a total of 20 to 40 lethal doses, developed a partial or complete adaptation to generalized Forssman reaction, and adaptation was associated with partial or complete modulation of Forssman antigen at the surface of the pulmonary and, in minor degree, renal endothelial and epithelial cells. These findings support the hypothesis that modulation of endothelial carbohydrate antigens contributes to adaptation of highly vascularized organs exposed to tolerable levels of allo- or xenoantibodies. (Am J Pathol 1995, 146:1260–1272)

The interaction of antibodies with cell surface antigens initiates tissue injury in hyperacute allograft[1–3] and xenograft[4,5] rejection, and in some diseases in which *in situ* formation of immune complexes occurs.[6] Our understanding of the morphological and kinetic aspects of these interactions derives from the study of well characterized antigen-antibody systems. We have reported that antibodies specific for an endothelial protein antigen, angiotensin-converting enzyme, induce *in vivo* a rapid redistribution of immune complexes at the surface of lung endothelial cells, then disappearance of angiotensin-converting enzyme from the endothelial cell surface (antigenic modulation).[7] This process was followed by shedding of angiotensin-converting enzyme immune complexes into the circulation, and by co-shedding of immunologically unrelated thrombomodulin.[8] After modulation of angiotensin-converting enzyme or thrombomodulin, endothelial cells were resistant to injury caused by specific antibodies.[7–9] This phenomenon is similar to graft adaptation or accommodation, a condition characterized by survival of highly vascularized organs despite the presence of antibodies and complement in the circulation.[10–12]

In the generalized Forssman (or heterophile) reaction induced by intravenous injection of rabbit anti-

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Forssman serum into guinea pigs, the antibodies bind primarily to the pulmonary endothelium, the first large capillary bed they encounter. The guinea pigs develop tachypnea, cough, ruffling of hair, hypotension, pulmonary hemorrhage and edema, and convulsions and die within minutes.[13] The antibodies bound to the pulmonary endothelium activate the direct pathway of complement that instigates the reaction.[14] In 1955 Spear[15] showed that guinea pigs injected with progressively increasing doses of Forssman antibodies can be desensitized and tolerate as many as 80 lethal doses (LD). This phenomenon is comparable with the adaptation to antibody- and complement-mediated injury occurring in rabbits injected with antibodies against angiotensin-converting enzyme or thrombomodulin,[7–9] and to adaptation of highly vascularized organs to humoral rejection.[11–12]

In this study we describe the localization of Forssman antigen in the lungs and kidneys of guinea pigs and the consequences of the interaction of Forssman antibodies with Forssman antigen *in vitro* and *in vivo* and discuss the mechanisms of adaptation to antibody.

Materials and Methods

Animals

Hartley female guinea pigs (200 to 250 g), male Holtzman rats (200 g), LVG hamsters, and male New Zealand White rabbits (from Charles River Laboratories, Wilmington, MA) were maintained with free access to Purina food and water.

Preimmune and Immune Sera

The preimmune and immune sera, and their designations, are listed in Table 1. The preparation of sheep and human erythrocyte stromata, used as Forssman-positive and Forssman-negative antigenic inocula, respectively, was previously described.[13] These were injected intravenously into rabbits. Rabbits and hamsters were immunized subcutaneously with guinea pig kidney tissue in Freund's adjuvant according to standard procedures. The animals were bled 14 days after the last immunization. The immunoglobulin (Ig)G fractions were prepared by ammonium sulfate precipitation (50%) and by protein A column chromatography. Fab fragments of the IgG, used for immunoelectron microscopy, were prepared with an Immuno Pure TM Fab Preparation Kit (Pierce Chemical Co., Rockford, IL).

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis and Immunoblotting

Lung and kidney homogenates of guinea pigs or rats were solubilized in a buffer (4.6% SDS, 20% glycerol, 1 mol/L Tris-HCl buffer at pH 6.8, and 0.1% bromphenol blue) and heated for 5 minutes in boiling water,[16] then resuspended in 8 to 25% SDS. The samples were subjected to electrophoresis (2 hours at 15 mA). The gels were stained in Coomassie blue. Some gels were transferred electrophoretically to nitrocellulose membranes (2 hours at 500 mA). These were reacted with relevant immune IgG at 1:500 and revealed with horseradish peroxidase (HRP)-conjugated protein A (Zymed Laboratories, San Francisco, CA), visualized with 0.02% diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) in 50 mmol/L Tris-HCl buffer (pH 7.6) containing 0.016% H_2O_2.

Elution of Forssman Antigen from Cells and Tissues and Characterization of the Eluates

Cultures of guinea pig or rat glomerular epithelial cells were prepared and characterized as previously described.[17] A total of 5.3×10^6 guinea pig or 3.6×10^6 rat cells were incubated with pure methanol at room temperature. The methanol was evaporated and the extract was resuspended in 1 ml of pure methanol. For elution of Forssman antigen from lung and kidney tissues, fresh-frozen unfixed 5-μ-thick sections were cut in a cryostat and incubated in 200 μl of PBS for 10 minutes at room temperature. The methanol and the PBS eluates were serially diluted, placed on

Table 1. *Preimmune and Immune Sera*

Host animal	Antigenic inoculum	IgG	Forssman antibodies	Designation
Rabbit (*Forssman*$^-$)	None	Preimmune	Absent	PRS
Rabbit	Sheep erythrocytes (*Forssman*$^+$)	Rabbit anti-sheep erythrocytes	Present	RbAShE
Rabbit	Guinea pig kidney (*Forssman*$^+$)	Rabbit anti-guinea pig kidney	Present	RbAGpK
Rabbit	Human erythrocytes (*Forssman*$^-$)	Rabbit anti-human erythrocytes	Absent	RbAHE
Hamster (*Forssman*$^+$)	Guinea pig kidney (*Forssman*$^+$)	Hamster anti-guinea pig kidney	Absent	HAGpK

nitrocellulose paper, air dried, incubated overnight at 4 C with RbAShE, followed by HRP-conjugated goat anti-rabbit IgG for 2 hours at room temperature. The reaction was visualized with 0.04% *o*-phenylenediamine (Sigma Chemical Co.) containing 0.005% H_2O_2 and then stopped by addition of 4 N H_2SO_4.

Cultures of Guinea Pig Aortic Endothelial Cells

Guinea pig aortic endothelial cells were isolated as described by Rone and Goodman.[18] Endothelial cells were removed from guinea pig aorta by gently scraping the intimal surface; alternatively, the vessel was cut into 2- to 4-mm rings and grown on culture plate in DMEM (GIBCO Laboratories, Grand Island, NY) supplemented with 10% guinea pig serum (GIBCO), glutamine (2 mmol/L), and penicillin/streptomicin (both 50 U/ml). Endothelial cells migrating from the explants were grown up and transferred by trypsinization. For experiments, cells (passages 2 to 6) were grown to confluence (10^5 cell/cm^2) on gelatin-coated coverslips and inserted in 24-mm tissue culture plates. Cultures formed cobblestone-like monolayers and had factor VIII-related antigen.[8,19]

Intravenous Injections of PRS or RbAShE into Guinea Pigs

Ten guinea pigs were rapidly injected intravenously with 12.6 to 21 mg of IgG PRS and sacrificed after 5 to 10 minutes. Ten guinea pigs rapidly injected with 12.6 mg of IgG RbAShE died within 10 minutes (average, 4.7 minutes). Five guinea pigs rapidly injected with 21 mg of IgG RbAShE died within 7 minutes (average, 4.1 minutes). Thus, 12.6 mg was considered the LD (Table 2). Eleven guinea pigs were desensitized with RbAShE according to the method described by Spear[15] (Table 3). Intravenous injections were given every 3 to 6 hours for 48 hours; the first and second doses were one-fourth LD, after which the dosage was gradually increased. When 4 to 6 LDs were tolerated, the amount of RbAShE was augmented more rapidly. Two guinea pigs received injections of comparable doses of PRS. Prolongation of the experiments was difficult because of the few remaining veins suitable for injection. Lungs and kidneys obtained immediately after sacrifice or death were processed for morphological and immunohistochemical studies.

Immunocytochemical and Immunohistological Localization of Forssman Antigen in vitro *and* in vivo

Light Microscopy

Fluorescein isothiocyanate (FITC)- or HRP-conjugated $F(ab')_2$ fragments of goat anti-rabbit IgG were purchased from Cappel Laboratories (West Chester, PA), FITC-sheep anti-guinea pig C3 from The BindingSite (Birmingham, UK), FITC-conjugated IgG fraction of goat antiserum to hamster IgG from Kirkegaard & Perry Laboratories (Gaithersburg, MD), and affinity-purified rabbit antibody to factor VIII from Calbiochem-Behring Corp. (La Jolla, CA). For direct immunofluorescence technique, the IgG fraction of RbAShE was conjugated with biotin[20] (Byotinyl-*N*-hydroxysuccinimide, Vector Laboratories, Burlingame, CA) and revealed with FITC-streptavidin purchased from Vector Laboratories, Inc.

The techniques used for localization of cell surface or intracellular antigens in cultured cells[21] and those used for the study of antibody-induced redistribution of cell surface antigens[17] have been described. The cells were studied without fixation or after fixation with

Table 2. *Results in Guinea Pigs Given a Single Rapid Intravenous Injection of Preimmune or Immune Sera*

No. of guinea pigs	IgG	Dose (mg)	Clinical response*	Lung immunohistochemistry			Lung morphology†	Kidney immunohistochemistry			Kidney morphology†
				RbIgG	FA	GpC3		RbIgG	FA	GpC3	
Group I											
10	PRS	21	None	0	++++	0	Normal	0	++++	0	Normal
Group II											
10	RbAShE	12.6 (LD)	++++ (died in ~4.7 minutes)	++/+++	++++	++/+++	++++	++	++++	+/++	++++
5	RbAShE	21	++++ (died in ~4.1 minutes)	++/+++	++++	++/+++	++++	++	++++	+/++	++++

RbIgG, rabbit IgG; FA, Forssman antigen; GpC3, guinea pig C3.
*Tachypnea, cough, ruffling of hair, nose scratching, and convulsion.
†Edema, hemorrhage, thickening of alveolar septa, accumulation of leukocytes in alveolar or renal capillaries, and infiltration of leukocytes and erythrocytes in alveolar spaces or renal interstitium.
0, absent; +, minimal and focal in amount and extent; ++, moderate and focal; +++, marked and diffuse; ++++, very marked and diffuse.

Table 3. *Results in Guinea Pigs Injected Intravenously with Preimmune or Immune Sera According to the Protocol Designed to Induce Adaptation*

Guinea pig	No. of injections	IgG	Last dose, mg (LD)	Total dose mg (LD)	Clinical response to last injection*	Lung immunohistochemistry			Lung morphology†	Kidney immunohistochemistry			Kidney morphology†
						RbIgG	FA	GpC3		RbIgG	FA	GpC3	
Group III													
1	9	PRS	63 (5)	92.4 (7.3)	None, S	0	++++	0	Normal	0	+++	0	Normal
2	10	PRS	126 (10)	300 (23.8)	None, S	0	++++	0	Normal	0	+++	0	Normal
Group IV													
3	8	RbAShE	63 (5)	92.4 (7.3)	++++, D	++	++	+	++++	+/++	+/++‡	0/+	Normal/+
4	9	RbAShE	63 (5)	94 (7.3)	++++, D	0/+	+	+/++	++++	+/++	+/++‡	0/+	Normal/+
5	8	RbAShE	63 (5)	92.4 (7.3)	++++, D	++	+/++	++	++++	+/++	+/++‡	0/+	Normal/+
6	13	RbAshE	63 (5)	172 (13.7)	++++, D	+/++	+	+	++++	+/++	+/++‡	0/+	Normal
7	15	RbAShE	126 (10)	282 (22.3)	+++, s	+/+	+	+/++	+++	+/++	+/++‡	0/+	Normal
Group V													
8	13	RbAShE	126 (10)	353 (28)	None, S	0/+	0/+	0	Normal/+	0	0/+§	0	Normal
9	16	RbAShE	189 (15)	349 (27.6)	None, S	0/+	0/+	0/+	Normal	0	0/+§	0	Normal
10	18	RbAShE	220 (17.5)	512 (40.6)	None, S	0	0	0	Normal/+	0	0/+§	0	Normal
11	18	RbAshE	154 (12.2)	450 (35.7)	+, S	0/+	0/+	0/+	Normal/+	0	0/+§	0	Normal

Additional explanations are provided in Materials and Methods and Results. RbIgG, rabbit IgG; FA, Forssman antigen; GpC3, guinea pig C3; S, survived; D, died; s, sacrificed.

*Tachypnea, cough, ruffling of hair, nose scratching, and convulsion.

†Edema, hemorrhage, thickening of alveolar septa, accumulation of leukocytes in alveolar or renal capillaries, and infiltration of leukocytes and erythrocytes in alveolar spaces or renal interstitium.

0, absent; +, minimal and focal in amount and extent; ++, moderate and focal; +++, marked and diffuse; ++++, very marked and diffuse; ‡+++ in glomeruli; §++/+++ in some glomeruli.

2 to 3.5% paraformaldehyde (PFA) for 20 minutes. Forssman antigen was detected by incubation with RbAShE, followed by FITC-goat antiserum to rabbit IgG. To establish the intracellular localization, some cell preparations were permeabilized (0.025% saponin or 0.1% Nonidet P-40), stained for Forssman antigen, and then incubated with rhodamine-conjugated wheat germ agglutinin (Molecular Probes, Eugene, OR), a marker of the Golgi apparatus.

Staining for Forssman antigen in sections of frozen lung and kidney obtained from normal guinea pigs and rats was performed as described,[22] with the following modifications. First, the effect of several fixatives (2% PFA, 2% periodate lysine paraformaldehyde, (PLP), 10% buffered formalin, and acetone) was studied. Second, before staining, some sections were digested for 10, 30, 60, and 120 minutes at 37 C with each of the following three enzymes: 1), 50 mU/ml endoglycoceramidase from *Rhodococcus* sp. G-74–2 (Seikagaku Kogyo Co., Nihonbashi-Honcho, Tokyo, Japan) in 10 mmol/L acetate buffer, pH 6.0 (some sections were digested with endoglycoceramidase in 10 mmol/L acetate buffer, pH 6.0, containing 0.1% m.w. of sodium taurodeoxycholine (Sigma Chemical Co.), which enhances the activity of the enzyme, at 37 C for 30 minutes and 1 and 2 hours; 2), 0.1 U/ml neuraminidase type III (Sigma Chemical Co.); and 3), 0.2 U/ml *n*-fucosidase (Sigma Chemical Co.) in 50 mmol/L sodium acetate buffer, pH 5.5, containing 9.0 mmol/L $CaCl_2$.

Guinea pig lungs and kidneys removed from naive guinea pigs or obtained from guinea pigs injected intravenously with PRS or RbAShE were fixed by perfusion with 2% PFA or by immersion in PFA or acetone. Forssman antigen was detected by indirect immunofluorescence technique as previously described.[22] Contiguous sections were first incubated with an avidin/biotin blocking kit (Vector Laboratories) followed by biotin-conjugated RbAShE and FITC-streptavidin. Rat lung and renal tissues, PRS, RbAHE, and HAGpK were used as controls. The amount and extent of immunofluorescence in lungs and kidneys was semiquantitatively graded on a scale from 0 to 4 (0, absent; +, minimal and focal; ++, moderate and focal; +++, marked and diffuse; ++++, very marked and diffuse). The interpretations were performed by two investigators without knowledge of the experimental conditions for the specimens.

Electron Microscopy

Cultured guinea pig glomerular epithelial cells were processed for immunocytochemistry as previously described.[21] In some experiments the cells were incubated with primary and secondary antibodies diluted in PBS containing 0.025% saponin. In other experiments the cells were fixed with 2% PLP for 5 minutes, washed in pure methanol for 10 minutes at 4 C, and then processed for visualization of Forssman antigen. For immunohistology, lungs were perfused with 2% PLP, dehydrated, and embedded in Epon.

Thin sections were first incubated with avidin/biotin blocking kits followed by biotin-conjugated RbAShE IgG and HRP-streptavidin (Vector Laboratories). Cultured cells incubated and lungs of guinea pigs injected with PRS, RbAHE, and HAGpK were used as controls.

Morphological Light and Electron Microscopy

Sections of lung and kidney tissues fixed in 10% buffered formalin and embedded in paraffin were stained with hematoxylin and eosin, periodic acid Schiff, and silver-methenamine. Cultured guinea pig glomerular epithelial cells and lung tissues were fixed in 2% glutaraldehyde and embedded in Epon 812. Thin sections, stained with uranyl acetate and lead citrate, were examined with a JEOL 100S electron microscope.[22]

Results

Characterization of the Immune Sera

On Western blots of guinea pig lung and kidney homogenates, RbAShE reacted with material in the region of 1.100 to 1.200 kd (Figure 1, lanes A and B) but not with rat lung and kidney homogenates (Figure 1, lanes G and H). Antisera without Forssman antibodies, RbAHE and HAGpK, reacted with lung and kidney components of higher molecular weight (Figure 1, lanes C and E (lung) and D and F (kidney)).

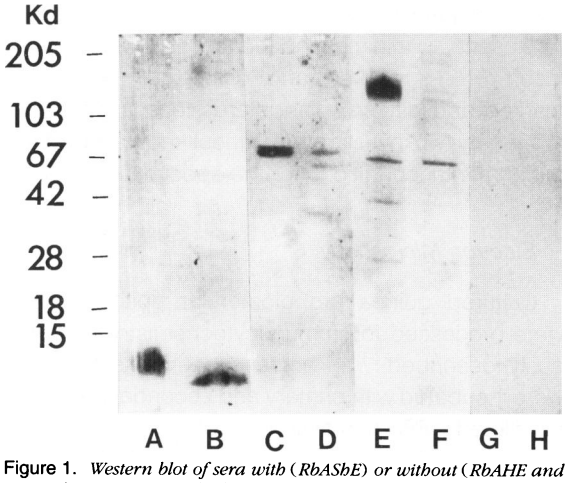

Figure 1. *Western blot of sera with (RbAShE) or without (RbAHE and HAGpK) Forssman antibodies and extracts of guinea pig (Forssman-positive) or rat (Forssman-negative) organs. RbAShE shows reactivity (1.100 to 1.200 kd) with guinea pig lung* (lane A) *or kidney* (lane B) *but not with rat lung* (lane G) *or kidney* (lane H). *RbAHE blotted with guinea pig lung* (lane C) *or kidney* (lane D) *and HAGpK blotted with guinea pig lung* (lane E) *or kidney* (lane F) *react with material of higher molecular weight.*

Localization of Forssman Antigen in Cultured Glomerular Epithelial and Aortic Endothelial Cells of Guinea Pig Origin and Experiments of Antibody-Mediated Antigen Redistribution

In unfixed glomerular epithelial cells, Forssman antigen was not detected by indirect immunofluorescence with RbAShE. After PFA fixation, the antigen was visualized at the cell surface, in a fine punctate pattern, corresponding to the microvilli (Figure 2a). After permeabilization, the antigen was found in cytoplasmic granules of various size. Double staining of permeabilized cells showed co-localization of rabbit IgG and wheat germ agglutinin in the Golgi apparatus. Immunoelectron microscopy of unpermeabilized PFA-fixed cells showed uniform distribution of Forssman antigen reaction product at the surface, especially on microvilli (Figure 2b, c). In permeabilized cells the reaction product was localized in the Golgi apparatus and in a large myelin-like structure. Incubation in methanol abolished the staining (not shown), presumably by elution as the extract contained Forssman antigen (Figure 3). PFA-fixed cells were not stained by PRS, RbAHE, and HAGpK used as primary antibodies.

Endothelial cells displayed a uniform diffuse distribution of Forssman antigen on the cell surface, although the amount per cell was variable (Figure 4A); alternatively, cells fixed in PFA containing detergent (3.5% PFA with 0.1% Nonidet P-40) revealed antigen distributed in a punctate globular pattern (Figure 4B). Incubation of cells in basal medium containing 2% RbAShE serum or its IgG fraction induced rapid (less than 5 minutes) redistribution of antigen into a punctate granular pattern, then loss from the cell surface. Simultaneously, cell margins retracted (Figure 4C). With continued exposure, for up to 24 hours, antigen-antibody complexes remained absent from the cell surface (Figure 4D); residual complexes were confined to the cell margins, in membrane blebs or vesicles (Figure 4E). The cellular margins remained retracted.

Localization of Forssman Antigen in Lungs and Kidneys of Naive Guinea Pigs

By both indirect and direct immunofluorescence, Forssman antigen was visualized in all pulmonary (Figure 5a) and renal (Figure 5b) vascular endothelia, in glomerular visceral and parietal epithelial cells, and

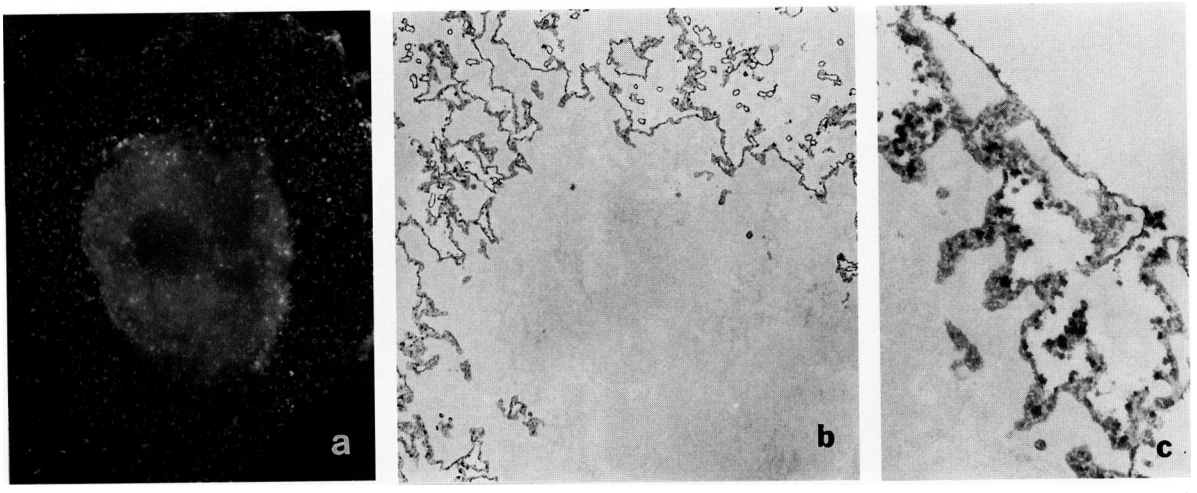

Figure 2. *Localization of Forssman antigen in cultured guinea pig glomerular epithelial cells.* a: *After fixation with 2% PFA, the antigen is localized by indirect immunofluorescence at the cell surface, mainly in microvilli.* b *and* c: *Direct immunoperoxidase electron microscopy of cells fixed in 2% PFA and 0.1% glutaraldehyde and not permeabilized. In* a, *the reaction product is localized at the cell surface, especially in microvilli. At higher magnification* (c), *granular aggregates of reaction product are localized at the surface of the plasma membrane. Magnification, ×1,000* (a), *×10,000* (b), *×25,000* (c).

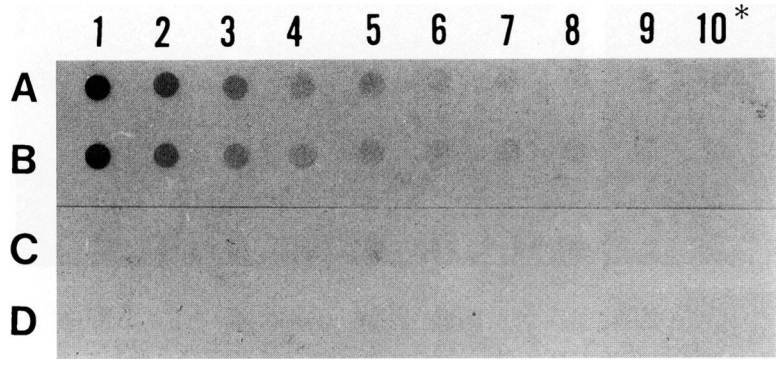

Figure 3. *Forssman antigen eluted by methanol from cultured glomerular epithelial cells of guinea pig origin. Dot blot analysis. In* A *and* B, *serial dilutions of the extract were placed on nitrocellulose paper and incubated with RbAShE followed by HRP-conjugated goat anti-rabbit IgG. Staining indicates the presence of Forssman antigen.* C *and* D *contain rat glomerular epithelial cell extract, used as control.* *, *Indicates the dilution titer* (2^{n*}).

in the cells of distal and collecting tubules (not shown). RbAGpK, containing both Forssman and basement membrane antibodies, stained endothelial and epithelial cells and the basement membranes. HAGpK, obtained from Forssman-positive hamsters, reacted only with the basement membranes. In unfixed, air-dried sections incubated with RbAShE, the deposits of rabbit IgG were fine and granular (++; not shown), but, in sections fixed by perfusion with 2% PFA, they were abundant, large, and granular (++++; Figure 5a). Sub-optimal fixation induced release of Forssman antigen from both the cell surface and cytoplasmic compartment; PBS eluates from sections of guinea pig lung and kidney contained Forssman antigen (Figure 6). Incubation of eluted sections with the Forssman-positive eluate, followed by fixation with PFA, did not reconstitute the original deposits of Forssman antigen. PRS, RbAHE, and HAGpK did not stain guinea pig or rat tissues, and rat lung and kidney eluates were consistently negative. Digestion of tissue sections with endoglycoceramidase greatly decreased or eradicated the staining (0/+), whereas neuraminidase and *n*-fucosidase only slightly decreased the intensity of staining (+/++; not shown).

Clinical Symptoms, Localization of Forssman Antigen, and Morphology of Lungs and Kidneys in Guinea Pigs Injected Intravenously with Preimmune or Immune IgG

Single and Rapid Intravenous Injection (Groups I and II, Table 2)

Injection with PRS (group I) had no apparent effect; in contrast, all guinea pigs injected with 12.6 or 21 mg of IgG from RbAShE (group II) developed tachypnea, cough, ruffled hair, nose scratching, and convulsions

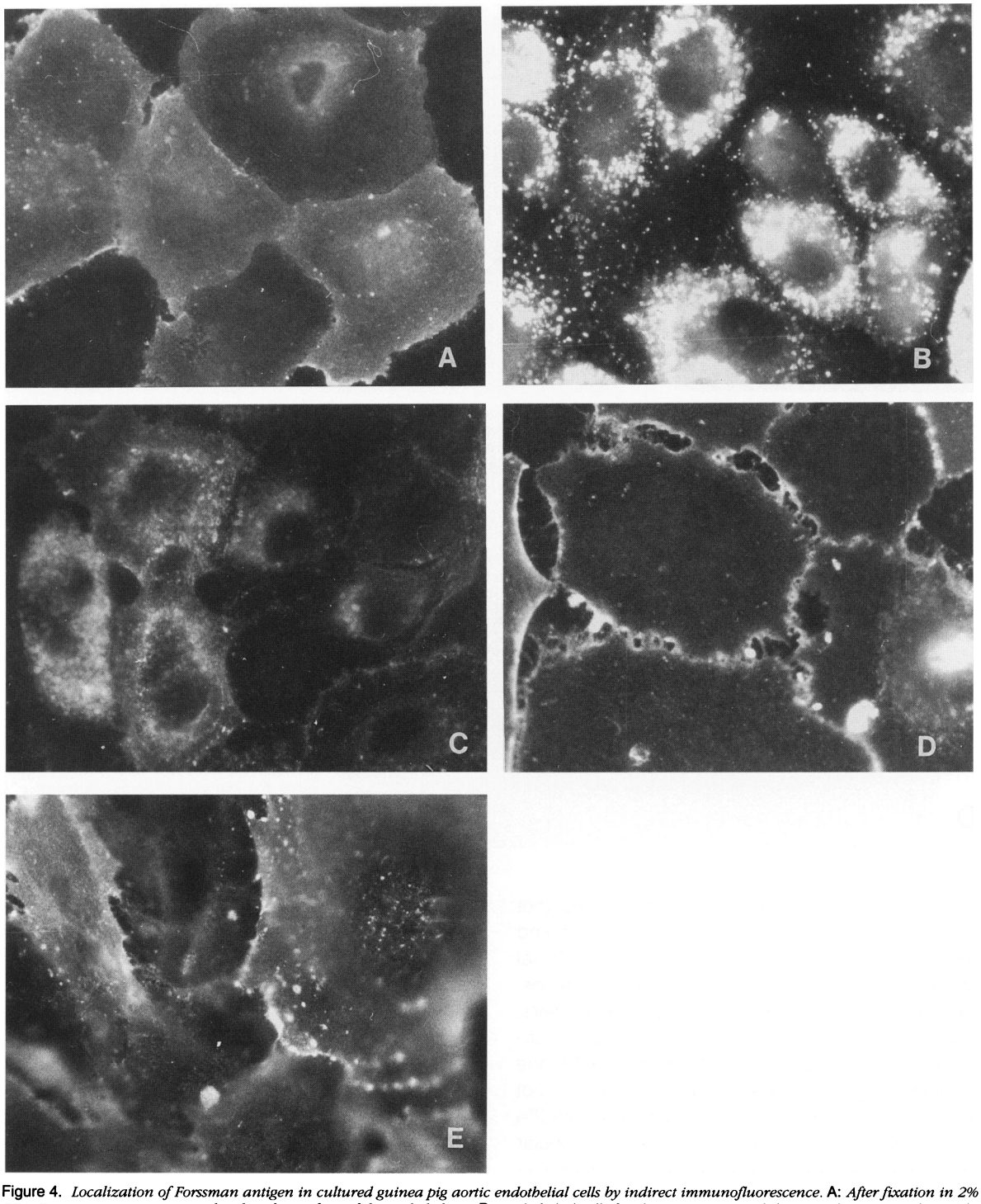

Figure 4. *Localization of Forssman antigen in cultured guinea pig aortic endothelial cells by indirect immunofluorescence.* **A:** *After fixation in 2% PFA, Forssman antigen is visualized at the surface of the endothelium.* **B:** *Endothelial cells fixed in 3.5% PFA and detergent (0.1% Nonidet P-40); punctate or globular distribution of Forssman antigen, mainly in the perinuclear region.* **C:** *Endothelial cells exposed to 2% RbASbE IgG in serum-free basal medium for 5 minutes at 37 C and then fixed in 2% PFA followed by FITC-goat anti-rabbit IgG. A patchy granular pattern of immune complexes is seen at the cell surface in the perinuclear region. The cellular margins appear scalloped, indicative of retraction. Some cells have already shed most of their immune complexes (right part of the frame).* **D:** *Endothelial cells incubated with RbASbE in basal medium for 24 hours and processed as described in* **C**. *Immune complexes have mostly been shed from the apical cell surface. Residual complexes are localized at the margins of the cells; critical focusing reveals that these are in membrane blebs or vesicles, shown at higher magnification in* **E**. *Magnification, ×600 (**A** to **D**), ×870 (**E**).*

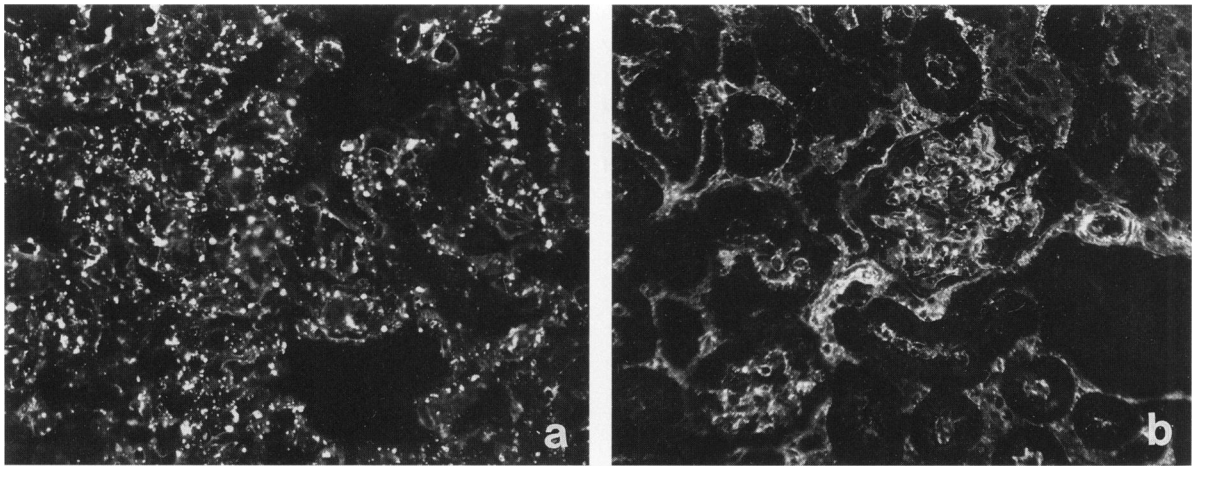

Figure 5. *Naive guinea pigs. Indirect immunofluorescence localization of Forssman antigen in lungs and kidneys fixed in 2% PFA. Granular deposits of rabbit IgG are seen in the walls of alveolar* (a) *and glomerular and peritubular* (b) *capillaries. Magnification, ×200.*

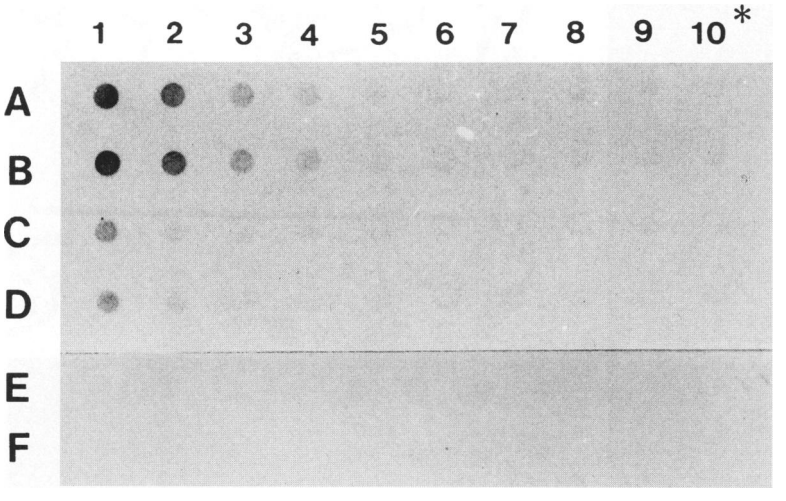

Figure 6. *Forssman antigen eluted by PBS from sections of guinea pig lungs* (A *and* B) *and kidneys* (C *and* D). E *and* F *contained rat lung and kidney eluates, respectively. The nitrocellulose paper was processed as described in the legend of Figure 3. *, Indicates dilution titer* (2^{n*}).

and died of pulmonary edema within 10 minutes (average, 4.4 minutes). Their lungs were enlarged, edematous, and hemorrhagic and there was bloody pleural effusion. Microscopy revealed swelling of the capillary endothelium with widespread adhesion of platelets and leukocytes, detachment of the endothelium from the alveolar basement membrane, edema of the alveolar septa, and intra-capillary deposits of fibrinoid material. Similar, but less severe, lesions were found in the endothelium of glomeruli and peritubular capillaries. Deposits of rabbit IgG and guinea pig C3 (++/+++) were focally distributed on the alveolar endothelium, peritubular and glomerular endothelium, and the endothelium of arteries and veins of both organs. The distribution and the amount of Forssman antigen detected by direct immunofluorescence were the same (++++) as in naive guinea pigs or guinea pigs injected with PRS (group I).

Multiple Injections Designed to Induce Adaptation (groups III, IV, and V, Table 3)

Guinea pigs 1 and 2, injected with PRS (group III), were unaffected.

Guinea pigs 3, 4, 5, and 6 (group IV) received 8 to 13 injections of RbAShE, developed pulmonary symptoms after each injection, and died of pulmonary edema after an injection of 63 mg of RbAShE (5 LD; total, 7.3 LD; Figure 7a). Deposits of rabbit IgG were decreased as compared with guinea pigs in group II (Table 2). Forssman antigen was also decreased (+/++) as compared with guinea pigs in groups I and II (Table 2) and III (Table 3). Rabbit IgG was found in numerous phagocytic, presumably septal, cells and alveolar macrophages (Figure 7b), and deposits of guinea pig C3 were minimal to moderate and focal (+/++). Rabbit IgG and Forssman antigen were also

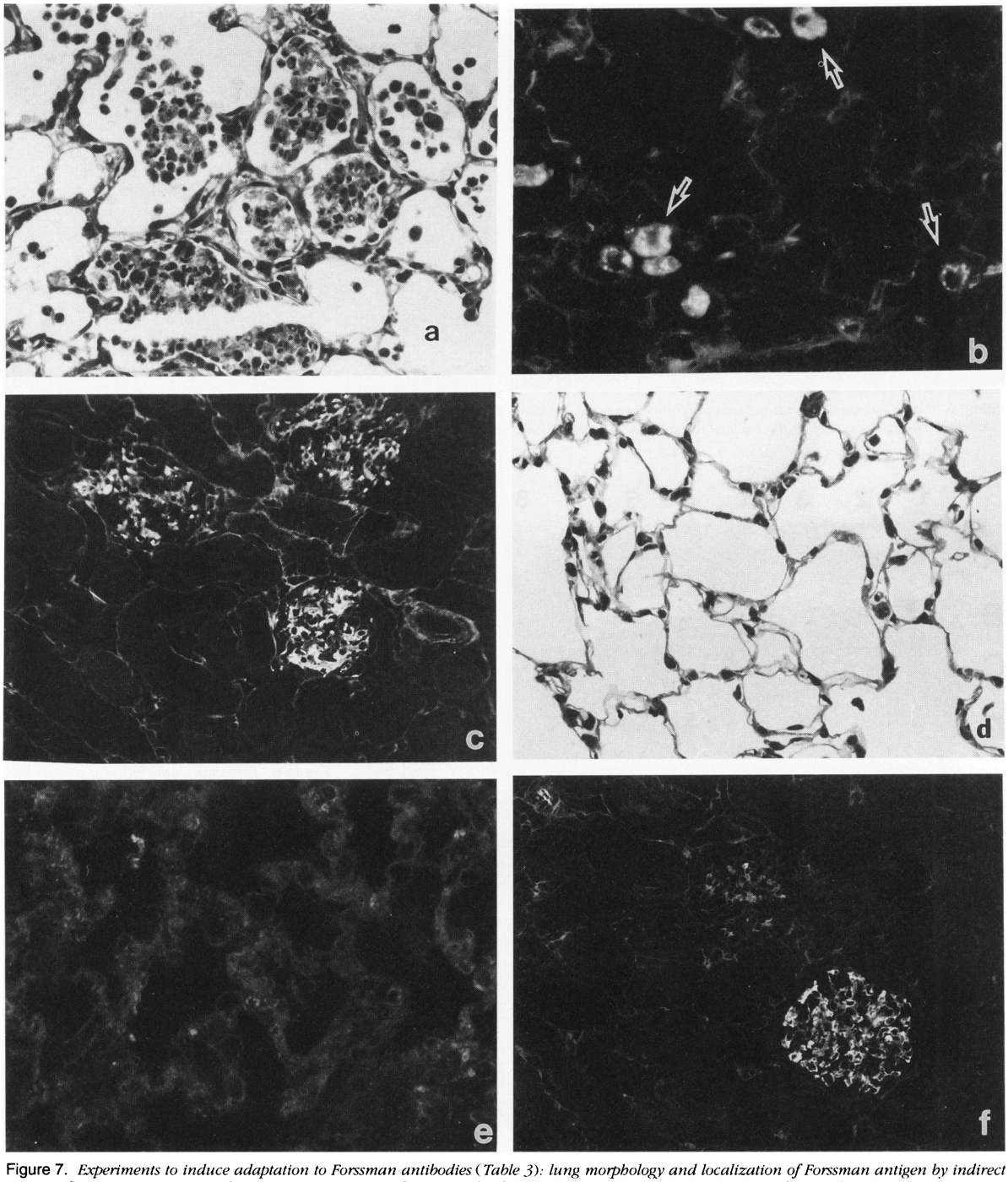

Figure 7. *Experiments to induce adaptation to Forssman antibodies (Table 3): lung morphology and localization of Forssman antigen by indirect immunofluorescence in tissues fixed with 2% PFA.* **a**: *Infiltration of inflammatory cells in the alveolar spaces of partially adapted guinea pig 7 (group IV). In partially adapted guinea pig 6 (group IV), the amount of rabbit IgG bound to alveolar capillary walls* (**b**) *and to renal peritubular capillaries* (**c**) *is diminished, as compared with naive guinea pigs (see Figure 5a, b). Abundant deposits of rabbit IgG are present in phagocytic cells and probably septal or alveolar macrophages* (**b**, **arrows**) *and in renal glomeruli* (**c**). **d**: *Normal lung morphology in completely adapted guinea pig 8 (group V). In completely adapted guinea pig 10 (group V), rabbit IgG is no longer detectable in the lung* (**e**) *and is also absent in peritubular capillaries and diminished or absent in some glomeruli* (**f**). *Magnification, ×200.*

decreased in the endothelium of peritubular capillaries (+/++) and in convoluted tubules but not in glomeruli (Figure 7c). Less severe changes were found in the lungs of guinea pig 7, which was sacrificed after an injection of 126 mg of RbAShE (10 LD; total, 22.3 LD); the amounts of rabbit IgG, C3, and Forssman antigen in its lungs and kidneys were comparable with those found in the other guinea pigs of group IV.

Guinea pigs 8, 9, 10, and 11 (group V) received 13 to 18 injections. The severity of pulmonary symptoms progressively decreased, and rapid injections of 126 to 220 mg of RbAShE (10 to 17.5 LD) were tolerated (total, 28 to 40.6 LD). The lungs (Figure 7d) and kidneys had minimal lesions or were normal. Deposits of rabbit IgG and Forssman antigen were not detected in the lungs (Figures 7e and 8b) or kidneys, with the exception of some glomeruli (++/+++; Figure 7f) in which Forssman antigen was retained in visceral epithelial cells (not shown). Deposits of guinea pig C3 were absent or minimal (0/+).

Discussion

Forssman antigen is a glycosphingolipid. The hydrophilic carbohydrates that confer the antigenic specificity lie along the surface of the plasma membrane and are attached to the hydrophobic ceramide region of the molecule anchored in the outer leaflet of the membrane.[23] The epitope, composed of 1 mol of glucose, 2 mol of galactose, and 2 mol of acetylgalactosamine,[24] is similar to blood group antigens[25] and to α-galactosyl, the principal xenoantigen of nonprimate mammals.[26–32] Helix pomata lectin binds to blood group A, α-galactosyl, and Forssman antigen.[33] Natural anti-α-galactosyl antibodies, present in high concentration in all humans,[26,27,30–34] are the main immunological barrier to transplantation of organs from New World monkeys[26,27,34] and from pigs.[29–33]

Forssman antigen is easily displaced and could be eluted by methanol or PBS from unfixed cells and tissues.[35] Optimal fixation was provided by 2 to 3.5% PFA. RbAShE reacted immunohistochemically or serologically with Forssman-positive cells and tissues or their extracts, and only such sera containing Forssman antibodies bound to endothelial cells and certain epithelial cells and could induce a generalized Forssman reaction when injected intravenously. Antibody binding was eradicated by digestion of tissues with endoglycoceramidase, which cleaves the linkage between the oligosaccharides and the ceramide.[36] Thus, the effects described were due to interaction of Forssman antibodies with the carbohydrate Forssman epitopes.

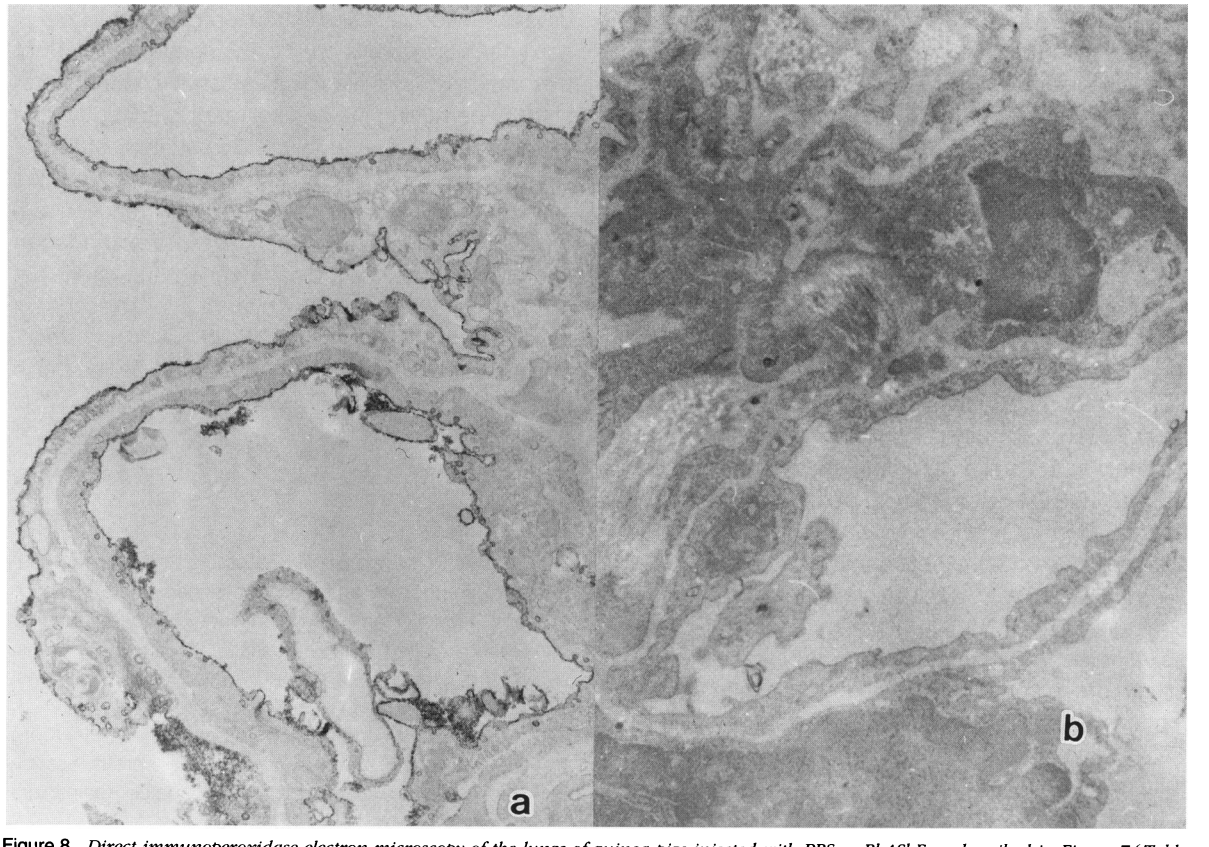

Figure 8. *Direct immunoperoxidase electron microscopy of the lungs of guinea pigs injected with PRS or RbAShE as described in Figure 7 (Table 3).* **a:** *In guinea pig 2 injected with PRS (group III), the reaction product is localized on the plasma membrane of alveolar endothelial and epithelial cells.* **b:** *In completely adapted guinea pig 10 (group V), the reaction product is absent from the plasma membrane of alveolar endothelial and epithelial cells. Magnification, ×10,000.*

In glomerular epithelial cells in culture, Forssman antigen was localized at the cell surface and also in the Golgi apparatus and in large cytoplasmic vacuoles, in agreement with studies performed by others in MDCK cells[35]; in endothelial cells, it was localized at the cell surface. Incubation of live endothelial cells with Forssman antibodies induced rapid redistribution of antigen-antibody complexes (clustering), reminiscent of that seen when cultured endothelial cells are exposed to divalent antibodies to surface protein antigens such as angiotensin-converting enzyme or thrombomodulin.[8] Shedding of Forssman antigen-antibody complexes from the cell surface appears to involve blebbing of plasma membrane and extrusion of vesicles or fragments; similar shedding of plasma membrane vesicles from oolemma had been described in rabbit oocytes exposed to angiotensin-converting enzyme antibodies.[37] In confluent monolayers of guinea pig endothelial cells, shedding was accompanied by retraction of cellular margins, which, if occurring in alveolar capillary endothelium, would result in pulmonary edema characteristic of the generalized Forssman reaction.

Guinea pigs rapidly injected with one LD of Forssman antibodies died within minutes with marked deposits of rabbit IgG and complement in their alveolar capillary endothelium (group II, Table 2); these were focal, suggesting that an acute vasoconstriction may have hindered the uniform distribution of antibodies in the pulmonary vasculature. Early signs of acute inflammation were already observed in these lungs, indicating that a rapid and massive antigen-antibody interaction activates complement,[14,38] and probably other mediators of inflammation,[39] but did not yet elicit antigenic modulation in the few minutes of survival. In contrast, guinea pigs initially injected with sublethal progressively increased amounts of antibodies (groups IV and V, Table 3) developed adaptation that was directly proportional to the degree of antigenic modulation. Complete adaptation required 27 to 40 LD injected over a period of 30 to 40 hours. Modulation of antigen at the surface of pulmonary endothelial cells was of critical importance for guinea pig survival. Similar adaptation occurs in a rabbit model in which depletion of antigen-antibody complexes from the plasma membrane of alveolar endothelium prevents fixation of C1q and activation of the complement cascade.[38]

The inflammatory lesions induced in the kidneys of guinea pigs by a single and rapid injection of antibodies (group II, Table 2) were associated with endothelial deposits of rabbit IgG and guinea pig C3 in the peritubular capillaries and, as observed in the lungs, Forssman antigen was not modulated. In contrast, partial (group IV) or complete (group V; Table 3) adaptation to antibody and complement was associated with antigenic modulation in the endothelium of peritubular capillaries and larger vessels, less in glomeruli, probably because Forssman antigen is in intracellular deposits of the podocytes. Persistence of Forssman antigen in glomeruli of guinea pigs of group V did not prevent their survival or induce a local inflammation, presumably because the antigen was intracellular. To explain those other glomeruli in which the amount of Forssman antigen was decreased, the possibility may be invoked that interaction of ligands with cell surface molecules inhibits their synthesis.[40]

Similarities between the generalized Forssman reaction and human hyperacute allograft rejection,[3] and differences between the Shwartzman reaction and experimental hyperacute allograft rejection have been described.[41] The generalized Forssman reaction,[13] hyperacute rejection of allografts in presensitized recipients,[1–3] ABO-incompatible allografts,[12,42,43] and discordant xenografts[4,5,44,45] are initiated by antibodies that bind to the vascular endothelium, activating complement and other mediators. In man and in laboratory animals, the severity of graft rejection may diminish when the levels of allo- or xeno-antibodies are decreased by pharmacological immunosuppression,[12,46–48] neutralization,[49] or absorption[50,51] of antibodies and plasmapheresis,[12,52] permitting the graft to adapt to antibody- and complement-mediated injury. By serial injections of tolerable amounts of antibodies in guinea pigs, the severity of the generalized Forssman reaction decreases, and the animals can also develop adaptation. We suggest that allo- or xenografts might become less vulnerable to hyperacute rejection, if exposed to tolerable levels of allo- or xeno-antibodies, by mechanisms of adaptation like those here described.

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