American Journal of Pathology, Vol. 137, No. 5, November 1990

Protein A-Gold Immunoelectron Microscopic Study of Amyloid Fibrils, Granular Deposits, and Fibrillar Luminal Aggregates in Renal Amyloidosis

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Glomeruli of archival renal biopsies, stored frozen at −70° C, from three patients with amyloid were examined by protein A-gold immunoelectron microscopy. In one with both fibrillar and granular deposits from a 'skin popper' drug abuser, the granular deposits were labeled with anti-IgG, while the fibrillar deposits were labeled with anti-amyloid-A (AA) protein and amyloid P component (AP), suggesting coexisting immune complex disease and AA due to different, but possibly related, pathogenesis. In studies using double-label immunostaining of primary amyloidosis-lambda light chain type (AL) and AA associated with Crohn's disease, AP occurred as widely separated single units along the amyloid fibrils and represented 1.5% and 6.5% of the total gold label in AL and AA, respectively, while the major fibril protein was labeled in single rows, similar to beads on a string. Fibrillar aggregates in the capillary lumens were labeled similarly by antisera to the major protein and AP and appeared to be contiguous with the fibrillar deposits at the glomerular basement membrane (GBM)–luminal interface, suggesting intravascular fibrillogenesis. (Am J Pathol 1990, 137: 1223–1231)

Two morphologically distinct types of deposits, one fibrillar and the other granular, have been described in tissues of patients with amyloid examined by electron microscopy.[1-3] In a previous study,[4,5] we observed four 'skin popper' drug addicts with subcutaneous abscesses, all of whom had protein A-associated amyloidosis (AA) as well as scattered granular deposits of immunoglobulin in glomeruli detected by immunofluorescence microscopy (Figures 1a and b) and fibrillar and granular deposits in the GBM ultrastructurally (Figure 1c). Because of the clinical features of chronic infection and the immunoglobulin nature of the granular deposits, we speculated that they represented immune complexes distinct from the AA fibril protein. However an alternative possibility, that the granular deposits might represent nonfibrillar form of AA protein, ie, preamyloid or degraded fibrils, as described by other investigators,[1-3] could not be resolved by the methods used. In the present study, immunoelectron microscopy was used to determine precisely the chemical and structural relationships of the two types of deposits.

Protein A-gold, a discrete, easily identified electron-dense marker, was chosen for the detection system because one antigen could be marked potentially by only one gold particle, allowing precise localization.

Materials and Methods

Archival diagnostic biopsy tissue stored frozen for up to 5 years at −60° to −70°C from three patients with amyloid were studied. All had been classified by immunofluorescence microscopy; two were AA, one associated with Crohn's disease and the other with skin abscesses in a drug addict, while the third patient had primary AL.

Immunoelectron Microscopy

Blocks of frozen tissue were fixed in freshly prepared 4% paraformaldehyde, 0.02% picric acid[6] in 0.1 mol/l (molar) sodium cacodylate buffer (pH 7.3) for 18 hours at 4°C, progressively dehydrated in ethanol (50%, 75%, 80%, 95%, and 100%) during 1 hour at 25°C, infiltrated in medium grade LR White resin (Ladd Research Industries, Inc., Burlington, VT) for 1 hour at 25°C, then embedded in the same resin in tightly capped gelatin capsules.[7] The polymerization was carried out at 50° to 55°C for 12 to

Supported in part by a Grant-in-Aid from the National Kidney Foundation of New York, New Jersey.

Accepted for publication June 29, 1990.

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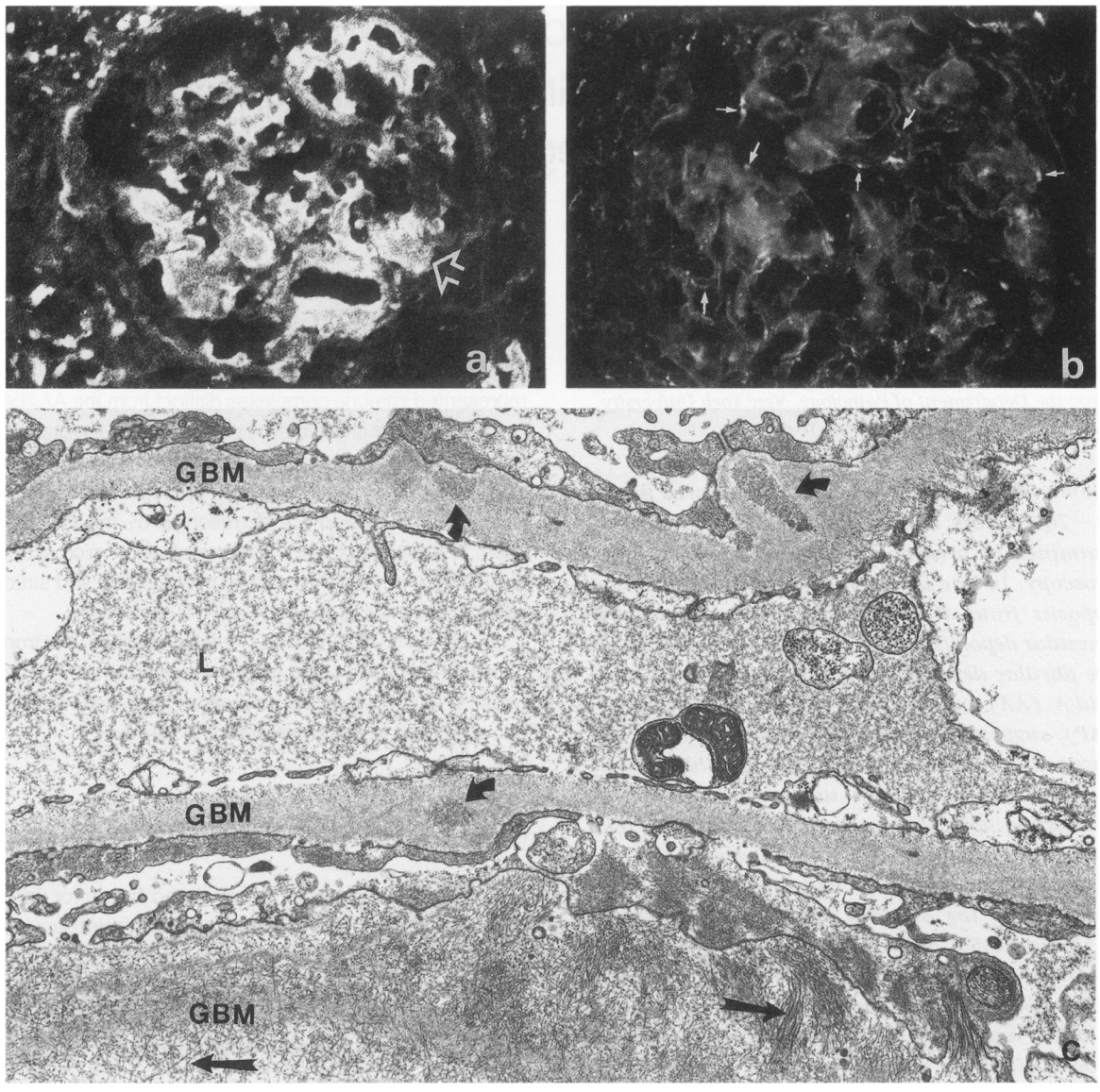

Figure 1. *Glomeruli in a diagnostic renal biopsy from a 'skin popper' drug abuser. Immunofluorescence micrographs (420×) of frozen sections show deposits reactive with anti-AA (large arrow) in* (a) *and small granular deposits reactive with anti-IgG (small arrows) in* (b). c: *An electron micrograph of tissue from the same biopsy, fixed in glutaraldehyde and osmium tetroxide, demonstrates both granular deposits (short arrows) and fibrillar deposits (long arrows) in the GBM. GBM, glomerular basement membrane; L, capillary lumen (Uranyl acetate and lead citrate, 14,900×).*

Table 1. *Density of Labeling over Two Types of Deposits and Structures in a Glomerulus from a 'Skin Popper' Drug Addict*

	Deposits		Structures		
Antibody	Granular	Fibrillar	GBM	Nuclei	Background
NRS	0.1 ± 0.1*	9 ± 6*	3.6 ± 1.6*	0.3 ± 0.1*	0.6 ± 0.1*
IgG	395 ± 146	15 ± 8†	11.0 ± 3.4†	0.2 ± 0.1*	0.4 ± 0.1*
AA	5 ± 4*	413 ± 99	1.4 ± 1.6*	0.1 ± 0.1*	3.1 ± 4.2*
AP	27 ± 15*	105 ± 29	21.5 ± 12*	0.1 ± 0.1†	0.5 ± 0.2†

Bound gold particles per μm^2 ± SD, determined by single label in parallel sections. In each horizontal column values identified with similar symbols (* or †) are not significantly different from one another. Nonidentified values are all significantly different ($P < 0.001$).

NRS, nonimmune rabbit serum; IgG, gamma globulin, heavy chain specific; AA, amyloid A-associated protein. AP, amyloid P component.

16 hours. Semithin sections (1 μm) were examined after staining with toluidine blue. Pale gold ultrathin sections of a well-preserved glomerulus were collected on bare thin-bar hexagonal 300 mesh nickel grids (Ladd) and air dried, then subjected to one of three types of immunolabel as follows.

(a) Single label[8]: Each grid was floated, section side down, on a drop of aqueous solution placed on a sheet of parafilm in a moisture chamber in the following sequence: bovine serum albumin (BSA; 1% BSA in 0.1 mol/l phosphate-buffered saline) for 2 hours at 25°C; primary antibody, ie, rabbit IgG anti-human antigens: anti-AA, 1:100; anti-AP, 1:10; anti-IgG (heavy chain specific), 1:10, anti-lambda light chain, 1:1000 (DAKO Co., Carpinteria, CA), for 15 hours at 4°C. The sections were washed serially by spinning on 10 drops of buffer (0.02% Tween in 0.1 mol/l TRIS buffered saline) on a sheet of parafilm adhered to the surface of a magnetic stirrer. After washing, each grid was placed on a drop of 10-nm protein A-gold at 1:50 dilution (Janssen Life Sciences Products, Piscataway, NJ) on a sheet of parafilm in a moisture chamber for 2 hours at 25°C, then washed serially by spinning on six drops of buffer and three drops of distilled water.

(b) Two-surface double label[9,10]: A section on the bare side of a formvar-coated nickel grid, was immunoreacted with anti-AA and labeled with 20-nm protein A-gold, 1:5 dilution (Cambridge Research Biochemicals Ltd., Cambridge, England). The formvar film then was dissolved away with chloroform to expose the unlabeled side, and the labeled side was protected by coating with formvar film. The freshly exposed side was immunoreacted with anti-IgG and labeled with 10-nm protein A-gold, 1:50 dilution (Janssen). After immunolabeling the second surface, the formvar film was dissolved away.

(c) One-surface sequential double label[11]: The surface of the section that had been incubated first with anti-AP and labeled by 10-nm protein A-gold, subsequently was floated for 16 hours at 4°C on a drop of rabbit anti-AA or anti-lambda light chain at high concentration (1:10) to block the unbound sites on the protein A molecules, followed by spinning through 10 drops of buffer and then labeling with 5-nm protein A-gold, 1:50 dilution (Janssen) in the manner described above.

Positive controls for reactions included similarly processed diagnostic biopsies with previously characterized immune complex glomerulonephritis exhibiting strong immunofluorescence staining for IgG. Negative controls included parallel sections incubated with nonimmune rabbit serum at the corresponding dilution instead of the primary antibody.

The immunolabeled sections were stained with saturated uranyl acetate in 50% ethanol for 8 minutes and washed in lukewarm 50% ethanol, 25% ethanol, and distilled water,[12] followed by Reynold's lead citrate for 0 to 4 minutes to optimize visualization of the fibrils without masking the gold label. The sections were examined at 80 KV and photographed with a Zeiss 10 A electron microscope (Carl Zeiss, Inc, Thornwood, NY).

Quantification of Immunolabel

The specificity of immunolabeling was determined by enumeration of gold particles (Tables 1 and 2) by a point-counting method.[13] A transparent 5-mm grid was superimposed on electron micrographs at 40,000× to 125,000× final magnification.

In the single labeling of parallel sections used in the case of the drug abuser with both granular and fibrillar deposits (Table 1), five micrographs were used for each structure and antibody. The density of particles labeling the different antibodies over various structures was obtained by dividing the number of gold particles by the area occupied by the structure.

In the one-surface sequential double-labeling method used to examine the case of primary AL and AA associated with Crohn's disease (Table 2), gold particles of each size (AP, 10 nm; major amyloid protein, 5 nm) were counted within an area. The percentage of AP was obtained by dividing the number of 10-nm gold particles by the total number of gold particles within a unit area. Ten areas per micrograph and five micrographs per patient were counted.

Results

Immunoreactivity of the Granular and Fibrillar Deposits in the Drug Abuser with Chronic Skin Abscesses

Single Labeling of Two Types of Deposits in Parallel Sections

As illustrated in Figure 2 and quantitatively tabulated in Table 1, the granular deposits were labeled mainly by

Table 2. *Density of Labeling over Amyloid Deposits in GBM and Fibrillar Aggregates in Capillary Lumens in Primary AL and in AA Associated with Crohn's Disease*

Diagnosis	Amyloid deposits	Luminal fibrillar aggregates	Background
AL	382 ± 56	96 ± 14	2.3 ± 1.4
(NRS)	1.1 ± 0.3*	0.5 ± 0.3*	0.9 ± 0.3*
AA	368 ± 78	106 ± 33	1.1 ± 0.1
(NRS)	10 ± 5.2*	14.5 ± 2.6*	1.5 ± 0.5*

Bound gold particles (AP plus AA or AL) per μm^2 ± SD, determined by one-surface sequential double label (anti-AP followed by anti-lambda in AL and anti-AP followed by anti-AA in AA secondary to Crohn's disease).

In each horizontal column values identified with * are not significantly different from one another. Nonidentified values are all significantly different ($P < 0.001$).

GBM, glomerular basement membrane; AP, amyloid P component; AL, primary amyloid-lambda light chain type; AA, amyloid A-associated protein; NRS, nonimmune rabbit serum.

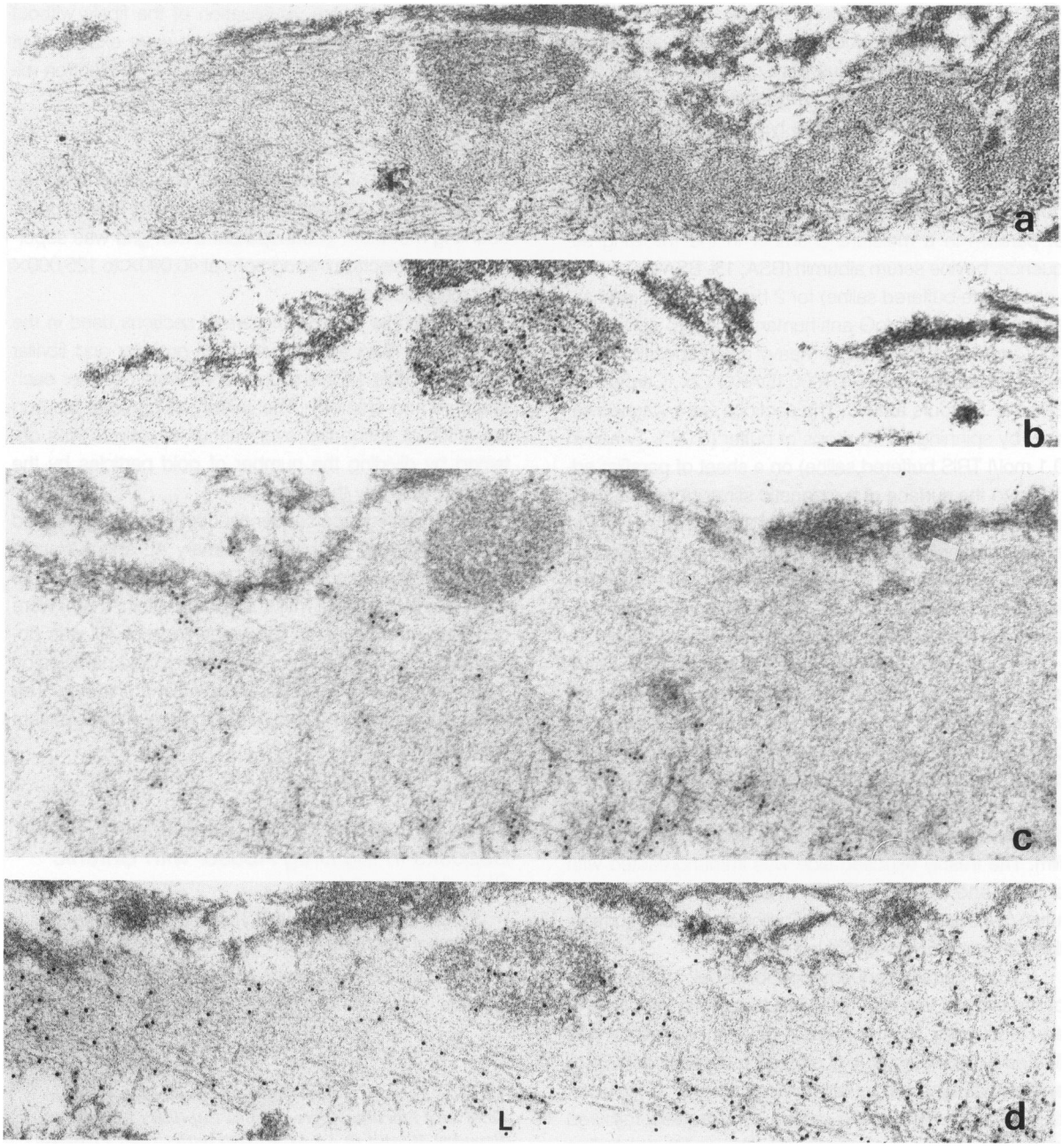

Figure 2. *Protein A-gold immunoelectron micrographs of the above biopsy, single labeled (10 nm gold), in parallel sections incubated with* (a) *nonimmune rabbit serum,* (b) *anti-IgG,* (c) *anti-AA, and* (d) *anti-AP. Granular deposits are mainly reactive with anti-IgG in* (b) *and the fibrillar deposits are mainly reactive with anti-AA in* (c) *and anti-AP in* (d). *Neither granular nor fibrillar deposits are labeled after incubation with nonimmune serum in* (a). *L: capillary lumen (Uranyl acetate and lead citrate, 63,000×).*

anti-IgG (Figure 2b). By contrast, the fibrillar deposits were labeled mainly by both anti-AA and anti-AP (Figures 2c and d). Control sections incubated with nonimmune serum showed no significant labeling of either the granular or the fibrillar deposits (Figure 2a).

Two-surface Double Label

A single field with both types of deposits illustrates the differential distribution of label in Figure 3. The multiple granular deposits were decorated with 10-nm gold bound to anti-IgG on one surface of the section, and the fibrillar deposits with the 20-nm gold bound to the anti-AA on the other surface of the section.

Quantification of Labels

The distribution of particle density in the two types of deposits, as determined by single label in parallel sections,

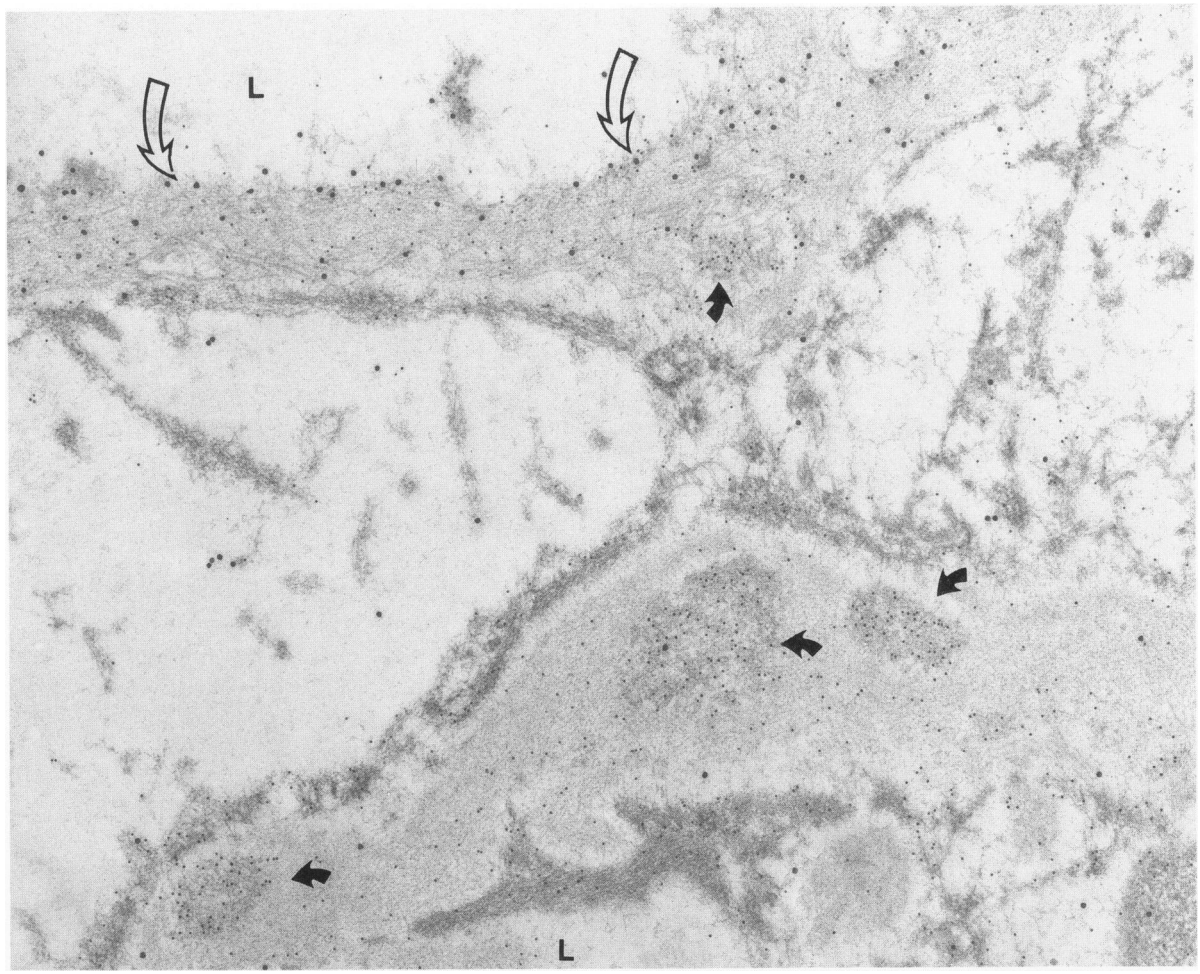

Figure 3. *Protein A-gold immunoelectron micrograph of the above biopsy, examined with two-surface double label, demonstrates that the granular deposits (solid arrows) are decorated with small gold particles (10 nm) bound to anti-IgG and the fibrillar deposits (hollow arrows) are decorated with large gold particles (20 nm) bound to anti-AA. L: capillary lumen (Uranyl acetate and lead citrate, 40,000×).*

is shown in Table 1. The fibrillar deposits were labeled with anti-AA to a greater degree than were the granular deposits (413 ± 99 *versus* 5 ± 4; $P < 0.001$). By contrast, the granular deposits were labeled with anti-IgG to a greater degree than were the fibrillar deposits (395 ± 146 *versus* 15 ± 8; $P < 0.01$). The binding of anti-AP to the granular deposits was not different from that in the GBM. Control reactions, in which nonimmune rabbit serum was substituted for immune serum, showed significantly lower binding as compared to the specific antibodies. Background staining was minimal. There was greater binding of anti-AP and anti-IgG in the GBM than in the control incubated with nonimmune rabbit serum.

Major Amyloid Protein and AP in Fibrils

Using the different methods, labeling of fibrils in AA associated with Crohn's disease and primary AL was examined.

Single Label

Amyloid P component was detected with quasi regularity at approximately 45-nm intervals along the AA fibrils in parallel arrays forming spicules (Figure 4a).

One-surface Sequential Double Label

Labeling of both AP and the major amyloid protein on the same surface of the section by different sized protein A-gold demonstrated their structural relationship along the same fibril in Figures 4a and b. The gold particles bound to the AA fibrils (Figure 4b) and AL fibrils (Figure 4c) were arranged in single rows along the fibrils, similar to beads on a string. As expected, there were fewer gold particles bound to AP than to the major protein constituent.

Quantification of Label Density

As determined by one-surface sequential double labeling of fibrils, the percentage of AP in both AA fibrils

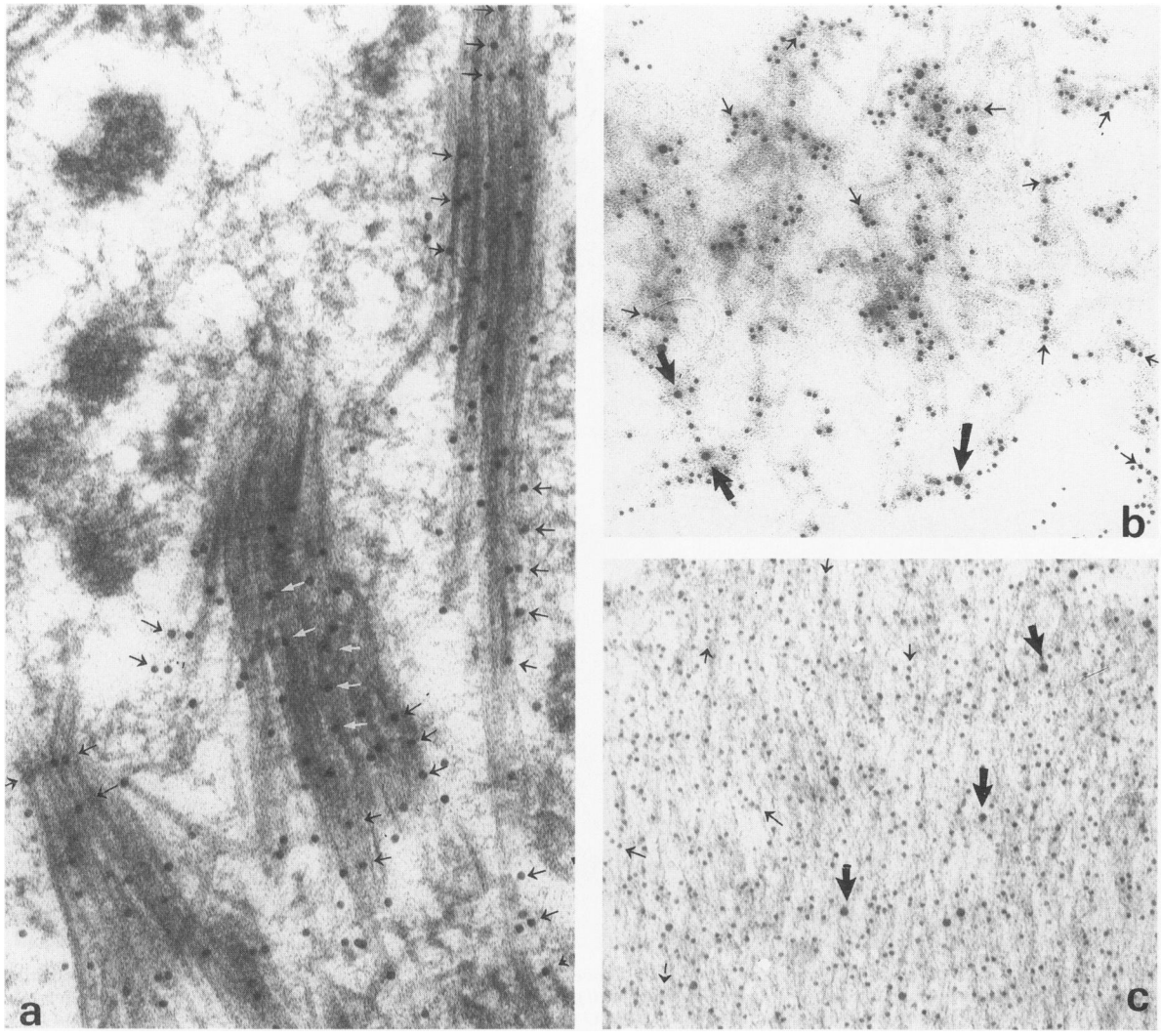

Figure 4. *Protein A-gold immunoelectron micrographs of a glomerulus with AA associated with Crohn's disease* (a, b) *and primary AL* (c). *In* (a) *the section examined with single label (10 nm) demonstrates the quasi periodicity of AP (small arrows) at 45-nm intervals along the parallel arrays of AA fibrils in a spicular formation. In* (b) *the same biopsy, examined with one-surface sequential double label, demonstrates that AP (10-nm gold probe, large arrow) occurs as widely spaced single units, while AA units (5-nm probe, small arrows) are closely spaced along the fibrils, as beads on a string. In* (c) *primary AL, examined with one-surface sequential double label, a similar arrangement of AP (10 nm) and lambda light chain (5 nm) is seen (Uranyl acetate and lead citrate in a and c, uranyl acetate only in b, ×125,000).*

and AL fibrils is obtained. The AA fibrils showed higher binding of AP than the AL fibrils (6.11% ± 0.78% *versus* 1.65% ± 0.28%; $P < 0.001$).

Luminal Fibrillar Aggregates

In the capillary lumens of glomeruli with AA and AL, there were fibrillar aggregates that appeared to be composed of short segments of curvilinearly arranged units at high magnification (Figure 5, top insert). The immunohistochemical nature of the luminal aggregates and their relationship to the amyloid deposits in the GBM was examined by single and double labeling.

Single Label

The density of gold particles in the luminal aggregates labeled with anti-lambda was in contrast to the paucity of particles in the background (Figure 5). High magnification of the GBM-luminal interface demonstrated that the fibrillar aggregates were contiguous with the amyloid fibrils in the GBM *via* the endothelial fenestrae (Figure 5, bottom insert). Similar luminal filamentous aggregates in AA were labeled by anti-AA. In both AL and AA, luminal aggregates were labeled also by anti-AP but proportionately less so with antibody to the major amyloid protein.

One-surface Sequential Double Label

The luminal aggregates were labeled by both anti-AP and anti-amyloid major protein, with the latter arranged in

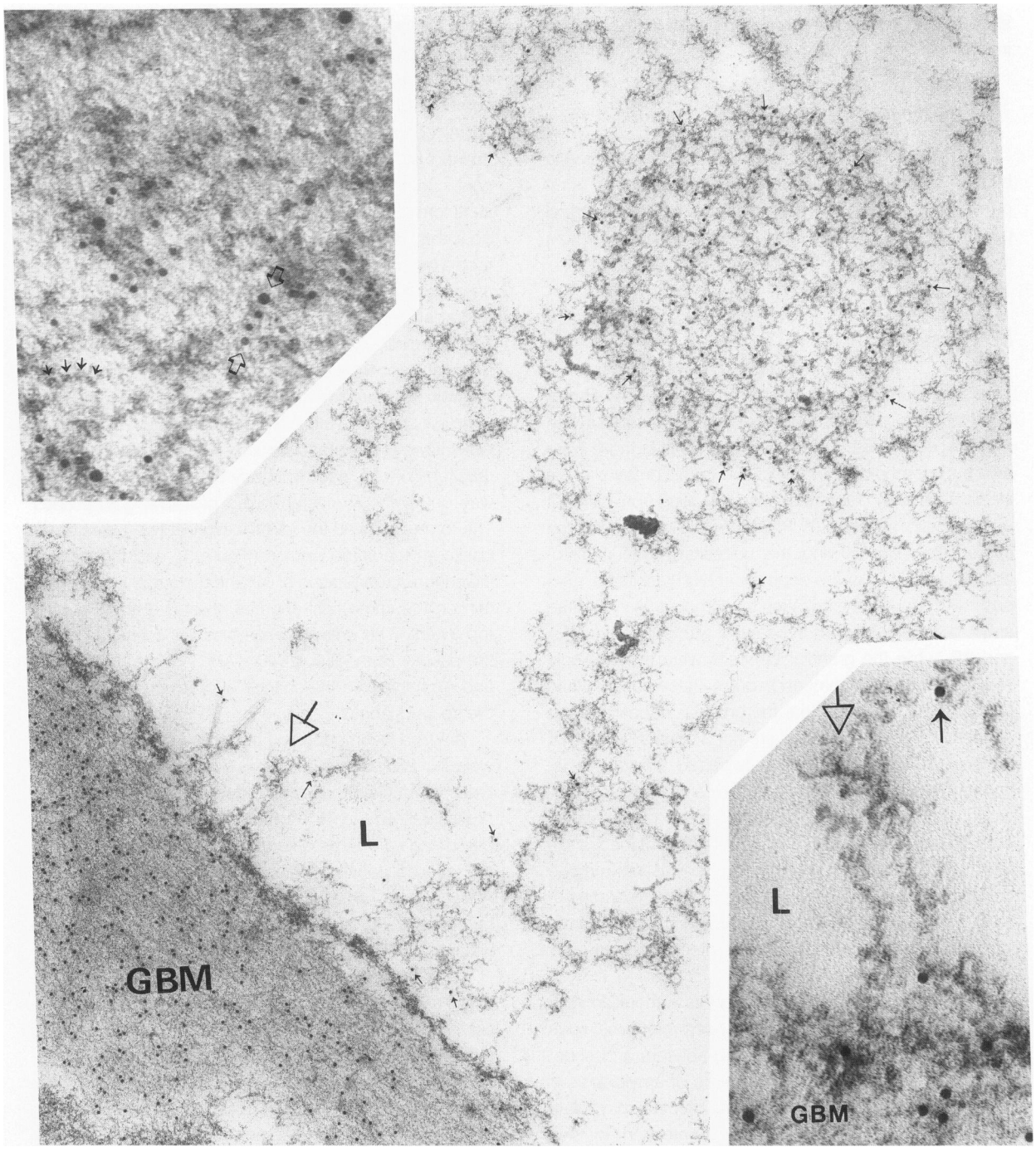

Figure 5. *Protein A-gold immunoelectron micrographs of a glomerulus with primary AL, examined with single label (10 nm), demonstrates that both the luminal fibrillar aggregate and the AL fibrils in the GBM are specifically labeled with anti-lambda antibody (arrows).* Bottom insert: *High magnification of the GBM-luminal interface (open triangle) demonstrates the continuity of the filamentous luminal aggregate to the amyloid fibrils in the GBM* via *endothelial fenestrae (arrow, 10 nm gold).* Top insert: *Same biopsy, examined with the one-surface sequential double label, demonstrates that the luminal fibrillar aggregates are labeled with both anti-AP (10 nm) and anti-lambda (5 nm). The top hollow arrow points to a single 10-nm gold probe labeling AP and the bottom hollow arrow points to three linearly arranged 5-nm gold probes labeling lambda. Small arrows point to the units forming a segment of a curvilinearly arranged filamentous aggregate, unexposed to the surface of the plastic section and consequently unlabeled by the gold probes. GBM, glomerular basement membrane; L, capillary lumen (Uranyl acetate and lead citrate, main micrograph 63,000×; inserts 250,000×).*

single rows and the former in single units (Figure 5, top insert).

Quantification of Label

As summarized in Table 2, there was apparent specific binding of antibody to the luminal aggregates, as compared to the infrequent labeling at these sites in control parallel sections that were reacted with nonimmune serum in both AL and AA.

Discussion

The concurrence of two types of deposits, one fibrillar and the other granular, in glomeruli of drug addicts with skin abscesses raised the question of whether these were different morphologic forms of the same protein, or instead, chemically different deposits resulting from different pathogenetic mechanisms. Protein A-gold immunoelectron microscopy, by allowing the necessary immunohistochemical analysis of the deposits at high resolution, successfully resolved the question by demonstrating that the amyloid fibrils were immunoreactive with antibody to AA and AP, while the granular deposits were reactive only with the antibody to immunoglobulin. These observations support the conclusion that the granular deposits in the glomeruli of the drug abuser are not a nonfibrillar form of AA protein, ie, preamyloid or degraded amyloid fibrils, and more likely are immune complexes resulting from different, but possibly related, pathogenetic mechanisms. Thus, in the case of drug addicts with subcutaneous abscesses induced by 'skin popping,' coexisting immune complex disease and amyloidosis may be a common occurence given the conditions of chronic antigenic stimulation and prolonged suppurative infection caused by staphylococci.

Immune complex glomerulonephritis without amyloid is a complication of other staphylococcal infections, such as endocarditis in intravenous drug abusers and shunt infections.[14] The presence or absence of amyloidosis in these different conditions of bacterial infection may relate to the duration, site, and intensity of infection. In 'skin poppers,' large surface areas may have abscesses for prolonged periods.[4] Of interest in this regard is the experimental murine model of AA produced during a period of 6 to 8 weeks by daily subcutaneous injections of casein and chemical irritants that cause extensive necrosis and abscesses.[15]

The issue of the coexistence of granular and fibrillar deposits is important in relation to the broader question of the mechanism and sites of fibril formation in amyloidosis, which is still unresolved. Other investigators, who noted both forms of deposits to be reactive with anti-AA detected by peroxidase-antiperoxidase immunoelectron microscopy, suggested that the nonfibrillar deposits may represent a preamyloid form of the protein before full assembly into mature fibrils.[1] Nonfibrillar deposits in the cerebral cortex of Alzheimer's and Down's patients, examined by immunogold labeling at the electron microscopic level, support the concept of preamyloid and the local formation of amyloid fibrils.[16] Still other investigators described nonfibrillar deposits reactive with anti-AA detected by the immunoperoxidase method in resolving renal amyloidosis and suggested that granular deposits are degradative products of amyloid fibrils.[3] Thus, because both possibilities exist, the determination of the nature of granular deposits coexisting with amyloid fibrils requires immunohistochemical analysis at the ultrastructural level.

In our study, we exploited double-labeling methods by either a two-surface reaction or by a one-surface sequential reaction in which each antibody bound to antigenic sites was detected by different-sized protein-A gold probes. The former allowed the identification of two types of deposits on the same section (Figure 3), and the latter allowed the colocalization of AP and the major amyloid protein on the same fibril (Figures 4b and c and Figure 5, top insert). The colocalization was successful because the paucity of the first label to AP (1.5% to 6.5%) in AL and AA minimized the chance of cross-contamination of the second label.

Amyloid P component, a glycoprotein with a molecular weight of 235,000 daltons,[17] has been found in all chemical types of amyloid thus far examined and constitutes approximately 6% to 14% of the amyloid fibril weight. The percentage of AP, as determined by the quantification of the labeling density in electron micrographs of AL and AA, was less (1.6% to 6.5%, respectively) than that shown by others using chemical methods (10% to 15%) of fibril weight.

In the AA fibrils arranged in parallel arrays on the surface of the section, periodicity of AP along the fibrils was observed (Figure 4a). However in most views the fibrils were arranged randomly in complex networks so that the precise configuration of AP on individual fibrils was not discernible in sections. Protein A-gold labels only the antibody bound to antigens exposed on the surface of the section, while deep fibrils unexposed are not accessible to the gold probe.

The nature of the filamentous aggregates observed in the lumens of glomerular capillaries with AL (Figure 5) and AA disease is uncertain. They resemble the finely granular and filamentous material in lumens described in an earlier comprehensive study of renal amyloidosis.[18] Our study with probes demonstrates the binding of anti-lambda or anti-AA to the amyloid fibrils in the GBM, as well as the fibrillar aggregates in the lumen and the filamentous material at the GBM-luminal interface in which the filaments

appear to be contiguous with fibrillar deposits *via* the endothelial fenestrae. These observations suggest the possibility of intravascular fibrillogenesis.

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