

Transcytosis of Albumin in Capillary Endothelium

A. J. Milici,* N. E. Watrous,* H. Stukenbrok,† and G. E. Palade‡

Departments of *Surgery and †Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract. We have used a variety of immunocytochemical procedures to localize albumin in transit through the capillary endothelium of the murine myocardium and thereby identify endothelial cell structures involved in albumin efflux. The most informative results were obtained with a protocol that included (a) removal of endogenous albumin by perfusion of the heart with PBS supplemented with 14 mM glucose, (b) perfusion of the heart vasculature with exogenous (bovine) albumin for various short time periods, (c) fixation of the vessels by formaldehyde-glutaraldehyde mixtures, (d) processing of fixed myocardium specimens through L. R. White embedding followed by sectioning, or direct thin frozen sectioning, and (e) reacting the surface of such specimens with antialbumin antibodies followed by gold-labeled secondary

antibodies. The results indicate that (a) monomeric albumin binds (with low affinity) to the luminal surface of the capillary endothelium, (b) it is restricted in transit through the endothelium to plasmalemmal vesicles, and (c) it appears in the pericapillary spaces <15 s after the beginning of its perfusion. No albumin concentration gradients, centered with their maxima on the exits from intercellular spaces, were detected at any time points, including the shortest ones (15 and 30 s) investigated. Additional information comparing monomeric vs. polymeric albumin transcytosis was obtained using albumin-gold complexes. The results are discussed in terms of vesicular transport of albumin across the endothelium and the relations of this type of transport to the postulated pore systems of the physiological literature.

WITHIN the cardiovascular system, albumin performs a variety of functions, including (a) maintenance of the oncotic pressure of the blood plasma (13), (b) regulation of the permeability of the microvasculature by forming a "fiber matrix" through interaction with the ectodomains of the proteins of the luminal plasmalemma of the endothelium and its associated structures (6), and (c) transport of fatty acids, steroids, hormones, and bilirubin within the circulation and between the plasma and interstitial fluid (2, 5, 27). In part, the carrier functions of albumin involve its own transport across the vascular endothelium, primarily at the level of the microvasculature as evidenced by its presence in varied but characteristic concentrations within the interstitial fluid and lymph associated with, or derived from, all microvascular beds.

At present, the pathways, forces, and mechanisms involved in albumin transport from the blood plasma to the interstitial fluid are issues on which there is no general agreement. Data obtained in physiological experiments have been interpreted in terms of albumin moving by diffusion and convection through a system of small (≤ 12 nm diam) and large (20–50 nm diam) pores (10, 15, 20) widely assumed to be located in the intercellular junctions of the endothelium (13, 20). Movement through these pores is presumably regulated by the fibrous matrix and controlled by concentration gradients and pressure differentials.

The role of the plasmalemmal vesicles of the endothelial cells in the transport of albumin and other solutes is still under debate. Although attempts to visualize directly the path-

way followed by albumin from the blood plasma to the interstitial fluid have implicated primarily these vesicles, the results cannot be considered definitive. For instance, with horseradish peroxidase (HRP)-labeled¹ antibodies, plasma proteins (including albumin) were localized primarily to plasmalemmal vesicles. The findings were taken to indicate vesicular transport of albumin (23, 30), but other pathways were not convincingly ruled out because of the excessive amounts and extensive spreading of the reaction products generated in the pericapillary spaces by the technique used. Albumin-gold (AuBSA) complexes were localized with remarkable specificity to the plasmalemmal vesicles of capillary endothelia by Ghitescu et al. (8). Binding was shown to be saturable and competitively by monomeric albumin, but transport across the endothelium was found to be unexpectedly slow. AuBSA complexes have large dimensions and probably behave like polymerized albumin, which may influence binding, dissociation, and even rates and pathways of transport.

In this paper we describe experiments in which we have used monomeric albumin as tracer and gold-tagged antibodies as detector. The results obtained indicate that, in murine myocardial capillaries, albumin is rapidly transported to the pericapillary spaces (exit time, <15 s) apparently exclusively through plasmalemmal vesicles.

1. *Abbreviations used in this paper:* AuBSA, albumin-gold; HRP, horseradish peroxidase; PBSG, PBS supplemented with 14 mM glucose; PBSO, PBS containing 1% ovalbumin.

Materials and Methods

Animals

Male Swiss albino mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighing 20–25 g were used in all experiments. All animals were allowed free access to food and water.

Materials

Reagents and other supplies were obtained as indicated below: PBS from Gibco, Grand Island, NY; BSA fraction V, ovalbumin grade V, polyvinylpyrrolidone (*M*, 10,000), polyvinyl alcohol (*M*, 10,000), paraformaldehyde and 3-3' diaminobenzidine from Sigma Chemical Co., St. Louis, MO; glutaraldehyde from Electron Microscopy Sciences, Fort Washington, PA; L. R. White resin, medium grade, from Ernest F. Fullam, Inc., Latham, NY; HRP goat anti-rabbit IgG, tannic acid, and BEEM capsules from Polysciences Inc., Warrington, PA; rabbit anti-BSA from CooperBiomedical, Inc., Malvern, PA; goat anti-rabbit IgG-Au₅ (GAR₅) from Janssen Life Sciences Products, Piscataway, NJ; sodium sulfite and polyethylene glycol (*M*, 20,000) from J. T. Baker Products, Phillipsburg, NY.

Experimental Protocol

Under light ether anesthesia, a thoracotomy was performed and a cannula, connected to a constant pressure perfusion apparatus, was inserted into the left ventricle. The right atrium was cut open to allow drainage during the rest of the procedure. The blood was then removed by perfusing the animal with 30–40 ml of PBS supplemented with 14 mM glucose (PBSG). The PBSG and all other solutions were warmed up to 37°C and oxygenated before use. The perfusion was carried out at a rate of 3.5 ml/min and at a pressure of 90 mmHg. Preliminary tests showed that this was sufficient to remove endogenous albumin from the microvasculature as well as from most of the pericapillary spaces. The vasculature was first perfused with PBSG containing 30 mg/ml BSA for 15 s, 30 s, 1 min, 2 min, 4 min, or 5 min (this step was omitted in the control experiments), and then with a mixture of 4% formaldehyde–0.5% glutaraldehyde in 0.1 M Na cacodylate-HCl buffer, pH 7.4. In some experiments this fixation step was preceded by a 1-min flush with PBSG. The myocardium was then removed, cut into small blocks which were immersed in the same fixative for up to 18 h. The blocks were then washed in 0.2 M Na cacodylate-HCl buffer, pH 7.4, (2 × 10 min) and processed as described below.

Immunogold Procedure

L. R. White-embedded Specimens. The washed tissue blocks were dehydrated through a series of graded ethanol solutions (70, 80, and 95%) starting at 4°C and immediately going to –20°C. They were subsequently infiltrated in a 70/30 mixture of L. R. White resin and 95% ethanol (1 h at –20°C) and then left overnight in 100% L. R. White resin (–20°C). The next day the blocks were transferred to fresh resin in BEEM capsules and the resin was allowed to polymerize at –20°C. Silver-to-gray thin sections were cut on an Ultracut E microtome (Reichert Scientific Instruments, Buffalo, NY) and were picked up on nickel grids. Sections on grids were then floated (sectionside down) on the following solutions: (a) PBS containing 1% ovalbumin (PBSO), 1 × 15 min; (b) rabbit anti-BSA, diluted 1:500² in PBSO, 1 × 90 min; (c) PBSO washes, 6 × 10 min; (d) goat anti-rabbit IgG-Au₅, diluted 1:500 in PBSO, 1 × 90 min; (e) PBSO washes, 6 × 10 min; (f) 2% OsO₄ in 0.1 M Na cacodylate-HCl buffer, pH 7.4, 1 × 15 min; (g) deionized distilled water washes, 3 × 2 min; (h) 1% tannic acid in 0.1 M Na cacodylate-HCl buffer, pH 6.5, 1 × 3 min; (i) 1% sodium sulfite in the same buffer, 1 × 1 min; (j) deionized distilled water washes, 3 × 1 min. Finally, the sections were lightly stained with lead citrate, and examined and micrographed in a Phillips 301 electron microscope.

Thin Frozen Sectioned Specimens. Washed tissue blocks were processed according to the method of Tokuyasu (28, 29, and personal communication), which requires tissue infiltration for 1–2 h with 50% polyvinylpyrrolidone containing 2.3 M sucrose in 0.1 M phosphate buffer, pH 7.4, followed by mounting on metal nails and freezing in liquid nitrogen. Thin frozen sections were cut on glass knives with an ultramicrotome provided with a FC-4 Cryo attachment (Reichert Scientific Instruments).

2. Standard ELISAs were carried out on the rabbit anti-BSA to find out to what extent it binds to murine albumin. The results indicate that there is limited cross-reactivity: the maximum dilutions for antigen recognition were 1:2,000 and 1:250 for bovine and murine albumin, respectively.

They were picked up on a drop of 1.92 M sucrose containing 0.75% gelatin in 9 mM phosphate buffer, pH 7.4, and placed on nickel grids. Sections on grids were then processed as follows: steps *a* to *f* as in the previous section, except that each washing step was reduced to 30 s; (g) 2% glutaraldehyde in PBS, 1 × 5 min; (h) PBS wash, 1 × 1 min; (i) 2% OsO₄ in deionized distilled water, 1 × 15 min; (j) deionized distilled water washes, 2 × 1 min; (k) 0.5% uranyl acetate in veronal acetate buffer, pH 6.0, 1 × 15 min; (l) 2.5% polyvinyl alcohol containing 0.0015% lead citrate, 2 × 2.5 min. The grids were air dried after removing excess fluid.

AuBSA Investigations. 7-nm gold particles were produced by a tannic acid–sodium citrate reduction of a chloroauric acid solution (17). Preliminary experiments showed that stable complexes could be obtained by mixing equal volumes of a gold particle suspension and a 3 mg/ml BSA solution followed by vortexing for several minutes and by adding polyethylene glycol to a final concentration of 0.01%. Excess albumin was then removed by pelleting the AuBSA particles at 150,000 *g*_w for 45 min followed by resuspension in PBS and repelleting. The final pellet was resuspended in a minimal amount of PBS and used immediately.

The experimental protocol using the AuBSA particles was essentially identical to that using the free albumin except that the AuBSA was perfused for up to 17 min, unbound AuBSA was removed with a 2-min PBSG flush, and animals were fixed by perfusion with 3% glutaraldehyde in 0.1 M Na-cacodylate-HCl buffer, pH 7.4. Myocardium tissue specimens were then processed, embedded, and sectioned for electron microscopy according to procedures previously described (16).

Immunoperoxidase Localization of BSA. AuBSA particles were prepared and perfused as described above except that the fixative in these experiments was 4% formaldehyde–0.5% glutaraldehyde in 0.1 M Na-cacodylate-HCl buffer, pH 7.4. After fixation, tissue blocks were cut into 20-μm-thick sections using an Oxford vibratome and the sections were processed through the following steps: (a) PBSO, 1 × 30 min; (b) rabbit anti-BSA, diluted 1:200 in PBSO, 1 × 2 h; (c) PBSO washes, 6 × 10 min; (d) HRP-goat anti-rabbit IgG, diluted 1:200 in PBSO, 1 × 2 h; and (e) PBSO washes, 6 × 10 min. The sections were then reacted for 20 min (22°C) in a modified Graham-Karnovsky incubation medium (9) that contained 1 mg/ml diaminobenzidine and 0.01% H₂O₂ (final concentration) in 0.1 M monobasic phosphate. After incubation, the sections were routinely processed for electron microscopy. Controls included omission of primary or secondary antibody and incomplete peroxidase reaction media.

Morphometry. To determine the percentage of labeled vesicles, we examined 10 random low magnification (18,000×) micrographs of L. R. White-embedded capillaries reacted as described above for each condition (controls, 15 s BSA perfusion and 1 min BSA perfusion) and counted in each case a minimum of 1,000 vesicles.

To determine if there was a gradient of albumin in the extravascular space in association with endothelial cell junctions, we examined 10 micrographs of cross-sectioned ultrathin frozen capillaries after either 30 or 15 s BSA perfusion. We selected narrow (~0.3 μm) pericapillary spaces on the assumption that this would increase the chances of detecting albumin gradients, if present, and excised therefrom samples that included the capillary basal lamina and the entire width of the pericapillary spaces. Two types of samples were collected from each micrograph: (a) 0.4 μm in length subtending an intercellular junction; (b) 0.5 μm in length located on each side of *a*. The number of gold particles in each sample was counted, the excised micrograph pieces were weighed, and particle counts were standardized to 1 μm² by dividing the total weight of each sample by the weight of a 1-μm² photographic paper standard.

Results

AuBSA Complexes

At all time points examined, AuBSA particles were found preferentially associated with luminal plasmalemmal vesicles. There was no extensive labeling of the luminal surface of the endothelium (Fig. 1 *A*) and occasional particles were seen in the infundibula leading to intercellular junctions but not within the junctions themselves. AuBSA particles were frequently found in slight depressions in the luminal plasmalemma, or dimples, as described by Ghitescu et al. (8). The particles were not removed from luminal plasmalemmal vesicles by flushing for several minutes with a protein-free

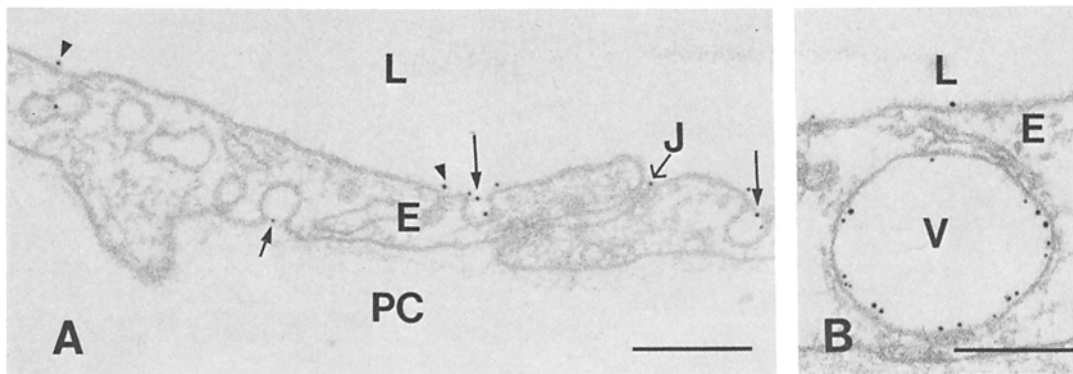


Figure 1. Myocardial capillaries perfused with AuBSA. (A) After a 16-min perfusion the plasmalemmal vesicles on the luminal front are preferentially marked by AuBSA (long arrows). The plasmalemma proper was sparsely labeled (arrowheads). Transport of tracer across the endothelium is limited and abluminal vesicles are only rarely labeled (short arrow). AuBSA particles are occasionally found in the luminal introit of intercellular junctions, but are not detected within or beyond these junctions. Bar, 0.2 μ m. (B) Limited field in the endothelium of a capillary perfused as above to illustrate a large vacuole (V) (presumably an endosome) containing AuBSA. Note the close association of the tracer with the vacuolar membrane. L, lumen; PC, pericapillary spaces; E, endothelium; J, intercellular junction. Bar, 0.2 μ m.

perfusate and did not appear to be transported rapidly or in large numbers to the extravascular space. AuBSA particles were infrequently seen in plasmalemmal vesicles associated with the abluminal front of the endothelium or in the pericapillary spaces. AuBSA particles were found in some coated vesicles/coated pits as well as in some large vacuoles presumed to be endosomes (Fig. 1 B).

Immunoperoxidase tests for albumin in AuBSA perfused capillaries colocalized HRP reaction product with gold particles in plasmalemmal vesicles on both the luminal and abluminal surface of the endothelium, thereby demonstrat-

ing that BSA remained on gold particles throughout the duration of the experiments (Fig. 2 A). Hence, the slow transcytosis rate observed can not be attributed to a loss of the albumin coating from the gold particles. AuBSA-labeled vesicles, apparently detached from both the luminal and abluminal endothelial plasmalemma, were occasionally found and their AuBSA particles had no detectable peroxidatic reaction product on their surface (Fig. 2 B). Unlabeled gold particles were also found in larger, apparently closed vesicular elements assumed to represent endosomes (Fig. 2 C). The peroxidatic reaction product allowed us to determine the ap-

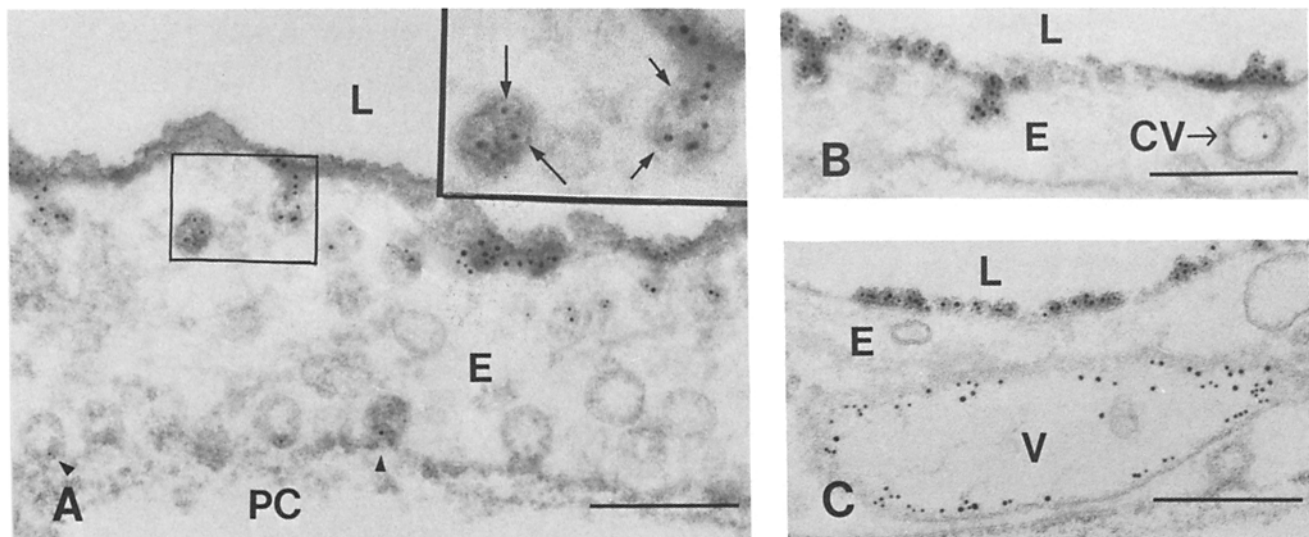


Figure 2. Immunoperoxidase localization of BSA in capillaries perfused with AuBSA. After a 12-min perfusion, treatment of the tissue with an anti-albumin antibody followed by an HRP-coupled secondary antibody demonstrates that albumin is still present on the gold particles. In the insert (the boxed area enlarged two times), the HRP reaction product reveals the approximate size of the albumin coat, in both negative (long arrow) or positive images (short arrow). The HRP reaction product to the albumin on the AuBSA marks a fraction of the total plasmalemmal vesicle population which includes vesicles opening on the abluminal front (arrowheads). Bar, 0.2 μ m. (B and C) Same type of specimens as in A to indicate that the anti-albumin has no access to either a large coated vesicle (CV) or a large vacuole (V) (endosome?) presumably because these structures are not open to either endothelial cell surface. Note that in this experiment the flushing of the capillary lumina (and pericapillary spaces) was not as thorough as in Fig. 1. Bar, 0.2 μ m.

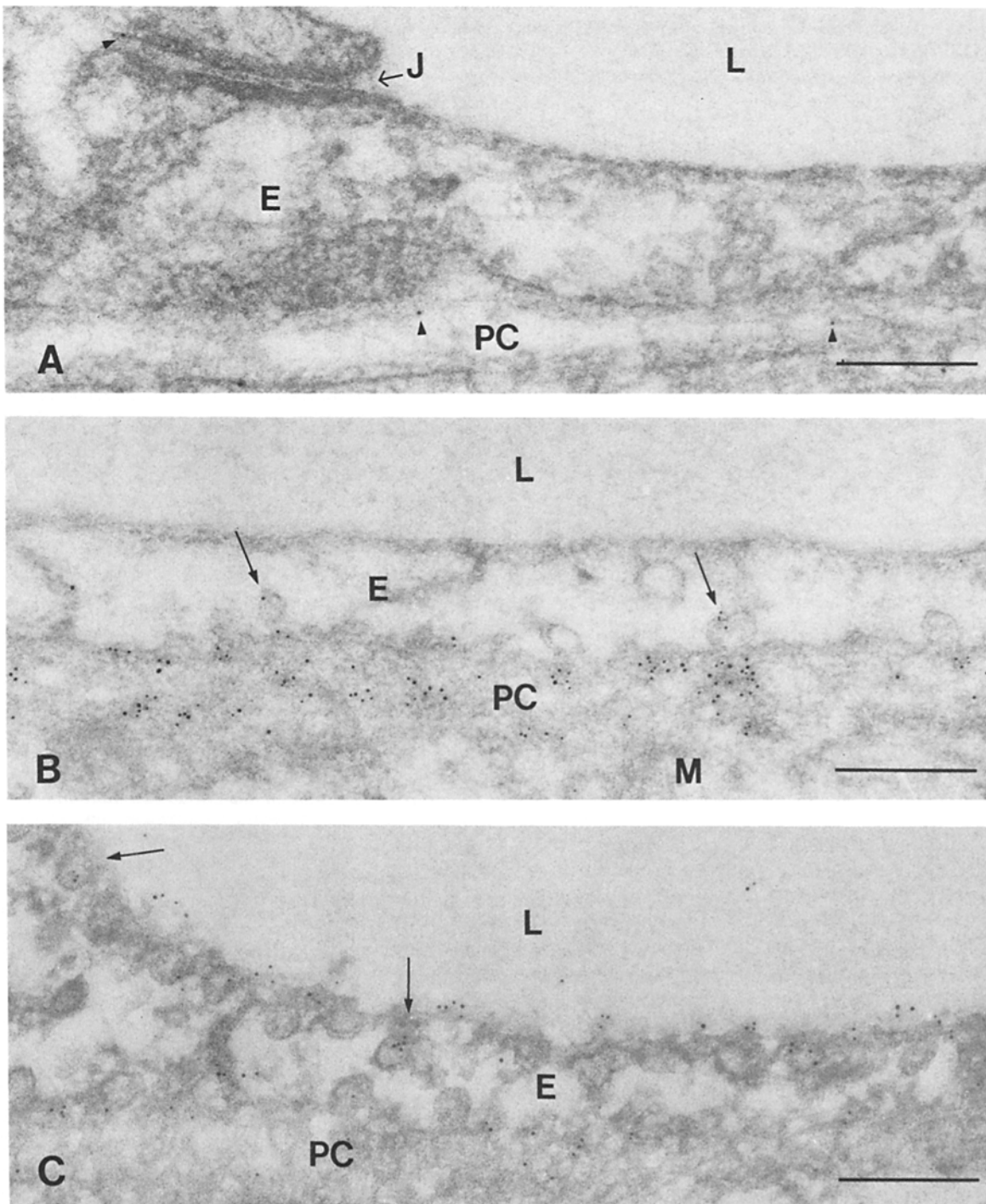


Figure 3. Immunogold localization of monomeric albumin in the endothelium and pericapillary spaces of myocardial capillaries in L. R. White-embedded specimens. (A) Control for the immunogold localization of BSA to show that a 10-min flush with PBSG removes most of the detectable albumin from the lumen, endothelium, and pericapillary spaces. The low background (gold particles marked by arrowheads) represents either nonspecific gold anti-albumin binding or binding to endogenous albumin not fully removed by flushing. Note the characteristic appearance of the intercellular junction (*J*) in this L. R. White-embedded specimen: extended collapse of the intercellular space and extended backing of the junction by fibrillar cytoplasmic condensations. Bar, 0.2 μ m. (B) Specimen perfused with albumin for 1 min followed by 1-min flush with PBSG. The micrograph shows labeling of plasmalemmal vesicles (*long arrows*) and pericapillary spaces with no labeling (in this field) of the luminal plasmalemma and adluminal plasmalemmal vesicles. We assume that labeling was removed from these sites by the 1-min flush that followed albumin perfusion. Bar, 0.2 μ m. *M*, myocardium. (C) After 1 min albumin perfusion without a subsequent flush, albumin is detected on the luminal plasmalemma and in plasmalemmal vesicles throughout the endothelium with heavier labeling in adluminal vesicles (*long arrows*). Albumin is also detected apparently randomly distributed in the pericapillary spaces. Bar, 0.2 μ m.

parent size of AuBSA particles both positively and negatively stained. The average size of the complex was found to be ~ 14.5 nm and that of the gold particles alone, ~ 7 nm.

Immunogold Localization of Exogenous, Monomeric Albumin

LR White Sections. In specimens embedded in L. R. White resin, albumin antigenicity is retained, but the structural preservation of the microvessels is suboptimal. Endothelial cells are generally intact, but the components of their cytoplasmic matrix are often coarsely precipitated. Moreover, cellular membranes are not clearly defined and occasionally show small-scale (<2 – 3 nm) discontinuities. Plasmalemmal vesicles, however, appear reasonably well preserved. These structural changes apparently occur after fixation, during resin polymerization. In surface-labeled specimens (L. R. White-embedded and thin frozen), the precision of antigen localization on sections can be expected to vary from 0 to ~ 27 nm (size of two antibody molecules plus the size of the gold particle).

The anti-BSA antibody was shown by ELISA to bind at low affinity to (endogenous) murine serum albumin. Therefore, to reduce background and exclude erroneous localizations of our tracer, BSA, we performed preliminary tests which established that a 10-min flush with a protein-free perfusate was sufficient to remove almost all of the detectable murine albumin from the vascular lumina, endothelium, and pericapillary spaces (Fig. 3 A). The result was a very low background labeling against which the perfused exogenous BSA could be unequivocally identified.

After BSA perfusion for >1 min, the tracer was detected on the luminal surface of the endothelium, in plasmalemmal vesicles, and in the pericapillary spaces, but the amount present in the latter was too high to allow a reliable identification of the structures involved in albumin transport across the endothelium. These experiments showed that monomeric BSA was much more rapidly transported than AuBSA, and prompted us to reduce stepwise the perfusion time of the tracer. Before that, however, we tested the effect of a 1-min PBSG flush introduced in the protocol after the tracer. The flush removed the BSA from the lumina and practically all plasmalemmal vesicles open on the luminal front of the endothelium (Fig. 3 B). BSA was still detected in plasmalemmal vesicles on the abluminal front and was present in the pericapillary spaces again in quantities large enough to preclude an accurate identification of the structures involved in its transport. These findings indicate that monomeric BSA binds to the endothelial surface (plasmalemmal vesicles included) with considerably lower affinity than AuBSA, which, as already mentioned, can be considered as a polymeric equivalent of BSA. When the 1 min PBSG prefixation flush was omitted, albumin was detected on the luminal surface of the endothelium, in plasmalemmal vesicles throughout the endothelial layer, and in the pericapillary spaces. In the latter location, however, there was an apparent decrease in tracer concentration (Fig. 3 C) probably reflecting a reduction by ~ 1 min in time available for albumin transport, in addition to the partial flushing of these spaces.

When the perfusion time was reduced to 30 s and the post-perfusion flush was omitted, we were able to localize albumin on the luminal aspect of the plasmalemma, in vesicles

on both the luminal and abluminal front of the endothelium and in all pericapillary spaces (Fig. 4 A). Albumin in vesicles on the abluminal front of the endothelium was generally at a higher concentration than in the adjacent extravascular space. No albumin was detected in the intercellular junctions, nor was a concentration of tracer seen in the intercellular spaces abluminal to the junctions and no tracer "clouds" were found in the pericapillary spaces centered on exits from endothelial intercellular spaces.

After further reduction in the BSA perfusion time to 15 s, the distribution of albumin was similar to that found at 30 s (Fig. 4 B) except that the amount of albumin in transit through the endothelium or already arrived in the pericapillary spaces appeared to be smaller.

Thin Sections of Frozen Specimens. With recently improved embedding and ultramicrotomy procedures, structural preservation of microvessels is considerably better in thin sections of frozen tissue than in L. R. White-processed specimens. In controls, labeling due to nonspecific binding or to residual endogenous murine albumin was very low (Fig. 5 A), yet higher than in corresponding L. R. White-embedded preparations, presumably because frozen sections are thicker.

In the capillaries of specimens processed after a 1-min perfusion with BSA (without a subsequent flush with PBSG), there was heavy labeling of the luminal aspect of the endothelial plasmalemma, extensive labeling of the plasmalemmal vesicles irrespective of their position within the endothelium, and minimal labeling of the intercellular junctions. There was also especially heavy and apparently randomly distributed labeling of the pericapillary spaces and on these accounts it was not possible to identify the structures involved in the transport of the tracer (Fig. 5 B).

When the perfusion time was reduced to 30 s (Fig. 6 A), less BSA was detected in the pericapillary spaces. In addition, the highest local concentrations of tracer were not associated with the abluminal end of the intercellular spaces but with segments of attenuated endothelium rich in plasmalemmal vesicles. Gold particles were observed on the luminal and abluminal aspect of endothelial cell junctions. However, labeling was not detected throughout the length of a junction, nor was a gradient of labeling detected in the extravascular space (Fig. 6 B) with a peak centered on the abluminal exit of any intercellular space.

After a further reduction in perfusion time to 15 s, the distribution of the tracer within the endothelium was comparable with that found at 30 s, but the labeling of the sub-endothelial spaces was clearly reduced and preferentially concentrated near open (apparently discharging) plasmalemmal vesicles on the abluminal front (Fig. 6 C). Intercellular junctions and exits from intercellular spaces were minimally labeled and gradients centered on junctions were not present.

All specimens were processed without a post-BSA flush, and at all time points examined, labeling of the luminal aspect of the plasmalemma was quite extensive and apparently randomly scattered. It was definitely not preferentially restricted to plasmalemmal vesicles and "dimples" open on the luminal front as in the case of AuBSA complexes in our experiments and in those of Ghitescu et al. (8). We assume that without a postperfusion flush, we detect BSA in both plasmalemmal vesicles and the fiber matrix that appears to cover the entire luminal surface of the endothelium.

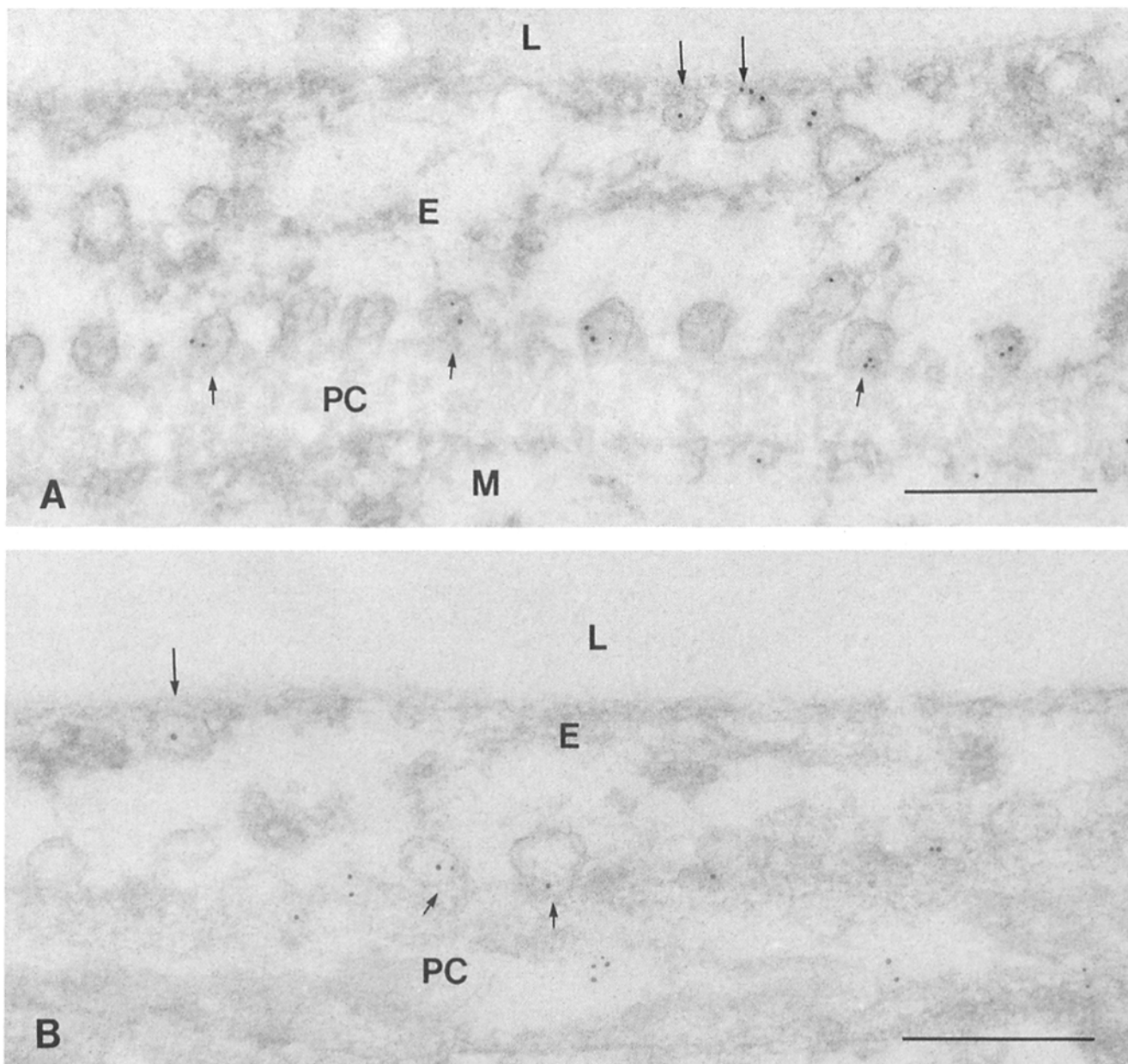


Figure 4. Immunogold localization of monomeric albumin in the endothelium and pericapillary spaces of myocardial capillaries in L. R. White-embedded specimens. (A) This micrograph demonstrates the general pattern of gold anti-albumin labeling after 30 s of albumin perfusion without a subsequent PBSG flush. Labeled vesicles are pointed out on both the luminal (*long arrows*) and abluminal (*short arrows*) front of the endothelium. Bar, 0.2 μm . (B) In this micrograph, the same pattern of labeling as in A is seen after only 15 s of albumin perfusion. Bar, 0.2 μm .

Morphometry

At all time points examined, BSA was detected in only a fraction ($\sim 16\%$, 8–45% range) of the total plasmalemmal vesicle population. Whereas this percentage ranged in different random micrographs, there was no difference observed in the percentage of labeled vesicles from 15 s to 1 min perfusion with BSA. The background labeling of vesicles in the controls was observed to be $\sim 3\%$ (0–7% range).

We measured 0.5 μm^2 of sectioned extravascular space in association with endothelial cell junctions and 1.1 μm^2 in areas away from the junctions and counted therein 82 and 234 gold particles, respectively. For easier comparison, we

normalized the figures to 1 μm^2 and in this case detected 164 gold particles in association with junctions and 212 gold particles in areas away from the junctions.

Discussion

Experimental Protocols

Albumin was chosen as the tracer for these experiments because of its physiological relevance and because its transport from the blood plasma to the interstitial fluid and to lymph is expected to occur at a higher rate in the myocardium than

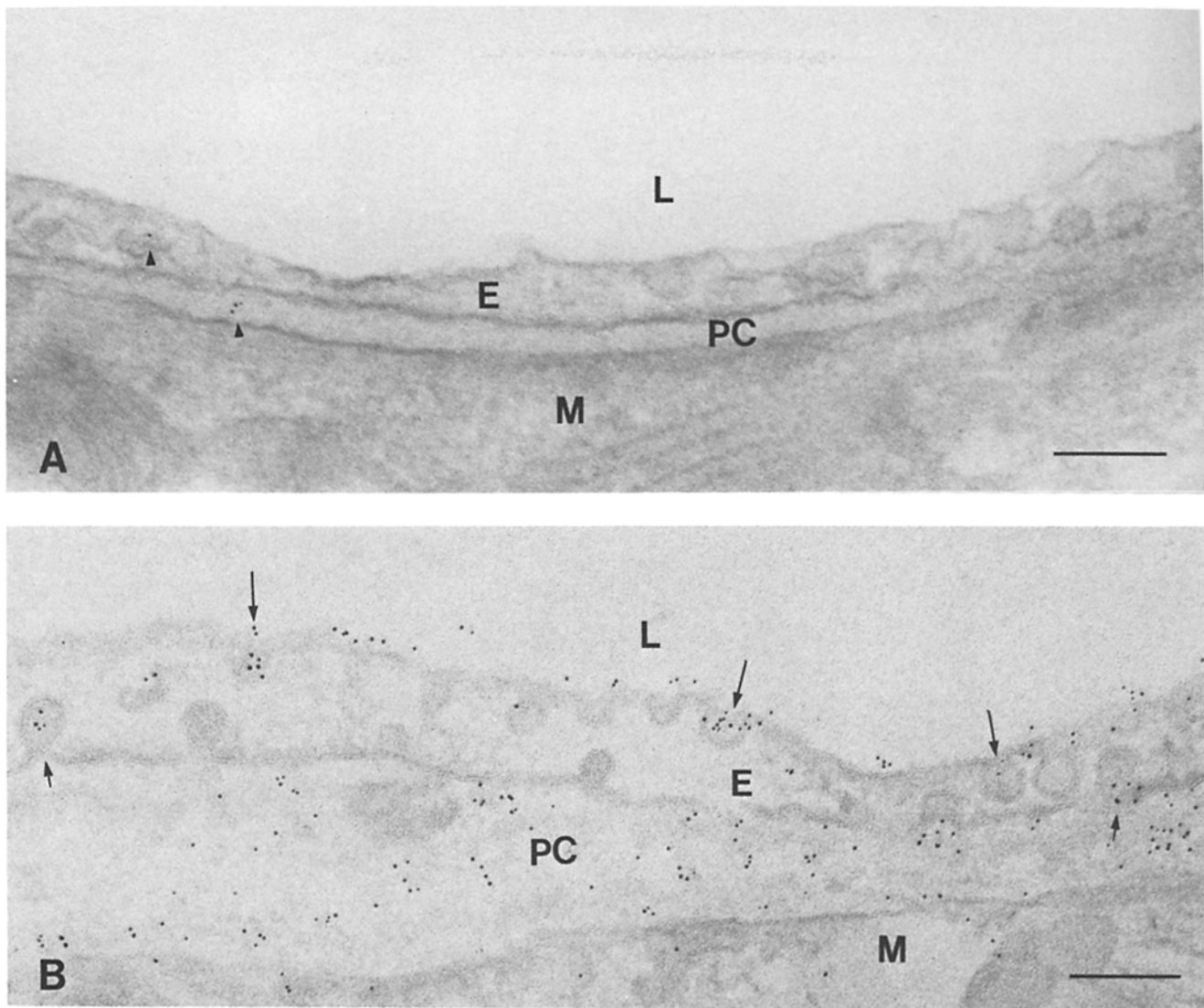


Figure 5. Immunogold localization of albumin in the endothelium and adjacent interstitium of myocardial capillaries in thin frozen sections. (A) This micrograph shows a control demonstrating the low background labeling (arrowheads) of the endothelium and pericapillary spaces. Bar, 0.2 μ m. (B) Specimen perfused with albumin for 1 min. Labeled vesicles on the luminal (long arrows) and abluminal (short arrows) surface are pointed out. Bar, 0.2 μ m.

in most other organs. Albumin represents $\sim 74\%$ of the total proteins of cardiac lymph, and the ratio plasma protein/cardiac lymph protein is 0.6 (7). For logistics reasons, we used an exogenous albumin (bovine) as a tracer instead of the endogenous (murine) albumin, hence the necessity of removing endogenous albumin from vascular lumina, capillary walls, and interstitia by appropriate flushing with protein-free PBSG.

BSA was administered as a bolus at the nearly physiological concentration of 30 mg/ml with the intent of obtaining a high signal and of minimizing the effects of low protein concentration in the perfusate. Old (13) as well as recent (14) data indicate that capillary permeability increases at a protein concentration $<0.01\%$ but is restored promptly (~ 30 s) to already normal levels when the concentration is increased to 1% (11). In our experiments an increase in capillary permeability may apply at the very beginning of BSA perfusions,

but it is expected to be transient and not to involve other than normal exit pathways.

AuBSA Complexes

We found that AuBSA complexes bind extensively and preferentially to plasmalemmal vesicles open on the luminal front of the endothelium, and to shallow depressions or dimples which may represent early stages in vesicle formation. They also bind to a more limited extent to the plasmalemma proper. In addition, AuBSA complexes were detected in small numbers in vesicles scattered throughout the endothelium, and in vesicles discharging on the abluminal front, but the latter were seen only occasionally after relatively long perfusion times: >10 min in our experiments; ~ 5 min, in those of Ghitescu et al. (8). AuBSA complexes were also detected in larger vesicles of irregular profiles tenta-

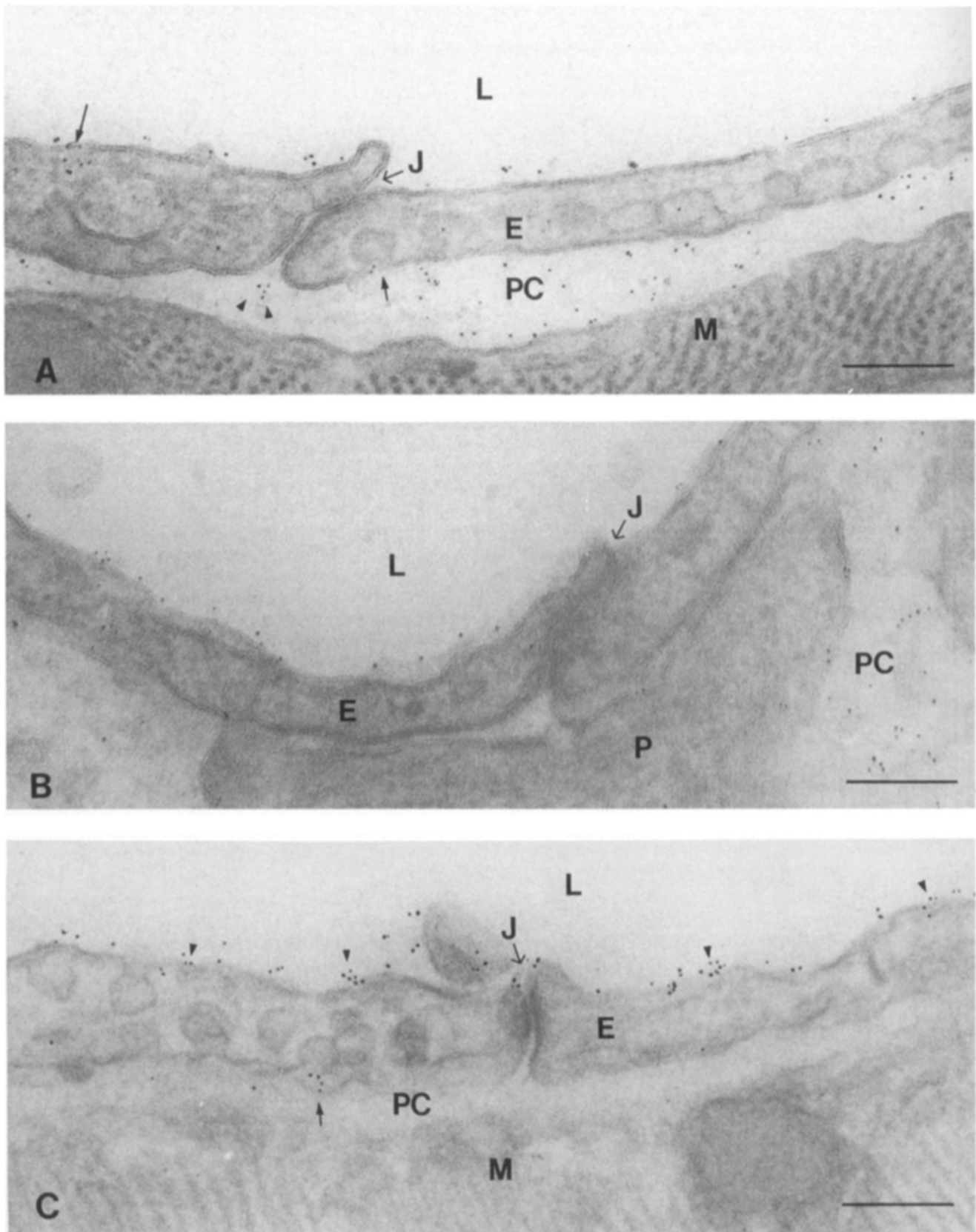


Figure 6. Immunogold localization of albumin in the endothelium and adjacent interstitium of myocardial capillaries in thin frozen sections. (A) After 30 s of albumin perfusion, labeled vesicles are found on both the luminal (*long arrow*) and abluminal (*short arrow*) front of the endothelium. No tracer is detected in the intercellular junction (*J*) and the subjacent intercellular space. Albumin is detected in the pericapillary spaces, but the concentration of gold anti-albumin in the sector immediately under the intercellular junction (*arrowheads*) is lower than in the rest of these spaces. Bar, 0.2 μ m. (B) Micrograph of another section from the specimen in A. It shows an endothelial

tively identified as endosomes. Except for a less pronounced labeling of the plasmalemmal vesicle membranes, our findings with AuBSA confirm those with AuBSA documented by Ghitescu et al. (8) for rat pulmonary capillaries. The results of our washing experiments indicate, however, that monomeric albumin binds with considerably less affinity than polymeric albumin (AuBSA) to the membrane of plasmalemmal vesicles.

The two-step experiments (first AuBSA perfusion and then reaction of vibratome sections with HRP-conjugated anti-BSA) expanded our findings by demonstrating that albumin forms around each gold particle a continuous, relatively thick coat that is retained during transport across the endothelium. AuBSA complexes found in endosomes and in many (but not all) plasmalemmal vesicles within the endothelial cytoplasmic matrix were not stained by the antibody, which is taken to mean that during specimen incubation the vesicles and vacuoles were not accessible to HRP-anti-BSA conjugates. The apparent diameter of the AuBSA complexes, revealed by the peroxidatic reaction, was found to average 14.5 nm, which would indicate that they can be used as exclusive probes for the postulated large-pore system (diam, >10 nm) of the capillary walls.

Monomeric Albumin

The most important findings, made possible by the use of monomeric albumin as tracer, can be listed as (a) extensive labeling of the luminal plasmalemma, (b) labeling of a fraction of the total population of plasmalemmal vesicles, (c) rapid (<15 s) and substantial transport of the tracer to the pericapillary spaces, (d) restriction of the tracer to plasmalemmal vesicles while in transit across the endothelium, (e) absence of detectable albumin gradients associated with intercellular junctions, and (f) rapid removal of the label by short PBSG flushes (1 min), hence apparently low-affinity albumin binding to the endothelium and associated plasmalemmal vesicles. The transport time, given as the interval between the start of tracer perfusion and the start of fixative perfusion, is an underestimate, because previous experiments (1) have established that ~6–9 s of fixative perfusion are required to reduce drastically the appearance of tracer-loaded vesicles on the abluminal front of the endothelium (the tracer used in those experiments was HRP). We assume that 20–25 s is probably not sufficient to fully dissipate gradients centered on the exits from intercellular spaces and that intercellular junctions are not preferentially flushed by the fixative. (We have shown that the latter does not flush the tracer from the luminal plasmalemma and plasmalemmal vesicles).

Monomeric vs. Polymeric Albumin

In addition to obvious differences in the rate of transport and amount of transported tracer, there are other differences between the behavior of monomeric and polymeric albumin

(AuBSA complexes) that deserve comment. The easy removal of monomeric albumin by relatively short flushes with PBSG suggests that the binding of albumin on the luminal surface and in plasmalemmal vesicles has a low association-dissociation constant. In contrast, the tenaciously bound AuBSA complexes cannot be displaced from the membrane of plasmalemmal vesicles by extensive flushing but the binding is comparable with monomeric albumin (8). The multifunctional character of AuBSA is probably the reason for the diversion of part of the tracer to endosomes and it may also explain the slow rate of transport.

Relations to Postulated Pores

Small pores. The results obtained with monomeric albumin suggest that the structural equivalents of the small pores are the plasmalemmal vesicles as far as macromolecular solutes are concerned. They probably function as transcytotic vesicular carriers, thereby accounting for the dissipative component of the plasma-interstitial fluid macromolecular efflux (21, 22); they may also form transient transendothelial channels that could account for part of the convective component of the exchanges. In addition, the results now obtained with monomeric albumin validate earlier findings coming from work done with either albumin (23, 30) or a series of proteins with peroxidatic activity ranging in mass from 1.8 to 50 kD (12³, 25, 26). In these cases, however, it was difficult to obtain generally accepted evidence because specimen fixation was slow (and tracers had time to diffuse), and because a detection procedure of low resolution (the diaminobenzidine-H₂O₂-peroxidase reaction) was used.

Large pores. The results we obtained with AuBSA complexes suggest that plasmalemmal vesicles also function as large pore equivalents, because they transport particles that dimensionwise (≥10 nm) qualify for exclusive large pore probes. Vesicles functioning as large pore equivalents may also form transendothelial channels, thereby providing a convective component to the transport of large (diam, ≥10 nm) particles. These interpretations, although clearly supported by our evidence, are still in need of confirmation by other laboratories using the same or similar tracers.

Further work is needed to find out whether the two pore functions are subserved by different populations or the same population of plasmalemmal vesicles and, in the second alternative, what mechanisms explain the ability of certain vesicles to take up and transport large particles. Although our findings strongly favor albumin transport via vesicles, they cannot provide an unambiguous answer as to the detailed mechanisms involved. The vesicles may function as shuttles from one front of the endothelium to the other (3, 18, 19) or may work as relays from one front to vesicles al-

3. Vesicles were labeled but the author concluded that the tracer left the lumina through the junctions.

intercellular junction (*J*) covered by a pericyte (*P*) and demonstrates that the concentration of tracer is considerably lower in the narrow space between the endothelium and the pericyte than in the rest of the pericapillary spaces. The narrow space would favor the maintenance of a tracer gradient if albumin would exit through the intercellular junction. Bar, 0.2 μm. (*C*) There is detectable albumin in the extravascular space in association with plasmalemmal vesicles (*short arrow*) after only 15 s of albumin perfusion. There is also extensive labeling of the plasmalemma proper (*arrowheads*). The introit to an intercellular junction (*J*) is labeled but the junction itself is free of tracer. Bar, 0.2 μm.

ready open at the other front as proposed by Clough and Michel (4) for frog mesenteric capillaries or they may establish transient transendothelial channels (25). Further work is needed to provide an answer to this question in mammalian myocardial capillaries. Additional work is also needed to define, in molecular terms, the interactions of albumin with both the plasmalemma proper and with the membrane of plasmalemmal vesicles. Work along these lines is already proceeding (24).

This work was supported by a National Institutes of Health grant (HL-17080) and a gift from the Nabisco-R. J. Reynolds Company.

Received for publication 1 June 1987, and in revised form 20 August 1987.

References

- Boyles, J., N. L'Hernault, H. Laks, and G. E. Palade. 1981. Evidence for vesicular shuttle in heart capillaries. *J. Cell Biol.* 91:418a. (Abstr.)
- Brodersen, R. 1978. Binding of bilirubin and other ligands to human serum albumin. In *Albumin, Structure, Biosynthesis, Function*. T. Peters and I. Sjöholm, editors. Pergamon Press, Oxford. 61.
- Bruns, R. K., and G. E. Palade. 1968. Studies on blood capillaries. II. Transport of ferritin molecules across the wall of muscle capillaries. *J. Cell Biol.* 37:277-299.
- Clough, G., and C. C. Michel. 1981. The role of vesicles in the transport of ferritin through frog endothelium. *J. Physiol. (Lond.)* 315:127-142.
- Corvol, P., and E. W. Bardin. 1973. Species distribution of testosterone binding globulin. *Biol. Reprod.* 8:277-284.
- Curry, F. E., and C. C. Michel. 1980. A fiber matrix model of capillary permeability. *Microvasc. Res.* 20:96-99.
- Drinker, C. K., M. F. Warren, F. W. Maurer, and J. D. McCarrel. 1940. The flow, pressure, and composition of cardiac lymph. *Am. J. Physiol.* 130:43-55.
- Ghitescu, L., A. Fixman, M. Simionescu, and N. Simionescu. 1986. Specific binding sites for albumin restricted to plasmalemmal vesicles of continuous capillary endothelium: receptor-mediated transcytosis. *J. Cell Biol.* 102:1304-1311.
- Graham, R. C., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. *J. Histochem. Cytochem.* 14:291-302.
- Grotte, G. 1956. Passage of dextran molecules across the blood-lymph barrier. *Acta Chir. Scand. Suppl.* 211:1-84.
- Huxley, V. H., and F. E. Curry. 1985. Albumin modulation of capillary permeability: test of an adsorption mechanism. *Am. J. Physiol.* 248: H264-H273.
- Karnovsky, M. J. 1967. The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. *J. Cell Biol.* 35:213-236.
- Landis, E. M., and J. R. Pappenheimer. 1963. Exchange of substances through the capillary walls. In *Handbook of Physiology*. Vol. II. Circulation. W. F. Hamilton and P. Dow, editors. American Physiological Society, Washington, D. C. 961-1034.
- Mason, J. C., F. E. Curry, and C. C. Michel. 1977. The effects of proteins upon the filtration coefficient of individually perfused frog mesenteric capillaries. *Microvasc. Res.* 13:185-202.
- Mayerson, H. S. 1963. The physiological importance of lymph. In *Handbook of Physiology*. Vol. II. Circulation. W. F. Hamilton and P. Dow, editors. American Physiological Society, Washington, D. C. 1035-1073.
- Milici, A. J., N. L'Hernault, and G. E. Palade. 1985. Surface densities of diaphragmed fenestrae and transendothelial channels in different murine capillary beds. *Circ. Res.* 56:709-717.
- Mühlpfort, H. 1982. The preparation of colloidal gold particles using tannic acid as an additional reducing agent. *Experientia (Basel)* 38:1127-1128.
- Palade, G. E. 1953. The fine structure of blood capillaries. *J. Appl. Phys.* 24:1424. (Abstr.)
- Palade, G. 1960. Transport in quanta across the endothelium of blood capillaries. *Anat. Rec.* 136:254. (Abstr.)
- Pappenheimer, J. R., E. M. Renkin, and L. M. Borrero. 1951. Filtration, diffusion and molecular sieving through pericapillary membranes. A contribution to the pore theory of capillary permeability. *Am. J. Physiol.* 167:13-46.
- Renkin, E. M., M. Gustafson-Sgro, and L. Sibley. 1987. Coupling of albumin flux to volume flow in skin and skeletal muscles of anesthetized rats. *Fed. Proc.* 46:349. (Abstr.)
- Renkin, E. M., P. D. Watson, C. H. Sloop, W. M. Joyner, and F. E. Curry. 1977. Transport pathways for fluid and large molecules in microvascular endothelium of the dog's paw. *Microvasc. Res.* 14:205-214.
- Schneeberger, E. E., and M. Hamelin. 1984. Interaction of serum proteins with lung endothelial glycocalyx: its effect on permeability. *Am. J. Physiol.* 247:H206-H217.
- Schnitzer, J., W. Carley, and G. Palade. 1988. Specific albumin binding to microvascular endothelium in culture: quantation and immunolocalization. *Am. J. Physiol.* In press.
- Simionescu, N., M. Simionescu, and G. E. Palade. 1973. Permeability of muscle capillaries to exogenous myoglobin. *J. Cell Biol.* 57:424-452.
- Simionescu, N., M. Simionescu, and G. E. Palade. 1978. Structural basis of permeability in sequential segments of the microvasculature of the diaphragm. II. Pathways followed by microperoxidase across the endothelium. *Microvasc. Res.* 15:17-36.
- Spector, A. 1975. Fatty acid binding to plasma albumin. *J. Lipid Res.* 16:165-179.
- Tokuyasu, K. T. 1986. Application of cryoultramicrotomy to immunocytochemistry. *J. Microsc. (Oxf.)* 143:139-149.
- Tokuyasu, K. T. 1986. Cryosections for immunocytochemistry. *Proc. 11th Int. Congr. Electron Microscopy*. 3:1977-1978.
- Yokota, S. 1983. Immunocytochemical evidence for transendothelial transport of albumin and fibrinogen in rat heart and diaphragm. *Biomed. Res.* 4:577-586.