Immunotargeting of Streptavidin to the Pulmonary Endothelium

Vladimir R. Muzykantov, Elena N. Atochina, Vitaly Gavriljuk, Sergei M. Danilov and Aron B. Fisher

Institute of Experimental Cardiology, Russian National Cardiology Research Center, Moscow, Russia; and Institute for Environmental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

We have observed previously that monoclonal antibody to angiotensin-converting enzyme (Mab 9B9) accumulates selectively in the lung after intravenous injection. The objective of the present work is the development of a universal system for targeting of drug or radiolabel to the lung, using biotinylated Mab 9B9 and streptavidin. **Methods:** Mab 9B9 was biotinylated with biotin succinimide ester (b-Mab 9B9), while streptavidin (SA) was radiolabeled with ^{125}I. Interaction between b-Mab 9B9 and SA has been estimated in solid-phase radioassay. Radiolabeled SA was conjugated with b-Mab 9B9 or with b-IgG and injected intravenously in rats or perfused in isolated rat lungs. **Results:** Radiolabeled b-Mab 9B9 biotinylated at biotin-to-antibody molar ratio 10 (b-Mab 9B9) retains its ability to accumulate in rat lungs after intravenous injection. Radiolabeled SA conjugated with b-Mab 9B9 accumulates in the lung tissue in perfused isolated rat lungs. About 20% of injected SA accumulates in the rat lung 1 hr after intravenous injection (localization ratio is 20, immunospecificity of the conjugate pulmonary uptake is 70). As compared with conjugate injection, stepwise intravenous injection of b-Mab 9B9 and radiolabeled SA leads to a marked reduction of SA pulmonary uptake. Maximal pulmonary uptake of Mab 9B9 has been observed 2–3 hr after intravenous injection, while 24 hr later, radioactivity in the lung was markedly reduced. In contrast to radiolabeled Mab 9B9 alone, radiolabeled SA conjugated with b-Mab 9B9 was retained in the lung for at least 48 hr. In concert with effective blood clearance of the conjugate, its prolonged lung retention leads to a marked increase in its lung-to-blood ratio: 80 for SA-b-Mab 9B9 versus 15–20 for Mab 9B9. **Conclusion:** Conjugation of Mab 9B9 with streptavidin enhances selective pulmonary uptake of the preparation, providing a background for intrapulmonary immunotargeting of various biotinylated agents.

Key Words: lung; endothelium; angiotensin-converting enzyme; biotinylated antibody; streptavidin

J Nucl Med 1994; 35:1358–1365

Avidin-biotin technology is widely accepted now in biomedical investigations (*1*). Avidin (MW 66 kD) and its analogue, streptavidin (MW 60 kD), are tetrameric proteins with a high-affinity biotin-binding site (Kd = 10^{15} M^{-1}) on each subunit (*2*). Almost any biomolecule may be biotinylated usually without significant loss of biological activity (*1*). Thus avidin-biotin technology offers a universal system for cross-linking, staining or targeting of biomolecules and is exploited extensively in vitro (*1*).

During the past 5 yr, several attempts have been made to introduce avidin-biotin technology to in vivo studies in laboratory animals and to clinical practice. The following applications for in vivo use of avidin-biotin technology have attracted special attention: selective elimination of various molecules, particles and cells from blood (*3–5*); study of blood cells in the circulation (*6–8*); radioimmunoimaging (*9–11*); drug targeting (*12–14*); and targeted stimulation of immune response (*15,16*).

The main principle for these approaches is selective binding of avidin or streptavidin to a defined cell, tissue or organ. Biotinylated affinity ligands to the target (i.e., biotinylated antibody, hormone, lectin, antigen, etc.) may be used for this purpose. Since avidin and streptavidin have four biotin-binding sites, biotinylated radiolabel, drug or antigen may be accumulated in the target. Furthermore, avidin-biotin technology provides an opportunity to develop a universal system for selective delivery of any biotinylated agent to the target using the same affinity carrier (i.e., biotinylated ligand-avidin complex).

Earlier we reported that monoclonal antibody (Mab) to angiotensin-converting enzyme (ACE) selectively accumulates in rat, cat, hamster and monkey lungs after systemic administration (*17*). Mab 9B9 does not induce complement-mediated injury to the endothelium in culture (Danilov SM, *unpublished data*) and does not result in damage to rat lung after systemic injection (*17*). ACE is localized on the luminal surface of the endothelial cell (*18*). Two important properties of ACE allow consideration of this enzyme as an appropriate target for selective drug delivery to the lung: (1) lung content of ACE is very high (*19*); and (2) pulmonary ACE is easily accessible from the bloodstream (*18*).

We have suggested that Mab 9B9 may be used for drug targeting to the pulmonary endothelium (*20*), as well as for radioimmunoimaging of the pulmonary vascular bed (*21*). To develop a universal targeting system for these purposes, we now have studied biodistribution and pulmonary uptake of biotinylated Mab 9B9 as well as of radiolabeled streptavi-

Received Jan. 31, 1994; revision accepted Apr. 18, 1994.

For correspondence or reprints contact: Vladimir R. Muzykantov, MD, PhD, IFEM, University of Pennsylvania Medical Center, One John Morgan Building, 36th St. and Hamilton Walk, Philadelphia, PA 19104-6068.

din conjugated with biotinylated Mab 9B9. Our results show that biotinylated Mab 9B9 provides specific pulmonary uptake of radiolabeled streptavidin. Moreover, tissue selectivity of the pulmonary uptake of streptavidin-Mab 9B9 complex is considerably higher than that of Mab 9B9 itself.

MATERIALS AND METHODS

Streptavidin and 6-biotinylaminocaproic acid N-hydroxysuccinimide ester were from Calbiochem (San Diego, CA); Na(^{125}-I) was from Amersham (Arlington Heights, IL); nonimmune mouse IgG and Sephacryl S-200HR were from Sigma (St. Louis, MO); and Iodogen was from Pierce (Rockford, IL). Male Wistar and Sprague-Dawley rats were used. Mouse Mab to human ACE (Mab 9B9) was obtained and characterized as described previously (*17*).

Biotinylation of Mab 9B9 and control mouse IgG was performed as described previously for polyclonal rabbit antibody to collagen (*22*). Briefly, an indicated amount of biotin ester in dimethylformamide (DMFA) was added to a solution of antibody (1–3 mg/ml in PBS, 7.4). After 1 hr incubation on ice, excess of biotin ester was removed by gel-filtration on Sephadex G-25.

Radiolabeling of antibody IgG and streptavidin was performed using Iodogen-coated tubes as described previously (*23*). Briefly, 100 μg of antibody or streptavidin was incubated with 100 μCi of Na^{125}I isotope in 100 μl of 200 m*M* borate buffered saline, pH 8.1, for 10 min on ice. Iodogen-coated tubes (10 μg/tube) were prepared according to manufacturer's recommendations by evaporating the chloroform solution of Iodogen by nitrogen gas. Excess isotope was removed by Sephadex G-25 gel-filtration. The percentage of bound radioactivity was 20%–40% and the final specific radioactivity was equal to 0.3–0.7 μCi/μg of protein. As revealed by precipitation with trichloroacetic acid, about 95% of radioactivity was associated with proteins.

Binding of radiolabeled b-Mab 9B9 to immobilized streptavidin and binding of radiolabeled streptavidin to b-Mab 9B9 was studied by direct radioassay in Costar microtiter plates (Cambridge, MA). Nonradiolabeled protein (streptavidin or b-Mab 9B9, 100–300 ng/well) was incubated in wells for 3–4 hr at room temperature and washed. After blocking of nonspecific binding sites by 0.1% solution of bovine serum albumin (BSA), dilutions of radiolabeled protein were added to wells, incubated for 1 hr, washed and counted in a Rack-Gamma counter (LKB, Sweden).

Perfusion of isolated rat lungs was performed as described previously (*24*). Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5 m*M* of glucose and 3% BSA (KRB-BSA) was used as a basic perfusate. Rats anesthetized with pentobarbital (50 mk/kg, intraperitoneally), were ventilated through a tracheostomy using a rodent respirator (Harvard Apparatus, Millis, MA) at 60 cycles per min with a 2-ml tidal volume and a 2-cm H_2O end-expiratory pressure. The chest was opened surgically and the right ventricle was cannulated. The left atrium was transected for the collection and recirculation of the perfusate. The lung was removed and placed into a thermostatically adjusted (37°C) chamber. Perfusion in the chamber was maintained with a peristaltic pump (Harvard Apparatus, Millis, MA) at flow rate 10 ml/min. Radiolabeled streptavidin-b-Mab 9B9 complex or its components were injected into the perfusate (0.5–2.0 μg per lung). After 30–60 min recirculating perfusion, perfusate was replaced by pure KRB-BSA and lung was perfused for an additional 10 min in the nonrecirculating mode (for washout of nonbound radioactivity). Radioactivity in the tissue was measured in a Rack-Gamma counter.

To study the biodistribution of the radiolabeled preparation, an injection was made into the tail vein. Each experimental point represents injection into three rats and results were calculated as mean + s.d., n = 3. After injection, animals were killed at indicated times. Internal organs were washed with saline and radioactivity in tissues was determined in a Rack-Gamma counter. The following parameters were used to characterize biodistribution: (1) Localization ratio (LR), ratio of radioactivity per g of tissue to that of blood, characterizing tissue selectivity of the targeting; (2) Percentage of injected dose per g of tissue (%ID/g), characterizing efficiency of the targeting; and (3) Immunospecificity (IS), ratio of tissue uptake of immune and nonimmune preparations (i.e., streptavidin-b-Mab 9B9 versus streptavidin-b-IgG), characterizing specificity of the targeting.

RESULTS

Table 1 shows the biodistribution in Wistar rats of radiolabeled Mab 9B9 modified with biotin N-hydroxysuccinimide ester at various biotin-to-antibody molar ratios in the mixture during biotinylation. At a high degree of biotinylation (biotin/antibody molar ratio in mixture >100), we observed a dramatic decrease in selective pulmonary uptake of Mab 9B9 (2.8% of dose/g versus 20.6%/g in control, $p < 0.01$). On the other hand, intensive biotinylation of Mab 9B9 leads to enhanced blood clearance of the modified antibody (1.1% of dose/g of blood versus 3.2%/g in control, $p < 0.01$). An increase in uptake by the spleen may be responsible for the rapid clearance of hyperbiotinylated Mab 9B9, since spleen-to-blood ratio was 1.46 for hyperbiotinylated Mab 9B9 versus 0.28 + 0.01 for nonbiotinylated Mab 9B9. Therefore, extensive biotinylation of Mab 9B9 leads to loss of its selective pulmonary uptake and to nonspecific uptake by tissues probably due to partial denaturation of the antibody.

Mab 9B9, biotinylated at a biotin-to-antibody molar ratio of 35 or less, retains its capacity to accumulate selectively in the lung (Table 1). For the rest of the study we used Mab 9B9 biotinylated at biotin-to-IgG ratio 15 (b-Mab 9B9). Figure 1A shows that b-Mab 9B9 binds specifically to immobilized streptavidin in vitro (Kd = $4*10^{13}$ M^{-1}). Vice versa, iodinated streptavidin binds specifically to immobilized b-Mab 9B9 (Kd = $2*10^{13}$ M^{-1}), but not to control non-biotinylated Mab 9B9 (Fig. 1B). Binding of radiolabeled streptavidin with biotinylated nonimmune mouse IgG, used as a control for in vivo studies (b-IgG) was the same as shown for biotinylated Mab 9B9 (data not shown).

To study immunotargeting of radiolabeled streptavidin to the pulmonary endothelium, we have used two models: perfusion of isolated rat lungs and systemic intravenous injection in rats. Figure 2A shows that radiolabeled streptavidin conjugated with b-Mab 9B9, but not with nonimmune b-IgG, accumulates in the lung tissue after perfusion of isolated rat lungs. Figure 2B shows pulmonary uptake of radiolabeled streptavidin (infusion of 0.5 μg SA per perfused lung) mixed with various doses of biotinylated Mab 9B9 1 hr before the perfusion. These results show specific, saturable, antibody-mediated accumulation of streptavidin in the pulmonary tissue. They further suggest that the antibody-to-streptavidin ratio should be no less than 1 for successful streptavidin targeting.

TABLE 1
Biodistribution of Biotinylated Mab 9B9 in rats

Biotin-to-IgG molar ratio		0	8.8	35	115
Blood	%ID/g	3.16 + 0.41	3.40 + 0.29	2.22 + 0.23	1.11 + 0.27
	LR*	1	1	1	1
Lung	%ID/g	20.57 + 1.94	18.19 + 3.59	14.47 + 0.37	2.83 + 0.74
	LR	6.52 + 0.28	5.42 + 1.53	6.56 + 0.74	2.69 + 1.20
Liver	%ID/g	1.37 + 0.16	1.64 + 0.26	1.25 + 0.05	0.85 + 0.03
	LR	0.44 + 0.11	0.48 + 0.12	0.56 + 0.15	0.79 + 0.20
Kidney	%ID/g	1.11 + 0.04	0.87 + 0.24	0.76 + 0.19	0.61 + 0.05
	LR	0.35 + 0.06	0.26 + 0.09	0.34 + 0.06	0.57 + 0.20
Spleen	%ID/g	0.86 + 0.07	0.84 + 0.05	0.68 + 0.10	1.55 + 0.91
	LR	0.28 + 0.01	0.25 + 0.01	0.30 + 0.02	1.46 + 0.11
Heart	%ID/g	1.11 + 0.29	0.98 + 0.31	0.77 + 0.06	0.40 + 0.04
	LR	0.36 + 0.14	0.29 + 0.07	0.34 + 0.02	0.38 + 0.04

*LR = localization ratio, a ratio of radioactivity per gram of tissue to that of blood. Iodine-125-labeled Mab 9B9 or its biotinylated analogues were injected in the tail vein of male Wistar rats (1 μg/rat). One hour later, animals were killed, internal organs were obtained, washed with saline, weighed and radioactivity in organs was measured in a Rack-Gamma counter. The data are presented as mean + s.d., n = 3 rats.

Table 2 shows biodistribution of the radiolabeled streptavidin, conjugated with nonlabeled b-Mab 9B9 or with b-IgG, 1 hr after intravenous injection of complexes into rats. Biodistribution of free radiolabeled streptavidin was similar to that of streptavidin-b-IgG conjugate (not shown). Very effective (about 20% ID/g) and selective (lung-to-blood ratio about 8) pulmonary uptake of streptavidin-b-Mab 9B9 conjugate was observed. There was no significant selective uptake of the immune preparation in any tissue, except the lung. Specificity of the pulmonary uptake, estimated as the ratio of tissue uptake of immune and nonimmune conjugates was extremely high (immunospecificity index about 50). Estimation of specificity of the pulmonary uptake by ratio of lung-to-blood indices for immune and nonimmune conjugates gives an even higher value (immunospecificity index about 70). The latter value may be more accurate because it accounts for differences in the blood level of immune and nonimmune preparations. To our knowledge, this is the highest parameter of immunospecificity for any tissue or antibody published to date.

Figure 3 shows the blood level and pulmonary uptake of streptavidin-b-Mab 9B9 conjugates prepared at various b-Mab 9B9-to-streptavidin ratios and injected in vivo. Similar to results obtained in the model of isolated rat lung (Fig. 2B), selective pulmonary uptake of streptavidin was observed in the range of antibody-to-streptavidin ratios 1–100. Maximal pulmonary uptake was observed at a ratio of about 2.5, corresponding to the antibody-to-streptavidin molar ratio of about 1. At lower antibody-to-streptavidin ratios, an increasing portion of streptavidin is not bound to b-Mab 9B9, while at a very high ratio, excess of free b-Mab 9B9 may inhibit pulmonary uptake of the conjugate. There were no significant changes in the blood level of the conjugates at various antibody-to-streptavidin ratios.

Figure 4 shows Sephacryl S-200HR gel-filtration of radiolabeled streptavidin and streptavidin mixed with b-Mab 9B9 at a ratio of 2.5. Free streptavidin peaks in fractions 19 and 20 (MW about 60 kD, BSA, according to calibration chromatography). Streptavidin-b-Mab 9B9 conjugate peaks in fraction 10, corresponding to the excluded volume of the column. There was no significant difference in pulmonary uptake of initial SA-b-Mab 9B9 mixture and purified conjugates (9.7 + 0.05% ID/g for initial mixture versus 10.4 + 2.1% ID/g for fraction 10, 40 min after injection of preparations).

It has been described recently that in some cases injection of radiolabeled streptavidin after injection of biotinylated antibody may help to increase target-to-blood ratio (two-step and three-step strategies, (*9–11*)). In our model, we have obtained the opposite result: injection of streptavidin 10 min after injection of b-Mab 9B9 leads to threefold reduction of pulmonary uptake of streptavidin (Figure 5A). Blood level of streptavidin even increases with this mode of administration. Thus, two-step streptavidin administration reduces lung-to-blood ratio: from 8.5 + 1.5 at injection of the conjugate to 1.98 + 0.55 with the two-step approach. Injection of streptavidin 1 hr after b-Mab 9B9 injection gave very similar results (lung-to-blood ratio 2.44 + 0.37).

Conjugation of streptavidin and b-Mab 9B9 in vitro (one-step injection) was performed with 150 μl PBS-BSA (i.e., final concentrations 10 μg/ml). In the two-step approach, conjugation has to occur in the blood (15-ml volume in rats). Therefore, in the two-step approach, concentrations of both reagents are 100 fold lower. However, the very high affinity constant of binding of b-Mab 9B9 and radiolabeled streptavidin (Fig. 1) raises the possibility that their concentrations in the ranges used both in vitro and in vivo might not affect efficiency of the conjugation. Figure 5B provides evidence for this suggestion: accumulation of streptavidin in isolated perfused rat lungs (perfusion volume 25 ml) did not depend on the mode of streptavidin addition. Addition of the streptavidin-b-Mab 9B9 conjugate to the perfusate or addition of streptavidin 10 min after the b-Mab 9B9 addition provided similar pulmonary uptake of radiolabeled streptavidin.

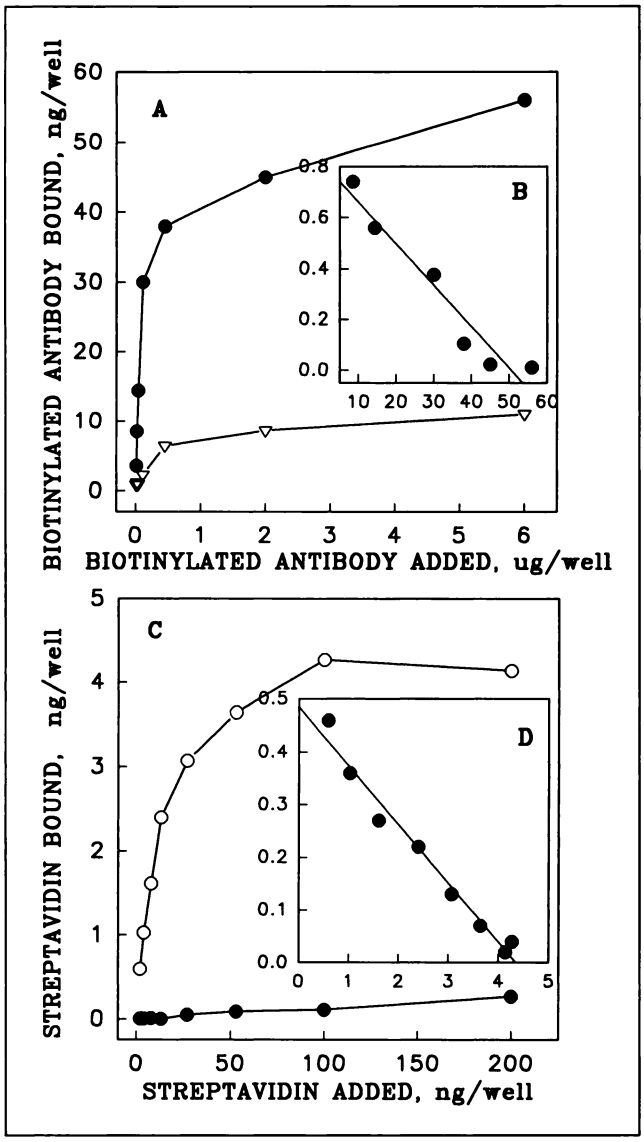

FIGURE 1. Estimation of interaction between b-Mab 9B9 and streptavidin in direct solid-phase radioassay. (A) Binding of radiolabeled b-Mab 9B9 with immobilized streptavidin (closed circles) or with BSA (triangles). (B) Binding analysis in Scatchard plot (x-axis: bound b-Mab 9B9 (ng/well); y-axis: bound-to-free b-Mab 9B9 ratio). (C) Binding of radiolabeled streptavidin with immobilized b-Mab 9B9 (open circles) or with control (nonbiotinylated Mab 9B9, closed circles). (D) Binding analysis in Scatchard plot (x-axis: bound streptavidin (ng/well); y-axis: bound-to-free streptavidin ratio).

Therefore, a comparison of the two models (systemic injection in rats versus perfusion of isolated rat lungs) suggests a previously unrecognized systemic effect that reduces pulmonary uptake of streptavidin when the step-wise procedure is used. A possibility is that streptavidin binding to biotinylated antibody in the circulation may be inhibited by some blood component (e.g., by endogenous biotin-containing compounds). To clarify this issue, we studied binding of radiolabeled streptavidin with immobilized b-Mab 9B9 in the presence of blood. Figure 6 demonstrates that normal rat blood inhibits streptavidin binding to biotinylated protein. Therefore, step-wise systemic administration of streptavidin and biotinylated antibody appears to reflect the effect of inhibition by blood in our model.

We have compared kinetics of the pulmonary uptake and blood clearance in Sprague-Dawley rats of radiolabeled streptavidin conjugated with biotinylated Mab 9B9 (antibody-to-streptavidin ratio is 2.5), as well as radiolabeled Mab 9B9. Our data (Figure 7) suggest two conclusions.

First, kinetics of the pulmonary uptake of the preparations differ dramatically. Initial accumulation of antibody in the lung is very fast (10%–12% ID/g is accumulated in the lung as soon as 5 min after injection). Three hours after injection, we observed maximal pulmonary uptake of antibody (13%–20% ID/g for various experiments). After this peak we observed a sharp decrease in lung radioactivity, probably due to shedding of the portion of endothelium-bound antibody from the cells. Very similar results were observed previously in Wistar rats (*17*) and in hamsters (Danilov SM, *unpublished data*).

Pulmonary uptake of radiolabeled streptavidin conjugated with biotinylated Mab 9B9 increased sharply during the first hour after injection (from 5% ID/g 5 min after injection to a maximal level of 17%–20% of dose/g 1 hr after injection). Unexpectedly, this maximal level of pulmonary uptake of streptavidin has a prolonged character. In contrast with radiolabeled antibody, we did not observe a significant decrease in the pulmonary level of targeted radiolabeled streptavidin 2 days after injection.

Second, kinetics of blood clearance of preparations also differ. Antibody clearance has a sharp two-phase character. The first phase is a very fast decrease in blood level of antibody followed by a slow elimination of circulating antibody. This results in blood levels equal to 0.59% + 0.01% ID/g of blood 24 hr after injection. Blood clearance of streptavidin-antibody conjugate is gradual, but very effective and provides a blood level equal to 0.27% + 0.06% ID/g of blood 24 hr after injection.

In concert with effective blood clearance of the conjugate, its prolonged pulmonary uptake provides a dramatic increase in radiolabeled streptavidin-Mab 9B9 lung-to-blood ratio, as compared with the radiolabeled Mab 9B9 lung-to-blood ratio (Figs. 7 and 8). At one hour after injection, the lung-to-blood ratio of streptavidin-b-Mab 9B9 is two times higher than that of antibody (Fig. 8A). One day after injection, the lung-to-blood ratio of streptavidin-b-9B9 was sevenfold higher than the corresponding parameter of Mab 9B9 (Fig. 8B). As Figure 8 shows, streptavidin conjugated with biotinylated nonimmune IgG does not accumulate in the lung and, therefore, enhancement of the pulmonary uptake by conjugation of streptavidin with biotinylated Mab 9B9 is not the result of nonspecific uptake of the conjugate by the pulmonary endothelium.

DISCUSSION

Since the first paper of Hnatovich et al. in 1987 (*9*), the number of publications exploring the avidin-biotin system for nuclear medicine and for drug targeting has increased

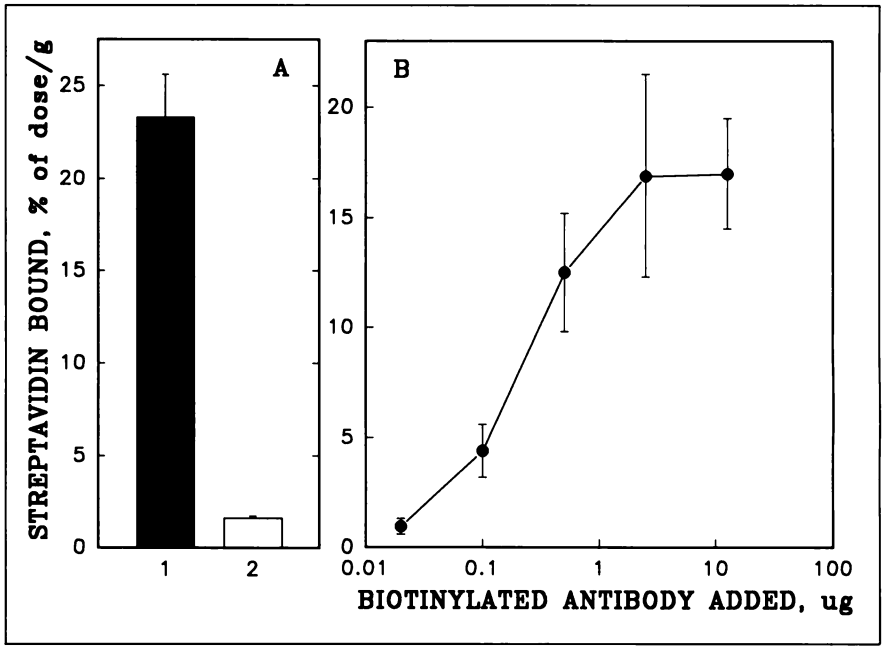

FIGURE 2. Perfusion of isolated rat lungs with SA-b-Mab 9B9 conjugate. (A) Pulmonary uptake of radiolabeled streptavidin conjugated with b-Mab 9B9 (1, closed bar) or with b-IgG (2, open bar) in isolated rat lungs. Lungs were perfused by recirculation for 45 min of 25 ml of KRB-BSA containing 0.5 μg of radiolabeled streptavidin and 1.5 μg of b-Mab 9B9 (b-IgG) and washed by 10-min nonrecirculating perfusion with KRB-BSA. The data are presented as mean + s.d., n = 3 rats. (B) Perfusion of isolated rat lungs with 0.5 μg of radiolabeled streptavidin conjugated with various doses of b-Mab 9B9. Lungs were perfused by recirculation for 30 min of 25 ml KRB-BSA containing indicated doses of the conjugate and washed by 10-min nonrecirculating perfusion with KRB-BSA. The data are presented as mean + s.d., n = 3 rats.

greatly. These publications have primarily used biotinylated Mabs as affinity carriers. Since Mabs are individual proteins with unique characteristics (e.g., degree of inactivation upon modification), biotinylation of antibody seems to be a critical step in the targeting. High degrees of biotinylation reduce the antigen-binding capacity of the antibody (*25*). Our results show that hyperbiotinylation not only reduces targeting to the lung, but also enhances elimination of antibody from the circulation (Table 1). Moreover, some other antibody functions, e.g., activation of the complement, may change upon biotinylation (*26*). Our results and analysis of the published data suggest that biotinylation at a biotin-to-antibody molar ratio in the reaction mixture about 10-20 provides biotinylated antibody possessing high affinity to streptavidin but does not significantly change antigen-binding capacity and biodistribution of antibody.

TABLE 2
Biodistribution of Radiolabeled Streptavidin Conjugated with Biotinylated Mab 9B9 or with Biotinylated IgG in Rats

		SA-b-Mab 9B9	SA-b-IgG	Immunospecificity*
Blood	%ID/g	2.46 + 0.43	3.34 + 0.26	0.7
	LR†	1	1	1
Lung	%ID/g	19.50 + 4.41	0.38 + 0.13	51.3
	LR	7.89 + 0.42	0.11 + 0.03	71.7
Liver	%ID/g	2.92 + 0.57	1.71 + 0.54	1.2
	LR	1.19 + 0.01	0.57 + 0.12	2.3
Kidney	%ID/g	0.79 + 0.07	0.99 + 0.07	0.7
	LR	0.32 + 0.03	0.30 + 0.05	1.1
Spleen	%ID/g	1.23 + 0.50	1.54 + 0.25	0.8
	LR	0.49 + 0.12	0.47 + 0.11	1.0
Heart	%ID/g	0.32 + 0.12	0.17 + 0.02	1.9
	LR	0.13 + 0.03	0.05 + 0.01	2.6

*Immunospecificity is the ratio of tissue levels for immune and non-immune preparations.

†LR = localization ratio is ratio of radioactivity per g of tissue to that of blood. Iodine-125 streptavidin (1 μg) was mixed with 2.5 μg of b-Mab 9B9 or with same amount of b-IgG 1 hr before intravenous injection in male Wistar rats. One hour after injection, radioactivity in tissues was estimated by the procedure described in the legend to Table 1. The data are presented as mean + s.d., n = 3 rats.

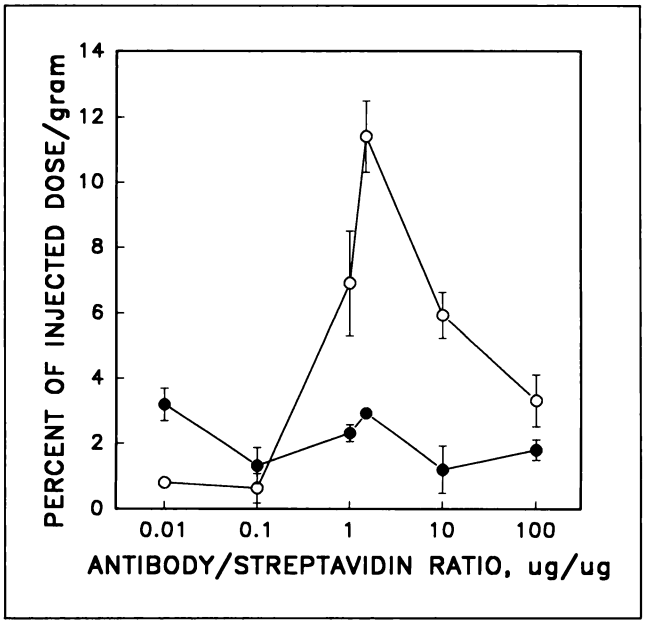

FIGURE 3. Pulmonary uptake (open circles) and blood level (closed circles) of radiolabeled streptavidin (1 μg/rat) conjugated with various doses of b-Mab 9B9 (0.01–100 μg/rat) 1 hr after intravenous injection in Wistar rats. The data are presented as mean + s.d., n = 3 rats.

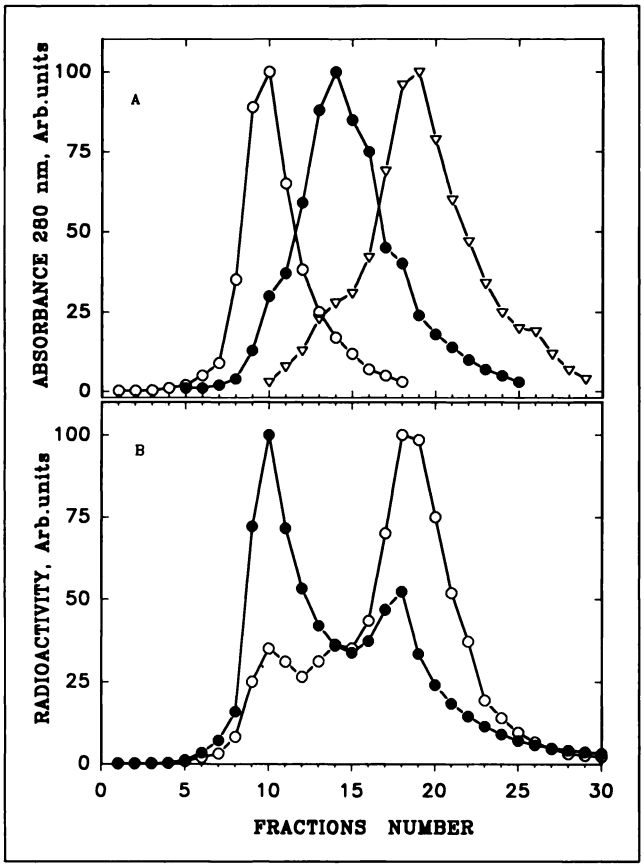

FIGURE 4. Gel-filtration of radiolabeled streptavidin on Sephacryl S-200HR. (A) Calibration chromatography using Dextran Blue (open circles, excluded volume), IgG (closed circles, 160 kD) and BSA (triangles, 60 kD). (B) Gel-filtrations of radiolabeled streptavidin (open circles) or radiolabeled streptavidin mixed with b-Mab 9B9 (closed circles).

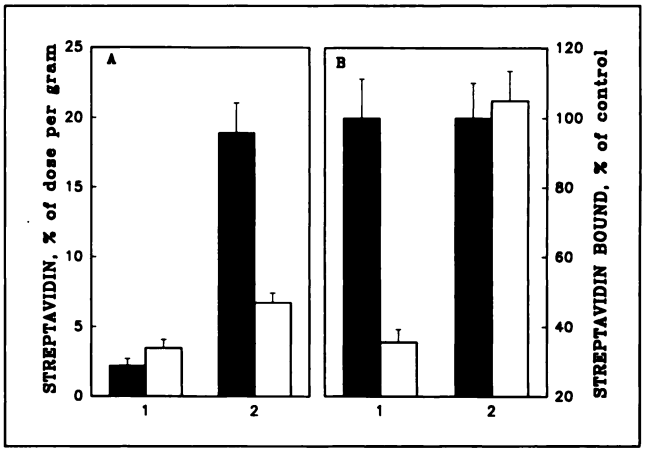

FIGURE 5. Comparison of one-step and two-step administration of radiolabeled streptavidin and b-Mab 9B9 in vivo and in perfusion of isolated rat lungs. Left panel: blood level (1) and pulmonary uptake (2) of streptavidin 1 hr after intravenous injection in Sprague-Dawley rats. Closed bars: 1 μg of streptavidin and 2.5 μg of b-Mab 9B9 were mixed in 150 μl of PBS-BSA 30 min before injection. Open bars: 1 μg of streptavidin was injected 10 min after injection of 2.5 μg of b-Mab 9B9. The data are presented as mean + s.d., n = 3 rats. Right panel: pulmonary uptake of streptavidin in vivo (1) and in perfused rat lungs (2). Closed bars: 1 μg of streptavidin and 2.5 μg of b-Mab 9B9 were mixed in 150 μl of PBS-BSA 30 min before injection or addition to perfusate. Open bars: 1 μg of streptavidin was injected 10 min after injection of 2.5 μg of b-Mab 9B9. The data are presented as percent of pulmonary uptake with one-step administration, mean + s.d., n = 3 rats.

There are various strategies for targeting biotinylated agents (e.g., radiolabel) using biotinylated antibody and avidin. These strategies have been developed for targeting to solid tumors localized in tissues. To increase the tumor-to-blood ratio of targeting, the use of a two-step or three-step administration of biotinylated antibody, avidin and biotinylated agent has been suggested (*9–11*). As a first step, biotinylated antibody or antibody-avidin conjugate should be injected. At some time after first injection, when accumulation in the target and blood clearance has occurred, biotinylated agent, e.g., radiolabel, should be injected. Due to low molecular size, this agent both reaches the target and is cleared from the bloodstream via the kidney rapidly. Various combinations of two- and three-step strategies provide a relatively high-target level of radioactivity at low blood radioactivity.

In our case, the target is normally exposed to the bloodstream, since ACE is a protein localized on the luminal surface of the endothelium (*18*). That is why we have very effective, selective and specific targeting of radiolabeled streptavidin conjugated with b-Mab 9B9. We also have explored a two-step strategy and injected streptavidin after biotinylated Mab 9B9. Unexpectedly, we observed a de-

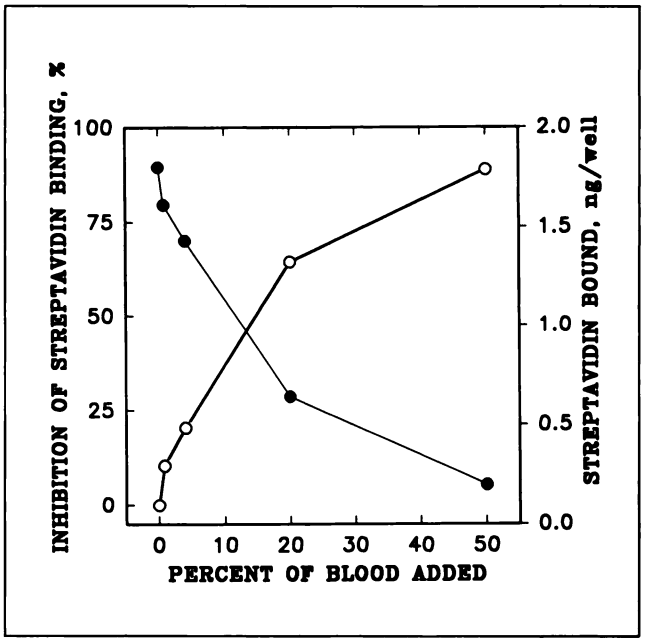

FIGURE 6. Normal rat blood inhibits binding of radiolabeled streptavidin to b-Mab 9B9 in vitro. The data are presented as binding of streptavidin to immobilized b-Mab 9B9 (ng/well, right scale, closed circles), and as a percent of inhibition of streptavidin binding to b-Mab 9B9 (left scale, open circles).

crease in the pulmonary uptake of streptavidin, both in terms of percent of dose per gram and the lung-to-blood ratio. Since we did not observe such a reduction in the perfusion of the isolated rat lung, some systemic effect may change targeting at a step-wise procedure. Our results clearly demonstrate that normal rat blood inhibits interaction of streptavidin with biotinylated protein. Therefore, in vivo interaction of streptavidin with biotinylated compounds may be restricted by blood. This appears to be of general interest for various areas of avidin applications in vivo (drug targeting, immunoimaging, blood clearance) and therefore, should be studied in detail to identify the agent responsible for inhibiting activity. Our preliminary data

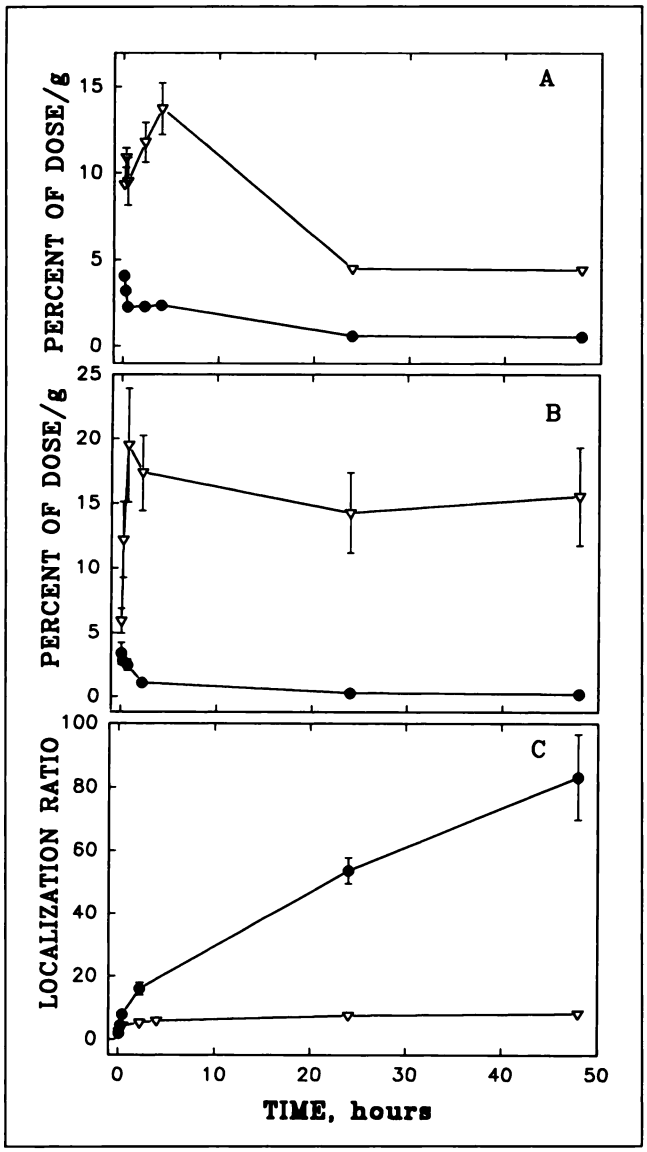

FIGURE 7. Kinetics of blood clearance, pulmonary uptake and lung/blood ratios of Mab 9B9 and streptavidin-b-Mab 9B9 conjugate in Sprague-Dawley rats. (A) Blood (circles) and lung (triangles) levels of Mab 9B9; (B) Blood (circles) and lung (triangles) levels of streptavidin-b-Mab 9B9; (C) Lung-to-blood ratios of Mab 9B9 (triangles) and streptavidin-b-Mab 9B9. The data are presented as mean + s.d., n = 3 rats.

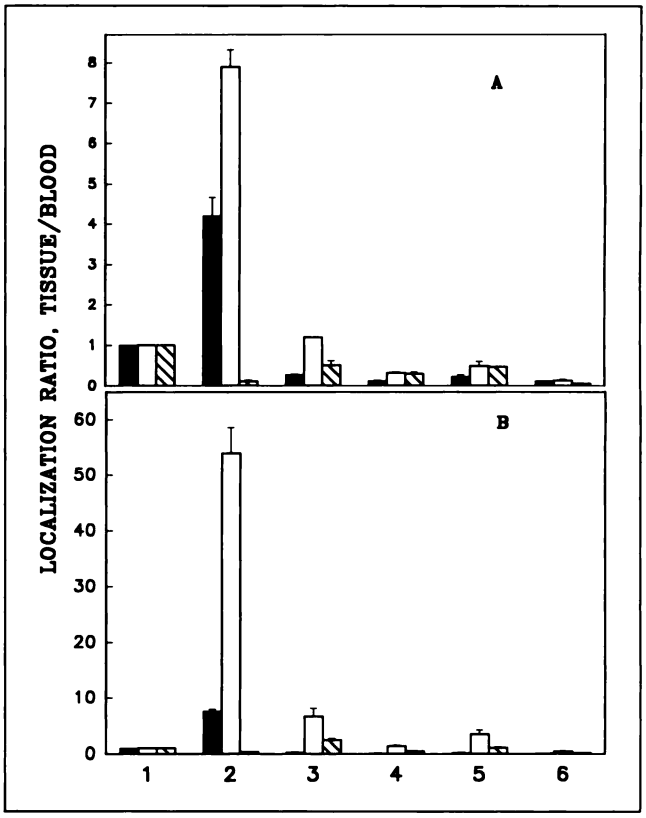

FIGURE 8. Tissue selectivity of biodistribution of Mab 9B9 (closed bars), streptavidin-b-Mab 9B9 (open bars) and streptavidin-b-IgG (striped bars) in Sprague-Dawley rats 1 hr (A) and 24 hr (B) after intravenous injection. 1 = blood; 2 = lung; 3 = liver; 4 = kidney; 5 = spleen; and 6 = heart. The data are presented as mean + s.d., n = 3 rats.

obtained in vitro suggest that the inhibiting effect of blood decreases at high doses of streptavidin. Presumably, the effect of blood may depend on a variety of factors, such as animal species, age and diet.

Therefore, targeting of streptavidin mediated by biotinylated antibody depends on several parameters. First, it is influenced by biotinylation of carrier antibody in terms of its antigen-binding capacity, biodistribution and affinity to streptavidin. Second, there are systemic effects of the organism, e.g., inhibition of avidin-biotin interaction by normal blood. Depending on the extent of such inhibition, the step-wise strategy may be inappropriate. Third, the properties of the target tissue are important. Tissue concentration of the antigen and its accessibility for the blood seem to be a most important parameter in terms of targeting. Our results obtained previously in hamsters show that antigen accessibility to the bloodstream is much more important for targeting, than total content of antigen in the organ (Danilov SM, *unpublished data*). Fortunately, ACE is normally localized on the luminal surface of endothelial cells and, therefore, is easily accessible to circulating antibody or conjugate. These circumstances allow very selective, effective and specific targeting to pulmonary ACE even with the one-step method of streptavidin administration.

In addition to accessibility for the bloodstream, the pathway for the antigen turnover and metabolism in the target tissue is an important parameter. Our preliminary results, obtained in the perfusion of isolated rat lungs, suggest that anti-ACE Mab 9B9, bound to the pulmonary endothelium at physiological temperature, disappears from the luminal surface of this cell by internalization. We suppose that internalization of streptavidin-b-Mab 9B9 complex is more effective than that of anti-ACE itself. It is known that the efficiency of internalization of various ligands increases with an increase in their molecular size, charge and valency of binding to receptors (*27*). This consideration helps to explain enhanced pulmonary uptake and prolonged pulmonary retention of streptavidin-b-Mab 9B9 conjugate in comparison with anti-ACE. Streptavidin-b-Mab 9B9 complex may be internalized by the cell more rapidly and effectively than the antibody itself.

In addition to an increase in molecular size, the number of antigen-binding sites per complex should be higher in the conjugate as compared with antibody. It also may increase the target uptake of the preparation due to increased valency of its binding to endothelium. Further work will focus on the mechanism of enhancement of targeting by conjugation of antibody with streptavidin.

Anti-ACE Mab 9B9 cross-reacts with human, monkey, hamster, cat and rat ACE and accumulates selectively in the lungs of these species (*17*). Therefore, effective, selective and specific targeting of streptavidin-b-Mab 9B9 conjugate looks encouraging as a tool for biomedical research. We suggest that visualization of pulmonary endothelial surface could provide novel insights for the study of lung function. Our studies in animal models show that pulmonary uptake of Mab 9B9 is altered with lung injury (*28,39*). Our preliminary observations in humans suggest that pulmonary uptake of Mab 9B9 in sarcoidosis patients differs from that in healthy volunteers (Danilov S, unpublished result). Therefore, visualization and quantitation of the pulmonary uptake of Mab 9B9 may be of diagnostic use. As another possibility, streptavidin-b-Mab 9B9 could be used for drug targeting to the pulmonary endothelium. Our preliminary results show that biotinylated superoxide dismutase-streptavidin-b-Mab 9B9 complex accumulates very selectively in rat lungs. This methodology could provide a new approach for local pulmonary antioxidant or other forms of enzyme therapy.

ACKNOWLEDGMENT

Dr. Vladimir Muzykantov has been supported in part by a fellowship from the Will Rogers Foundation and RO1 grant (HL-41939) from the National Heart, Lung and Blood Institute.

REFERENCES

1. Wilchek M, Bayer E. The avidin-biotin complex in bioanalytical research. *Anal Biochem* 1988;171:1–32.
2. Green M. Avidin. In: Anfunsen C, Edsall J, Richards F, Eds. *Advances in protein chemistry*, vol. 29. New York: Academic Press; 1975;85–133.
3. Klibanov A, Martynov A, Slinkin M, et al. Blood clearance of radiolabeled antibody by lactosamination, treatment with biotin-avidin or anti-mouse IgG antibodies. *J Nucl Med* 1988;29:1251–1256.
4. Sinitsyn V, Mamontova A, Chekneva E, et al. Rapid blood clearance of biotinylated IgG after infusion of avidin. *J Nucl Med* 1989;30:66–69.
5. Ogihara-Umeda I, Sasaki T, Nishigori N. Active removal of radioactivity in the blood circulation using biotin-bearing liposomes and avidin for rapid tumor imaging. *Eur J Nucl Med* 1993;20:170–172.
6. Suzuki T, Dale G. Biotinylated erythrocytes: in vivo survival and in vitro recovery. *Blood* 1987;70:791–795.
7. Hoffman-Fezer G, Masehke H, Zeitler H, et al. Direct in vivo biotinylation of erythrocytes as an assay for cell survival studies. *Ann Hematol* 1991;63: 214–217.
8. Cavill I, Trevett D, Fisher J, Hoy T. The measurement of the total volume of red blood cells in man: a nonradioactive approach using biotin. *Brit J Hematol* 1988;70:491–493.
9. Hnatovich D, Virzi F, Ruskovski M. Investigations of avidin and biotin for imaging applications. *J Nucl Med* 1987;28:1294–1302.
10. Paganelli G, Belloni C, Magnani P, et al. Two-step tumour targeting in ovarian cancer patients using biotinylated monoclonal antibodies and radioactive streptavidin. *Eur J Nucl Med* 1992;19:322–329.
11. Del Rosario R, Wahl R. Biotinylated iodo-polylysine for pretargeted radiation delivery. *J Nucl Med* 1993;34:1147–1157.
12. Philpott G, Kulczycky A, Grass E, Parker C. Selective binding and cytotoxicity of rat basophilic leukemia cells with immunoglobulin E-biotin and avidin-glucose oxidase conjugate. *J Immunol* 1980;125:1201–1209.
13. White R, Lowrie L, Stork J, et al. Targeted enzyme therapy of experimental glomerulonephritis in rats. *J Clin Invest* 1991;87:1819–1827.
14. Bickel U, Yoshikawa T, Landaw E, et al. Pharmacologic effects in vivo in brain by vector-mediated peptide drug delivery. *Proc Natl Acad Sci USA* 1993;90:2618–2622.
15. Skee D, Barber B. Studies of the adjuvant-independent antibody response to immunotargeting. *J Immunol* 1993;151:3557–3568.
16. Magnani M, Chiaramantini L, Vittoria E, et al. Red blood cells as an antigen-delivery system. *Biotechnol Appl Biochem* 1992;16:188–194.
17. Danilov S, Muzykantov V, Martynov A, et al. Lung is a target organ for monoclonal antibody to angiotensin-converting enzyme. *Lab Invest* 1991; 64:118–124.
18. Erdos E. Angiotensin-converting enzyme and the changes of our concepts through the years. *Hypertension* 1990;16:363–370.
19. Lieberman I, Sastre M. Angiotensin-converting enzyme in postmortem human tissues. *Lab Invest* 1981;48:711–727.
20. Muzykantov V, Martynov A, Puchnina E, Danilov S. In vivo administration of glucose oxidase conjugated with monoclonal antibody to ACE: targeting into the lung. *Am Rev Respir Dis* 1989;136:1464–1473.
21. Danilov S, Martynov A, Klibanov A, et al. Radioimmunoimaging of lung vessels: an approach using ^{111}In-labeled monoclonal antibody to ACE. *J Nucl Med* 1989;30:1686–1692.
22. Muzykantov V, Sakharov D, Smirnov M, et al. Immunotargeting of erythrocyte-bound streptokinase provides local lysis of fibrin clot. *Biochim Biophys Acta* 1986;884:355–363.
23. Hiemish H, Gavriljuk V, Atochina E, et al. Purification of radiolabeled monoclonal antibody to ACE significantly improves specificity and efficacy of its targeting to the lung. *Nucl Med Biol* 1993;20:435–441.
24. Fisher A, Dodia C, Linask J. Perfusate composition and edema formation in isolated rat lungs. *Exp Lung Res* 1980;1:13–21.
25. Panutich A, Baturevich E, Kolesnikova T, Ganz T. The effect of biotinylation on the antigenic specificity of anti-defensin monoclonal antibodies. *J Immunol Meth* 1993;158:237–242.
26. Jokiranta T, Meri S. Biotinylation of monoclonal antibodies prevents their ability to activate the classical pathway of complement. *J Immunol* 1993; 151:2124–2131.
27. Brown V, Greene M. Molecular and cellular mechanisms of receptor-mediated endocytosis. *DNA Cell Biol* 1991;10:399–409.
28. Muzykantov V, Puchnina E, Atochina E, et al. Endotoxin reduces specific pulmonary uptake of radiolabeled monoclonal antibody to ACE. *J Nucl Med* 1991;32:453–460.
29. Atochina E, Hiemish H, Muzykantov V, Danilov S. Systemic administration of PAF in rat reduces specific pulmonary uptake of circulating monoclonal antibody to ACE. *Lung* 1992;170:349–358.