objection in that the drug may not be presented in the same physical state, since in the liposomal triamcinolone is present as a molecular dispersion in lipid micelles (17). Under these circumstances the possibility of a dissolution-dependent disposition of triamcinolone in the control preparation would seriously affect the pharmacokinetic comparison of the two dosage forms. Finally, a physical admixture of drug, lipids, and calcium chloride solution could, potentially, promote the spontaneous formation of an undetermined number of liposomes when agitated. If this happened, the control would not be totally free of drug-containing liposomes. Thus, while the triamcinolone solution in polyethylene glycol–water used in this study may not be qualitatively similar to the liposomal dosage form, it was designed to present the model compound in an intravenous dosage form that was miscible with plasma water. The comparison of the pharmacokinetics of triamcinolone in liposomal and solution dosage forms is, thus, analogous to other comparative studies (*e.g.*, bioavailability) of a therapeutic agent in different formulations which may not (and often do not) contain the same excipients. If it is assumed that the presence in blood of exogenous lipids (used in the preparation of the liposomes) and polyethylene glycol in the concentrations used did not influence blood flow rate significantly, then the differences in β and Vd_β observed in this study are due probably to the favorable physicochemical properties (especially lipid solubility) of the liposomal entity.

Results of the present study indicate that liposome-encapsulated triamcinolone enhances tissue distribution of the drug in the rabbit. The results also suggest that, given by the intravenous route, neutral multilamellar liposomes could serve as carriers for chemotherapeutic agents whose efficacy depends on sustained blood levels and deep tissue distribution.

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Acrylic Microspheres *In Vivo* IX: Blood Elimination Kinetics and Organ Distribution of Microparticles with Different Surface Characteristics

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Abstract □ The elimination of microparticles from blood after intravenous injection is dependent on the surface characteristics of the particles. The half-life in blood increases from 44 to 84 min after modification of surface-localized human serum albumin with polyethylene glycol. Irrespective of the surface properties, particles are localized in the reticuloendothelial system, mainly in the liver and spleen. In preimmunized mice, the distribution of particles is somewhat altered, *i.e.*, the liver and lung uptake is significantly higher in preimmunized animals than in untreated animals. The rate of phagocytosis of particles with different surface characteristics has also been studied *in vitro* with isolated mouse peritoneal macrophages. This technique gives a good correlation with the *in vivo* results; thus particles with a short half-life in mice are rapidly phagocytosed by the macrophages *in vitro*.

Keyphrases □ Microparticles—polyacrylamide, blood elimination kinetics, organ distribution, effect of surface characteristics, mice □ Elimination—polyacrylamide microparticles from blood, kinetics, organ distribution, effect of surface characteristics, mice □ Delivery systems—polyacrylamide microparticles, blood elimination, kinetics, organ distribution, mice

Immobilized systems in the form of small beads or particles have recently been introduced as carriers of enzymes *in vivo* (1). Some of these systems are characterized as biodegradable (*e.g.*, liposomes), while others are slowly metabolized (*e.g.*, acrylic particles). Irrespective of the type of particles, these systems show great promise as a tool for "active targeting" of enzymes and other macromolecules to specific cells or organs in the body. However, one of the

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drawbacks of using such particles as a carrier system is the lack of "directability," because the reticuloendothelial system (RES), mainly the liver, spleen, and bone marrow, dominates the uptake of intravenously injected particles. It is well recognized that this uptake must be circumvented to realize the dream of "active targeting" using particulate carrier systems.

Within a few hours after intravenously injecting massive amounts of particles, none are left free in the circulatory system [*e.g.*, microparticles of polyacrylamide have a half-life of 40–60 min in blood (2)]. This efficacy in identifying the foreign nature of such particles in the presence of particles that are normal to blood must depend on surface characteristics. The present study investigates the possibility of changing the rate of phagocytosis of the microparticles in order to make them more suitable as carriers for enzymes.

EXPERIMENTAL

Materials—Human serum albumin[1], IgG[1], rabbit anti-(human serum albumin) globulin[2], L-asparaginase[3], polyethylene glycol monoethyl ether[4] (mol. wt. 1900), [^{14}C]salicylic acid[5], and [^{14}C]paraformaldehyde[5] were used without further purification. Acrylamide[6], *N,N'*-methylenebisacrylamide[6], *N,N,N',N'*-tetramethylethylenediamine[7], tris(hydroxymethyl)aminomethane[7], and other chemicals were analytical grade.

Preparation of Microparticles—Microparticles[8] were prepared and characterized using a reported method (1). A solution of acrylamide (6% w/v) and *N,N'*-methylenebisacrylamide (2% w/v) in 0.1 *M* KCl–0.005 *M* sodium phosphate buffer (pH 7.4), which also contained the protein to be immobilized, was homogenized in an organic phase consisting of toluene–chloroform (4:1 v/v). Ten milliliters of the aqueous phase was emulsified with an homogenizer in 200 ml of the organic phase with a detergent[9] immediately after the catalyst system, consisting of *N,N,N',N'*-tetramethylethylenediamine and ammonium persulfate, had been added to the monomer solution. For the experiments described, microparticles were prepared from human serum albumin, L-asparaginase, or polyethylene glycol-modified human serum albumin by dissolving 0.5–2 g, 96 mg, or 0.5 g of the substrate, respectively, in 10 ml of the monomer solution. After polymerization, the microparticles were freed from the organic phase by repeated washings with buffer and physiological saline. Prior to injection, the particles were carefully dispersed in physiological saline.

^{14}C-Labeled particles were prepared as described by Sjöholm and Edman (2). Radioactivity was measured by liquid scintillation counting. The counting efficiency was calculated with an external standard.

Functional Capacity of Immobilized Human Serum Albumin—The functional capacity of albumin in microparticles was determined as previously described (3). Microparticles with albumin were incubated with [^{14}C]salicylic acid. The amount of albumin binding with salicylic acid was calculated from the binding degree and from a standard curve derived by equilibrium dialysis with different albumin concentrations.

Preparation of Tissue Samples for the Determination of the Distribution of Microparticles—^{14}C-Labeled microparticles in physiological saline (0.2 ml) were injected intravenously or intraperitoneally in the mice. The dose corresponded to 0.5 mg of lyophilized microparticles. The different particles used are specified in Table I. After 6 hr, 24 hr, 1, and 2 weeks, five animals were killed by cervical dislocation. Five mice which were not given any microparticles were used as a control group. The organs were immediately removed, weighed, and prepared for radioactivity measurements. The whole spleen, both lungs, one kidney, and 0.2–0.3 g of the liver were each dissolved in 2 ml of a tissue solubilizer[10] in a counting vial. After treatment at 40° for 12 hr (yielding a clear solution), 2-propanol (0.4 ml) and 30% hydrogen peroxide (0.8 ml) were added to minimize color quenching. A scintillation cocktail[11] (10 ml) was then added, and the samples were counted after adapting to the dark and appropriate temperature.

Determination of the Disappearance Rate of Microparticles from the Circulatory System—Male mice[12] weighing 18–22 g were used. The microparticles (0.5 mg dry weight) were injected intravenously in the tail vein, in a total volume of 0.2 ml. The characteristics of the particles used are summarized in Tables I and II.

Blood samples (75 μl) were collected with a heparinized microtube from the orbital plexus 5, 10, 15, 20, 30, 45, 60, 90, 120, 240, and 300 min after dosing. Only two blood samples were drawn from each mouse; thus, the cumulative blood volume withdrawn never exceeded 10% of the total blood volume. Background values were obtained in the same manner from animals not given microparticles. The animals were unrestrained and had free access to food and water.

The whole blood was digested with 1 ml of a solution consisting of tissue solubilizer[10] and 2-propanol (1:1) in a counting vial. Hydrogen peroxide (30%, 0.5 ml,) was added and the vial was left at 40° for 15 min. A scintillation cocktail[11] was then added and after appropriate equilibration to darkness and temperature, the samples were counted in the liquid scintillation counter.

Assay of Native and Immobilized L-Asparaginase—L-Asparaginase activity was determined from the amount of ammonia produced by reaction with the substrate, L-asparagine, at 37° as earlier described (1). After addition of the ammonia color reagent, the absorbance was determined at 500 nm. Appropriate enzyme and substrate blanks were included in all assays. A standard curve was prepared with known amounts of ammonium sulfate. One unit of activity is defined as the amount of enzyme catalyzing the formation of 1.0 μmole of ammonia/min at 37°.

Autoradiography—Whole-body autoradiography was performed using the method of Ullberg (4). The ^{14}C-labeled microparticles in 0.2 ml of physiological saline were injected in the tail vein. After 24 hr the animals were anesthetized with ether and killed by immersion in hexane–solid carbon dioxide (−78°). With an aqueous gel of carboxymethylcellulose as the medium, the mice were mounted on a large microtome stage. Several 20- or 60-μm saggital sections were cut through the whole body and fixed on adhesive tape. The sections were cut at −15 to −20° and freeze-dried. The slices were pressed against photographic films, which were exposed for up to 6 months. After developing, the films were compared with the corresponding tissue sections.

Determination of Particle Size—The size of the particles was measured from photographs taken by scanning electron microscopy as described by Höglund and Morein (5). The particles were fixed at 4° with glutaric dialdehyde for ~20 hr. Postfixation was done in neutralized 1% osmium tetroxide for 1 hr. The samples were rinsed in saline and freeze-dried. The dehydrated specimens were coated with palladium–gold alloy in an evaporator and analyzed in a scanning electron microscope[13] at 30-keV accelerating voltage.

Immunization Procedures—The mice were injected with L-asparaginase (5 IU) in native form intramuscularly and/or subcutaneously. The enzyme was administered in Freund's complete or incomplete adjuvant. The animals received booster injections after 5, 10, 20, and 30 days. Blood was collected from the orbital plexus and centrifuged at 4° to obtain serum. Each mouse received a total dose of 20–25 IU of L-asparaginase during the study. The antibody titer was assayed by double immunodiffusion in agar gel as described by Ouchterlony (6).

Preparation of Polyethylene Glycol-Modified Human Serum Albumin—Covalent attachment of monomethoxy polyethylene glycol (mol. wt. 1900) to human serum albumin was effected using the procedure of Abuchowski *et al.* (7). Cyanuric chloride (5.5 g, 0.03 mmole) was dissolved in 400 ml of anhydrous toluene and monomethoxy polyethylene glycol (mol. wt. 1900) (19 g, 0.01 mmole) was added to the solution. The 2-*O*-methoxypolyethylene glycol-4,6-dichloro-*s*-triazine (activated polyethylene glycol) formed was precipitated several times with petroleum ether until it was free of cyanuric chloride. The activated polyethylene glycol was coupled to human serum albumin by dissolving the two reactants in sodium borate buffer (0.1 *M*, pH 9.2). The reaction was allowed to proceed for 2 hr at 4°. The fraction of modified primary amino groups was determined by the trinitrobenzenesulfonate method of Ha-

[1] KABI AB, Stockholm, Sweden.
[2] DAKO AB, Copenhagen, Denmark.
[3] Crasnitin; obtained as a gift from Bayer (Sverige).
[4] Aldrich Europe Co.
[5] Amersham, England.
[6] Eastman Kodak Co.
[7] Sigma Chemical Co.
[8] U.S. Patent 4,061,966.
[9] Pluronic F 68.
[10] Soluene 350, Packard Instrument Co.
[11] Dimilume 30, Packard Instrument Co.
[12] NMRI strain.
[13] JSM-U3 microscope, JEOL.

Table I—Characteristics of the Microparticles

Preparation	Protein Immobilized	mg of Protein Incorporated / mg of Dry Particles[a]	Size Distribution[b] <300 nm	301–750 nm	751–1050 nm	1051–1800 nm	Mean Particle Diameter, nm
I	None	—	30%	49%	11%	10%	520
II	None	—	9%	26%	36%	29%	860
III	Human serum albumin	0.05[c]	33%	64%	3%	—	290
IV	Human serum albumin	0.17[c]	not determined				
V	Polyethylene glycol-modified human serum albumin	0.13[d]	21%	70%	9%	—	370
VI	L-Asparaginase	0.02	not determined				

[a] The dry weight of the microparticles was determined after lyophilization. [b] Particle diameter. The size distribution was determined by scanning electron microscopy; 350–550 particles were counted from each preparation. [c] The values were obtained by determination of the functional capacity of albumin as described in the text. [d] The high degree of polyethylene glycol substitution changed the functional capacity of the albumin used. The amount of protein incorporated was therefore determined by amino acid analysis.

beeb (8). Protein concentrations were determined by the biuret method (9), since the method by Lowry *et al.* (10) gives erroneously high values with polyethylene glycol-modified albumin (7). A double immunodiffusion test was used (6) to study the interaction between polyethylene glycol-modified human serum albumin and rabbit anti-(human serum albumin).

Cultivation of Mouse Peritoneal Macrophages—Cells from the peritoneal cavity of unstimulated adult male mice were harvested with 3 ml of cold (4°) phosphate-buffered saline. The cells were further suspended in 10 ml of cold (4°) buffer and mildly spun down at 180×*g* for 10 min. The pellet was resuspended in a nutrient medium[14] containing 10 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4. The medium was supplemented with benzylpenicillin (100 U/ml), streptomycin (10 μg/ml), and 20% fetal calf serum. The cells were seeded on 50-mm plastic petri dishes and cultured at 37° in 5% CO_2 in air at 85% humidity. After incubation for 45 min, nonadherent cells were removed by washing with buffer, and 5 ml of fresh medium was added. The cells were incubated for an additional 24 hr before use.

Measurement of Phagocytosis of Microparticles—^{14}C-Labeled microparticles (0.25 mg dry weight in 50 μl of phosphate-buffered saline) with a radioactivity of ~50,000 dpm were added to a petri dish containing 1–2 × 10^6 mouse peritoneal macrophages, and the phagocytosis was studied at 37° or 0°. After 2, 4, and 6 hr, the tissue culture medium was aspirated, and the tissue cultures were rinsed with saline (0.154 *M* NaCl) six times to remove unattached microparticles. The contents of the dried dishes were digested for 8 hr at room temperature in 1.5 ml of 0.5 *M* NaOH (11). A 100-μl portion of the digest was removed by aspiration for protein determination; the rest was quantitatively transferred to a liquid scintillation vial, and the radioactivity was determined.

Estimation of the Rate of Particle Aggregation—A light-scattering spectroscopy instrument[15] capable of estimating the size of particles, aggregates, or other particulate matter within the size range of 0.10–3 μm was used to measure the relative size of different microparticle batches and to follow the aggregation of microparticles in the presence of specific antisera. Microparticles and protein solutions were diluted in phosphate-buffered saline, which was passed through a membrane filter (pore size 0.22 μm) prior to use. In a typical experiment, particles of the same size containing human serum albumin, L-asparaginase, or no protein at all were diluted to the same absorption at 450 nm. The initial value for 450 μl of the sample was recorded by the instrument and 100 μl of a solution of antiserum [*i.e.*, rabbit anti-(human albumin serum) containing 3.6 mg of IgG/ml] or buffer was added with subsequent recording of the light scattering. All measurements were done in triplicate.

RESULTS

Blood Clearance of Microparticles with Different Surface Characteristics—When a protein is immobilized in microparticles, it will not only be localized within the gel structure but will also protrude from the matrix and, thus, will be partly localized on the surface of the microparticles (12). To investigate if surface-localized protein would influence the survival of microparticles in blood, four different preparations were made (Table I). After intravenous injection the blood life span of microparticles was followed with time (Fig. 1). In Table II some experimental values calculated from the curves in Fig. 1 are summarized. The experimental points obtained during the first 90 min were fitted to a straight line by linear regression (with time as the independent variable and the concentration in the circulatory system as the dependent variable). The 5-min values were omitted, since the initial mixing in the whole blood volume apparently was not complete at this time. From the slope of the line, k, the half life ($t_{1/2}$) was estimated from the expression:

Table II—Blood Elimination Parameters of the Microparticles[a]

Preparation	$t_{1/2}$, min	V_d, ml	CL, ml/min
I	61	2.6	0.024
III	44	2.1	0.034
IV	33	2.5	0.052
V	84	2.5	0.020
Mean ± *SEM*		2.4 ± 0.1	

[a] Half-lives, distribution volumes, and clearance values.

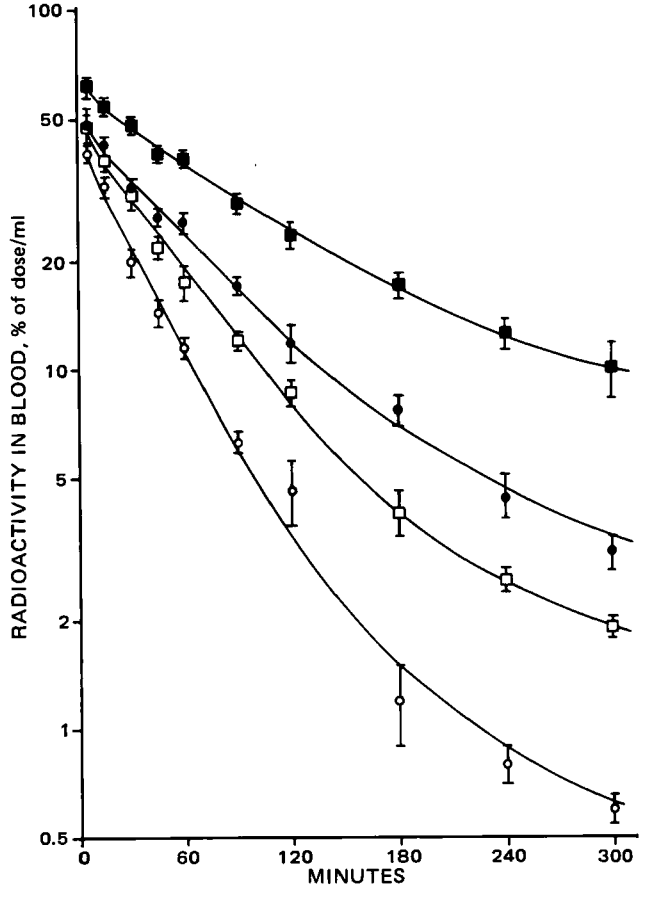

Figure 1—*Disappearance of ^{14}C-labeled microparticles from blood after intravenous injection in mice. Radioactivity is calculated in percent of the total dose (log scale) retained in 1 ml of blood. Key: (●) empty particles; (○) particles containing 0.17 mg of albumin/mg of particles; (□) particles containing 0.05 mg of albumin/mg of particles; and (■) particles containing polyethylene glycol-modified albumin.*

[14] F-10 medium, GIBCO BIO-CULT.
[15] Nanosizer, Coulter, England.

$$t_{1/2} = \frac{\log 2}{k} \quad \text{(Eq. 1)}$$

From the intercept on the y-axis (C_0^g) and the dose given, the apparent volume of distribution, V_d, was calculated:

$$V_d = \frac{\text{dose}}{C_0^g} \quad \text{(Eq. 2)}$$

From $t_{1/2}$ and V_d, the clearance from the blood was estimated according to:

$$CL = \frac{0.693 \times V_d}{t_{1/2}} \quad \text{(Eq. 3)}$$

Intravenously injected empty microparticles (I) were rapidly cleared from the circulatory system with a half-life of 61 min. Particles with immobilized human serum albumin (0.05 mg of protein/mg of lyophilized particles, III), were eliminated with a half-life of 44 min. The elimination rate was further accelerated when the protein content in the particles was increased; *e.g.*, particles with 0.17 mg of protein/mg of dry weight (IV) were eliminated from the circulatory system with a half-life of 33 min.

In an attempt to block the influence of the surface-localized proteins on the extraction rate of particles from the circulatory system, polyethylene glycol-modified human serum albumin was immobilized (V). Seventy percent of the primary amino groups of albumin were derivatized with activated polyethylene glycol. The antigenicity of the derivative was checked by double immunodiffusion. In accordance with the results of Abuchowski *et al.* (7), no precipitation occurred when ≥50% of the primary amino groups were modified. As a consequence, the survival time of these particles in blood was significantly prolonged ($t_{1/2}$ = 84 min) as compared with albumin-containing particles (33 min).

Distribution of Microparticles with Different Surface Characteristics—The distribution pattern of the microparticles was followed quantitatively in mice for 2 weeks after intravenous injection (Table III). The surface properties of the microparticles had no effect on the gross distribution pattern. The microparticles were concentrated in the spleen and liver, and initially also in the lungs. These results agree with those found earlier with empty polyacrylamide particles (2) except for the lung uptake. Earlier, only small amounts of radioactivity were found in the lungs after injection of massive doses (4.1 mg) of empty microparticles in mice. In this study, 4–30% of the radioactivity was found in the lungs 6 hr after injection. However, in 24 hr a redistribution could be seen, with elimination of radioactivity from the lungs and a corresponding uptake of radioactivity in the liver.

The differences in the results between this and the previous study (2) were thought to be an effect of the microparticle size, since larger particles were used in this study. In the earlier study, the mean particle size was estimated to 0.25 μm compared with mean values of 0.29–0.86 μm in this study (Table I). The effect of the particle size on the organ distribution was checked by comparing two preparations of empty microparticles with mean diameters of 0.52 and 0.86 μm (I and II, respectively). As expected, the batch with the larger particles was retained to a greater extent in the lungs than the batch with the smaller mean particle diameter. Thus, 6 hr after intravenous injection, 30% of II and 9% of I were found in the lungs. Two weeks after the injection of small microparticles (I), no radioactivity was detected in the lungs, while 10% of the large microparticles (II) still remained in the lungs.

Uptake of Microparticles by Mouse Peritoneal Macrophages—The dependence of the elimination rate of microparticles from blood in mice on the proteins immobilized in the microparticles was also studied *in vitro*. A system with mouse macrophages was designed, in which the phagocytosis of ^{14}C-labeled microparticles with different immobilized proteins was followed. A nontoxic dose (0.25 mg dry weight of particles) was added to a monolayer of the macrophages cultivated in a petri dish. The extent of phagocytosis was estimated from the radioactivity content in the macrophages after different time periods and related to the protein content, which represents a measure of viable cells. The dose chosen was the lowest found to give the maximal uptake in ~1–2 × 10^6 cells.

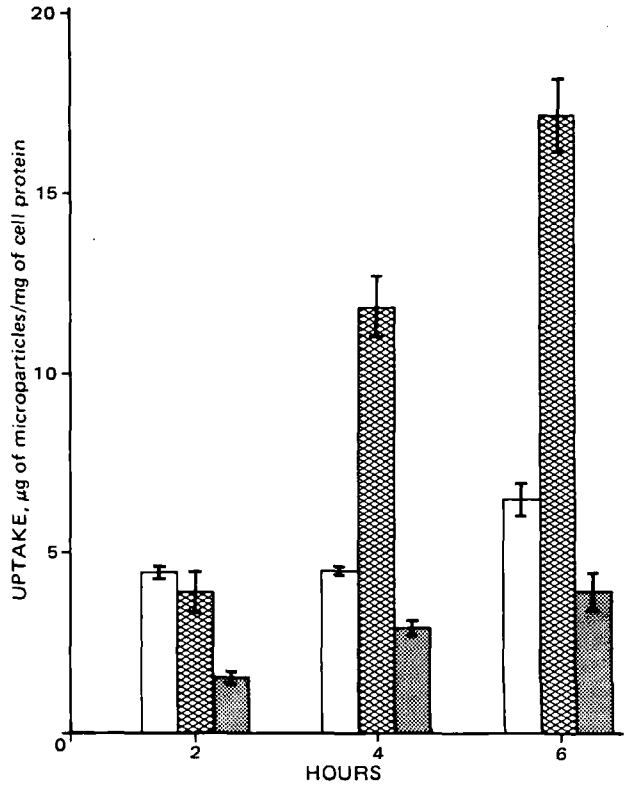

Figure 2—*Rates of uptake of microparticles with different surface characteristics by mouse peritoneal macrophages. All values represent the mean ± SEM of triplicate incubations, corrected for unspecific interaction between cells and particles. The uptake was calculated as the amount of microparticles (μg)/mg of cell protein. Key: (□) ^{14}C-Labeled empty microparticles (I); (▨) albumin-containing particles (IV); (■) particles with polyethylene glycol-modified albumin (V).*

The results from the cell studies are summarized in Fig. 2. The rate of uptake of empty microparticles was slower than that of particles containing human serum albumin, but faster than that of particles in which the amino groups of albumin had been chemically modified with polyethylene glycol. The unspecific adsorption of particles to the macrophages, as measured at 0°, was low and insignificant. As is apparent, the results correlate well with those found in the *in vivo* studies.

Disappearance Rate from Blood of Microparticles with L-Asparaginase in Preimmunized Mice—The mice were immunized with

Table III—Organ Distribution of Microparticles with Different Surface Characteristics [a]

Time, days	Organ	Preparation I	III	IV	V
0.25	Liver	38.4 ± 1.2	57.1 ± 1.4	47.3 ± 1.2	48.4 ± 2.5
	Spleen	8.3 ± 0.3	17.4 ± 1.0	13.2 ± 0.5	16.5 ± 0.4
	Lung	30.5 ± 0.9	9.9 ± 0.4	15.3 ± 0.4	3.6 ± 0.2
1	Liver	42.3 ± 1.6	61.7 ± 0.9	54.1 ± 2.1	56.5 ± 2.2
	Spleen	8.8 ± 0.4	13.9 ± 0.8	12.9 ± 0.4	16.2 ± 0.4
	Lung	19.6 ± 0.5	2.4 ± 0.5	6.1 ± 0.7	2.4 ± 0.2
7	Liver	65.2 ± 1.3	65.7 ± 3.1	63.0 ± 1.2	60.9 ± 1.1
	Spleen	10.7 ± 0.1	13.0 ± 0.4	12.7 ± 0.6	13.6 ± 0.9
	Lung	7.3 ± 0.9	0.6 ± 0.09	2.6 ± 0.3	0.2 ± 0.02
14	Liver	62.1 ± 2.1	62.5 ± 0.8	53.9 ± 1.0	54.8 ± 1.1
	Spleen	9.6 ± 0.6	12.0 ± 0.6	9.7 ± 0.3	13.1 ± 0.2
	Lung	10.3 ± 0.9	0.5 ± 0.03	2.8 ± 0.2	0.1 ± 0.01

[a] *Distribution of microparticles after intravenous injection in mice. Each value is expressed as percent of the injected dose and represents the mean ±SEM of five animals.*

Table IV—Organ Distribution of Microparticles Containing L-Asparaginase [a]

Time, days	Organ	Untreated Mice	Immunized Mice
0.25	Liver	41.2 ± 0.2	49.4 ± 2.7
	Spleen	17.6 ± 1.0	4.6 ± 0.2
	Lung	5.7 ± 0.4	12.3 ± 2.7
1	Liver	43.3 ± 0.9	53.2 ± 1.9
	Spleen	13.6 ± 1.0	9.6 ± 0.3
	Lung	1.8 ± 0.2	7.2 ± 1.3
7	Liver	43.0 ± 3.5	54.6 ± 4.1
	Spleen	10.3 ± 1.0	9.6 ± 1.5
	Lung	0.0	0.3 ± 0.03
14	Liver	36.4 ± 2.5	46.3 ± 2.6
	Spleen	7.9 ± 1.0	12.2 ± 2.1
	Lung	0.3 ± 0.03	0.3 ± 0.02

[a] Distribution of ^{14}C-labeled microparticles containing L-asparaginase after intravenous injection in preimmunized and untreated mice. The figures show means ±*SEM* obtained from four or five animals.

soluble L-asparaginase as described above. The antibody titer, as detected by double immunodiffusion according to Ouchterlony (6), was 1:1 to 1:2. Only small doses of microparticles with L-asparaginase could be given. After intravenous injection of 0.5 mg of particles containing 1.2 IU of L-asparaginase, three of eight mice died within 15–20 min. After half the original dose (0.25 mg dry weight of microparticles) was administered, 3 of 18 mice died. The microparticles were very rapidly cleared from the circulatory system with a half-life of <10 min.

The quantitative distribution of the particles in surviving mice was followed in the liver, spleen, and lungs over a period of 2 weeks (Table IV). The values obtained from the lungs of preimmunized mice 6 hr postinjection showed that 12% of the injected dose was found in this organ compared with 6% in the controls (nonimmunized mice). The liver uptake of the injected dose was 8–11% greater for preimmunized animals during the entire study.

After intraperitoneal injection in preimmunized mice, the microparticles were localized in the lymph nodes draining the peritoneal cavity and the central lymphatic vessels, as shown by the autoradiogram (Fig. 3) taken 1 day after injection. The quantitative distribution of the L-asparaginase-containing particles in different organs was followed over a period of 1 week. The total recovery was low, 7% of the injected dose being found in the liver and spleen after 6 hr compared with 17% in the controls. After 1 week, 26 and 35% of the injected dose was recovered in these organs in preimmunized and nonimmunized animals, respectively.

The high lung uptake of L-asparaginase particles after intravenous injection in preimmunized mice is probably due to aggregate formation between the particles and circulating antibodies. To study the rate of formation of particle aggregates, an *in vitro* system was set up to simulate the interaction between specific antibodies and particles containing the corresponding antigen. In this study, anti-(human serum albumin) and albumin particles were used to simulate the *in vivo* condition and the particle size was followed with a light-scattering spectroscope (Fig. 4). As expected, incubation with the specific antibody gave a rapid increase in particle size. The apparent mean diameter increased from 0.6 to 2 μm after a 15-min incubation with the specific antibody and to >3 μm after 60 min. There was no interaction between anti-(human albumin globulin) and empty particles or with particles containing L-asparaginase under the conditions used. Furthermore, the interaction between the albumin particles and anti-albumin serum was inhibited by adding soluble human serum albumin to the solution.

Reticuloendothelial System (RES) Blockade—The capacity of the RES to phagocytose foreign particles from the circulation and an attempt to block such phagocytosis were investigated by repeated intravenous injections of empty polyacrylamide microparticles. In the first study, 0.5 mg of unlabeled empty particles was injected intravenously, and after 6 half-lives a second 0.5 mg dose of ^{14}C-labeled particles was injected. The second study was performed by injecting 2.5 mg (dry weight) particles, with the second dose (0.5 mg) given 16 hr later. This time point was chosen for two reasons. First, there are no particles in the blood after 16 hr. Second, it is known (13) that 24 hr after injection of particles there is an increasing number of phagocytosing cells in the RES, especially in the liver, signifying that the phagocytic capacity is increased. Considering these facts, we chose a time point between 6–24 hr, namely 16 hr.

Irrespective of the size of the first dose (0.5 or 2.5 mg), the RES retained its capacity to extract particles from the blood. Moreover, the blood clearance and organ uptake of microparticles in blockaded animals were almost the same as in normal animals. Thus, the liver and spleen uptake in blockaded animals corresponded to 60–70% of the injected dose, whereas the uptake in the lungs was increased to ~10–15% of the dose. The half-life ($t_{1/2}$) of the particles in blood was 50–60 min in the blockaded animals, the same as in untreated animals.

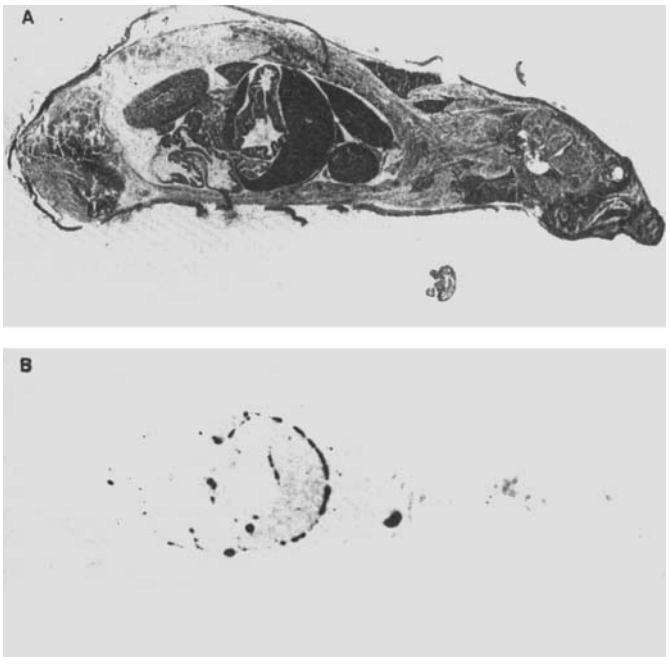

Figure 3—*Whole body autoradiogram (B) of a mouse 1 day after intraperitoneal injection of ^{14}C-labeled microparticles containing L-asparaginase (50,000 dpm in 0.5 mg). The mouse was immunized against L-asparaginase. (A) Corresponding tissue section, 20 μm.*

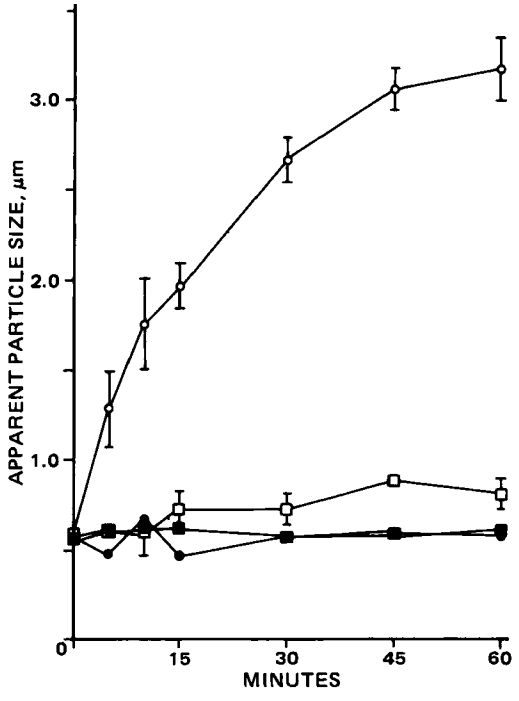

Figure 4—*Interaction between antialbumin globulin and particles with immobilized proteins. The immobilized proteins were albumin (○) and L-asparaginase (□); empty particles (●) were used as controls. In one study (■), albumin particles and antialbumin globulin were incubated in a solution containing free albumin (2×10^{-4} M). The interaction was measured as an increase in the apparent particle size. All values are mean ± SD from triplicate determinations.*

DISCUSSION

When immobilized in acrylic microparticles, proteins are partly entrapped within the polymeric network and partly fixed in the polymer threads forming the particles (12). As a consequence, a fraction of the immobilized macromolecules are localized on the surface and protrude from the particles. This phenomenon has been utilized to detect specific cell-surface receptors and for cell separation purposes after immobilization of appropriate cell-reactive agents in the microparticles (14).

Microparticles containing proteins are rapidly cleared from the circulatory system after intravenous injection. Furthermore, the rate of elimination from the incubation is significantly affected by the protein immobilized in the particles and depends on the amount of protein localized on the particle surface. The results indicate that the phagocytosis of the microparticles apparently is mediated by some receptor mechanism that makes it possible for the macrophages in the RES to discriminate between different surface characteristics and also to estimate the extent of the foreign nature of the particles. The data in Fig. 1, moreover, show that the "estrangement" of the microparticles mediated by the surface-localized proteins can be camouflaged by polyethylene glycol, manifested as an increased circulating lifetime. As a result of the modification with polyethylene glycol, the half-life of microparticles with human serum albumin in blood is increased from 33 to 84 min. This dramatic change may be due to different effects imparted by the modification of the surface properties: a decrease in the foreign nature, changed affinity for water, increased size, or a combination of these effects. The work of Abuchowski *et al.* (7) has shown that polyethylene glycol alters the immunological properties of bovine serum albumin and increases the circulating time of bovine catalase in mice. Polyethylene glycol has also been used in conjugates with antigen to suppress reaginic antibody response (15). It is thus evident that important determinants on the surface are quenched by the modification. However, the long half-life, which significantly exceeded that of microparticles containing no protein ($t_{1/2}$ = 61 min), indicates that other factors may also be involved, *e.g.*, physicochemical surface properties. In this case, the changed size distribution of the microparticles cannot be of any significance, as the size of the particles containing the modified albumin was approximately the same.

The survival time of microparticles in blood was drastically decreased in preimmunized mice, which in this case was a result of aggregation and a concomitant accelerated phagocytosis by the macrophages of the RES, especially the Kupffer cells. The liver uptake of the injected dose was 10% greater in immunized mice as compared with the controls. A plausible explanation to this difference is that Kupffer cells phagocytose larger particles in preference to smaller ones (16). *In vitro* experiments with particles containing albumin and anti-albumin globulin support such an interpretation. Thus, the apparent mean diameter of the particles increased from <1 μm to 3 μm within 15 min. A higher lung uptake in immunized animals gives further support to this hypothesis.

In the experiments presented only modest changes of the tissue distribution were observed, although a substantial change in the clearance rate of different particles was detected. This indicates that the RES, especially the liver and spleen, has an important and dominating role in particle clearance. It is likely that this phenomenon is representative for all particulate matter of submicron size.

Many papers describe attempts to block the RES (17, 18). These studies have shown that the capacity of the RES to eliminate foreign particles from the circulatory system is high. It was, moreover, necessary to inject massive and toxic doses of particles, ghost red blood cells, or liposomes to obtain a blockade. Pretreatment of mice with 0.5 mg of particles, which is the threshold dose required to produce morphological changes in the liver and spleen, did not affect the rate of elimination of subsequently injected particles. However, the practical use of an RES blockade is limited, because the treatment impairs the innate immunity of the recipient.

The results clearly demonstrate that alteration of the surface characteristics of the microparticles can lead to an increased circulation time *in vivo*. The finding is important because it constitutes a means of designing a carrier for macromolecular drugs in order to actively target drugs to specific cells in the vascular system *in vivo*.

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