VESICLE INTERACTIONS WITH POLYAMINO ACIDS AND ANTIBODY: IN VITRO AND IN VIVO STUDIES

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Artificial spherules or vesicles of 900 Å in diameter formed from phosphatidylcholine and gangliosides and enclosing $^{99m}TcO_4^-$ (standard preparation) survive intact in the circulation of the mouse. Polyamino acids and protein have been incorporated into and onto the vesicles; such vesicles remain intact as determined by diffusion dialysis studies and by electron paramagnetic resonance studies of vesicles enclosing spin label. In studying the distribution of polyamino acid-vesicles and protein vesicles in vivo, it was found that the latter distribute differently from standard vesicles or free protein alone whereas aromatic polyamino acid-vesicles concentrate in the liver and spleen to a greater extent than standard vesicles. We conclude that the permeability and stability characteristics of vesicles may be preserved when they are modified by the addition of protein or polyamino acids and that such modification of vesicles may be associated with an alteration of their fate in vivo. The potential exists to use vesicles as carriers of radiopharmaceuticals and other drugs and to direct the vesicles preferentially to tissue targets in vivo.

Vesicles about 900 Å in diameter and composed of phosphatidylcholine and gangliosides have been shown to survive intact in the circulation of the mouse for periods up to 46 min and the distribution and fate of these vesicles after intravenous injection have been investigated by enclosing gamma-emitting radionuclides within their cavities (*1*). This paper describes the introduction of either polyamino acids or IgG globulin into or onto the lipid membrane of artificially prepared vesicles. The survival and permeability of these vesicles in vitro and their fate after injection in the mouse are also reported.

The experiments described represent preliminary studies designed to test whether molecules such as antibodies or trophic hormones could be attached to vesicles enclosing diagnostic or therapeutic agents with the hope of causing the spherules to adhere to or combine with specific target sites. Chains of nonpolar polyamino acids were studied since it was believed that they might be incorporated at right angles to the membrane plane in a manner analogous to the central segment of proteins in biologic membranes (*2,3*) and with the hope that molecules having recognition sites specific for certain antigens or organs might be attached later to the outer exposed part of the incorporated polyamino acid chain.

MATERIALS AND METHODS

Materials. Phosphatidylcholine (lecithin) was prepared by the method of Singleton, et al (*4*) and further purified by chromatography on silicic acid. Gangliosides were purchased from Sulpelco, Bellefonte, Pa. (04-0632) and phosphatidylethanolamine from Calbiochem, La Jolla, Calif. (524625). Poly-l-phenylalanine (P-6,886, MW 3,550) poly-l-leucine (P-5,762, MW 4,400) poly-l-lysine (P-0879, MW 2,000) and poly-l-alanine (P-5,512, MW 3,000), were purchased from Sigma Chemical Co., St. Louis, Mo. Poly-l-tyrosine (Catalog No. 4,145, MW 700) was purchased from Nutritional Biochemicals, Cleveland, Ohio. The intravesicular radionuclide marker ^{99m}Tc as pertechnetate ($^{99m}TcO_4^-$), half-life 6 hr, was eluted from a ^{99}Mo-molybdenum generator (New England Nuclear Corp., Boston, Mass.). Sodium [^{125}I]-iodide used to label protein was obtained from the same source. Harden M. McConnell, Stanford University, kindly supplied us with the spin label tempocholine chloride (N,N-Dimethyl-N-(2′,2′,6′,6′,-tet-

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tramethyl-4′-piperidyl)-2-acetoxyethanyl ammonium bromide); and N-cyclohexyl-N′-(3-(4 methyl morpholineum) ethyl carbodiimide p-toluene sulfonate (CMC)) was purchased from Aldrich Chemicals, Milwaukee, Wis.

Standard vesicle preparation. Standard vesicles were prepared according to the method described by McDougall, et al (*1*). Forty-four μM lecithin in ethanol and 4 μM gangliosides in chloroform/methanol (1/1) were evaporated to dryness in a round-bottomed flask using a Buchler flash evaporator. The dried lipids were suspended in 1.3 ml salt buffer consisting of 3.5 mM NaH_2PO_4, 1.5 mM NaH_2PO_4, 145 mM NaCl, 15 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 10 mM dextrose adjusted to a pH of 7.1; $^{99m}TcO_4^-$ (when used as an intravesicular marker) was added to the suspension in a volume of 0.1 ml. The solution was sonicated with a probe sonicator until the turbidity cleared, signaling the formation of vesicles; this usually occurred within 5 min. Vesicles were separated from free radiopharmaceutical on a Sephadex G-25 (fine) column. The diameter of vesicles prepared by this technique was about 900 Å as determined by their scattering effect on a laser beam (*5*). Sonication for 60 min resulted in vesicles of of about 500 Å.

In experiments where antibody was complexed to vesicles using the carbodiimide reaction, 15 μM phosphatidylethanolamine was an added ingredient of the standard lipid mixture.

Incorporation of polyamino acids into vesicles. Vesicles incorporating polyamino acids were formed by adding 2 mg of a polyamino acid (poly-l-phenylalanine, poly-l-tyrosine, poly-l-leucine, poly-l-lysine, or poly-l-alanine) to the lecithin and gangliosides prior to evaporation of the organic solvents. The mixture was then treated as previously described; $^{99m}TcO_4^-$ was used as an intravesicular marker for all polyamino acid vesicles. Separation of vesicles from free polyamino acids was achieved by Sephadex G-25 (fine) column chromatography. In the case of poly-l-tyrosine, the amount present in vesicles was assayed by the method of Lowry, et al (*6*).

Binding of IgG globulin to vesicles. A preparation of ^{125}I-labeled human immunoglobulin G (IgG) containing antithyroglobulin (*7*) was used for protein-binding experiments. To study nonspecific adherence, 0.6 μg of this preparation was incubated for 30 min at room temperature with preformed vesicles in 0.5 ml salt buffer. Unbound IgG globulin was separated from globulin-vesicle complex by chromatography on Sepharose 6B (column 16 × 1.8 cm). Fifty fractions, each of 0.5 ml, were collected and radioactivity (^{125}I) counted. The fraction of IgG binding to vesicles was calculated from the total radioactivity in those samples containing vesicles divided by the total radioactivity added.

Iodine-125-IgG was also bound to vesicles (standard preparation with added phosphatidylethanolamine) using the carbodiimide reaction in which carboxyl groups on the antibody are activated and react with nucleophilic groups such as NH_2 on phosphatidylethanolamine (*8,9*); 25 μg of ^{125}I-IgG in 5 ml 0.9% saline were incubated for 30 min with 2 mg CMC. The reaction was quenched by adding 5 drops 1 M acetate buffer at pH 4.75, and the activated IgG was incubated with vesicles for 30 min. Separation of vesicles from free IgG was achieved with column chromatography using Sepaharose 6B, again monitoring ^{125}I radioactivity of recovered fractions.

Vesicle nomenclature. Material enclosed within the vesicle cavity is designated with a subscript e, as with $^{99m}TcO_{4_e}^-$-vesicles. Vesicles formed in the presence of a material that is both inside the vesicle and in the wall are designated with a subscript c, as with poly-l-tyrosine$_c$-vesicles. When a material is adsorbed onto or inserted into the vesicle wall after vesicle formation, the subscript a is used, as with IgG_a-vesicles when IgG is added to the vesicle by nonspecific adherence. Material reacted with the vesicle is designated by the subscript r, as when IgG is added to the vesicle wall by the CMC reaction (IgG_r-vesicle).

Characterization of vesicle preparation in vitro. Electron paramagnetic resonance (EPR) spectra were made on vesicle preparations to test the integrity of the vesicles. Tempocholine chloride (TCC) was used as the spin label and EPR spectra were obtained using a Varian Model E-4 Spectrometer set at 0°C. Vesicles enclosing spin label were prepared by adding tempocholine chloride (0.016 M) to the salt buffer at the time of sonication. The signal of spin-label vesicles was compared with the signal from vesicles treated with 0.01% Triton X-100 and also after separate treatment with 0.035 M ascorbate (*10*). Triton ruptures the vesicles, releasing any enclosed tempocholine, and changes the EPR signal from a protected to an unprotected one. Ascorbate reduces tempocholine outside vesicles and abolishes this outside (unprotected) signal. Values for EPR spectra are reported as relative amplitudes. The amplitude of untreated vesicle preparations is given a value of one and the height of vesicles treated with Triton X-100 or ascorbate is expressed as a multiple of the amplitude of untreated specimens.

As a test for vesicle permeability in vitro, diffusion dialysis was used to measure the rate of release of $^{99m}TcO_4^-$ into a dialysate bath (*1*); 0.3–0.5 ml of vesicles enclosing $^{99m}TcO_4^-$ were dialyzed against 50 ml of phosphate-buffered saline for 1 hr.

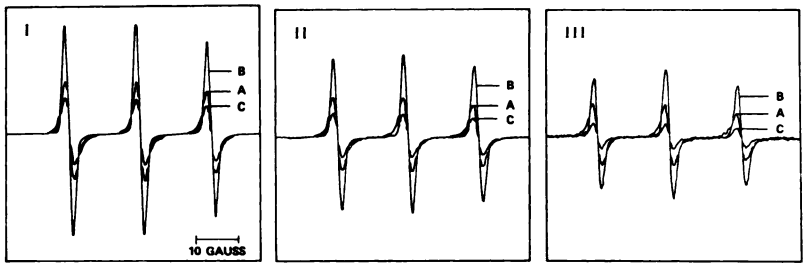

FIG. 1. Electron paramagnetic resonance signals from (I) poly-1-lysine$_c$-vesicles (A) untreated (relative amplitude 1.0), (B) treated with Triton X-100 (relative amplitude 2.2), (C) treated with ascorbate (relative amplitude 0.7); (II) poly-1-tyrosine$_c$-vesicles (A) untreated vesicles (relative amplitude 1.0), (B) treated with Triton X-100 (relative amplitude 2.2), (C) treated with ascorbate (relative amplitude 0.6); and (III) standard vesicles reacted with carbodiimide and IgG globulin (A) untreated vesicles (relative amplitude 1.0), (B) treated with Triton X-100 (relative amplitude 2.1), (C) treated with ascorbate (relative amplitude 0.4).

One-milliliter samples of the dialysate were removed at 1, 2, 5, 10, 15, 20, 25, 30, 45, and 60 min. At 60 min the dialysis bag was removed, all samples were counted, and the rate of $^{99m}TcO_4^-$ release was calculated.

Experimental design of in vivo studies. The distribution of standard vesicles in mice was compared with that of polyamino acid$_c$-vesicles, IgG$_a$-vesicles, and IgG$_r$-vesicles. In all experiments C$_3$H mice (bred at Stanford University, average weight 20 gm) were anesthetized with an intraperitoneal injection of 1.2 mg pentobarbitone. The various vesicle preparations were injected into the tail vein of the mouse in volumes ranging from 0.1 to 0.3 ml. In vivo distribution of vesicles was found to be independent of volume administered when the latter was in the range 0.1–0.8 ml. The distribution of the polyamino acid$_c$-vesicles was studied using $^{99m}TcO_4^-$ as an intravesicular marker. The distribution of protein-vesicles was monitored by a dual-label technique using $^{99m}TcO_4^-$ as an intravesicular marker and ^{125}I-protein as a vesicle wall marker. Mice were sacrificed at 5 and 30 min, and the organs, plus blood and urine specimens, were taken as previously described (*1*). Radioactivity was expressed as percent of total administered dose per organ and percent per gram of tissue. The amount of $^{99m}TcO_4^-$ radioactivity in the stomach was used as an index of vesicle permeability to intravesicular $^{99m}TcO_4^-$ (*1*).

RESULTS

Protein determinations on poly-1-tyrosine$_c$-vesicles showed that approximately 20% (0.3–0.4 mg) of the added polyamino acid was incorporated in the vesicle bilayer. The total volume of internal cavities of the vesicles was estimated by calculating the volume of one vesicle and multiplying by the number of vesicles in each preparation; it was calculated that only approximately 1/1,000 of the buffer solution was inside of vesicles. Thus only about 0.1% of the polytyrosine would be intravesicular.

The results of electron paramagnetic resonance studies done on both poly-1-tyrosine$_c$ and poly-1-lysine$_c$-vesicles enclosing the spin-label tempocholine

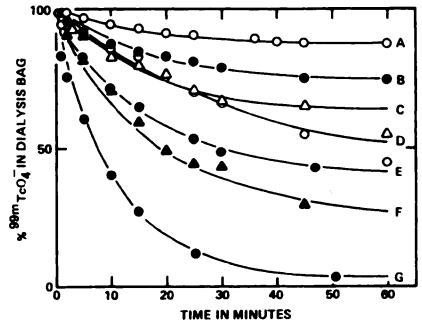

FIG. 2. Rate of loss of radioactivity from vesicles during dialysis. (A) poly-1-tyrosine$_c$-vesicles, (B) standard vesicles, (C) poly-1-phenylalanine$_c$-vesicles, (D) poly-1-leucine$_c$-vesicles, (E) poly-1-lysine$_c$vesicles, (F) poly-1-alanine$_c$-vesicles, and (G) free $^{99m}TcO_4^-$ (no vesicles).

are shown in Fig. 1. For both preparations the height of the signal was increased by the addition of Triton X-100 and reduced but not abolished by the addition of ascorbate. (Ascorbate reduces extravesicular TCC.) These findings indicate that the polyamino acid$_c$-vesicles were intact prior to addition of detergent.

The relative permeabilities in vitro of the various vesicle preparations to enclosed $^{99m}TcO_4^-$ are shown in Fig. 2. After 60 min of dialysis of standard vesicles, 74% of the radioactivity in the vesicles remained in the dialysis bag. Compared with standard vesicles, poly-1-tyrosine$_c$-vesicles were less permeable, while phenylalanine$_c$ poly-1-lysine$_c$, poly-1-leucine$_c$, and poly-1-alanine$_c$-vesicles all were more permeable to enclosed $^{99m}TcO_4^-$.

Table 1 shows the distribution of standard vesicles and polyamino acid$_c$-vesicles in mouse tissues 5 min after their intravenous injection. Compared with standard vesicles, the localization of ^{99m}Tc in liver was greatly augmented for poly-1-phenylalanine$_c$-vesicles and moderately higher for poly-1-tyrosine$_c$-vesicles. The distribution pattern of poly-1-leucine$_c$, poly-1-lysine$_c$, and poly-1-alanine$_c$-vesicles was similar to that of standard vesicles. Relative to the blood levels, the liver and spleen uptake was very much higher for poly-1-phenylalanine$_c$-vesicles than for any of the other preparations (Table 1B).

Following simple mixing of ^{125}I-IgG to preformed

TABLE 1. DISTRIBUTION OF STANDARD VESICLES AND POLYAMINO ACID$_c$-VESICLES IN C$_3$H MICE 5 MIN AFTER ADMINISTRATION

	Standard*	Poly-1-phenyl-alanine*	Poly-1-tyrosine†	Poly-1-leucine*	Poly-1-lysine*	Poly-1-alanine*
A. Percent dose per gram of tissue‡						
Liver	15.5 ± 0.6	53.9 ± 3.1	25.7	19.2 ± 1.5	13.3 ± 0.7	11.5 ± 0.5
Blood	7.5 ± 0.8	2.4 ± 0.7	5.2	4.9 ± 0.6	8.1 ± 0.5	12.4 ± 1.0
Spleen	15.0 ± 2.3	51.3 ± 6.3	32.2	12.9 ± 4.9	21.6 ± 4.7	20.2 ± 1.6
Kidney (L)	5.2 ± 0.5	1.5 ± 0.1	8.1	2.0 ± 0.1	6.2 ± 0.9	4.6 ± 0.4
Kidney (R)	5.2 ± 0.7	1.6 ± 0.1	8.3	2.0 ± 0.1	6.4 ± 0.9	4.6 ± 0.3
Heart	3.1 ± 0.7	0.9 ± 0.3	2.0	2.0 ± 0.4	2.9 ± 0.4	5.9 ± 1.1
Lung	5.8 ± 1.4	2.1 ± 0.2	4.7	4.2 ± 0.5	6.9 ± 0.5	10.0 ± 0.9
Stomach	9.1 ± 1.5	1.3 ± 0.1	2.8	6.7 ± 1.5	12.2 ± 1.3	10.4 ± 2.8
Muscle	1.3 ± 0.6	0.4 ± 0.3	0.7	0.9 ± 0.1	1.9 ± 0.2	2.2 ± 0.1
Fat	0.5 ± 0.3	0.2 ± 0.1	0.8	0.5 ± 0.4	1.7 ± 0.7	0.8 ± 0.2
B. Ratios of distribution of vesicles (calculated from Table 1A)						
Liver/blood	2.1	22.5	4.9	3.9	1.6	0.93
Spleen/blood	2.0	21.4	6.2	2.6	2.7	1.6
Muscle/blood	0.17	0.17	0.13	0.18	0.23	0.18
Stomach/blood	1.2	0.54	0.53	1.4	1.5	0.84

* Mean value (± 1 s.d.) in three mice.
† Mean value in two mice.
‡ Mean wet organ weights (gm) were: liver 1.67, spleen 0.14, right kidney 0.20, left kidney 0.19, heart 0.14, lung 0.34, and stomach 0.27.

standard and poly-1-tyrosine$_c$-vesicles, the percentage ^{125}I bound calculated from the amount of radioactivity eluted with the vesicle fractions after Sepharose 6B column chromatography was 1.3% ± 0.7 (s.d.) and 4.2% for standard and poly-1-tyrosine$_c$-vesicles, respectively. The in vivo distribution of IgG$_c$-vesicles was compared with that of free ^{125}I-IgG (Table 2). Iodine-125-IgG$_a$-poly-1-tyrosine$_c$-vesicles concentrated in the liver and spleen with a pattern similar to that of standard vesicles but the distribution of free ^{125}I-IgG was quite different, the latter showing higher blood levels and much lower amounts in liver and spleen. A relative high blood level was observed after injection of all vesicles containing IgG and there were correspondingly low liver/blood ratios (Table 2B) although not as low as those associated with injection of free ^{125}I-IgG.

Phosphatidylethanolamine$_c$-vesicles were compounded with IgG using the CMC reaction and purified by Sepharose 6B chromatography. Using this technique 12% of the ^{125}I was recovered with the vesicles. These vesicles were concentrated to some extent in the liver and spleen in vivo (Table 2) while blood levels remained high relative to those observed with vesicles devoid of protein (Table 2B). Dialysis experiments using ^{125}I-IgG$_r$-$^{99m}TcO_4^-$-vesicles showed that more than 50% of the $^{99m}TcO_4^-$ was released within 1 hr. On the contrary, no ^{125}I was released from these vesicles during dialysis. For these reasons, ^{125}I is a more reliable marker for the in vivo distribution of such vesicles than is ^{99m}Tc. Electron spin resonance studies showed that IgG$_r$-vesicles remain intact. Triton X-100 increased the tempocholine signal while ascorbic acid treatment of vesicles decreased the spin-label signal (Fig. 1).

DISCUSSION

Polyamino acids and proteins have been incorporated into lipid bilayer vesicles and electron paramagnetic resonance studies show that such vesicles remain intact. Vesicles formed with added poly-1-phenylalanine or poly-1-tyrosine are less permeable to enclosed $^{99m}TcO_4^-$ than standard vesicles but when injected intravenously the former are removed to a greater extent by liver and spleen. In contrast, vesicles made with polyamino acids without an aromatic ring were not associated with an increased liver and spleen uptake while permeability of such vesicles to pertechnetate was increased. We have observed that smaller lipid vesicles are taken up to a greater extent by the liver (*1*). Vesicles formed with aromatic polyamino acids might confer stability to vesicles in a manner similar to that induced by cholesterol (*1*).

IgG globulin has been bound nonspecifically to vesicles and also has been coupled directly to them using the CMC reaction. The nonspecific binding of proteins to membranes usually involves the mingling of hydrophobic chains of protein with the bilayers of the membranes (*2,11,12*). This phenomenon could be involved in the nonspecific binding of proteins to vesicles observed in our studies. In the CMC reac-

TABLE 2. DISTRIBUTION OF IgG, IgG_a-VESICLES, IgG_r-VESICLES, AND PHOSPHATIDYLETHANOLAMINE$_e$-VESICLES IN C_3H MICE SACRIFICED 5 MINUTES AFTER ADMINISTRATION

	^{125}I-IgG	^{125}I-IgG$_a$-vesicles	^{125}I-IgG$_a$-poly-l-tyrosine$_e$-vesicles	^{125}I-IgG$_r$-phosphatidyl-ethanolamine$_e$-vesicles	$^{99m}TcO_4^-$$_e$-phosphatidyl-ethanolamine$_e$-vesicles
A. Percent dose per gram tissue*					
Liver	6.9	19.5	23.0	29.7	15.6
Blood	22.7	19.5	19.4	16.2	9.4
Spleen	7.7	35.8	28.5	41.1	15.9
Kidney (L)	7.5	5.5	9.5	7.8	5.9
Kidney (R)	5.9	5.2	8.4	7.6	6.0
Stomach	4.6	2.7	3.2	2.7	13.6
Heart	6.6	4.2	3.6	7.7	3.7
Lung	57.8	17.8	13.3	10.9	7.2
Muscle	1.4	1.8	3.5	2.8	1.7
Fat	0.4	0.8	2.7	2.0	0.6
B. Ratios of distribution of vesicles (calculated from Table 2A)					
Liver/blood	0.30	1.0	1.2	1.8	1.7
Spleen/blood	0.34	1.83	1.5	2.5	1.7
Lung/blood	2.54	0.91	0.69	0.67	0.77

* Mean wet organ weights (gm) were: Liver 1.46, spleen 0.13, right kidney 0.21, left kidney 0.20, heart 0.13, lung 0.35, and stomach 0.25.

Values given are mean of two animals per point.

tion the terminal carboxyl group of the immunoglobulin is activated; this group is the site of fixation to the vesicles, probably to polar phosphamino groups in phosphatidylethanolamine. Other investigators have postulated that the phosphamino group in membranes (i.e., membranes containing phosphatidylethanolamine) can take part in chemical reactions (*13*).

Our studies should be regarded as prototypes for modification of vesicles by addition of specific molecules which have the potential of conferring unique properties upon them, thus enabling the use of vesicles as carriers of radiopharmaceuticals and other drugs which might preferentially attach to or combine with specific tissue targets in vivo.

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