ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1988, p. 966–970
0066-4804/88/070966-05$02.00/0

Vol. 32, No. 7

In Vitro Lysis of the Bloodstream Forms of *Trypanosoma brucei gambiense* by Stearylamine-Bearing Liposomes

HIROSHI TACHIBANA,[1] EISAKU YOSHIHARA,[2] YOSHIMASA KANEDA,[1] AND TAIJI NAKAE[2]*

Department of Parasitology[1] and Institute of Medical Sciences,[2] School of Medicine, Tokai University, Bohseidai, Isehara, Kanagawa 259-11, Japan

Received 4 November 1987/Accepted 24 March 1988

Cytolytic activity of liposomes consisting of stearylamine and phosphatidylcholine (SA/PC-liposomes) was examined in vitro against the bloodstream forms of *Trypanosoma brucei gambiense*. More than 99% of the cells (2×10^6/ml) were killed within 30 min by treatment with 15 mol% SA/PC-liposomes (100 μM total lipids). As few as 1.2×10^{12} liposomes per ml (equivalent to 2 nM liposome) showed trypanocidal activity. Fluorescence microscopy of cells treated with the dansylated SA/PC-liposomes suggested that the liposomes bound to and accumulated on the cell surface, eventually damaging the plasma membrane. SA/PC-liposomes showed no significant hemolysis when incubated with human and mouse erythrocytes under conditions that killed >99.9% of the *T. b. gambiense* trypomastigotes. Human leukocytes were also shown to be less susceptible to SA/PC-liposomes than *T. b. gambiense*. These results may point to a new direction in strategy for therapy of African trypanosomiasis.

African trypanosomiasis is caused by infection with certain salivarian trypanosomes, e.g., *Trypanosoma brucei gambiense*. Some 35 million people and 25 million cattle in Africa are at risk of infection with these trypanosomes (22). Curative and/or preventive agents for African trypanosomiasis are still far from satisfactory. Although a few drugs are in use, severe adverse effects and frequent appearances of drug-resistant protozoa are encountered. Moreover, no trypanocidal drug is available that can be used routinely for the prevention of transmission during blood transfusion (9).

We reported that liposomes consisting of stearylamine and phosphatidylcholine (SA/PC-liposomes) cause extensive hemolysis of rabbit erythrocytes but less hemolysis of human erythrocytes (25). These liposomes were found to be cytotoxic to *Trypanosoma cruzi*, which is the causative agent of South American trypanosomiasis (26). Therefore, the effect of SA/PC-liposomes in vitro on the bloodstream form of *T. b. gambiense* was tested. As will be shown, the liposomes did kill *T. b. gambiense* cells very efficiently.

MATERIALS AND METHODS

Parasite. *T. b. gambiense*, strain Wellcome, obtained from the Department of Protozoology, Research Institute for Microbial Diseases, Osaka University, was maintained in female ICR mice for several years in this laboratory by passages repeated at 3- to 4-day intervals. Whole blood from the peak parasitemic mice was collected by cardiac puncture and centrifuged at 150 × *g* for 15 min. The supernatant was recentrifuged at 400 × *g* for 3 min. The pellet was washed three times with Dulbecco modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS) and resuspended to 2.2×10^6 cells per ml. Contamination with blood cells in this suspension never exceeded 1%.

Blood cells. Heparinized whole blood was washed twice with 10 volumes of an ice-cold solution of 140 mM NaCl–10 mM sodium phosphate, pH 7.4. The washed cells were resuspended in DMEM containing 10% FCS and used for the hemolysis assay immediately. Human mononuclear (MN) and polymorphonuclear (PMN) leukocytes were isolated as follows. Heparinized fresh whole blood from volunteers (3.5 ml) was overlaid on 3 ml of Mono-Poly Resolving Medium (Flow Laboratories, Inc., McLean, Va.) and centrifuged at 300 × *g* for 30 min. MN and PMN leukocytes, located at the plasma-medium interface and below this interface, respectively, were separated and washed twice with DMEM containing 10% FCS by centrifugation at 400 × *g* for 2 min. Homogeneity of MN and PMN leukocytes appeared to be more than 99% as determined by differential count.

Preparation of liposomes. Liposomes were prepared as described (25). Briefly, a mixture of egg yolk phosphatidylcholine (PC; Sigma Chemical Co., St. Louis, Mo.; type V-E, approx. 99%) and stearylamine (SA; Wako Pure Chemical Industries Ltd., Osaka, Japan; >90% [wt/wt]) in chloroform was dried in a tube, and the tube was kept in an evacuated desiccator. The dried lipid film was suspended in a solution of 150 mM NaCl–10 mM Tris hydrochloride, pH 7.4. The suspension was subjected to sonic oscillation for 5 min in a Branson sonifier (model 200) equipped with a microtip at 50°C (Branson Sonic Power Co., Danbury, Conn.). The amount of liposome was expressed as total lipid content as a sum of SA and phospholipid(s).

Experimental procedures. The liposomes were mixed with 9 volumes of the cell suspension, and the mixture was incubated at 37°C. Viable cells were counted under a light microscope after mixing with 0.04% (wt/vol) erythrosin B (15). Hemolysis was determined by quantifying the released hemoglobin at 540 nm as described (25).

To compare the cytolytic activity of various polycationic agents, *T. b. gambiense* cells (8×10^5, 360 μl) were mixed with 40 μl of a solution containing 0.15 M NaCl and 10 mM Tris hydrochloride, pH 7.4, and a polycationic agent (15 mol% SA/PC-liposomes [1 mM], polymyxin B, or streptomycin), and the mixture was incubated at 37°C for 30 min. The cell suspension was mixed with an equal volume of 0.04% erythrosin B or 0.2% trypan blue, and viable cells were scored as described above.

Fluorescence labeling. *N*-Dansyl phosphatidylethanolamine (*N*-dansyl-PE) was synthesized by the method of Waggoner and Stryer (23). The fluorescence-labeled liposome consisted of PC/SA/*N*-dansyl-PE in a molar ratio of 82:

* Corresponding author.

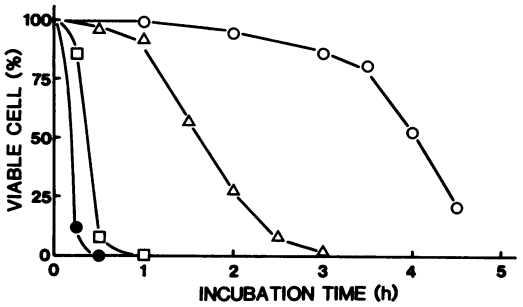

FIG. 1. Effect of liposome concentration on trypanocidal activity. *T. b. gambiense* (8×10^5 cells, 360 μl) was mixed with 40 μl of various concentrations of 15 mol% SA/PC-liposomes. The mixture was incubated at 37°C, and viable cells were counted as described in Materials and Methods. Final lipid concentrations were 5 (○), 10 (△), 50 (□), and 100 (●) μM.

15:3 or PC/*N*-dansyl-PE in a molar ratio of 97:3. Twenty microliters of the labeled liposomes (5 mM lipids) was mixed with 180 μl of the cell suspension (1.5×10^7 cells per ml), and the mixture was incubated at 37°C. Part of the mixture was withdrawn and mixed with a quarter volume of 10% (vol/vol) Formalin. Fixed cells were examined immediately under a Nikon XF-EFD2 fluorescence microscope (Nippon Kogaku K.K, Tokyo, Japan).

RESULTS

Effect of SA/PC-liposomes on the viability of *T. b. gambiense*. *T. b. gambiense* (8×10^5 cells) was incubated with the liposomes consisting of 15 mol% SA and 85 mol% PC (15 mol% SA/PC-liposomes), and their viability was examined (Fig. 1). More than 99% of the cells were killed within 30 min of incubation with SA/PC-liposomes (100 μM lipids). The trypanocidal activity of the liposomes depended on their concentration and was detectable even at 5 μM lipids. Lag times were observed before the viable cells began to decrease at liposome concentrations of less than 10 μM lipids, suggesting that accumulation of a critical number of liposomes is essential to elicit cytolytic activity.

The cells were also treated with liposomes containing different amounts of SA. As shown (Fig. 2), cytotoxic activity of the liposomes became stronger with increasing SA content. Plots of the reciprocal of the time required to kill 50% of the cells versus moles percent SA were linear. Since the intercept of the line with the abscissa indicated about 5 mol%, liposomes containing less than 5 mol% SA were theoretically inactive (Fig. 2, inset).

To examine how SA/PC-liposomes damage the parasite, the interaction of the cells with SA/PC-liposomes containing *N*-dansyl-PE was examined. When the cells were incubated with 15 mol% SA/PC-liposomes for 15 min, all fluoresced strongly, exhibiting the binding and accumulation of SA/PC-liposomes at the cell surface (Fig. 3A and D). Simultaneously, most cells were irreversibly deformed, becoming rounded, yet some cells (unfixed) were still alive. At 30 min, most cells were swollen and ruptured (Fig. 3B and E). On the other hand, cells incubated with the liposomes without SA for 1 h showed no significant fluorescence (Fig. 3C and F). The deformation and loss of dye-excluding ability of the SA/PC-liposome-treated cells were irreversible even after SA/PC-liposomes were washed out, since the viable number of the SA/PC-liposome-treated and subsequently washed cells appeared to be comparable to the viable number of SA/PC-liposome-treated unwashed cells (0.1 to 0.25%).

To gain insight into the mechanism of action of SA/PC-liposomes, the effect of other polycationic agents, such as streptomycin and polymyxin B, as well as SA/PC-liposomes was investigated. *T. b. gambiense* cells were mixed with 100 μM SA/PC-liposomes containing 15 μM SA and incubated at 37°C for 30 min. Viability of the SA/PC-liposome-treated cells dropped to less than 0.25%, whereas the viability of the streptomycin- or polymyxin B-treated cells was nearly unchanged, as examined by erythrosin B staining and trypan blue staining (Table 1). Increasing the concentration of streptomycin up to 150 μM gave the same result. The morphological appearance of the streptomycin- or polymyxin B-treated cells under the above conditions was indistinguishable from that of untreated cells (not shown). To know whether the morphological alteration was specific to the SA/PC-liposome treatment or simply due to rupture of the cell, *T. b. gambiense* cells were subjected to osmotic

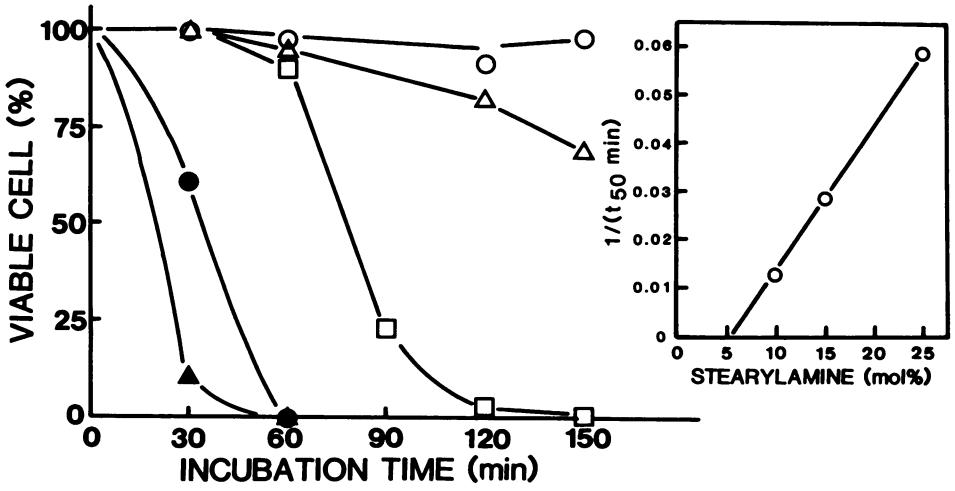

FIG. 2. Effect of SA density in the liposome membrane on trypanocidal activity. Parasites (6×10^5 cells, 270 μl) were mixed with 30 μl of the liposome suspension (200 μM lipids) containing 0 (○), 5 (△), 10 (□), 15 (●), or 25 (▲) mol% SA. The mixture was incubated at 37°C for various times, and viable cells were counted. Inset: Relationship between SA density and reciprocal of time required to kill 50% of the parasites ($1/t_{50}$ min).

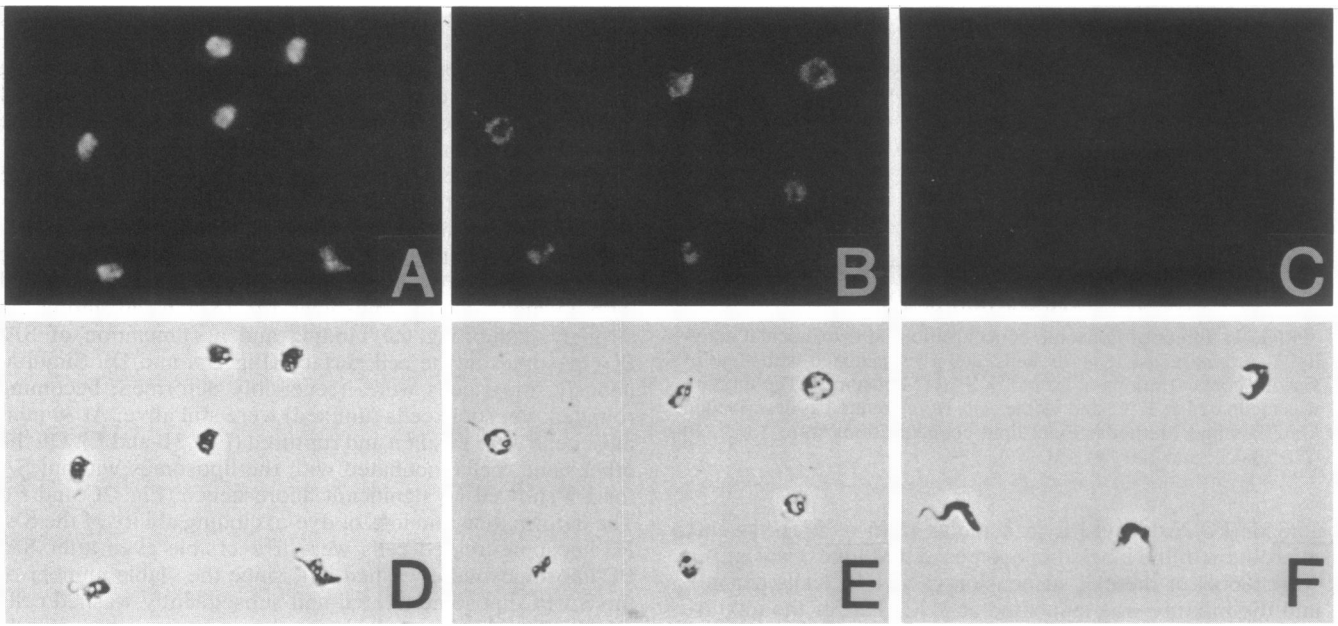

FIG. 3. Fluorescence (A, B, C) and phase-contrast (D, E, F) photomicrographs of *T. b. gambiense* treated with SA/PC-liposomes. Experimental details are described in Materials and Methods. All magnifications, ×520. Cells were incubated with 15 mol% SA/PC-liposomes with *N*-dansyl-PE for 15 min (A and D) or 30 min (B and E). (C and F) Cells incubated with liposomes made of PC and *N*-dansyl-PE for 60 min.

lysis by suspension in 2 M sucrose and subsequently by 20-fold dilution in distilled water. Over 90% of the cells were dead, judged as described above, but all the dead cells maintained a cell shape similar to that of intact cells. These results suggested that the SA/PC-liposome-mediated lysis of *Trypanosoma* cells is not due to simple adherence of the polycationic agent to the cell surface, the presence of fatty acid-containing polyamine, or mechanical damage of the plasma membrane.

Effect of SA/PC-liposomes on blood cells. Human or mouse erythrocytes were incubated with 15 mol% SA/PC-liposome (100 μM lipids), and hemolysis was measured. Less than 1% of these erythrocytes were lysed after 4 h of incubation (Table 2). The effect of SA/PC-liposomes on human MN and PMN leukocytes was also examined (Fig. 4) by incubating with 25 μM lipids (Fig. 4A) or 100 μM lipids (Fig. 4B) of 15 mol% SA/PC-liposomes. These concentrations of SA/PC-liposomes killed *T. b. gambiense* rapidly. However, 25 and 100 μM lipids of SA/PC-liposomes showed no significant damage to MN cells after 2 h (Fig. 4A and B), whereas about half of the PMN leukocytes were killed by treatment with 100 μM lipids of the liposomes for 70 min (Fig. 4B).

TABLE 1. Comparison of the cytolytic activities of various polycationic agents

Agent[a]	Final concn (μM)	% Viable cells[b]
Control (none)		100
SA/PC-liposomes	15	<0.25
SA/PC-liposomes[c]	15	<0.25
Polymyxin B	15	100.9
Streptomycin	15	110.5
Streptomycin	150	100.9

[a] Erythrosin B was used for the dye test unless indicated otherwise.
[b] Calculated from the number of viable cells per 400 cells counted.
[c] Trypan blue was used for the dye test.

DISCUSSION

This study showed that liposomes containing 5 μM lipids (15 mol% SA in PC) killed 50% of *T. b. gambiense* within 4 h (Fig. 1). Since an average particle weight of the unilamellar liposomes such as those used in this study was estimated to be 2 megadaltons (20), the number of liposomes needed to kill half of the cells within 4 h appeared to be 1.2×10^{12}/ml (2 nM liposomes). Human erythrocytes treated under conditions that killed over 99.9% of *T. b. gambiense* showed no significant hemolysis. Human MN and PMN leukocytes also appeared to be less susceptible to SA/PC-liposomes than *T. b. gambiense*. Thus, it is our hope that this kind of liposome preparation can be used to prevent transmission of the parasite via blood transfusion. However, it remains a possibility that other cells in the hosts might be even more susceptible to the liposomes in vivo. Since mouse erythrocytes are as resistant to SA/PC-liposomes as human erythrocytes, further studies with mice should answer the remaining questions.

Since SA/PC-liposomes containing phosphatidylserine became less hemolytic, the polycationic nature of the liposome seems to be essential for cytolytic activity, as suggested earlier (25). Treatment of trypanosomes with SA alone

TABLE 2. Effect of 15 mol% SA/PC-liposomes on human and mouse erythrocytes[a]

Erythrocytes	Hemolysis[b] (%)			
	1 h	2 h	3 h	4 h
Human	0	0	0.3	0.3
Mouse	0	0.2	0.2	0.6

[a] Erythrocytes (final hematocrit, 10%) were incubated with 15 mol% SA/PC-liposomes (100 μM lipids) at 37°C.
[b] Full hemolysis (100%) was the absorption at 540 nm of erythrocytes mixed with 0.1% Triton X-100 (25).

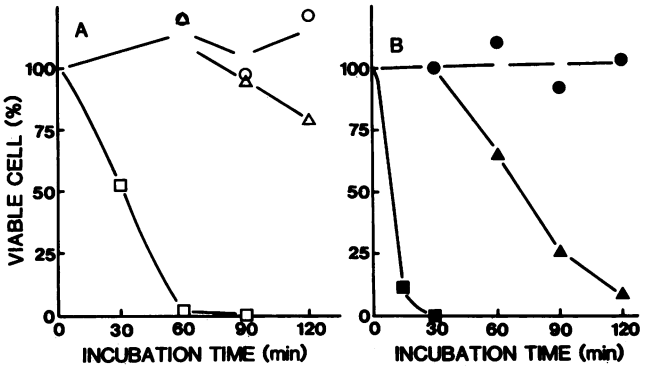

FIG. 4. Effect of SA/PC-liposomes on MN and PMN leukocytes. Nine volumes of MN leukocytes (8×10^5 cells, 360 μl), PMN leukocytes (4×10^5 cells, 360 μl), or *T. b. gambiense* (8×10^5 cells, 360 μl) were mixed with 1 volume of 15 mol% SA/PC-liposomes and incubated at 37°C. Viable cells were counted as described in Materials and Methods. (A) MN leukocytes (○), PMN leukocytes (△), and *T. b. gambiense* (□) treated with 25 μM lipids of 15 mol% SA/PC-liposomes. (B) MN leukocytes (●), PMN leukocytes (▲), and *T. b. gambiense* (■) treated with 100 μM lipids of 15 mol% SA/PC-liposome.

(without elaboration into the liposome; equivalent to 100 μM lipids in 15 mol% SA/PC-liposomes) showed no significant trypanocidal activity (data not shown). Evidently, the incorporation of SA into the liposome membrane (forming a polycationic liposome) is important to induce trypanocidal activity.

Why is *T. b. gambiense* highly susceptible to SA/PC-liposomes? We observed that SA/PC-liposomes induce swelling and lysis of *T. cruzi* (26). When the susceptibility of the three developmental forms of *T. cruzi* was compared, the trypomastigote appeared to be the most susceptible, followed by the amastigote and epimastigote. This order of susceptibility is in accord with the order of their negative surface charges (7, 8, 19).

There is a question whether loss of dye-excluding ability means death of the cells. The loss of dye-excluding ability of the SA/PC-liposome-treated cells was confirmed by the more commonly used trypan blue exclusion test (Table 1). Our preliminary data showed that treatment of *Pseudomonas aeruginosa*, susceptible to aminoglycoside, with 30 mol% SA/PC-liposomes reduces CFU by several orders of magnitude. This reduction of CFU counts is not due to the aggregation of bacterial cells, as the same extent of reduction was confirmed after dissociation of the liposomes by nonionic surfactant treatment or by extensive shearing (P. Viljanen and T. Nakae, manuscript in preparation).

Although the mechanism of action of SA/PC-liposomes on *Trypanosoma* organisms is not clear at present, our preliminary observations suggested that SA/PC-liposomes fuse with the plasma membrane and the SA molecule(s) acts on it. Lines of evidence to support this assumption are as follows. (i) Cells treated with polycationic antibiotics, such as streptomycin or polymyxin B, are neither killed nor deformed (Table 1), although these molecules are presumably attached on the negatively charged external surface of the plasma membrane (10). (ii) Since polymyxin B appeared to be ineffective, fatty polyamine does not always satisfy the conditions required for cytotoxicity. (iii) The typical shape of *Trypanosoma* cells was maintained when the cells were disrupted osmotically. Thus, we assume that SA molecules are translocated from the liposome membrane to the plasma membrane, probably by the membrane fusion, and act on the shape-maintaining machinery, eventually causing cell lysis.

Studies on the effect of drug carrier liposomes against leishmaniasis (1–6, 11–14, 24) and malaria (16–18, 21) showed that lower amounts of drug were more effective in the liposomal form than the free form and that adverse effects were fewer. Since the liposome membrane reported here can damage the target by itself, it is free from shearing. One alternative use of SA/PC-liposomes would be to encapsulate antiparasite drugs in the intraliposomal cavity. Such liposomes might damage the target synergistically by the effects of SA molecules and the encapsulated drug(s).

We hope that the study reported here will facilitate research on development of cytotoxic liposomes for practical use.

ACKNOWLEDGMENTS

We thank H. Ikigai of Jikei University for valuable suggestions for preparation of *N*-dansyl-PE.

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan; Tokai Medical research grants; and a grant from the General Organization of Research in Tokai University.

LITERATURE CITED

1. **Alving, C. R., E. A. Steck, W. L. Chapman, Jr., V. B. Waits, L. D. Hendricks, G. M. Swartz, Jr., and W. L. Hanson.** 1978. Therapy of leishmaniasis: superior efficacies of liposome-encapsulated drugs. Proc. Natl. Acad. Sci. USA **75**:2959–2963.
2. **Alving, C. R., E. A. Steck, W. L. Chapman, Jr., V. B. Waits, L. D. Hendricks, G. M. Swartz, Jr., and W. L. Hanson.** 1980. Liposomes in leishmaniasis: therapeutic effects of antimonial drugs, 8-aminoquinolines, and tetracycline. Life Sci. **26**:2231–2238.
3. **Alving, C. R., E. A. Steck, W. L. Hanson, P. S. Loizeaux, W. L. Chapman, Jr., and V. B. Waits.** 1978. Improved therapy of experimental leishmaniasis by use of a liposome-encapsulated antimonial drug. Life Sci. **22**:1021–1026.
4. **Alving, C. R., G. M. Swartz, Jr., L. D. Hendricks, W. L. Chapman, Jr., V. B. Waits, and W. L. Hanson.** 1984. Liposomes in leishmaniasis: effects of parasite virulence on treatment of experimental leishmaniasis in hamsters. Ann. Trop. Med. Parasitol. **78**:279–286.
5. **Black, C. D. V., G. J. Waston, and R. J. Ward.** 1977. The use of Pentostam liposomes in the chemotherapy of experimental leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. **71**:550–552.
6. **Chapman, W. L., Jr., W. L. Hanson, C. R. Alving, and L. D. Hendricks.** 1984. Antileishmanial activity of liposome-encapsulated meglumine antimonate in the dog. Am. J. Vet. Res. **45**:1028–1030.
7. **De Carvalho, T. U., T. Souto-Padróon, and W. De Souza.** 1985. *Trypanosoma cruzi*: surface charge and freeze-fracture of amastigotes. Exp. Parasitol. **59**:12–23.
8. **De Souza, W., C. Arguello, A. Martinez-Palomo, D. Trissl, A. Gonzales-Robles, and E. Chiari.** 1977. Surface charge of *Trypanosoma cruzi*. Binding of cationized ferritin and measurement of cellular electrophoretic mobility. J. Protozool. **24**:411–415.
9. **Gutteridge, W. E.** 1985. Existing chemotherapy and its limitations. Br. Med. Bull. **41**:162–168.
10. **Lanham, S. M., and D. G. Godfrey.** 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp. Parasitol. **28**:521–534.
11. **New, R. R. C., and M. L. Chance.** 1980. Treatment of experimental cutaneous leishmaniasis by liposome-entrapped Pentostam. Acta Trop. **37**:253–256.
12. **New, R. R. C., M. L. Chance, and S. Heath.** 1981. Antileishmanial activity of amphotericin and other antifungal agents entrapped in liposomes. J. Antimicrob. Chemother. **8**:371–381.
13. **New, R. R. C., M. L. Chance, and S. Heath.** 1981. The treatment of experimental cutaneous leishmaniasis with liposome-en-

trapped Pentostam. Parasitology **83**:519–527.
14. **New, R. R. C., M. L. Chance, S. C. Thomas, and W. Peters.** 1978. Antileishmanial activity of antimonials entrapped in liposomes. Nature (London) **272**:55–56.
15. **Phillips, H. J., and J. E. Terryberry.** 1957. Counting actively metabolizing tissue cultured cells. Exp. Cell Res. **13**:341–347.
16. **Pirson, P., R. Steiger, and A. Trouet.** 1982. The disposition of free and liposomally encapsulated antimalarial primaquine in mice. Biochem. Pharmacol. **31**:3501–3507.
17. **Pirson, P., R. F. Steiger, A. Trouet, J. Gillet, and F. Herman.** 1980. Primaquine liposomes in the chemotherapy of experimental murine malaria. Ann. Trop. Med. Parasitol. **74**:383–391.
18. **Smith, J. E., P. Pirson, and R. E. Sinden.** 1983. Studies on the kinetics of uptake and distribution of free and liposome-entrapped primaquine, and of sporozoites by isolated perfused rat liver. Ann. Trop. Med. Parasitol. **77**:379–386.
19. **Souto-Padrón, T., T. U. De Carvalho, E. Chiari, and W. De Souza.** 1984. Further studies on the cell surface charge of *Trypanosoma cruzi*. Acta Trop. **41**:215–225.
20. **Szoka, F., Jr., and D. Papahadjopoulos.** 1980. Comparative properties and methods of preparation of lipid vesicles (liposomes). Annu. Rev. Biophys. Bioeng. **9**:467–508.
21. **Trouet, A., P. Pirson, R. Steiger, M. Masquelier, R. Baurain, and J. Gillet.** 1981. Development of new derivatives of primaquine by association with lysosomotropic carriers. Bull. W.H.O. **59**:449–458.
22. **Turner, M. J.** 1982. Biochemistry of the variant surface glycoproteins of salivarian trypanosomes. Adv. Parasitol. **21**:69–153.
23. **Waggoner, A. S., and L. Stryer.** 1970. Fluorescent probes of biological membranes. Proc. Natl. Acad. Sci. USA **67**:579–589.
24. **Weldon, J. S., J. F. Munnell, W. L. Hanson, and C. R. Alving.** 1983. Liposomal chemotherapy in visceral leishmaniasis: an ultrastructural study of an intracellular pathway. Z. Parasitenkd. **69**:415–424.
25. **Yoshihara, E., and T. Nakae.** 1986. Cytolytic activity of liposomes containing stearylamine. Biochim. Biophys. Acta **854**: 93–101.
26. **Yoshihara, E., H. Tachibana, and T. Nakae.** 1987. Trypanocidal activity of the stearylamine-bearing liposome in vitro. Life Sci. **40**:2153–2159.