boring recombinant plasmids nor the phage preparations containing the monomeric polyoma DNA insert induced any tumors.

As in the studies of mouse infectivity, the dimer-containing phage materials showed the most biologic activity (18 percent tumor response) of the various recombinant materials tested, probably reflecting the ability of these molecules to generate intact polyoma genomes by recombinational mechanisms. The mouse studies showed that the potentially infectious dimer-containing phage DNA did not lead to infection when administered as infected *E. coli* in the latent period (*2*). While this mode of infection was not studied in the hamster system, the extremely low efficiency of lambda phage production in the absence of aerobic conditions, as would be the case in the tissues of the animal, makes it quite likely that the same reduction in activity would have been seen.

Whereas the experiments presented are in need of extension to other host-vector combinations, they do add to the reassuring conclusions of the earlier mouse infectivity studies (*1*, *2*). The findings that no tumors were induced with the viable plasmid-containing bacteria not only provides further evidence for the safety of cloning viral genomes in *E. coli* but also provides for the safety of cloning other postulated oncogenic gene segments.

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References and Notes

1. M. A. Israel, H. W. Chan, W. P. Rowe, M. A. Martin, *Science* **203**, 883 (1979).
2. H. W. Chan, M. A. Israel, C. Garon, W. P. Rowe, M. A. Martin, *ibid.*, p. 887.
3. M. Fried, B. Klein, K. Murry, J. Tooze, W. Boll, C. Weissmann, *Nature (London)* **279**, 811 (1979).
4. B. E. Eddy, S. E. Stewart, R. Young, G. B. Mider, *J. Natl. Cancer Inst.* **20**, 747 (1958); M. Stoker, *Br. J. Cancer* **14**, 679 (1960).
5. M. A. Israel, H. W. Chan, S. L. Hourihan, W. P. Rowe, M. A. Martin, *J. Virol.* **29**, 990 (1979).
6. M. A. Israel, D. Simmons, S. L. Hourihan, W. P. Rowe, M. A. Martin, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
7. A large plaque variant (LP) of polyoma virus [M. Vogt and R. Dulbecco, *Virology* **16**, 41 (1962)] was provided by Dr. T. Benjamin.
8. Hamsters inoculated with live *E. coli* or with phage particles were held under P4 physical containment conditions, while hamsters receiving DNA were held under P3. All host-vector systems were EK2.
9. This calculation is based on the assumption that the ratio of particles to infectivity is 300.
10. S. B. Levy, N. Sullivan, S. Gorbach, *Nature (London)* **274**, 395 (1978).

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Sporozoite-Induced Malaria: Therapeutic Effects of Glycolipids in Liposomes

Abstract. *Liposomes containing neutral glycolipids with a terminal glucose or galactose, when injected intravenously, prevented the appearance of erythrocytic forms of malaria (*Plasmodium berghei*) in mice previously injected with sporozoites. Inhibitory glycolipids included glucosyl, galactosyl, or lactosyl ceramide. Inhibition was not observed with liposomes containing ceramide, phosphocholine ceramide, sulfogalactosyl ceramide (sulfatide), or ganglioside G_{M1}. Liposomes containing glycolipids did not inhibit infection transmitted by injecting blood containing erythrocytic stages of malaria. These results may have therapeutic implications in the treatment of malaria. Analysis of the mechanism of interference with the life cycle of malaria by liposomal glycolipids may yield information about the interactions of parasites with cellular membranes.*

Liposomes consist of closed concentric spheres of phospholipid membrane. Upon intravenous injection, liposomes accumulate preferentially in the liver, mainly in Kupffer cells, and the spleen [see (*1*)]. We and others demonstrated in rodents that injection of drug-laden liposomes could be used for the treatment of experimental *Leishmania donovani* infections of Kupffer cells (*2*). In the present study we used liposomes to treat experimental sporozoite-induced *Plasmodium berghei* infections of hepatocytes in mice.

When malaria parasites (sporozoites) are injected into mammals by the bite of an anopheline mosquito, the parasites travel to the liver of the host. The sporozoites remain inside hepatocytes for a period of days (exoerythrocytic stage) before emerging as exoerythrocytic schizonts capable of invading erythrocytes. Animals that have erythrocytic forms are said to have "patent" infections, and the preceding interval, which follows injection of sporozoites by mosquitoes, is called the prepatent period (*3*). Antimalarial drugs are usually classified according to their effects on a particular stage of the plasmodial life cycle; for example, primaquine and related drugs act primarily against parasites in the liver, whereas chloroquine affects parasites in the erythrocytes (*4*). In the experiments described herein we demonstrate that liposomes containing certain membrane glycolipids, without additional drugs, interfere with the malarial life cycle during the prepatent period and prevent the appearance of erythrocytic forms of the parasite.

Plasmodium berghei (either ANKA or NK65 strain) was cycled through *Anopheles stephensi* mosquitoes and golden Syrian hamsters. Salivary glands were isolated from the most heavily infected mosquitoes 18 to 25 days after they had taken a blood meal, according to the method of Bosworth *et al.* (*5*). The glands were triturated in a glass syringe and the sporozoites were counted in a hemocytometer. The sporozoites were suspended in Medium 199 (1 to 2 $\times$ 10^5 per milliliter), and 0.1 to 0.2 ml of suspension was injected intravenously into each mouse (ICR, Walter Reed strain). In some experiments, 0.1 ml of infected blood was used to transmit the infection. The infected blood was drawn from a mouse with a patent infection 1 week after it had been injected with 3 $\times$ 10^4 sporozoites; the blood contained 0.19 parasite per 10^3 erythrocytes.

The lipids and their sources were as follows: dimyristoyl phosphatidylcholine and mixed beef brain ceramide (Sigma); cholesterol (Calbiochem); dicetyl phosphate (K and K Laboratories); galactosyl, glucosyl, and lactosyl ceramides (Miles Laboratories); sulfatide (Applied Sciences Laboratories); and ganglioside G_{M1} (Supelco).

Liposomes, swollen in 0.15*M* NaCl, were prepared by previously described standard procedures (*6*). The liposomes consisted of dimyristoyl phosphatidylcholine, cholesterol, and dicetyl phosphate in molar ratios of 1:0.75:0.11, respectively, plus 100 μg of ceramide lipid (except in the case of sphingomyelin) per micromole of phosphatidylcholine. When phosphocholine ceramide (sphingomyelin) was used, it replaced an equivalent molar amount of phosphatidylcholine, so that phosphatidylcholine, sphingomyelin, cholesterol, and dicetyl phosphate were in molar ratios of 0.8:0.2:0.75:0.11, respectively. The phosphatidylcholine, or phosphatidylcholine plus sphingomyelin, was 10 m*M* with respect to the 0.15*M* NaCl used for swelling. On the basis of Coulter counter analysis of similar preparations, the liposomes had a broad hyperbolic size distribution (*7*). Although most of the liposomes were small (1.5 μm or less), most of the surface area and volume were due to large (> 1.5 μm) liposomes (*7*). The liposomes were diluted approximately sevenfold with 0.15*M* NaCl and centri-

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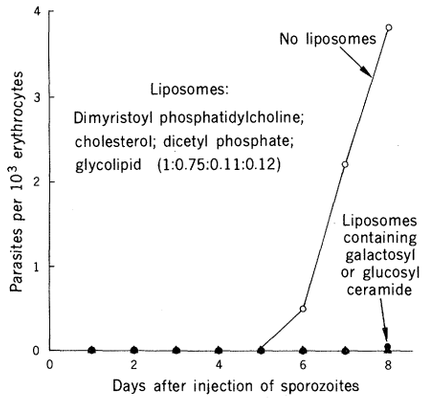

Fig. 1. Inhibition of erythrocytic parasites after injection of liposomes containing glycolipids. The animals were injected intravenously with liposomes 1 day after sporozoite injection. Each point represents the mean of 10 or 11 animals.

fuged at 27,000*g* at 20°C for 10 minutes. The supernatant (containing small liposomes with about 30 percent of the original phospholipid) was discarded. The pellet was suspended in a volume of 0.15*M* NaCl amounting to one-fourth of the original swelling volume. Mice were injected intravenously with 0.15 to 0.2 ml of the final liposome suspension either 1 day after injection of sporozoites or 1 day after injection of infected blood.

Figure 1 shows that between days 5 and 8, when the average erythrocytic parasite count in untreated animals was rising rapidly, erythrocytic parasites were barely detectable in animals treated with liposomes containing either galactosyl or glucosyl ceramide. At early stages of infection the number of parasites may be too low to quantitate accurately, but in these experiments the infection was considered patent even if only one parasite was detected per 10^5 erythrocytes. Patency did not occur before day 4, and after day 9 animals with nonpatent infections did not develop patent infections. Treatment with liposomes containing galactosyl ceramide markedly diminished the number of animals that developed patent infections. As shown in Table 1, 79 percent of untreated animals developed patent infections, whereas only 7 to 15 percent of animals treated with liposomes containing galactosyl, glucosyl, or lactosyl ceramide developed such infections. The carbohydrate group was necessary for inhibition of patency, because both phosphocholine ceramide and ceramide alone were ineffective. Sulfatide (3-sulfogalactosyl ceramide) and ganglioside G_{M1} [galactose-*N*-acetylgalactosamine - galactose(*N* - acetylneuraminic acid)-glucose-ceramide] also were ineffective. Each of the last two compounds has a strong negative charge either attached to, or in the vicinity of, the terminal galactose, and this may have reduced the effectiveness of the molecules against sporozoites.

The activity of the liposomes containing galactosyl ceramide was not due to an effect against blood forms of the malaria parasite that emerged from the liver. Figure 2 shows that treatment with galactosyl ceramide liposomes did not inhibit parasitemia in animals in which the parasite was transmitted by injecting blood containing erythrocytic forms of *P. berghei* from animals with patent infections.

Hepatic cells from several species have a lectin that recognizes exposed galactose groups of desialated glycoproteins (*8*). The present study was based on the rationale that liposomes containing membrane glycolipids might become associated with the lectin on the plasma membranes of hepatocytes. We had hoped to use lectin-mediated recognition of liposomes as a method of targeting drug-loaded liposomes to hepatocytes during the exoerythrocytic stage of sporozoite-induced malaria infection. The experiments described herein demonstrate that the liposomes themselves interfered with the malarial life cycle during the prepatent period. This observation provides indirect evidence that an intracellular hepatic lectin that recognizes galactose or glucose might play a role in hepatic infection with *P. berghei*. The lectin that occurs on the surface of mammalian hepatocytes also occurs on microsomes, Golgi apparatus, and lysosomes (*9*). Liposomes containing glycolipids might block the parasite by interaction with the intracellular lectin. A preliminary experiment indicated that liposomal glycolipids did not have a prophylactic antimalarial effect (*10*), and it

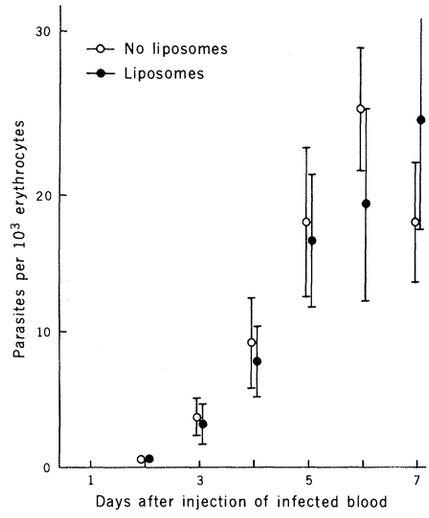

Fig. 2. Absence of liposomal-inhibition of erythrocytic parasites in animals injected with infected blood. Liposomes containing galactosyl ceramide were administered to 14 mice 1 day after injection of infected blood. The control group consisted of 12 mice that received saline instead of liposomes. Each point represents the mean ± standard deviation.

seems unlikely, therefore, that the glycolipids competed with sporozoites for the lectin present on the plasma membrane.

Data from other studies suggest that liposomes containing galactosyl lipids may be recognized by, and internalized by, mammalian cells. Uptake of liposomes by rat liver in vivo was enhanced by ganglioside G_{M1} or asialoganglioside G_{M1}, and the enhanced uptake was blocked by asialofetuin [which is recognized by rat hepatocyte lectin (*11*)]. Upon injection into mice, liposomes with EDTA in the aqueous phase were accumulated to a greater extent by the liver when the liposomes contained galactosyl ceramide compared with those that contained gangliosides (including ganglio-

Table 1. Influence of saccharide moieties on inhibitory effects of ceramide lipids in liposomes. The liposomes consisted of dimyristoyl phosphatidylcholine, cholesterol, and dicetyl phosphate, plus the indicated lipid. Controls received only saline.

Ceramide lipid	Number of animals*		Patency (%)	Average prepatent period (days)†
	With patent infections	Injected		
Control (no liposomes)	49	62	79	5.1 ± 1
Glucosyl ceramide	2	28	7.4	6.0
Galactosyl ceramide	6	40	15	7.3 ± 1.6
Lactosyl ceramide	1	7	14	6.0
Ceramide	6	7	86	6.3 ± 0.8
Phosphocholine ceramide (sphingomyelin)	5	7	71	6.2 ± 0.4
Sulfogalactosyl ceramide (sulfatide)	5	7	71	5.8 ± 0.8
Ganglioside G_{M1}	5	7	71	6.4 ± 0.9

*Animals were examined for patency daily during 3 to 8 days after injection of sporozoites. Patency was defined as at least 1 parasite per 10^5 erythrocytes. †Values given as mean ± standard deviation.

side G_{M1}) (*12*). It has been demonstrated also that liposomes that become associated with cells via glycolipid-lectin interactions may be taken up by fusion with the cells. Fusion of liposomes with HeLa cells in tissue culture was markedly enhanced by galactosyl or lactosyl ceramide in the liposomes (*13*).

From a therapeutic standpoint, under the conditions we used, successful treatment of sporozoite-induced malaria with liposomal glycolipids was an all-or-none phenomenon. Of 74 animals that did not develop patent infections at 10 days, all remained alive and apparently free of "clinical" malaria after 40 to 46 days. These data suggested that the liposome-injected animals had been cured (*14*).

In the few animals (7 to 15 percent) that did develop patent infections despite treatment with liposomes containing appropriate glycolipids, the average prepatent period was not significantly different from that of untreated animals, or that of animals that were treated with liposomes containing ineffective glycolipids (Table 1). The reason for the apparent complete lack of efficacy of liposomal glycolipids against the infection in 7 to 15 percent of the animals is not clear.

Treatment with liposomes containing glycolipids is particularly interesting because extremely high dilutions of liposomes were therapeutically effective, and there was an apparent lack of acute toxicity (that is, lethality) of undiluted liposomes (*15*).

In chemotherapy of leishmaniasis, the liposomal phospholipid and surface charge had strong influences on efficacy of treatment (*2*). Further experiments may reveal the influence, if any, of liposome composition on suppression of sporozoite-induced malaria.

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References and Notes

1. D. A. Tyrrell, T. D. Heath, C. M. Colley, B. E. Ryman, *Biochim. Biophys. Acta* **457**, 259 (1976).
2. C. R. Alving, E. A. Steck, W. L. Chapman, Jr., V. B. Waits, L. D. Hendricks, G. M. Swartz, Jr., W. L. Hanson, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2959 (1978); C. R. Alving, E. A. Steck, W. L. Hanson, P. S. Loizeaux, W. L. Chapman, Jr., V. B. Waits, *Life Sci.* **22**, 1021 (1978); C. R. Alving and E. A. Steck, *Trends Biochem. Sci.* **4**, N175 (1979); C. D. V. Black, G. J. Watson, R. J. Ward, *Trans. R. Soc. Trop. Med. Hyg.* **71**, 550 (1977); R. R. C. New, M. L. Chance, S. C. Thomas, W. Peters, *Nature (London)* **272**, 55 (1978).
3. P. C. C. Garnham, *Malaria Parasites and Other Haemosporidia* (Blackwell, Oxford, 1966).
4. T. Butler, K. S. Warren, A. A. F. Mahmoud, *J. Infect. Dis.* **133**, 721 (1976).
5. A. B. Bosworth, I. Schneider, J. E. Freier, *J. Parasitol.* **61**, 769 (1975).
6. C. R. Alving and R. L. Richards, *Immunochemistry* **14**, 373 (1977).
7. C. R. Alving, D. H. Conrad, J. P. Gockerman, M. B. Gibbs, G. H. Wirtz, *Biochim. Biophys. Acta* **394**, 157 (1975).
8. G. Ashwell and A. G. Morell, *Adv. Enzymol.* **41**, 99 (1974).
9. J. R. Riordan, L. Mitchell, M. Slavik, *Biochem. Biophys. Res. Commun.* **59**, 1373 (1974); W. E. Pricer, Jr., and G. Ashwell, *J. Biol. Chem.* **251**, 7539 (1976).
10. An experiment was performed to determine if liposomal glycolipids could have a prophylactic effect in the inhibition of malarial infection. Liposomes were injected into a total of 28 mice 1, 5, and 7 days prior to sporozoite injection. Under these conditions the liposomes did not have any detectable inhibitory effect.
11. A. Surolia and B. K. Bachhawat, *Biochim. Biophys. Acta* **497**, 760 (1977).
12. M. M. Jonah, E. A. Cerny, Y. E. Rahman, *ibid.* **541**, 321 (1978).
13. R. W. Bussian and J. C. Wriston, Jr., *ibid.* **471**, 336 (1977).
14. To test further for viable parasites in the livers of mice that did not develop patent infections, minced livers or collagenase-treated livers from liposome-treated mice that were free of patent infections 42 to 45 days after sporozoite injection were injected into fresh uninfected mice [D. A. Foley, J. Kennard, J. P. Vanderberg, *Exp. Parasitol.* **46**, 179 (1978)]. Minced liver from four mice or collagenase-treated liver from two mice free of patent infections were injected intraperitoneally into a total of 40 uninfected mice (24 mice received 12 to 30 mg of minced tissue; 16 mice received 7×10^5 to 9.5×10^6 collagenase-treated cells). None of the mice injected with "infected" liver had a patent infection 21 days after injection. This assay also tested for any small numbers of viable parasites that might have been present in the blood that was in the injected liver.
15. In animals treated with 0.15 ml of liposomes containing galactosyl ceramide, a dose-response analysis showed that a 1:1,000,000 dilution of liposomes still had half the effectiveness of undiluted liposomes. There was no mortality among 42 uninfected animals injected with 0.1 to 0.25 ml of liposomes containing normal saline (24 animals were injected with liposomes with galactosyl ceramide, and 18 animals were injected with liposomes lacking glycolipid). None of the injected animals showed any signs of distress within 24 hours after injection.
16. The skillful technical assistance of Cynthia Skelton and John Shaker is gratefully acknowledged.

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Determination of the Pore Size of Cell Walls of Living Plant Cells

Abstract. *The limiting diameter of pores in the walls of living plant cells through which molecules can freely pass has been determined by a solute exclusion technique to be 35 to 38 angstroms for hair cells of* Raphanus sativus *roots and fibers of* Gossypium hirsutum, *38 to 40 angstroms for cultured cells of* Acer pseudoplatanus, *and 45 to 52 angstroms for isolated palisade parenchyma cells of the leaves of* Xanthium strumarium *and* Commelina communis. *These results indicate that molecules with diameters larger than these pores would be restricted in their ability to penetrate such a cell wall, and that such a wall may represent a more significant barrier to cellular communication than has been previously assumed.*

The mechanical strength and contiguous nature of plant cell walls imparts structural rigidity to the entire plant. It also creates a biological problem, since plant cells must sense their environment through this large, porous network. Thus, the diameter of the pores, or capillaries, in the cell wall must ultimately impose a restriction on the size of molecules (nutrients, toxins, herbicides, elicitors, genetic elements, and viruses and other pathogens) with which the cells can interact. Despite the importance of the size of these pores, very little information is available concerning the capillary diameters in the cell walls of higher plants. The pores easily allow the passage of salts, sugars, and amino acids, since these substances can move freely in the plant (*1*). In addition, phytohormones can be translocated from cell to cell through the cell wall space (*2*). The

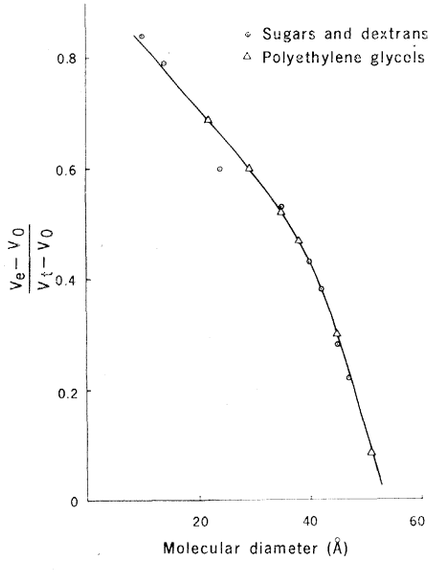

Fig. 1. Relationship between molecular radius and elution behavior during gel chromatography. A Bio-Gel P-10 column (200 to 400 mesh; 1 by 115 cm) was equilibrated at 23°C, with water as the solvent. Radii of sucrose, stachyose, and dextrans were calculated from known molecular weights or were taken from the literature; the points for the various PEG polymers were positioned on this graph on the basis of their elution behavior, and radii were obtained thereby. V_e is eluted volume, V_0 is void volume, and V_t is total volume.

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