difference in specific activities is taken into account.

In a separate set of experiments the cytoplasmic extracts, incubated with the iodinated and tritiated tracer, were centrifuged on glycerol gradients (*15*) in the presence and absence of 0.4*M* KCl. The low-salt 8*S* form (Fig. 3a) is totally transformed into the 4*S* form by 0.4*M* KCl (Fig. 3b) (*16*). The radioactivity bound to both the 8*S* and 4*S* forms is almost completely displaced by DES. The glycerol density gradients of the cytoplasmic extracts incubated with [^{3}H]estradiol (not shown) were identical to those incubated with the iodinated tracer with two exceptions: there was less bound radioactivity and more nonspecific binding of [^{3}H]estradiol than of ^{125}I-labeled estradiol.

The charcoal assay and glycerol gradient analysis in concert with the competition by the nonsteroidal estrogen DES show that [16α-^{125}I]iodoestradiol binds to the estrogen receptor. Unlike the results obtained by Katzenellenbogen *et al.* (*3*) with ^{125}I-labeled hexestrol, there was no evidence for increased nonspecific binding sites in the uterus when compared to [^{3}H]estradiol. The specific binding of the ^{125}I-labeled estradiol compares favorably with that of [^{3}H]estradiol, indicating that more sensitive assays than that obtainable with [^{3}H]estradiol could be performed with this high-specific-activity tracer. In addition, ^{125}I-labeled estradiol does not bind to the testosterone-estradiol binding globulin present in human plasma (*11*). This protein (*17*) contaminates most human tissue preparations, complicating the detection of sex steroid receptors. The use of the ^{125}I-labeled estradiol as a ligand in such assays will eliminate the "nonspecific" binding caused by this protein. Kinetic experiments and Scatchard analyses (*18*) indicate that in rat uterine cytosol the binding constant, K_d, of ^{125}I-labeled estradiol, is very close ($K_d = 2.7 \times 10^{-10}$) to that of [^{3}H]estradiol ($K_d = 1.6 \times 10^{-10}$) (*11*).

Its binding characteristics, stability, and ease of preparation, as well as the economic and technical advantages in determining radiation from a gamma emitter as compared to a beta emitter, make [16α-^{125}I]iodoestradiol the compound of choice for experimentation with the estrogen receptor. The studies in the rat in vivo demonstrating that ^{125}I-labeled estradiol is concentrated in the uterus, a tissue containing the estrogen receptor, provides an impetus for experiments to determine whether this steroid labeled with ^{131}I could be used in vivo for the radioimaging of estrogen receptor-containing breast tumors. It has also been found that ^{125}I-labeled estradiol binds to antibodies to estradiol conjugated at positions 3, 6, and 17 of the steroid nucleus and thus can be used as a ligand for the radioimmunoassay of estradiol (*11*).

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Molecular Cloning of Polyoma Virus DNA in *Escherichia coli:* Oncogenicity Testing in Hamsters

Abstract. *Inoculation of suckling hamsters with 2 × 10^8 live cells of* Escherichia coli *K12 strain χ1776, carrying the complete genome of polyoma virus in a recombinant plasmid, failed to induce tumors in any of 32 recipients. Also, lambda phage DNA and particles with a monomeric insert of polyoma DNA did not induce tumors. Purified recombinant plasmid DNA, as well as phage particles and DNA containing a head-to-tail dimer of polyoma DNA, showed a low degree of oncogenicity, comparable to that of polyoma DNA prepared from mouse cells. These findings support the previous conclusions, based on infectivity assays in mice, that propagation of polyoma virus DNA as a component of recombinant DNA molecules in* E. coli *K12 reduces its biologic activity many orders of magnitude relative to the virus itself.*

We have recently reported the results of a series of risk assessment experiments involving derivatives of *Escherichia coli* K12 bearing recombinant DNA that contains the complete genome of polyoma virus (*1, 2*). In those experiments, the circular polyoma viral DNA was converted to the linear form by cleavage with single-cut restriction enzymes, ligated to plasmid or lambda phage vectors, and propagated in *E. coli* K12. The *E. coli* that contained recombinant DNA, as well as the purified recombinant DNA, were tested for their ability to produce polyoma infection. Although the recombinant molecules contained complete polyoma genomes which were infectious when enzymatically excised from the recombinant molecules, *E. coli* carrying these molecules consistently failed to induce polyoma infection, even when massive numbers were fed or injected into mice, a highly sensitive indicator system for productive polyoma infection. Similar results were recently reported by Fried *et al.* (*3*).

As a further step in evaluating the biologic activity of the polyoma-plasmid and polyoma-lambda recombinant DNA host-vector systems, we have tested their ability to induce tumors in suckling

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hamsters. These animals are highly sensitive to tumor induction by polyoma virus (*4*); and further, intact virions are not required for tumorigenesis, since we have recently shown that naked DNA (*5*) and even subgenomic fragments (*6*) of viral DNA can induce tumors in them. Thus, inoculation of hamsters provides a bioassay system that can detect biologic activity of a subgenomic portion of the viral DNA, thereby complementing and extending the mouse infectivity assays.

The recombinant DNA materials we tested (*1, 2*) include plasmid pBR322 containing the complete polyoma genome inserted at the Eco RI or Bam HI site, and λgtWES · λB phage with monomeric or head-to-tail dimeric inserts of the complete polyoma genome at the Eco RI site. The LP strain of polyoma (*7*) was used as the source of polyoma DNA; this strain is quite tumorigenic in hamsters, the mean tumor-producing dose (TPD_{50}) being 4×10^3 plaque-forming units of virus (*5*). The recombinant plasmid systems were studied by inoculation of hamsters with live *E. coli* carrying the recombinant molecules or with purified recombinant DNA before and after treatment with restriction enzymes. The phage system was studied by injection of hamsters with recombinant-containing particles or DNA.

Experimental materials were inoculated subcutaneously into the backs of 1-day-old hamsters. The animals were observed for tumor formation for 5 months (*8*). Table 1 gives the results of the inoculations with live χ1776 carrying the recombinant plasmids. None of the 32 animals developed tumors after injection of 2×10^8 live bacteria; assuming a copy number of ten plasmids per cell, the inoculum contained an amount of polyoma DNA equivalent to that contained in 1700 TPD_{50} of polyoma virus (*9*). Since ten copies per cell is a minimum estimate, and since the χ1776 may have multiplied after injection, the actual dose of polyoma DNA per hamster may have been considerably larger. The dose of χ1776 administered to the baby hamsters is close to the maximum that could be tolerated, since the hamsters were ill for 2 days after the injection; furthermore, this inoculum corresponds to the mean lethal dose of χ1776 for suckling mice (*10*).

The tumorigenicity of recombinant DNA's, recombinant phage particles, and nonrecombinant virus-derived polyoma DNA is shown in Table 2. Uncleaved, supercoiled plasmid DNA's induced tumors in 2 of 27 recipients. When the recombinant plasmids were cleaved with the restriction enzyme used for their construction, the preparations induced tumors in 9 of 17 animals. This activity is equivalent to that of linear forms of polyoma DNA prepared from infected mouse cells (*5, 6*) (Table 2, last line). No tumors developed in the hamsters receiving lambda phage DNA containing the monomeric inserts, but 3 of 16 hamsters receiving recombinant molecules containing the dimeric insert developed tumors. Similarly, injection of phage particles resulted in tumors in 2 of 12 hamsters injected with dimer-containing particles, and no tumors in the case of the monomeric insert. In general, the oncogenic activity of the intact recombinant DNA and phage materials was comparable to, or less than, that of an equivalent amount of virus-derived polyoma DNA. This, in turn, is four to five orders of magnitude less than the activity of polyoma virus itself.

With regard to the relevance of these studies to risk assessment, the tests with live *E. coli* are clearly the most pertinent in that they are the only tests that in any way mimic a possible naturally occurring event. The tests with purified DNA and phage particles are important in allowing much larger amounts of DNA to be tested than can be administered in live *E. coli*, and, in our view, they are of value chiefly for indicating the extreme limits of what might be obtained after injection of intact organisms. The fact that the recombinant DNA molecules were biologically active when injected as cell-free materials, but not when contained in the live *E. coli* host, suggests that the bacterial cell constitutes an effective barrier against the transfer of its DNA to eukaryotic cells.

Since polyoma virus is so highly oncogenic in hamsters, and since noninfectious subgenomic segments of polyoma DNA are oncogenic in this host, these experiments constitute a "worst case" analysis; that is, the experimental design maximizes the chances for obtaining positive results. Thus, it is all the more striking that neither the live χ1776 har-

Table 1. Inoculation of suckling hamsters with χ1776 carrying polyoma DNA in recombinant plasmids. Plasmids pPB5 and pPB6 are recombinant plasmids consisting of pBR322 with the complete polyoma genome inserted at the Bam HI site in the two possible orientations; likewise, pPR18 and pPR21 are pBR322 with the polyoma genome inserted in both orientations at the Eco RI site (*1*). Equal numbers of organisms in the mid-log phase of growth from each of the cultures were suspended in saline; portions of the suspension containing 2×10^8 live *E. coli* K12 were injected subcutaneously in the backs of 1-day-old hamsters. Animals were examined for tumors twice weekly over a 5-month period.

Inoculum	Tumors*
χ1776 (pPB5) + χ1776 (pPB6)	0/23
χ1776 (pPR18) + χ1776 (pPR21)	0/9

*Ratio of the number of hamsters with tumors to the number of hamsters treated.

Table 2. Evaluation of the tumorigenicity of recombinant and nonrecombinant polyoma DNA's in suckling hamsters. DNA's were diluted in saline to give the equivalent of 0.45 to 0.5 μg of polyoma DNA per 0.03 ml of inoculum. Day-old Syrian hamsters were inoculated subcutaneously and checked twice weekly for the development of tumors over a 5-month period. The DNA's of polyoma plasmids pPR18, pPR21, pPB5, and pPB6 (see Table 1) were tested either in the supercoiled configuration (uncleaved) or after cleavage with the restriction enzyme used for the insertion (*1, 6*). Lambda-polyoma monomer recombinants λ-PY3 and λ-PY63 contain the polyoma genome in opposite orientations; the two phages or their DNA's were combined in equal proportions for the injections. The dimer-containing phage λ-PY30-5B (*2*) was separated from the monomer-containing phages by density-gradient centrifugation prior to injection or extraction of DNA. Phage particle inocula contained 10^{10} or 7×10^9 plaque-forming units, respectively, per 0.03 ml for the monomer- and dimer-containing phages. Nonrecombinant polyoma DNA was isolated from mouse cells infected with large-plaque polyoma virus (*5*). Tumors in animals inoculated with recombinant DNA were invariably subcutaneous tumors at the site of inoculation; the great majority of those examined histologically were fibrosarcomas.

Inoculum	Number of hamsters with tumors/number tested			
	DNA			Phage particles
	Uncleaved	Cleaved		
		Eco RI	Bam HI	
	Recombinant DNA			
Plasmid system				
pPR18 + pPR21	1/11	6/9		
pPB5 + pPB6	1/16		3/8	
Phage system				
Monomer insert (λ-PY3 + λ-PY63)	0/20			0/8
Dimer insert (λ-PY30-5B)	3/16			2/12
	Nonrecombinant DNA			
Polyoma DNA	4/73 (19 percent)	29/64 (45 percent)	11/35 (31 percent)	

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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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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