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11. We acknowledge Dr. Aaron B. Lerner (personal communication), who has independently demonstrated selective toxicity of L-dopa using a different technique.
12. M.W. thanks Dr. Thomas B. Fitzpatrick, who was responsible for initiating interest in the melanoma problem, for many helpful discussions. Supported in part by NIH grant CA 06516.

14 April 1977

Bacteriophages in Live Virus Vaccines: Lack of Evidence for Effects on the Genome of Rhesus Monkeys

Abstract. *Four juvenile rhesus monkeys were inoculated with 10^{12} plaque-forming units of the bacteriophage ϕV1 isolated from live virus vaccines. After ϕV1 had been cleared from the blood, DNA's were isolated from the livers and kidneys and analyzed for the presence of bacteriophage by plaque assays, and for the presence of ϕV1 DNA by DNA-DNA reassociation kinetics. No evidence was found for persistence of the bacteriophage or for replication of the phage genome in these rhesus monkeys.*

In 1972 Merrill reported that bovine serums contained bacteriophages as contaminants (*1*). This finding was confirmed by Chu *et al.* (*2*), and it was subsequently shown that live virus vaccines frequently contained bacteriophages, presumably due to contamination in the serums used to grow the cells in which the vaccines were produced (*3*). Further studies showed that the three bacteriophage plaque types isolated from 161 lots of vaccines were indistinguishable by physical and immunological assay (*4*). Our work was, therefore, limited to the original vaccine isolate designated ϕV1.

Previous studies with ϕX174 as a prototype bacteriophage had indicated that no biological or clinical effects were noted in mice, monkeys, or humans on exposure to high titers of the bacteriophage (*5*); however, more recent evidence has suggested that ϕX174 can replicate in monkey livers and human lymphocytes, and can become associated, perhaps transiently, with the host genome (*6*).

Because of the above findings with ϕX174, we attempted to determine whether ϕV1 injected into monkeys had any effect on the host genome or, indeed, if this bacteriophage were capable of replicating in a primate host. Our data show no evidence for replication or persistence of the ϕV1 genome by bacteriophage titration with the use of DNA's isolated from these monkeys or by nucleic acid hybridization studies. These results suggest that ϕV1 might play a useful role as a safe vector in recombinant DNA research.

Four 6- to 8-week-old rhesus monkeys were studied for presence of neutralizing antibody (*K* value) to ϕV1 as described for other bacteriophages (*7*). All were antibody-negative. One week later, a partial hepatectomy and unilateral nephrectomy were performed on each animal. These tissues served as preinoculation baseline materials. Biopsies of these organs were assayed and found to be negative for the presence of ϕV1 by means of the agar overlay technique with *Escherichia coli* C-3000 (*3*). DNA was extracted from the remaining tissue by a modification of the Marmur method (*8*). Three weeks later, after recovery from surgery, each monkey received 1×10^{12} plaque-forming units (PFU) of ϕV1 intravenously in a volume of 1 ml.

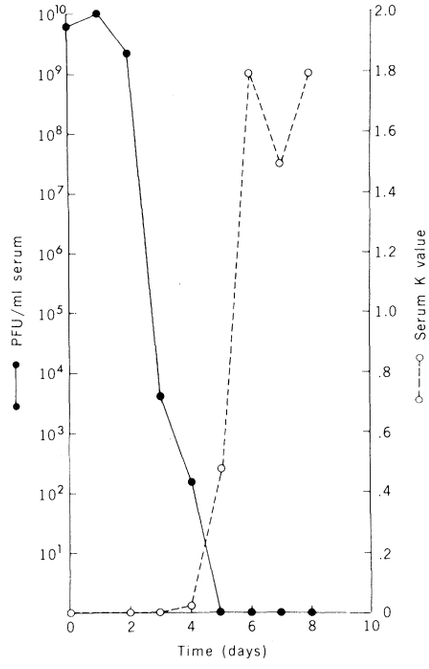

Fig. 1. Clearance of ϕV1 from the peripheral circulation (●) and antibody response (*K* value) to ϕV1 (- - ○ - -) after inoculation of 1×10^{12} PFU into a juvenile rhesus monkey. For inoculation, ϕV1 purified by CsCl banding, was grown in *E. coli* strain K12 F$^-$ (CR 63) at a multiplicity of 0.1 in a volume of 2 liters. The titer of the bacteriophage was ascertained as described (*11*).

Serum samples were obtained 15 minutes after inoculation and daily thereafter. A clearance pattern for ϕV1 typical of that seen for the monkeys in our study and the antibody response to this bacteriophage are shown in Fig. 1. The phage is cleared from the circulation by 5 days, and antibody begins to appear by 4 days after inoculation. Nine days after the phage inoculation the animals were killed. The remaining liver and kidney and the serum of each animal were assayed individually for the presence of ϕV1, and DNA was extracted from livers and kidneys.

The organ DNA's before and after inoculation were assayed by the plaque method on *E. coli* C-3000 for the presence of infectious ϕV1. No evidence of bacteriophage was found in any of the DNA's at any of the dilutions tested (undiluted to 10^{-6}). Thus, there was no intact, infectious ϕV1 present in the samples which might reanneal in the hybridization reaction mixture.

Quantitation of viral DNA copies by acceleration of reassociation had been described by Gelb *et al.* (*9*). In order to use this approach, we first examined the reassociation behavior of radioactively labeled ϕV1 DNA in the presence of an excess of unlabeled salmon sperm DNA. It reassociated with a $C_0t_{1/2}$ of 2.17×10^{-2} (C_0t is the concentration of nucleotide in moles per liter times the time in seconds) and followed typical second order kinetics [see Fig. 2 (open circles) and Fig. 3]. A reconstruction experiment to show the effect of adding copies of unlabeled ϕV1 DNA on the reassociation is illustrated in Fig. 2. The control reaction contained the equivalent of 7.64 copies per cell of ^{33}P-labeled ϕV1 DNA, calculated by the formula of Gelb *et al.* (*9*), with the use of salmon sperm DNA at absorbance A_{260} of 19 *A* units per milliliter and a ratio of 7.26×10^4 for the molecular weights of monkey DNA to ϕV1 DNA. Addition of known amounts of unlabeled ϕV1 DNA resulted in an acceleration equivalent to the number of copies of ϕV1 DNA added (Fig. 2). In the reannealing experiments reported below, ratios of unlabeled monkey cell DNA to ^{33}P-labeled ϕV1 DNA were such that we would see a 1.4-fold increase in $C_0t_{1/2}$ if there were one copy of ϕV1 DNA per cell.

We analyzed DNA from organs removed from monkeys before and after inoculation with ϕV1. Representative data are shown in Fig. 3. In no case for any of the four monkeys tested was there

an acceleration of reassociation. These data indicate that there is less than one copy per cell of ϕV1 DNA in the DNA preparations isolated from monkeys that were inoculated with 10^{12} PFU of ϕV1.

These experiments suggest that ϕV1 DNA does not replicate in monkey liver cells under our experimental conditions, in contrast to similar experiments with the bacteriophage ϕX174 (*6*), which indicated that ϕX DNA may be present in amounts of up to 100 copies per cell after intravenous inoculation to juvenile monkeys. Under the experimental conditions used we could not detect, however, the presence of a few cells containing ϕV1 DNA, either free or chromosome-associated, in a large background of noncarrier cells, or the presence of partial copies of ϕV1 DNA in small amounts.

The lack of evidence for a genetic interaction between the genomes of ϕV1 and nonhuman primates is important to the argument against possible past health hazards when ϕV1 was present in vaccines. The negative data are of equal interest when considering potential current and future health hazards associated with vectors proposed for use in recombinant DNA research. ϕV1 offers the advantage of a prokaryotic vector for which we find no evidence of replication or integration in nonhuman primates under conditions which would be most favorable for such events to occur. Thus, the possibility of human infections from a ϕV1 vector would seem to be extremely remote.

Previous experiments in our laboratory showed that DNA from phage ϕX174 may replicate in monkey liver when 10^{11} PFU's were administered intravenously to juvenile monkeys undergoing liver regeneration (*6*). However, one might expect that ϕX174 is better suited for replicating in a foreign host since its genome is small, single-stranded, and circular and thus may be more stable in a foreign system. In contrast, ϕV1 is a more complicated bacteriophage. The genome is a linear double-stranded DNA whose molecular size is 27×10^6 daltons (*4*). Repeated attempts to infect spheroplasts prepared from *E. coli* strains C, C-3000, and K12, *Salmonella typhimurium* strain Q22, and *Pseudomonas aeruginosa* with ϕV1 DNA were unsuccessful, while ϕX174 DNA was able to productively infect spheroplasts of the same bacteria in parallel experiments. Whether the inability to plaque ϕV1 DNA with the use of spheroplasts is a reflection of its increased susceptibility to shear or whether some factor necessary for infectivity is contained in the intact phage but not in its isolated DNA is not known.

Our experiments suggest that the bacteriophage which had been found in live virus vaccines did not appear to replicate in or to integrate into the DNA of nonhuman primates when 10^{12} PFU were administered intravenously to animals with regenerating livers. These results are consistent with long-term holding experiments (*10*) in which a variety of animals including nonhuman primates were inoculated with ϕV1, and no adverse effects have been noted after 2 years.

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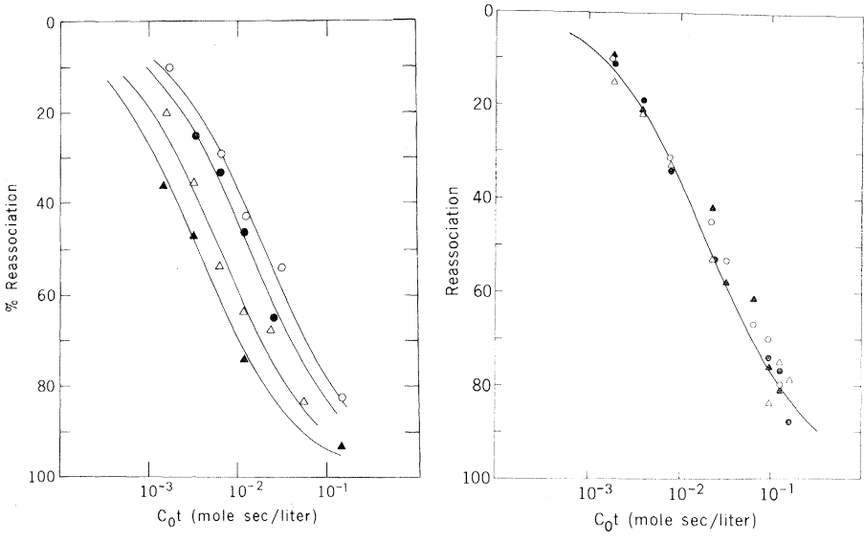

Fig. 2 (left). Reassociation of ^{33}P-labeled ϕV1 DNA in the presence of increasing amounts of unlabeled ϕV1 DNA. *Escherichia coli* C-3000 was grown in 100 ml of media containing 2.3 mg of KH_2PO_4, 0.1 percent glucose, 0.01 mg of thymidine per milliliter, and 0.1 mc of ^{33}P-labeled orthophosphate per milliliter, to a concentration of 5×10^8 per milliliter. After a 10-minute exposure to mitomycin C (0.05 mg/ml), the cells were resuspended in fresh media lacking ^{33}P and infected with ϕV1 (10^{10} PFU). ^{33}P-Labeled ϕV1 was purified by sedimenting onto a CsCl cushion (ρ = 1.535 g/ml) at 82,000 g for 3 hours (Beckman SW 27 rotor) and centrifuging the sharp visible band to equilibrium in CsCl. Infectivity and radioactivity coincided at ρ = 1.535 g/ml. DNA having a specific activity of more than 10^6 count/min per microgram was isolated by phenol extraction of purified bacteriophage. Reassociation kinetics were monitored by determining resistance to the single-strand specific nuclease S1 from *Aspergillus oryzae* (*12*) of ^{33}P-labeled ϕV1 DNA (10,000 count/min), sheared to a uniform size of 5.5*S* in an alkaline sucrose gradient; the sheared DNA was then denatured and incubated for 0 to 96 hours at 68°C in a volume of 2 ml containing 0.003*M* EDTA, 0.01*M* tris, *p*H 7.8, 1*M* NaCl, 19 A_{260} units of sheared salmon sperm DNA, and increasing amounts of sheared unlabeled ϕV1 DNA: (○) 7.64 copies of ^{33}P-labeled ϕV1 DNA, and no additional ϕV1 DNA, $C_0t_{1/2} = 2.17 \times 10^{-2}$; (●) 3.4 copies per cell of ϕV1 DNA was added, giving a reassociation rate that was increased 1.4-fold, corresponding to 3.3 copies per cell; (△) 17 copies per cell added, 3.2-fold acceleration corresponding to 17 copies per cell; and (▲) 34 copies per cell added, giving a 5.6-fold acceleration, corresponding to 35 copies per cell. Fig. 3 (right). Reassociation kinetics of ^{33}P-labeled ϕV1 DNA in the presence of sheared DNA (24 A_{260} unit/ml) prepared from monkey B-6663 (●) liver, before inoculation; (▲) kidney, before inoculation; (○) liver, after inoculation; and (△) kidney, after inoculation. Reassociation was performed as described in the legend of Fig. 2, except that lower concentrations of ^{33}P-labeled ϕV1 DNA were used in order to increase the sensitivity of the reaction. Reassociation of monkey DNA was monitored in a parallel reaction mixture by absorbancy measurements; reiterated DNA was already reassociated at the early time points, unique sequence DNA was reassociating during the time course of the experiment, and was essentially complete at the termination of the experiment.

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7 February 1977; revised 29 March 1977