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Bacteriophages, Vaccines, and People: An Assessment of Risk (40208)

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In 1973 we published the first evidence that the live virus vaccines (measles, mumps, polio and rubella) manufactured prior to that time frequently contained bacteriophages at low concentrations (1). Federal Regulations for purity in effect at that time stipulated that "viral vaccines shall not contain extraneous agents". The problem of vaccine purity notwithstanding, the most serious question was whether the presence of viable phages in the vaccines constituted a threat to the public health. Faced with the consequences of prohibiting the distribution of all live viral vaccines until phages could be eliminated from them, the Commissioner of the Food and Drug Administration (FDA), after consultation with an *ad hoc* expert committee, published regulations which in effect allowed phages to continue to be present in the vaccines for a limited time. During this period work would be done to define the scope of the problem, to resolve the question of safety, and to develop methods for the elimination of phages from vaccines without adversely affecting the effectiveness of the vaccines (2). That administrative decision was based on a benefit-to-risk assessment which attempted to weigh the theoretical risks of phages against the well-documented benefits of the vaccines for man. The theoretical risks have been discussed previously (3, 4). They include both indirect and direct effects of phages on humans: (1) the induction of a toxin by phages in appropriate bacterial hosts followed by a disease which is due to the toxin; and (2) the induction of changes in human cells which could then lead to any of a variety of diseases. The basis for considering the first risk is the now well-documented relationship between corynebacteria and phages in the production of the toxin which causes diphtheria. The second set of risks is based on two reports which show direct effects of phages on mammalian cells *in vitro*. In one investigation, the virus λ was used at multiplicities of infection (MOI) of about 10^5 phages per cell to transduce human galactosemic cells in order to correct the deficiency of the enzyme UDPG-transferase (5). The second study showed that after phage T7 was inoculated at high MOI onto hamster cells, T7 DNA sequences could be found in the cell nuclei (6). No studies have been reported in which phages have been injected into man or animals with the main purpose of assessing in a prospective manner any adverse effects upon the health of the subjects. Indeed, the discovery of phages and their lytic action by D'Herelle stimulated attempts to use phages as therapeutic agents for the control of bacterial infections (7, 8). Later they were considered convenient, safe biologic tools that could be used in man to study host defense mechanisms such as the kinetics of viral clearance from the circulation, primary antibody responses, and other immunological phenomena (9). Questions of phage pathogenicity for man were not considered relevant in those studies because, by definition, phages were restricted in their host range to bacteria and were not expected to cause disease in man.

In the remainder of this presentation we will review efforts that have been made to attempt to answer the question of whether or not phages in vaccines have been harmful to people. The studies were restricted to phage φV-1, since, as described in the preceding paper, it was the only phage that we isolated from vaccines (10).

Materials and methods. Phage preparation. A concentrated stock of φV-1 was prepared as previously described (10), and had a titer of 2.8×10^{13} PFU/ml. The stock was diluted in fetal bovine serum (FBS) to give a final concentration of 9.2×10^{10} PFU/ml for the small animal inoculations, and 9.2×10^{11}

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PFU/ml for the monkey inoculations.

Bacterial lysate. E. coli B was grown overnight in 100 ml of tryptone broth, pelleted, resuspended in 16 ml of 0.15 *M* NaCl, 0.05 *M* Tris pH 8, 60,000 μm/ml lysozyme, 4% EDTA, and allowed to lyse at 4° for 1 hr. The lysate was diluted 1:100 into FBS for the animal inoculations.

Endotoxin assays. The FBS, tryptone broth, *E. coli* lysate, and phage dilutions were assayed for the presence of endotoxin using the limulus amebocyte lysate procedure as previously described (11).

Animal studies. Preliminary studies (unpublished results) with φV-1 were initiated shortly after its isolation from vaccines to determine whether the phage was capable of causing gross of microscopic pathology in a limited number of small laboratory animals and two infant rhesus monkeys (*Macaca mulatta*). More extensive studies with larger numbers of animals were then undertaken with a more concentrated and purified preparation of φV-1. The larger study reported here consisted of 311 newborn hamsters, 319 newborn mice, 41 suckling rabbits, 57 suckling guinea pigs, and 16 infant and juvenile rhesus monkeys. The animals of each species except the monkeys were divided into four approximately equal groups which were inoculated with 100 μl of: (a) Saline, (b) tryptone broth, (c) *E. coli* lysate, and (d) 9×10^9 PFU of φV-1. All 16 monkeys were inoculated with 9×10^{11} PFU of φV-1 in a volume of 1 ml. The hamsters, guinea pigs and mice had intraperitoneal inoculations, while the rabbits and monkeys had intravenous inoculations. All the animals were counted daily and those which died were examined by a pathologist for gross and microscopic lesions unless the animals had been cannibalized or necrosis was too extensive. The small animals were held for a 2-year observation period at which time those remaining were sacrificed and examined by a pathologist for gross and microscopic lesions.

Cytogenetic studies. The possible induction of chromosome abnormalities by φV-1 was investigated using cell cultures derived from fresh human blood, primary human embryonic kidney, human diploid fibroblast cell lines (WI-38, M, P. W, and 98-4), human lymphoblastoid cell lines (JC & PB), muntjac fibroblast cell line (MJ), and *Peromyscus eremicus* cell lines (2656 and 2352). Cells were cultured in Eagle's minimal essential medium with 10% FBS or in McCoy's 5A medium with 20% FBS. All sera were tested for phages using the agar overlay technique with *E. coli* C-3000 as the bacterial host.

Standard procedures were used in the preparation of metaphases for analysis (12, 13). G-banding studies were done on 35 cells, using standard techniques (14). Control cultures included the following inoculation conditions: (a) tryptone broth; (b) cell culture growth medium; and (c) *E. coli* lysate. Phage (10^8–5×10^{10} PFU/culture) or control inocula were added to the cultures in a volume of 25–100 μl, and chromosome harvests were from 24 to 72 hr later.

Results. The endotoxin assays were positive for all samples tested. Only those dilutions of materials used in the inoculations were assayed, and the results were as follows: FBS 128 ng/ml. *E. coli* lysate 512 ng/ml; φV-1 256 ng/ml; and tryptone broth 40 ng/ml.

The initial animal studies (unpublished results) revealed no gross abnormalities, including, tumors, in any of the animals, and there were no differences in the survival of the small animals injected with phage when compared with saline controls. Microscopic examination of sections of brain, lung, liver, lymph nodes, and kidneys of the 26 small animals sacrificed at the end of a 1½ year observation period also failed to show any distinctive pathologic lesions with the exception of one nude mouse inoculated with 1.5×10^7 PFU of φV-1 which showed marked lymphoid hyperplasia of the spleen. The data from the larger study reported here confirmed the results of our preliminary experiment in that there were no significant differences in the long-term survivals when animals inoculated with φV-1 were compared with normal saline controls. The acute deaths (<72 hr) were due to cannibalism and inoculation trauma, and Table I shows the numbers of such deaths in each of the inoculated groups of hamsters and mice. The only acute deaths in the other species were 5 of 17 suckling guinea pigs inoculated with tryptone broth. When the number of acute deaths in mice inoculated with φV-1 was compared with the control groups by the χ^2 test, there was a

significant difference ($P < 0.025$) between ϕV-1 and the saline-inoculated group. The tryptone broth group also differed significantly from the saline group. A similar analysis of the acute deaths in the hamsters showed that the ϕV-1, saline, and *E. coli* lysate groups all differed significantly from the tryptone broth group which had the lowest number of acute deaths of any inoculated group. The acute deaths were eliminated from the data for Fig. I which shows the percent (%) deaths for the controls and phage-inoculated hamsters during the 2-year observation period. The inoculated mice showed the same death pattern as the hamsters. In both the mice and the hamsters, the group inoculated with *E. coli* lysate showed the lowest number of deaths after the first 72 hr. No significant differences in the % deaths were noted among the rabbit and guinea pig groups until the 16th month when there were excess deaths in those rabbits inoculated with normal saline. One monkey died secondary to trauma from a cage-mate. The histopathologic lesions found in the animals that died during the study were generally of the same types and frequency in the phage-inoculated animals and in the controls. For example, lymphoma was found in 1 of 78 hamsters in the tryptone broth group and 1 of 60 in the ϕV-1 group.

TABLE I. SUMMARY OF ACUTE DEATHS (<72 hr) AFTER INOCULATION OF NEWBORN HAMSTERS AND MICE.

Animals	Inoculum[a]	# deaths/# inoculated (%)
Hamsters	Saline	12/92 (13)
	Tryptone broth	2/80 (2)
	E. coli lysate	15/65 (23)
	ϕV-1 (9×10^9 PFU)	14/74 (19)
Mice	Saline	6/57 (11)
	Tryptone broth	17/71 (24)
	E. coli lysate	14/85 (16)
	ϕV-1 (9×10^9 PFU)	30/106 (28)

[a] Intraperitoneal inoculations within the first 24 hr of birth with a volume of 0.1 ml.

Since the chromosomal aberration rates among the controls were not significantly different when tested by the χ^2 method, they were combined. Table II shows that in these systems ϕV-1 did not induce statistically significant gross chromosomal changes in either number or structure than observed in control cultures. G-banding studies on 35 cells also failed to reveal chromosomal abnormalities that could be ascribed to any effect of ϕV-1 on the inoculated cells.

Discussion. In the evaluation of potential risks associated with phages in vaccines, *in vivo* human data would be of special relevance. We had hoped to be able to identify field trial populations of vaccine recipients who had received specific lots of vaccines, some of which contained phages. A retrospective assessment of adverse health effects could then have been made, and correlations with phages in the vaccine could have been attempted. Unfortunately, two such populations could not be differentiated because vaccine lots with and without phage were used in the same study groups, and there was no record of which lot was given to each vaccine recipient. Because of the absence of such human data, we have attempted to evaluate the vaccine phage isolate, ϕV-1, for its potential to induce abnormalities in both *in vivo* and *in vitro* systems.

Investigations to determine the origin of phages in vaccines incriminated the bovine serum used as a nutrient for the cell culture as the most important source (1, 15). Data developed in a number of laboratories (15–17) suggested that in addition to the C-3000 and K-12 *E. coli* strains, the bacterial host spectrum used to detect phages in sera

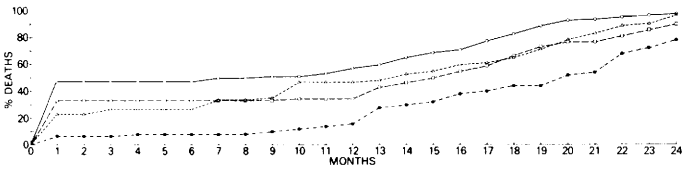

FIG. 1. Time course for cumulative deaths of newborn hamsters inoculated with ϕV-1 and control materials. Acute deaths (>72 hr) were eliminated from the calculations because they were due to cannabilism and inoculation trauma. Animals were inoculated with 1.0 ml intraperitoneally of normal saline (80 animals) (○——○), ϕV-1 (9×10^9 PFU) (60 animals) (△- - -△), tryptone broth (78 animals) (□- - -□), or *E. coli* lysate (50 animals) (●-·-●).

TABLE II. SUMMARY OF CHROMOSOMAL ABNORMALITIES OF CELL CULTURES EXPOSED TO ϕV-1.

Cell culture	# Cells examined	Abnormality (%) Hyperdiploid	Breaks and gaps	Structural[a]	Other[b]
Human blood					
control[c]	139	1 (0.7)	2 (1)	0	1 (0.7)
ϕV-1	59	1 (2)	2 (3)	0	1 (2)
Human embryonic kidney					
control	23	1 (4)	0	0	0
ϕV-1	100	4 (4)	2 (2)	1 (1)	1 (1)
Human fibroblast					
control	1004	2 (0.2)	22 (2)	1 (0.1)	0
ϕV-1	548	1 (0.2)	12 (2)	0	0
Human lymphoblastoid					
control	1650	0	15 (0.9)	0	0
ϕV-1	1075	0	12 (1)	0	0
Muntjac fibroblast					
control	200	42 (21)	8 (4)	15 (7.5)	2 (1)
ϕV-1	232	17 (7)	3 (1)	11 (5)	0
Peromyscus fibroblast					
control	1600	8 (0.5)	31 (2)	0	0
ϕV-1	800	4 (0.5)	20 (2)	0	0

[a] Dicentrics, rings, translocations, etc.
[b] Pseudodiploids and uncategorizeable.
[c] Combined (after tests for homogeneity) from separate analyses. Of cultures exposed to either: cell culture medium; tryptone broth; or C-3000 lysate.

and vaccines should be expanded to include *Pseudomonas aeruginosa, Salmonella typhimurium,* and *Enterobacter cloacae.* We have used these five bacterial strains to assay for phages in every lot of live virus vaccine that was released by the Bureau of Biologics (BB) since 1974, and in no instance have we recovered phages. In addition, attempts to detect vaccine phages on other known phage hosts such as *Staphylococcus aureus, Bacillus cereus,* and *Proteus morgangii* were unsuccessful. We take this to indicate that when bovine sera are screened for phages before use in vaccine production the problem of phages in vaccines can be controlled.

The animal inoculations failed to show a difference in the types or frequency of histopathologic lesions in the phage-inoculated animals when compared to controls. The reason for mice and hamsters inoculated with normal saline showing the highest % deaths during the first month, or for the *E. coli* lysate inoculated animals showing the highest survival is unknown. It is of interest, however, to note that the *E. coli* lysate inoculum (0.1 ml) did contain the largest amount of endotoxin (51.2 ng), and that the % death for both mice and hamsters appears to be inversely related to the amount of endotoxin in the inoculum of the various groups of animals. In both the mice and hamster studies, ϕV-1 inoculated animals fell within the limits set by normal saline and tryptone broth. This suggests that ϕV-1 did not have any significant effect on the survival of these animals. Support for this interpretation comes from the fact that the nonhuman primates continue to be free of discernible disease and no unusual deaths have occurred.

The Muntjac cultures were included in the cytogenetic studies because they represent the simplest known mammalian cytogenetic system; and the identification of consistent low frequency abnormalities was, therefore, greatly facilitated. The Peromyscus cultures were studied because the short arms of the chromosomes of this species are entirely heterochromatic. Thus, we could evaluate possible differential effects of ϕV-1 on heterochromatin compared with euchromatin. None of the cytogenetic systems studied showed any effect attributable to ϕV-1. It is also of interest to note that endotoxin did not induce chromosomal abnormalities even when present in the cultures at a concentration of 10 ng/ml.

None of the *in vivo* or *in vitro* results to date suggest that ϕV-1 has posed a health hazard

to vaccine recipients who may have received it along with the intended vaccine virus. The nonhuman primates inoculated with ϕV-1 will continue to be evaluated for many years for effects that were not evident during this phase of the study. We had the opportunity to evaluate only ϕV-1, and we cannot rule out the possibility that other phages may have been present in vaccines in the past. On the basis of isolation results from sera and vaccines (15–17), however, the variety of phages that might have been inoculated into humans does not seem to be as broad as initially supposed.

We consider the possibility of ϕV-1 in vaccines having induced health abnormalities to be remote primarily because of the findings presented in this report and the recent study which showed no detectable interaction between ϕV-1 and the DNA of monkeys (18). In addition, it should be recalled that we found ϕV-1 in vaccines licensed and distributed in the United States at concentrations no greater than 20 PFU/ml. Therefore, it is highly improbable that the exposure to the few phages (1–20 PFU) in vaccines would have added significantly to the natural burden to which humans are exposed in their daily environment.

Summary. The bacteriophage ϕV-1 isolated from live virus vaccines in 1973 was evaluated with respect to its ability to induce disease in small laboratory animals and nonhuman primates. Cytogenetic studies were also undertaken to evaluate the potential of ϕV-1 as a clastogen. The phage caused neither an increased death rate nor more histopathologic lesions than were found in controls. Similarly, the chromosomal aberration rate in cell cultures inoculated with ϕV-1 was not different from controls. On the basis of these studies and the fact that no more than 20 PFU/ml were found in vaccines, it is unlikely that ϕV-1 posed a health hazard to vaccine recipients who received it in the past. Since 1974 detectable phages such as ϕV-1 have been absent from the live virus vaccines released in the United States.

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