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A Therapeutic Antiviral from an Extract of λ Infected *E. coli* (Phagicin).* (30603)

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Inhibition of viral cytopathogenicity and viral replication by materials derived from bacteria has been reported(1,2). The most recent reports have dealt primarily with the presence of cytopathic effects in cultures treated with lysates of some bacteria such as *Corynebacterium* and *E. coli* K-12(3,4). Since the bacteria involved are known to contain prophages in their genome, it seemed important to determine whether bacteriophage might be responsible for the antiviral activity against animal viruses.

The *E. Coli* K-12 was chosen as a model system because its metabolism and genetic structure are well known and it harbors a temperate phage lambda which has been intensively studied. In this system it is possible to document phage-specific immunity, interference and phage conversion. Although there is to date no evidence of interference between bacteriophages and animal viruses it seemed to us worthwhile to study the possibile activity of *E. coli* lysates containing bacteriophage against herpes and vaccinia viruses.

Methods and materials. Bacterial strains. The strains of *E. coli* K-12 (λ) and *E. coli* λ sensitive used in this investigation were obtained from Dr. W. Dempsey, Biochemistry Dept., University of Florida.

Media. Media used for propagation of bacterial strains was Difco Pen Assay Broth. Same media containing 0.8% agar, 0.5% NaCl and 10^{-3} M Mg $SO_4 \cdot 7H_2O$ was used for the agar overlay technique.

Preparation of phage lysates. A stock of phage was obtained by U.V. induction of an exponentially growing culture of *E. coli* K-12 (λ). The lysate was filtered, stored at 4°C and subsequently used as an inoculum to infect *E. coli* λ sensitive for production of high titer lambda phage lysates. The phage stock used throughout this work was prepared by the agar overlay technique(5). Young cultures of a lambda sensitive strain of *E. coli* were suspended in distilled water containing 10^{-3} M Mg $SO_4 \cdot 7H_2O$ and 0.5% NaCl, to a concentration of 10^7 cells per ml. One ml of the bacterial suspension was infected with λ phage, placed in 9 ml of soft agar, allowed to absorb for one hour at 46°C and poured over a hard trypticase soy agar base in 32 oz bottles. The bottles were incubated at 36°C for 18 hours at which time the soft agar layer was removed, broken and suspended in Pen Assay Broth. The soft agar and bacterial debris were removed by cenrifugation and the supernatant fluid was filtered through millipore filters in the cold. A final filtration was done through a sterile 0.45 μ millipore, and this lysate was used for antiviral activity assays both *in vivo* and *in vitro*. The phage titer of each lysate was determined by counting the number of plaques produced by a diluted sample of the phage lysate on a layer of sensitive indicator strain.

For biochemical determinations the plaques were washed with lambda diluent(6) consisting of 1 M Tris buffer pH 7.5; 0.12 g of Mg SO_4, $7H_2O$; 4 g NaCl and 0.05 g gelatin per liter. In nucleic acid analyses, an aliquot of the lysate was incubated with dilute alkali at 37°C for 18 hours followed by acidification with trichloroacetic acid. The RNA was measured in the acid soluble supernatant with the orcinol reaction(7). The DNA was ex-

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tracted from the residue with 0.5 M perchloric acid at 70°C for 20 minutes and analyzed by the procedure of Burton(8). Protein was determined by the method of Lowry (9).

Irradiation of the phage lysate was done with a 15 watt G.E. germicidal lamp at 15 cm for 30 minutes.

Virus strains. The herpes simplex strain GCA3 kindly donated by Dr. M. J. Fruitstone, Variety Children's Hospital, Miami, Fla. and a strain of vaccinia virus donated by Dr. G. E. Gifford, University of Florida, were used.

Stocks of each virus were prepared from 32 oz chick embryo monolayer cultures, harvested and stored in glass sealed vials at —90°C. Monkey kidney Medium A was used for the initial growth of chick embryo monolayers. For plaquing assay the monolayers were kept in maintenance media consisting of: 5 ml yeast extract (1%); 5 ml Lactalbumin hydrolyzate (1%); 5 ml Proteose peptone (1%); 5 ml of sodium bicarbonate (2.5%) per 100 ml of Gey's Balanced Salt solution.

Antiviral assay. The antiviral activity of the lysate was studied using the plaque inhibition assay: 2 oz chick embryo monolayer cultures were inoculated with 1 ml of the phage lysate dilution plus 1 ml of maintenance media with an appropriate number of plaque forming units of herpes or vaccinia virus. The bottles, 5 per dilution, were incubated stationary at 36 until plaques reached maximum size and before the appearance of secondary plaques; approximately 30 hours for herpes simplex and 48 hours for vaccinia virus. The bottles were stained with crystal violet and plaques were counted under a photographic enlarger at a magnification of 10×. *In vivo* studies of herpes simplex keratitis were done by the method of Kaufman(10). Virus was inoculated in the rabbit cornea; 48 hours after infection the animals were randomized into similar groups of 12. The material tested was applied every 2 hours and evaluation of the ulcers was done at the third day of treatment. The scoring was done as follows:

1 = ulcers covering ¼ of cornea
2 = ulcers covering ½ of cornea
3 = ulcers covering ¾ of cornea
4 = ulcers covering entire cornea.

TABLE I. Antiviral Activity of λ Phage Lysate.

Exp	Final dilution of lysate in culture	No. of plaques: Vaccinia	No. of plaques: Herpes simplex
1	1:2 *	0	0
	1:4	0	0
	1:8	0	0
	1:16	0	0
	1:20	0	32
	Control	257	456
2	1:2 †	0	
	1:20	35	
	1:200	85	
	Control	136	

* 5.7 × 10^8 PFU of λ phage/ml.
† 6.38 × 10^7 PFU of λ phage/ml.

Results. Antiviral activity. The antiviral activity of the lysate is easily documented by the plaque inhibition assay in chick embryo monolayers with herpes simplex and vaccinia as the challenge virus (Table I). Table I also shows the relative concentration of the phage lysate needed to suppress plaques completely and those concentrations below which the lysate was no longer effective. In these experiments both virus and phage dilutions were added simultaneously but the effect of adding the phage lysate before and after infection was also studied. The results as tabulated in Table II indicated that the lysate is active when added as long as 6 hours after infection but higher concentrations are required for activity. A bacterial extract pre-

TABLE II. Antiviral Activity of λ Phage Lysate.

Exp	Final dilution of lysate in culture	Time of addition	No. of plaques: Vaccinia	No. of plaques: Herpes simplex
1	1:2*	0	0	0
	1:2*	+6	0	0
	Control	0	110	100
	E. coli control†	0	112	
		6	107	
	E. coli control	0		95
		6		98
2	1:2‡	−12	7	
	1:4	−12	12	
	1:8	−12	14	
	Control	0	113	

* 2.09 × 10^8 PFU of λ phage/ml.
† Bacterial extract from *E. coli* λ sensitive.
‡ 4.4 × 10^8 PFU of λ phage/ml.

TABLE III. Effect of λ Phage Lysate on Herpetic Corneal Ulcers (McKrae Strain).

	Ulcer grade
IDU .5%	0 ±0
Phage lysate	.75 ± .30
Control*	2.35 ± .49

Phage lysate contains 1.55×10^8 PFU/ml.
* Phage lysate diluent.

pared from *E. coli* λ sensitive, but not containing phage, is not effective and the activity of the lysate was still present in chick embryo monolayers that had been preincubated with the phage lysate, although the bottles were washed thoroughly prior to infection.

The activity of the lysate was not altered by overnight dialysis against maintenance media in the cold or U.V. irradiation which rendered the lambda particles non-infective.

In vivo this material has significant therapeutic antiviral activity. The effect of treatment of rabbit corneal ulcers with the phage lysate is shown in Table III. An IDU control was included for comparison and to determine the relative potency of the lysate. The activity, although not equal to IDU is indeed significant, despite the crude nature of the preparation.

Mode of action. Experiments were done to ascertain whether the antiviral activity of the phage was due to direct inactivation of the virus, blocking of the absorption process, or inhibition of replication.

To test any hindrance of the adsorption process, chick embryo monolayers were incubated with phage lysate for 12 hours. The bottles were washed, inoculated with herpes simplex and allowed to absorb at 37 for 2 hours. The supernatant was then poured off and assayed for any remaining virus. In the control cultures, at zero time, we obtained 207 plaques. The supernatant of these cultures, when plated 2 hours later, gave no plaques. All of the virus had been adsorbed, as expected. In the preincubated bottles, no plaques were observed, nor was there any virus present in their supernatant. The data suggest that the virus was adsorbed but did not replicate within the cell.

Preliminary studies suggest that the lysate not only suppressed intracellular virus, but might actually inactivate it. The intracellular activity of the phage lysate is further confirmed by the fact that the lysate inhibits plaque formation when applied 6 hours after infection. Since the virus studied can pass directly from cell to cell, the material must act to inhibit virus replication within the cell. In addition, it has been shown that the activity is retained intracellularly by the cells since preincubation of cells with the phage lysate prior to infection also resulted in suppression of plaques (Table II).

Chick embryo monolayers were infected with herpes simplex and incubated with the phage lysate. When plaques were visible on the controls without lysate, half of all the bottles were stained. The remainder of the infected treated bottles were washed thoroughly to remove all lysate and were incubated with fresh media for an additional 30 hours. The phage lysate not only temporarily suppressed virus growth but prevented the subsequent reactivation of virus, at least for the period of observation. Further studies of the mechanism of action are in progress.

We have not observed any toxicity of the lysate *in vivo* or *in vitro*. Normal noninfected rabbit corneas have been treated every 2 hours for 7 days without developing any signs of irritation or toxicity. *In vitro,* the results have been similar. Dilutions of the phage lysate which completely suppress herpes and vaccinia plaques were tested for toxicity. Young chick embryo cells were incubated with the lysate 24 hours after seeding and observed for several days. The cells grew to a confluent monolayer, and there was no obvious cell death or quantitative hindrance of growth.

Nature of the active compound. The data presented indicate that the activity of this material resides either in the lambda particles or other substance produced during phage replication. Experiments were conducted to determine more precisely the nature of the active substance.

The possibility that bacterial endotoxin might be responsible for the antiviral activity appeared to be ruled out by the fact that nonphage containing bacterial extract made from

TABLE IV. Antiviral Activity of λ Phage Lysate Fractions.

Final dilution of lysate in culture	No. of plaques	
	Vaccinia	Herpes simplex
Supernatant fraction		
1:2	0	0
1:4	4	0
1:8	6	0
Pellet fraction		
1:2	15	49
1:4	25	140
1:8	45	185
Phage lysate control		
1:2	0	0
1:4	1	0
1:8	5	0
Virus control	87	107

the same bacteria that produced the phage was ineffective both *in vitro* and *in vivo*.

Interferon was considered as another possibility. The phage lysate was tested as a possible inducer for interferon production by the method of Gifford, Toy and Lindenmann (11). Chick embryo monolayers were incubated for 6 hours with the phage lysate as an inducer. The lysate was decanted, fresh medium was introduced, and the bottles were incubated for an additional 24 hours. This fluid and an appropriate phage lysate control were dialyzed against .1 M HCL/KCL pH_2 buffer for 24 hours in the cold, followed by dialysis against 2 changes of maintenance media. This material was then assayed for interferon activity against vaccinia virus, and no interferon activity was detectable in the fluid of the lysate-preincubated bottles.

Since the active substance did not appear to involve either interferon or endotoxin, studies were done to determine whether activity resided in the intact phage particles. The phage particles were removed from the supernatant by centrifugation at 30,000 rpm for 30 minutes. The pellet was reconstituted to the original volume with Pen Assay Broth, and the activity of both fractions was tested against herpes simplex and vaccinia viruses. The results tabulated in Table IV showed that the activity was primarily in the supernatant rather than in the pellet fraction. Since no countable phage was present in the supernatant, it can be concluded that the activity of the lysate is not due to the infective lambda phage *per se*, but to a soluble material present in the lysate and produced as a result of phage infection.

The activity is not destroyed by deoxyribonuclease or ribonuclease. The active substance is non-dialyzable, sensitive to heat and not affected by U.V. irradiation.

Studies on the characterization of this material are now in progress.

Discussion. The experiments show that an antiviral agent with activity both *in vivo* and *in vitro* can be obtained from lysates of *E. coli* after infection with lambda phage. Although it has been shown that the activity resides in the soluble fraction of the lysate rather than in the infective lambda particle *per se*, production of the therapeutically active antiviral agent is associated with the process of phage replication, as such activity is not found in lysates of uninfected lambda sensitive strains. Because of its origin the term *phagicin* is proposed for this antiviral agent.

The apparent lack of toxicity of this active substance *in vivo* and *in vitro* and its biological origin places this material in a unique position within antiviral agents. It is possible that some of the antiviral activity previously attributed to bacterial extracts could be due to the production of *phagicin* associated with spontaneous lysis of lysogenic cells, but this is the first time that any substance linked to the production of a bacterial virus has been shown to inhibit the multiplication of animal viruses.

Phagicin has unique characteristics that may distinguish its activity from that described for other bacterial extracts(3,4). It is found in the soluble fraction of the lysate; preincubation of chick cells with this material protects the cell against viral infection even after it has been removed from the culture; and unlike other agents, it does not prevent young cells from multiplying normally and forming a confluent layer. The long lasting protection of cells, the solubility, and the apparent lack of toxicity indicate that although *phagicin* may not be the same as the antiviral substances observed in bacterial extracts, its unique properties make it a promising agent for further investigation.

Summary. A new antiviral agent with activity both *in vivo* and *in vitro* has been obtained from lambda infected *E. coli.*

A study of the antiviral activity of the lysate in chick embryo monolayers shows that this material is effective in completely suppressing both vaccinia and herpes simplex when added before and as long as 6 hours after infection, and the activity is retained by chick embryo monolayers that have been preincubated with the lysate and were washed thoroughly prior to infection.

The lysate does not block absorption of the virus, but rather inhibits intracellular replication. The activity of the lysate is not due to the infective lambda particle *per se* but to a soluble protein fraction.

In vivo, this material has significant therapeutic activity in the treatment of rabbit corneal ulcers.

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Chloramphenicol and L-Phenylalanine Therapy in Experimental *Klebsiella pneumoniae* Infection in Mice.* (30604)

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Ingestion of L-phenylalanine has been shown to reverse one of the early toxic effects of chloramphenicol on human bone marrow, namely, the cytoplasmic vacuolization of erythrygones(1). The relationship of these vacuoles to the aplastic anemia associated with chloramphenicol treatment is not clear. Before investigating the possibility that prophylactic administration of phenylalanine might prevent the later more serious toxic effects of chloramphenicol, it was necessary to evaluate the effect of administration of the amino acid upon the antibacterial efficacy of chloramphenicol.

The need for the study was also suggested by the possibility that the cytotoxic effect of chloramphenicol in clinical situations and the antibiotic activity of the drug might involve a common mechanism. In addition, Woolley(2) reported that phenylalanine inhibits the antibacterial action of chloramphenicol *in vitro.*

Materials and methods. The following materials in varying combinations were injected into Swiss White male mice (25 $\pm$ 2 g initial weight) in each test. A strain of *Klebsiella pneumoniae* isolated from the blood culture of a patient was used throughout. The organism was maintained and diluted in brain-

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