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Effect of the Interferon Inducer, Dextran Phosphate, on Influenza Virus Infection in Mice[1] (38960)

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Interferon inducing activity of phosphorylated polysaccharides was reported in previous papers (1, 2). These polysaccharides are phosphomannans excreted by *Hansenula*, a phosphogalactomannan extracted from *Aspergillus* and various chemically phosphorylated polysaccharides (1, 2). Although the titer of circulating interferon induced by these polysaccharides in rabbits was lower than that by other well-known inducers including poly I·poly C and endotoxin (3–7), these polysaccharide inducers were characterized by extremely low toxicities in various experimental animals. These findings suggest that if the polysaccharide inducers could be shown to be effective against any infection model in animals, clinical application might be considered. Another polysaccharide, dextran, already has been used as a blood plasma substitute in clinical studies. Based upon these considerations, the antiviral effect of a dextran phosphate was examined on influenza virus infection in mice.

Materials and Methods. Mice. The dd strain of white mice weighing 14–16 g were used throughout the study. They were obtained from the Central Farm of Tohoku University where care was taken to prevent infections with Sendai virus and *Mycoplasma.*

Virus. The kumamoto strain of influenza A2 virus (H_2N_2) was used for the infection. A mouse adapted virus strain was propagated once in the allantoic cavity of embryonated eggs and 48 hr allantoic fluid was used as the inoculum. The egg infectious titer (EID_{50}) of the fluid was $10^{9.0}$/ml, which corresponded to $10^{5.0}$ LD_{50} in mice, inoculated intranasally, by our standard inhalation procedure (8).

For the interferon assay, the Indiana strain of vesicular stomatitis virus (VSV) which was propagated in the thymidine-kinase-less mutant strain of L cell cultures (L-1D cells) and stored at −80°, was used as a challenge virus. The 50% tissue culture infectious dose (TCD_{50}) was $10^{7.0}$/ml in the same L cells.

Phosphorylation of dextran. Dextran, molecular weight of 40,000 by Sephadex-gel filtration, was purchased from Pharmacia Co., Uppsala, Sweden. This dextran is made up approximately of 90% β-1-6 linkages. The phosphorylation of dextran was carried out by the method of Whistler and Towle with slight modification (9). Briefly, the phosphorylation mixture was prepared as follows: Tetrapolyphosphoric acid (3 g) was added to 20 ml of dimethylformamide and 6.4 ml of tri-*n*-butylamine, and the mixture was stirred to complete solution. Dextran (1 g) was added slowly with stirring, and the mixture then stirred at 120° for 6 hr with exclusion of moisture. After cooling to 25°, the viscous solution was added to 5 vol of ethanol and precipitated by addition of several drops of saturated sodium chloride solution. Dextran tri-*n*-butylamine phosphate was collected by centrifugation, redissolved in 200 ml of water, and the solution was adjusted to pH 9–10 with 1 *N* sodium hydroxide solution. The liberated tri-*n*-butylamine was removed by repeated evaporation under reduced pressure. The solution was repeatedly dialyzed against distilled water and the

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sodium salt of the phosphorylated polysaccharide was precipitated by addition of 5 vol of ethanol. The precipitate was washed twice with 99.5% ethanol, and dried in a vacuum desiccator over calcium chloride. By the above procedure, 1.4 g by dry weight of dextran phosphate (DP 40) was obtained. It was stable at 4° for 2 mo. The LD_{50} of DP 40 in mice after ip injection was 4.5 g/kg.

Other compounds used. Virazole, 1 β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (10), was kindly provided by Dr. R. W. Sidwell, ICN Nucleic Acid Research Institute, Irvine, California, USA, and *Escherichia coli* endotoxin was prepared in our laboratories by Westphal's method (11).

Virus inoculation procedure. Virus infection was achieved by inhalation of influenza A2 virus. A vaponephrine type nebulizer was connected to the compressor to spray about 10 ml of diluted allantoic fluid in 20 min. This procedure resulted in the inoculation of 1/500 ml of the fluid per mouse (8). The virus dose per mouse is specified for each experiment.

Determination of virus titer in mouse lung by EID_{50}. Infected mouse lungs were disrupted in sterile sea sand with mortar and pestle and made up to a 10% suspension with nutrient broth. Serial tenfold dilutions of the supernatant were inoculated allantoically into groups of four embryonated chicken eggs. Allantoic fluids were tested for hemagglutinin (HA) activity after incubation for 72 hr at 36°. EID_{50} titers were calculated for the 10% suspension according to Reed and Meunch.

HA inhibition (HAI) tests. The techniques recommended by the Committee on Standard Serological Procedures in Influenza Studies was followed to determine the HAI titers.

Calculation of lung consolidation grade. The grade of virus-induced lung consolidation was determined according to the method of Horsfall with a modification (12). Briefly, five mice in the treated group and seven mice in the control group were killed and bled on the respective day after intranasal inhalation of influenza virus. Before autopsy, the mice were irrigated by injection into the heart of M/100 phosphate buffer (pH 7.2) to remove red blood cells from lung tissues. The consolidation score was calculated by an average of the numerical scores obtained for each mouse. To obtain a numerical score for each mouse, the following six possible disease categories were defined; survival without pulmonary consolidation = 0, 20% of lung consolidated = 1, 40% of lung consolidated = 2, 60% of lung consolidated = 3, 80% of lung consolidated = 4, and death or 100% lung consolidated = 5.

Interferon induction in mice. For the interferon induction and assay, a minor modification of Lampson's procedure was followed (13). Briefly, mice were injected intraperitoneally with DP 40, and at appropriate intervals serum or lung specimens were taken for antiviral assay. Assays for antiviral activity were carried out with L-1D cells grown in Eagle's MEM with 10% bovine serum at 37° for 3 days. Serial two-fold dilutions of the specimens were made with Eagle's MEM containing 5% calf serum. Each dilution was added to four monolayer culture tubes of mouse L-1D cells and incubated at 37° for 20 hr. Then the culture was washed with Hanks' balanced solution and the culture medium was replaced by new medium containing 100 TCD_{50} of VSV and incubated again for 18 hr at 37°. At this time the inhibitory effect on viral cytopathic effect (CPE) was observed. The interferon titer was expressed by the reciprocal of the maximum dilution capable of preventing the viral CPE.

Results. Effect of DP 40 on influenza virus infection in mice. Employing the same administration schedule, 6 groups of 12 mice were treated with DP 40 (3 groups, 250, 25, and 2.5 mg/kg, respectively), dextran (2 groups, 250 and 25 mg/kg, respectively) and *E. coli* endotoxin (1 group, 1 mg/kg) (Table I). These inducers dissolved in 0.5 ml of saline were given ip twice before infection (−3 and −1 hr), twice after infection (+1 and +3 hr) and once daily for 4 consecutive days after virus infection. Another group of 36 mice receiving 0.5 ml of saline at the same times and served as a control. All of the mice were challenged with 10 LD_{50} of influenza virus intranasally.

As shown in Table IA, whereas all of the

TABLE I. EFFECT OF DEXTRAN PHOSPHATE ON INFLUENZA INFECTION[a] IN MICE.

Tested materials[b]	Dose (mg/kg)	No. of mice	Survival days	% Survival
A. When challenged with 10 LD_{50}				
Dextran phosphate 40	250	12	11, 11, 11, 11, 13, 13, 14, S, S, S, S, S[c]	42[d]
Dextran phosphate 40	25	12	12, 13, 13, 13, 14, 18, 20, 20, 20, 20, S, S	16.7[e]
Dextran phosphate 40	2.5	12	12, 12, 13, 13, 18, 18, 20, 20, 20, 22, 22, 22	0
Dextran 40	250	12	11, 11, 12, 12, 13, 14, 14, 16, 20, 20, 22, 22	0
Dextran 40	25	12	12, 12, 13, 13, 14, 14, 15, 16, 16, 20, 20, 20	0
E. coli endotoxin	1	12	11, 12, 12, 12, 14, 14, 15, 16, 18, S, S, S	25[d]
Saline		36	11, 11, 11, 11, 11, 12, 12, 12, 12, 12, 12, 12, 12, 12, 12, 13, 13, 13, 13, 13, 13, 13, 14, 14, 14, 14, 14, 15, 15, 16, 16, 20, 20, 20	0
B. When challenged with 3 LD_{50}				
Dextran phosphate 40	150	20	11, 12, 12, 14, 14, 14, S, S, S, S, S, S, S, S, S, S, S, S, S, S	70[d]
Dextran phosphate 40	75	20	12, 12, 12, 14, 14, 14, 14, S, S, S, S, S, S, S, S, S, S, S, S, S	65[d]
Saline		40	10, 10, 10, 10, 10, 10, 10, 10, 10, 10, 10, 10, 10, 11, 11, 11, 11, 11, 12, 12, 12, 12, 12, 12, 12, 12, 13, 13, 13, 13, 13, 13, 14, 14, 14, 14, S, S, S, S	10

[a] Virus was given by inhalation.

[b] The administration of tested materials was done at 3 and 1 hr before virus infection, 1 and 3 hr after virus infection, and was continued once daily for 4 consecutive days.

[c] Survived more than 30 days.

[d] Significant difference between control and treated; $P = <0.005$.

[e] Significant difference between control and treated; $P = 0.025$.

control mice receiving either saline (36 mice) or dextran (two groups, 24 mice) died (within 22 days), an apparent protective effect on the life span was observed in the groups treated with 250 mg/kg and 25 mg/kg doses of DP 40. The survival rate of mice treated with 250 mg/kg of DP 40 was 42%, which was better than that of endotoxin treatment (25%). When the challenge dose of influenza virus was lowered to 3 LD_{50}, DP 40 was shown to be more effective than it was against the larger challenge dose. As shown in Table IB, 2 groups of 20 mice treated with DP 40 at a concentration of 150 and 75 mg/kg survived at a rate of 70 and 65% for more than 30 days after virus infection. With such a low dose challenge, however, the survival rate of mice receiving saline was 10%.

Effect of single-administration of DP 40 on influenza infection in mice. Taking the interferon inducing activity of DP 40 into consideration, the effect of a single-administration was examined with two doses (300 and 100 mg/kg) which were given ip 1 hr before infection. Twenty mice were used for each dose and 30 mice, served as controls (Fig. 1). As a positive control, the antiviral substance, virazole (30 mg/kg) was given ip to another 10 mice at 24 and 1 hr before, 1 and 3 hr after virus infection, and once daily for 4 consecutive days. All of the mice were challenged with 10 LD_{50} of influenza virus intranasally. As shown in Fig. 1, all of control mice died within 11 days, and a single-administration of DP 40 produced 35 and 10% survival rates at concentrations of 300 and 100 mg/kg, respectively. In comparison virazole treatment gave a 50% survival rate. The significance of the differences between control and DP 40 (300 mg/kg) or virazole treated groups was $P < 0.005$.

Development of lung consolidations after DP 40 treatment of influenza infected mice. A total of 48 mice were infected intranasally with 10 LD_{50} of influenza virus. Twenty of these mice were given a single ip dose of 250 mg/kg of DP 40 and the rest, served as controls. DP 40 was administered at 3 and 1 hr before virus infec-

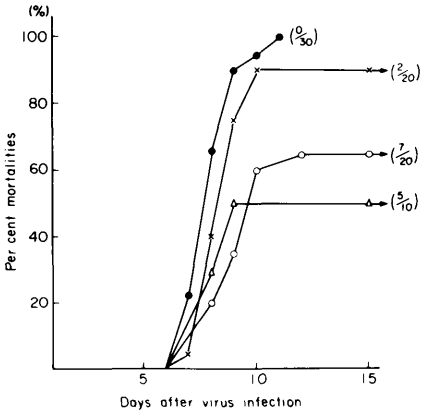

FIG. 1. Effect of a single-administration of dextran phosphate (DP 40) on the survival of mice infected with influenza A_2 virus. Two doses of DP 40, 300 (○——○) and 100 mg/kg (×——×), and saline (●——●) were injected ip 1 hr before infection. As a positive control 30 mg/kg dose of virazole (△——△) was used under the administration schedule described in the text. All of the mice received 10 LD_{50} of influenza A_2 virus intranasally.

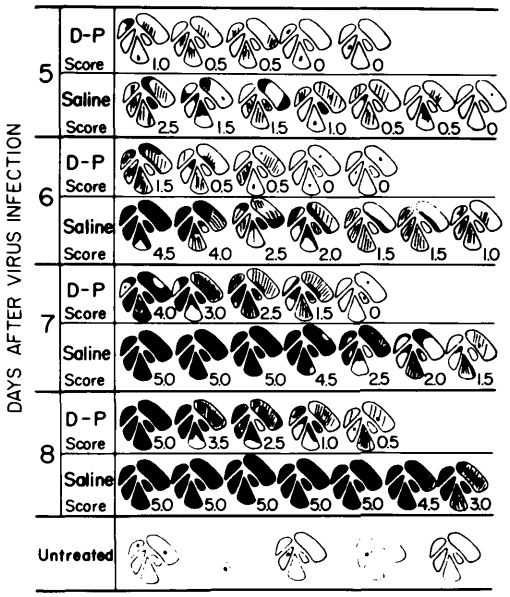

FIG. 2. Effect of DP 40 on the development of lung consolidation in influenza virus infected mice. Mice were infected intranasally with 10 LD_{50} of influenza A_2 virus and received 250 mg/kg of DP 40 twice before and after infection respectively and daily for 4 consecutive days. The grade of virus-induced lung consolidation was determined according to the method of Horsfall with a modification. In the illustration of consolidation, darkened areas indicate the portion of full consolidation and shaded areas indicate, partial consolidation. The numbers under each lung indicate their consolidation score. The calculation of the consolidation score was detailed in Materials and Methods.

tion, 1 and 6 hr after virus infection, and once daily for 4 consecutive days. Five mice of the treated group and seven of the controls were sacrificed at daily intervals from day 5 to day 8 for the calculation of lung consolidation scores. As shown in Fig. 2, although signs of lung lesions were found in all control mice on day 6 (mean consolidation score: 2.5), almost no lesions were found in the treated group (mean score: 0.5). On day 8, lungs of the control group showed almost complete consolidation (mean score: 4.6), whereas the development of consolidation in the treated group (mean score: 2.5) was comparable to that of the control on day 6. The difference between control and treated groups was significant ($P < 0.02$) when the 6- and 8-day results were compared. Thus, about a 2-day delay in consolidation score was found in the treated group.

Virus and antibody titrations in mice treated with DP 40. The effect of DP 40 on the growth of influenza virus in mouse lung and a production of serum neutralizing antibody were examined. Two hundred mice were used, one-half for the titration of virus growth, and the other half, for HAI antibody titration. The administration schedule of dextran phosphate was the same as that in the survival experiment and all of the mice were infected intranasally with 10 LD_{50} of virus.

Ninety mice of the first group were divided into three subgroups, and each received an ip injection of 250 mg/kg of DP 40, the same amount of dextran and 0.5 ml of saline, respectively. Five mice from each subgroup were sacrificed on the day after virus infection (day 1) and at 2-day intervals, from days 2 to 10, for virus titration. The results which are shown in Fig. 3, indicate a delay of the initial growth and an almost tenfold reduction of the maximum titer in the treated subgroup. On day 4, the growth of virus of the two control subgroups receiving dextran and saline, reached a maximum titer of $10^{7.2}$/ml EID_{50}. In con-

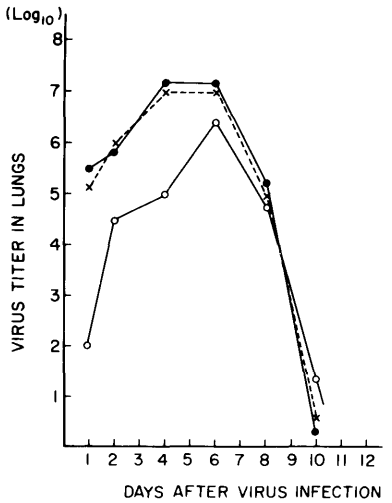

FIG. 3. Effect of DP 40 on the virus growth in influenza virus infected mice. Mice infected with 10 LD_{50} dose of influenza A_2 virus received 250 mg/kg of dextran phosphate (○——○), the same amount of dextran (●——●) and saline (×- - -×) under the multiple administration schedule. The EID_{50} was calculated by the method of Reed and Meunch.

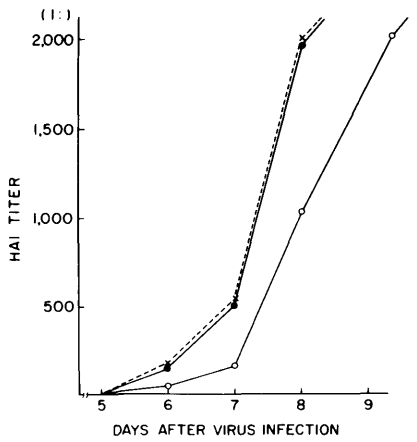

FIG. 4. Effect of DP 40 on the production of HAI antibody in influenza infected mice. ○——○: 250 mg/kg of dextran phosphate; ●——●: dextran (250 mg/kg dose) as a control; ×- - -×: 0.5 ml of saline injected, under the multiple administration schedule.

trast, the maximal virus titer in the DP 40-treated subgroup was $10^{6.0}$ EID_{50}/ml and occurred on day 6.

Ninety-six mice of the second group were also divided into three subgroups and treated with dextran phosphate, dextran, and saline. Eight mice from each group were killed at daily intervals from day 6 to day 9 and HAI antibody titrations were performed on their sera (Fig. 4). Antibody responses of the three subgroups were first detected on day 6. However, the serum antibody titer of dextran phosphate-treated mice was lower than those of the other mice on days 6, 7, and 8 and the HAI production curve of the treated group revealed a 1-day delay when compared with the two controls.

Mechanism of antiviral effect. To test the possibility of direct interaction with the virus particles, 10^6 TCD_{50} of VSV or 10^7 EID_{50} of influenza A2 virus (H_2N_2) were mixed with 1 mg of DP 40/ml in serum-free Eagle's MEM and incubated for 3 hr at 37°. Control virus preparations free from the compound were included. The initial and residual virus contents were determined by titrating the VSV samples in L-1D cultures and the influenza samples in the chorioallantoic cavity of chick embryos. As a result, DP 40 did not reveal any virucidal effect on VSV and influenza virus. In the same culture system, DP 40 did not show any inhibitory action on the growth of VSV and influenza virus, when examined at 1 mg/ml. Therefore, the evidence for interferon production was sought in serum and lung (10% saline extract) when ten mice were given an ip dose of 250 mg/kg DP 40. The data shown in Fig. 5 reveal a relatively low antiviral titer in plasma which was maximum at 13 hr. In contrast, antiviral titer in the lung reached almost 1000 units/ml at 19 hr. The interferon nature of the antiviral activity was documented by the following criteria: (i) the antiviral effect was species specific and failed to protect either rabbit (RK-13) or human (HeLa-S3) cells from VSV challenge; (ii) the activity was left in the supernatant after centrifugation at 100,000*g* for 2 hr; (iii) the activity was relatively stable to prolonged incubation at pH 2.0 at 4°; and (iv) it was readily lost during trypsin treatment.

Discussion. In the various interferon inducers so far reported (3–7), some essential common features have been noticed. One of them is a stable long-chain backbone on which negative charges are placed in a regular and dense sequence. The second important feature of the inducer is high mo-

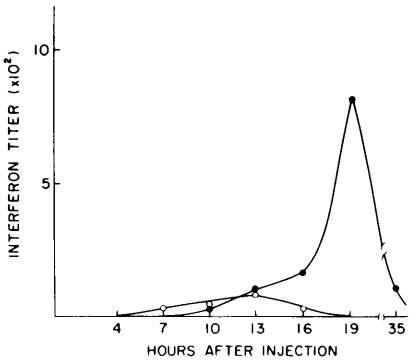

FIG. 5. Interferon inducing activity of DP 40 in mice. From mice receiving a 250 mg/kg ip dose of DP 40, serum and lung specimens were obtained on indicated days. Serial twofold dilutions of serum and 10% lung suspension were made with maintenance medium (Eagle's MEM containing 5% calf serum) and interferon activity was assayed in L-1D cell-VSV system. The interferon titer was expressed by the reciprocal of the maximum dilution capable of preventing the viral CPE. ○——○: serum interferon; ●——●: lung interferon.

lecular weight as has been established with poly I·poly C (14), pyran copolymer (7), and polyacrylic acid (15). With these considerations in mind, DP 40 (MW 40,000) was synthesized (1, 2) and has been shown to induce interferon in rabbits (2) and mice.

In the initial stage of this study, because of the low stability of the lyophilized preparation of DP 40, further antiviral evaluation was abandoned. However, recent improvements in preparing the compound by repeated dialysis followed by thorough lyophilization and storage at refrigerator temperature, allowed us to initiate antiviral experiments with a preparation of constant inducing activity.

Studies of the prophylactic effect of ip administered interferon inducers on influenza infection in mice generally have failed to be protective. These inducers include a double-stranded RNA (poly I·poly C) (16) and a low molecular substance (cp-20961) (17). A small but significant effect was found with chloriteoxidized oxyamylose (COAM) (18) given ip against influenza PR 8 (H_0N_1) infection, with tilorone (19) given orally against influenza A/Eq and B/Mass, and with a double-stranded RNA originating from phage MU 9 (20) against Sendai virus infection in mice. In our laboratory, a double-stranded RNA extracted from *Lentinus edotes* was also found to be effective against A/SW 15 and A2/Kumamoto virus infection in mice (3, 21) even when given ip, but not against PR 8 infection in the same mice. Based upon such experience, several inducers in our own hands have been screened for their antiviral effect in mice infected with the more susceptible A2/Kumamoto strain.

As a result, a series of phosphorylated compounds were found to be effective in prolonging the life span of infected mice, and a dextran phosphate with 40,000 MW (DP 40) was chosen for further studies because of its extremely low toxicity (22). Acute LD_{50} of DP 40 in mice was calculated to be 4.5 g/kg after ip injection, more than 4 g/kg after intramuscular and subcutaneous injections. After iv injection, however, the LD_{50} was 150 mg/kg probably because of its high viscosity.

When DP 40 was given in repeated manner, the survival effect of the compound was not only reproducible, but the analyses of virus titer, antibody response, and interferon response in treated mouse clearly indicated a 1-day delay of virus effects in comparison with untreated controls. Moreover, a substantial difference between the treated and the untreated mice was found in the development of lung consolidation. Furthermore, rales of treated mice did not increase from day 8, suggesting earlier termination of influenza pneumonitis in these mice.

The present study indicates but does not finally establish that protection against influenza was mediated by the interferon induced by DP 40. Additional suggestive evidences to support such a causal correlation would be: (a) protection by DP 40 but not by dextran against a broad range of virus infections of mice; and (b) greatest protection against interferon sensitive viruses.

Summary. Intraperitoneal administration of dextran phosphate (MW 40,000) or DP 40, an interferon inducer, was shown to increase the survival rate of mice infected with 10 LD_{50} dose of influenza A_2 virus

(H_2N_2). In the treated mice, a 1-day delay was evident in the virus growth in lung, and production of HAI antibody, when compared to the nontreated or dextran-treated controls. More significant was the 2-day delay in the development of lung consolidation, which leads to 40% survival of the treated mice. Mediation of the protection by the interferon induced by DP 40 was indicated.

1. Suzuki, S., Suzuki, M., and Imaya, M., *Japan. J. Microbiol.* **15,** 485 (1971).
2. Suzuki, S., Suzuki, M., Imaya, M., and Chaki, F., *Japan. J. Microbiol.* **16,** 1 (1972).
3. Tsunoda, A., Suzuki, F., Sato, N., Miyazaki, K., and Ishida, N., *Prog. Antimicrob. Anticancer Chemother.* **II,** 832 (1970).
4. Lampson, G. P., Tytell, A. A., Field, A. K., Nemes, M. M., and Hilleman, M. R., *Proc. Nat. Acad. Sci.* **58,** 782 (1967).
5. Stinebring, W., and Youngner, J. S., *Nature (London)* **204,** 712 (1964).
6. Toy, S. T., and Gifford, G. E., *Proc. Soc. Exp. Biol. Med.* **126,** 787 (1967).
7. Merigan, T. C., *Nature (London)* **214,** 416 (1967).
8. Ishida, N., Kosaka, Y., and Sasaki, H., *Tohoku J. Exp. Med.* **71,** 151 (1959).
9. Whistler, R. L., and Towle, G. A., *Arch. Biochem. Biophys.* **135,** 396 (1969).
10. Sidwell, R. W., Huffman, J. H., Khare, G. P., Witkowski, J. T., and Robins, R. K., *Science* **177,** 705 (1972).
11. Westphal, O., and Luderitz, O., *Angew. Chem.* **66,** 407, (1954).
12. Horsfall, F. L., *J. Exp. Med.* **70,** 209 (1939).
13. Lampson, G. P., Tytell, A. A., Nemes, M. M., and Hilleman, M. R., *Proc. Soc. Exp. Biol. Med.* **121,** 377 (1966).
14. Tytell, A. A., Lampson, G. P., Field, A. K., Nemes, M. M., and Hilleman, M. R., *Proc. Soc. Exp. Biol. Med.* **135,** 917 (1970).
15. De Somer, P., De Clercq, D., Billiau, A., Schonne, E., and Claesen, M., *J. Virol.* **2,** 886 (1968).
16. Nemes, M. M., Tytell, A. A., Lampson, G. P., Field, A. K., and Hilleman, M. R., *Proc. Soc. Exp. Biol. Med.* **132,** 776 (1969).
17. Hoffman, W. W., Korst, J. J., Niblack, J. F., and Cronin, T. H., *Antimicrob. Ag. Chemother.* **3,** 498 (1973).
18. Billiau, A., Desmyter, J., and De Somer, P., *J. Virol.* **5,** 321 (1970).
19. Krueger, R. F., and Mayer, G. D., *Science* **169,** 1213 (1970).
20. Nemes, M. M., Tytell, A. A., Lampson, G. P., Field, A. K., and Hilleman, M. R., *Proc. Soc. Exp. Biol. Med.* **132,** 784 (1969).
21. Tsunoda, A., and Ishida, N., *Ann. N.Y. Acad. Sci.* **173,** 719 (1970).
22. Suzuki, M., in preparation.

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