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Effect of Puromycin on Virus and Endotoxin-Induced Interferonlike Inhibitors in Rabbits.* (30731)

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Since the discovery that bacterial endotoxins as well as viruses may induce interferonlike viral inhibitors in experimental animals(1,2), there have been indications that the endotoxin-induced inhibitor (EII) and virus-induced interferon (VII) are not identical in physical properties and that the processes of their intracellular formation are different. Thus crude EII has been shown to be different from VII in being more labile to heat and acid(2), and the molecular weight of EII has been estimated to be considerably greater than that of VII(3).

In addition, it has been reported that the induction of interferon by viruses in cell cultures(4,5) and in rabbits(6) was found to be inhibited by actinomycin D and hence at least partially dependent on the transcriptive function of cell DNA. On the other hand, the induction of EII in rabbits was not found to be affected by actinomycin D (6). These results were interpreted to mean that endotoxin and virus induced the production of interferons in a different manner and that perhaps EII was largely preformed.

This paper reports further efforts to test this hypothesis. The production of EII and VII following inoculation of endotoxin and virus was measured in rabbits in which protein synthesis had been largely blocked by puromycin(7). It was thought that if the production of VII was inhibited by puromycin, as has been shown in cell cultures(8), whereas the production of EII was not inhibited, further evidence would be provided that EII is largely preformed, in contrast to VII.

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Materials and methods. Details on materials and methods used, unless hereafter specified, were as described elsewhere(6). Small albino rabbits weighing 320-470 g were used because of the cost of puromycin.† These were fasted for 24 hours prior to experimentation. Puromycin was dissolved in phosphate-buffered saline (PBS, pH = 7.2) and administered intraperitoneally (i.p.). Preliminary experiments showed that 13.3 mg of the drug per 100 g weight of the animal was the minimum dose necessary to maintain more than 95% inhibition of protein synthesis for at least 2 hours. Therefore, a total dose of 40 mg per 100 g of body weight divided into bihourly subdoses was used in experiments lasting 2.5 and 4.5 hours (*i.e.*, 2 injections of 20 mg per 100 g of body weight and 3 injections of 13.3 mg per 100 g of body weight, respectively). In the case of experiments lasting 7.5 hours, 13.3 mg per 100 g of rabbit was administered bihourly for as long as required (*i.e.*, 4 injections). To induce EII and VII, *E. coli* (0113) endotoxin‡ (0.5 μg in PBS) or Sindbis virus pellet suspended in PBS (about 10^9 p.f.u.) was injected intravenously (i.v.) 0.5 hour after the first injection of puromycin(6). To monitor protein synthesis, C^{14}-leucine,§ in dosages of 10 μc/100 g rabbit weight was injected i.v. 0.5 hour before sacrifice. Control animals received PBS rather than puromycin.

To measure EII or VII, serum samples were obtained by cardiac puncture. Serial 2-fold dilutions were made and 0.3 ml was

† Nutritional Biochemicals Corp.

‡ Provided through courtesy of Dr. A. I. Braude.

§ New England Nuclear Corp., uniformly labeled, specific activity > 200 mc/mM.

TABLE I. Effect of Puromycin on Induction of Interferon by Sindbis Virus.

Hr after virus inoculation	Inhibitory titers*	Mean	α†	% inhibition of protein synthesis‡
	Control (no puromycin)			
4	16, 64, 512, 1024	404		
7	128, 1024, 2048, 2048	1312		
	Puromycin-treated			
4	2, 2, 2, 8	3.5	.014	99.4
7	0, 2, 2, 64	17	.014	98.7

* Expressed in the reciprocals of the highest dilutions. Each figure represents a sample from a different animal.

† Probability that the observations of the control and treated groups are chosen at random from the same population as defined by the Wilcoxon rank-sum test (Table A-20, Dixon, W. J. & Massey, F. J., Introduction to Statistical Analysis, 1957).

‡ Calculated on the basis of C^{14}-leucine incorporation in livers of 2 control and 2 puromycin-treated animals as described in *Materials and methods.*

incubated with duplicate rabbit embryo cell cultures for 18 hours, after which they were challenged with vesicular stomatitis virus (VSV). Inhibitory titers were expressed as the highest dilutions calculated to inhibit the cytopathic effect of VSV in 50% of the cultures as calculated by the Reed-Muench method(2).

To measure protein synthesis, the incorporation of C^{14}-leucine into liver protein was assayed. At the indicated time interval, the liver was obtained from a sacrificed animal and a homogenate was made. To 0.2 ml of the homogenate, 2 ml of 10% trichloroacetic acid (TCA) were added. After centrifugation, the supernate and the precipitate were counted separately by liquid scintillation method, and the ratio of the C^{14} radioactivity in the 10% TCA precipitate and the supernate was taken as the index of protein synthesis of the sample(9). The per cent inhibition of protein synthesis was estimated according to the following formula: % inhibition of protein synthesis $= (1 - [I_t/I_c]) \times 100\%$ where I_t is average index of protein synthesis of the puromycin-treated group; I_c is average index of protein synthesis of the control group.

Results and discussion. 1. Effect of puromycin on the virus induction of interferon (VII) (Table I): Two groups of 4 animals were pretreated with puromycin while 2 similar groups served as controls. Each animal then received Sindbis virus intravenously. Four and 7 hours thereafter, one treated and one control group were bled and sacrificed for determination of serum inhibitory titers after inactivation of residual Sindbis virus(6) and for estimation of inhibition of protein synthesis.

As seen from the Table, high titers of interferon were evident in control sera at 4 hours and these were even higher at 7 hours, in agreement with previous experience (6). In contrast, the sera from puromycin-treated animals showed greatly reduced inhibitory titers. Whatever residual interferon activity was evident may be attributed to residual protein synthesis, although the possibility that virus induces some preformed interferon which does not require protein synthesis cannot be entirely excluded.

2. Effect of puromycin on endotoxin induction of interferonlike inhibitor (EII) (Table II): The design of the experiment was similar to the above described. Two groups of 4 animals each were treated with puromycin while 2 served as controls. Each animal then received 0.5 μg endotoxin i.v. Two and 4 hours thereafter, one treated and one control group were bled and sacrificed for titration of serum inhibitory activity and measurement of puromycin effect on protein synthesis.

It is evident from the Table that in contrast to the induction of VII, these rabbits were able to form EII which titered in the same range and appeared at the same time as in the control animals irrespective of puromycin treatment. It is evident that although inhibitory activity was evident in the 2- and 4-hour serums, it was not as pronounced as that of VII. These titers are comparable to

TABLE II. Effect of Puromycin on Induction of Interferonlike Inhibitor (EII) by Endotoxin.

Hr after endotoxin	Inhibitory titers*	Mean	a†	% inhibition of protein synthesis‡
	Control (no puromycin)			
2	4, 4, 32, 32	19		
4	<2, 16, 64, 128	52		
	Puromycin-treated			
2	4, 8, 16, 16	11	.44	99.6
4	8, 32, 64, 128	58	.44	96.3

* Expressed in the reciprocals of dilution. Each figure represents a sample from a different animal.

† Probability that the observations of the control and treated groups are chosen at random from the same population as defined by the Wilcoxon rank-sum test (Table A-20, Dixon, W. J. & Massey, F. J., Introduction to Statistical Analysis, 1957).

‡ Calculated on the basis of C^{14}-leucine incorporation in livers of 2 control and 2 puromycin-treated animals as described in *Materials and methods*.

those obtained in 1 kg rabbits(2), but the peak appears to be at 4 rather than at 2 hours.

These experiments, taken together with previous reports(6), suggest strongly that the induction of interferon by viruses in animals, just as in cell cultures, requires new protein synthesis; while endotoxin releases an interferonlike inhibitor without requiring the transcriptive function of cell DNA or new protein synthesis. The relationship of these two inhibitors to one another both chemically and physiologically remains to be elucidated.

Summary. Puromycin was found to inhibit interferon formation in rabbits induced by Sindbis virus. It did not inhibit the induction of an interferonlike inhibitor by bacterial endotoxin. It is suggested that the latter inhibitor is present in rabbits mainly in a preformed state and its release apparently does not require new protein synthesis. On the other hand, the production of the virus-induced interferon in rabbits requires such metabolic function.

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