Inhibition of Biologic Activity of Poly I:Poly C by Human Plasma (34492)

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Polyinosinic-polycytidylic acid, a synthetic double-stranded RNA, is known to be a potent stimulus to interferon production (1–3) and inhibitory to certain animal tumors (4). In view of these facts, it has been widely suggested that poly I:poly C might be useful in the treatment of human viral diseases or tumors. Studies in our laboratories, to be published elsewhere, have demonstrated poly I:poly C to be a potent pyrogen in rabbits (5). Therefore, we attempted to induce human and rabbit leukocytic pyrogan release *in vitro*, but noted that human plasma inhibited the pyrogenicity of poly I:poly C. This report deals with experiments designed to examine this phenomenon.

Materials and Methods. Poly I:poly C was prepared under sterile conditions in pyrogen-free physiological saline at a concentration of 1 mg per ml (4) and diluted to 0.2 mg/ml in modified Hanks' solution[2] (MHS) for subsequent use. Sterility was verified by culturing for 72 hr in thioglycolate broth.

Human plasma was obtained fresh daily and anticoagulated with either ACD (14%) or heparin (10 units/ml). Fresh rabbit plasma was obtained for each experiment by intracardiac puncture and anticoagulated with 20 units/ml of heparin or 14% ACD. The larger quantity of heparin (20 units/ml) was needed to prevent clotting in rabbit plasma. Horse, chicken, and fetal calf serums were obtained from commercial sources in the frozen state. Guinea pig and dog serums had been frozen for 1–2 months prior to use, but mouse and monkey serums were obtained fresh.

Rabbit white blood cells were obtained from anticoagulated whole blood by centrifugation at 900 rpm for 40 min at 4° and aspiration of the buffy coat at the plasma–red cell interface. White blood cell counts were performed with an electronic particle counter[3] and differential counts were done on 200 Wright-stained cells.

To stimulate leukocyte pyrogen release *in vitro*, 100×10^6 neutrophils and monocytes (PWBC) were suspended in 10 ml of solution containing 15–30% homologous plasma, 0.03 mg/ml poly I:poly C, and MHS. After 16 hr of incubation at 37° in a shaking incubator, the suspension was centrifuged at 3500 rpm for 30–60 min at 4°. The supernatant fluid was decanted, cultured in thioglycolate broth, and, if sterile, stored at 4° until used.

Occasionally, it was desirable to inactivate differentially poly I:poly C or the leukocytic pyrogen. Pancreatic-RNAse and T_1-RNAase[4] were added in a final concentration of 0.02 mg/ml and 30 units/ml, respectively, and incubated for 1 hr at 37° to destroy poly I:poly C. Leukocytic pyrogen was destroyed by incubation with RNAase-free trypsin (1) for 4 hr at 37° (6). A standard rabbit fever assay was used to detect pyrogen (7), and all solutions to be tested were usually injected into two or more rabbits. Details of care of rabbits and recording of temperatures have been previously reported (8).

Results. The supernatant fluid from a mixture of rabbit cells, rabbit plasma, and

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[2] Sodium 139.5 meq/liter, potassium 17 meq/liter, chloride 139 meq/liter, phosphate 9 meq/liter, pH 7.5, and glucose 200 mg/100 ml.

[3] Coulter Electronics, Hialeah, Florida.

[4] Worthington Biochemical Co., Freehold, New Jersey.

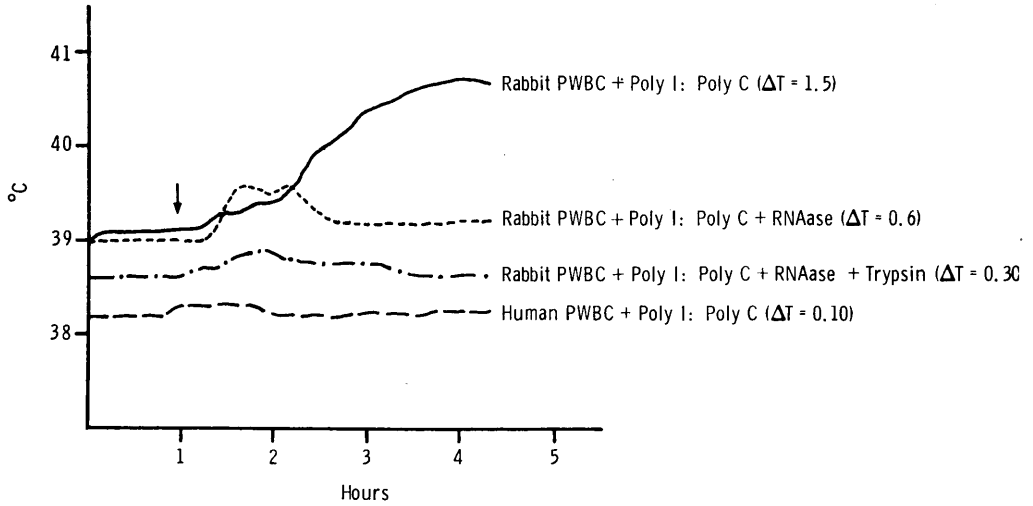

FIG. 1. Supernatant fluid from rabbit PWBC incubated in rabbit serum with poly I:poly C for 16 hr produced a marked febrile response (—) when injected into rabbits (ΔT 1.5°). This same supernatant fluid, if treated with pancreatic and T_1-RNAase, produced a residual but markedly decreased fever (ΔT = 0.6°) typical of fever responses by leukocytic pyrogen (- - - -). Further treatment with trypsin abolishes completely the pyrogenicity of this supernatant fluid (ΔT = 0.3° (—•—). Human PWBC incubated with human plasma and poly I:poly C induces no fever without prior treatment with RNAase and trypsin (ΔT 0.1°) (- -). Arrow indicates the time of injection of each solution.

poly I:poly C after 16 hr of incubation at 37° induced a marked febrile response which began within ½ hr after injection (Fig. 1). Rabbit cells or plasma alone did not induce fever. Enzymatic hydrolysis of the poly I:poly C destroyed most of the pyrogenicity of the suspension, leaving a curve typical for endogenous pyrogen; *i.e.*, abrupt onset, peaking with in 1 hr, and rapid return to baseline (9). Subsequent treatment with typsin further reduced the pyrogenicity indicating the presence of a pyrogenic protein, presumably leukocyte pyrogen (6). In striking contrast, human PWBC and plasma incubated with polyI: poly C were nonpyrogenic without prior RNAase or trypsin treatment (Fig. 1). Despite many attempts under varying conditions, we have been unsuccessful in inducing the formation of leukocytic pyrogen (LP) from human PWBC by poly I: poly C activation.

Preliminary experiments indicated that human plasma and serum, rather than the PWBC, were responsible for the decreased febrile response. Therefore, experiments were designed to investigate this property of human plasma. Either 15% (diluted in MHS) or 100% plasma (or serum) without PWBC was used in all subsequent experiments. Poly I:poly C was added to the mixtures at a final concentration of 0.03 mg/ml. The mixture was incubated for variable periods of time at 37°. One and one-half milliliters of the dilute plasma (serum) or 1.0 ml of the whole plasma (serum) solutions were injected intravenously into the ear vein of rabbits, and temperatures were recorded. The maximum temperature rise (ΔT) during the 3 hr after injection was used as a measure of the pyrogenicity of each injected solution. A ΔT of less than 0.3° is not considered significant.

The results of several experiments comparing human and rabbit serums and plasma anticoagulated with ACD or heparin are shown in Table I. All preparations were incubated for 16 hr. The data show that human plasma or serum has the capacity to destroy the pyrogenicity of poly I:poly C in contrast to rabbit serum or plasma which does not have this capacity.

To test whether this was a unique property of human blood, serums from eight different species were incubated in dilute concentra-

TABLE I. The Effect of Human and Rabbit Plasma and Serum on Pyrogenicity of Poly I:Poly C after 16 hr of Incubation at 37°.

Test solution	No. of rabbits	Dose poly I:poly C injected per rabbit (mg)	ΔT[a] (°)
Poly I:poly C in MHS[b]	9	0.045	1.58
Dilute[c] rabbit plasma (ACD)	6	0.045	1.42
Dilute[c] rabbit plasma (heparin)	3	0.045	1.40
Dilute[c] rabbit serum	5	0.045	1.74
Undiluted rabbit plasma (ACD)	3	0.030	1.62
Undiluted rabbit plasma (heparin)	3	0.030	1.40
Undiluted rabbit serum	2	0.030	1.90
Dilute[c] human plasma (ACD)	8	0.045	0.19[d]
Dilute[c] human plasma (heparin)	6	0.045	0.15[d]
Dilute[c] human serum	5	0.045	0.13[d]
Undiluted human plasma (heparin)	3	0.030	0.28[d]

[a] ΔT = mean maximum temperature rise (°) over baseline during a 3-hr period.
[b] MHS = modified Hanks' solution.
[c] Plasma or serum diluted to 15% in MHS.
[d] ΔT of less than 0.3° are not meaningful.

tion (15%) with poly I:poly C (0.03 mg/ml) for 16 hr and injected into rabbits. Control rabbits received injections of plasma containing no poly I:poly C. It is of interest that chicken and, to some extent, fetal calf serums also inactivate poly I:poly C pyrogenicity in rabbits (Table II).

In another series of experiments, undiluted plasma and whole blood were anticoagulated with ACD or heparin and incubated for short periods of time with poly I:poly C (0.03 mg/ml) and injected into rabbits (Table III). Before injection, whole blood suspensions were centrifuged at 2500 rpm at 4° for 15 min to remove red blood cells, and aliquots of supernatant plasma were injected. Whole blood and plasma anticoagulated with ACD destroyed the pyrogenicity of poly I:poly C within 2 hr, whereas heparinized human blood was considerably less effective after 4 hr (Table III). Since heparinized and ACD plasma destroyed all poly I:poly C pyrogenicity by 16 hr (Table I), the heparin inhibition is obviously time-dependent.

TABLE II. The Effect of Sera from Various Species on Poly I:Poly C Pyrogenicity.[a]

Species	No. of rabbits	ΔT[b] (°)
Rabbit	5	1.74
Human	5	0.13[c]
Mouse	2	1.55
Dog	4	1.46
Guinea pig	2	1.87
Horse	2	2.05
Monkey	2	1.65
Chicken	2	0.25[c]
Fetal calf	7	0.95
Control[d]	4	0.10

[a] Poly I:poly C (0.03 mg/ml) incubated in 15% sera and modified Hanks' solution for 16 hr at 37°.
[b] Mean maximum temperature rise (°) over baseline during a 3-hr period.
[c] ΔT of less than 0.3° are not meaningful.
[d] Serums from various species containing no poly I:poly C were injected into rabbits.

Neomycin is frequently added to tissue cultures stimulated with poly I:poly C (10) to produce interferon. To test whether neomycin, like heparin, might inhibit the action of human plasma on the pyrogenicity of poly I:poly C, mixtures of human plasma (ACD), neomycin (300 μg/ml) and poly I:poly C (0.03 μg/ml) were prepared and incubated for 16 hr at 37°. Neomycin completely eliminated the capacity of dilute (15%) human plasma to inactivate poly I:poly C pyrogenicity and depressed the effects of whole human plasma (Table III). It is of interest that neomycin is a polybasic com-

TABLE III. The Effect of Anticoagulant and Duration of Incubation (37°) on the Pyrogenicity of Poly I:Poly C in Human Plasma.

	Duration of incubation (hr)	No. of rabbits	Dose of poly I:poly C per rabbit (mg)	ΔT[a] (°)
ACD				
Undiluted plasma + poly I:poly C	1	3	0.030	1.50
Undiluted plasma + poly I:poly C	2	3	0.030	0.18[b]
Undiluted blood + poly I:poly C[c]	1	2	0.030	1.17
Undiluted blood + poly I:poly C[c]	2	3	0.030	0.22[b]
Dilute plasma + poly I:poly C	16	8	0.045	0.19[b]
Dilute plasma + poly I:poly C + neomycin[d]	16	6	0.045	1.99
Undiluted plasma + poly I:poly C + neomycin[d]	16	3	0.030	0.72
Heparin				
Undiluted plasma + poly I:poly C	4	2	0.030	1.27
Undiluted blood + poly I:poly C[c]	2	2	0.030	0.90
Undiluted blood + poly I:poly C[c]	4	4	0.030	1.58

[a] Mean maximum temperature rise (°) over baseline during 3-hr period after injection.

[b] ΔT of less than 0.3° is not meaningful.

[c] All red blood cells were removed by centrifugation before injection of 1 cc of plasma into each rabbit.

[d] Neomycin 300 μg/ml.

pound and may act by stabilization of the RNA molecule (11).

Using dilute (15%) ACD plasma, other preparations containing poly I:poly C (0.03 mg/ml) were incubated at 4, 27, and 37° for 16 hr and injected into rabbits. The data show that the depression of pyrogenicity occurs only at 37° (Table IV).

The effects of time and temperature on this inhibition suggest an enzymatic reaction. To test this hypothesis, poly I:poly C containing ^{14}C-labeled inosinic acid and tritium-labeled cytidylic acid were incubated in whole and diluted human serum at 37°. At zero, 1, 2, 4, 6, and 16 hr after initiation of incubation, aliquots were removed, and trichloroacetic acid was added (final concentration 10%). The precipitate was removed by centrifugation, and the radioactivity of both the precipitate and supernatant fraction was counted in a scintillation counter. The percentage of acid-soluble ^{14}C-labeled inosinic acid soluble in TCA and ^{3}H-labeled cytidylic acid was calculated. Single nucleotides and short oligonucleotides will remain in solution, but long-chain polynucleotides (greater than 10–12 nucleotides) will precipitate with TCA. Tritium was rendered acid-soluble after treatment with human serum, indicating destruction of the poly C molecule (Table V). The poly I was much less affected by human serum, since, even after 16 hr of incubation, most of the labeled carbon remained in the precipitate. Rabbit serum, in contrast, had minimal effect on either of the two RNA strands. Comparable experiments with serums from several other animal species, using

TABLE IV. The Effect of Temperature of Incubation upon the Pyrogenicity of Poly I:Poly C[a] in in Human Plasma.

Temperature of incubation (°)	No. of rabbits	ΔT[b] (°)
4	3	1.41
27	3	1.71
37	8	0.19[c]

[a] Poly I:poly C (0.03 mg/ml) was incubated in 15% plasma (ACD) and MHS for 16 hr.

[b] Mean maximum temperature rise (°) during a 3-hr period after injection.

[c] Temperature rise of less than 0.3° is not meaningful.

TABLE V. Percentage of Acid-Soluble Labeled Poly I:Poly C after Incubation with Human and Rabbit Sera for Variable Periods at 37°.

	Incubation (hr)					
	0	1	2	4	6	16
No.[a]						
^{3}H	4.5	48	68	85	88	91
^{14}C	2.4	2.8	2.5	4.2	4.5	7.9
Bal.[a]						
^{3}H	4.0	55	50	67	78	92
^{14}C	2.3	2.4	2.9	3.7	4.4	7.1
GF[b]						
^{3}H	8.0	72	73	73	72	
^{14}C	2.1	1.5	2.3	2.2	3.2	
JN[b]						
^{3}H	8.9	74	69	72	72	
^{14}C	0.8	1.9	1.5	2.2	3.1	
Rab.[c]						
^{3}H	0.4	0.5	0.9	1.5	1.9	4.5
^{14}C	2.1	2.3	2.7	2.8	3.4	3.9

[a] Fifteen percent human serum containing poly I:poly C at 0.03 mg/ml. Initials denote different normal donors.

[b] Whole human plasma containing poly I:poly C at 0.03 mg/ml.

[c] Fifteen percent rabbit serum containing poly I:poly C at 0.03 mg/ml.

nonlabeled poly I:poly C and measuring optical density of the acid-soluble material, confirmed the data of Table II.

Discussion. The data presented in Table V strongly suggest that human serum or plasma is capable of rapid enzymatic degradation of poly I:poly C, although we have not attempted to isolate an enzyme as such. The presence of an RNAase in human plasma and sera is well established (12, 13). Bovine pancreatic RNAase, which is generally used as a standard reference for human blood RNAase, specifically attacks single-stranded polycytidylic acid rapidly, but acts slowly on the poly C portion of the poly I:poly C complex. This enzyme is also known to be inhibited by heparin (14–17). These similarities between pancreatic RNAase and the human plasma poly I:poly C inhibitors make it even more likely that a serum RNAase is responsible for the destruction of poly I:poly C. However, it has been reported that comparable amounts of pancreatic-type RNAase are present in the serums of the other species, such as rabbit and monkey, that do not inactivate. It must be considered that the nature of the enzymatic action responsible for the effects reported here is not clear. The inhibition of pyrogenic activity of poly I:poly C by human serum is associated with an abolition of the capacity of this agent to induce the formation of interferon in both tissue culture and mice (18).

For maximum interferon production, the complex poly I:poly C molecule is required (1). If the destruction of poly I:poly C in human beings is very rapid, then higher doses might be needed to be therapeutically effective. On the other hand, such doses may be prohibitively toxic. Poly I:poly C is quickly cleared from the bloodstream of animal species whose serums do not rapidly hydrolize this agent. The critical question in therapy is whether the compound can reach the effective sites in sufficient concentration before it is hydrolized.

The potential application of poly I:poly C in the treatment of human disease should take into account the data presented in this paper. The exciting therapeutic possibilities raised by these compounds should spur the search for other agents which will possess the interferon-inducing capacities but be resistant to the inhibitory effects of human sera.

Summary. Human serum and plasma have been shown to abolish the pyrogenicity of poly I:poly C. Chicken serum and, to a lesser extent, fetal calf serum also possess this inhibitory effect, while serums of six other animal species do not alter the pyrogenic properties of this compound. The inhibitory effects are time- and temperature-dependent and suggest that they are enzymatic in origin.

1. Field, A. K., Tytell, A. A., Lampson, G. P., and Hilleman, M. R., Proc. Nat. Acad. Sci. U. S. **58**, 1004 (1967).

2. Vilcek, J., Ng, Mun H., Friedman-Kien, A. E., and Krawciw, T., J. Virol. **2**, 648 (1968).

3. Youngner, J. S. and Hallum, J. V., Virology **35** 177 (1968).

4. Levy, H. B., Law, L. W., and Rabson, A. S., Proc. Nat. Acad. Sci. U. S. **62**, 357 (1969).

5. Wolff, S. M., Sheagren, J. N., and Levy, H. B., in preparation.

6. Rafter, G. W., Collins, R. D., and Wood, W. B., J. Exp. Med. **111**, 831 (1960).

7. Root, R. K., Nordlund, J. J., and Wolff, S. M., submitted for publication.

8. Wolff, S. M., Mulholland, J. H., and Ward, S. B., J. Lab. Clin. Med. **65**, 268 (1965).

9. Atkins, E. and Huang, W. C., J. Exp. Med. **107**, 383 (1958).

10. Billiau, A., Buckler, C., Levy, H. B., Dianzani, F., Uhlendorf, C., and Baron, S., "Proc. Third Int. Conf. Interferon," Lyon, France (1969), Academic Press, New York.

11. Dianzani, F., *in* "The Second Conference on Anti-Viral Substances," New York Acad. Sci., in press.

12. Houck, J. C. and Berman, L. B., J. Appl. Physiol. **12**, 473 (1958).

13. Gupta, S. and Herriott, R. M., Arch. Biochem. Biophys. **101**, 88 (1963).

14. Connolly, J. H., Herriott, R. M., and Gupta, S., Brit. J. Exp. Pathol. **43**, 402 (1962).

15. Zollner, N. and Fellig, J., Am. J. Physiol. **173**, 224 (1953).

16. Houck, J. C., Biochim. Biophys. Acta **26**, 649 (1957).

17. Roth, J. S., Arch. Biochem. **44**, 265 (1953).

18. Baron, S. and Levy, H. B., unpublished observations.

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