Effect of Polyribonucleotides on In Vitro Platelet Aggregation Induced by Acid-soluble Collagen and Other Aggregating Agents

By Robert Rosenstein, Leo R. Zacharski, and Kendall A. Smith

This investigation was conducted in an attempt to determine if a reported case of disseminated intravascular coagulation (DIC) in a patient receiving polyribonucleotides could be related to an effect of polyribonucleotides on platelet aggregation. It was determined that polyribonucleotides produced a decrease in latency and an increase in the rate of platelet aggregation induced by acid-soluble collagen (ASC) at 37°C and 24°C. All activity resided in the poly(I) strand, which produced a significant effect in a concentration as low as 0.1 µg/ml of plasma (0.02 µmole phosphorus/liter). At concentrations up to 0.25 mg/ml no activity was observed with polyribadenyl acid-polyribouridylic acid, while polyriboguanylic acid had an inhibitory effect in concentrations down to 2.5 µg/ml (0.05 µmole P/liter). Similarly, moieties of poly(I), inosine, and inosine 3'- and 5'-monophosphoric acid lacked activity. Inhibition of ASC polymerization by ascorbic acid diminished the effect of poly(I) on platelet aggregation, while poly(I) alone enhanced ASC polymerization. Since poly(I) was found not to affect platelet aggregation induced by particulate collagen, it was concluded that it enhanced ASC-induced platelet aggregation by promoting collagen polymerization. Poly(I) in high concentrations (about 0.05 mg/ml) was found to inhibit platelet aggregation induced by adenosine diphosphate, epinephrine, and ristocetin. The inhibitory effect, however, did not appear incompatible with the appearance of a thrombotic disorder since plasma concentrations in vivo probably fall rapidly below the effective inhibitory concentration. The reported presence of a soluble form of collagen (procollagen) in human serum and the identification in arterial thrombi of collagen that may be derived from this source provide a possible link between poly(I) and DIC.

The synthetic double-stranded polyribonucleotide, polyribonucleotides-polyribocytidylic acid [poly(I)·poly(C)] is a potent inducer of interferon production both in vivo and in vitro. Interest has arisen in administering poly(I)·poly(C) to humans since interferon, and perhaps poly(I)·poly(C) itself, possesses antitumor activity in animal tumor systems and possibly in some human cancers. Administration of poly(I)·poly(C) to humans is associated with several effects in addition to interferon production, including induction of transient fever, lymphopenia, and neutrophilia, and changes in blood coagulability.
A hypercoagulable state was observed by Cornell et al. following poly (I)·poly (C) injection in a single patient who had acute lymphoblastic leukemia in relapse. The individual received the highest dose of poly (I)·poly (C) (10 mg/kg) administered to patients reported in their series. Manifestations of the coagulation defect included a prolongation of the prothrombin and thrombin times, reduction in platelet count, and elevation of the level of fibrin split products. These changes were interpreted as indicative of disseminated intravascular coagulation.

The finding in this patient led to a systematic evaluation of the effects of poly (I)·poly (C) on several clotting parameters in vitro. We report the effects of this substance on platelet aggregation.

**MATERIALS AND METHODS**

*Preparation of Platelet-rich Plasma*

Blood was collected from fasting healthy human volunteers by the two-syringe technique through siliconized needles, and it was anticoagulated with 0.1 volume of 3.5% sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 170 g for 5 min and platelet-poor plasma (PPP) by centrifugation at 3200 g for 15 min at 4°C. Whole blood and plasma were handled entirely in plastic syringes, tubes, and pipettes, except that PRP was stored at 4°C in 0.5-mI aliquots in siliconized glass cuvettes. Platelet counts on the PRP were between 200,000 and 300,000.

*Materials*

Particulate collagen was prepared from fresh, cleaned human Achilles tendon obtained at autopsy by a modification of the method of Neuman. Tendon, in a ratio of 1 g to 30 ml of normal saline, was homogenized at 4°C in a refrigerated blender. Large particles were removed by centrifugation at 4 g for 10 min. The supernatant was filtered three times through three layers of gauze. This filtrate was used in the experiments. Then 4-ml samples of the collagen preparation were dialyzed against 2-liter volumes of distilled water with four changes over a 24-hr period. The samples were then desiccated and the dry weights determined.

Acid-soluble bovine skin collagen (ASC) in buffered citrate solution was obtained from Worthington Biochemicals, Freehold, N.J. For polymerization studies, the ASC was dialyzed against 0.05 M acetic acid at room temperature and centrifuged at 4°C at 100,000 g for 60 min. Poly (I)·poly (C), poly (I), poly (C), polyriboadenylic acid-polyriboylidylic acid [poly (A)·poly (U)], and polyriboguanylic acid [poly (G)] were obtained from Miles Laboratories, Elkhart, Ind. Ristocetin was obtained from Abbott Laboratories, North Chicago, Ill., and inosine and the sodium salts of inosine 3’- and 5’-monophosphate were obtained from the Sigma Chemical Co., St. Louis, Mo. These reagents were dissolved in or diluted with 0.85% saline.

The preparation of poly (I)·poly (C) that had been utilized in patients by Cornell et al. contained as preservatives 0.8 mg/ml methyl paraben and 0.2 mg/ml propyl paraben. These preservatives were found to interfere with platelet aggregation in vitro in concentrations as low as 1/10 of that present in the preparation administered to patients. Therefore, polyribonucleotide preparations devoid of preservatives were used in this investigation. Adenosine diphosphate (ADP), epinephrine bitartrate, and ascorbic acid were also obtained from Sigma. L-ascorbic acid was dissolved in distilled water; ADP was dissolved in Owren’s buffer and adjusted to pH 7.35; and epinephrine bitartrate was dissolved in Tyrode’s buffer and adjusted to pH 7.35. All dilutions of polyribonucleotides and solutions of other reagents were made on the day of use and stored at 4°C. Buffer solution for polymerization studies contained NaCl (140 mM), Tris (25 mM), EDTA (0.3 mM), and glucose (5 mM) adjusted to pH 7.4.

*Techniques*

Platelet aggregation was studied according to the turbidimetric technique of Born with the use of Payton Dual Channel Aggregometers (Payton Associates, Buffalo, N.Y.) and appropriate
recorders. The 0.5-ml samples of PRP were warmed to the experimental temperature (either room temperature or 37°C) for 5 min prior to being placed in the aggregometer. The samples were then stirred at 900 rpm. Light transmission through PPP represented 100% aggregation. To ascertain the degree of aggregation induced by an aggregating agent, the light transmission through PRP following addition of a reagent was measured and expressed as a percentage of light transmission through PPP. The tangent of the steepest portion of an aggregation curve was the measure of the rate of aggregation. Latency was defined as the time between addition of reagent and a positive deflection in the aggregation curve. To minimize addition and dilution artifacts, the concentrations of the solutions of most aggregating agents were adjusted so that they could be introduced into the PRP in volumes of 10 μl. Aggregating agents were introduced by positive displacement pipettors (Micro/pettor, Scientific Manufacturing Industries, Emeryville, Calif.).

Precipitation of ASC, like platelet aggregation, was studied by a photometric method with the Payton Aggregometer. Light transmission through buffer solution was utilized to obtain a zero baseline. Light transmission through a buffer solution containing ASC in a concentration compatible with the particular experiment and which had been incubated at 37°C until no further change in optical density (OD) could be detected was used to set the upper limit of the recorder. Changes with time in the OD of a collagen solution were then read and expressed in arbitrary OD units. The resulting plots were similar to those of Wood and Keech.

Data were analyzed by the two-sample Student's t test.

RESULTS

Effect of Single- and Double-Stranded Homopolymers of Polyribonucleic Acid on Platelet Aggregation Induced by Soluble Collagen

In order to test the effect of poly (I)·poly (C) on platelet aggregation, this substance was incorporated into tests of platelet aggregation in which aggregation was induced by a variety of aggregating agents. It was noted that poly (I)·poly (C) potentiated platelet aggregation induced by ASC. As seen in Table 1, poly (I)·poly (C) produced a significant decrease in latency and an increase in peak rate of platelet aggregation. An attempt was made to determine if this was

<table>
<thead>
<tr>
<th>Table 1. Platelet Aggregation induced by ASC at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polyribonucleotide Added</strong></td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>Concentration (mg/ml)</td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>Latency (sec)</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>SD</td>
</tr>
<tr>
<td>p*</td>
</tr>
<tr>
<td>Maximum aggregation (%)</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>SD</td>
</tr>
<tr>
<td>p</td>
</tr>
<tr>
<td>Peak rate aggregation (°/%,30 sec)</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>SD</td>
</tr>
<tr>
<td>p</td>
</tr>
</tbody>
</table>

*Different from response obtained in absence of polyribonucleotide.
†No aggregation 1/3.
a common property of double- and/or single-stranded homopolymers of polyribonucleic acid.

Poly (A) - poly (U) was without effect. Of three single-stranded homopolymers tested, only poly (I) potentiated the effect of ASC. The potency of poly (I), when present as part of the hybrid helix poly (I) - poly (C), did not appear to be influenced by combination with the inactive poly (C). Poly (G), unlike the other polymers, exerted a significant inhibitory effect \( p < 0.03 \) on the rate of platelet aggregation induced by soluble collagen. This inhibition was observed with a concentration of poly (G) as low as 2.5 \( \mu \text{g/ml} \) (equivalent to 0.54 \( \mu \text{mole P/liter} \). It was noted that, based on the poly (I) content, the concentration of poly (I) - poly (C) used in this experiment was at the upper end of the dose–response curve (Fig. 1) but well below a concentration of poly (I) - poly (C) that could still produce significant potentiation of ASC-induced platelet aggregation (Fig. 2). Since the concentrations of the various polymers tested

---

**Fig. 1.** Effect of poly (I) on platelet aggregation induced by fixed concentration of ASC at 37°C. Each point represents the mean of five determinations. ASC concentration \( 2.1 \times 10^{-7} \text{mg/ml} \) was minimal threshold in the absence of poly (I). PRP was incubated with poly (I) for 5 min before addition of ASC. •, latency; ○, percentage aggregation; ●, peak rate of aggregation; *, different from response to 1000 \( \times 10^{-5} \) mg/ml poly (I), \( p \leq 0.05 \).

**Fig. 2.** Effect of moieties of poly (I) on percentage platelet aggregation induced by ASC at 37°C. •, poly (I) - poly (C); ○, inosine; ●, inosine 3'-monophosphate; □, inosine 5'-monophosphate; ± SD. \( N = 6 \).
were similar to that of poly (I), their potencies would have to differ from that of poly (I) by more than an order of magnitude for an effect not to have been seen.

**Effect of Moieties of Poly (I)**

Moieties of poly (I) in concentrations comparable to effective concentrations of the polymer were tested against a threshold dose of ASC. All moieties examined failed to potentiate the effect of the ASC on platelet aggregation (Fig. 2).

**Characteristics of the Effect of Poly (I) on Platelet Aggregation Induced by ASC**

The threshold dose of poly (I) for the potentiation of platelet aggregation by ASC was 0.1–0.2 μg/ml of plasma, or 0.11–0.44 μmole P/liter (Fig. 1). Since minimal threshold concentrations of ASC were used in these experiments, it is possible that with higher concentrations of ASC a lower threshold for poly (I) could have been seen. As the concentration of poly (I) was increased (i.e., in approximately twofold increments), there was a gradual increase in the rate of aggregation and a gradual decrease in the latency of aggregation. By contrast, the dose–response relationship for percentage aggregation plateaued rapidly, with the maximum aggregation produced with less than twice the threshold dose. When the concentration of ASC was varied and the concentration of poly (I) kept constant, or when poly (I) was omitted from the experiment, the dose–response relationship for latency appeared to plateau at the same time as that for percentage aggregation, both curves plateauing before that for rate of aggregation. The cause of this difference is not known. In any event, when percentage aggregation was used to define the influence of poly (I) and the concentration of poly (I) was maintained constant, it was noted that poly (I) appeared simply to increase the potency of ASC by more than 24-fold.

**Influence of Poly (I) on Platelet Aggregation Induced by Particulate Collagen**

The dose–response curves for platelet aggregation with particulate collagen were similar to those obtained with ASC. Unlike the response to ASC, however, when a dose of particulate collagen was chosen which gave a latency of 90 sec or greater, maximum aggregation was not seen. Poly (I) did not influence these findings. By contrast, with soluble collagen, maximum aggregation could be obtained after response latencies of 200 sec either in the absence or presence of poly (I). The difference in maximum latency which could be followed by maximal aggregation was probably related to the time required for the polymerization of the ASC, since collagen must be in a polymeric state before it can aggregate platelets. Poly (I), in a concentration which produced pronounced potentiation of platelet aggregation induced by ASC, failed to influence rate, degree, or latency of platelet aggregation induced by particulate collagen. Thus poly (I) did not
Table 2. Effect of Ascorbic Acid on Optical Density (OD) ASC in Tris Buffer, pH 7.4, at 37°C

<table>
<thead>
<tr>
<th>Concentration of Ascorbic Acid</th>
<th>N</th>
<th>2 mM</th>
<th>4 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>% maximum OD at 15 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mM</td>
<td>3</td>
<td>63.8 ± 32.1*</td>
<td>89.7 ± 11.5</td>
</tr>
<tr>
<td>4 mM</td>
<td>3</td>
<td>25.4 ± 23.9</td>
<td>4.6 ± 10.2†</td>
</tr>
<tr>
<td>Relative peak rate OD Δ</td>
<td></td>
<td>6.0 ± 2.2</td>
<td>0.10 ± 0.01</td>
</tr>
</tbody>
</table>

*Mean ± SD.
†Different from control, p ≤ 0.001.
‡Different from control, p ≤ 0.05.

appear to act by promoting the interaction of platelets and polymerized collagen.

Influence of Ascorbic Acid on the Potentiation by Poly (I) of Aggregation Induced by ASC

We postulated that enhancement by poly (I) of platelet aggregation by ASC might be due to an ability of poly (I) to accelerate collagen polymerization. The effect of ascorbic acid which retards polymerization of monomeric collagen15 was therefore studied.

As shown in Table 2, ascorbic acid was capable of retarding the OD change of a solution of ASC incubated in buffer at 37°C, reflecting inhibition of collagen polymerization. As shown in Table 3, a concentration of ascorbic acid which was itself without significant effect on platelet aggregation induced by particulate collagen produced significant inhibition of aggregation induced by ASC in the presence of poly (I).

Effect of Poly (I) · Poly (C) and Poly (I) on Polymerization of Soluble Collagen

At 37°C, poly (I) · poly (C) produced a significant increase in the initial rate of polymerization of ASC (Fig. 3A). A similar result was obtained with the use

Table 3. Effect of Ascorbic Acid on Platelet Aggregation Induced by ASC and Particulate Collagen in the Presence of Poly (I)

<table>
<thead>
<tr>
<th></th>
<th>ASC†</th>
<th>Particulate Collagen†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Ascorbic Acid</td>
<td>With Ascorbic Acid</td>
</tr>
<tr>
<td>Latency (sec)</td>
<td>40.5 ± 8.3§</td>
<td>122.4 ± 76.4</td>
</tr>
<tr>
<td>Peak rate of aggregation (% Δ30 sec)</td>
<td>26.7 ± 10.7</td>
<td>5.4 ± 5.7§</td>
</tr>
<tr>
<td>Maximum aggregation (%)</td>
<td>71.6 ± 14.0</td>
<td>**</td>
</tr>
</tbody>
</table>

*Incubated with ascorbic acid for 30 min prior to addition to PRP. Final concentration of ascorbic acid in PRP, 0.5 mM.
†Concentration used was subthreshold in the absence of poly (I) but produced a submaximal effect in the presence of poly (I).
‡Concentration used produced a submaximal effect in the presence or absence of poly (I).
§Mean ± SD.
|| Different from control, p ≤ 0.01.
¶Different from control, p ≤ 0.001.
**Rate of aggregation too slow in some instances to ascertain peak level.
of poly (I), even when the collagen solution was incubated at 23°C (Fig. 3B). The technique utilized for measuring collagen polymerization required the use of ASC concentrations well in excess of those utilized in platelet aggregation. It is reasonable, however, to assume that poly (I) - poly (C) and poly (I) act in the same manner in more dilute solutions of ASC.

**Effect of Poly (I) and Poly (I) - Poly (C) on the Response to Platelet Aggregating Agents Other Than Collagen**

Both poly (I) and poly (I) - poly (C) inhibited some parameters of platelet aggregation induced by epinephrine (2 × 10⁻⁶ M), ADP (5 × 10⁻⁶ M), and ristocetin (1.05 mg/ml), but only in a concentration equivalent to at least 0.05 mg/ml of poly (I). While this concentration of poly (I) was probably less than peak plasma levels in vivo, it was approximately 500 times that which was capable of altering ASC-induced aggregation.

**DISCUSSION**

The apparent induction of disseminated intravascular coagulation in a patient with acute lymphoblastic leukemia in relapse upon administration of poly (I) - poly (C) led to the study of the effects of this substance on certain parameters of blood coagulation. Several possible mechanisms for initiation of blood coagulation were considered, including possible activation of factor XII, induction of tissue factor production by leukocytes, and potentiation of platelet aggregation. No effect of poly (I) - poly (C) on factor XII activation (both in the
presence and absence of collagen) has been observed.\textsuperscript{16} In fact, coagulation times became prolonged in the presence of poly (I) \cdot poly (C), illustrating the anticoagulant properties previously attributed to this substance.\textsuperscript{17} By contrast, poly (I) \cdot poly (C) was found to be a potent activator of tissue factor synthesis by cultured leukocytes (unpublished observations).

Results of the present experiments indicate that poly (I) and poly (I) \cdot poly (C) can potentiate platelet aggregation induced by ASC but do not by themselves serve as aggregating agents. This ability to potentiate aggregation does not appear to be a common property of either single- or double-stranded polynucleotides as it was not shared by poly (C), poly (A) \cdot poly (U), or poly (G). Indeed, poly (G) produced the opposite effect and inhibited platelet aggregation induced by ASC. The multimeric configuration of poly (I) was necessary for potentiation of platelet aggregation by ASC since moieties of poly (I) were without effect.

The lack of effect of poly (I) on platelet aggregation induced by particulate collagen together with an inhibitory effect of poly (I) on platelet aggregation induced by agents other than ASC suggests that the poly (I) does not exert its potentiating effect through an action on the platelets but rather on the ASC. This conclusion is supported by the finding that poly (I) enhances the rate of polymerization of ASC, microfibrils being required for the initiation of platelet aggregation.\textsuperscript{3,13,14} In addition, ascorbic acid, which inhibited collagen fibril formation in solutions of ASC, also inhibited the effect of poly (I) on platelet aggregation induced by ASC but not that induced by particulate collagen. Thus, poly (I) may potentiate the effect of ASC in platelet aggregation by virtue of its ability to potentiate collagen fibril formation. This action of poly (I) appears similar to that of dextran as described by Bygdeman and Tangen,\textsuperscript{10} although poly (I) is apparently much more potent.

Poly (I) has been found to possess anticoagulant properties in vitro.\textsuperscript{17} The concentration reported to be required for this effect, however, is at least 400 times that which potentiates platelet aggregation induced by ASC. At the same time, the anticoagulant property has been found to be shared by poly (G), a substance which, as shown in the present study, inhibits ASC-induced platelet aggregation. Therefore, the mechanism by which poly (I) affects coagulation appears to be unrelated to the mechanism by which it affects ASC.

Poly (I) \cdot poly (C) as well as poly (A) \cdot poly (U) and poly (G) are interferon inducers,\textsuperscript{13,18} while only poly (I) or poly (I) \cdot poly (C) potentiate ASC-induced platelet aggregation. This finding suggests that there is no relationship between the two phenomena. At the same time, poly (I) \cdot poly (C), unlike poly (A) \cdot poly (U), is pyrogenic\textsuperscript{5,19} and is lethal in mice,\textsuperscript{5,20} producing extensive tissue damage. Thus, we cannot dismiss the possibility that a relationship exists between the mechanism by which poly (I) \cdot poly (C) induces its toxic effects and the mechanism by which it affects ASC.

In speculating on a relationship between poly (I) and poly (I) \cdot poly (C) effects in vitro and in vivo, it must be borne in mind that when administered as single-stranded homopolymers both poly (I) and poly (C) are essentially without effect in vivo,\textsuperscript{21} while in some instances the double-stranded complex may be without effect in vitro.\textsuperscript{17} It is likely, however, that the single-stranded polymers
are the active moieties in vivo as well as in vitro since the requirement for
double-stranded molecules in vivo is due to the relative resistance of such mole-
cules to rapid enzymatic degradation.\textsuperscript{21}

Jaffe and Deykin\textsuperscript{13} have reported that as they increased the concentration of
monomeric collagen, the lag phase or latency of platelet aggregation remained
constant despite increasing the percentage of platelet aggregation. They indi-
cated that this occurred because, at the doses of collagen they employed, the
rate of polymerization of monomers to microfibrils was independent of the
concentration of collagen—polymerization of monomers being a requirement
for the initiation of platelet aggregation. By contrast, in the present study, we
observed that latency of aggregation did decrease as ASC concentration was
increased from the threshold level. This decrease, it may be argued, could have
been due to the presence of microfibrillar collagen in our ASC preparation.
Jaffe and Deykin\textsuperscript{13} pointed out that there was no significant difference between
microfibrillar and macrofibrillar or particulate collagen in the ability to aggre-
grade platelets. Therefore, as the concentration of ASC was increased in the
present study, there might simply be a concomitant increase in the concentra-
tion of the microfibrillar collagen. Such an explanation, however, is not com-
patible with our findings.

Thus with a fixed concentration of ASC, we found that by increasing the con-
centration of poly (I) we decreased the latency for platelet aggregation. Since
we also demonstrated that poly (I) had no effect on platelet aggregation in-
duced by particulate collagen, we could only conclude that the decrease in
latency was related to an effect of poly (I) on monomeric collagen. Supporting
this conclusion was our finding that ascorbic acid, which inhibited polymeriza-
tion of ASC when used in a concentration which did not inhibit platelet aggre-
gation induced by particulate collagen, nearly eliminated the potentiating effect
of poly (I) on platelet aggregation induced by ASC. The latter finding tends
to diminish but not eliminate the possibility that poly (I) acts by promoting the
adhesion of platelets to monomeric collagen.

The finding of an inhibitory effect of high concentrations of poly (I) on plate-
et aggregation induced by agents other than ASC is not surprising since it has
been reported that certain polyribonucleotides can inhibit cell adhesion.\textsuperscript{22} This
effect, however, is not incompatible with the appearance of a thrombotic dis-
order since plasma concentrations in vivo probably fall rapidly below the in-
hibitory level.\textsuperscript{6}

The potentiating effect of poly (I) on polymerization of ASC might relate to
the development of disseminated intravascular coagulation in a patient receiv-
ing poly (I) · poly (C) if, indeed, a soluble form of collagen exists in the circula-
tion. While under physiologic conditions collagen itself has not been found in
solution, its precursor, procollagen, has been found.\textsuperscript{23,24} Moreover, procollagen
or a fragment thereof has been identified in human serum in a concentration as
high as 80 \( \mu \)g/100 ml.\textsuperscript{23} Thus since procollagen peptidase acts extracellularly to
convert procollagen to tropocollagen,\textsuperscript{24} the potential exists for the formation
of tropocollagen in the circulation. Indeed, collagen, which cannot be ac-
counted for by invading fibroblasts, has been identified in human arterial thrombi.\textsuperscript{26} This collagen, it has been postulated, may be derived from circulat-
ing dissolved collagen. Therefore it appears that a relationship could exist between poly (I) and disseminated intravascular coagulation through an effect of poly (I) on a form of circulating collagen.

REFERENCES


Effect of polyribonucleotides on in vitro platelet aggregation induced by acid-soluble collagen and other aggregating agents

R Rosenstein, LR Zacharski and KA Smith