Positive Limulus Amoebocyte Lysate Reactions with Polyriboinosinic Acid-Polyribocytidyllic Acid

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The annealed copolymer polyriboinosinic acid-polyribocytidyllic acid reacted with Limulus amoebocyte lysate to cause gelation at a concentration approximately 2,000-fold or greater than bacterial endotoxins. This copolymer was pyrogenic in rabbits and demonstrated hypochromicity, but no significant correlation was noted among Limulus amoebocyte lysate reactivity, pyrogenicity, and hypochromicity. Like endotoxin, polyriboinosinic acid-polyribocytidyllic acid did not react with purified Limulus coagulogen. Similar concentrations of the homopolymers polyriboinosinic acid and polyribocytidyllic acid were negative or significantly below the Limulus amoebocyte lysate reactivity of the copolymer and essentially nonpyrogenic. Thus, polyriboinosinic acid-polyribocytidyllic acid is a compound in addition to endotoxin that effects a positive Limulus amoebocyte lysate test.

Some studies investigating the specificity of the Limulus amoebocyte lysate (LAL) test conclude that the LAL test is specific for endotoxin (8, 13, 14), but other studies have shown that compounds other than endotoxin cause a positive LAL test (6, 11, 17). Previous research in this laboratory demonstrated a positive LAL reaction with polyriboinosinic acid-polyribocytidyllic acid [poly(I)-poly(C)] (6); however, other investigators have challenged these findings (2, 10). Work in other laboratories has shown that poly(I)-poly(C) is pyrogenic in rabbits, but single-stranded homopolymers poly(I) and poly(C) are not pyrogenic (9, 12). In this study, LAL tests and rabbit pyrogen tests were performed on homopolymers and on poly(I)-poly(C) formed after annealing the homopolymers in an attempt to resolve the controversy. Additionally, several different preparations of poly(I)-poly(C) were tested with purified Limulus coagulogen (16) as well as with LAL and the rabbit pyrogen test.

MATERIALS AND METHODS

Nucleotides. Eight different lyophilized preparations of poly(I) and poly(C), having sedimentation coefficients ranging from 2.5 to 13.2, were obtained from P-L Biochemicals (Milwaukee, Wis.) and Miles Laboratories (Elkhart, Ind.). Poly(I) and poly(C) designated as numbers 1 through 6 were part of a "Molecular Size Kit" offered by P-L Biochemicals. Two Molecular Size Kits of the same lot were obtained and tested with different lots of Limulus amoebocyte lysate. None of the homopolymer preparations were guaranteed to be sterile or pyrogen free.

Poly(I)-poly(C) was obtained from P-L Biochemicals (lot 547291), Miles Laboratories (lot 12), and Boehringer Mannheim Corp., Indianapolis, Ind. (lot 7375111). These lyophilized preparations, with the exception of the P-L Biochemicals poly(I)-poly(C), also were not guaranteed to be sterile or pyrogen free.

Annealing process. Solutions of poly(I) and poly(C) were prepared in pyrogen-free 0.15 M NaCl at a concentration of 0.003 M (1.10 mg of poly(I) per ml and 1.02 mg of poly(C) per ml). Annealing was accomplished by mixing equal quantities of one poly(I) and one poly(C) that were of comparable molecular size, then heating the mixture at 44°C. Annealing was confirmed by determining the hypochromic effect at 250 nm (1, 3). The entire annealing process was done in capped pyrogen-free plastic tubes (Falcon Plastics, Oxnard, Calif.).

LAL test. LAL tests were performed on all single-stranded and double-stranded preparations in pyrogen-free 0.15 M NaCl by a previously described method (6). The test results were scored as follows: clear or slight flocculation, negative; increased turbidity, 1+; increased viscosity and granules adherent to the side of the tube, 2+; and a firm gel that remained adherent to the bottom of the tube when inverted 180°, 3+. All samples were titrated to the highest dilution yielding a 3+ reaction at 1 h. The two lots of LAL used were prepared as previously described (6), and each could detect 0.5 ng of Escherichia coli endotoxin per ml (15) (the endotoxin used by the Food and Drug Administration as their reference endotoxin) with a 3+ reaction.

Homopolymers and poly(I)-poly(C) annealed in this laboratory were tested at concentrations the same as those used for the annealing process. Poly(I)-poly(C) preparations obtained elsewhere were dissolved in pyrogen-free 0.15 M NaCl at a concentration of 1 mg/ml for LAL testing.

Pyrogen testing. Male New Zealand white rabbits, weighing 1.8 to 2.2 kg, obtained from the National Institutes of Health colony, were used to test the
pyrogenicity of homopolymers (numbers 1 to 6) and poly(I)-poly(C) (numbers I to VI) preparations as previously described (19). Samples for intravenous injection into a marginal ear vein were prepared by making 1:5 dilutions of each solution in pyrogen-free 0.15 M NaCl. One or two rabbits were injected with 2 ml of poly(I) or poly(C) (approximately 20 µg/kg), and four to nine rabbits were injected with 1 ml of poly(I)-poly(C) (approximately 10 µg/kg). Rabbits were given a larger amount of the homopolymers than the copolymers since previous studies had shown the former to be nonpyrogenic and the latter to be pyrogenic in rabbits (9). Temperatures of the rabbits were measured electronically every 12 min for a 5-h period after injection. The average temperature change at each 12-min interval was calculated and plotted. Each rabbit was used only once and received an injection of only one compound.

Poly(I)-poly(C) preparations purchased as double-stranded nucleotides were tested by injecting 1 ml of a 1.25-µg/ml solution in pyrogen-free 0.15 M NaCl into two rabbits. For each preparation, the average fever index (15) was determined.

**Limulus coagulogen.** Purified *Limulus* coagulogen (16) was a kind gift of Robert Seid and Darrell Liu of the Bureau of Biologies, Food and Drug Administration. The coagulogen was dissolved in pyrogen-free 0.05 M tris(hydroxymethyl)aminomethane-0.01 M CaCl₂, pH 7.5, to a concentration of 5 mg/ml. Trypsin (lot 37J628; Worthington Biochemical Corp., Freehold, N.J.) was used as a positive control, and Food and Drug Administration reference endotoxin (15) was used as a negative control. Tests with the coagulogen were performed on the purchased poly(I)-poly(C) preparations in the same manner as the LAL test with coagulogen replacing LAL.

**Statistical methods.** The Spearman rank correlation coefficient was used to compare LAL reactivity, pyrogenicity, and hypochromicity (4).

**RESULTS**

Homopolymers poly(I) and poly(C) were either nonreactive or minimally reactive in the LAL test as compared with the annealed copolymer, poly(I)-poly(C) (Table 1). All annealed poly(I)-poly(C) preparations showed significantly increased (a 2-tube or greater increase) LAL endpoints compared with the homopolymers and showed the hypochromic effect (Table 1). The concentration of poly(I)-poly(C) required to effect a positive LAL test ranged from approximately 2 µg/ml (a 1:512 dilution of a 1-mg/ml solution) to 250 µg/ml (a 1:4 dilution of a 1-mg/ml solution) (Table 1). LAL tests on the second batch of poly(I) and poly(C) were consistent with the first experiment.

Pyrogen testing showed a similar pattern: single-stranded homopolymers did not produce significant fever, but all double-stranded poly(I)-poly(C) preparations produced significant fever (Fig. 1). There were no significant correlations among LAL reactivity, pyrogenicity, and percentage of hypochromicity. However, all poly(I)-poly(C) preparations produced both significant fever and a significantly increased LAL endpoint.

The three purchased poly(I)-poly(C) preparations were pyrogenic and LAL reactive, but were not reactive with *Limulus* coagulogen. LAL endpoints and fever indices (FI) for the three preparations were: P-L Biochemicals LAL, 1:256 (FI, 2.78); Miles LAL, 1:64 (FI, 4.10); Boehringer LAL, 1:8 (FI, 1.84).

**DISCUSSION**

Information about the specificity of LAL to react with materials other than bacterial endotoxins is important for the interpretation of a positive test. In 1973, this laboratory reported that poly(I)-poly(C) at a concentration of 0.1 µg/ml effected a positive LAL test (6). In 1974, Niwa et al. concluded that poly(I)-poly(C) was
negative in the LAL assay at a concentration of 100 µg/ml (10). However, a year later the same group reported that poly(I)·poly(C) at a concentration of 2.8 µg/ml resulted in a positive LAL assay at “50% of the maximum clot” (11). Cooper reported that the commercially prepared poly(I)·poly(C) was inactive with LAL (2). The results of the current study corroborate the previous report from this laboratory and establish poly(I)·poly(C) as a compound that activates LAL to cause gelation.

The concentration of poly(I)·poly(C) required to produce a positive LAL test varied with the preparation. Concentrations from 2 to 250 µg/ml among the different preparations of poly(I)·poly(C) were required for a positive LAL test.

The results of studies by Niwa et al. and by this laboratory of the reactivity of poly(I)·poly(C) with LAL are within this range (5, 11). However, the initial report from this laboratory indicated that 0.1 µg of the poly(I)·poly(C) preparation per ml caused a positive LAL test, which is below the lower concentration of the present study by a factor 20 (6). The preparation of poly(I)·poly(C) used in the initial study was either unusually reactive with LAL or contaminated with substances that could react with LAL, such as endotoxin.

The relatively broad range of concentrations of the different preparations of poly(I)·poly(C) to effect a positive LAL test is similar to that of bacterial endotoxins. Each endotoxin prepara-
tion varies in its reactivity with LAL (5). However, there is a significant correlation between LAL reactivity and pyrogenicity in rabbits among different endotoxins (5). In the current study, a significant correlation among LAL reactivity, pyrogenicity in rabbits, and hypochromicity among six different preparations of poly(I)-poly(C) was not found. Thus, the nature of the copolymers responsible for the gelation of LAL was not apparent from this study.

There are structural similarities between the lipid A moiety of bacterial endotoxin and polynucleotides. Lipid A is composed of diglucosamine units linked by phosphodiester bridges with fatty acids attached to the amino and hydroxyl groups of the diglucosamine units (7). On the other hand, the polynucleotide is composed of ribose units linked by a phosphate bridge with nucleic acids attached to the carbohydrate moiety (18). Thus, the fatty acids in lipid A would have a counterpart of a nucleic acid in the polynucleotide. This partial homology between these two compounds may give some insight into the similar properties of pyrogenicity and LAL reactivity.

Poly(I)-poly(C) may activate LAL by a mechanism similar to that of bacterial endotoxins but at a significantly greater concentration. Poly(I)-poly(C), like endotoxin, failed to react with purified Limulus coagulogen (16). Thus, poly(I)-poly(C) is activating some component of LAL which then reacts with the coagulogen to cause gelation. This may be the same component activated by endotoxins. However, approximately a 2,000-fold or greater concentration (wt/vol) of poly(I)-poly(C) than endotoxin is required to produce a positive LAL test. Thus, it would be easy to separate purified preparations of endotoxin and poly(I)-poly(C) by the amount of the preparation required to effect a positive LAL test. However, given a positive LAL test with an unknown solution, either endotoxin or poly(I)-poly(C) could cause the positive test.

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LITERATURE CITED


