Vol. 84, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1978 Pages 809-815

ENZYMATIC DEGRADATION BY THE SERA OF VARIOUS ANIMAL SPECIES OF PENICILLIUM CHRYSOGENUM MYCOPHAGE DOUBLE-STRANDED RNA

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Received July 25,1978

Summary. The enzymatic degradation of dsRNA by the sera of a number of animal species was studied using a quantitative polyacrylamide gel assay procedure. Species ranking of activity correlate well with known biological effects of dsRNA. Human serum showed unusually high activity, and a comparison was made between it and bovine ribonuclease A using penicillium chrysogenum mycophage double-stranded RNA and yeast sRNA substrates.

Introduction. Prior exposure of the synthetic dsRNA analog Poly I·Poly C to serum results in a large diminuation in its interferon inducing ability. Nordlund, et al. (1) have shown a differential effect, after exposure of Poly I·Poly C to sera from various animals, on reducing pyrogenic response in rabbits suggesting enzymatic degradation. Human serum showed the greatest activity.

Systematic attempts to quantitate serum degradation using acid precipitation ribonuclease assays have led to contradictory results. Stern (2) demonstrated activity in the sera of a number of animal species. However, the ranking he obtained showed no correlation with the pyrogenic results of Nordlund, et al. (1). Human serum was only moderately active where rat and rabbit showed the greatest enzymatic activity. A more recent paper by Körner (3) reports a total lack of degradative activity in human serum.

The present report demonstrates conclusively the existence of enzymatic activity in the sera of a number of species against PCMdsRNA[1] using a quantitative gel electrophoresis assay procedure. Substantial enzymatic activity was shown in human serum and studied further by ΔA_{260} and viscosity.

[1] Penicillium chrysogenum mycophage double-stranded RNA.

0006-291X/78/0843-0809$01.00/0

Enzymatic Degradation of dsRNA by Human Serum. A linear relationship between % dsRNA degraded and nl human serum added per µg dsRNA was obtained after 15 minute incubations in standard reaction mixtures (Fig. 1) using the gel-assay procedure. The products of the reaction migrate rapidly as a broad band and are completely off the gel in the 3 hour running time used in the assays. The reaction products, if double stranded, are $< 5 \times 10^5$ daltons, which is the estimated lower limit of retention on the gels using unreacted PCMdsRNA and reovirus dsRNA as markers (4). It has been shown that the interferon inducing capacity of PCMdsRNA (5) drops off rapidly as the molecular weight of sonicated fragments approaches 1.0×10^5 daltons.

Comparisons between Human Serum and Ribonuclease A Activities Against PCMdsRNA and Yeast sRNA. The kinetics of degradation of PCMdsRNA and sRNA by a known dilution of human serum (0.17 ul/ml) and a known concentration of bovine ribonuclease A (320 ng/ml) using ΔA_{260} to follow the reactions are compared in Fig. 2. The concentration of ribonuclease A was the minimal amount needed to show a measurable effect by ΔA_{260} and gives up to 22% degradation when assayed by gel electrophoresis after 15 minutes incubation at 37°C. This amount of ribonuclease A attacks yeast sRNA to such a great extent that a mathematical extrapolation had to be made to obtain the initial pseudo-zero order portion of the kinetic curve. In contrast human serum degrades yeast sRNA much less than ribonuclease A at a concentration (0.17 µl/ml) where PCMdsRNA is degraded 19% after 15 minute incubation at 37°C in the gel assay (Fig. 1).

In a separate set of experiments it was determined that 0.4 ng/ml of ribonuclease A degraded yeast sRNA at about the same rate as 0.17 µl/ml of human serum under the same set of conditions. At 0.4 ng/ml of ribonuclease A in the standard reactions there is no indication of degradation of PCMdsRNA by either ΔA_{260} or the gel assays.

Fig. 3 shows the kinetics of degradation of PCMdsRNA monitored by viscosity change by a 1.0 µl/ml dilution of human serum and 4 ng/ml of ribonuclease A. The decrease in viscosity is much greater for the serum firmly establishing a greater activity of human serum against dsRNA than bovine ribonuclease A relative to yeast sRNA as a substrate.

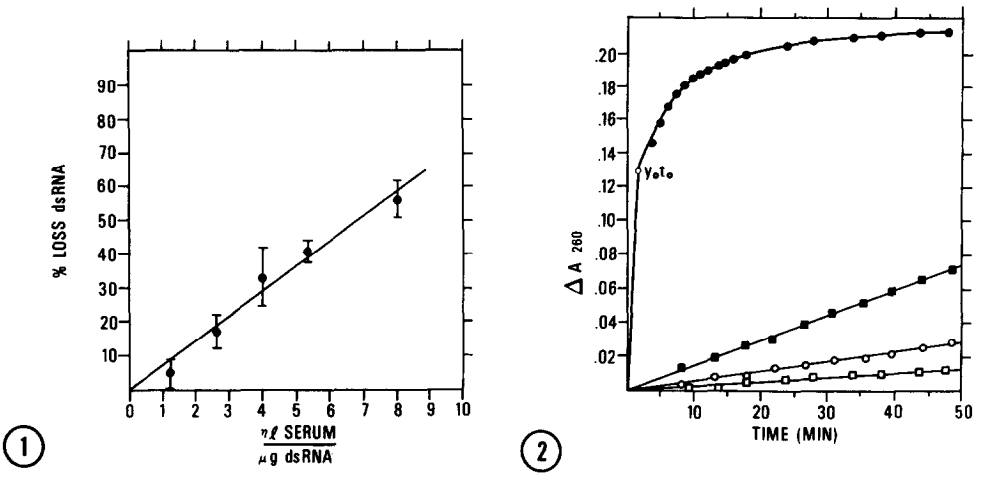

Figure 1. Degradation of PCMdsRNA by Human Serum: Gel Electrophoresis Assay. The isolation and characterization of PCMdsRNA has been described elsewhere (11). Electrophoresis on 2 1/2% polyacrylamide gels was done in precision machined supersil 4 mm x 65 mm tubes, 6 ma/tube for 3 hours. Band profiles at 260 mμ were obtained using a Cary 15 spectrophotometer equipped with a motor driven scanning device in the cell compartment. Areas were calibrated with known concentrations of PCMdsRNA. Tubes were scanned again after 90° rotation, and areas averaged. Differences of +5% in area for the same band after rotation were not acceptable. Duplicate runs in separate tubes were always made. Reaction conditions: 200 μl total reaction volume, buffer: 0.15M NaCl .016M Na citrate, 10^{-4} M $MgCl_2$, pH 7.0; PCMdsRNA concentration 62 μg/ml; 37° incubation for 15 minutes. Reactions quenched in ice water, then immediately assayed or frozen and stored in liquid N_2 for future analysis. 50 μl of reaction mixture were loaded on the gels. Four different samples obtained from four male volunteers after overnight fasting were run. Error bars represent ±S.D. from the mean at each point.

Figure 2. Comparison of the Degradation of Yeast sRNA and PCMdsRNA by Human Serum and Bovine Ribonuclease A: Absorbancy Change Assay. A_{260} was followed using a modified Hitachi spectrophotometer (11). Reaction volumes 2 ml; temperature 37° C. Starting A_{260} = 1.0 for both yeast sRNA (General Biochem) and PCMdsRNA. Bovine ribonuclease A was obtained from Sigma. Buffer: 0.15M NaCl; .016M Na citrate, 10^{-4} M $MgCl_2$, pH 7.0 ●—● Ribo A(320 ng/ml), sRNA. Experimental points fitted by computer to the functions $y(t) = m - (m-y_o)e^{-k'(t-to)}$ for $t > t_o$, where $y(t) = A_{260}$ at time t. Optimal parameters of m = 0.21; k' = 0.13; t_o = 1.5 and y_o = 0.3 were obtained. Reasonable fit could not be obtained to an expression of the form $y(t) = m(1-e^{-k't})$ strictly analogous to a first order rate expression. The line y(t) = yo/to t with slope yo/to is taken to represent the unobserved initial velocity. The rest of the data are fitted to least squares lines. For the purpose of comparison, activities are taken to be linearly related to the slopes ΔA_{260}/min. □—□ Ribo A (320 ng/ml) dsRNA: ■—■ human serum (170 nl/ml), sRNA: O—O human serum (170 nl/ml) dsRNA.

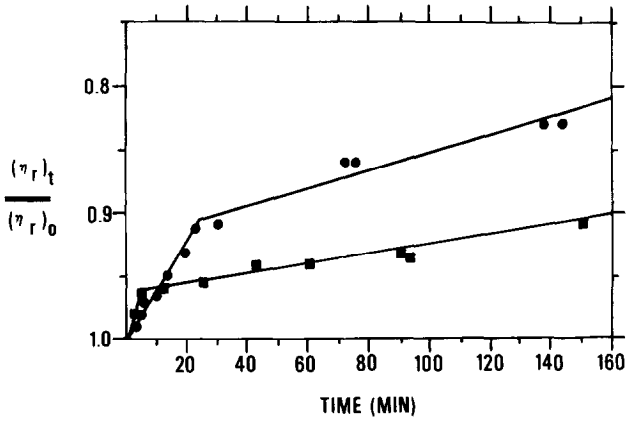

Figure 3. Degradation of PCMdsRNA by Human Serum and Bovine Ribonuclease A. Viscosity determinations were made in a Canon Uberlohde semimicro mixing viscometer at 30° C. $(\eta r)_t/(\eta r)_o$ = reduced viscosity time t/reduced viscosity time o; 2.0 ml reaction volumes. Buffer: 0.15M NaCl, .016M Na citrate, 10^{-4} M $MgCl_2$, pH 7.0. PCMdsRNA 143 µg/ml. ●—● dsRNA plus 1 µl/ml human serum. ■—■ dsRNA plus 4 ng/ml bovine ribonuclease A.

The results of the ΔA_{260} assays can now be quantitated and are displayed in Table 1. It follows that human serum is about 140 times more active than bovine ribonuclease A against dsRNA relative to yeast sRNA as a substrate. Bordon, et al. (6) have isolated an enzymatic fraction from human serum and have reported 700 times more activity for it than bovine ribonuclease A relative to sRNA using the same sort of absorbancy change assay and comparison presented here.

Species Comparison of Serum Activity. Comparisons between different species were made using the gel-assay procedure. The results of this study, along with data on pyrogenic effect (1) and total RNAase activity assayed against sRNA (7), taken from the literature are displayed in Table 2.

There is no correlation (except for guinea pig which has unusually high levels) between degradative activity against PCMdsRNA and total serum ribonuclease, strongly suggesting some specificity of that component in the sera most active against dsRNA. There is excellent agreement between the results of Nordlund, et al. (1) using pyrogenic response and the results of our gel-assay confirming their conclusion that varying degrees of enzymatic degradation in different sera are

Table 1. Comparison of Activities of Human Serum and Bovine Ribonuclease A Using Yeast sRNA and PCMdsRNA Substrates

Sample	Activity (ΔA_{260}/min)		Activity dsRNA/Activity sRNA
	PCMdsRNA	Yeast sRNA	
Serum	5.8×10^{-4}	1.5×10^{-3}	0.39
Ribonuclease A	2.4×10^{-4}	8.6×10^{-2}	2.8×10^{-3}

responsible for the differential pyrogenic responses they had observed. Only guinea pig serum deviates markedly for some unknown reason.

The large degree of activity of human serum, especially in comparison to rat, mouse, and rabbit is inverse to these species' ability to respond to dsRNA as an interferon inducer (8), indicating a relationship between the degree of serum enzymatic activity of a species toward dsRNA and this biological response.

Dog serum gave unique results in the gel assay. Even at serum concentrations as high as 13.4 µl/ml in the reaction mixture, very limited digestion was observed producing only very large double-stranded reaction products $>5.0 \times 10^5$ daltons. It is suggested that the severe toxic reaction of dogs to a single iv dose of 1 mg/kg of dsRNA (9) is in part due to the inability of this animal's serum to efficiently degrade this molecule to other than large fragments.

Severe toxic responses are difficult to compare because of the variety of dosages and administration routes used by different investigators. In general, toxic responses at high dosages are most severe in species that respond best to dsRNA as an interferon inducer (mice, rabbits, and rats). The only effect in monkeys at iv dosages of 1.0 mg/kg was elevated alkaline phosphatase levels (9).

Conclusions. We have shown for the first time a good correlation between differential biological response (especially pyrogenicity and interferon induction) as observed by others and ranking of dsRNA degradative capacity in the sera of various species as determined by gel electrophoresis. These results strongly support the intuitive view that the primary

Table 2. Species Comparisons of Various Serum Activities

	dsRNA(1) Degradation	ΔT(2) Pyrogenicity	Non-specific Ribonuclease Activity (3)
Human	7.67 ± 1.8	.13	2.8
Pig	1.09 ± .14	-	3.8
Cow	1.08 ± .09	-	-
F. Calf	1.05	.95	-
Monkey	.79 ± .06	1.65	-
Mouse	.64 ± .06	1.55	2.6
Rat	.58 ± .09	-	-
Rabbit	.49 ± .02	1.74	3.6
Horse	.37 ± .09	2.05	1.6
Dog	-	1.46	4.2
G. Pig	10.2 ± 3.6	1.87	13.0

(1) Reactions were incubated at 37°C for 15 minutes, varying the dilution of serum for each animal and quenched. Reaction volume 200 µl, buffer 0.15M NaCl, .016M Na citrate, 10^{-4} M $MgCl_2$, pH 7.0. After electrophoresis (50 µl aliquots) % loss dsRNA was calculated from areas under the bands by comparison with appropriate controls. For each species (except fetal calf serum) at least 3 separate sera samples from 3 separate animals were run. Values given are the average slopes of the plots of % degradation vs. nl serum/µg dsRNA and, therefore, have the units 100X µg dsRNA degraded/nl serum/15 min. (for human serum, see Fig. 1). The data were fitted to least squares lines passing through the origin. The error limits are ± S.D. for the slopes of these lines. At least five points were taken to determine each line. The concentration of dsRNA (~62 µg/ml) was kept reasonably constant for all the runs.

(2) ΔT values obtained from Nordlund, et al. (1), reported error limits ±0.3°C.

(3) Activity as reported by Lechner and Deque-Magalhaes, (11) in units/min/ml serum with sRNA as substrate.

factor responsible for the degree and duration of response in any one species is the half life of dsRNA as significantly large fragments in the serum.

The lack of correlation of enzymatic activity and known biological activity, and the disagreement on the existence and/or degree of degradation by serum found in earlier studies,

can be attributed to two primary causes. First, the assays (both ΔA_{260} and acid precipitation) detect degradation that is rather extensive and somewhat removed from initial endonuclease clippages that render the dsRNA biologically inactive. Limited digestion to fragments ~1.0 x 10^5 daltons, would be difficult to detect by ΔA_{260}. Also this size fragments precipitate and would be counted as unreacted substrate in the acid precipitation assay (10). Secondly, these assays in the presence of large amounts of serum protein can lead to significant artifacts as demonstrated by Körner (3) for the acid precipitation assay.

Acknowledgments. We wish to thank Drs. Norris Allen and Tats Matsuoka of the Lilly Research Laboratories for providing us with animal sera, and Dr. Robert Wolen of the Lilly Clinic for the human serum samples.

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