IMMUNOLOGICAL STUDIES ON THE INTERACTION OF POLYADENYLIC ACID AND HUMAN LIVER RIBONUCLEASE

Edward A. Neuwelt*, William A. Gahl and Carl C. Levy

Baltimore Cancer Research Center, NCI
Laboratory of Molecular Biology
3100 Wyman Park Drive
Baltimore, Maryland 21211

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SUMMARY The effect of polyadenylic acid, a potent inhibitor of mammalian and bacterial RNAses, on the binding of human liver RNAse to its antibody was studied. To do this, a human liver RNAse antibody was immobilized on Sepharose 4B. Examination of the ability of the enzyme to bind to the immobilized anti-RNAse in the presence or absence of polyadenylic acid indicated that enzyme-antibody binding was more sensitive to the presence of polyadenylic acid than was enzyme activity. Furthermore, the effect of polyadenylic acid on enzyme-antibody binding was specific since neither polycytidylic acid nor polyuridylic acid had much effect on the antigenicity of the enzyme. The metal cation, Mg^{2+}, and the polyamine, spermidine, but not putrescine, readily reversed the effects of polyadenylic acid on enzyme-antibody binding.

The ubiquitous nature of polyadenylic acid at the 3' termini of a number of mRNAs, as well as viral RNAs, has been clearly established (1-4). The role that this polyribonucletoide plays in cellular metabolism is, however, far from clear. Recent evidence has indicated that a number of ribonucleases, human and bacterial, are inhibited competitively by poly(A) (5-7). It has been shown, moreover, that poly(A) may function in the maintenance of the biologic activity of RNA molecules to which the polynucleotide is linked covalently (8-10). In view of this, we have suggested that poly(A) may act to maintain the structural and functional integrity of a mRNA by inhibiting its hydrolysis by cellular RNAse (5,11,12). In the present study, it was of interest to determine if poly(A), in addition to its role as a competitive inhibitor of RNAse activity, was also changing or masking the antigenicity of the RNAse. Since enzyme

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Abbreviations: NRS-Sepharose, normal rabbit serum conjugated to Sepharose; AB-Sepharose, antibody conjugated to Sepharose; poly(A) polyadenylic acid; poly(C), polycytidylic acid; poly(U), polyuridylic acid.

* Present address: Division of Neurosurgery, University of Texas Southwestern Medical School, Dallas, Texas 75235.

catalytic sites generally are not antigenic (13), and, indeed, antigenic sites are known to exist away from the catalytic site in bovine pancreatic RNAse (14), such inhibition of enzyme antibody binding might suggest that poly(A) affects more than just the catalytic site. Studies with human liver RNAse did, in fact, demonstrate such an effect. Since the polyamine, spermidine, as well as some metal cations, have been shown to reverse the inhibition of human liver RNAse activity induced by poly(A) (7), the ability of these same agents to reverse the effects of poly(A) on enzyme antigenicity was also examined.

MATERIALS AND METHODS

Purified human liver RNAse was prepared as described elsewhere (7). Cyanogen bromide-activated Sepharose 4B was purchased from Pharmacia, Piscataway, New Jersey. Putrescine and spermidine were obtained from Calbiochem, La Jolla, California. The sources of all synthetic polynucleotides and the procedures used in the purification of yeast RNA have been described elsewhere (6).

Assay of human liver ribonuclease - Human liver RNAse activity was measured as described previously (7).

Preparation and detection of antibody to human liver ribonuclease - Antibody to human liver RNAse was prepared and detected as described elsewhere (15).

Preparation of immunoadsorbent - Pre- and post-immunization sera (3 ml), obtained as described elsewhere (15), were dialyzed against a solution containing 0.1 M $NaHCO_3$ and 1.0 M NaCl and then independently coupled to 12 g of CN Br-activated Sepharose 4B as described previously (19), except that the coupling reaction was carried out for 2 h at 25°C. The immobilized antiserum will be referred to as AB-Sepharose and the pre-immunization serum conjugated to Sepharose will be referred to as NRS-Sepharose.

Standard reaction mixture - The standard reaction mixture consisted of 4.5 units of enzyme, 80 µmoles of Tris-HCl buffer (pH 7.5), 0.5 mg of bovine serum albumin, and polyribonucleotide in a volume of 0.5 ml. In experiments in which the reversal of the effects of poly(A) on antibody was studied, spermidine or putrescine, or Mg^{2+} was added to the reaction mixture following the addition of the polyribonucleotide. Then 0.15 ml of either AB- or NRS-Sepharose, suspended in 0.15 ml of 0.005 M Tris-HCl buffer (pH 7.5) was added to the mixture. After incubation at 4°C for 30 min, the Sepharose was allowed to settle and a 0.3 ml aliquot of the supernatant solution was assayed for ribonuclease activity as described above. The difference in ribonuclease activity between the supernatant solution of the AB- or NRS-Sepharose-containing tube and the supernatant solution of the tube containing the same reagents but lacking AB- or NRS-Sepharose, gave a measure of the amount of RNAse activity bound by Sepharose-conjugated serum. Corrections were made when necessary for alteration of enzyme activity by the polyribonucleotides and the other reagents added to the standard reaction mixture. All experiments were run in duplicate, and duplicate values which varied by more than 10% were discarded.

Immunodiffusion studies - Ouchterlony slides were prepared with 1% agarose, as described previously (16), and each well contained 10 µl of either enzyme (12 µg/ml), whole serum, or poly(A) (2.5 mg/ml).

Table 1

Amount of human liver RNAse bound by AB-Sepharose and NRS-Sepharose at 0.08 M Tris-HCl and 0.005 M Tris-HCl [1]

Buffer	Enzyme bound (Δ O.D. 260 nm)	
	AB-Sepharose	NRS-Sepharose
0.08 M Tris-HCl, pH 7.5	1.31	0.11
0.005 M Tris-HCl, pH 7.5	0.71	0.54

[1] The reaction mixture is outlined in the METHODS section. The same amount of enzyme activity was used at 0.08 M Tris-HCl as at 0.005 M Tris-HCl.

RESULTS

Preliminary studies had indicated that human liver RNAse could bind non-specifically to Sepharose with or without its conjugation to rabbit serum. To determine the extent to which this binding was a factor in the binding of liver RNAse to the immunoadsorbent, control reaction mixtures containing NRS-Sepharose were run routinely in all experiments. As shown in Table 1, a large amount of human liver enzyme was bound non-specifically to NRS-Sepharose at low buffer concentrations (0.005 M) but did not bind significantly when the buffer concentration was raised to 0.08 M. With respect to AB-Sepharose, considerably less liver RNAse was bound at the lower buffer concentration than at the higher one. Consequently, all studies were conducted in 0.08 M Tris-HCl buffer (pH 7.5), to minimize non-specific binding and to increase antibody binding.

To establish the effects of poly(A) on enzyme antibody binding, different amounts of the polynucleotide were added to the enzyme before the addition of the immunoadsorbent in the standard reaction mixture (Fig. 1). As the concentration of poly(A) increased, enzyme binding to antibody decreased such that at the final concentration of poly(A) tested, there was a 60% inhibition of enzyme-antibody binding. This would suggest that the polynucleotide is either

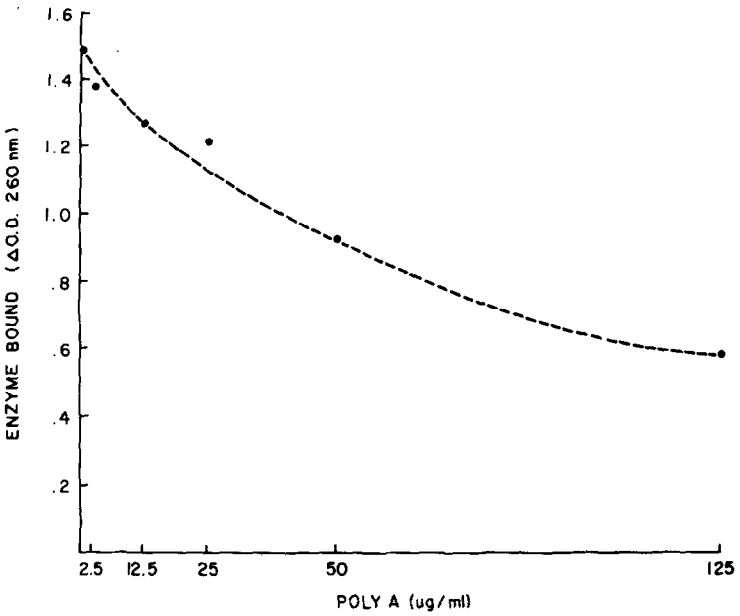

Fig. 1. The effect of varying concentrations of poly(A) on enzyme-antibody binding. The reaction mixtures were prepared as in the METHODS section, except that the amount of poly(A) added to each reaction mixture was varied from 2.5 µg to 125 µg. When NRS-Sepharose was used instead of AB-Sepharose and the concentration of poly(A) varied as above, the mean amount of enzyme bound had an activity of 0.06 absorbance units.

able to mask or in some manner induce a change in the antigenic sites of the liver RNAse.

Since the inhibition of RNAse activity by poly(A) can be reversed by spermidine (5-7), the effects of this polyamine on enzyme-antibody binding in the presence or absence of poly(A) were studied. As is apparent (Fig. 2), in the absence of poly(A), no matter how the spermidine concentration was varied, there was no effect on enzyme-antibody binding. However, the inhibition of enzyme-antibody binding induced by the polynucleotide was reversed in direct relation to the concentration of the polyamine up to a concentration of 10^{-3} M As the concentration of spermidine went beyond this, the reversal effect plateaued. At 2×10^{-3} M, approximately 80% of the inhibition of enzyme-antibody binding was reversed by the polyamine. It should be noted that the

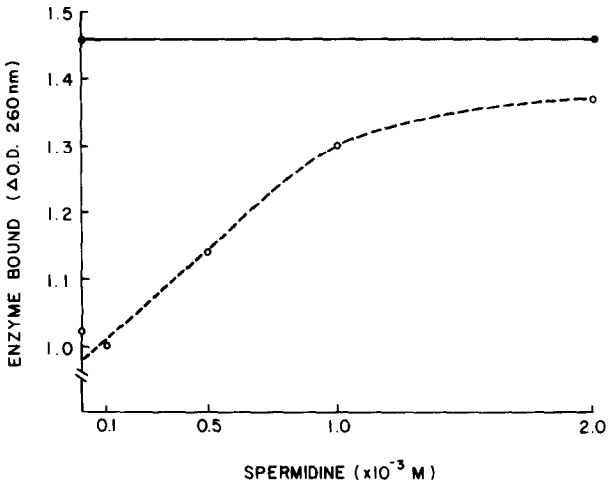

Fig. 2. The reversal by spermidine of poly(A)-induced inhibition of enzyme-antibody binding. The reaction mixtures were prepared as described in the METHODS section, except that 25 µg of poly(A) was added to each tube, followed by the addition of varying amounts of spermidine, ranging from 10 to 200 nmoles (O---O). Controls were prepared as above, except that poly(A) was not added (O——O). When both the experimental and the control tubes contained NRS-Sepharose rather than AB-Sepharose, the mean amount of enzyme bound had an activity of 0.04 absorbance units.

reversal of enzyme-antibody binding begins to plateau at that concentration of spermidine (10^{-3} M) which was reported previously to give maximal restoration of human liver RNAse activity following poly(A) inhibition (7).

The studies described thus far have indicated that the effects of poly(A) and spermidine on enzyme-antibody binding parallel the effects of these two compounds on enzyme activity reported elsewhere (7). It is interesting to note, however, that the buffer concentration chosen for the present studies has been reported to be almost as effective in reversing poly(A) inhibition of enzyme activity as was spermidine (7). Yet the same amount of poly(A) used in those studies (i.e., 25 µg/ml) inhibited enzyme-antibody binding by 40% (Fig. 3). This suggests that enzyme-antibody binding may be a more sensitive indicator of the presence of poly(A) than is RNAse activity. Furthermore, since both poly(A) and poly(U), at the same concentrations, had an inhibitory effect of less than 10% on enzyme-antibody binding, this would suggest that

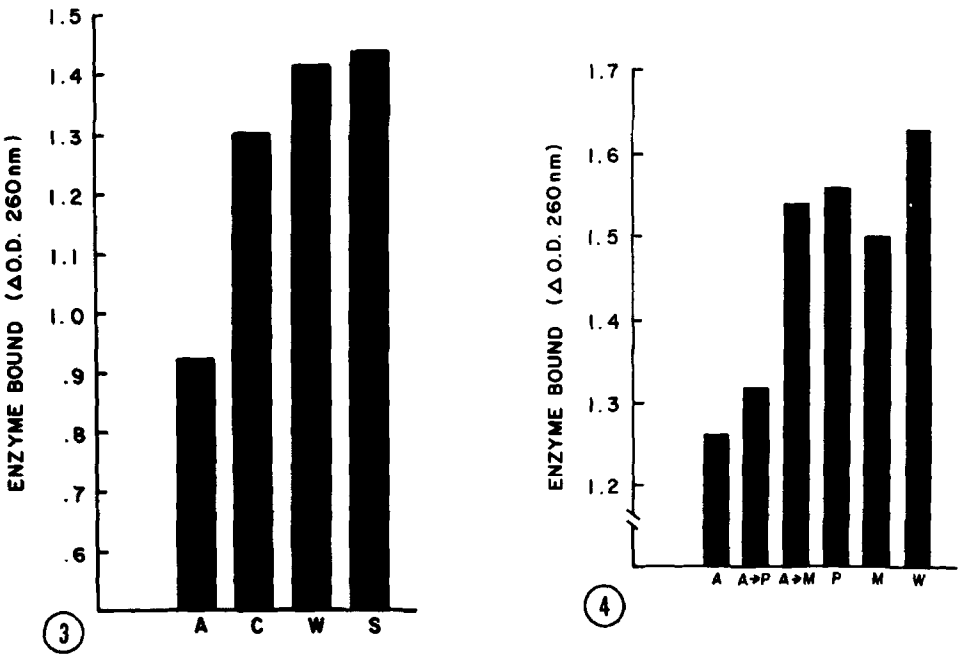

Fig. 3. Comparison of the effects of poly(A), poly(C), and spermidine on enzyme-antibody binding. The reaction mixtures were prepared as described in the METHODS section except that 25 µg of either poly(A) (A), poly(C) (C), or 50 nmoles of spermidine (S) was added after the addition of enzyme. Control tubes containing the standard reaction mixtures were prepared, except that water (W) was added in place of the above indicated additions.

Fig. 4. Effect of putrescine and Mg^{2+} on poly(A)-induced inhibition of RNAse binding to antibody. The reaction mixtures were prepared as described in the METHODS section, except that control tubes received either 25 µg of poly(A) (A), or 50 nmoles of either putrescine (P), or Mg^{2+} (M) or water (W). The experimental tubes received 25 µg of poly(A), followed by either 50 nmoles of putrescine (A → P) or 50 nmoles of Mg^{2+} (A → M).

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the effects of poly(A) were specific. Thus, enzyme-antibody binding is a sensitive and specific means to detect the presence of poly(A), even at buffer concentrations at which enzyme activity is only minimally inhibited by the polynucleotide.

To determine if the effects of spermidine on the reversal of enzyme-antibody binding were unique, other cations at similar concentrations were examined. As shown in Fig. 4, Mg^{2+} appeared to be as effective as was spermidine in reversing the effects of poly(A). Another polyamine, putrescine, on the other hand, was considerably less effective. These results also appear to

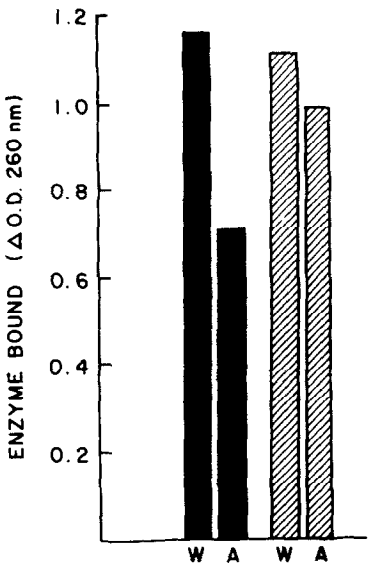

Fig. 5. The effect of the order of addition of poly(A) and AB-Sepharose on enzyme-antibody binding. The reaction mixtures were prepared as in the METHODS section except that 25 µg of poly(A) (A) was added either before (■) or after (▨) the addition of immunoadsorbent. Control reaction mixtures received water (W) instead of poly(A).

parallel the observations made elsewhere with Mg^{2+} and putrescine reversal of polynucleotide inhibition of liver RNAse activity (7).

In all the studies described, the order of addition of the various components of the reaction mixture was strictly maintained. That is, AB-Sepharose was added to the standard reaction mixture after poly(A). If this order was reversed such that AB-Sepharose was added to the reaction mixture before the addition of poly(A), there was significantly less inhibition of enzyme-antibody binding (Fig. 5). This would indicate that under the conditions utilized, antibody and poly(A) appear to be competitive binders of enzyme, and whichever compound binds to the enzyme first seems to remain bound.

To eliminate the possibility that the effects of poly(A) on enzyme-antibody binding were due to polynucleotide binding to antibody rather than to enzyme, [^{3}H]poly(A) was added to both NRS- and AB-Sepharose, as well as to

unconjugated Sepharose 4B. The results of this experiment showed that the same amount of [^{3}H]poly(A) bound to both NRS- and AB-Sepharose as bound to unconjugated Sepharose 4B. Immunodiffusion studies also failed to demonstrate any reaction of poly(A) with liver RNAse antibody. Thus, the effects of poly(A) on enzyme-antibody binding do not appear to be due to the polynucleotide binding to the antibody, but rather to the binding of poly(A) to enzyme. The demonstration of a poly(A)-human liver RNAse complex has been clearly demonstrated by Frank et al. (7).

DISCUSSION

Although poly(A) is known as a potent inhibitor of RNAse activity (5,6, 11,12), an attempt has been made in the present study to examine the interaction of poly(A) and RNAse from a somewhat different perspective; one that would determine if the polynucleotide also affects the antigenicity of the enzyme. It appears from the studies described above that poly(A) does indeed markedly inhibit the binding of human liver RNAse to antibody, which suggests some type of change in antigenicity of the enzyme. Although the precise cause of this change has not been determined, it is significant, nevertheless, that the antigenic effects were present at a buffer concentration at which poly(A) inhibition of enzyme activity was barely detectable. Thus, enzyme-antibody binding appears to be considerably more sensitive to the presence of poly(A) than is inhibition of RNAse activity. Furthermore, the immunology effect of poly(A) is quite specific, since neither poly(U) nor poly(C) has much effect on RNAse binding to antibody.

To determine if the effect of poly(A) on RNAse antigenicity was a generalized phenomenon, the effect of poly(A) on the binding of several other human RNAses to their respective antibodies was studied. With only minor variations, poly(A) was able to inhibit the binding between human spleen (15), pancreas and plasma RNAses (6) and their respective antibodies. Since the human plasma and pancreas RNAses do not appear to have any antigenic sites in common with the human liver RNAse (15), whatever the mechanism of poly(A)'s

effects, that mechanism functions with enzymes that are catalytically and immunologically very different.

It should be noted, moreover, that the same substances (i.e., spermidine and Mg^{2+}) that have been reported to reverse poly(A) inhibition of RNAse activity (7) were also able to reverse the inhibition by poly(A) of enzyme-antibody binding, and with the same relative efficacy. The reversal of the immunological effects of poly(A), then, closely paralleled the reversal of the inhibitory effects on enzyme activity by the polynucleotide.

REFERENCES

1. Darnell, J. E., Wall, R. and Tushinski, R. J. (1970) Proc. Natl. Acad. Sci. U.S.A. 68, 1321-1325
2. Edmonds, M., Vaughan, H. M. and Nakazato, H. (1970) Proc. Natl. Acad. Sci. U.S.A. 68, 1336-1340
3. Mendecki, J., Lee, S.-Y. and Brawerman, G. (1970) Proc. Natl. Acad. Sci. U.S.A. 68, 1331-1335
4. Kates, J. (1970) Cold Spring Harb. Symp. Quant. Biol. 35, 743-762
5. Levy, C. C., Schmukler, M., Frank, J. J., Karpetsky, T. P., Jewett, P. B., Hieter, P. A., LeGendre, S. M. and Dorr, R. G. (1975) Nature 256, 340-342
6. Schmukler, M., Jewett, P. B. and Levy, C. C. (1975) J. Biol. Chem. 250, 2206-2212
7. Frank, J. J. and Levy, C. C. (1976) J. Biol. Chem. 251, 5745-5751
8. Spector, D. H. and Baltimore, D. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2983-2987
9. Huez, G., Marbaix, G., Hubert, E., Leclercq, M., Nudel, U., Soreq, H., Salomon, R., Lebleu, B., Revel, M. and Littauer, U. Z. (1974) Proc. Natl. Acad. Sci. U.S.A. 72, 3143-3146
10. Marbaix, G., Huez, G., Burny, A., Cleuter, Y., Hubert, E., Leclercq, M., Chantrenne, H., Soreq, H., Nudel, U. and Littauer, U. Z. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3065-3067
11. Hieter, P. A., LeGendre, S. M. and Levy, C. C. (1976) J. Biol. Chem. 251, 3287-3293
12. Levy, C. C. (1975) Life Sciences 17, 311-316
13. Bussard, A. E. Antibodies to Biologically Active Molecules (ed. B. Cinader) Pergamon Press, Oxford, 1967, p. 412
14. Pelichova, H., Suzuki, T. and Cinader, B. (1970) J. Immunol. 104, 195-202
15. Neuwelt, E. A., Frank, J. J. and Levy, C. C. (1976) J. Biol. Chem. 251, 5752-5758
16. Neuwelt, E., Stumpf, D., Austin, J. and Kohler, P. (1971) Biochim. Biophys. Acta 236, 333-346
17. Neuwelt, E., and Kohler, P. (1973) Immunochemistry 10, 767-771