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Synergistic fibrinolysis: Combined effects of plasminogen activators and an antibody that inhibits α_2-antiplasmin

(thrombolysis/synergism/inhibitor)

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ABSTRACT **To improve the efficacy of plasminogen activators, we produced a monoclonal antibody (RWR) that inhibits human α_2-antiplasmin (α2AP). In addition to inhibiting α2AP in plasma, RWR binds to and inhibits fibrin cross-linked α2AP and reproduces the "spontaneous" clot lysis that is the hallmark of human α2AP deficiency. By inhibiting the inactivation of plasmin by α2AP, RWR interacts synergistically with plasminogen activators to increase the potency (for 50% clot lysis) of urokinase by 80-fold, tissue plasminogen activator by 27-fold, and streptokinase by 20-fold. Yet, for a given amount of fibrinolysis, the combination of RWR and lower doses of plasminogen activator leads to less fibrinogen consumption than is obtained with higher, equipotent doses of plasminogen activator alone. These results suggest a strategy for increasing the efficacy of plasminogen activators. More generally, this approach to amplifying enzymatic activity by immunoneutralizing an inhibitor may be useful in other biologic processes that are rigidly governed by inhibitors.**

To improve thrombolytic therapy, it will be necessary to increase the rate and extent of clot lysis without inducing a systemic lytic state. One strategy has been to find synergistic combinations of plasminogen activators. Unfortunately, *in vitro* studies have failed to demonstrate synergy between activators and *in vivo* studies have demonstrated synergy only within a narrow dosage range (reviewed in ref. 1).

Another strategy for improving thrombolytic therapy has been to alter plasminogen activators, usually with the goal of modifying their fibrin-binding properties (reviewed in ref. 2). Although this approach is promising, early efforts have been hampered by difficulties with construct instability, expression level, nonfunctional domains, etc.

Previous efforts to improve thrombolytic therapy have all but ignored α_2-antiplasmin (or α_2-plasmin inhibitor) (α2AP), the most important inhibitor of clot lysis *in vivo*. In part, this neglect is due to the belief that the chief function of α2AP in thrombolysis is to inhibit circulating plasmin and prevent a systemic lytic state (3). However, Sakata and Aoki (4) have demonstrated that α2AP, by virtue of its cross-linking to fibrin, is the critical determinant of the clot's resistance to lysis. Their results are substantiated by the observation that clots from α2AP-deficient patients lyse spontaneously, even though these patients show no sign of a systemic lytic state (5, 6).

Given these findings we reasoned that an α2AP inhibitor might increase the efficacy of plasminogen activators. To obtain a specific, high-affinity inhibitor, we generated a monoclonal antibody against human α2AP. This antibody (RWR) interacts synergistically with plasminogen activators and markedly increases clot lysis. More importantly, the combined use of a plasminogen activator and this α2AP inhibitor results in less fibrinogen consumption for a given amount of lysis than does an equipotent dose of the activator alone. These results challenge the conventional view of the role of α2AP in thrombolysis and suggest an innovative strategy for improving plasminogen activator therapy.

MATERIALS AND METHODS

Materials. Materials were purchased from the following suppliers: Protein A-Sepharose, Sigma; human α2AP, American Diagnostica (Greenwich, CT); affinity-purified goat anti-(mouse Fab') (GAMFab), Cappel Laboratories; thrombin, Parke-Davis; Superose-12 column, Pharmacia; tissue plasminogen activator (t-PA), BioResponse (Hayward, CA); urokinase, Abbott; streptokinase, Hoechst-Roussel; pristane (2,6,10,14-tetramethylpentadecane), Pfaltz & Bauer; murine antibody isotyping kit, Boehringer Mannheim. Chromogenic substrates S-2251 (*H*-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride) and S-2288 (*H*-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide dihydrochloride) were purchased from Kabi Vitrum (Helena Laboratories); mice, A/J and CAF strains, were from The Jackson Laboratories; and microtiter plates were from Becton Dickinson Labware.

Preparation of Proteins. α2AP was purified from multidonor frozen plasma by affinity chromatography as described by Wiman (7) followed by fast-protein liquid chromatography on a Superose-12 column. This purification process yielded a single band on SDS/PAGE (8). Plasminogen was isolated from plasma according to the protocol of Deutsch and Mertz (9) and was activated with urokinase (7). Determinations of α2AP and plasminogen concentrations were based on absorption coefficients (A^{1cm}_{280nm}) for a 1% solution of 7.03 and 17.0, respectively (10, 11).

Preparation of Murine Monoclonal Antibodies. Five A/J mice were immunized intraperitoneally and subcutaneously with 10 μg of human α2AP in complete Freund's adjuvant and were given booster injections 1 month later with 2 μg in incomplete Freund's adjuvant. After 2 months, antibody titers were determined by solid-phase radioimmunoassay (12). The mouse showing the highest titer was hyperimmunized with α2AP, 5 μg (aqueous) intravenously and 10 μg intraperitoneally, in incomplete Freund's adjuvant 4 days prior to fusion and again with 5 μg (aqueous, intravenously) 3 days prior to fusion. The splenocytes and murine SP2/0 cells were fused as described (13). Hybridomas were selected by a solid-phase radioimmunoassay with α2AP immobilized in wells of polyvinylchloride microtiter plates (12) by using GAMFab that had been radioiodinated (specific activity, $\approx$7 μCi/μg; 1 Ci = 37 GBq) by the chloramine-T method (14). Hybridomas were cloned by limiting dilution and were expanded into ascites in pristane-primed CAF mice. Antibody was isolated by protein A-Sepharose.

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Abbreviations: α2AP, α_2-antiplasmin; t-PA, tissue plasminogen activator; GAMFab, goat anti-(mouse Fab'); IU, international unit(s).

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Inhibition Studies. The ability of RWR to inhibit the inactivation of plasmin by α2AP was tested in a chromogenic substrate assay (S-2251) described by Wiman (7). α2AP (0–20 nM) was mixed with 0.3 mM S-2251 and plasmin (100 nM) in 0.1 M phosphate buffer (pH 7.3) to a total of 1 ml. The rate of change in optical density at 405 nm was automatically recorded every 10 s on a Hewlett-Packard 8451A spectrophotometer and a standard curve was constructed that related α2AP concentration to the rate of product formation in a linear fashion (r = 0.98). Next, α2AP (20 nM) was mixed with RWR (0.875–350 nM) and incubated with 0.3 mM S-2251 in 0.1 M phosphate buffer (pH 7.3) for 2 hr at 25°C. Plasmin (100 nM) was quickly added and the rate of change in optical density was used to calculate the amount of residual α2AP activity.

Antibody Binding to Plasma Clots. Fresh-frozen human plasma (100 μl) pooled from four random donors was mixed with a 100-μl solution of thrombin (1 unit/ml) and calcium chloride (4 mM) in 50 mM Tris-buffered saline (pH 7.4) in test tubes (75 × 12 mm) and incubated in a water bath for 2 hr at 37°C. The clots were then treated with 100 μl of 0.1% iodoacetamide for 15 min, washed with 2 ml of 10 mM phosphate-buffered saline (pH 7.4), and compressed to extrude unbound proteins. After the clots were incubated overnight at 4°C with monoclonal antibody RWR or a control antibody of the same isotype [anti-digoxin antibody 40–160 (15)], they were washed again with buffer and incubated with ^{125}I-labeled GAMFab (100,000 cpm) for 1 hr at room temperature. After an additional washing, the amount of antibody bound to the plasma clots was determined by γ emission counting.

Time-Related Lysis of Plasma Clots by t-PA. Fifty microliters of fresh-frozen, multidonor human plasma was mixed with trace amounts of ^{125}I-labeled fibrinogen in test tubes (75 × 12 mm) and clotted with a 50-μl solution of thrombin (1 unit/ml) and $CaCl_2$ (50 mM) in 10 mM Tris-buffered saline (pH 7.4) in a 37°C water bath for 2 hr. The clots were counted in a γ-counter and RWR (0 or 250 nM), control anti-digoxin 40–160 (0 or 250 nM), or t-PA [0 or 1 international unit (IU)] was added to the clot in each tube. The total vol of each tube was brought up to 1 ml with 10 mM Tris-buffered saline azide with 2 mM $CaCl_2$. At various intervals a 200-μl aliquot of supernatant was sampled and γ-radiation was counted. The percentage lysis was computed from the initial radioactivity in the clot and the radioactivity released into the supernatant as soluble fibrin degradation products.

To study the effect of RWR on lysis by "endogenous" plasminogen activator, radiolabeled plasma clots were made as described above. Then RWR or the control antibody (final concentration, 500 nM) was added to the clots in each tube (in quadruplicate) and the total vol was brought up to 500 μl with Tris-buffered saline azide containing 2 mM $CaCl_2$. At various intervals, an aliquot of supernatant was sampled and γ-radiation was counted in a γ-counter to compute percentage lysis.

Synergism Studies. The interaction between t-PA and RWR was studied as suggested for combinations of agents by Loewe (16) and Berenbaum (17, 18). First, the dose–response curves for both agents were constructed (18) to determine the isoeffective doses of t-PA and RWR. Then, fractional amounts of the isoeffective doses of the two agents were combined and their joint effect on clot lysis was determined. An isobole was plotted that graphs the amount of each agent, alone or in combination, that produced an equivalent amount of lysis in a given period of time. (The graphical determination of synergy, additivity, and antagonism is shown in Fig. 5*B Inset*.)

To perform the synergism studies, we clotted 25 μl of plasma with trace amounts of ^{125}I-labeled fibrinogen (as described in the previous section). The clots were counted in a γ-counter and then incubated at 37°C with various amounts of t-PA (0–0.03 IU) or RWR (0–500 nM) alone, or in fractional combination. The percentage lysis at 48 hr was determined as described above.

Plasma Clot Lysis by Plasminogen Activators. One hundred-microliter radiolabeled plasma clots were made as described above. The clots were compressed and washed to remove unbound proteins. To the clot in each tube was added 200 μl of fresh-frozen plasma, 75 μl of a plasminogen activator [urokinase (0.31–160 IU), t-PA (0.31–120 IU), or streptokinase (0.41–100 units)], and RWR (1.15 nmol) in Tris-buffered saline (pH 7.4) or Tris-buffered saline alone to make a total vol of 325 μl. The tubes were incubated at 37°C for 1 hr. Then 75 μl of ice-cold phosphate-buffered saline azide (containing 5000 kallikrein inhibitor units of aprotinin per ml) was added to each tube to halt plasminolysis. An aliquot of the liquid supernatant was removed and counted in a γ-counter, and the percentage of clot lysis was determined. The residual fibrinogen concentration in each tube was immediately assayed in triplicate by the sodium sulfite method (19). To confirm these results, fibrinogen levels in the t-PA experiments were also measured by a modified Clauss assay (20).

The specific activities of the t-PA and urokinase were 4.05 × 10^{-5} and 12.1 × 10^{-5} nmol/IU as measured by an S-2288 assay according to the manufacturer's data sheet. Streptokinase was used as supplied by the manufacturer.

RESULTS

Characterization of the Antibody. Of the 21 monoclonal antibodies obtained from the fusion, 7 were selected because of their binding in a solid-phase radioimmunoassay and expanded into ascites. After purification by protein A affinity chromatography, the antibodies were tested for their ability to inhibit the inactivation of human α2AP by plasmin. One of these antibodies, RWR ($Ig\gamma_1$-κ serotype), showed a dose-related inhibition of the interaction between α2AP and plasmin (Fig. 1) achieving 50% inhibition of α2AP at a concentration of 14 nM or at a 0.7:1 molar ratio of RWR to α2AP.

Binding of Antibody to α2AP Cross-Linked to Plasma Clots. Because antibody RWR was capable of binding to and inhibiting α2AP in solution, it was tested for the ability to bind to α2AP that had been cross-linked to fibrin by activated factor XIII. After plasma clots had been treated with 0.1% iodoacetamide to inhibit activated factor XIII [which catalyzes the reversible cross-linking of α2AP to fibrin (21)], they were washed and incubated with various amounts of RWR or a control antibody of the same isotype [anti-digoxin monoclonal antibody 40–160 (15)]. Antibody bound to the plasma

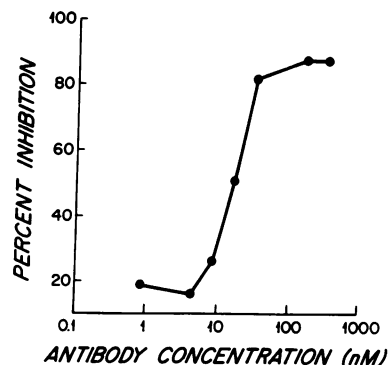

FIG. 1. Inhibition of α2AP by RWR. α2AP (final concentration, 20 nM) was mixed with various amounts of RWR and S-2251 (final concentration, 0.3 mM) for 2 hr at 25°C. Plasmin was added (final concentration, 100 nM) and the percentage of residual α2AP activity was determined. Fifty percent inhibition of α2AP occurred at an antibody concentration of 14 nM.

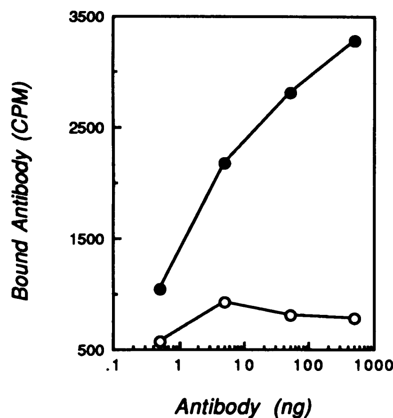

FIG. 2. Binding of RWR to α2AP cross-linked to fibrin (4) in a plasma clot assay. Washed plasma clots were incubated with various concentrations of RWR (solid circles) or an anti-digoxin control antibody (open circles) of the same isotype (15). After the washing, monoclonal antibody bound to the clot was detected with ^{125}I-labeled GAMFab. The means of triplicate observations are shown.

clots was detected with ^{125}I-labeled GAMFab. Fig. 2 shows that, compared with the control antibody, RWR bound in a dose-dependent fashion to α2AP cross-linked to the fibrin clot.

Enhancement of "Endogenous Lysis" by Antibody RWR. The clinical hallmark of human α2AP deficiency is the spontaneous lysis of blood clots *in vitro* (5, 6). This apparently results from the uninhibited action of plasmin generated by endogenous plasminogen activator (22). Because RWR binds to soluble and fibrin cross-linked α2AP, we tested the ability of RWR to reproduce this spontaneous clot lysis. Fig. 3 shows that, compared with the control antibody (anti-digoxin 40–160), RWR caused increasing "endogenous" or spontaneous lysis. Thus, RWR reproduced the spontaneous clot lysis seen in human α2AP deficiency.

Clot Lysis by t-PA and RWR. Since t-PA preferentially activates fibrin-bound plasminogen to plasmin, and fibrin-bound plasmin is relatively resistant to the inhibitory effects of α2AP (23), it might be expected that clot lysis by t-PA would not be significantly affected by an α2AP inhibitor. To test this hypothesis, the rate of fibrinolyis was measured in plasma clots treated with RWR or a control antibody alone, or RWR or the control antibody in combination with t-PA. During the relatively brief time of this experiment (5 hr; cf. Fig. 3), RWR alone did not cause more lysis than the control antibody alone (Fig. 4). The combination of the control antibody and t-PA caused slightly more clot lysis. However, RWR and t-PA in combination almost completely lysed the clot. The marked enhancement of clot lysis by the combination of RWR and t-PA suggested that the two agents interact synergistically.

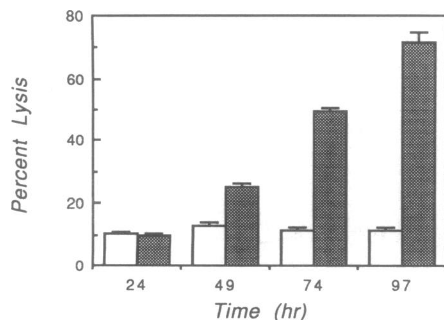

FIG. 3. Enhancement of "endogenous" clot lysis by monoclonal antibody RWR. Plasma was clotted with trace amounts of ^{125}I-labeled fibrinogen. The clots were incubated with RWR (stippled bars) or a control antibody (anti-digoxin 40–160; open bars). At the indicated times, the percentage clot lysis was determined. The means and SEMs of quadruplicate observations are shown.

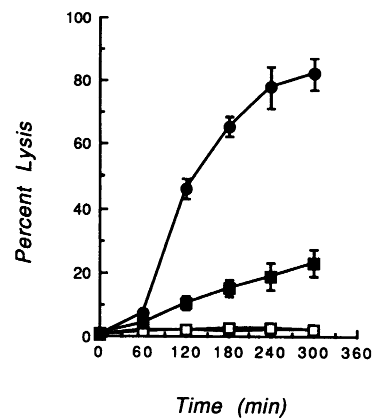

FIG. 4. Plasma clot lysis by t-PA and monoclonal antibody RWR or a control antibody. Clots were incubated with a 250 nM concentration of RWR (open circles), control antibody alone (open squares) (note that these two curves are superimposed), or a 250 nM concentration of RWR plus 5 IU of t-PA (solid circles) or control antibody plus 5 IU of t-PA (solid squares). At the indicated times, the percentage clot lysis was determined. The data represent the means and SEMs of observations in triplicate.

Synergistic Interaction Between t-PA and RWR. We studied the hypothesis of synergy between RWR and t-PA by applying the pharmacologic criteria of Loewe (16) and Berenbaum (17, 18). To avoid mathematical assumptions about the dose–response curves of t-PA and RWR, it was necessary to determine an isoeffective dose for each (i.e., the amount of each agent that produces an equivalent effect). Fig. 5*A* shows the dose–response curve for t-PA compared with that for RWR. A t-PA dose of 0.03 IU/ml (1.2×10^{-3} nM) and a 500 nM dose of RWR were found to be isoeffective.

The next step in the analysis was to test the effect on clot lysis of various fractional combinations of RWR and t-PA. The results were then plotted on an isobole, a graph that depicts the isoeffective doses of the two agents alone or in combination. For the purpose of illustration, a hypothetical isobole is shown in Fig. 5*B (Inset)*. If the isoeffective doses of the two agents in combination lie along a straight line drawn between the isoeffective doses of the two agents alone, the agents are additive. If the isoeffective doses lie along a convex line at a greater distance from the origin than from the line of additivity, the agents are antagonistic. But if the two agents in combination lie along a concave line closer to the origin than to the line of additivity, the agents are synergistic.

The empirical isobole for these experiments is shown in Fig. 5*B*. Solid circles represent the doses of t-PA and RWR alone or in combination that produced equivalent mean lysis (defined as 35% ± 1%). Open circles represent, for the purpose of illustration, the dose combinations of RWR and t-PA that caused even greater mean lysis (41–100%). The fact that such small doses of the two agents in combination produce equal or greater lysis than much larger doses of the two agents alone indicates a potent synergistic interaction between RWR and t-PA.

Effect of RWR on the Potency of Plasminogen Activators. The effect of α2AP inhibition on the potency of urokinase, streptokinase, and t-PA was studied in a quantitative clot lysis assay. The results suggest that inhibition of α2AP strikingly increases the potency of all three plasminogen activators. Fig. 6 shows the amount of each activator (logarithmic scale) necessary to produce clot lysis in the presence and absence of RWR. A comparison of the amount of urokinase necessary to produce 50% lysis indicates that RWR

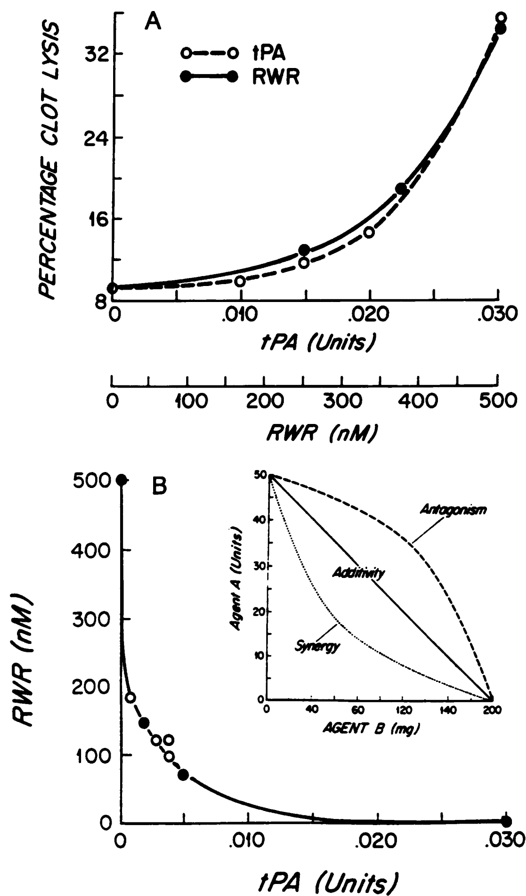

FIG. 5. The synergistic effects of t-PA and RWR on plasma clot lysis. To determine the effect of combinations of t-PA and RWR on lysis, we constructed an isobole as recommended by previous studies (16–18). (*A*) First, isoeffective doses of t-PA and RWR were determined. In these studies, t-PA (0.03 IU/ml; 1.2×10^{-3} nM) (open circles) and 500 nM RWR (solid circles) produced an equivalent percent lysis (35.5 ± 3.1 and 34.3 ± 5.1; mean ± SEM, observations in triplicate). (*B*) (*Inset*) Hypothetical isobole [cf. Berenbaum (18)], demonstrating synergy, additivity, and antagonism (see *Results* for explanation). (*B*) Experimental isobole illustrating the interaction between t-PA and RWR in clot lysis. Dose combinations of RWR and t-PA that produced mean clot lysis equivalent to lysis with these agents alone are indicated by solid circles. Open circles denote dose combinations showing still higher mean lysis (40–100%). Compared with each agent alone, combinations of RWR and t-PA are strongly synergistic.

increases the potency of urokinase by ≈80-fold. A similar comparison for t-PA and streptokinase demonstrates that RWR increases the potency of these activators by about 27-fold and 20-fold.

Effect of α2AP Inhibition on the Degradation of Human Fibrinogen. It has been hypothesized that nonspecific proteolysis of clotting factors such as fibrinogen may be associated with a higher risk of bleeding during thrombolytic therapy (24). To study the effect of α2AP inhibition on the specific degradation of fibrin compared with the nonspecific degradation of fibrinogen, we determined the amount of fibrinogen consumption as a function of fibrinolysis. Fig. 7 shows the effects on fibrinogen levels of RWR and the three plasminogen activators (with and without RWR). In clots treated with urokinase alone, fibrinogen levels fell quickly as a function of lysis (Fig. 7*A*). However, when the clots were treated with urokinase and RWR there was a notable preservation of fibrinogen despite even greater clot lysis. A similar, though less striking, preservation of fibrinogen was seen for t-PA (Fig. 7*B*) even when, for confirmation, fibrinogen was measured by a different

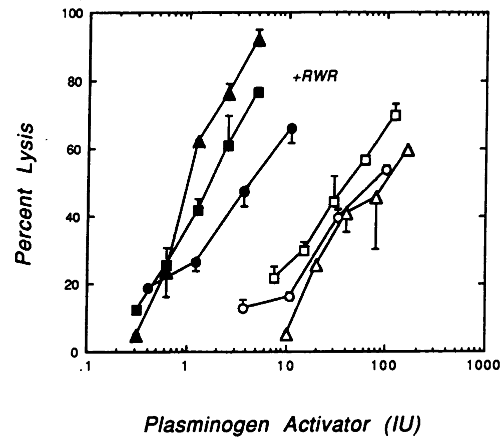

FIG. 6. The effect of monoclonal antibody RWR on the potency of plasminogen activators in a plasma clot lysis assay. Radiolabeled plasma clots were suspended in plasma containing various amounts of urokinase (triangles), t-PA (squares), or streptokinase (circles), with (solid symbols) or without (open symbols) monoclonal antibody RWR. The means ± SEM are shown.

method (20) (data not shown). For streptokinase also there was a greater preservation of fibrinogen, as a function of lysis, in the presence of antibody RWR (Fig. 7*C*).

DISCUSSION

Our *in vitro* clot lysis experiments demonstrate that an inhibitor of α2AP markedly increased the fibrinolytic potency of urokinase, streptokinase, and t-PA. The enhanced lysis results from synergy between the α2AP inhibitor and a plasminogen activator, as was rigorously shown for t-PA. The synergy is due to the fact that both agents increased the concentration of plasmin by different mechanisms. Yet in spite of the potent fibrinolysis produced by the plasminogen activators in combination with RWR, there was less fibrinogenolysis with the combined agents than with a larger, equipotent dose of plasminogen activator alone.

In previous models of fibrinolysis (3), the chief role assigned to α2AP has been to inactivate circulating plasmin and prevent a systemic lytic state. Thus, it may be surprising that an α2AP inhibitor can increase clot lysis without increasing fibrinogenolysis. Perhaps the inhibitory antibody, although selected for binding to free α2AP, was a better inhibitor of fibrin-bound α2AP. Since this antibody augmented clot lysis by a fibrin-selective agent such as t-PA as well as that by the nonselective activators urokinase and streptokinase, it appears that fibrin-bound α2AP plays a critical role in determining the rate of lysis by any exogenous plasminogen activator. This observation extends the earlier finding that fibrin-bound α2AP is the chief deterrent to clot lysis by the endogenous plasminogen activator t-PA (4).

Antibody RWR appeared to have a greater effect on the lytic potency of urokinase and t-PA than on that of streptokinase. This difference may be due to the fact that α2AP is a slow inhibitor of both urokinase and t-PA but has no significant effect on the streptokinase–plasmin complex (10, 25, 26). It also appeared that α2AP inhibition augmented the fibrinolytic potency of urokinase more than it did t-PA. This is consistent with previous findings that t-PA is relatively less sensitive to the inhibitory effects of α2AP, probably because of its fibrin binding ability and preferential activation of fibrin-bound plasminogen (23). The antibody's pronounced enhancement of urokinase-induced clot lysis also magnified its fibrinogen-sparing effects with this plasminogen activator.

At the doses routinely used for thrombolytic therapy, even fibrin-selective plasminogen activators like t-PA can cause

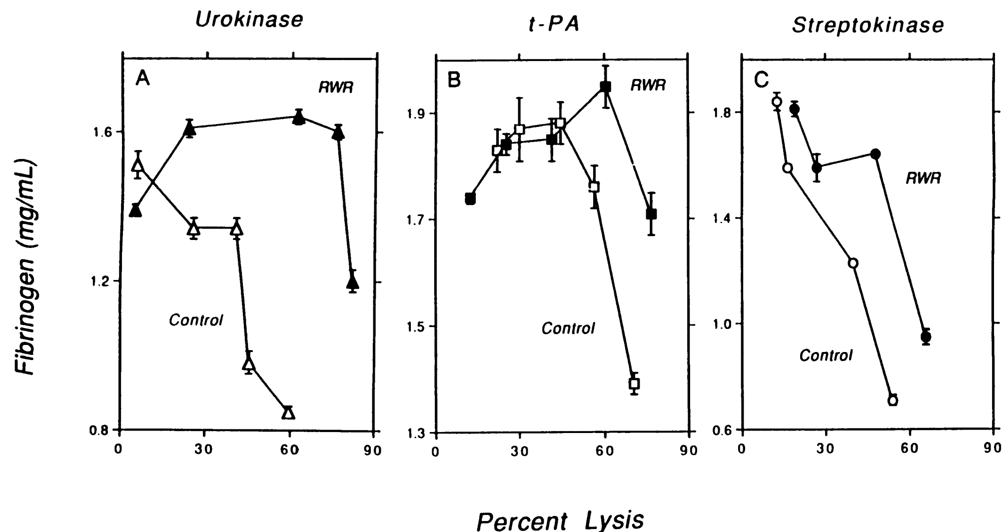

FIG. 7. Residual fibrinogen concentrations as a function of plasma clot lysis with and without α2AP inhibition by RWR. The plasma clot lysis experiments were performed as described in Fig. 6, with various amounts of urokinase (triangles in *A*), t-PA (squares in *B*), or streptokinase (circles in *C*) with (solid symbols) or without (open symbols) monoclonal antibody RWR. After 1 hr the residual fibrinogen concentration was measured in triplicate by the sodium sulfite precipitation method (19). The means ± SEM are shown.

widespread activation of plasminogen and degradation of clotting factors such as fibrinogen. Since this may increase the odds of hemorrhagic complications (24), researchers have sought to identify synergistic combinations of agents that could enhance fibrinolysis without increasing fibrinogenolysis. Although synergy between plasminogen activators has been described within a narrow dosage range in some *in vivo* experiments, results have been inconsistent and efforts to demonstrate synergy *in vitro* have been unsuccessful (1). However, our demonstration of synergy between the α2AP inhibitor and t-PA suggests that the combination of these agents can significantly increase the potency and specificity of thrombolytic therapy.

In a more general sense, our results demonstrate that a monoclonal antibody can be used to augment the catalytic function of an enzyme by neutralizing an inhibitor. This approach could be particularly useful in biologic processes that are tightly governed by inhibitors. Because coagulation is a finely balanced system in which the effects of enzymes (generally serine proteases) are pitted against the effects of inhibitors [frequently serpins (serine protease inhibitors)], pathological alterations in clotting can be treated by augmenting enzyme activity or by neutralizing an inhibitor. In these experiments, we have exploited both approaches—enzyme activation and inhibitor neutralization—to modulate a model thrombotic state.

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1. Collen, D., Stump, D. C. & Gold, H. K. (1988) *Annu. Rev. Med.* **39,** 405–423.
2. Haber, E., Quertermous, T., Matsueda, G. R. & Runge, M. S. (1989) *Science* **243,** 51–56.
3. Francis, C. W. & Marder, V. J. (1986) *Annu. Rev. Med.* **37,** 187–204.
4. Sakata, Y. & Aoki, N. (1980) *J. Clin. Invest.* **65,** 290–297.
5. Aoki, N., Saito, H., Kamiya, T., Koie, K., Sakata, Y. & Kobakura, M. (1979) *J. Clin. Invest.* **63,** 877–884.
6. Miles, L. A., Plow, E. F., Donnelly, K. J., Hougie, C. & Griffin, J. H. (1982) *Blood* **59,** 1246–1251.
7. Wiman, B. (1981) *Methods Enzymol.* **80,** 395–408.
8. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
9. Deutsch, D. G. & Mertz, E. T. (1970) *Science* **170,** 1095–1096.
10. Moroi, M. & Aoki, N. (1976) *J. Biol. Chem.* **251,** 5956–5965.
11. Robbins, K. C., Summaria, L., Elwyn, D. & Barlow, G. H. (1965) *J. Biol. Chem.* **240,** 541–550.
12. Eshhar, Z. (1985) in *Hybridoma Technology in the Biosciences and Medicine*, ed. Springer, T. A. (Plenum, New York), pp. 3–41.
13. Galfre, G., Howe, S., Milstein, C., Butcher, G. W. & Howard, J. (1977) *Nature (London)* **266,** 550–552.
14. Greenwood, F. C. & Hunter, W. M. (1963) *Biochem. J.* **89,** 114–123.
15. Mudgett-Hunter, M., Anderson, W., Haber, E. & Margolies, M. N. (1985) *Mol. Immunol.* **22,** 477–488.
16. Loewe, S. (1957) *Pharmacol. Rev.* **9,** 237–242.
17. Berenbaum, M. C. (1981) *Adv. Cancer Res.* **35,** 269–335.
18. Berenbaum, M. C. (1985) *J. Theor. Biol.* **114,** 413–431.
19. Rampling, M. W. & Gaffney, P. J. (1976) *Clin. Chim. Acta* **67,** 43–52.
20. Vermylen, C., De Vreker, R. A. & Verstraete, M. (1963) *Clin. Chim. Acta* **8,** 418–424.
21. Mimuro, J., Shigeru, K. & Aoki, N. (1986) *J. Clin. Invest.* **77,** 1006–1013.
22. Aoki, N., Sakata, Y. & Ichinose, A. (1983) *Blood* **62,** 1118–1122.
23. Matsuo, O., Rijken, D. C. & Collen, D. (1981) *Thromb. Haemostasis* **45,** 225–229.
24. Verstraete, M. & Collen, D. (1986) *Blood* **67,** 1529–1541.
25. Zamarron, C., Lijnen, H. R. & Collen, D. (1984) *Thromb. Res.* **35,** 335–345.
26. Wiman, B. (1980) *Thromb. Res.* **17,** 143–152.