Immunohistochemical Study of Thrombolytic Mechanisms

By N. Back, R. Hiramoto and J. L. Ambrus

In previous reports\textsuperscript{1-3} various methods, including radiotracer studies, revealed the capacity of plasmin to adsorb selectively to preformed venous and arterial clots. Components of the fibrinolytic system were labeled with iodine\textsuperscript{131} and their distribution studied following intravenous administration. The highest degree of adsorption to fibrin clots was exhibited by human plasminogen activated with streptokinase (SK) or urokinase (UK). Spontaneously activated human plasmin or chloroform-activated bovine plasmin exhibited less localizing capacity. The inactive precursor plasminogen also localized in clots to some extent. On the other hand, plasminogen activators as UK and SK given alone behaved as the radio-iodinated albumin control.

Both experimentally and clinically\textsuperscript{1,4-6} clot lysis following plasmin infusion was found to proceed long after the disappearance of any measurable circulating fibrinolytic activity. Radioactivity measurement revealed the presence of a plasmin-antiplasmin complex which seems to release free plasmin to the fibrin substrate.

The studies with I\textsuperscript{131}-labeled plasmin preparations involve two sources of error. First, since even the purest plasmin preparations available at present contain various protein impurities, the danger exists that part of the I\textsuperscript{131}-labeled material which was found to localize in fibrin clots actually represented impurities rather than plasmin itself. Second, it was shown\textsuperscript{1} that radioiodination alters enzymatic activities to some extent and what was measured actually was the distribution of partially denatured enzymes. For this reason it was decided to repeat these experiments using immunofluorescent technics.

Materials and Methods

Preparation of Antisera

Antisera against various components of the fibrinolytic system were prepared in mature male albino rabbits. The antigens and doses used are indicated in table 1. Preparative methods, sources of components, methods of activation, assays and units were described previously\textsuperscript{5,6} and are also indicated in table 1. One RPMI unit of plasmin activity dissolves a 0.3 per cent purified human fibrin clot made with 1 NIH unit of purified thrombin in 2 minutes at 45 C. and pH 7.2. One RPMI unit of plasminogen converts into 1 RPMI unit of plasmin at an optimal concentration of streptokinase. Animals received the antigens intravenously 3 times a week for 3 weeks and blood was collected 1 month after the first injection. The antisera were adsorbed with rat liver sediments to remove...
Table 1

<table>
<thead>
<tr>
<th>Antigen Preparation</th>
<th>Dose (3 Times a Week for 3 Weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptokinase*-activated human plasmin†</td>
<td>100 U/Kg.</td>
</tr>
<tr>
<td>Urokinase†-activated human plasmin†</td>
<td>100 U/Kg.</td>
</tr>
<tr>
<td>Urokinase†</td>
<td>10,000 U/Kg.</td>
</tr>
<tr>
<td>Streptokinase*</td>
<td>10,000 U/Kg.</td>
</tr>
<tr>
<td>Human plasminogen†</td>
<td>100 U/Kg.</td>
</tr>
<tr>
<td>Chloroform-activated bovine plasmin§</td>
<td>100 U/Kg.</td>
</tr>
<tr>
<td>Spontaneously activated human plasmin</td>
<td></td>
</tr>
</tbody>
</table>

*Purified Streptokinase obtained from Merck Sharp and Dohme Co., West Point, Pa.
†Human Urokinase obtained from Parke Davis Co., Detroit, Mich.
§Chloroform activated bovine plasmin obtained from Parke Davis and Co., Detroit, Mich.
||Spontaneously activated human plasmin obtained from Michigan State Laboratories, Lansing, Mich., and prepared in this laboratory.5,6

One RPMI unit of plasmin activity dissolves a 0.3 per cent purified human fibrin clot made with 1 NIH unit of purified thrombin in 2 minutes at 45 C. and pH 7.2. One RPMI unit of plasminogen converts into 1 RPMI unit of plasmin at an optimal concentration of streptokinase.

Fluorescent materials that stain tissue in a nonspecific manner.7 One ml. of antiserum was added to washed rat liver homogenate sediments prepared from 5 ml. of a 20 per cent suspension. The adsorption was made at room temperature for 30 minutes with constant agitation on a wrist-action shaker. Adsorptions were made in this manner for all antisera. The presence and homogeneity of the antibody preparations were analyzed using the Ouchterlony plate technic.8

**In Vivo Clot Formation**

Femoral and jugular vein clots were prepared in anesthetized dogs with canine, bovine and human fibrinogen by a previously described technic.9 A section of the vein was isolated and artery clamps placed on both proximal and distal ends. All side branches of the vessel were ligated except one into which was inserted a fine polyethylene cannula. Dog blood clots were formed by retaining blood in the ligated portion of the vessel and introducing purified human thrombin* (1 NIH unit) through the cannula. Bovine clots were made by replacing the blood from the isolated vessel with purified bovine fibrinogen followed by the human thrombin. In like manner introduction of human fibrinogen permitted the formation of human fibrin clots. The proximal clamps were removed ¼ hour following clot formation and 1 hour later the distal clamps replaced with semiconstricting ligatures to prevent the escape of the clot. Methods for the preparation of purified fibrinogen were reported previously.6

The plasmin or activator preparations (antigens) were injected intravenously and clot removed 1 hour later. The 1 hour time was established previously as the time for maximal uptake by clots of the enzymes.7 Control clots were excluded from the circulation prior to enzyme injection, removed and treated in a manner identical to that of the experimental clots.

**Indirect Immunofluorescent Technic**

The indirect staining technic of Coombs10 was employed as represented schematically in figure 1. The blood clots with antigen localized onto the surface were quick-frozen,

*Purified bovine thrombin supplied by Parke Davis Co.
sectioned, and serial sections mounted on glass slides. The slides then were incubated with
the specific antiserum for 1 hour, washed, and then incubated for 1 more hour with fluo-
rescein-labeled horse antirabbit gamma globulin reagent. Control slides were incubated
with unlabeled horse antirabbit gamma globulin before exposure to the labeled horse
antirabbit gamma globulin to demonstrate specific blocking of localization of the latter
by the unlabeled antibodies. The slides were washed once again and observations on
the fixation of the fluorescein-labeled reagent made on a Reichert ultraviolet microscope.
The degree of fluorescence on experimental clots was compared with that of control clots.
Comparisons also were made amongst the three different types of clots.

Results

Ouchterlony Gel Precipitation Data

Figure 2 diagrammatically represents the precipitation lines obtained with
the various preparations. A single line was obtained with the activators UK
and SK. Two definite bands formed when the UK-plasmin antiserum was
applied against the antigens UK-plasmin and plasminogen. Similar cross-re-
actions against these antigens were obtained when plasminogen and SK-
plasmin antisera were used. It was of interest to note that spontaneously ac-
tivated human plasmin antisera formed only one band with UK-plasmin, SK-
plasmin, spontaneously activated human plasmin, and plasminogen antigens.
Figure 3 shows an experiment in which human serum containing antibodies
against SK-plasmin is placed into the central well and various concentrations
of SK into the surrounding wells. Precipitation bands are seen up to an SK
**Fig. 2.—Diagram of Ouchterlony gel precipitation experiment.**

concentration of 2500 Christensen units\(^5\)\(^,\)\(^6\) per ml. Lower SK levels are apparently not sufficient to produce visible precipitation lines.

**Degree of Fluorescence on Clots**

Table 2 summarizes the degree of fluorescence (on an arbitrary scale) observed on human fibrin clots in dogs. Antisera to SK-human plasmin, UK-human plasmin and spontaneously activated plasmin indicated strong localization onto all three types of clots. Localization of chloroform-activated bovine plasmin occurred to a lesser extent. This was true for all three types of fibrin clots used. Significant localization of human plasminogen occurred in clots. On the other hand, SK or UK, in the doses employed, did not localize to any significant extent. It is of interest to note, that no localization of anti-SK antibodies occurred on clots demonstrating heavy localization of SK-activated human plasmin. This may be due to the relatively small amounts of SK used in these preparations. Control clots excluded from the circulation during infusion of the antigens showed no fluorescence when exposed to any of the antisera. Clots treated in vitro with the fibrinolytic enzymes also showed a high degree of localization, whereas fluorescence on clots following treatment with activators was minimal.

Figure 4 is a composite of microphotographs of clots removed from dogs receiving a variety of plasmin preparations. Clots 1–4 are from dogs that received SK-human plasmin, UK-human plasmin, spontaneously activated human plasmin and human plasminogen respectively. All clots demonstrate localization. Extensive diffusion of the plasmin and plasminogen into the clot meshwork can be seen. Adsorption of plasmin onto the endothelial surface of the vessel also can be seen in photographs 1, 2 and 4.
Fig. 3.—Photograph and diagram of Ouchterlony gel precipitation experiment.

DISCUSSION

The indirect immunofluorescent technic has been applied to the study of thrombolytic mechanisms. Antisera against components and activators of the fibrinolysis system have been produced successfully in experimental animals. Immunodiffusion studies have revealed the heterogeneity of various plasmin preparations, a finding reported already by others.\textsuperscript{12} Antigenic similarities were found amongst the various plasmins activated by a variety of activators. Distribution studies using presently available plasmin preparations will continue to be hampered by the presence of contaminants. However, the immunologic approach described here tends to confirm results obtained with radioisotope-labeled enzymes.\textsuperscript{1}

Antibodies against SK have been demonstrated using Ouchterlony gel diffusion technics. Anti-SK antibodies have been studied in immune sera.\textsuperscript{13-16} Only a single precipitating band was observed against SK and UK. In man, SK is highly antigenic, whereas no antigenicity has been reported against human UK. The low levels of SK and UK used in activating the human plasminogen were insufficient to produce antibodies in the rabbit injected with the activated plasmins, and explains, in part, the failure of SK or UK antisera to localize onto clots perfused with SK- or UK-human plasmin. On Ouchterlony plates antibodies produced in man following treatment with SK-plasmin formed precipitin bands with high concentrations of SK, but not with concentrations below 2500 Christensen units/ml.
Table 2.—In Vivo Localization of Components of Fibrinolysin System onto Fibrin Clots in Dogs

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>ANTI-SK PLASMIN</th>
<th>ANTI-SK</th>
<th>ANTI-SK SPONTANEOUS PLASMIN</th>
<th>ANTI-UK PLASMIN</th>
<th>ANTI-UK BOVINE PLASMIN</th>
<th>ANTI-PLASMINOGEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-HUMAN PLASMIN 100 u/kg</td>
<td>4+</td>
<td>0</td>
<td>4+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STREPTOKINASE 1x10^6 u/kg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UK-HUMAN PLASMIN 100 u/kg</td>
<td>3+</td>
<td></td>
<td>4+</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UROKINASE 1x10^6 u/kg</td>
<td></td>
<td>0</td>
<td>0</td>
<td>1+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPONTANEOUS HUMAN PLASMIN 100 u/kg</td>
<td>4+</td>
<td>0</td>
<td>4+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BOVINE PLASMIN 100 u/kg</td>
<td></td>
<td>0</td>
<td>1+</td>
<td>2+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUMAN PLASMINOGEN 100 u/kg</td>
<td>±</td>
<td>2+</td>
<td>3+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Use of immunofluorescent technic has permitted confirmation and extension of previous observations that all types of fibrinolytic enzymes adsorb to some extent to fibrin clots in contrast to activators (UK, SK) which showed no such localizing abilities. The indirect "sandwich" fluorescent method is considered more sensitive than the direct method, permitting use of unconjugated sera or protein. Use of unlabeled fibrinolytic enzyme preparations offers advantages over the previously used radioactive tracer technic study because (a) histologic localization of the antibody can be traced, (b) greater specificity can be obtained, and (c) the protein to be studied is in unaltered form. Previous radioisotope studies suffered from the disadvantage of alterations and partial inactivation of the enzyme preparations following radioiodination. Both the previous and present study share a mutual disadvantage, that of using preparations known to be antigenically heterogenous. Nevertheless, the similarity of the results in spite of differences in sources of errors tends to confirm the validity of the conclusions. It is unlikely that impurities which represent probably only a portion of the proteins in the enzyme preparation would represent the majority of the antigenicity.

In many instances, the enzymes administered appeared to penetrate into the central portions of the clots. Thus, visual demonstration has been provided, perhaps for the first time, that plasmin not only adsorbs onto the surface of, but diffuses into, the clots.
Fig. 4.—Microphotographs of immunohistologic preparations of clots removed from dogs 1 hour after injection with various plasmin preparations: (1) SK-human plasmin, 100 RPMI U/Kg.; UK-human plasmin, 100 RPMI U/Kg.; (3) spontaneously activated human plasmin, 100 RPMI U/Kg.; (4) human plasminogen, 100 RPMI U/Kg.

In many immunohistologic sections, the intimal lining of the blood vessels appeared to fluoresce strongly following administration of plasmin. This may suggest the adsorption of the enzyme onto a fibrin film covering the vascular intima, a concept that has both proponents and opponents. The possibility cannot be excluded that fibrin present on the vessel wall might have formed as a result of the procedure used to initiate thrombosis or due to the post-mortem handling of the tissue. This question presently is under investigation.

**Summary**

The indirect immunofluorescent staining technic has been employed to study the localization onto fibrin clots of components of the fibrinolysin system and its activators. Immunodiffusion technics revealed the heterogeneity of the various enzyme preparations used. The activated fibrinolytic enzyme preparations were found to localize onto and diffuse into the matrix and core of the clots. High degree of localization was seen with streptokinase-, urokinase- and spontaneously activated human plasmin, as well as human plasminogen. Chloroform-activated bovine plasmin localized to a lesser extent. No differences were observed in the results whether the fibrin clots were of human, canine or bovine origin.

**Somario in Interlingua**

Indirecte technicas de tincturation immunofluorescentic esseva empletate in studiar le localisation, super coagulos de fibrina, de componentes del
IMMUNOHISTOCHEMICAL STUDY OF THROMBOLYTIC MECHANISMS

systema de fibrinolysina e de su activatores. Technicas immunodiffusional revelava le heterogeneitate del vari preparatos enzymatic usate. Esseva trovate que le activate preparatos de enzyma fibrinolytic se localisa super le matrice, diffundente se ad in illo e le interior del coagulos. Un alte grado de localisation esseva notate in le caso de plasmina human activate per medio de streptocinase, per medio de urocinase, o spontaneemente, sed etiam in le caso de plasminogeno human. Plasmina bovin activate per medio de chloroform se localisava minus extensemente. Nulle differentias esseva observate in le resultatos secundo que le coagulos de fibrina esseva de origine human, canin, o bovin.

ACKNOWLEDGMENT

We wish to express our appreciation to Paul Gandel (Brown University) who assisted in this project while holding a summer fellowship at the Roswell Park Memorial Institute.

REFERENCES

15. Ambrus, J. L., Ambrus, C. M., Sokal, J. E., Back, N., and Metzger, R.:


Nathan Back, D.Sc., Department of Biochemical Pharmacology, State University of New York at Buffalo, School of Pharmacy, Buffalo, N. Y.

Raymond Hiramoto, Ph.D., Department of Pediatrics, St. Jude's Hospital, Memphis, Tenn.

Julian L. Ambrus, M.D., Ph.D., Roswell Park Memorial Institute, Buffalo, N. Y.
Immunohistochemical Study of Thrombolytic Mechanisms

N. BACK, R. HIRAMOTO and J. L. AMBRUS