## Antigenic Studies on Human Plasminogen

Although the human serum protease, plasmin (fibrinolysin), and its inactive precursor, plasminogen (profibrinolysin), have been studied extensively[1], attempts to determine the antigenic properties of plasminogen preparations have not been reported. This communication presents evidence for the inhibition of the proteolytic activity of streptokinase (SK)-activated human plasminogen by rabbit antiserum prepared against purified human plasminogen (PHP) and for the antigenic heterogeneity of the plasminogen preparation.

Human plasminogen was prepared according to KLINE[2] from Human Fraction III and lyophilized. The final preparation contained 90 $\mu$g N/mg, and 25 $\mu$g N of this material, when activated by SK, had a proteolytic activity equivalent to that of 11 $\mu$g crystalline trypsin (Worthington). The major part of this preparation migrated as a single component on paper electrophoresis at pH 8·6 in 0·075 $\mu$ Veronal buffer; however, 10–15% appeared as a second component constantly.

Two adult New Zealand white rabbits were injected intravenously with 5 mg PHP at weekly intervals for three weeks. Samples of serum at the fourth week indicated precipitin titers of only 1:100 by the ring test. At this time 1 ml of a preparation of PHP in the FREUND and MCDERMOTT[3] adjuvant (8·4 mg PHP/ml) was injected subcutaneously. After two weekly injections of this antigen the serum from each animal gave a precipitin titer of 1:10000. These animals, as well as two similar untreated animals, were exsanguinated; the pooled sera were designated, respectively, immune rabbit serum (IRS) and normal rabbit serum (NRS).

Each of these pools of serum was tested for its effect on the proteolytic activity of SK-activated PHP by the casein digestion method previously described[4]. Essentially, this method consists in the determination of trichloroacetic acid soluble products in the digest by the FOLIN-CIOCALTEAU phenol procedure. Color values were read at 650 m$\mu$ in a Lumetron Colorimeter. Table I shows an increased proteolytic activity over that exhibited by PHP alone in the PHP-serum mixtures containing either IRS or NRS. Less activity was recorded with the PHP-IRS mixture than with the PHP–NRS, but the results did not conclusively demonstrate an inhibition by the rabbit antiserum. These findings were to be expected, since whole rabbit serum itself contains considerable plasminogen which is activated by PHP–SK mixtures[5].

To eliminate the complicating effects of the presence in whole serum of rabbit plasminogen, both the IRS and NRS were fractionated by the ethanol method of NICHOL and DEUTSCH[6]. The rabbit proteolytic proenzyme was found to be localized largely in their precipitate B (beta- and gamma$_1$-globulin) while the antibody-containing fraction, precipitate C (gamma$_2$-globulin) was virtually free of rabbit plasminogen. (Subsequent studies in this laboratory by continuous flow electrophoresis of rabbit serum have confirmed the occurrence of plasminogen in the beta-gamma$_1$-globulin area. It is significant that no normal inhibitors for rabbit or human plasmin were observed in the slower migrating gamma-globulins.)

*Table I*

Effect of whole normal and immune rabbit sera and of fractions therefrom on the proteolytic activity of SK-activated human plasminogen

| Contents of Digestion Mixture[7] | Optical Density |
|---|---|
| PHP[8] | 0·370 |
| PHP + immune rabbit serum (IRS) (0·4 ml) | 0·505 |
| PHP + normal rabbit serum (NRS) (0·4 ml) | 0·710 |
| PHP + ppt B[9] from NRS (0·684 mg N) | too high to read |
| PHP + ppt B from IRS (0·696 mg N) | too high to read |
| PHP + ppt C[9] from NRS (0·752 mg N) | 0·370 |
| PHP + ppt C from IRS (0·776 mg N) | 0·130 |
| PHP + ppt C from IRS (0·752 mg N) (incubated 30 min at 37°C before activation with SK) | 0·115 |

A repetition of tests with the casein digestion procedure, using the immune gamma-globulin (IGG) and normal gamma-globulin (NGG), in place of the original whole sera, showed that the IGG exhibited a definite inhibitory action on the proteolytic activity of SK-activated PHP, while the NGG did not affect the result (Table I). Specific precipitates were formed with IGG and not with NGG. The precipitates were not centrifuged off before enzyme assays, and hence it was demonstrated that the plasminogen in the precipitated complex either could not be activated by SK, or the activity of the plasmin formed was inhibited. Incubation of IGG and PHP for 30 min at 37°C before activation reduced the final activity only about 3%. Similar results were obtained at other antibody concentrations suggesting that the major activity of the antibody was to inhibit plasmin rather than its activation by SK. However, further study of this point is required to elucidate this mechanism.

It was necessary to prepare a new lot of antiserum to proceed with further quantitative studies. The materials used were as described above: 5 rabbits were immunized by three weekly subcutaneous injections of PHP in the FREUND-MCDERMOTT adjuvant (total, 25 mg PHP/animal). Pooled serum from these animals, collected on the fourth week, had a 1:5000 precipitin titer by the ring test. A pooled serum sample collected from 5 normal animals was demonstrated to have no precipitins for PHP. Both pools of sera were fractionated as above to obtain normal gamma-globulin (NGG) and immune gamma-globulin (IGG) as the NICHOL-DEUTSCH precipitate C. (This fractionation procedure has been subsequently used extensively in this laboratory to obtain rabbit plasminogen preparations. In several instances precipitate C has contained some proteolytic activity which could be activated by SK-human plasminogen preparations.)

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[5] W. M. MEYERS and K. L. BURDON, Amer. J. Physiol. *190*, 303 (1957).

[6] J. C. NICHOL and H. F. DEUTSCH, J. Amer. chem. Soc. *70*, 80 (1948).

[7] One ml 6% casein (Hammarsten quality) and 0·2 ml Varidase (500 units SK), in addition to the indicated components, were included in the digestion mixture. All reagents were prepared in *M*/10 phosphate buffer (pH 7·8) and the total volume was 3 ml. Incubation was at 37°C for 30 min. Except where stated the serum or its fractions and SK were added simultaneously.

[8] Purified Human Plasminogen from Human Fraction III by method of KLINE[2]. All digests contained 33 $\mu$g PHP Nitrogen.

[9] Prepared by ethanol fractionation method of NICHOL and DEUTSCH[6].

Results of further tests with these materials are summarized in Table II. Since the PHP was only approximately 50% soluble under the conditions used, it was necessary to conduct quantitative assays with the soluble portion only. However, the proteolytic activity of the soluble and insoluble portions of this preparation per mg nitrogen were essentially the same. The indicated amounts of soluble PHP were mixed, in duplicate, with 1 ml of 2% NGG or 1 ml of 2% IGG in a total volume of 3 ml. All solutions were prepared in $M/10$ phosphate buffer at pH 7·8, centrifuged at high speed, and filtered to remove insoluble material. The PHP-globulin preparations were incubated 30 min at 37°C and then kept at 4°C for 24 h. At the end of this time all tubes were centrifuged at 2–4°C and the supernatants saved for enzymatic analysis and checks for antigen (An) or antibody (Ab) excess by the ring test. The specific precipitates were washed three times with cold 0·15 $M$ NaCl and total nitrogens determined (micro-Kjeldahl). (No precipitate was obtained with the NGG and hence no values on this are included in Table II.) Proteolytic activity assays were carried out on the supernatants and on a solution containing corresponding amounts of soluble PHP only.

*Table II*
Effect of normal and specific immune rabbit gamma-globulins on Human plasminogen*

| µg Plasminogen N | Proteolytic Activity (Opt. Density) | | | Antigen (An)-Antibody (Ab) analyses | |
|---|---|---|---|---|---|
| | Plasminogen only | Supernatant | | | |
| | | Plasminogen + NGG | Plasminogen + IGG | µg N in An-Ab ppt. | Supernatant Composition |
| 5·3 | 0·040 | 0·040 | 0·002 | 139 | Ab |
| 10·6 | 0·080 | 0·085 | 0·035 | 241 | Ab, An |
| 15·9 | 0·200 | 0·210 | 0·050 | 296 | Ab, An |
| 26·5 | 0·330 | 0·335 | 0·110 | 352 | Ab, An |
| 42·4 | 0·560 | 0·570 | 0·270 | 360 | An |

* Conditions of assay given in text.

The data (Table II) indicate that the presence of IGG reduced the proteolytic activity of the supernatants markedly, while NGG had no effect. The portion of the plasminogen preparation responsible for proteolytic activity could be virtually completely precipitated in the region of large Ab excess. The results further suggested that, since both An and Ab were present in the supernatants over a broad zone, PHP probably contains more than one antigenic component. This was confirmed by using the OUDIN agar-diffusion technique[10] which demonstrated that the soluble PHP contained at least 4 antigenic components reacting with IGG. When three whole normal human sera were checked individually against IGG, multiple rings were also formed.

It was of interest to determine if IGG affected human blood clot lysis. One ml of 2% IGG in saline was mixed with 1 ml whole fresh human blood and allowed to clot. Streptokinase (1000 units) was injected into the center of these clots, as well as into two sets of control clots, one containing only saline and blood and the other 2% NGG and blood. Clot lysis was virtually complete after 90 min in the control tubes, while in those containing IGG very little lysis had taken place after 24 h. Although this is an extremely crude procedure it did furnish evidence for the inhibition of fibrinolysis in whole blood by IGG.

[10] J. OUDIN, Meth. med. Res. *5*, 335 (1952).

Thus the KLINE procedures for preparation of PHP yielded immunologically heterogeneous preparations; however, a specific antiserum was obtained which inhibited the proteolytic and fibrinolytic activity of human plasmin and formed specific precipitates. This heterogeneity makes further immunochemical studies on this plasminogen preparation of doubtful significance and renders an immunochemical assay for plasminogen impossible at this time.

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*Résumé*

Les auteurs ont préparé, chez le lapin, les anticorps specifiques inhibiteurs des activités fibrinolytiques et protéolytiques du plasminogène humain activé par la streptokinase. Le plasminogène humain purifié est un antigène hétérogène.

## Cholesterol Blood Levels in Rh Sensitized Women Treated with Rh Hapten

In this laboratory we are engaged in an attempt to assess the value of oral administration to the Rh negative sensitized woman of the Rh hapten (a red cell fraction described previously by CARTER[1] and CARTER *et al.*[2]). Although several workers, including HOWE and RUSTIGIAN[3] and OSBORN[4], have reported inability to confirm *in vitro* observations on the Rh hapten, this inability due, it would seem, to technical variations, BARNARD[5] and GOLDSMITH[6] independently have successfully confirmed this phase of the work. WOLF *et al.*[7] stated: 'A number of our observations suggest that the term Rh hapten may correctly be applied to the extract described by CARTER'. Although WOLF *et al.*[7], MARSTERS *et al.*[8], and SPURLING *et al.*[9] have been unsuccessful in the clinical use of Rh hapten to prevent erythroblastosis, these failures due to use of too little of the fraction too late in pregnancy, EHRENBERG[10] and SCHUBERT and GRUNBERG[11] have reported clinical successes.

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