PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 411-414 (1976)

# Production of Antiserum to Human Properdin and Demonstration of Antigenic Differences between the Native and Activated Protein[1] (39222)

JOE O. MINTA[2]

(Introduced by H. Z. Movat)

*Department of Pathology, Division of Experimental Pathology, and Institute of Immunology, University of Toronto, Toronto, Canada*

The properdin system has now been established as an alternative pathway for activating the terminal complement sequence with the generation of cytotoxic activity and phlogistic products (1-4). The constituents of the properdin system recognized to date include Properdin, Factors B, D, and C3 (5-14).[3] This pathway is activated by aggregates of IgA, guinea pig $\gamma_1$ globulin and complex polysaccharides, e.g., zymosan, inulin, dextran, endotoxin (1, 7, 15-17), and has been implicated in a variety of host defense mechanisms (18-24).

Conventional procedures for purification of properdin have resulted in the isolation of the protein in its activated state (5, 25-29). Using a reversed application of the technique of affinity chromatography we have recently isolated properdin from human serum in its precursor form (30). In this paper, data will be presented showing that there are certain antigenic determinants in precursor properdin which are lacking in activated properdin. This finding would tend to suggest that activation of properdin may be associated with a conformational change or a limited proteolytic cleavage of the molecule to yield the activated product.

*Methods and materials.* Normal human serum from healthy donors was kindly supplied by the Canadian Red Cross Society (Toronto).

Serum was immunochemically depleted of properdin, as described earlier (27, 29), by passage through an immunoadsorbent column of Sepharose 4B covalently linked to anti-properdin (IgG fraction). One-tenth milliter of the properdin-depleted serum (RP) was diluted with 0.9 ml of 0.01 *M* phosphate-buffered saline, pH 7.4, in 0.14 *M* NaCl (PBS) and emulsified in an equal volume of Complete Freunds Adjuvant (CFA). The emulsion was injected intradermally into the back of an albino rabbit at multiple sites (0.1 ml/site). The rabbit was boosted 4 weeks thereafter by injecting intramuscularly 0.5 ml of the emulsion into each thigh. Antiserum was harvested 10-14 days later. The IgG fraction of the rabbit anti-RP antiserum was purified by precipitation with 40% ammonium sulfate followed by chromatography on a DEAE cellulose column equilibrated with phosphate buffer $\mu$-0.02, pH 7.5. The unadsorbed protein peak containing the IgG fraction (anti-RP IgG) was concentrated to 10 mg/ml and dialyzed against 0.2 *M* borate buffer, pH 8.6.

Properdin in its activated form ($\bar{P}$) was purified from human serum by the procedure of Pensky *et al.* (5). Properdin in its native form (P) was prepared from fresh serum by the reversed application of the affinity chromatography technique (30). Essentially, fresh human serum was diluted at 4° with 0.005 *M* sodium phosphate buffer, pH 7.5, to a conductance of 3.6 mmho/cm and gently stirred for 30 min. The properdin-rich precipitate formed (Prf) was solubilized in 0.3 *M* NaCl and passed through an immunoadsorbent column of Sepharose 4B

[1] This work was supported by the Canadian Medical Research Council (Grant No. MA 5063) and the Ontario Heart Foundation.

[2] Research Scholar of the Canadian Heart Foundation.

[3] P is precursor properdin; $\bar{P}$ is activated properdin; Factor B is synonymous with C3 proactivator (C3PA), glycine-rich $\beta$-glycoprotein (GBG), and $\beta_2$-glycoprotein II; Factor D is synonymous with C3PA convertase and GBGase; KCNS, potassium thiocyanate; RP is serum immunochemically depleted of properdin; Prf is properdin-rich precipitate obtained by diluting serum at pH 7.5 and 4° to a conductance of 3.6 mmho/cm; $P_{ff}$ is the properdin-free protein peak eluted from Sepharose anti-RP column with 3 *M* KCNS.

covalently linked to anti-RP IgG. Properdin passed through the column unadsorbed and the bound proteins were washed with 1.0 *M* NaCl and then eluted with 3 *M* KCNS. The eluted proteins were concentrated by ultrafiltration and dialysed against 0.01 *M* phosphate buffered saline, pH 7.4, in 0.14 *M* NaCl (PBS).

Antiserum to $\bar{P}$ and P were raised in rabbits by injecting each rabbit intradermally at multiple sites (0.1 ml/site) with 1 ml solution of the antigen (100 $\mu$g protein/1 ml PBS) emulsified in an equal volume of complete Freunds adjuvant (CFA). The rabbits were boosted 4 weeks later by immunization and subcutaneous injections of the same dose of the emulsion. The rabbits were bled 10–14 days after the second injection and several days thereafter. Antiserum to P was also prepared by immunizing rabbits with Prf in the manner described above. The anti-Prf obtained was rendered monospecific for properdin by absorption with RP or with the properdin-free protein ($P_{ff}$) eluted from the Sepharose anti-RP globulin column with potassium thiocyanate.

Immunodiffusion and immunoelectrophoresis were carried out on microscope slides coated with 1% agarose in 0.05 *M* barbital buffer, pH 7.5 (29). The reactions were incubated in a humid chamber at room temperature for 24–48 hr, and then washed for 1 day with 0.4 *M* NaCl, followed by an overnight rinse in distilled water. The slides were dried, stained with Amido Black, destained and photographed.

*Results and discussion.* The antisera to precursor preoperdin (P) obtained either by immunization of rabbits with P or with Prf followed by absorption with $P_{ff}$ were monospecific. Each reacted with P and $\bar{P}$ and yielded one precipitin arc after immunoelectrophoresis of whole human serum concentrated twofold (Fig. 1). The immunoelectrophoretic mobility of P was similar to properdin in whole serum but different from $\bar{P}$, which was cathodal. However, in some preparations of P, small amounts of a cathodal species resembling the $\gamma_2$ mobility of $\bar{P}$ was seen. On Ouchterlony analysis, only one precipitin band was obtained when anti-P or anti-$\bar{P}$ was diffused against P, $\bar{P}$, or concentrated human serum. When anti-$\bar{P}$ was used in the center well, reactions of identity were seen between $\bar{P}$ and P, P and NHS, and $\bar{P}$ and NHS (Fig. 2a). However, when anti-P was placed in the center well, a reaction of identity was obtained only between P and NHS. Reactions of partial iden-

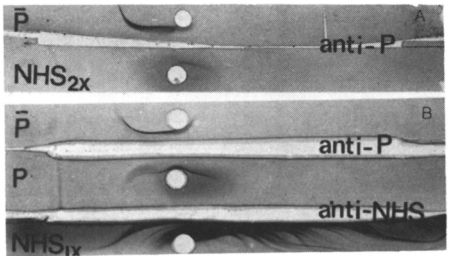


FIG. 1. (A) Immunoelectrophoresis showing the mobilities of highly purified activated properdin ($\bar{P}$) and properdin in twofold concentrated serum ($NHX_{2x}$). The anode is to the right. (B) Immunoelectrophoresis showing the mobilities of highly purified preparations of activated properdin ($\bar{P}$) and precursor properdin (P). $NHS_{1x}$ is normal human serum (unconcentrated). The anode is to the right.

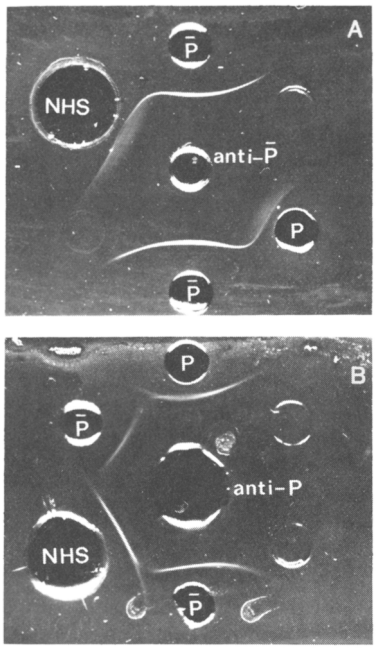


FIG. 2. Immunodiffusion pattern of purified preparations of activated properdin ($\bar{P}$), precursor properdin (P), and properdin in twofold concentrated serum (NHS). In Fig. 2A (upper panel) the center well contains rabbit antiserum to human $\bar{P}$. In Fig. 2B (lower panel), the center well contains rabbit antiserum to human precursor properdin (P).

tity were formed between P and $\bar{P}$ and between NHS and $\bar{P}$ (Fig. 2b) with spur formations from the NHS and P wells extending over the precipitin line formed between $\bar{P}$ and anti-P. This would tend to suggest that certain antigenic determinants of P may be deficient in $\bar{P}$. In addition to earlier observtions that $\bar{P}$ is a $\gamma_2$ protein while P is a $\beta_2$ protein, it may be concluded that the antigenic deficiency in $\bar{P}$ may be a reflection of the release of a fragment from P upon activation to $\bar{P}$. It is quite possible that activation of P to $\bar{P}$ may not be associated with a proteolytic cleavage but is due to alteration in the configuration of the molecule in such a way that certain antigenic determinants become concealed in the interior of the molecule. However, recent evidence that P has a sedimentation rate of 6.1 S and subunit molecular weight of 59,000 and is thus greater than $\bar{P}$, whose sedimentation rate and molecular weight are 5.2 S and 54,000, respectively, makes the last possibility unlikely. The evidence from these immunochemical studies raises the possibility of the presence in human serum of an enzyme capable of effecting activation of P, a process which may involve cleavage of the molecule with the release of a minor fragment from the major active fragment ($\bar{P}$). The possible relationship of this enzyme to properdin convertase (31) and C3 nephritic factor (32) is under investigation.

*Summary.* Antisera were raised in rabbits to human properdin in the precursor form (P) and in the activated state ($\bar{P}$). On Ouchterlony analysis using the anti-$\bar{P}$, reactions of complete identity were obtained between P, $\bar{P}$, and properdin in twofold concentrated serum (NHS). However, when anit-P was used, a reaction of identity was obtained only between P and NHS, and a reaction of partial identity was formed between P and $\bar{P}$ and between properdin in NHS and $\bar{P}$, suggestive of the fact that certain antigenic determinants in P may be lacking in $\bar{P}$. The results indicate that activation of precursor properdin may involve proteolytic cleavage and/or conformational alterations of the molecule.

The author thanks Miss E. Kunar and Miss D. Mann for their valuable technical assistance, and Dr. D. M. Wrobel and Mrs. I. Macdonald of the Canadian Red Cross Society for a generous supply of blood.

1. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W. and Wardlaw, A. C., Science **120,** 279 (1954).
2. Lepow, I. H., *in* "Immunochemical Approaches to Problems in Microbiology" (M. Heidelberger and O. J. Pfescia, eds.) p. 280. Rutgers University Press, New Brunswick, N. J. (1960).
3. Lepow, I. H., *in* "Progress in Immunology I" (B. Amos, ed.) p. 609. Academic Press, New York (1972).
4. Muller-Eberhard, H. J., *in* "Progress in Immunology II" (L. Brent and J. Holborow, eds.) Vol. 1, p. 173. North-Holland Publishing Co., Amsterdam (1974).
5. Pensky, J., Hinz, C. F., Jr., Todd, E. W., Wedgwood, R. J., Boyer, J. T., and Lepow, I. H., J. Immunol. **100,** 142 (1968).
6. Pensky, J., Wurz, L., Pillemer, L., and Lepow, I. H., Z. Immun. Forsch. **118,** 329 (1959).
7. Gotze, O., and Muller-Eberhard, H. J., J. Exp. Med. **134,** 90s (1971).
8. Goodkofsky, I., and Lepow, I. H., J. Immunol. **107,** 1200 (1972).
9. Alper, C. A., Goodkofsky, I., and Lepow, I. H., J. Exp. Med. **137,** 424 (1973).
10. Blum, L., Pillemer, L., and Lepow, I. H., Z. Immun. Forsch. **118,** 349 (1959).
11. Muller-Eberhard, H. J., and Gotze, O., J. Exp. Med. **135,** 1003 (1972).
12. Hunsicker, L. G., Ruddy, S., and Austen, K. F., J. Immunol. **110,** 128 (1973).
13. Cooper, N. R., J. Exp. Med. **137,** 451 (1973).
14. Goodkofsky, I., Stewart, A., and Lepow, I. H., J. Immunol. **111,** 187 (1973).
15. Sandberg, A. L., Gotze, O., Muller-Eberhard, H. J., and Oster, A. G., J. Immunol. **107,** 920 (1973).
16. Gerwuz, H., Shin, H. S., and Mergenhagen, S. E., J. Exp. Med. **128,** 1049, (1968).
17. Marcus, R. L., Shin, H. S., and Mayer, M. M., Proc. Nat. Acad. Sci. USA **68,** 1351 (1971).
18. Johnson, F. R., Agnello, V., and Williams, R. C., Jr., J. Immunol. **109,** 141, (1972).
19. Gallin, J. I., Clark, R. A., and Frank, M. M., Clin. Immunol. Immunopathol. **3,** 334 (1975).
20. Wardlaw, A. C., and Pillemer, L., J. Exp. Med. **103,** 553 (1956).
21. Wedgwood, R. J., Ginsberg, H. S., and Pillemer, L., J. Exp. Med. **137,** 424, (1956).
22. Hinz, C. F., Jr., Jordon, W. S., Jr., and Pillemer, L., J. Clin. Invest. **35,** 453 (1956).
23. Gotze, O., and Muller-Eberhard, H. J., N. Engl. J. Med. **286,** 180 (1972).
24. Alper, C. A., *in* "Progress in Immunology" (B. Amos, ed.), Vol. 1, p. 609. Academic Press, New

York, 1972.
25. Gotze, O., and Muller-Eberhard, H. J., J. Exp. Med. **139,** 44 (1974).
26. Fearon, D. T., Austen, K. F., and Ruddy, S., J. Exp. Med. **139,** 355 (1974).
27. Minta, J. O., and Lepow, I. H., J. Immunol. **111,** 286 (1973).
28. Minta, J. O., and Lepow, I. H., Immunochemistry **11,** 361 (1974).
29. Minta, J. O., J. Immunol. **114,** 1415 (1975).
30. Minta, J. O., Fed. Proc. **34,** 981 (1975).
31. Stitzel, A. E., and Spitzer, R. E., J. Immunol. **112,** 56 (1974).
32. Vallota, E. H., Gotze, O., Spiegelberg, H. L., Forristal, J., West, C. D., and Muller-Eberhard, H. J., J. Exp. Med. **139,** 1249 (1974).

Received July 25, 1975. P. S. E. B. M. 1975, Vol. 151.