In view of the physical as well as biochemical heterogeneity of the subcellular particles of polymorphonuclear leukocytes, we examined other cells under identical conditions to compare the distributions of particles and the associated enzyme activities. As shown in Fig. 3, the particles comparable to peak III particles of polymorphonuclear leukocytes were entirely absent from macrophages and liver cells. However, peaks physically comparable to peak II of the polymorphonuclear particles were found in both macrophage and liver cell homogenates. The unstimulated normal lung macrophages and BCG-stimulated macrophages showed practically similar distribution patterns. Unlike the polymorphonuclear leukocytes where acid phosphatase appeared exclusively in peak I, the acid phosphatase in macrophages and liver was equally associated with both of the lighter peaks, peaks I and II. Most of the lysosomal enzymes in macrophages and liver cells were associated with the major peak II. Nevertheless the distribution of enzymes in the major peak of these cells was not uniform and indicated the presence of further heterogeneity. In addition to the differences in the distribution of enzymes between subcellular particles of polymorphonuclear leukocytes and the other three cells there were also quantitative differences. Alkaline phosphatase activity of polymorphonuclear leukocytes was 20 times higher than that of macrophages and liver lysosomes. Peroxidase and trypsin-like activities were negligible in normal macrophages as well as BCG-stimulated macrophages, although trypsin-like activity was present in liver lysosomes. On the other hand, catheptic activity, compared to polymorphonuclear leukocyte, was higher in macrophage lysosomes.

It is concluded that the six cationic proteins of polymorphonuclear granular fractions are exclusively confined to a group of unique, heavy, intensely eosinophilic particles that are poor in enzymes, such as acid phosphatase, alkaline phosphatase, and lysozyme, and are evidently absent from normal macrophages, BCG-stimulated macrophages, and liver cells. Concomitant with the absence of these particles, the group of six cationic components is not found in the cytoplasmic organelles of these three cell types.

Note added in proof: Since this manuscript was submitted, we have had the privilege of reading the manuscript of M. Baggiolini, J. G. Hirsch, and C. de Duve (*J. Cell. Biol.*, in press). Their work on the distribution of enzymes in polymorphonuclear leukocyte granules was in part known to us during our investigation of the localization of cationic proteins in granules.

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Hemophilia A: Polymorphism Detectable by a Factor VIII Antibody

Abstract. *Plasma from 54 patients with hemophilia A was tested for neutralizing activity with a human antibody to factor VIII. The plasma from 52 patients had no demonstrable neutralizing activity. Two plasma samples had neutralizing activity equivalent to that of normal plasma despite the lack of factor VIII clotting activity. Apparently, most patients with hemophilia A do not synthesize factor VIII, whereas a few synthesize an inactive molecule with a presumed genetic structural mutation of the active site but with antigenic determinants in common with normal factor VIII. Thus, hemophilia A is a disease caused by more than a single genetic mechanism.*

Hemophilia A is considered a hereditary defect resulting in the decreased synthesis of factor VIII. If a molecule with similar antigenic determinants but without an active site were present in hemophilic plasma, an antibody to factor VIII might combine with a "defective molecule" and, therefore, antibody neutralization studies could be used to detect the presence or absence of such a molecule. A relatively small number of patients with hemophilia A have been so tested with variable results (*1*). We have studied a large population of hemophiliacs and report results indicating that a few patients with hemophilia A have antibody-neutralizing activity in their plasma.

Plasma from hemophiliacs was prepared by high-speed centifugation of nine volumes of venous blood added to one volume of a balanced citrate anticoagulant. Plastic syringes and tubes were used. Serum from a patient with a spontaneous factor VIII inhibitor, which had been characterized as an IgG immunoglobulin (*2*), was heated for 30 minutes at 56°C and then absorbed twice with a 25 percent $Al(OH)_3$ gel. The absorbed serum was then saturated with 40 percent $(NH_4)_2 SO_4$, and the precipitate was isolated by high-speed centrifugation and redissolved in normal saline. The inhibitor was further purified by zone electrophoresis on starch and stored at −20°C. The concentration of factor VIII in plasma of patients with hemophilia A was determined by the two-stage method of Pool and Robinson (*3*). Residual factor VIII in the second incubation mixture of the antibody-neutralization test was measured by a one-stage factor VIII assay based upon the partial thromboplastin time (*4*).

Samples were tested for material that neutralized factor VIII antibody as follows. The antibody was diluted in normal plasma to a point (1:7) where all of the antibody was neutralized when incubated at room temperature for 90 minutes. When the antibody was similarly diluted in buffer or in a single prototype hemophilia A plasma, there

Table 1. Differentiation of two types of hemophilia A by antibody neutralization tests. The normal plasma was a pooled sample. Mixture No. 1 consisted of inhibitor diluted 1:7 in test material. Mixture No. 2 consisted of 0.3 ml of normal plasma plus 0.3 ml of mixture No. 1 or buffer as indicated. A control for mixture No. 2, consisting of equal parts of buffer and normal plasma, was always included to account for deterioration of factor VIII over the 90-minute incubation period. If there had been no deterioration in activity, the expected residual factor VIII activity of this equal part mixture would be 50 percent.

No. determinations	Test material in mix 1	Factor VIII (%)	Mix 2	Residual factor VIII in mix 2 (%)	
				Mean ± S.D.	Range
16*	Normal plasma	100	Mix 1	31.6 ± 8.8	15–45
			Buffer	32.5 ± 7.7	17.5–40
16*	Buffer	0	Mix 1	5.0 ± 1.7	3–9
52†	Factor VIII deficient plasma type I)	Mean = 2.2 (< 1 – 17.5)	Mix 1	5.3 ± 1.7	3–9
6‡	Factor VIII deficient plasma (type II) C.W.	1.4	Mix 1	26	
4‡	Factor VIII deficient plasma (type II) J.T.	6	Mix 1	36	

* Separate determinations. † Individual patients. ‡ Separate determinations on each individual.

was no neutralization of antibody, as judged by subsequent incubation of a sample of the first incubation mixture with an equal volume of normal plasma for 90 minutes at 22°C (mixture No. 2) and measurement of factor VIII activity. If the test plasma added to the first incubation mixture did not contain antibody neutralizing material, then a large amount of residual antibody would inactivate the factor VIII added in the second mixture and result in a low factor VIII activity. Conversely, if the test plasma contained antibody neutralizing material, then there would be no residual antibody and a high factor VIII activity would be present in the second mixture.

To establish that residual factor VIII activity in the second mixture depended upon the concentration of factor VIII in the test material, normal plasma was diluted with buffer to give known concentrations of factor VIII, and these diluted plasmas were tested for neutralizing material as described above. The percentage of residual factor VIII in mixture No. 2 (Y) was then plotted against the percentage of factor VIII activity in the test material (X). The best-fitting straight line was calculated for 55 points by the method of least squares, and a positive regression line with b equal to 0.276 ($P < .01$) was obtained (*5*). This showed that the residual factor VIII activity in the second mixture was directly proportional to the amount of factor VIII activity in the test material. Because antigen-antibody reactions are stoichiometric, the percentage of residual factor VIII activity in the second mixture was therefore directly proportional to the amount of antibody neutralizing material in the test plasma.

Normal human plasma contained a molecule which resulted in complete neutralization of the factor VIII antibody (Table 1). Plasma from 52 patients (50 families) with hemophilia A failed to neutralize the antibody and was not significantly different from the buffer controls. The concentration of factor VIII in these patients varied from less than 1 percent to 17.5 percent (mean, 2.2 percent). These patients have been designated type 1. The mean residual factor VIII in the second mixture for all 52 patients was 5.3 ± 1.7 percent with the 99 percent prediction interval for the individual being 0.2 to 10.4 percent.

Plasma from two patients with factor VIII activity of 1.4 and 6 percent completely neutralized the inhibitor on repeated testing and with several samples taken at different times. The mean residual factor VIII obtained with their plasma did not differ significantly from the mean obtained wtih normal plasma. Since the residual factor VIII in these two patients greatly exceeded the 99 percent prediction interval of the type 1 hemophilic population, the probability that these patients are members of that hemophilic population is less than .01. If the results from these two patients are included in the calculations for the total hemophilic population, the mean residual factor VIII for the 54 patients would be 6.3 ± 5.7 percent. Even then the 99 percent prediction interval would not include the two outliers.

These two outliers could not be differentiated in any other way from the type 1 hemophilia A population. They were both males with congenital hemorrhagic disease, and both had normal bleeding times as judged by the Ivy method (*6*). They were, however, the only affected individuals in their families. The mother of C.W. has a factor VIII concentration of 75 percent, and he has one female sibling with a factor VIII concentration of 82 percent. The family of J.T. was unavailable for study, but he has four maternal uncles and ten male first cousins without hemorrhagic disease.

These data mean to us that a very small percentage of patients with hemophilia A do not have a quantitative decrease in factor VIII but synthesize an inactive molecule which has antigenic determinants in common with normal factor VIII. This is analogous to hemophilia B (hereditary lack of factor IX activity) in humans (*7*), tryptophan synthetase deficiency in *Neurospora* and bacteria (*8*), and catalase deficiency in mice (*9*). In all of these situations two mutants have been described: (i) those with enzymatic deficiency who produce an abnormal molecule which can be detected by immunochemical techniques; and (ii) those with enzymatic deficiency who do not produce a molecule with antigenic cross reactivity.

Thus, hemophilia A appears to be another example of an enzymatic deficiency resulting from two genetic mechanisms. The majority of patients apparently do not synthesize factor VIII, whereas a small minority synthesize an inactive factor VIII molecule with antigenic determinants in common with normal factor VIII. These two types of hemophilia A can be differentiated by a test for neutralization of antibody to factor VIII (*10*).

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