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The Electrophoretic Analysis of the Third Component of Complement (C3) in Dried Bloodstains

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ABSTRACT: The polymorphic serum protein C3, which is the third component of the classical complement system, has been analyzed by agarose gel electrophoresis and immunofixation. It is sufficiently stable that phenotyping can be performed from dried bloodstains maintained at room temperature for at least two weeks or kept frozen for at least one month. Because its rate of migration differs from that of group specific component (Gc) in Tris-glycine buffer, both genetic markers can be simultaneously phenotyped.

KEYWORDS: pathology and biology, genetic typing, electrophoresis, genetic markers, complement, C3, group specific component, immunofixation, bloodstains

Because many proteins are genetically polymorphic, their analysis can be significant in forensic serology studies. Electrophoresis of such blood proteins as phosphogluconutase, glyoxalase I, esterase D, adenosine deaminase, adenylate kinase, and hemoglobin has become a relatively common technique because of its usefulness in helping to determine the probable source of a bloodstain.

The complement system represents a group of serum proteins that participate in the cytolysis of cellular antigens by specific antibodies. It is present in all mammalian sera. Upon activation it becomes important in chemotactic, opsonic, and anaphylactic phenomenon. Complement is also involved in immune adherence and viral neutralization.

The third component of complement, C3, is present in serum at a concentration of approximately 1.6 mg/mL. It is composed of two peptide chains that are held together by three disulfide bonds. The C3 protein behaves like a β_2 globulin and has a molecular weight of 180 000. Human C3 polymorphism has been determined previously by prolonged agarose electrophoresis [*1*]. The allotypes of C3 differ in net surface charge at pH 8.6 but have identical hemolytic complement activity. There are three common phenotypes of C3 that are controlled by a pair of autosomal alleles. At least 15 additional rare variant phenotypes have been reported [*2*]. The three common phenotypes were designated FF, FS, and SS by Alper and Propp [*1*]. These correspond to phenotypes 1-1, 1-2, and 2-2 as described by Azen and Smithies [*3*], who studied C3 polymorphism by high-voltage starch gel electrophoresis. There is a difference in gene frequencies among the major races of man. The $C3^S$ gene is very

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common among whites, blacks, and Asians. However, the $C3^F$ gene is less common in Asians and blacks than in whites [*1*]. A study of 464 Japanese individuals revealed that 97.41% were phenotyped as SS, while there were no individuals with the SF or FF phenotype. The remaining 2.59% were phenotyped as SF_{rare} [*3*]. Alper and Propp, [*1*] have determined that among U.S. blacks the gene frequencies of $C3^F$ and $C3^S$ are 0.1 and 0.9, respectively; furthermore, U.S. whites have gene frequencies of 0.25 and 0.75, respectively.

The position to which C3 has migrated following electrophoresis can be determined by using an agarose overlay containing sensitized sheep red blood cells and an excess of all complement components other than C3. Following incubation at 37°C, a lytic zone can be recognized that corresponds to C3 hemolytic activity. Alternately, C3 can be determined by immunofixation [*4,5*]. The present report demonstrates that C3 polymorphism can be phenotyped in dried bloodstains by immunofixation, and furthermore that C3 and group specific component (Gc) can be simultaneously determined in a group system.

Materials and Methods

Blood Samples

Blood samples were obtained from volunteers either by venipuncture or by finger pricks. Serum was prepared from whole blood following clot formation and retraction by centrifugation. Serum was then aliquoted and frozen at −20°C until it was analyzed. Dried blood samples were prepared by placing whole blood on No. 8 cotton thread followed by air drying. The stained threads were either kept at room temperature or frozen at −20°C for varying periods until analysis. Each volunteer was phenotyped electrophoretically for C3, first by using fresh serum and subsequently with a dried bloodstained thread.

Sample Preparation

Before analysis, bloodstained threads were extracted with a minimal amount of distilled water for periods ranging from 40 min to 24 h. The older the stain, the longer an extraction became necessary. The best results were obtained when the liquid extract contained at least 20 mg protein per millilitre. Four microlitres of the liquid extract were then placed directly into the wells in the gel. Alternately, if fresh serum was analyzed it was first diluted 1:2 with gel buffer and 4 μL were placed directly into the wells.

Buffers

The tank and gel buffer consisted of 0.29*M* glycine and 0.037*M* trizma base, adjusted to pH 8.4 with 0.1*M* hydrochloric acid.

Gel

The 1.0-mm-thick gel was prepared with low electroendosmosis agarose (relative electrophoretic mobility of an uncharged polymer compared with a standard $[-m_r] < 0.1$) (Sigma Chemical Co., St. Louis, MO) and undiluted tank buffer to a final concentration of 1% w/v.

Electrophoretic Conditions

When C3 was phenotyped the gel was electrophoresed for 6 h by using a constant 10 V/cm across the gel. When C3 and Gc were simultaneously electrophoresed, the time of the run was shortened to 3 h.

Immunofixation

The position of C3 was determined by immunofixation using the method of Ritchie and Smith [5]. Goat antihuman C3 was purchased from Cappel Laboratories (Cochranville, PA) and was used at a dilution of 1:6 in phosphate buffered saline, pH 7.2. Immunofixation was allowed to proceed for approximately 20 to 24 h in a humidified chamber maintained at 25°C.

Drying and Staining

Following immunofixation the plates were washed for 3 h in saline and then transferred to distilled water for at least an additional 4 h to remove any nonprecipitated protein within the gel. The plates were then air-dried overnight or alternatively placed in a forced-air oven at 50°C for 20 to 30 min. The dried gels were then stained with Coomassie Blue (0.1% w/v) in methanol:water:glacial acetic acid (5:4:1). Gels were destained in the same solution without the dye.

Results

Electrophoresis of serum samples was performed for periods of time varying between 2 and 10 h. It was found that 6 h of electrophoresis provided optimal resolution of the fast (F) and slow (S) isozymes of C3. We have confirmed that the inclusion of calcium (lactate) in the tank buffer tends to slow down the migration of the two common C3 isozymes [1]. All gels were consequently run in the absence of calcium. Figure 1 illustrates the separation of the C3 fast

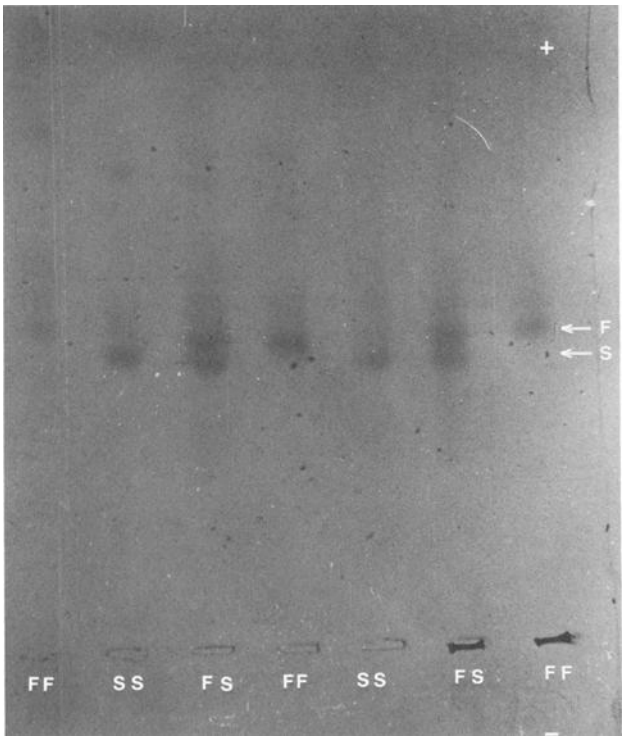

FIG. 1—*Immunofixation following electrophoresis of dried bloodstains for 6 h reveals the three phenotypes of C3: FF, FS, SS. Isozymes* F *(fast) and* S *(slow) are labeled.*

and slow isozymes in extracts of one-week-old bloodstains that were maintained in the frozen state until analysis. The results are highly reproducible; however, prolonged extraction of older stains was required to ensure that sufficient protein (2 to 6 μg of C3) was placed into each sample well (see Materials and Methods). When samples were frozen and thawed twice before analysis, the C3 isozymes changed their mobility, becoming positioned more anodally but retaining their positions relative to each other (Fig. 2). Our findings indicate that two freeze-thaw cycles stimulate cleavage of a C3 fragment, which retains C3 antigenicity, resulting in altered mobility of the larger remaining fragment. We are trying to determine if these two fragments correspond to C3a and C3b, which are known to result from activation of C3 by incubation with immune complexes or inulin. Slightly cathodal to these secondary bands, a prozone effect can be observed. These proteins presumably represent cleavage products, since degradation products of C3, produced by incubating normal human serum at elevated temperature, migrate significantly more anodically. The C3 isozymes migrate cathodically relative to Gc. Figure 3 illustrates the results of the simultaneous analysis of C3 and Gc following a 3-h electrophoretic run. The two isozymes of Gc can be observed, as well as the C3 fast and slow bands. Bands representing cleavage products of the two C3 isozymes can also be seen. Despite the fact that C3 is present in high concentrations in blood, it is not found

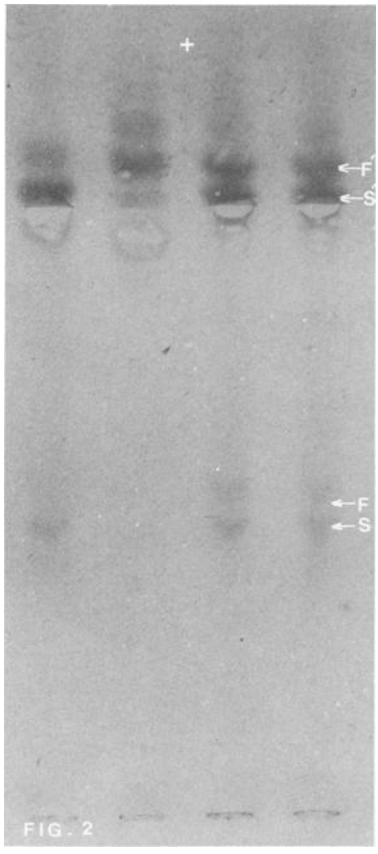

FIG. 2—*C3 analysis of samples that have been frozen and thawed twice reveals that this treatment results in more anodically migrating proteins having C3 antigenicity. These are presumably C3 cleavage products. Phenotyping can be performed on these isozyme fragments as well as on the native isozymes.*

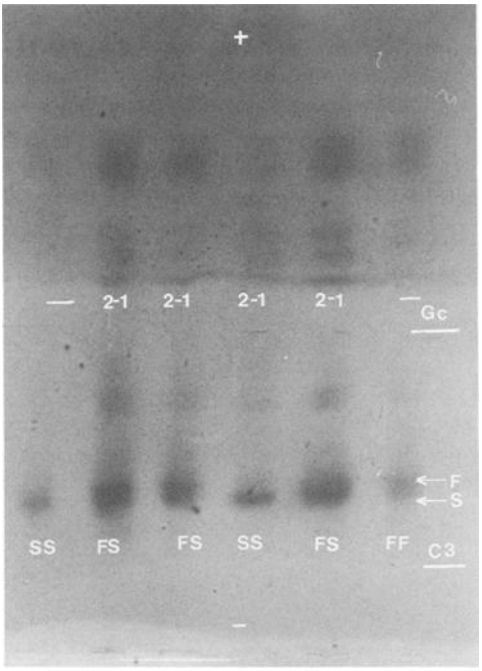

FIG. 3—*Following a 3-h electrophoretic run and immunofixation. both C3 and Gc can be simultaneously phenotyped. Resolution of C3 is poorer during this short run but within acceptable limits.*

in all tissues. We were unable to find C3 in semen, in agreement with the findings of Tauber et al [*6*].

Discussion

Several methods have been described for the analysis of C3 in fresh blood or serum, including electrophoresis on cellulose acetate membranes [*7*]. We have now described a rapid, economical, and reproducible method that allows C3 phenotyping in dried bloodstains by agarose gel electrophoresis. The immunofixation procedure allows phenotyping of a dried stain in less than two days. The C3 is sufficiently stable (for at least two weeks) so that C3 polymorphism can be used for forensic science purposes. Further studies are now being conducted to determine up to what time the genetic markers can still be detected and phenotyped.

The gene frequencies of $C3^S$ and $C3^F$ differ among different populations. In Denmark, Germany, Finland, Norway, Spain, and Sweden and for U.S. whites, the average gene frequency values of $C3^S$ and $C3^F$ calculated from the results of different population studies are 0.795 and 0.200, respectively. The rare genes have a frequency of 0.005. These results are in fairly close agreement with those of Alper and Propp [*1*], who determined gene frequencies of 0.75 and 0.25, respectively, for U.S. whites. Japanese, U.S. Asians, and U.S. blacks have significantly higher levels of $C3^S$ and lower levels of $C3^F$. The frequency of $C3^F$ found in the U.S. black population is 20 to 50% lower than that found among white populations. The $C3^F$ gene is completely absent among Japanese and American Asians. This fact makes C3 a valuable ethnic marker. If the very rare $C3^F$ alleles are neglected and it is assumed that $C3^F$ and $C3^S$ constitute a codominant two-allele system in Hardy-Weinberg equilibrium, then the average probability of exclusion is given by $pq(1 - pq)$ [*8*]. Thus for U.S. whites and blacks if we use

the gene frequency data of Alper and Propp [*1*], these values equal 0.1523 and 0.0819, respectively.

The probability that two individuals, chosen at random from U.S. whites, will have the same C3 phenotype is given by

$$Q = \sum_{i=1}^{n} Pi^2$$

where Pi represents the phenotype frequency of the ith phenotype and n is equal to the number of phenotypes within the system [*8*]. This value is 0.688 and 0.461 for U.S. blacks and whites, respectively. Thus the discrimination probability, which equals $1 - Q$, is equal to 0.312 and 0.539, respectively. Because the C3 phenotyping can be conducted simultaneously with Gc phenotyping, the discrimination probability is given by $1 - Q_1Q_2$, where Q_1 is the match probability of C3 and Q_2 is the match probability of Gc. These values equal 0.598 and 0.800 for U.S. blacks and whites, respectively, using the data provided by Selvin for Gc [*8*]. We have been unable to find any sex difference in gene frequencies of the C3 alleles in our limited population study of 30 individuals, nor has any difference ever been reported by others in previous population studies. Preliminary studies indicate that C3 can also be phenotyped simultaneously with transferrin; further studies are now under way to develop this group system.

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