Genetic Polymorphism of C'3(β_{1C}-Globulin) in Human Serum

Abstract. *Genetic polymorphism of the third component of human complement and its breakdown products has been detected in human serum by high-voltage starch-gel electrophoresis. Six phenotypes were observed in a study of 113 randomly chosen Caucasians. Their inheritance is controlled by four codominant alleles at an autosomal locus. The gene frequencies in this study were* $C3^1$, *0.21*; $C3^2$, *0.77*; $C3^3$, *~0.01; and* $C3^4$, *~0.004.*

The capacity of starch-gel electrophoresis to resolve protein mixtures has led to the discovery of genetically determined polymorphisms in human serum, such as haptoglobin (*1*) and transferrin (*2*). With few exceptions, these observations were made under standard conditions (*1, 3*), and relatively low-voltage gradients were used. We decided to investigate high-voltage starch-gel electrophoresis in the search for new genetically determined serum protein variants, since our early experience (*4*) and that of other workers (*5*) suggested improved resolution of serum proteins, especially group specific component, when high voltage was used. As a result of these studies, we have found a genetically determined polymorphism of the third component of complement (C'3 or β_{1C}-globulin) and its breakdown products in human serum. These findings are presented here as a preliminary report; the details will be published later (*4*).

After electrophoresis of fresh human serums under special conditions of high voltage (*6*), a series of sharply defined zones that stained for protein (but not for lipid) appeared in two regions of the electrophoretic pattern migrating behind α_2 macroglobulin and transferrin, respectively (Fig. 1, A and B). Under these conditions, the haptoglobins in region B became indistinct and usually did not interfere with the band patterns.

Proteins in region A were relatively unstable and temperature-sensitive, since they diminished rapidly or disappeared when the serums were stored for 24 hours at room temperature, but were at least partially maintained for several years when the serums were stored in liquid nitrogen. Even when the serums were frozen at −20°C, the concentration of stainable proteins in region A diminished or disappeared after several months of storage (Fig. 1, left sample of 2–2). Incubation experiments done at room temperature for 24 hours demonstrated the simultaneous loss of stainable proteins in region A and intensification of the bands in region B. This suggests that the proteins in region A were converted to those in region B by a degradative process.

Different patterns of bands were observed with serums from different individuals, as shown in Fig. 1. A consistent and simple interpretation of the band patterns is that they represent the proteins determined by alleles at a single locus. Each gene is postulated to control two pairs of bands. A pair is present in both regions A and B, and each includes a heavily staining band and a more rapidly migrating lighter-staining band; the light band in region A is very faint. In some heterozygotes (1–2 and 2–3), heavy bands in region B corresponding to one allele may overlap the lighter bands corresponding to the other allele. Consequently, if region B alone is examined, this may rarely cause confusion in determining phenotypes. The use of fresh serums is advisable so that region A, which is free from this complication, can also be examined. We observed that the patterns of proteins in region B from serums stored at −20°C remain stable for at least 6 months. When serums were stored at room temperature for 24 hours (with sodium azide to prevent bacterial growth), the phenotypes did not change.

In an investigation of serums from 113 healthy, randomly chosen Caucasians, different patterns of bands representing six phenotypes were observed. Representative band patterns of four of the phenotypes and the change in one after storage are shown in Fig. 1. Simple autosomal inheritance of two frequent codominant alleles (1 and 2) and two relatively rare alleles (3 and 4) is postulated to explain the protein patterns found. The frequencies of the observed phenotypes fit those expected by the Hardy-Weinberg equilibrium if one assumes four autosomal alleles at a single locus (Table 1). We suggest the symbol *C3* for the locus, and $C3^1$, $C3^2$, $C3^3$, and $C3^4$ for the alleles discovered so far. A rare fifth allele, probably present in the population studied, was detected by the appearance of bands migrating faster than those controlled by gene 1. This possible fifth allele was found in one individual after the study was completed. As yet, we do not have family data to support its presumed relationship to this genetic system.

Family studies were consistent with

Table 1. Distribution of serum C'3 phenotypes in a study of a total of 113 randomly chosen adult Caucasians (61 males and 52 females). There were no significant differences between the frequencies of phenotypes observed and those expected from the Hardy-Weinberg equilibrium, if one assumes four autosomal alleles. The frequencies of the phenotypes according to sex were not significantly different. The number of individuals of each type is listed, and the percentage of that type is given in parentheses. Gene frequencies were $C3^1$, 0.21; $C3^2$, 0.77; $C3^3$, ⌣ 0.01; and $C3^4$, ⌣ 0.004.

C'3 phenotypes							
1–1	1–2	2–2	1–3	2–3	1–4	3–3 2–4	3–4 4–4
			Observed				
4(3.5)	38(33.6)	68(60.2)	1(0.9)	1(0.9)	1(0.9)	0	
			Expected				
5.1	37.1	67.7	0.5	1.7	0.2	0.7	

Table 2. The serum C'3 phenotypes in 29 American Caucasian families with 91 children. Some families were selected because a member possessed an uncommon phenotype.

Type of mating	No. of families observed	C'3 phenotypes						
		1–1	1–2	2–2	1–3	2–3	1–4	2–4
1–1 × 1–2	1	3	1					
1–1 × 1–2	2	2	1	1				
1–2 × 2–2	15		27	28				
2–2 × 2–2	6			18				
1–1 × 2–3	1		2		1			
1–2 × 2–3	1					1		
2–2 × 2–3	1					1		
1–1 × 2–4	1		1				1	
1–2 × 2–4	1			1			1	1

the hypothesis of autosomal codominant transmission of four alleles (Table 2). In addition, seven pairs of maternal and cord blood serums were studied. The genotypes of the newborns were completely expressed and, in two instances, differed from that of the mother. This indicates that the protein in the cord blood was synthesized by the infant and not derived from the mother by placental transmission.

We were unable to identify this variable protein system with the known serum polymorphisms of haptoglobin, transferrin, group specific component, or Ag (*7*). We suspected that the protein might be the C'3 component of complement, since other workers had described the marked thermolability and spontaneous conversion of C'3 in serum to multiple faster-migrating components α_{2D} and β_{1A} (*8*, *9*). In addition, the relatively high concentration of our protein in adult and cord serums, as indicated by the intensity of staining of the zones, correlated with previous observations on C'3 (*10*). Therefore, we studied serums (from patients with acute glomerulonephritis) containing very low concentrations of C'3, along with other defects of the complement system (*11*). The components in regions A and B were virtually absent. We also studied a serum containing a normal concentration of C'3 but low concentrations of C'2 and C'4 components of complement (*11*) [from a patient with hereditary angioneurotic edema (*12*)]. The concentration of stainable proteins in region B was normal. Starch-gel immunoelectrophoresis (*13*) was then performed on serum from a 1–2 heterozygote. Specific immune precipitation was observed in regions A and B overlying the variable bands, identifying them as C'3. [The breakdown products of C'3, although antigenically deficient when compared to the parent molecule, share some common antigens (*9*).] An experiment was performed in a similar fashion on the serum known to be deficient in C'3 (and in the proteins described here), and there was no immune precipitation in regions A and B.

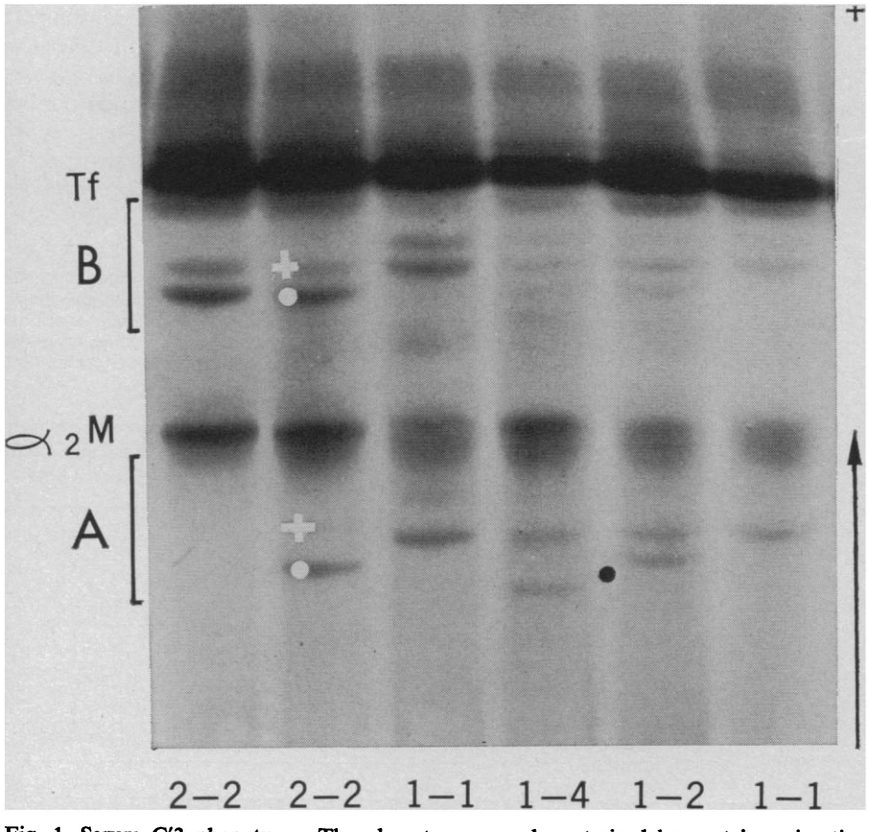

Fig. 1. Serum C'3 phenotypes. The phenotypes are characterized by proteins migrating in two regions: (A) behind alpha$_2$ macroglobulin (α_2M) and (B) behind transferrin (*Tf*). Six serum samples with four phenotypes (1–1, 1–2, 2–2, and 1–4) were used in this experiment; the left sample of 2–2 had been stored at −20°C for several months. In the fresh 2–2 serum, the major C'3 components are shown by dots and the minor ones by crosses. Proteins in region A are probably the C'3 components of complement and those in region B, its breakdown products. The major protein band determined by the allele $C3^3$ migrates in the position indicated by the small black dot between samples 1–4 and 1–2; that is, between the proteins corresponding to $C3^2$ and $C3^4$.

This evidence establishes with a reasonable degree of certainty that the genetically determined polymorphism described is of C'3 (Fig. 1, region A) and its breakdown products (Fig. 1, region B). It should be noted that Ropartz (*14*), using methods of agglutination-inhibition, has also described a polymorphism of C'3 in human serum. However, he described no family studies and the frequencies of the phenotypes (inhibitor +, inhibitor −) did not correspond to those we observed. Using agar-gel electrophoresis, Wieme and Demeulenaere observed a slowly migrating variant of C'3 in the serums of four individuals (*15*) from three generations of one family. Similar observations were made on the serum of a single individual with idiopathic thrombocytopenia (*16*).

The concentration of C'3 was the highest [approximately 120 mg/100 ml (*17*)] of all the complement components of serum, and this component has many important functions in the immune mechanisms (*18*). Thus, the genetically determined polymorphic nature of C'3 may prove useful for further studies in genetic and complement research.

Note added in proof: After we had submitted this manuscript for publication, similar data obtained by agarose-gel electrophoresis were published by Alper and Propp (*19*).

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References and Notes

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6. Details of the development of high-voltage starch-gel techniques will be described elsewhere (*4*). The system employs a starch-gel tray with two water jackets, 14° to 17°C on the front and back. The gel is 32.5 cm long by 3 mm thick. A typical gel was made with 125 g of starch (from Connaught Medical Research Laboratories, Toronto, Canada) and 1000 ml of buffer that contained 40 ml of tris-hydrochloride stock solution (0.2*M* HCl with tris base added to *p*H 8.9). The Stock solution was diluted fivefold to make the bridge solution. As a result of accidental and long-standing contamination of our distilled water system with tap water, we found magnesium to be essential for optimum resolution of the C'3 component of complement. Therefore, $MgCl_2$ (0.002*M* in final concentration)

was added to the buffer at the time the gel was made and when the bridge solution was prepared; $FeSO_4$ (0.00001*M*) was also added to sharpen the transferrin zone. Electrophoresis was done in the vertical position for 6 to 7 hours and the voltage gradient set at 16 volt/cm across the gel.
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Chemical Carcinogenesis: Persistence of Bound Forms of 2-Fluorenylacetamide

Abstract. *The persistent binding of metabolites of hepatic carcinogen, 2-fluorenylacetamide, to glycogen and to DNA in a new population of liver cells, hyperplastic nodules, and to glycogen in liver cancer cells weeks to months after the carcinogen was removed from the animals' diet is indicated by spectrophotometric, chromatographic, and mass spectrographic data. This persistence of binding does not appear to occur in the nonhyperplastic or nonneoplastic liver surrounding the nodules or the cancer.*

For many different tissues, the required exposure time to a variety of carcinogens is considerably less than the shortest interval in which malignant cells can be detected with current techniques. Such observations have suggested that the rapid induction by cancer-inducing agents of irreversible tissue changes akin to mutations, which, by subsequent selection, lead to malignant neoplastic transformation.

In order to study the molecular pathology of liver carcinogenesis, we have fed rats a diet containing a carcinogen, 2-fluorenylacetamide (2-FAA), which induces a new population of liver cells, namely hyperplastic nodules (*1*). The hyperplastic nodules appear to be a site of origin for liver cancer and are: (i) quite uniform in morphologic and biochemical properties; (ii) large enough for gross identification and for isolation to enable appropriate biochemical, morphologic, and biological studies to be made. These two criteria are deemed essential for the analysis of the cellular and molecular events during carcinogenesis (*2*).

Male, white Wistar rats (Carworth Farms) (150 to 200 g) were fed the basal diet containing 2-FAA under conditions which lead to the induction of large hyperplastic nodules and hepatocellular carcinoma (*1*). Then, unless specified otherwise, all rats were fed the basal diet without the added 2-FAA for a minimum of 4 weeks before their being killed by guillotine. After decapitation, samples of hyperplastic nodules and carcinoma as well as of the nonhyperplastic or nonneoplastic liver surrounding these lesions were removed for histological study (*1*) and for extraction of glycogen (*3*) or DNA (*4*). Liver from control animals fed only the basal diet was examined similarly.

Purified glycogen (*3*) was extracted an additional three times with ethyl ether. After being dried, portions were dissolved in water and examined spectrophotometrically (Cary model 15 recording spectrophotometer) or hydrolyzed.

Glycogen from hyperplastic nodules, both before and after methanolysis or hydrolysis (see below), consistently showed a region of increased absorption between 265 and 305 nm, while glycogen from normal control liver or from the surrounding nonhyperplastic liver showed no absorption peak in this region (Fig. 1). Analysis of the small amount of glycogen from liver cancer many months after the inducing carcinogen 2-FAA had been removed from the diet showed an absorption spectrum similar to that from the nodule, whereas the glycogen from the surrounding nonneoplastic liver showed no such increased absorption (Fig. 1). Glycogen from control livers allowed to react with *N*-acetoxy-*N*-2-fluorenylacetamide (*N,O*-diacetyl-2-fluorenylhydroxylamine) (*5*) in vitro under N_2 at 37°C for 8 hours showed an absorption spectrum similar to that noted in either nodular or cancer glycogen.

Purified glycogen from hyperplastic nodules or from liver cancer was hydrolyzed for 20 hours at 70°C with 0.5*N* HCl in methanol or for 1 hour at 100°C in either 1.0*N* HCl or 1.0*N* H_2SO_4. This hydrolyzate, when separated with thin-layer chromatography (*6*), showed a minor component in addition to the major constituent, glucose. This extra spot had an R_F value different from that for glucose and was readily visualized as an area of fluorescence with a 254 nm lamp. Chromatograms of hydrolyzates of glycogen from the liver surrounding either hyperplastic nodules or carcinomas or of glycogen from liver of control animals did not have this component. The absorption spectrum of this extra component was similar to the spectra of *N*-acetoxy-2-FAA after it had been hydrolyzed and chromatographed in the same way as the glycogen from the nodule.

Polyacetate and trimethylsilyl esters of glycogen hydrolyzed by methanolysis were subjected to gas chromatography and mass spectrometry (*7*). In hydrolyzed glycogen from hyperplastic nodules and liver cancer, components which have retention times and mass numbers different from glucose were present. Some of those additional mass numbers suggest persistent binding of an aromatic compound to glucose within the glycogen.

Glycogen prepared from the livers of rats fed the diet with 2-FAA for 3 weeks, the basal diet for 1 week, and the carcinogen-containing diet for 2 additional weeks had an absorption spectrum very similar to that of glycogen from the hyperplastic nodules. Glycogen from animals fed this latter dietary regimen became radioactive when 9-^{14}C-*N*-hydroxy-2-FAA was fed or injected.

The above indicates that 2-FAA or its derivative [presumably esters of *N*-