The genetic variants of group-specific component (vitamin D-binding protein) possess different binding characteristics for immobilized Cibacron Blue 3-GA

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The elution profiles of several variants of the Gc protein have been studied after chromatography on immobilized Cibacron Blue 3-GA. The allele products belonging to the Gc1 type were retarded and eluted with a V_e/V_0 at 1.5, as previously reported for the Gc1-1 phenotype [Chapuis-Cellier, Gianazza & Arnaud (1982) *Biochim. Biophys. Acta* 709, 353–357]. The allele products belonging to the Gc2 type were further retarded (V_e/V_0 at 2.6), and both Gc1 and Gc2 allele products were clearly separated in heterozygous individuals. This observation allows the isolation and purification of Gc variants in heterozygous individuals which carry the combination Gc1 variant–Gc2, Gc1–Gc2 variant, or Gc1 variant–Gc2 variant. In contrast, the corresponding holo-proteins did not bind to the gel and were eluted in the void volume. This suggests that the interaction of Gc with immobilized Cibacron Blue 3-GA involves the binding site of the protein for 25-hydroxycholecalciferol and that the dye behaves as a 'pseudo-ligand' for the protein. In addition, our data suggest that the different elution profiles of the variants could reflect a different affinity of the gene products for the dye.

Discovered by Hirschfeld in 1959, group-specific component (Gc) is a protein present in human plasma at a concentration of 20–50 mg/dl (Putnam, 1977). Recent interest comes from the observation that its biological role is the transport of vitamin D metabolites in plasma (Daiger *et al.*, 1975), and that it is also bound to the membrane of certain cells, including circulating B-lymphocytes (Petrini *et al.*, 1983). In addition, tissue actin binds Gc with high affinity (Haddad, 1982). Gc protein is highly polymorphic, and since the original observation of Hirschfeld *et al.* (1960), refined methodology combining thin-layer i.e.f.–p.i.f. (Chapuis-Cellier *et al.*, 1980) has revealed more than 60 different genetic variants (Dykes *et al.*, 1983). Nevertheless, only three of these alleles, designated Gc1F, Gc1S and Gc2, occur at polymorphic frequencies in most of the populations studied so far (Dykes *et al.*, 1981). The structural basis for this

genetic polymorphism is as yet unknown, and this can be partly explained by the difficulty inherent in the purification of the protein and the very low yields reported by several authors (Bowman, 1969; Bouillon *et al.*, 1976). We recently proposed a new technique for Gc purification from human plasma (Gc1-1 phenotype), which is based on its affinity for immobilized Cibacron Blue 3-GA (Chapuis-Cellier *et al.*, 1982). In the present paper we show that the genetic variants of Gc exhibit different binding characteristics for the immobilized dye, which permits separation and purification of the two allele products in heterozygous individuals. This should help to elucidate the basis for the extensive polymorphism of this protein.

Experimental procedures

Materials

Blood samples were obtained from individual donors and collected in citrate/SBTI as described by Harpel (1973). After centrifugation at 200g at 4°C for 15 min, the supernatant was decanted and dialysed overnight at 4°C against 0.03 M-sodium phosphate buffer, pH 7.0. Radioactively labelled 25(OH)D₃ {25-hydroxy[26(27)-methyl-³H]chole-

Abbreviations used: Gc, group-specific component (vitamin D-binding protein); SBTI, soya-bean trypsin inhibitor; 25(OH)D₃, 25-hydroxycholecalciferol; i.e.f.–p.i.f., isoelectric focusing–print immunofixation.

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calciferol, sp. radioactivity 10Ci/mmol} was obtained from Amersham, Arlington Heights, IL, U.S.A. When the radiolabelled product was to be used as a tracer, a molar ratio of 25(OH)D₃ to Gc globulin of 1:2500 was employed. Saturation of the plasma with 25(OH)D₃ (Roussel Laboratories, Paris, France) was performed with a 5-fold molar excess over Gc globulin concentration. The Gc concentration was determined by rocket immunoelectrophoresis (Laurell, 1966) using standards from Calbiochem-Behring (La Jolla, CA, U.S.A.).

Methods

Gc phenotyping. This was performed on serum by i.e.f.-p.i.f. as described previously (Chapuis-Cellier *et al.*, 1980; Dykes *et al.*, 1983). Gc phenotypes were identified by comparison with controls available at the Minneapolis War Memorial Blood Bank, Minneapolis, MN, U.S.A.

Chromatography on immobilized Cibacron Blue 3-GA. All chromatographic experiments were performed at room temperature. For large-scale preparative experiments, 80 ml of plasma were applied to a column (2.5 cm × 100 cm; Pharmacia,

Piscataway, NJ, U.S.A.) containing 400 ml of Affigel Blue (50–100 mesh; concentration of ligand, 4.5 µmol of dye/ml of gel; a gift from Bio-Rad Laboratories, Richmond, CA, U.S.A.), equilibrated in 0.03 M-phosphate buffer, as described previously (Gianazza & Arnaud, 1982a). For small-scale preparative experiments, a smaller column (1.0 cm × 15 cm) containing 25 ml of gel was used, and 2 ml plasma samples were applied.

Miscellaneous techniques. Thin-layer i.e.f. was performed in a pH range 3.5–8.0 or 4.0–6.5 on 0.5 mm-thick gels as previously described (Gianazza & Arnaud, 1981). P.i.f. (Arnaud *et al.*, 1977), fused rocket immunoelectrophoresis (Svendsen, 1973) and immunoelectrophoresis (Grabar & Williams, 1953) were performed by using antisera from Dako (Accurate Chemical and Scientific Co., Westbury, NY, U.S.A.). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970) and Laurell (1966) rocket immunoelectrophoresis were performed by using standard procedures. Immunological detection of Gc bands in the fractions eluted from the column was performed by immunoblotting after transfer

Fig. 1. Elution profile of the Gc proteins from the plasma of a Gc1-2 individual. Plasma (80 ml) was applied to a column containing 400 ml of immobilized Cibacron Blue 3-GA. (a) A_{280} profile. (b) Fused rocket immunoelectrophoresis in 1% (w/v) agarose in barbital buffer, pH 8.6. Samples (2 µl) were applied in each hole. Bottom layer, anti-Gc antiserum (10 µl/ml); top layer, anti-(total human) antiserum (20 µl/ml). (c) I.e.f.-p.i.f. strip of the concentrated Gc peak 2 (A, fractions 92–134) and peak 1 (B, fractions 50–76) compared with the plasma of the same subject (C). Note that A contains the major band of Gc2 and B the two major bands of Gc1, together with minor bands which do not appear in plasma (C) because of a concentration effect.

on to nitrocellulose as described by Towbin *et al.* (1979), a rabbit anti-Gc antiserum (1:500 dilution) and a swine peroxidase-labelled anti-rabbit antibody (1:1000), both from Dako, being used.

Results

When homozygous Gc1 plasma was run on the large Affigel Blue column, fused rocket immunoelectrophoresis showed that the apoprotein was clearly retarded when compared with other proteins and appeared with a V_e/V_0 of 1.5, as previously reported (Chapuis-Cellier *et al.*, 1982). In contrast, when plasma from a heterozygous Gc1-2 subject was run on the same column, Gc was eluted in two peaks (Fig. 1), which were clearly separated from each other. Analysis of these peaks by i.e.f.-p.i.f. after pooling and concentration showed that the first peak included the two major bands characteristic of Gc1, whereas the second included the major band of Gc2, in addition to other minor bands which appeared because of the concentration of the proteins studied (Fig. 1). Analysis of each individual fraction by thin-layer i.e.f. followed by specific detection of the Gc bands by the sensitive immunoblot technique (used instead of i.e.f.-p.i.f. because of the lower Gc concentration in these fractions) showed that the two bands of the Gc1 allele product were not separated during the elution process (Fig. 2). Gc1 and Gc2 were further purified to apparent homogeneity as previously described (Chapuis-Cellier *et al.*, 1982) by two additional steps of gel filtration and ion-exchange chromatography respectively (Fig. 3). The yield was similar to that reported previously, i.e. 30% for Gc1 and 38% for Gc2, assuming an identical expression of the two allele products in the total Gc value of plasma.

Further experiments were then performed on a small scale by using a 1.0 cm $\times$ 15 cm column containing 25 ml of gel and 2 ml plasma samples. Three homozygous phenotypes (Gc 1F-1F, 1S-1S and 2-2) and seven heterozygous phenotypes (Gc 1F-1S, Gc 1F-2, Gc 1S-2, Gc 1A10-1S, Gc 1A10-2, Gc 1C10-2 and Gc 1S-2A2) were studied. The results of these experiments are indicated in Fig. 4. It shows that the variants designated as 1 (1F, 1S, 1A10, 1C10) all are clearly eluted in front of the variants designated as 2 (2 and 2A2). We did not see any difference in the elution profile of the 1F, 1S, and 1A10 variants, either in the homozygous (1F-1F, 1S-1S) or in the heterozygous (1F-1S, 1F-2, 1S-2, 1A10-1S, 1A10-2) combinations. In contrast, the 1C10 variant was slightly more retarded than the other 1 variants, and the 2A2 variant was less retarded than the 2 variant.

Finally, the position of elution of the holo form of the variants was analysed by incubating the

Fig. 2. Immunoblot after i.e.f. of the fractions corresponding to the first Gc peak

A 10 μ l portion of each fraction was applied on the i.e.f. gel. The electric transfer was performed on nitrocellulose sheets as described by Towbin *et al.* (1979). The first layer contained a rabbit anti-Gc antiserum (1:500) and the second layer contained a peroxidase-labelled swine anti-rabbit antiserum (1:1000), both from Dako. The complexes were revealed with diaminobenzidine. The fraction numbers correspond to those of Fig. 1.

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the Gc1 and Gc2 allele products from a Gc1-2 heterozygous subject

These were purified separately from the pools from the Cibacron Blue column by two additional chromatography steps, i.e. gel filtration and ion-exchange chromatography (Chapuis-Cellier *et al.*, 1982). 1, Gc1 reduced; 2, Gc1 unreduced; 3, Gc2 reduced; 4, Gc2 unreduced; 5, high- M_r standards; 6, low- M_r standards. The running gel was 5–20% (w/v) polyacrylamide with 4% stacking gel. A 2 μ g portion of Gc protein was loaded and the gel was stained with silver. Note the difference in M_r between reduced and unreduced samples due to the unfolding of the protein.

Fig. 4. Composite picture showing the elution profiles on immobilized Cibacron Blue 3-GA of several Gc variants in different combinations as determined by fused rocket immunoelectrophoresis

Plasma (2 ml) was deposited on a column containing 25 ml of gel. The top part of the Figure indicates the A_{280} profile.

Fig. 5. Elution profile of the holo form of Gc2 detected by incubation with $25(OH)D_3$

Plasma (2 ml) was incubated with $[^3H]25(OH)D_3$ (D_3/Gc ratio: 1:2500) and run on the same column as in Fig. 4. Radioactivity and A_{280} were measured in each fraction, numbers for which are the same as in Fig. 4.

plasma with trace amounts of radiolabelled $25(OH)D_3$ and the radioactivity counted in each tube. In every case, including the homozygous 2 phenotypes (Fig. 4), the radioactivity eluted in front of the position of the major Gc1 apoprotein peak (Fig. 5). Similarly, saturation of a Gc-2 homozygous sample with a 5-fold molar excess of $25(OH)D_3$ shifted the elution peak of Gc to a

position identical with that of the holo form (results not shown).

Discussion

The interaction of the different Gc allele products with immobilized Cibacron Blue 3-GA appears to be of great practical importance. Indeed, it permits clear separation of the two allele products of the heterozygous subjects studied providing that one of these belongs to the 1 type and the other to the 2 type. It should be kept in mind that Gc1 and Gc2 were named according to their anodal (Gc1) or cathodal (Gc2) migration by Hirschfeld *et al.* (1960), and that further analysis by isoelectric focusing showed that Gc1 was separated into two major bands, whereas Gc2 included only one major band (Van Baelen *et al.*, 1978) (although minor bands are always present in each of these allele products, see Fig. 1). For further details on the genetic nomenclature of the Gc phenotypes, see Constans & Cleve (1979). The basis for the genetic polymorphism of the Gc system has not yet been elucidated, although Svasti *et al.* (1979) have recently reported differences in the peptide maps between Gc1 and Gc2. The interest of the separation procedure that we report here lies in the fact that Gc1 and Gc2 are the most frequent allele products in Caucasian populations (the allele frequencies of Gc1 and Gc2 being about 0.997; see Dykes *et al.*, 1983), and the chances of finding another variant in the homozygous state are extremely low. By contrast, if an uncommon Gc1 variant is associated with the Gc2 allele, or conversely, if a Gc2 variant is associated with the Gc1 allele, it should be easy to separate the variant allele product on the basis of the interaction described above.

The mechanism of the interaction of Gc with the immobilized dye is difficult to explain. We have shown previously that the retardation of Gc1 affected only the apoprotein, when the holo form did not apparently interact with the dye and was eluted in the void volume together with proteins of similar M_r and/or pI (Chapuis-Cellier *et al.*, 1982). We have extended this observation in the present work and shown that all the holoproteins, regardless of their phenotype, were eluted in the same position, i.e. in the void volume of the column, and therefore did not appear to interact significantly with the dye. This suggests that the interaction between the dye and Gc involves the binding site for vitamin D_3 metabolites. Similar findings have been reported concerning the binding of albumin to Cibacron Blue. It was first thought that this binding necessitated the availability of the binding sites for bilirubin (Leatherbarrow & Dean, 1980), although it was later shown by Metcalf *et al.* (1981)

to be fatty acids. Similarly, a series of enzymes which have NAD, NADP or ATP as coenzymes strongly bind to the Blue dye, and this interaction is abolished by addition of the cofactor (reviewed by Dean & Watson, 1979). Thompson *et al.* (1975) have observed a similarity in the tertiary structure between the Blue dye and NAD(P). It is therefore possible that Cibacron Blue competes with 25(OH)D₃ by a similar mechanism, although any definite conclusion should await direct measurement of the binding constants of the holo and apo forms of Gc to Cibacron Blue. These constants have been recently determined for albumin and albumin-fatty acid complexes by using affinity electrophoresis (Johnson *et al.*, 1980; Metcalf *et al.*, 1980).

Finally, the different interaction of the genetic variants of Gc with the dye is even more difficult to explain. The existing differences in the pI of the variants could play a role in this separation, but several facts argue against this. First, we have shown that there is a weak correlation ($r = 0.42$) between the pI of the proteins and their elution profile from the Blue column under the conditions used (Gianazza & Arnaud, 1982b). Second, all the holo forms of the different variants are co-eluted in the same position, i.e. in the void volume of the column, despite the fact that the isoelectric points of Gc1 and Gc2 are still clearly different after saturation with 25(OH)D₃ (Van Baelen *et al.*, 1978; Emerson *et al.*, 1984). Third, the 1F, 1S and 1A10 allele products, which all display different isoelectric points, had elution profiles indistinguishable from one another when run on the same column. Furthermore, the two bands of the Gc1 allele product were not separated during the elution process despite their different pI. Our results therefore suggest that the Gc2 allele products display a higher affinity for the immobilized dye than the Gc1 variants, although other mechanisms, involving the tertiary structure or the sugar content of the molecule, cannot be excluded.

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