THE RELATIONSHIP OF GLYCINE-RICH β-GLYCOPROTEIN TO FACTOR B IN THE PROPERDIN SYSTEM AND TO THE COBRA FACTOR-BINDING PROTEIN OF HUMAN SERUM*

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Glycine-rich β-glycoprotein (1), or GBG,[1] is a heat-labile 6.2S pseudoglobulin of normal human serum. It was first recognized by its reaction in fresh serum during immunoelectrophoresis with antiserum to one of its fragments, a 4.2S protein, glycine-rich γ-glycoprotein or GGG (2). It subsequently became clear that this fragment was the same as β_2-glycoprotein II, described earlier by Haupt and Heide (3) as a native serum protein. Antiserum prepared against the parent molecule, GBG, also revealed an α_2-mobility fragment, glycine-rich α-glycoprotein or GAG (1).

During studies of a patient with type I essential hypercatabolism of C3 (4), increased susceptibility to infection with pyogenic bacteria, and defects of complement-mediated functions, it was observed that these functions could be restored by a 5–6S heat-labile β-pseudoglobulin fraction of normal serum (5). Since C1, C4, and C2 were in normal concentrations in his serum, it was recognized that his abnormalities involved a pathway to C3 activation alternative to the classical mechanism. The properdin system is such a pathway (6) and was shown over a decade ago to involve a hydrazine-sensitive protein, factor A (7), and a heat-labile protein, factor B (8), in addition to properdin itself and Mg^{2+}. The concentration of properdin protein was normal in the patient's serum, but the activity of the properdin system was nevertheless low as judged both by zymosan assay (6) and the lack of bactericidal activity for a properdin-sensitive strain of Shigella (9).

GBG was undetectable in the patient's serum, but an enzyme, GBGase, was found which rapidly cleaved added GBG (10, 11). His serum lacked a normal inhibitor for this enzyme; the inhibitor was a 6S heat-labile β-pseudoglobulin. Other proteins miss-

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[1] *Abbreviations used in this paper:* C3PA, C3 proactivator; CoF, cobra venom factor; GAG, glycine-rich α-glycoprotein; GBG, glycine-rich β-glycoprotein; GGG, glycine-rich γ-glycoprotein; RB, serum depleted of factor B.

ing from his serum included the C3b inactivator (12) and a 5S heat-labile β-pseudoglobulin capable of forming a 9S complex with a 7S protein in cobra venom, CoF, such that the complex inactivated C3 (13, 14).

It is the purpose of this report to define the relationship between GBG and properdin factor B and to determine whether GBG reacts directly with CoF to form a C3-inactivating complex. The genetic polymorphism in GBG (15) was used toward these ends. In humans, there are two common alleles designated Gb^F and Gb^S (for the fast and slow electrophoretic mobilities of the GBG gene products) and two rare alleles, Gb^{F_1} and Gb^{S_1}, which synthesize very fast and very slow GBG. A brief report of these studies has been presented (16).

Materials and Methods

Protein Purification.—GBG was prepared as described previously (1). C3 was purified by the method of Nilsson and Müller-Eberhard (17). CoF was isolated from the venom of the Indian hooded cobra (*Naja naja*) by the procedure described by Müller-Eberhard and coworkers (13). All preparations were free of contaminants as judged by immunoelectrophoresis, agarose gel electrophoresis, and polyacrylamide gel electrophoresis.

Radioiodination.—Purified proteins were labeled with ^{125}I by the iodine monochloride method (18). Specific activities ranged from 6×10^4 to 3×10^5 cpm/μg. Radioisotope not bound to protein was removed by exhaustive dialysis. The final dialysis was against 0.15 M Veronal-buffered saline at pH 7.4 containing 2×10^{-3} M Mg^{2+}.

Electrophoresis.—Agarose gel electrophoresis was performed on either a standard apparatus (19) for short separations or a modified device (20) for prolonged electrophoresis. For most experiments, the electrophoresis and gel buffers consisted of 0.05 M Veronal at pH 8.6 containing 2×10^{-3} M Mg^{2+}. For immunoelectrophoresis (21), antibody troughs were cut in the agarose gel between the migration paths of adjacent samples, antiserum was added, and patterns were developed overnight. In experiments involving ^{125}I-labeled proteins, the agarose gels after electrophoresis were fixed in methanol:water:acetic acid (45:45:10), washed in water, and dried. Autoradiography was then carried out on Kodak No-screen X-ray film.

Polyacrylamide gel electrophoresis (22) was performed with 5.5% acrylamide in the same apparatus as that used for short agarose gel electrophoresis (19). Mg^{2+} was added to all buffers to a concentration of 2×10^{-3} M. For autoradiography, the acrylamide gel, after fixation of separated proteins and washing, was covered with hot liquid 1% agarose in distilled waer and dried to a thin film.

Pevikon electrophoresis was carried out by the method of Müller-Eberhard (23) with 0.05 M Veronal buffer at pH 8.6 containing 2×10^{-3} M magnesium acetate. Separation was continued for 72 h at 4°C at 400 V. The eluates from 1-cm segments of the block were concentrated 15-fold.

Factor B Assay.—The assay for factor B in the properdin system was carried out as described previously (24). The assay depends on restoration of the ability of zymosan to inactivate C6 in serum depleted of factor B by heating at 50°C for 30 min (RB).

GBG Quantitation.—GBG was measured by the electroimmunoassay of Laurell (25). In some instances, peak heights were used without reference to standards (relative concentration). In other cases, absolute concentrations were determined.

Gel Filtration.—Gel filtration was carried out on 2.5×100 cm columns of Sephadex G-200. Column and elution buffers were 0.15 M Veronal-buffered saline at pH 7.4 with 2×10^{-3} M Mg^{2+}. To assess the extent of binding of ^{125}I-labeled CoF by serum, the gel filtration elution patterns of labeled CoF in buffer and in serum were plotted so that the lighter half of the

major (7S) peak coincided. The difference between the heavier parts of the serum and buffer curves were then determined by subtraction. Plotting the difference yielded a normally distributed 9S peak. The weight of this 9S peak divided by the weight of the buffer 7S peak gave the decimal ratio of bound to total (unbound) labeled CoF.

Specific Precipitation.—Specific rabbit antisera to GBG, CoF, and ceruloplasmin were added to fresh normal human serum (1 ml antiserum:0.075 ml or 0.040 ml human serum) which had previously been incubated for 30 min at 37°C with labeled or unlabeled CoF or GBG. These mixtures were incubated at 37°C for 1 h and overnight at 4°C. 1 ml of goat antiserum to rabbit proteins (including immunoglobulins) was then added and the mixtures were further incubated for 1.5 h at 37°C and overnight at 4°C. Specific precipitates were separated by centrifugation and washed twice in cold 0.15 M phosphate-buffered saline. The precipitates and the supernatants combined with their respective washes were counted in a gamma scintillation counter.

RESULTS

The Relationship Between GBG and Factor B in the Properdin System.—(*a*) As shown in Fig. 1, increasing amounts of GBG added to the RB resulted in increasing inactivation of C6 on subsequent incubation with zymosan. It is evident that the two fractions from the same chromatographic run produced similar dose-response curves. This was consistent with behavior of GBG as factor B.

(*b*) Sera containing different variants of GBG showed partial resolution of these variants after prolonged electrophoresis in Pevikon. It can be seen in Fig. 2 that there was a good correlation between factor B activity and GBG protein concentration. In the case of Gb FF serum, there was some anodal extension of factor B activity beyond the GBG protein peak.

(*c*) It is evident from Table I that the serum of the patient with type I essen-

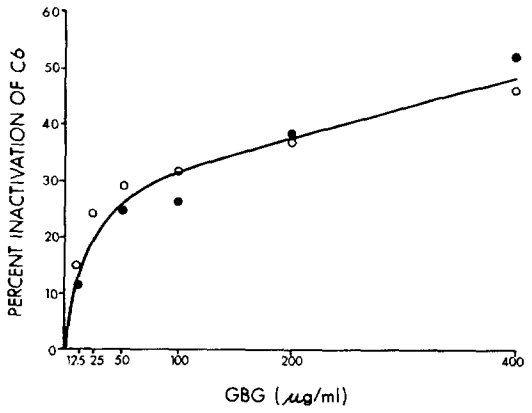

FIG. 1. Factor B activity in two fractions from the final DEAE-Sephadex elution step in the purification of GBG. Fraction 128 (●) was from the early portion of the elution peak, whereas fraction 175–176 (○) was from the end portion. Both fractions were free of detectable contaminants. GBG concentrations are expressed as micrograms per milliliter in the final reaction mixtures.

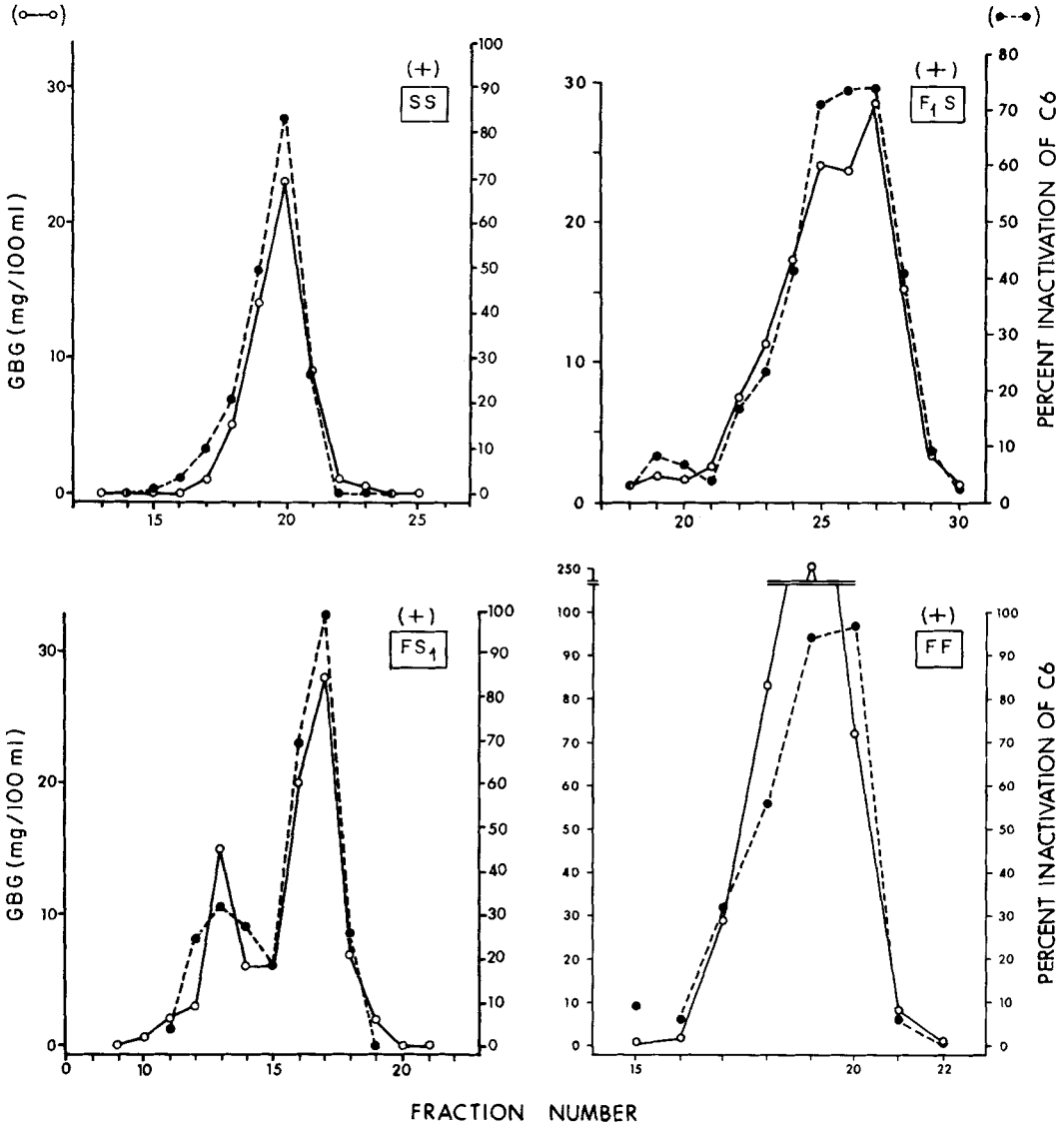

FIG. 2. Factor B activity (●) and GBG concentration (○) in fractions from Pevikon electrophoresis of sera containing different genetic variants of GBG. The anode was at the right in each instance. Gb types of the sera were: SS (upper left), F_1S (upper right), FS_1 (lower left), and FF (lower right).

tial hypercatabolism of C3 lacked significant factor B activity. After addition of GBG, factor B activity was restored but was progressively lost during incubation with the patient's serum at 37°C for 30 min. As determined by immunoelectrophoresis, the added GBG was completely cleaved by this time.

TABLE I

Interaction of Serum from the Patient with Type I Essential Hypercatabolism of C3 with GBG

RB	Buffer*	Patient's serum	Normal human serum	GBG‡	Preincubation at 37°C	C_6 inactivation by zymosan
ml	*ml*	*ml*	*ml*	*ml*	*min*	*%*
0.1	0.1	—	—	—	—	4
0.1	0.05	0.05	—	—	—	4
0.1	0.05	—	0.05	—	15	48
0.1	0.05	—	0.05	—	30	48
0.1	0.05	—	0.05	—	60	45
0.1	0.05	—	—	0.05	—	56
0.1	0.05	—	—	0.05	30	57
0.1	0.05	—	—	0.05	60	56
0.1	—	0.05	—	0.05	—	53
0.1	—	0.05	—	0.05	15	9
0.1	—	0.05	—	0.05	30	−3

* Veronal-buffered saline, pH 7.4, ionic strength 0.15, containing 1.5×10^{-4} M Ca^{2+} and 5×10^{-4} M Mg^{2+} and 0.05% gelatin (Kind and Knox Gelatin Co., Camden, N. J., type 2138).

‡ Final concentration 30 mg/100 ml in reaction mixtures.

Does GBG Form a C3-Inactivating Complex with CoF?—(*a*) Fractions from prolonged electrophoresis in Pevikon of sera containing different variants of GBG were tested for their ability to convert ^{125}I-labeled C3 in the presence of CoF on subsequent incubation at 37°C. Fig. 3 shows the distribution of C3 inactivation in four such sera. In no instance did this coincide in distribution with GBG protein, nor did it show evidence of the bimodality of the GBG variants. In general, the mediation of C3 inactivation by CoF had the approximate electrophoretic mobility of Gb S_1 to S.

(*b*) Evidence for complex formation between GBG and CoF in whole serum was sought by adding CoF to sera containing the rare fast and slow GBG variants in amounts roughly equimolar to GBG concentration. During immunoelectrophoresis, such sera produced clear-cut double arcs with anti-GBG (Fig. 4) but the precipitin arcs of CoF were single, the same as those produced by CoF in buffer, and did not correspond in any way to the GBG arcs in the same serum. There was no conversion of GBG after the addition of CoF and incubation at 37°C for 30 min.

(*c*) A mixture of [^{125}I]CoF and unlabeled CoF in a total amount equimolar to GBG content was added to normal serum and the mixture was incubated at 37°C for 30 min. During gel filtration through Sephadex G-200, as shown in Fig. 5, the [^{125}I]CoF formed three peaks. That appearing with the void volume (19S) was probably aggregated material since it was also present in buffer alone. The major peak (7S) corresponded to uncomplexed CoF. The middle peak (9S) represented a complex with a human serum protein, since it was not

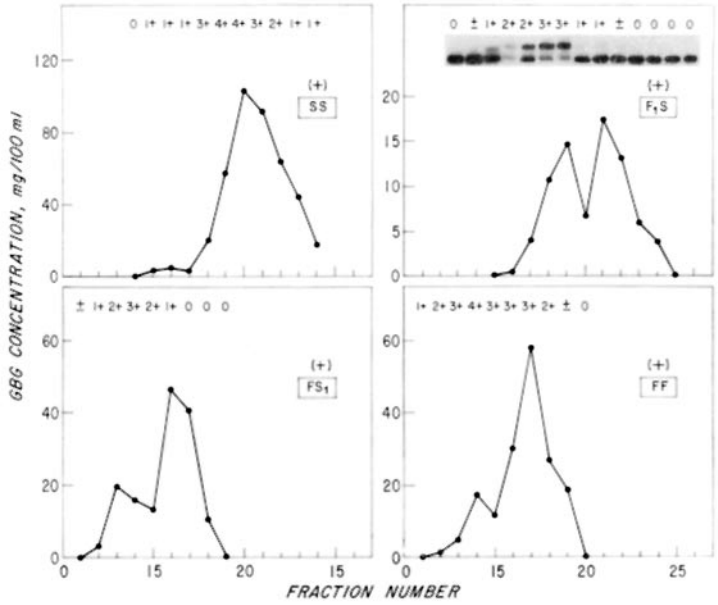

FIG. 3. GBG concentration and serum mediation of CoF inactivation of C3 in fractions from Pevikon electrophoresis of sera containing different genetic variants of GBG. The anode was at the right in each instance. Gb types of the sera were: SS (upper left), F_1S (upper right), FS_1 (lower left), and FF (lower right). The distribution of the serum mediator of C3 inactivation by CoF was assessed by incubating the electrophoretic fractions (in Mg^{2+}) with purified CoF and ^{125}I-labeled C3 and subjecting the mixtures to agarose gel electrophoresis and autoradiography. The autoradiographic patterns themselves are shown for Gb F_1S. Native C3 is the slower band; inactivation is reflected by conversion to the more rapid band (C3i). For this and the remaining sera, C3 inactivation was graded on a scale of 1–4+, shown above each of the GBG distribution patterns.

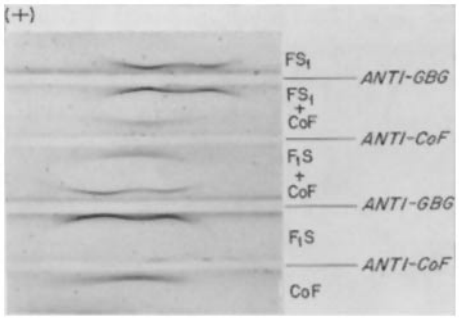

FIG. 4. Prolonged immunoelectrophoresis in agarose gel. The anode was at the left.

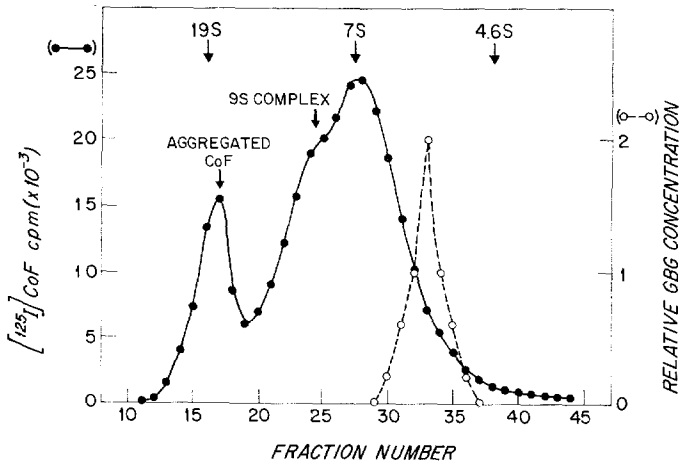

FIG. 5. Gel filtration through Sephadex G-200 of serum incubated with [^{125}I]CoF and unlabeled CoF at a final CoF concentration of 50 mg/100 ml. GBG concentrations were determined in all fractions. The positions of the three major protein peaks of the serum are indicated.

demonstrable during gel filtration of CoF in buffer and comprised 4% of labeled CoF added. GBG was eluted as a single symmetrical 6S peak, identical with that observed during gel filtration of fresh serum without added CoF. The possibility that GBG complexed with CoF might be antigenically "masked" was ruled out by immunochemically quantitating GBG in serum to which either CoF or buffer was added. The concentration of GBG in the first instance was 31 mg/100 ml and in the second it was 32.5 mg/100 ml.

(*d*) Labeled and unlabeled CoF were added to normal serum samples to a final concentration of 1 mg/100 ml or 50 mg/100 ml. If CoF and GBG in the serum formed a complex, it should be possible to precipitate a significant portion of the labeled CoF with antiserum to GBG. As shown in Table II, at neither concentration of CoF was this the case. Antiserum to GBG precipitated the same percentage of the [^{125}I]CoF as did anticeruloplasmin. When labeled GBG was added to serum and unlabeled CoF was added at either 1 or 50 mg/100 ml, the results were similar (Table II) in that labeled GBG was not precipitated by antiserum to CoF, although the percentage label precipitated at a CoF concentration of 1 mg/100 ml was 2½ times that at 50 mg/100 ml. It may be, therefore, that the anti-GBG used contained antibody to the protein capable of binding to CoF. The much lower percentage precipitation of labeled CoF in serum at 1 mg/100 ml compared with the percentage at 50 mg/100 ml may be explained by antigenic masking of CoF in complex with the binding protein.

(*e*) Although most preparations of GBG when incubated with CoF and ^{125}I-labeled C3 in the presence of Mg^{2+} did not result in C3 conversion as

judged by agarose gel electrophoresis and autoradiography, they all had factor B activity. A single preparation did induce C3 conversion in the presence of CoF. This preparation was studied for its interaction with CoF in polyacrylamide gel electrophoresis.

Fig. 6 shows that when this preparation was mixed in molar excess with labeled CoF, a complete slowing of the electrophoretic mobility of the CoF

TABLE II

Precipitation of ^{125}I-Labeled CoF or GBG in Serum by Antisera to GBG, CoF, and Ceruloplasmin

Antiserum to	CoF* + NHS (1 mg/100 ml)‡	CoF + NHS (1 mg/100 ml)‡ + GBG*	CoF* + NHS (50 mg/100 ml)‡	CoF + NHS (50 mg/100 ml)‡ + GBG*
	%§	%§	%§	%§
GBG	2.0	73.6	0.9	82.4
Ceruloplasmin	2.9	2.2	2.3	0.8
CoF	23.5	5.6	89.0	1.6

* Labeled with ^{125}I.

‡ Concentrations in parentheses are final CoF concentrations in serum.

§ Percent offered radioactivity precipitated.

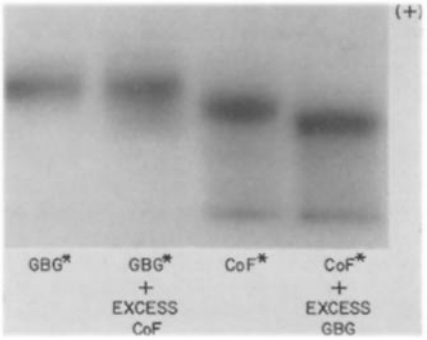

FIG. 6. Autoradiography of polyacrylamide gel electrophoresis of mixtures of purified ^{125}I-labeled and unlabeled CoF and GBG. Labeled proteins are indicated by asterisks.

occurred. When, on the other hand, a molar excess of CoF was added to this preparation of GBG labeled with ^{125}I, most of the GBG remained unaltered in electrophoretic mobility. A small portion of the radioactivity migrated with the mobility of complexed CoF.

(*f*) A partially purified preparation of GBG, free of GBGase inhibitor but containing the CoF-binding protein, was incubated in the patient's serum for 30 min at 37°C. This resulted in complete cleavage of the GBG and, as indicated earlier, complete destruction of factor B activity. When ^{125}I-labeled C3 was added to this mixture and it was incubated for an additional 30 min at 37°C,

no more C3 conversion occurred than was observed in normal serum (Fig. 7). However, when CoF was added, C3 conversion did occur.

Estimates of the Concentration in Normal Serum of the CoF-Binding Protein.—(*a*) The previous experiments suggested that some preparations of GBG were contaminated with small amounts of protein capable of complexing with CoF. To determine approximately the concentration of this CoF-binding protein in normal serum, labeled CoF was added to two sera in varying final concentrations from 25 mg/100 ml to 0.5 mg/100 ml and incubated for 30 min at 37°C. The mixtures were then subjected to polyacrylamide gel electrophoresis and autoradiography. Fig. 8 shows that even at a CoF concentration as low as 0.5 mg/100 ml some of the CoF remained in free form. If the complex is equimolar, as is suggested by the presence of only a single slowed band, then the concentration of CoF-binding protein was less than 0.5 mg/100 ml.

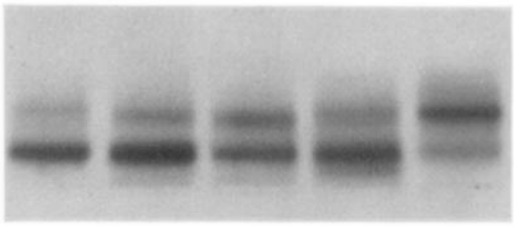

FIG. 7. Autoradiography of agarose gel electrophoresis of (from left to right): (*a*) ^{125}I-labeled C3 (C3*) incubated with normal human serum for 30 min at 37°C; (*b*) a partially purified preparation of GBG containing the CoF-binding protein incubated at 37°C for 30 min and then with C3* in buffer for an additional 30 min; (*c*) the same as the preceding except that CoF was added in the second incubation; (*d*) the same as *b* except that serum from the patient with type I essential hypercatabolism of C3 was substituted for buffer (to supply GBGase) in the first incubation; his serum contains no detectable CoF-binding protein; (*e*) the same as the preceding except that CoF was added in the second incubation. Note that C3 conversion greater than in normal human serum alone occurs only in *c* and *e* (in the presence of CoF) indicating preservation of CoF-binding protein activity despite conversion of GBG. We have no explanation for the apparent increase in CoF-binding protein activity after incubation in the patient's serum.

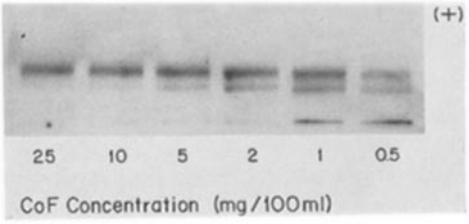

FIG. 8. Autoradiography of polyacrylamide gel electrophoresis of mixtures of ^{125}I-labeled CoF with serum at different final concentrations of CoF. The faster band represents free CoF, the slower is CoF complexed with a serum protein.

(*b*) One interpretation of the experiment shown in Fig. 5 is that CoF added to a final serum concentration of 50 mg/100 ml (equimolar to the GBG content) is very much in excess of the binding capacity of the serum. Thus, the preponderant 7S peak represented uncomplexed CoF and only 4% of CoF was bound. To test this hypothesis, ^{125}I-labeled CoF was added to normal serum to a concentration of 0.17 mg/100 ml, below the CoF-binding capacity estimated in the previous experiment. Fig. 9 shows that under these conditions all of the labeled CoF shifted to the 9S area during gel filtration and the 7S peak was completely absent. Thus, the CoF-binding capacity of normal serum was between 0.17 and 2 mg/100 ml or under 6.5% of the concentration of GBG.

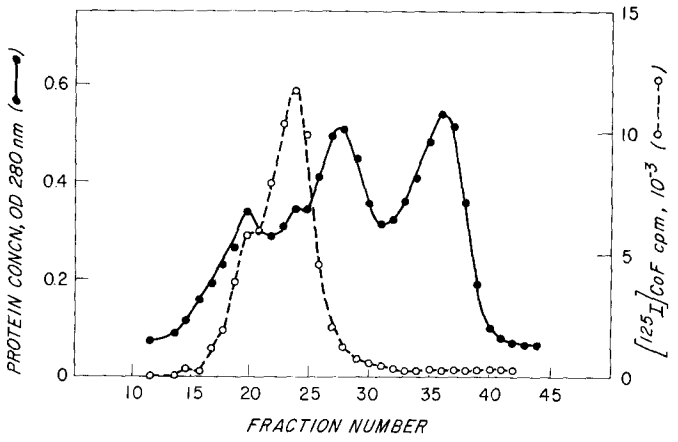

FIG. 9. Gel filtration through Sephadex G-200 of serum incubated with [^{125}I]CoF at a final CoF concentration of 0.17 mg/100 ml. Compare with Fig. 5 and note the complete shift of labeled CoF from the 7S to the 9S position.

DISCUSSION

Three lines of evidence have been presented which clearly establish that GBG is the previously described factor B in the properdin system. (*a*) Purified preparations of GBG restored the factor B activity of serum heated at 50°C for 30 min. The observation that two fractions from different parts of the final elution peak of GBG from DEAE-Sephadex had similar factor B activities per microgram of protein provides additional confirmation of this identity. (*b*) Factor B activity paralleled GBG protein concentration in the electrophoretically separated genetic variants. Since specific genetic variation occurs independently at the various loci that determine the structure of individual proteins, if activity parallels protein distribution for variants, particularly rare ones, then the activity must be a function of the molecule in question. This has recently been shown to occur in the case of genetic variants of C3, even for their minor gene-specific anodal components (26). (*c*) GBGase in the serum of the

patient with type I essential hypercatabolism of C3 destroyed factor B activity as it cleaved GBG.

The anticomplementary activity of snake venom has been a subject of investigation throughout virtually the entire history of the study of complement itself. It was noted in 1894 that putrefaction occurred very rapidly in persons who died of snake bites and that their serum lacked the bactericidal activity of normal serum (27). Flexner and Noguchi in 1902 (28) showed that the venoms of many snakes destroyed complement activity in vitro. Only two components of complement were known in 1912, when Ritz (29) showed that the action of venom was on neither. This observation prompted him to define a third component. It was only much later when purification techniques were developed allowing further separation of this third component that the specific target protein of venom action could be delineated. This was identified as C3 (in modern terms) by Klein and Wellensieck (30). The attack was shown by Nelson (31) to be indirect, involving a heat-labile serum protein. Müller-Eberhard and co-workers (13, 14) further characterized the serum protein as a 5S β-pseudoglobulin, demonstrated that a complex was formed between this protein and the venom factor (CoF) and showed that this complex bore C3-cleaving activity.

Since the serum of the patient with type I essential hypercatabolism of C3 lacked the CoF-binding protein, the possibility that GBG was this protein was considered earlier (5). However, since cobra venom added to normal serum failed to alter the gel filtration characteristics of GBG, this seemed unlikely. The present studies provide considerable evidence that these two proteins are distinct. (*a*) The distribution in electrophoretic fractions of the mediator of C3 inactivation by CoF differed from that of GBG in sera containing GBG variants. (*b*) The distribution of CoF in sera containing GBG variants in immunoelectrophoresis differed completely from the distribution of GBG. (*c*) No alteration in the size of GBG could be detected on gel filtration of serum incubated with labeled CoF despite complex formation of the latter with a serum protein. (*d*) It was not possible to precipitate CoF added to serum by anti-GBG, nor GBG by anti-CoF. (*e*) Most preparations of GBG did not promote C3 conversion by CoF; the one preparation that did showed a small amount of contamination with a CoF-binding protein. (*f*) GBGase cleaved GBG and destroyed factor B but did not destroy CoF-mediated conversion of C3. The lack of CoF binding by factor B has also been observed by Hunsicker and his colleagues (32) in whole serum and in partially purified form.

It appears that the CoF-binding protein is a trace serum protein occurring at a concentration less than 6.5% of that of GBG. It is clear that GBG and the major protein of the C3 proactivator (C3PA) isolated by Götze and Müller-Eberhard (33) are the same. The observations in this report make it likely that contamination of C3PA preparations by traces of CoF-binding protein would not be detectable by standard electrophoretic or immunoelectrophoretic techniques. We previously reported that C3PA preparations had the activity of

factor B in the properdin system (24) and the present observations are entirely consistent with this finding. Similar dose-response relationships were observed while testing C3PA and GBG preparations for factor B activity.

Despite the multiple serum protein deficiencies in the patient with type I essential hypercatabolism of C3 (4, 5, 10–12), the only protein thus far shown to be subject to proteolytic attack in his serum in GBG. This cleavage was found in the present study to be accompanied by loss of factor B activity. The identification of GBG with factor B thus helps to clarify the patient's abnormalities in activities of the properdin system and to reemphasize the importance of the properdin system in the maintenance of host resistance to infection.

Our experiments do not rule out the participation of GBG, its fragments, or other serum proteins in the attack of CoF-binding protein-CoF complex on C3 or on later-acting complement components. Similarly, we have not yet identified the mediator of the attack by GBG or one of its fragments on C3 (10). This mediator, apparently missing from the serum of the patient with type I essential hypercatabolism of C3, was not present in the partially purified preparation containing GBG and CoF-binding protein.

If, as seems to us to be the case, factor B is distinct from the CoF-binding protein, the role of the latter in the properdin or alternate pathway to C3 inactivation remains to be defined. The present experiments do not rule out the possibility that the CoF-binding protein is related to and perhaps intimately involved in the properdin pathway. Indeed, this protein, as well as factor B, is undetectable in the serum of the patient with type I essential hypercatabolism of C3.

SUMMARY

Factor B activity of the properdin system was found to be identical with purified glycine-rich β-glycoprotein (GBG) but was distinct from the normal human serum protein capable of forming a C3-inactivating complex with a protein from cobra venom (CoF). Factor B activity coincided with electrophoretically separated GBG genetic variants, whereas the CoF-binding protein did not. GBGase destroyed factor B as it cleaved GBG but did not destroy the C3-inactivating activity of the CoF-binding protein. During incubation of serum with CoF, GBG did not change in molecular size, nor was there any coincidence in the immunoelectrophoretic mobilities of CoF and GBG. It was not possible to precipitate labeled CoF incubated with serum by anti-GBG, nor labeled GBG from serum incubated with CoF by anti-CoF.

The CoF-binding capacity of serum was 2 mg/100 ml or less or under 6.5% of the serum concentration of GBG. When labeled CoF was added to serum below the binding capacity, complete complexation of the CoF was demonstrated, whereas CoF was largely uncomplexed when CoF was added in amounts equimolar to GBG.

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