IMMUNOGLOBULIN-SULFATED POLYSACCHARIDE INTERACTIONS

Binding of Agaropectin and Heparin by Human IgG Proteins*

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Immunoglobulin molecules serve as humoral and cellular receptors of the immune response by their capacity to bind antigen and to interact with other biological substances. Considerable information concerning the antibody and effector functions of immunoglobulins has come from studies on the homogeneous (monoclonal) protein products of plasma-cell and related malignancies (reviewed in [1]). Homogeneous immunoglobulins that interact with the acid polysaccharides agaropectin (2–4), carrageenan (4), or heparin (5–10) have been identified among human myeloma proteins and Waldenström macroglobulins. The extent, specificity, and functional significance of these interactions, however, are presently unknown.

We have employed several types of immunochemical and physicochemical methods to demonstrate the interaction of certain monoclonal proteins of the major immunoglobulin class, IgG, with agar and/or heparins derived from several biological sources. 8 such proteins were identified among 100 monoclonal IgG proteins examined. The capacity of certain IgG proteins to distinguish molecular heterogeneity among heparins was shown by the patterns of reactions with heparins of defined molecular form. These results provide new information concerning the nature and extent of IgG-heparin interactions which, because of their distinct chemical specificity, may have potential physiological significance.

Materials and Methods

Materials. Serum samples containing monoclonal immunoglobulins were obtained from patients with multiple myeloma or benign monoclonal gammopathy. Pooled normal human IgG (Cohn Fraction II γ-globulin) was obtained from Mann Research Laboratories, Mann Chemical Corp, Louisville, Ky. Commercial sources of heparin were as follows: sodium heparin derived from porcine intestinal mucosa, Riker Laboratories, Inc., Northridge, Calif. (Lipoheparin), Abbott Diagnostics, Diagnostic Products, North Chicago, Ill. (Panheparin), and Inolox Corp., Biomedical Div., Glenwood, Ill. (Stage 14 heparin); sodium heparin derived from bovine lung, Upjohn Co., Kalamazoo, Mich. (Heparin Sodium Injection U.S.P.) and ICN Nutritional Biochemicals Corp., Cleveland, Ohio (Heparin-Sodium, 100 U/mg); 3H-labeled heparin (0.732 mCi/mg), New England Nuclear, Boston, Mass. Mercuripapain, hog pepsin, DNA, and RNA were obtained from Worthington Biochemical Corp., Freehold, N. J.; D-(+)-glucosamine free base, D-(+)-glucosamine HCl, and chondroitin sulfate, ICN Nutritional Biochemicals Corp.;

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glucuronic acid, California Foundation for Biochemical Research; galactosamine HCl and hyaluronic acid, Mann Research Laboratories; N-acetyl-d-glucosamine, Calbiochem-Behring Corp., American Hoechst Corp. San Diego, Calif.; dextran (Blue dextran 2000) and Sepharose 4B, Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.; and polyethylene glycol 6000, Fisher Scientific Co., Pittsburgh, Pa. (Carbowax PEG 6000).

Methods. IgG proteins and other protein constituents were isolated from serum specimens by zone (block) electrophoresis on polyvinyl chloride (Pevikon; KemaNord, Stockholm, Sweden). Monoclonal IgG protein preparations consisted of ~85–95% homogeneous IgG as determined by immunochromatographic analyses with monospecific anti-heavy chain and with anti-\(\kappa\) and anti-\(\lambda\) light chain antisera. IgG proteins were subclassified allotypically or chemically on the basis of analyses performed by Dr. Arthur G. Steinberg, Case Western Reserve University, Cleveland, Ohio and Dr. Blas Frangione, New York University Medical Center, New York, respectively. The methods for the preparation and isolation from IgG of papain-derived Fc and Fab fragments, pepsin-derived F(\(\text{ab}'\))\(_2\) and F(\(\text{ab}'\)) fragments, and heavy and light polypeptide chains were as described previously (11, 12). The absence of any remaining intact IgG in these preparations was confirmed immunochromatically. Carbamylated and acetylated derivatives of IgG were prepared by use of potassium cyanate (13) and acetic anhydride (14), respectively.

The methods for preparing multi-chain (macromolecular) rat skin heparin (relative molecular mass \([M_r]\) >900,000), the chemically-derived single-chain form \([M_r] \sim 60,000\), and the enzymatically-derived depolymerized forms (designated "partial", \(>20,000\) and \(8,000-20,000 M_r\) were as described (15–17). One sample of multi-chain rat skin heparin served as the source of the single-chain and depolymerized heparin components. The molecular weights of the depolymerized rat skin heparins were estimated by gel filtration through a column of Bio-Gel A 15M calibrated with heparin standards of 23,000, 10,000, and 6,000 \(M_r\) (18) (kindly furnished by Dr. Robert D. Rosenberg, Harvard Medical School, Boston, Mass.) and with the single-chain form of rat skin heparin. Four samples of porcine intestine heparin of defined molecular weight and functional activity, also furnished by Dr. Rosenberg, consisted of two \(\sim20,000-M_r\) species, one of high affinity and one of low affinity for antithrombin, and two \(\sim7,000-M_r\) components, one of high affinity and one of low affinity for antithrombin (19, 20).

Protein concentrations were determined by a modification of the Folin-Ciocalteu method (12), and heparin concentrations by the metachromasia at \(A_{500 \text{ nm}}\) of Azure A dye (19). Optical density measurements were made using a Gilford spectrophotometer.

Immunoelectrophoretic and immunodiffusion analyses were performed in 2% (wt:vol) agar or 1% (wt:vol) agarose gels prepared in a 20 mM sodium barbital buffer, pH 8.6, ionic strength 0.02. For certain experiments, immunodiffusion analyses were performed in 1% (wt:vol) agarose gels prepared in a 50 mM sodium barbital buffer, pH 8.6, containing 3% polyethylene glycol 6000.

Electrophoretic analyses on cellulose acetate or agarose gel membranes were performed with a Microzone apparatus (Beckman Instruments, Inc., Fullerton, Calif.). Membranes were stained for protein or mucopolysaccharide by Ponceau S or toluidine blue (0.1% in 0.5% acetic acid), respectively.

For affinity chromatography and solid-phase assay experiments, a monoclonal IgG protein was immobilized on cyanogen bromide-activated Sepharose 4B (21) at an initial ratio of 1.7 mg of protein:1 ml of gel, as determined by measuring the amount of protein that remained uncoupled. Sepharose, similarly activated under alkaline conditions by cyanogen bromide and then rendered inactive by treatment with ethanalamine, served as a control for affinity column and solid-phase assays. Scatchard plots (22) of IgG-heparin associations were constructed from data obtained by adding a constant amount of \(^3\)H-labeled heparin (10^6 cpm) and increasing amounts of bovine lung heparin to 0.24-ml samples of IgG-Sepharose gel (containing 0.40 g of protein) or 0.24 ml of ethanalamine-Sepharose gel suspended in 2 ml of 25 mM sodium phosphate buffer, pH 7.4. The samples were agitated occasionally during a 16-h incubation period at 7, 23, 32, and 56°C, centrifuged at 300 g for 5 min in a temperature-controlled centrifuge, and the radioactivity of the supernatant fluid determined. Data points were derived

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1. Abbreviation used in this paper: \(M_r\), relative molecular mass.
from the averages of triplicate samples for each concentration of heparin minus the nonspecific adsorption of heparin by the ethanolamine-Sepharose control samples.

Dialfiltration analyses (23) of heparin-IgG binding behavior were performed using an Amicon Model 8MC ultrafiltration chamber equipped with an XM100A membrane (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) (cut-off level, $M_w \sim 100,000$). Bovine lung heparin (10 $\mu$g/ml) was infused as a constant rate into an ultrafiltration cell containing a 2-ml solution of IgG protein (2 mg) dissolved in a 25 mM sodium phosphate buffer, pH 7.4. The protein solution was stirred continually, and chamber pressure (~5 lb/in$^2$) maintained to ensure a constant flow rate of 6 ml/h. After 100 $\mu$g of heparin was added to the cell, the heparin concentration in the ultrafiltrate was measured by the metachromasia of Azure dye at $A_{500}$ nm.

### Results

**Recognition of Agar-binding Proteins.** Monoclonal IgG human immunoglobulins that interact with agar were recognized by the characteristic dense white precipitate formed in agar gels by serum specimens from certain patients with multiple myeloma or benign monoclonal gammopathy. For example, during the process of serum analysis by immunoelectrophoresis, a dense, white rocket-shaped precipitate, located immediately cathodal to the application well, appeared after electrophoresis but before the antiserum trough was filled (Fig. 1). Although the location of the rocket precipitate corresponded to that of the subsequent immune precipitin reactions, the relatively anodal electrophoretic mobility of the IgG proteins in the agar gel at pH 8.6 did not correspond to the more cathodal location of the proteins subjected to electrophoresis at the same pH either on a cellulose acetate membrane or in an agarose gel. Further, no precipitate was evident immediately after electrophoresis in agarose gels. After fractionation of serum proteins by zone electrophoresis, only the IgG-containing fractions formed precipitates in agar gels identical to those observed for the whole serum. Single radial diffusion in agar gels led to the formation of a dense white precipitin ring around the application well, which had a diameter proportional in size to the concentration of IgG protein. Although the isoelectric points of the agar-binding IgG proteins were typically high (as shown by their extreme cathodal electrophoretic mobility at pH 8.6), other monoclonal proteins of identical mobility did not have this property. Six agar-binding IgG proteins were identified among 100 monoclonal IgG-containing serum specimens. No agar-binding proteins were found

![Fig. 1. Precipitation of an IgG protein in a 2% agar gel. In this immunoelectrophoretic analysis of a serum specimen containing a monoclonal IgG protein (CA), a photograph of the reaction was obtained immediately after electrophoresis for 45 min at 50 V (0 h) and at the designated times (24 h and 48 h) after the antiserum trough was filled with an anti-IgG antiserum. The locations of the polyclonal IgG-anti-IgG and the monoclonal IgG-anti-IgG precipitin reactions are indicated by arrows on the 24-h and 48-h patterns, respectively.](image)
in 26 serum specimens containing monoclonal IgA proteins or among 20 with IgM, 5 with IgD, and 1 with IgE monoclonal proteins.

IgG-Agar Interaction. The extent of precipitation of IgG proteins in agar gels was influenced by ionic strength and temperature; no precipitate formed in gels containing >50 mM NaCl and the precipitate was denser in those slides incubated at 7°C than in those incubated at 37°C. Precipitation with agar occurred with the pepsin-derived F(ab')2 fragment but not with F(‘ab’), the papain-derived Fc or Fab fragments, or the isolated heavy or light chains.

Recognition of Heparin-binding IgG Proteins. Because some IgG proteins precipitated in agar but not in agarose, the presence of the acidic polysaccharide constituent of agar, agarpectin (24), was implicated as the cause of this interaction. Another interacting ligand, heparin, was found by testing the capability of serum CH (containing an agar-binding monoclonal IgG protein) to form precipitin-like reactions with other hexoses, hexosamines, and acidic macromolecules. When serum CH was tested by comparative double-diffusion analyses in 1% agarose gels containing 3% polyethylene glycol 6000 (25) against chondroitin sulfate, dextran, DNA, galactosamine, glucosamine, glucosaminic acid, D(+)glucosamine HCl, glucuronic acid, heparin, hyaluronic acid, and RNA (in concentrations from 0.15 to 5.0 mg/ml), a precipitin-like reaction was obtained only with heparin. That the monoclonal IgG protein contained in serum CH was solely responsible for the reaction with heparin was readily demonstrated by double diffusion and immunoelectrophoretic analyses in agarose gels (Fig. 2).

Among 100 monoclonal IgG-containing serum specimens, 8 formed precipitin-like reactions with heparin (including 5 of 6 serums which reacted with agar) and, in each case, the reaction could be localized to the monoclonal IgG protein. The three additional heparin-binding IgG proteins did not bind agar but only one agar-binding protein failed to bind heparin.

Fig. 2. Demonstration of heparin-IgG interaction in 1% agarose gels containing 3% polyethylene glycol 6000. (a) Double-diffusion analysis: the central well contained serum specimen CH and the six outer wells (1, 2, 3, 4, 5, and 6) contained sodium heparin derived from porcine intestinal mucosa at concentrations of 5.0, 2.5, 1.3, 0.6, 0.3, and 0.1 mg/ml, respectively. (b) Immunelectrophoresis: the antigen well contained serum specimen CH and the upper and lower antisera troughs contained bovine lung heparin (4.0 mg/ml) and an anti-IgG antiserum, respectively.
**Heparin-IgG Interaction in Agarose Gels.** The presence of both heparin and protein in the precipitin-like lines formed in agarose gels was confirmed by staining duplicate gels with toluidine blue and Coomassie blue, respectively. This reaction between the IgG proteins and heparin was visualized after 16-24 h at 7°C; however, when commercial (pharmacologic) sources of bovine lung or porcine intestine heparin were used, the lines disappeared at 37°C or when washed in solutions containing >50 mM NaCl. In contrast, the reaction with rat skin heparin occurred at 37°C and was stable under physiological conditions.

All eight IgG proteins formed precipitin-like reactions in agarose gels with each of the heparin preparations tested. The nature of these reactions was markedly different, depending on the source and molecular form of the heparin (Fig. 3). The precipitin-like reactions between IgG proteins and three different pharmacologic sources of heparins derived from bovine lung or porcine intestinal mucosa were less sharp and only partially identical to those obtained with multi-chain rat skin heparin (Fig. 3 A). Furthermore, multiple precipitin-like bands resulted from interactions between the IgG proteins and some commercial heparin preparations. The bands formed from the interaction of the IgG proteins with multi-chain rat skin heparin and with single-chain rat skin heparin gave reactions of identity; the precipitin-like reaction of the commercial heparins were also only partially identical to those of the single-chain species (Fig. 3 B). The partially depolymerized ~50,000-Mr form was distinguishable from the more extensively depolymerized ~20,000-Mr species (Fig. 3 C). The precipitin-like reaction given by the lower molecular weight component was comparable to that of commercial porcine heparin. One of the two precipitin-like bands formed between IgG and porcine heparin gave a reaction of partial identity with the band formed between IgG and the ~20,000-Mr depolymerized rat skin heparin. The IgG proteins could also differentiate between the porcine heparins of defined molecular weight; the reactions given by the 7,000-Mr material were partially identical to those of the 20,000-Mr species (Fig. 3 D). The reactions of the IgG proteins with heparins of comparable molecular weight having high or low affinity for antithrombin were identical.

Precipitin-like reactions of protein CA with both types of rat skin heparins (but not with the commercial heparins) were evident after bovine lung heparin had been added to serum CA; no reactions with commercial or rat skin heparins occurred after addition of multi-chain rat skin heparin.

**Demonstration of Heparin Binding by IgG Proteins.** An interaction between heparin and IgG could be demonstrated electrophoretically at a pH of 3.0 to maximize the electrophoretic differences of the two constituents (15). Protein CA combined with increasing amounts of heparin gave protein-mucopolysaccharide complexes of increasingly anodal electrophoretic mobilities. Under comparable conditions, no complexes resulted when polyclonal IgG was substituted for protein CA. Heparin-IgG interaction could also be shown by diafiltration experiments employing membranes which almost completely prevented the passage of IgG and yet only slightly impeded the flow of heparin. Three monoclonal IgG proteins of similar electrophoretic mobility were studied: an agar- and heparin-binding protein (protein CA), a heparin-binding protein (protein MA), and a protein that bound neither agar nor heparin (protein BR). Under comparable conditions, heparin was retained only in the ultrafiltration cells containing proteins MA and CA. The amounts of heparin
FIG. 3. Comparison of the interactions in agarose gels of monoclonal IgG-containing sera with preparations of bovine, porcine, and rat skin heparins. The center well in each pattern contained serum specimens CA or CAR as indicated; the concentration of the monoclonal IgG protein in each serum was ~30 mg/ml. (A) The outer wells 1 and 4 contained multi-chain (macromolecular) rat skin heparin (2.5 mg/ml); well 2, bovine lung heparin, Upjohn Co. (0.3 mg/ml); well 3, Stage 14 heparin, Inolex Corp., Biomedical Div. (0.3 mg/ml); well 5, porcine intestine heparin, Abbott Laboratories (0.3 mg/ml); and well 6, bovine lung heparin, ICN Nutritional Biochemicals Corp. (1 mg/ml). (B) The outer wells 1 and 4 contained multi-chain rat skin heparin (2.5 mg/ml); wells 2 and 5, single-chain rat skin heparin (2.5 mg/ml); well 3, porcine intestine heparin (0.3 mg/ml); and well 6, bovine lung heparin (1 mg/ml). (C) The outer wells 1 and 4 contained single-chain rat skin heparin (2.5 mg/ml); well 2, partially depolymerized heparin (Mr ~ 50,000, 1 mg/ml); wells 3 and 5, extensively depolymerized heparin (Mr ~ 20,000, 1 mg/ml); and well 6, porcine intestine heparin (0.3 mg/ml). (D) The outer wells 1 and 4 contained single-chain rat skin heparin (2.5 mg/ml); well 2, high antithrombin affinity, low molecular weight porcine intestine heparin (Mr ~ 7,000, 0.7 mg/ml); well 3, high antithrombin affinity, high molecular weight porcine intestine heparin (Mr ~ 20,000, 0.7 mg/ml); and well 6, low antithrombin affinity, low molecular weight porcine intestine heparin (Mr ~ 7,000, 0.7 mg/ml). The 1% (wt:vol) agarose gels were prepared in a 50 mM sodium barbital buffer, pH 8.6, containing 3% polyethylene glycol 6000.
in the ultrafiltrate of cells containing either no protein (control) or proteins MA, CA, and BR were 29.4, 0.9, 4.0, and 35.4 µg, respectively.

The binding of heparin by protein CA was also shown by affinity chromatography. 1 ml of the protein CA-Sepharose column (protein density, 1.7 mg/ml of packed gel) bound ~200 µg of heparin, retaining virtually 100% of applied material as long as this amount was not exceeded. The same sites on the column bound both ³H-labeled and unlabeled heparin, as evidenced by the lack of further binding of ³H-labeled heparin after pretreatment with an excess of unlabeled heparin (1 mg/ml of column volume). The bound heparin was completely eluted with 1 M NaCl; only 55% of the heparin could be eluted with 0.15 M NaCl. A Scatchard-type analysis of the binding at 7, 24, and 37°C to immobilized protein CA of ³H-labeled heparin and unlabeled bovine lung heparin revealed a biphasic curve. The association constant of the high-affinity sites was temperature sensitive (7°C, 1.3 × 10⁸ M⁻¹; 24°C, 0.6 × 10⁸ M⁻¹; 37°C, 0.3 × 10⁸ M⁻¹); the lower affinity sites were more numerous and less affected by increased temperature, and had a binding constant ~10% that of the high-affinity sites.

The addition of heparin to solutions of certain IgG proteins altered the characteristic $A_{280}$ nm (but not $A_{600}$ nm) as observed when heparin interacts with other proteins (26). Although heparin alone has no absorbance at this wavelength, a sharp increase in optical density of a 1 mg/ml solution of protein MA occurred when heparin was added. The maximum effect was observed with ~50 µg of heparin per ml of protein solution (Fig. 4). No changes in absorbance occurred when heparin was added to solutions of polyclonal IgG or to solutions of the IgG protein BR (data not shown).

Precipitation of IgG Proteins by Heparin. Heparin and protein coprecipitated when heparin was added to solutions of heparin-binding IgG proteins. This effect was quantitated turbidimetrically at $A_{600}$ nm by determining the protein content of the precipitate or supernatant. In the case of serum, a decrease in concentration after addition of heparin was documented by densitometry after analytical electrophoresis of the serum. The precipitation by heparin of IgG proteins directly from serum

![Fig. 4. Ultraviolet absorbance of IgG. The absorbance of 1 mg/ml solutions of the monoclonal IgG protein MA (○) and polyclonal IgG (Fraction II γ-globulin) (▲) in a 25 mM sodium phosphate buffer, pH 7.4, was compared at $A_{280}$ nm in the presence of varying concentrations of bovine lung heparin. The Δ OD represents the percent difference in absorbance of the protein solutions after addition of heparin.](image-url)
was facilitated by adding 3% polyethylene glycol to the reaction mixture (25). The quantitative nature of the precipitin-like reactions of two heparin-binding proteins (CA and MA) is illustrated in Fig. 5.

The amounts of proteins CA and MA precipitated by bovine lung heparin were affected by the ionic strength and temperature of the reaction mixture. Maximum precipitation occurred in solutions of low ionic strength. As shown in Table I, the amount of protein MA precipitated by heparin decreased progressively as the ionic strength of the solution was increased by the addition of NaCl. When the temperature was increased from 7 to 24°C, the extent of protein precipitated by heparin also decreased. The addition of urea to a final concentration of either 3 or 6 M to a solution of protein CA and heparin (in 25 mM sodium phosphate buffer, pH 7.4) produced a 28 and a 47% inhibition, respectively, of protein precipitation. The

![Graph](image)

**Fig. 5.** Precipitation of monoclonal IgG proteins by heparin. 100 μg of the IgG proteins CA (○), MA (▲), and BR (●) in a 50 mM sodium phosphate buffer, pH 7.4, containing 4% polyethylene glycol 6000, were incubated for 15 min at 7°C with 0.7, 13.5, 27, 54, or 270 μg of bovine lung heparin. The samples were centrifuged at 300 g for 5 min and the resulting precipitates were washed three times with the buffer solution, dissolved in 1 M NaOH, and the protein content was determined.

| NaCl | Protein precipitated* | Inhibition of precipitation |
|------|-----------------------|-----------------------------|
| mM   | μg                    | %                           |
| —    | 71                    | 0                           |
| 25   | 53                    | 25                          |
| 50   | 50                    | 29                          |
| 75   | 37                    | 47                          |
| 125  | 23                    | 67                          |

* The complete reaction mixtures (1 ml) contained 25 mM sodium phosphate buffer, pH 7.4, 4% polyethylene glycol 6000, 13.5 μg bovine lung heparin, 100 μg IgG protein MA, and concentrations of NaCl from 25 to 125 mM. Each mixture was incubated at 7°C for 15 min, centrifuged at 300 g for 5 min, and the protein content in the recovered precipitate determined.
precipitation of protein CA by both chain forms of rat skin heparin, but not by commercial sources of bovine lung or porcine intestinal heparin, occurred under physiological conditions of ionic strength and temperature. When ionic strength was increased by adding NaCl in concentrations from 25 to 150 mM or when the temperature was raised from 7 to 24°C, the precipitation of IgG by multi-chain rat skin heparin decreased by <25%.

The interaction of monoclonal IgG proteins with the acid polysaccharides heparin and agaropectin was not affected by chemical reduction and alkylation of the native IgG molecule. Carbamylation or acetylation of the IgG protein CA reduced its positive charge and abolished its capability to bind heparin (and agar).

Localization on the IgG Molecule of the Heparin-binding Site. The Fab (but not the Fc) fragments of proteins CA and MA were precipitated by the multi-chain and single-chain rat skin heparins. Further, the dimeric fragment F(ab')$_2$ of protein MA retained its heparin-binding reactivity, giving a precipitum curve similar to the intact molecule (Fig. 5). Immobilized bovine lung heparin was the ligand used to investigate the heparin-binding capacity of the pepsin-derived dimeric and monomeric fragments of protein MA. The data for the F(ab')$_2$ and F(ab') fragments of protein MA (Table II) indicate that both components retained their heparin-binding activity; reduction of the dimeric F(ab')$_2$ fragment of protein MA to the F(ab') monomer led to decreased

| Table II |
|-------------------|
| Binding of a Monoclonal IgG Protein and Its Pepsin-derived Fragments to Immobilized Heparin |

| Protein     | Amount added* | Amount bound to heparin-Sepharose |
|-------------|---------------|----------------------------------|
|             | µg            | µg                               | %     |
| IgG         |               |                                  |
|            | 30            | 25‡                             | 83    |
|            | 40            | 35                               | 88    |
|            | 90            | 70                               | 78    |
|            | 170           | 120                              | 71    |
|            | 400           | 210                              | 53    |
| F(ab')$_2$ | 50            | 40                               | 80    |
|            | 60            | 40                               | 67    |
|            | 120           | 90                               | 75    |
|            | 225           | 110                              | 49    |
| F(ab')     | 60            | 40                               | 67    |
|            | 90            | 60                               | 67    |
|            | 150           | 90                               | 60    |
|            | 260           | 90                               | 35    |

* Designated amounts of the native IgG protein MA and its dimeric F(ab')$_2$ and monomeric F(ab') pepsin-derived fragments were incubated at 7°C for 15 min with 0.5 ml of bovine lung heparin-Sepharose or ethanolamine-Sepharose in a 50 mM sodium phosphate buffer, pH 7.4. The samples were centrifuged at 300 g for 5 min and the protein concentrations in the supernatant fluids were measured.

‡ Corrected for nonspecific absorption of heparin to ethanolamine-substituted Sepharose.
precipitation with heparin. In contrast with the intact protein, the Fab fragments of certain IgG proteins (e.g., CA and CH) were not precipitated by commercial heparins.

Characteristics of Representative IgG Proteins. Several immunochemical and physicochemical properties of 11 monoclonal IgG proteins were studied (Table III). Six of the nine agar-/heparin-binding proteins were of the IgG1 subclass. Activity was independent of the IgG light chain type, solubility property, or electrophoretic mobility. Euglobulins may precipitate nonimmunologically with acid polysaccharides (3). Although proteins MA, SU, and GO are not euglobulins, they also bound heparin or agar; conversely, the euglobulin protein BO bound neither. Evidence for a cross-reacting idiotypic determinant (27) among the heparin-binding IgG proteins was obtained with an anti-idiotypic antiserum prepared against the crystallizable monoclonal IgG heparin-binding protein DOB (6). By immunodiffusion analyses, the eight heparin-binding IgG proteins each formed a precipitin reaction with the anti-DOB antiserum (kindly supplied by Dr. Lisa A. Steiner, Massachusetts Institute of Technology, Cambridge, Mass.), which had been absorbed extensively with pooled normal IgG (Cohn Fraction II) or with monoclonal IgG proteins of the same VH region subgroup (VIII) as protein DOB (28). When tested in similar fashion, this antiserum did not react with any of 52 non-heparin-binding monoclonal IgG proteins (see Fig. 6 for representative analysis). Further, the precipitin reactions between the anti-DOB antiserum and the heparin-binding IgG proteins CA and CAR were inhibited by the presence in agarose gels of multi-chain rat skin heparin; by contrast and under identical conditions, heparin did not affect the precipitin reactions between proteins CA and CAR and an antiserum prepared against the Fc fragment of Cohn Fraction II IgG.

Discussion

The interaction of 6 of 100 monoclonal IgG proteins with agarpectin, the highly acidic sulfated polysaccharide constituent of agar (24), was implicated by the precip-

### Table III

| IgG protein | Heavy chain subclass | Light chain type | Euglobulin property* | Electrophoretic mobility‡ | Reactivity | Agar | Heparin§ |
|-------------|---------------------|-----------------|----------------------|--------------------------|------------|------|--------|
| CA          | γ₁                  | κ               | I                    | -2.0                     | +          | +    |        |
| CAR         | γ₁                  | λ               | I                    | -1.6                     | 0          | +    |        |
| CH          | γ₁                  | κ               | I                    | -2.2                     | +          | +    |        |
| BO          | ND                 | κ               | I                    | -0.7                     | 0          | 0    |        |
| BR          | γ₁                  | λ               | S                    | -1.8                     | 0          | 0    |        |
| GO          | ND                 | κ               | S                    | -1.6                     | +          | 0    |        |
| LE          | γ₁                  | κ               | S                    | -0.5                     | 0          | +    |        |
| MA          | γ₁                  | λ               | S                    | -1.4                     | 0          | +    |        |
| OA          | γ₁                  | κ               | I                    | -2.2                     | +          | +    |        |
| SU          | γ₁                  | κ               | S                    | -1.6                     | +          | +    |        |
| VA          | γ₁                  | κ               | I                    | -2.2                     | +          | +    |        |

* Solubility of IgG protein in deionized, distilled water: S, soluble; I, insoluble.
‡ Distance from application site after electrophoresis on an agarose gel membrane in 25 mM sodium barbital buffer, pH 8.6, for 100 min at 150 V.
§ Bovine lung heparin.
|| Not determined.
Fig. 6. Demonstration of cross-reacting idiotype among heparin-binding IgG proteins. Immunodiffusion analyses in agar gels of monoclonal heparin-binding proteins (1 mg/ml) IgGα CA, IgGα VA, and IgGα CAR and non-heparin-binding proteins IgGα TIL, IgGα BR, and IgGα MeG. (Left) The center well contained a monospecific anti-IgG antiserum. (Right) The center well contained an antiserum which was prepared against the heparin-binding IgG protein DOB (6, 28) and absorbed with Cohn Fraction II γ-globulin. The 2% (wt:vol) agar gels were prepared in a 20 mM sodium barbital buffer, pH 8.6, containing 3% polyethylene glycol 6000.

IgG and anomalous electrophoretic behavior of such proteins in agar but not in agarose. This interaction involved the divalent F(ab')2 molecule; no precipitation occurred with monovalent F(ab'), Fab, or Fc fragments, or with the isolated heavy or light chains of the agar-binding IgG proteins.

Another acidic polysaccharide, heparin, was identified as a ligand interacting with five of the six agar-binding IgG proteins and with three non-agar-binding IgG proteins. The interaction between heparin and IgG resulted from a binding between the two compounds and was not attributable to a solvent-exclusion effect (29) by the polyanionic carbohydrate that would have allowed only protein to precipitate. Heparin-IgG interaction was also demonstrated through other studies, including: the quantitative precipitation of IgG by heparin; the electrophoretic demonstration of mucopolysaccharide-protein complexes; the prevention by IgG of the ultrafiltration of heparin through membranes of appropriate permeability; the enhancement by heparin of the optical density at A280 nm of IgG; and affinity chromatography and solid-phase assays.

The binding of heparin by human monoclonal immunoglobulins has been attributed to antibody specificity of the proteins for heparin (5-10). This interaction can also result from salt bonding between the acidic polysaccharide heparin (or agaropectin) and certain positively charged IgG proteins. All heparin-binding monoclonal IgG proteins so far detected have had relatively high isoelectric points; desulfation of heparin results in loss of reactivity with IgG (10). Although the complementarity of charge between the basic IgG protein and the acidic ligands suggests an electrostatic interaction, other monoclonal IgG proteins, e.g., protein BR (Table III), had equivalent electrophoretic mobilities at pH 8.6 and yet failed to bind heparin (or agar). Heparin-IgG interactions were also demonstrable at a pH of 10 when the IgG molecule was present as a negatively charged species. Thus, the overall charge of the protein cannot be the sole explanation for this interaction. It may, instead, represent only one criterion for binding which requires, in addition, a specific IgG conformation (or charge distribution), as occurs for induced antibodies raised against highly charged antigens (30). Studies of model systems have shown that heparin affinity for other plasma proteins, e.g., anti-thrombin and lipoprotein lipase,
involves more specificity than just the presence of cationic groups on the binding proteins (20, 29, 31, 32).

Considerable functional and chemical heterogeneity among commercial heparin preparations has been documented through studies of the anti-coagulant and protein-binding properties of heparin in other interacting systems. Heparins vary in the number of N-sulfated glucosamine moieties per mole, and can be fractionated into two distinct species based upon high and low affinity for antithrombin and on anticoagulant activity (18-20, 31). Heparin prepared from rat skin is a multi-chain proteoglycan with $M_r >900,000$ (15) and can be converted chemically to a single-chain species with $M_r \sim 60,000$ (16) or depolymerized enzymatically to material of molecular weights (7,000-25,000 $M_r$) comparable to commercially available forms of heparin (17, 20). These structural differences may account for the capacity of heparin-binding IgG proteins to distinguish between the multi-chain and single-chain rat skin heparin preparations and the lower molecular weight depolymerized rat skin heparin or commercial heparins and between bovine and porcine heparins (see Fig. 3). Thus, subpopulations of molecules within a monoclonal IgG heparin-binding population may differentiate molecular species of heparin.

The site(s) on the Fab portion of the IgG molecule involved in binding agaropectin or heparin has not been definitely established. The virtual identity in primary structure of the heavy and light chain constant domains of monoclonal IgG proteins of the same subclass and light chain type, respectively, and the demonstration of heparin-binding by monoclonal IgM and IgA proteins (5-10) implicate the heavy and/or light chain variable (V) domains as the major site(s) of such interaction. The presence of common structural V region similarities among our heparin-binding IgG proteins was evidenced immunochemically by the demonstration that these proteins share a cross-reactive idiotypic antigenic determinant (27).

None of the eight patients whose serum contained monoclonal heparin-binding IgG proteins had received heparin or had evident disorders of blood coagulation. That monoclonal heparin-binding IgG proteins may be a minor constituent of normal IgG and have functional significance was suggested by our demonstration of heparin-binding IgG in serum specimens from two individuals who required, for anticoagulation, therapeutically inordinately large doses of heparin. In both cases, this activity was localized to relatively cathodally migrating, IgG-containing fractions. The physiological or pathological significance of heparin-IgG interactions is also evidenced by the molecular events associated with heparin-induced platelet aggregation (34) and thrombocytopenia (35).

Because the nature of heparin-binding by certain IgG proteins appears similar to that of other heparin-interacting proteins, the conformational changes induced by such binding may also allosterically affect the IgG molecule, thus influencing its function as a humoral or cellular receptor. The availability of purified heparins of defined chemical and biologic specificity and of monoclonal IgG proteins with

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2 Although the heparin-binding protein DOB has a deletion of 15 residues in the hinge region of the heavy chain and lacks heavy-light interchain disulfide bridges (28), features of the $V_H$ sequence that might account for this activity are not as yet evident. The deletion of the hinge region is not likely associated with heparin-binding because none of our IgG proteins are known to share this feature and, further, the IgG protein MoG (kindly supplied by Dr. Allen B. Edmundson, University of Utah, Salt Lake City, Utah), which contains an identical 15-residue hinge region deletion (33), is devoid of heparin-binding activity.
heparin-binding properties should provide the material necessary to define further the structural basis and biological significance of these interactions.

Summary

The interaction of immunoglobulins with certain acidic polysaccharides was demonstrated by the binding of the sulfated glycans agaropectin and heparin by certain human IgG proteins. Heparin-binding IgG proteins can distinguish between the molecular forms of heparin derived from porcine intestine, bovine lung, and rat skin. The major specificity of these proteins is for native and certain high molecular weight subunit components of rat skin heparin. The interactions with multi-chain and single-chain rat skin heparin are stable under physiological conditions and involve the Fab and, more specifically, the Fv region of the IgG molecule. These reactions occur as a result of an electrostatic interaction between cationic sites on certain IgG proteins and anionic sulfate residues of agaropectin or heparin. The characteristics of heparin-IgG interaction resemble those of heparin with other plasma proteins, the interactions of which have biological significance.

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