Atypical Glycosylation of an IgG Monoclonal Cryoimmunoglobulin*

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The NH2-terminal amino acid sequence of the heavy chain of the IgG monoclonal cryoimmunoglobulin Ger was determined. Evidence for the glycosylation of the first heavy chain hypervariable region of this protein was found. The inability of the deglycosylated Fab fragment of Ger to inhibit cryoprecipitation provides direct evidence that the presence of an additional sialic acid residue in a heavy chain's first hypervariable region can account for the cryo properties of this protein. This is the first convincing description of a molecular defect that explains the atypical low temperature solubility of a monoclonal cryoimmunoglobulin.

The presence of reversibly cold-insoluble immunoglobulins (cryoimmunoglobulins) is associated with a wide variety of human diseases (1–3). In many cases, it is thought that the physical properties of these proteins are directly related to some of their pathological effects. Indirect evidence suggests that the low temperature-induced aggregation of these proteins is most commonly a consequence of electrostatic interactions between cryoimmunoglobulin molecules (4). This is in marked contrast to other solubility-related diseases such as sickle cell anemia where apolar contacts between protein molecules in the aggregated state seem to be responsible for the abnormal association of hemoglobin S molecules (5). Evidence that cryoglobulins precipitate as a consequence of polar interactions includes inhibition of precipitation by neutral salts (4, 6), chemical modification studies of charged residues (7), and the magnitude of the thermodynamic parameters associated with the solubilization process (8). The results of these studies are open to alternate interpretation, however, because of the complex effects of salts and chemical modification upon proteins. It is clearly necessary to establish a structural basis for the atypical electrostatic properties of cryoimmunoglobulins (9, 10). In at least three cases, the reduced and alkylated heavy chains of monoclonal IgG cryoglobulins have been found to manifest retardation upon polyacrylamide gel electrophoresis in sodium dodecyl sulfate when compared to noncryoglobulin IgG heavy chains (11). This abnormality is not associated with atypical heavy chain molecular weights and has therefore been suggested to arise from either an altered polarity or a conformational effect (11). In this work we examine the sequence of one of these IgG cryoglobulin heavy chains and describe for the first time a defect in molecular polarity that can be convincingly connected to the abnormal solubility of a monoclonal cryoimmunoglobulin.

MATERIALS AND METHODS

Preparation of Immunoglobulins—Cryoimmunoglobulins were isolated by repetitive cold precipitation and redissolution followed by molecular sieve chromatography as described previously (9, 10). The protein Ger was judged pure by immunodiffusion, immunoelectrophoresis, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis after reduction and alkylation. Noncryoglobulin immunoglobulin was isolated from sera of patients with multiple myeloma and purified by molecular sieve and ion-exchange chromatography.

Sequence Analysis—The primary structure of the NH2-terminal portion of the Ger heavy chain was determined using identical approaches to those described previously (11). In addition, peptide mixtures were subjected to 1) reversed-phase HPLC on a 15-cm × 4-mm Varian MCH-10 column using a gradient of 100% 0.1% trifluoroacetic acid to 75% acetonitrile, 25% trifluoroacetic acid, and 2) gel filtration HPLC on a Varian TSK 5,000 30-cm × 0.8-mm column in 4 M guanidine HCl, 10 mM NaH2PO4, 1 mM EDTA, pH 6.5. In both cases chromatography was monitored both at 235 and 280 nm.

Miscellaneous Methods—The preparation of heavy chains, IgG Fab and Fc fragments, enzymatic removal of carbohydrate, and assay of cryoprecipitation were performed as described previously (10). Neutral sugars and hexosamines in the Ger Fab fragment were determined by gas chromatographic analysis by the method of Porter (12). Circular dichroism spectra were obtained with a JASCO J500A spectropolarimeter employing 1-mm cells. Fluorescence was measured with an SLM 4000 spectrofluorometer employing 8-nm slits and excitation at 285 nm.

RESULTS AND DISCUSSION

Sequence Analysis of IgG Ger Fab—The IgG cryoglobulin Ger was selected for sequence analysis since it displays the most dramatic differences with normal IgG in electrostatic properties as determined by isoelectric point (7.4 versus 7.8–8.9 for cold-soluble IgG) and potentiometric titration curve (not illustrated). Furthermore, this protein has been shown to have an abnormality in its heavy chain that has been localized to the NH2-terminal half by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13). We therefore focused on this region in sequence analysis.

NH2-terminal amino acid sequencing of the Ger heavy chain and succinylated Ger heavy chain in the presence of carrier [14C]carboxyamidomethylated pancreatic trypsin inhibitor were consistent with NH2-terminal blockage, presumably by pyrrolidone carboxylic acid. Removal of the pyrrolidone carboxylic acid from the succinylated heavy chain was effected by enzymatic deblocking employing calf liver pyroglutamate aminopeptidase (EC 3.4.11.8) as previously described (14). The heavy chain was succinylated, cleaved with cyanogen bromide, and subjected to gel filtration on Sephacryl S-200 in 5 M guanidine HCl. The orientation of the resulting specimen is consistent with a carbohydrate residue being at the completed chain. The orientation of the resulting specimen is consistent with a carbohydrate residue being at the reduced chain terminus.

The abbreviation used is HPLC, high performance liquid chromatography.
peptides was made tentatively by comparison of their NH2-terminal sequences with other human immunoglobulin IgG heavy chains. The single fragment which proved refractory to direct sequencing was treated with pyroglutamate amino peptidase and found to represent the NH2-terminal amino acid. Direct sequencing of the first 20 positions of CN1 placed it at the NH2-terminal position 70. Initially pyroglutamate amino peptidase-treated CN2 was found to represent the NH2-terminal peptide. The peptide next was digested with staphylococcal protease V-8 and the resulting peptides were separated by reversed-phase HPLC. The sequences of three peptides were determined through their respective COOH termini and with one exception (see below) were fully consistent within their whole integer amino acid compositions, although the characteristic depressed yield of methionine as homoserine lactone was observed. The fourth peptide contained an NH2-terminal Glx and its amino acid composition was consistent with the NH2-terminal position 70. Initially pyroglutamate amino peptidase-treated CN2 was found to represent the NH2-terminal peptide. The NH2-terminal sequence of the deblocked heavy chain provided overlaps for three peptides and the selective cleavage of the succinylated heavy chain by trypsin at Arg28 and further sequencing provided an overlap with the staphylococcal protease peptide. The overlap of the CN2 and CN1 peptides was made by identities in invariant positions and recovery of an arginine containing heptapeptide, spanning positions 67-73, from a tryptic digest of the intact succinylated heavy chain separated by preparative cation-exchange chromatography. The sequence of the first 94 residues of the Ger heavy chain is presented in Fig. 1 with the sequence of the prototypic human IgG heavy chain Newm. In the course of sequencing the unblocked peptides recovered from the staphylococcal protease V-8 digest of CN2, a failure to detect a phenylthiohydantoin that would represent position 33 was noted on initial HPLC analyses. Furthermore, a 50% reduction in repetitive yield (~93% calculated for leucine and soleucine) was noted after this position. Back hydrolysis of the phenylthiohydantoin-derivative at position 33 yielded Asx, consistent with the presence of a single Asx in the integral amino acid composition for the peptide. These findings taken together with the anomalously high molecular weight of the Fd region of Ger (11) and the presence of a Tyr and Ser immediately COOH-terminal to the Asx, a common site of carbohydrate attachment, suggested that the heavy chain might be glycosylated at this position. Carbohydrate analyses of the CN2 peptide showed it to possess (mole of carbohydrate/mole of peptide) mannose:galactose:N-acetyl-gluconamime:fucose:siacilic acid in a 3:2:2:4:1:2:2.5 ratio, fully consistent with immunoglobulin-type glycosylation. This Ger glycosylation site is within the first hypervariable region. Although glycosylation of immunoglobulin light chain hyper-variable regions has occasionally been reported (15-17), it has only rarely been encountered in heavy chain hypervariable regions (16).

The variable region of the Ger light chain was also completely sequenced using automated sequence technology and found to be of the \( \alpha \) subgroup III type. Extended NH2-terminal sequencing and sequencing of succinylated triglycine peptides and peptides produced by cleavage with V8 protease permitted the complete resolution of the 115 amino acid light chain sequence. This sequence was found to be DIVMTQVPALTLSVPSPEGATLSRACRQSISSNLAYQYQKPCQQAPRL武LIYAASTRATGIPARFGSGSTFTLTISSLQSEDFAVYYQYYPDDWPPITFGQTRLEIKRTVAAASPV. There is no evidence in this sequence for an N-type glycosylation signal. Furthermore, carbohydrate composition analyses showed the light chain to be devoid of appreciable (variation from other "normal" light chains) amounts of carbohydrate.

Relationship of Glycosylation to Cryoprecipitation—Although the finding of an abnormal glycosylation event in the first hypervariable region of Ger suggests that this may be related to its abnormal solution properties, experimental evidence of this hypothesis is required. This is especially true since there is no reported evidence of cryoglobulin properties in other abnormally glycosylated immunoglobulins (15-17). Initially, attempts were made to inhibit the cryoprecipitation of this protein by enzymatic removal of the total carbohydrate. It was possible to remove 60-80% of the covalently bound carbohydrate, but the resultant deglycosylated molecule displayed further decreases in solubility at all temperatures examined, presumably due to an overall reduction in protein solubility. Analysis of various protein fragments in fact revealed reduction of carbohydrate in both Fab and Fc regions. We therefore performed the following experiment to explore the selective effect of the hypervariable region glycosylation. The presence of high molar ratios of Ger Fab was found to inhibit cryoprecipitation (Fig. 2). Using a mixture of endo- and exoglycosidases, we were able to remove approximately 80% of the carbohydrate in the Fab region. The terminal sialic acid residues could also be quantitatively removed by treatment with neuraminidase. It is shown in Fig. 2 that both of these deglycosylated Fab fragments have lost the ability to inhibit cryoprecipitation of intact Ger. No evidence for conformational changes in the deglycosylated Fab fragments could be detected by CD or fluorescence, suggesting that the selective removal of the carbohydrate itself was responsible for this loss of inhibiting ability. This argues strongly, although indirectly, that the presence of this carbohydrate produces additional electrostatic contacts between the charged sialic acid residues and a positively charged Ger residue(s) in the solid phase that leads to the abnormal cold insolubility of this

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**Fig. 1.** The NH2-terminal sequence of the heavy chain of Ger and the prototypic IgG heavy chain Newm (24). Regions of homology are boxed, the complementarity determining regions (CDR) are indicated and the presumptive glycosylation site is starred. The trypsin succinylated (ST), staphylococcal protease V-8 (V8), and cyanogen bromide (CN) cleavage sites are shown by arrows.
protein. Five Fab fragments from monoclonal noncryoglobulin IgG proteins and a Fab from another IgG cryoglobulin and their glycosidase-treated forms had no effect on the precipitation of Ger.

This work presents the first definitive description of a molecular defect in a monoclonal cryoglobulin that appears to be responsible for abnormal solubility properties. Although the crystal structure of two monoclonal IgG cryoglobulins has been determined (18, 19), the molecular basis of their cryoglobulin behavior remains unknown. In the case of IgG Ger, an abnormal glycosylation event in the heavy chain first hypervariable region apparently produces low temperature insolubility. This is the direct result of the generation of a glycosylation recognition site in this region, presumably as a random by-product of the normal process of the generation of immunoglobulin diversity by somatic recombination or mutational processes (20). We have shown previously that glycosylation of some proteins can lead to dramatic reductions in solubility as an immediate consequence of the formation of a few novel intermolecular contacts in the solid phase (21).

The generality of this finding is unknown. In at least two cases, sialic acid has been shown to be directly involved in the cryoglobulin and cold agglutination properties of monoclonal IgM cryoglobulins (22, 23). Enzymatic removal of sialic acid from the intact molecules caused a loss of cold-induced insolubility for both proteins, but abnormal glycosylation has not been demonstrated. At least one other IgG cryoglobulin manifests a retardation in electrophoretic mobility of its heavy chain as seen with Ger, but this protein has a substan-

Lastly, reduced sialic acid content (approximately 20% of normal) (11). In addition, previous sequencing of cryoimmunoglobulin heavy and light chains has produced no evidence of unusual glycosylation, but rather the presence of rare substitutions in relatively constant portions of variable regions (14). It thus appears most probable that any of a wide number of small structural changes are capable of reducing overall immunoglobulin solubility to the extent that cryoglobulin behavior is produced. We have previously shown that the significant temperature dependence of cryoglobulin solubility is an intrinsic property of immunoglobulin molecules generally (8). The induction of large changes in solubility produced by subtle structural modification is similar to that observed with hemoglobin S and other modified hemoglobin S (5) and is presumably a further reflection of the delicate balance of intermolecular and solvent forces that maintain protein solubility.