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Immunology

Structural studies of human IgD: Isolation by a two-step purification procedure and characterization by chemical and enzymatic fragmentation

(Fab$_\delta$ and Fc$_\delta$ fragments/hinge region/lymphocyte receptor)

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ABSTRACT **A myeloma IgD immunoglobulin (designated WAH) that was present in high concentration in plasma (≈3.5 g/dl) was purified in >90% yield by a two-step procedure of ammonium sulfate precipitation plus AcA 34 gel filtration. Although the plasma had been stored for 2 years without the addition of a proteolytic inhibitor, no "spontaneous" degradation was apparent and the isolated IgD remained structurally intact. However, the purified IgD showed extreme susceptibility *in vitro* to various proteolytic enzymes; e.g., Fab$_\delta$ ($M_r \approx 47{,}000$) and Fc$_\delta$ ($M_r \approx 80{,}000$) fragments were generated quantitatively after only 10 min of incubation with papain in the absence of cysteine. By combining limited enzymatic digestion, reductive cleavage, and cyanogen bromide fragmentation, several series of well defined fragments corresponding to the different regions and domains of the IgD molecule were generated. These fragments are useful for physical, chemical, and immunological studies, as well as for the sequence determination of the IgD δ chain. A model of the IgD molecule was derived from such studies and from overlapping of the series of fragments. The possible existence of an extra constant domain in the δ chain appears unlikely in view of our finding of an extended hinge region of about 50 residues which can be cleaved off the amino terminus of the papain Fc$_\delta$ by brief treatment with trypsin. In addition to a distinct stretch of carbohydrate attachment sites, the δ-chain hinge region contains a segment unusually rich in electrical charge. This charged segment is responsible for the lability of IgD to spontaneous degradation and may be related to its biological role as a B lymphocyte receptor.**

Although IgD was discovered as a fourth class of human immunoglobulins nearly 15 years ago (2, 3), detailed structural studies have previously been greatly hampered because of several unfavorable factors. First, the very low incidence of IgD myeloma, the short survival time of the patients, and the comparatively low serum concentration of myeloma IgD all restricted the chance of collecting an IgD sample in sufficient quantity to complete the necessary work (4). Second, the notorious susceptibility of most IgD proteins to "spontaneous" degradation either *in situ* or during fractionation further reduced the availability of intact IgD (5, 6). Third, the earlier failure to demonstrate any significant biological function for IgD detracted from interest in it (7). Nevertheless, preliminary studies revealed some interesting structural features of IgD molecules (8, 9). Thus, IgD, especially its Fc$_\delta$ region, is very rich in carbohydrate. Its heavy chain (δ chain) has a molecular weight suggesting either a five-domain structure (10) or a four-domain structure with an "extended" hinge region (5). The presence in IgD of a single inter-heavy-chain disulfide bond also appears to be unique among human immunoglobulins (11).

In 1973, IgD was demonstrated to be a major class of receptor immunoglobulins on human B lymphocytes despite its low serum concentration (12). This observation prompted much new research that led to the finding of an IgD homologue on B cells in many animals (13). Evidence is rapidly accumulating to support the idea that membrane IgD plays a key role in lymphocyte activation, differentiation, and proliferation (14).

After rather uninformative exploratory studies using several other IgD proteins available to us in small amounts and already degraded to Fab and Fc fragments (Lov, Pa), our interest was renewed by the finding that a large amount (12–24 g) of intact IgD could be purified by a simple two-step procedure from the plasma of a single patient (WAH). This gave us the opportunity to explore the structural and functional uniqueness of the last class of human immunoglobulins that remains to be elucidated. Physical, chemical, and immunological studies of the WAH IgD are underway; in this communication we report some recent results that give new information about the structure of an intact IgD molecule.

MATERIALS AND METHODS

Purification of WAH IgD. Two units of an IgD plasma (WAH), with a total volume of ≈750 ml, were kindly sent to us 3 years ago by J. H. Keffer (St. Francis Hospital, Miami, FL). Zone electrophoresis performed at the time of collection revealed a marked elevation in the γ1 region. The plasma was collected without the addition of ε-aminocaproic acid (εAhx), an inhibitor of plasminogen activator, but was immediately frozen. For purification of the IgD, one unit of frozen WAH plasma was melted directly into 200 ml of 0.15 M NaCl (pH 7.5) solution containing 80 mM εAhx as a protease inhibitor. Saturated ammonium sulfate solution (4.1 M at 25°C) was added dropwise into the ice-chilled plasma solution, and the precipitate was collected at each stage when the ammonium sulfate concentration reached 1.5, 2.1, and 2.5 M. Each precipitate was redissolved in 0.1 M Tris·HCl/0.15 M NaCl, pH 8.0, containing 20 mM εAhx and applied to an AcA 34 column (5 × 100 cm) in the same buffer. Fractions were assayed by immunodiffusion and immunoelectrophoresis for IgD, IgG, IgM, IgA, α_2-macroglobulin, fibrinogen, haptoglobin, ceruloplasmin, transferrin,

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; εAhx, ε-aminocaproic acid. Abbreviations for classes, fragments, regions, and domains of immunoglobulins accord with official WHO recommendations for human immunoglobulins published in ref. 1.

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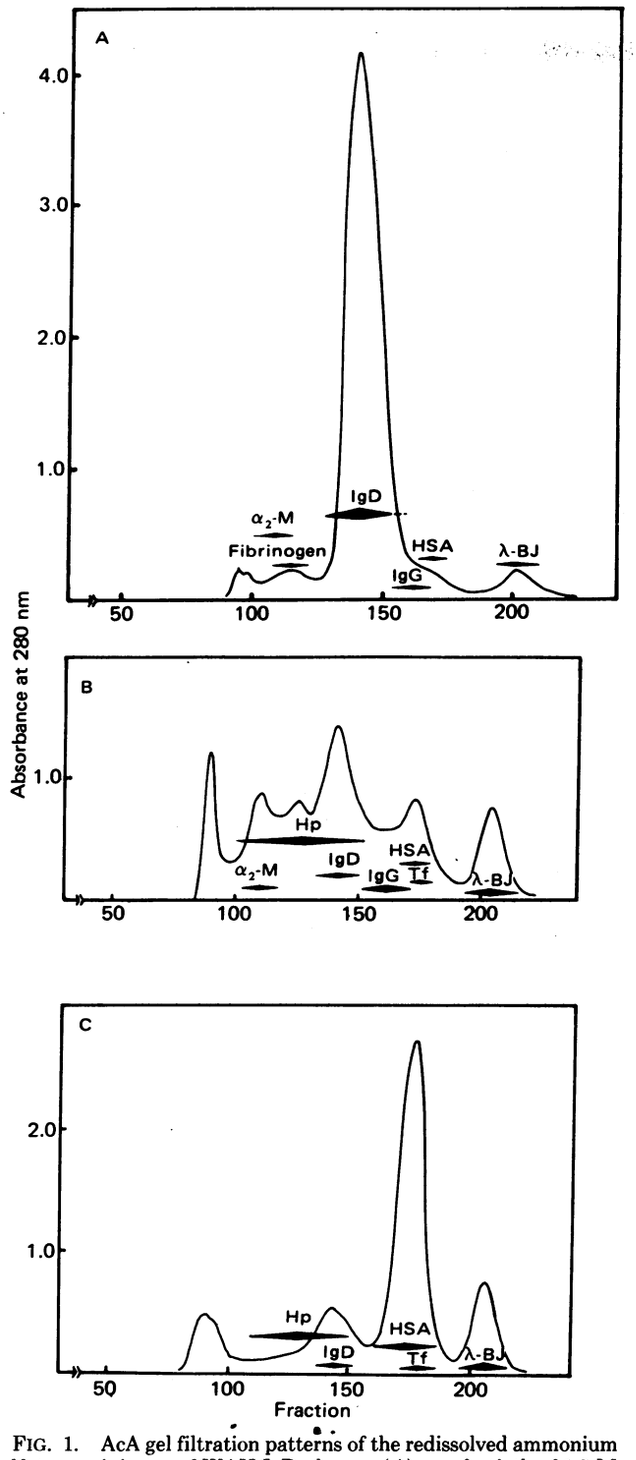

FIG. 1. AcA gel filtration patterns of the redissolved ammonium sulfate precipitates of WAH IgD plasma: (*A*) one-fortieth of 1.5 M precipitate; (*B*) one-sixth of 2.1 M precipitate; and (*C*) one-half of 2.5 M precipitate. Approximately 400 mg of protein from each pool was applied to a 5 × 90 cm column equilibrated in 0.1 M Tris·HCl/0.15 M NaCl, pH 8.0, with 20 mM ϵAhx. The fraction size was controlled at 7.5 ml; the flow rate was adjusted to about 60 ml/hr. The immunodiffusion results against different plasma proteins are shown by shaded bars according to their strength of reaction: α_2-M, α_2-macroglobulin; Hp, haptoglobin; Tf, transferrin; HSA, human serum albumin; λ-BJ, λ-Bence Jones protein.

serum albumin, and λ-Bence Jones protein. The purity of the IgD-positive pool was checked by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis and zone electrophoresis.

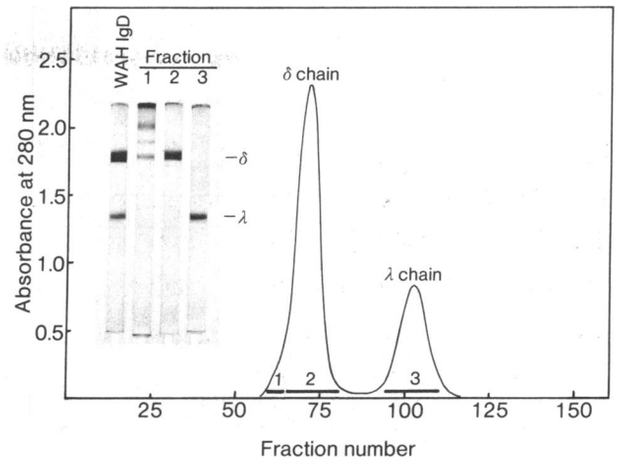

FIG. 2. Separation of WAH δ chain and λ chain. Fifty milligrams of partially reduced and carboxymethylated WAH IgD was dialyzed into 0.1 M Tris·HCl/0.15 M NaCl/8 M urea, pH 8.0, and applied to a 2.5 × 100 cm Sephadex G-150 column in the same buffer. The fraction size was controlled at 2.5 ml; the flow rate was adjusted to about 12 ml/hr. The purity of separated δ chain (fraction 2) and λ chain (fraction 3) was assessed by $NaDodSO_4$/polyacrylamide gel electrophoresis, with reduced WAH IgD as a control. A small amount of δ-chain aggregate (fraction 1) was visible.

Reductive Cleavage of WAH IgD. The purified WAH IgD (λ) was partially reduced with 10 mM dithiothreitol for 1 hr at 25°C and then alkylated with iodoacetate or ethyleneimine. The δ chain and λ chain were separated by gel filtration with Sephadex G-150 in the same Tris buffer with 8 M urea. The isolated reductive subunits were either renatured by removing urea or were subjected to complete reduction and alkylation.

Papain Digestion of WAH IgD. The IgD was dialyzed into 20 mM NaH_2PO_4/0.15 M NaCl, pH 7.5, with 2 mM EDTA. Papain (2× crystallized, Worthington) was activated immediately before use by resuspending the crystals in phosphate buffer containing 10 mM cysteine. The suspension was incubated at 25°C for 10 min and centrifuged; the supernatant was collected and its absorbance at 280 nm was adjusted to between 5.0 and 7.5 (i.e., 2–3 mg of papain per ml). The activated papain was added to 1% IgD solution at an enzyme/substrate ratio of 1:100 (wt/wt). The digestion was allowed to proceed at 37°C for 10 min before it was stopped by addition of iodoacetate to a final concentration of 2 mM. The papain Fc_δ and Fab_δ fragments in the digestion mixture were separated by gel filtration through a Sephadex G-100 column in the same phosphate buffer.

Tryptic Digestion of Papain Fc_δ Fragment. Purified papain Fc_δ fragment in phosphate buffer was digested with 1:100 (wt/wt) trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone, Worthington) at 25°C for 15 min. After the digestion was stopped by addition of soybean trypsin inhibitor, the digestion mixture was fractionated with a Sephadex G-100 column. Fractions containing the nonabsorbing hinge peptides were detected by the fluorescamine assay.

Cyanogen Bromide Cleavage. Samples for the cyanogen bromide cleavage were first dialyzed against distilled water and lyophilized; the protein was then dissolved in 70% formic acid to make a 0.5–1% solution. CNBr was added in 800-fold excess for each methionine residue, and the reaction was allowed to proceed at room temperature for 3 hr. The reaction was halted by diluting the solution with 8- to 10-fold amount of distilled water, and the digest was immediately freeze-dried in a desiccator.

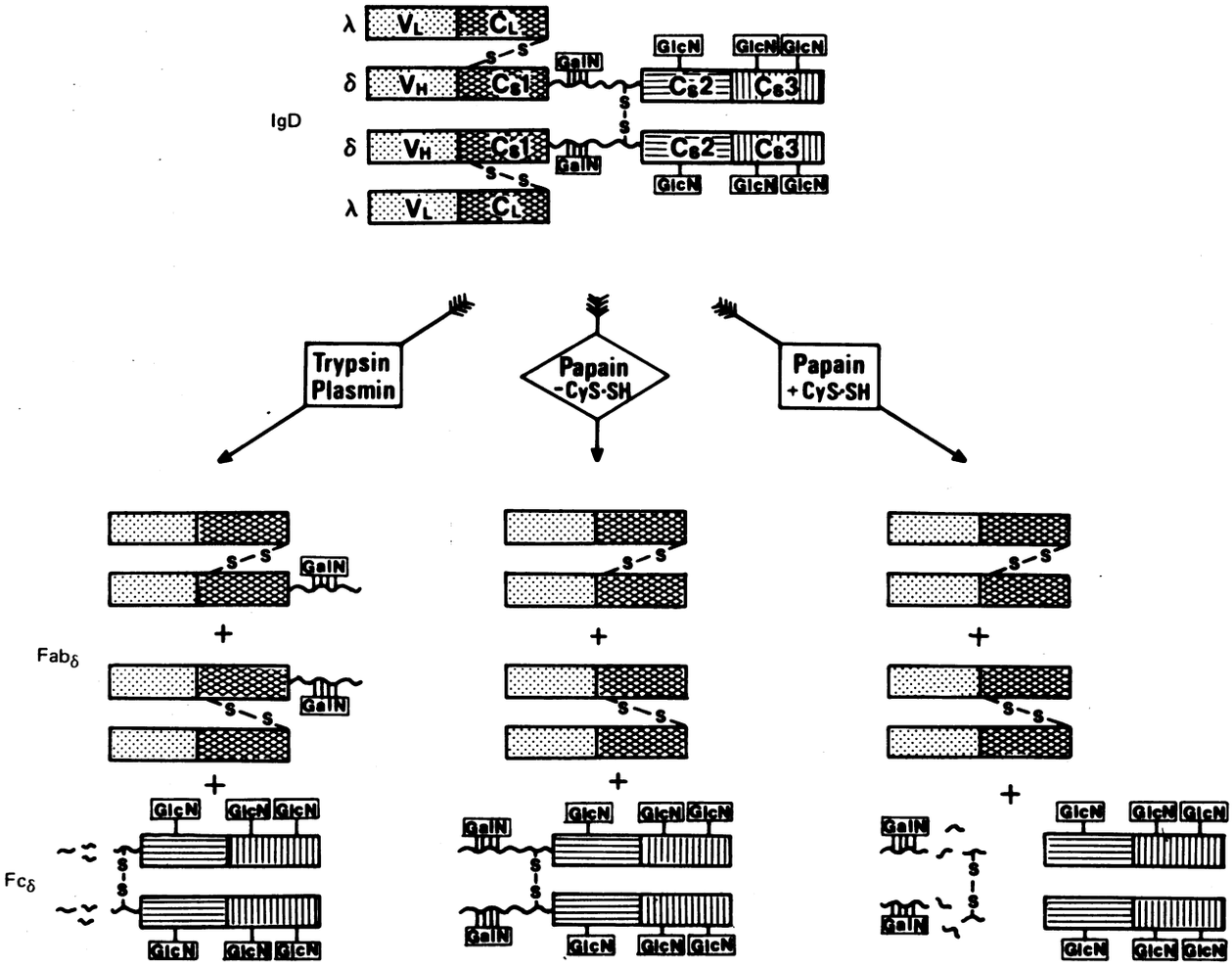

FIG. 3. Enzymatic fragmentation of human IgD and structural characteristics of the proteolytic fragments. Individual domains are denoted by separate shading. Trypsin, plasmin, and probably other trypsin-like serum proteases cleave the IgD molecule according to the left-hand pathway to generate two galactosamine-containing Fab_δ fragments and an Fc_δ fragment with all the glucosamines. Several peptides lacking carbohydrate are excised from the hinge region and are missing from these two tryptic fragments. Preactivated papain, on the other hand, cleaves the IgD molecule by the middle pathway to form two carbohydrate-free Fab_δ fragments and an Fc_δ fragment retaining all the oligosaccharides. The hinge region attached to the amino terminus of this papain Fc_δ fragment can easily be removed by brief tryptic digestion to form a tryptic Fc_δ fragment indistinguishable from the tryptic Fc_δ fragment derived directly from the IgD molecule. In the presence of cysteine (>10 mM), the papain Fc_δ fragment is extensively degraded at its amino terminus, as shown in the right-hand pathway.

RESULTS

Purity and Integrity of Isolated WAH IgD. By using the two-step purification procedure of ammonium sulfate precipitation plus AcA 34 gel filtration, we isolated undegraded WAH IgD in high yield. Fig. 1*A* shows a typical gel-filtration pattern of the redissolved 1.5 M precipitate. From the absorbance at 280 nm, IgD comprises 80% of the proteins precipitated at this molar concentration. Immunodiffusion indicated no contamination by α_2-macroglobulin, fibrinogen, haptoglobin, transferrin, or serum albumin and only a trace of IgG at the trailing end of the IgD peak. Less than 5% of the total IgD remained in the 1.5 M supernatant, as shown in Fig. 1 *B* and *C*, which give the gel-filtration patterns of the 2.1 M and 2.5 M precipitates. IgD-positive fractions from these two pools were heavily contaminated with haptoglobin, which was the major contaminant if the whole plasma was fractionated directly by AcA 34 gel filtration. In summary, approximately 12 g of purified IgD was obtained from one unit of WAH plasma by this procedure.

Despite the fact that WAH IgD plasma had been stored for 2 years without the addition of ϵAhx, no spontaneous fragmentation was apparent and the isolated IgD remained structurally intact. This is illustrated in Fig. 2, which shows the separation of the WAH δ chain and λ chain after the isolated IgD was partially reduced and alkylated. The δ chain appeared as a single band on $NaDodSO_4$/polyacrylamide gel electrophoresis with a molecular weight estimated to be 66,000 ± 1000 SEM.

Proteolytic Fragmentation of WAH IgD. The integrity of the purified WAH IgD does not indicate unusual stability reflecting unique structural characteristics such as a new subclass of IgD, for during *in vitro* studies, the WAH IgD was found to be extremely susceptible to limited cleavage to Fab and Fc-like fragments by various proteolytic enzymes, including trypsin, plasmin, papain, pepsin, and staphylococcal protease. Of these, trypsin and papain were found most useful for structural studies. As shown schematically in Fig. 3, the Fab_δ fragment generated by trypsin retains all the GalN oligosaccharides, while the tryptic Fc_δ fragment has all the GlcN oligosaccharides. However, several small nonglycopeptides were deleted from the hinge region in this digestion pathway. In contrast, two carbohydrate-free Fab_δ fragments and an Fc_δ fragment that had all the carbohydrate were generated by papain digestion in the absence of cysteine. If cysteine was included in the di-

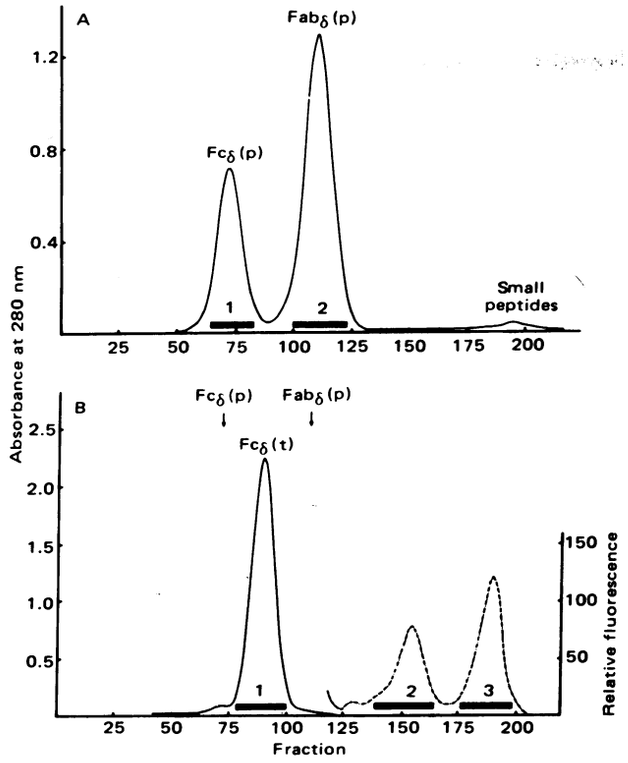

FIG. 4. (*A*) Gel filtration pattern of the papain digest of WAH IgD. Fifty milligrams of purified WAH IgD was digested with papain, and the digest was applied to a Sephadex G-100 (2.5 × 90 cm) column equilibrated in 20 mM NaH_2PO_4/0.15 M NaCl, pH 7.5. The fraction size was controlled at 2.5 ml; the flow rate was adjusted to 15 ml/hr. Papain fragments Fc_δ(p) and Fab_δ(p) were recovered in fractions 1 and 2, respectively. Little or no degradative peptides were detected either by absorbance at 280 nm or by fluorescamine assay. (*B*) Gel filtration of the tryptic digest of WAH papain Fc_δ fragment. Sixty milligrams of WAH papain Fc_δ fragment was digested with trypsin and fractionated under the same conditions described in *A*. Tryptic Fc_δ fragment, Fc_δ(t), was recovered in fraction 1; it has an elution position between those of papain Fc_δ and Fab_δ fragments. Fractions 2 and 3 were detected by fluorescamine assay; they contain hinge region glycopeptide and nonglycopeptides, respectively.

gestion mixture, the papain Fc_δ fragment was further degraded at its amino terminus, yielding several peptides including one containing GalN.

Based on this information, the WAH IgD was subjected to papain digestion without cysteine. As shown in Fig. 4*A*, stoichiometric amounts of papain Fc_δ and Fab_δ fragments were generated after incubation for only 10 min and were easily separated by gel filtration. The molecular weights estimated for papain Fc_δ and Fab_δ are 80,000 and 47,000, respectively. Brief treatment with trypsin transformed the papain Fc_δ fragment into a smaller tryptic Fc_δ fragment that has a molecular weight of ≈64,000 (Fig. 4*B*). The hinge region was excised from the amino terminus of papain Fc_δ by this treatment and was cleaved into a galactosamine-containing glycopeptide and several nonglycopeptides.

General Structure of Human IgD. By combining proteolytic fragmentation with cyanogen bromide cleavage, we have isolated a series of defined fragments that correspond to the different regions and domains in the WAH IgD molecule. Preliminary physical and chemical studies of these fragments have provided new insight on the general structure of the human IgD molecule, which is summarized in Fig. 5.

The existence of an extra constant domain proposed because of the 66,000-dalton size of the δ chain is unlikely in view of the long hinge region that extends between the $C_\delta1$ and the $C_\delta2$ domains. Our amino acid analysis and partial sequence analysis of the hinge region peptides indicate that the δ hinge region is about 50 residues long and that it contains only one hydrophobic residue (a leucine, which is near the amino terminus) and is unexpectedly low in proline (three or four residues). The amino-terminal side of the hinge region is a glycopeptide segment about 20 residues long in which there are four or five GalN carbohydrate units attached through *O*-glycosidic linkage mainly to the threonine side chains. Papain cleaves IgD near the amino terminus of this glycopeptide, whereas trypsin cleaves at the carboxy terminus. The carboxy-terminal side of the hinge is free of carbohydrate but includes a segment of about 24 residues that is unusually rich in electrical charge; there are 10 negative charges contributed by 10 glutamic acids and 10 positive charges contributed by 7 lysines and 3 arginines. The inter-δ-chain disulfide bond is located slightly beyond this segment and was found to be the fourth residue of the tryptic Fc_δ fragment. This highly charged segment of the hinge region is probably responsible for the lability of IgD to spontaneous degradation; this segment escaped detection by earlier workers because it contains multiple cleavage sites susceptible to trypsin-like proteases which disintegrate it into small peptides.

DISCUSSION

Apparent Stability of WAH IgD. IgD is unique among the immunoglobulins in its extreme susceptibility to limited proteolysis. It will undergo spontaneous degradation into Fab and Fc-like fragments during conventional isolation and storage procedures unless ϵAhx, an inhibitor of plasminogen–plasmin activation, is added to the plasma promptly after collection to prevent the formation of plasmin. Thus, it was surprising that the isolated WAH IgD remained structurally intact after 2 years of storage of the frozen plasma without the addition of ϵAhx.

The possibility that the WAH IgD represents an "atypical" molecule or a new subclass or allotype was ruled out because: (*i*) the purified IgD showed the usual *in vitro* sensitivity to various proteases; (*ii*) its hinge region contains multiple sites that are readily accessible to trypsin or plasmin cleavage; and (*iii*) by tryptic peptide mapping, the WAH δ chain appears to share most, if not all, the constant region peptides with another δ chain isolated from an IgD (Lov) which did show the usual *in vivo* susceptibility to spontaneous degradation.

The apparent *in vivo* stability of the WAH IgD could be accounted for by any of the following possibilities: (*i*) the two-step purification procedure reported in this communication provides a quick and efficient separation of WAH IgD from plasma proteases; (*ii*) the patient's plasma had an abnormally low level of plasminogen/plasmin; (*iii*) the patient had an abnormally high level of plasmin inhibitors, such as the antiplasmin that was recently discovered (15); or (*iv*) the patient might have a defective plasminogen–plasmin activation system.

Possible Structural and Functional Correlation of IgD. The natural design of the δ-chain hinge region makes serum IgD readily susceptible to rapid cleavage by serum proteases into Fab_δ and Fc_δ fragments. Because membrane-bound IgD seems more stable and needs more drastic conditions for cleavage (such as higher enzyme concentration and longer incubation time), we assume that the multiple cleavage sites in the hinge region of receptor IgD remain hidden until a proper signal is evoked—for example, by the binding of specific antigens. Either the removal of the Fab region may expose a second binding site in the Fc region, which enables the latter to interact with another membrane component (16), or the antigen–Fab complex that is split off may itself interact with the exposed Fc. In

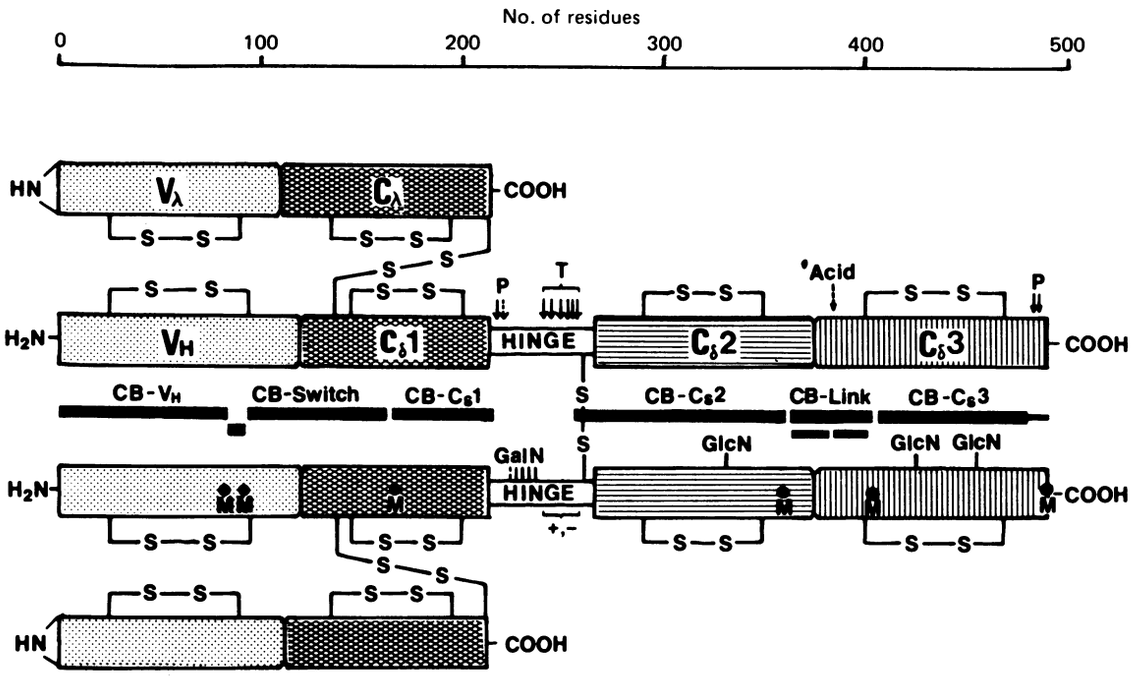

FIG. 5. Tentative structural model of IgD WAH. This IgD molecule has the basic four-chain structure of immunoglobulins; its light (λ) chain is blocked whereas its δ chain has a free amino terminus. The WAH δ chain consists of one variable domain (V_H) and three constant domains ($C_\delta1$, $C_\delta2$, and $C_\delta3$) with an extended hinge region located between the $C_\delta1$ and the $C_\delta2$ domains. An inter δ-λ disulfide bond is located near the V-C switch region and one inter δ-δ disulfide bond is located near the carboxy terminus of the extended hinge. Six methionines (denoted M) were found in the WAH δ chain; two methionines are present in the variable domain near its second half-cystine; four methionines were found in the constant region, one within the disulfide loop of $C_\delta1$ domain, one near the carboxy terminus of $C_\delta2$ domain, one in the $C_\delta3$ domain close to its first half-cystine, and one as the carboxy-terminal residue of the δ chain. Three large CNBr peptides were generated from the papain Fab_δ fragment of δ chain; they are designated as CB-V_H, CB-switch, and CB-$C_\delta1$ to indicate their location in the δ chain. Three large CNBr peptides, similarly designated as CB-$C_\delta2$, CB-link, and CB-$C_\delta3$, were generated from the tryptic Fc_δ fragment. The CB-$C_\delta3$ peptide showed carboxy-terminal heterogeneity due to enzymatic chipping when Fc_δ fragment was prepared, whereas the CB-link peptide was partially cleaved at an acid-labile Asp-Pro bond into two pieces. As in human IgA1, all the GalN oligosaccharides are clustered within the hinge region; three glucosamine-containing carbohydrate units are located in the tryptic Fc_δ fragment, one in CB-$C_\delta2$ and two in CB-$C_\delta3$.

either case, the interaction may result in the transmission of a signal to the interior of B lymphocytes. In addition, several small peptides are released from the IgD hinge region into the surrounding medium. Because of their unique sequences and unusual distribution of electrical charges, these peptides may serve as special signals to T lymphocytes or macrophages and thus may be involved in B cell–T cell–macrophage interactions.

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