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Abstract

Preparations of IgG2b purified from several mouse hybridoma clones were highly susceptible, compared to other subclasses, to peptic digestion under conditions usually used to prepare F (ab')2 fragments. Analyses of the digestion products revealed that no F (ab')2 was produced and that the main product was a Fab-like fragment. Demonstration of the hinge disulfides in the Fc portion clearly indicated that in IgG2b the primary peptic cleavage occurs on the NH2-terminal side of the inter-heavy chain disulfide bridge. The resulting Fab failed to bind with antigen, suggesting the importance of the CH1-hinge region in maintaining the native conformation of the antigen-binding site.

KEYWORDS: monoclonal antibody, immunoglobulin G2b, f (ab')2, peptic digestion, maleimide compound

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Unique Features of Monoclonal IgG2b in the Cleavage Reaction with Pepsin

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Preparations of IgG2b purified from several mouse hybridoma clones were highly susceptible, compared to other subclasses, to peptic digestion under conditions usually used to prepare $F(ab')_2$ fragments. Analyses of the digestion products revealed that no $F(ab')_2$ was produced and that the main product was a Fab-like fragment. Demonstration of the hinge disulfides in the Fc portion clearly indicated that in IgG-2b the primary peptic cleavage occurs on the NH_2-terminal side of the inter-heavy chain disulfide bridge. The resulting Fab failed to bind with antigen, suggesting the importance of the C_H1-hinge region in maintaining the native conformation of the antigen-binding site.

Key words : **monoclonal antibody, immunoglobulin G2b, $F(ab')_2$, peptic digestion, maleimide compound**

It has been useful in many studies to prepare antibody fragments lacking the Fc portion while retaining the capacity to bind antigens. Preparation of such fragments has been achieved by controlled protease digestion of purified IgG (1, 2). With the advent of hybridoma technology (3), the use of monoclonal antibodies to obtain antibody fragments has increased in frequency and necessity. One problem with monoclonal antibodies, however, is that their susceptibility to a protease varies greatly depending on the subclass to which the antibody of interest belongs (4, 5). Knowledge of the digestibility of individual subclasses is essential to attain a satisfactory fragmentation.

In an attempt to prepare a peroxidase-conjugated monoclonal IgG for intracellular localization of the corresponding antigen, we employed a conjugation method in which the enzyme is first modified with a maleimide compound, then conjugated to the reduced peptic fragment of IgG (6, 7). Since the reaction involves the sulfhydryl group derived from the inter-heavy chain disulfide linkage in the hinge region, the simple and logical procedure is to use the $F(ab')_2$ fragment obtained by the peptic digestion of monoclonal IgG. Preliminary digestion of several monoclonal antibodies isolated in this laboratory showed that some clones are extremely susceptible to pepsin and give an unusual fragmentation pattern as analyzed by SDS-PAGE. Subclass assignment revealed

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that all these clones belong to IgG2b.

In this study we show that mouse IgG2b is rapidly degraded by pepsin as compared to other IgG subclasses, with concomitant loss of antigen-binding capacity. We further demonstrate that the primary peptic cleavage site of IgG2b occurs on the NH_2-terminal side of the inter-heavy chain disulfide linkage by presenting direct evidence that hinge disulfides reside on Fc fragments after cleavage.

Materials and Methods

Mouse monoclonal IgG. According to a standard procedure (8, 9), seveal clones of hybridoma were selected after cell fusion between mouse myeloma cells (X63Ag8. 653)(10) and spleen cells from BALB/c mice which had been immunized with a retrovirus (11, 12). Culture supernatants of cloned cells were used to determine the subclass of IgG by the double immunodiffusion procedure (Ouchterlony). Three hybridoma clones (14H10, A-11, 15G-11) producing IgG2b, one clone (E-1) producing IgG2a and one clone (C-1) producing IgG1 were used in this study. Monoclonal IgGs were purified from ascites fluid obtained from the BALB/c mice inoculated with each hybridoma by the use of affinity chromatography on protein A-Sepharose 4 B (Pharmacia Fine Chemicals)(13).

Peptic digestion. The purified monoclonal IgG was dialyzed against 0.1 M acetate buffer (pH 4.5) at 4°C, and then, in the presence of 0.1 M NaCl, allowed to react with pepsin (2X crystallized; Sigma Chemical Co.) at an enzyme : globulin ratio of 1 : 25. The mixture was incubated at 37°C for various lengths of time, and to stop the reaction, the pH of the solution was brought to 8.0 with 1N NaOH. The digestion product was fractionated with a column (2×55 cm) of Sephacryl S-200 equilibrated with 0.1 M sodium borate buffer (pH 8.0). The IgG subfragments in the peptic digest and in the Sephacryl S-200 fractions were analyzed by SDS-PAGE (14). Distribution of the Fab and Fc fragments in the fractions was determined by the double immunodiffusion procedure with rabbit anti-mouse Fab and Fc sera.

Measurement of antibody activity. A dot blotting assay was performed to measure the antibody activity associated with IgG and the peptic subfragments. They were incubated at 37°C for 60 min with the corresponding antigen blotted onto nitrocellulose discs (3 mm in diameter) which were subsequently coated with bovine serum albumin. This and the following steps were performed in the wells of a microtiter plate. After washing the discs, the bound IgG fragments were detected by the avidin-biotin complex system (Vectastain, Vecter Lab., Inc.) using biotinylated anti-mouse IgG antibody as a second antibody. The peroxidase reaction was developed by the addition of 4-chloro-1-naphthol solution (0.5 mg/ml) containing 0.05% H_2O_2.

Maleimide conjugation. To determine the sulfhydryl groups in the hinge region, the IgG peptic fragments separated by Sephacryl S-200 column chromatography were conjugated to horseradish peroxidase (HRP) using a maleimide compound (6). HRP (6 mg/ml) in 0.1 M phosphate buffer (pH 7.0) was incubated with the N-hydroxysuccinimide ester of N-(4-carboxycyclohexylmethyl)-maleimide (Zieben Chemical Co., Tokyo) at 30°C for 60 min to introduce maleimide groups to HRP, and then subjected to gel filtration on a column of Sephadex G-25 to remove the unreacted reagents. The maleimide-HRP was allowed to react at 4°C for 16 h with the sulfhydryl groups of the peptic subfragments which had been reduced with mercaptoethylamine, and the conjugates were separated from unbound HRP by gel filtration on a Sephacryl S-200 column. The peaks for HRP-conjugated IgG subfragments and free HRP were deduced from differential absorbance at 280 nm and 403 nm.

Results

Two monoclonal IgG preparations, 14 H-10 (IgG2b) and E-1 (IgG2a), were treated with pepsin for various lengths of time and their respective time courses of digestion were compared by SDS-PAGE with or without reduction by 2-mercaptoethanol (Fig. 1). When analyzed under non-reducing conditions, a remarkably high susceptibility of

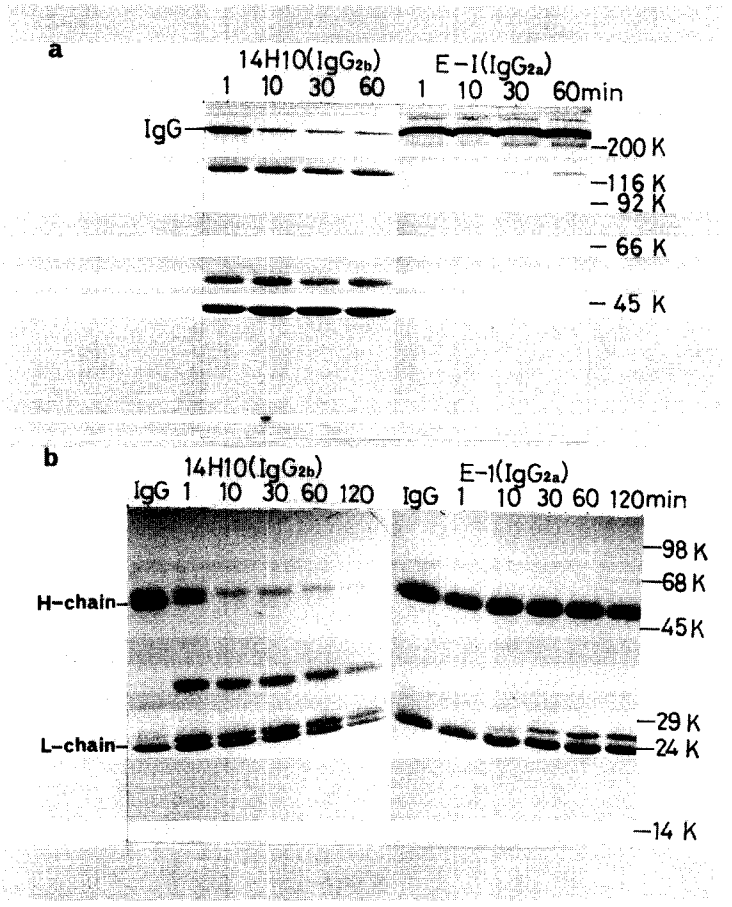

Fig. 1 Time course of peptic digestion monitored by SDS-PAGE. Samples were removed at the times indicated and subjected to electrophoresis in 7.5% polyacrylamide gel without reduction (a) and in 14% acrylamide gel with reduction (b). Positions of molecular weight markers are indicated on the right.

IgG2b to pepsin was evident (Fig. 1a). A large proportion of IgG2b was degraded within 10 min of incubation, whereas most IgG2a remained undigested even at 60 min under identical conditions. The hydrolysis rate of monoclonal IgG1 was similar to that of IgG2a (data not shown)(16, 17). The apparent molecular weight of the fragment (*ca.* 120,000) generated from IgG2a after 30 min agrees with the size expected for $F(ab')_2$, which is normally the primary product of pepsin digestion. However, the fragmentation pattern of IgG2b is clearly aberrant in those two stable fragments were observed in the Mr 40-50 K region. Under reducing conditions, IgG2b yielded 4 fragments including intact heavy and light chains (Fig. 1b). The fragments with molecular weights of 35 K and 25 K are likely to originate from the heavy chain. A significant decrease in the mobility of the heavy chain band was noted in the course of digestion (see Discussion). The unusual susceptibility and the anomalous cleavage products appear to be common to this particular IgG subclass (IgG2b) since essentially the same results were obtained with other clones of IgG2b (A-11 and 15 G-11)(9).

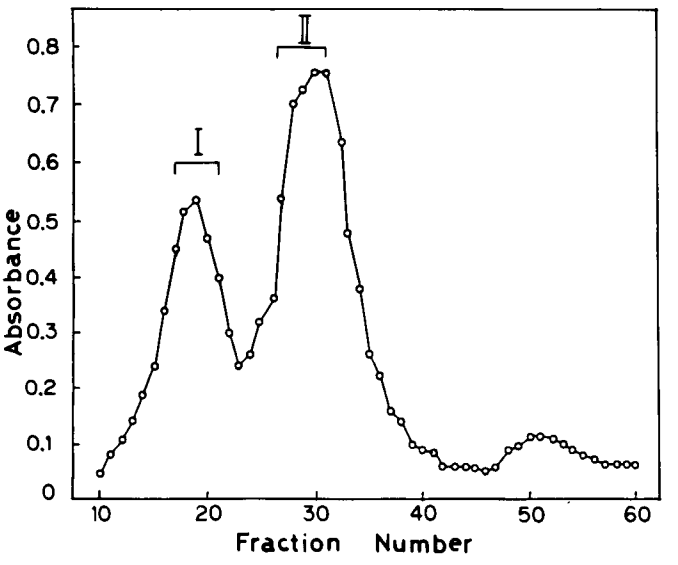

Fig. 2 Fractionation of IgG2b peptic digest on Sephacryl S-200. Peak fractions indicated by brackets were pooled and dialyzed against appropriate buffers for subsequent experiments.

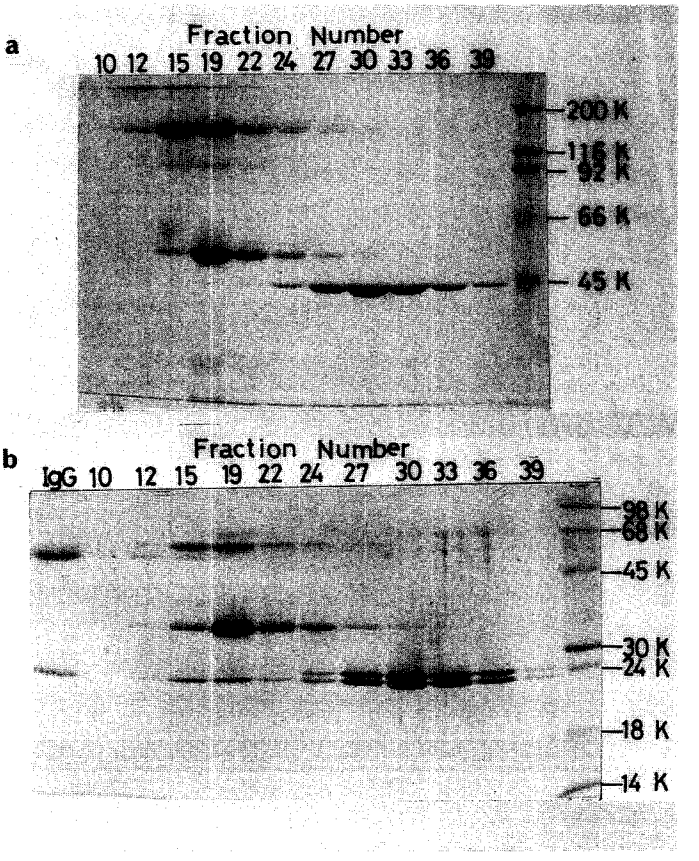

Fig. 3 SDS-PAGE analysis of Sephacryl S-200 fractions. Aliquots from corresponding fractions in Fig. 2 were run in 10% polyacrylamide gel without reduction (a) and in 14% polyacrylamide gel with reduction (b).

To further analyze the mode of peptic cleavage of IgG2b, purified IgG2b (14 H10) was treated with pepsin for 30 min, and hydrolysis products were fractionated with a column of Sephacryl S-200 (Fig. 2). Fractions of the first two peaks were subjected to double immunodiffusion analysis against monospecific antisera to Fab and Fc fragments of mouse IgG. The first peak (peak I) contained both antigenic determinants, whereas peak II showed a strong reaction only against anti-Fab. Non-reducing SDS-PAGE of the Sephacryl fractions (Fig. 3 a) revealed that peak II consisted of a single fragment of Mr 44 K which was dissociated into polypeptides of Mr 24 K (L-chain) and Mr 25 K upon reduction (Fig. 3 b). These results indicate unequivocally that peptic digestion of monoclonal IgG2b mainly produces Fab-like fragments instead of $F(ab')_2$. The presence of the Fc portion in peak I was corroborated by the existence of a fragment which behaves like Mr 58 K or Mr 32 K under non-reducing or reducing conditions, respectively.

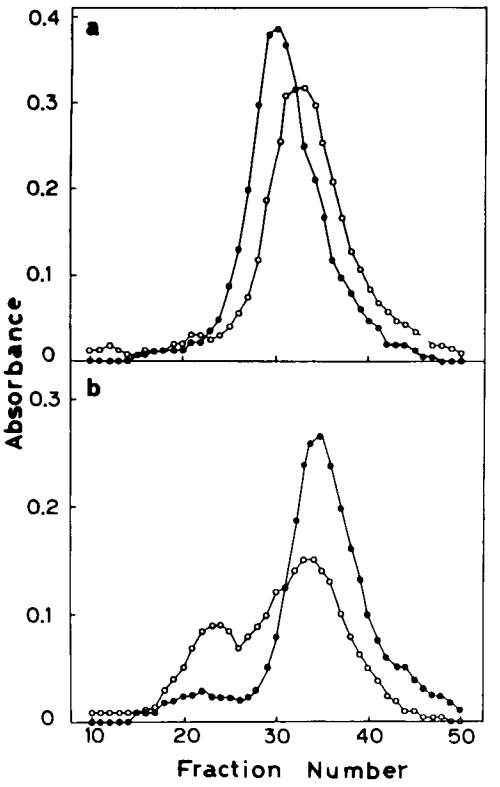

Fig. 4 Gel filtration of HRP-conjugates of fractionated peptic fragments. The maleimide derivative of HRP was incubated with pooled fractions of Sephacryl S-200 peak II (a) or peak I (b), and the mixture was fractionated again on Sephacryl S-200. The difference in elution volumes of peroxidase in **a** and **b** was due to different fraction size. Absorbance at 280 nm (○), absorbance at 403 nm (●).

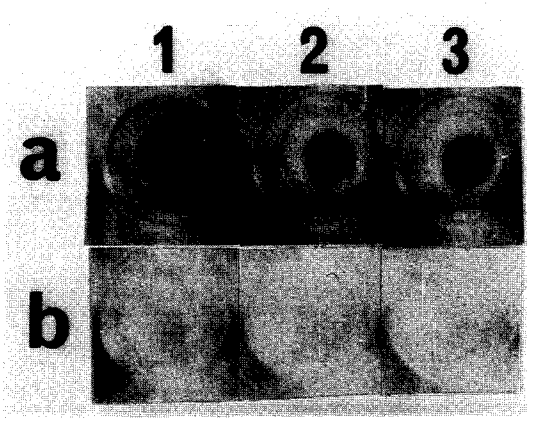

Fig. 5 Measurement of antibody activity associated with Fab. An equal amount of retrovirus antigen was adsorbed on the filter and incubated either with undigested IgG2b (a) or Sephacryl S-200 peak II (b) diluted to $10^1\times(1)$, $10^2\times(2)$, $10^3\times(3)$. The protein concentration of undiluted antibody was 0.1 mg/ml.

The procedure for selective conjugation of peroxidase (HRP) with $F(ab')_2$ utilizing the reduced disulfides in the hinge region (6) was employed to locate the peptic cleavage site of monoclonal IgG2b with respect to the inter-heavy chain disulfide bridge. The pooled fractions for peaks I and II (Fig. 2) were allowed to react separately with maleimide-HRP, and the conjugates were separated on Sephacryl S-200 (Fig. 4). No conjugate was formed between HRP and the Fab fragment (peak II in Fig. 2)(Fig. 4a). As revealed by absorbance at 403 nm, HRP was eluted exclusively as a monomer. With peak I (Fc), however, in addition to the

prominent monomer peak, a significant proportion of HRP was found in the peak of higher molecular weight, implying the presence of the HRP-Fc complex (Fig. 4b). Therefore, we conclude that the primary cleavage of mouse monoclonal IgG2b by pepsin occurs at the NH_2-terminal side of the hinge disulfides.

Finally, the antigen-binding activity of the peptic Fab fragment of monoclonal IgG2b (14H10) was estimated using a solid phase enzyme-linked immunosorbent procedure (Fig. 5). No binding of Fab to the antigen-coated filter was detected at any dilution, while intact IgG2b gave a positive reaction.

Discussion

We have shown that IgG2b purified from several mouse hybridoma clones is extremely susceptible to pepsin cleavage. It has been known for some time that human IgG subclasses are differently affected by pepsin digestion (2, 15). The highly susceptibile nature of IgG2b to pepsin was also noted in mouse myeloma IgG (4, 5) and, more recently, in mouse hybridoma IgG (16, 17). Our results confirmed these observations and further demonstrated that in IgG2b the principal peptic cleavage occurs at the NH_2-terminal side of the hinge disulfides, generating Fab fragments with no antigen-binding capacity. This conclusion was substantiated by the presence of the hinge sulhydryl groups on the Fc fragment (Fig. 4).

It should be pointed out that peptic digestion of IgG2b releases a substantial amount of a fragment of Mr 120 K (Fig. 1a) which is eluted from Sephacryl S-200 in the first portion of peak I (Figs. 2 and 3a). This fragment appears to dissociate into intact light and heavy chains under reducing conditions (Fig. 3b), thus excluding the possibility of its being $F(ab')_2$. The formation of 120 K fragments is most likely to be the consequence of asymmetric glycosylation of serine residues of IgG2b that are located at the NH_2-terminal side of the hinge disulfide bonds (17, 18). Glycosylation at this site may confer resistance against proteolysis, so that preferential cleavage of the nonglycosylated heavy chain yields Fab and Fab/c (120 K fragment). This notion was supported by the fact that the heavy chain band released from the 120 K fragment migrates slightly slower than the original main component, implying the presence of additional glycosylation on this subset of heavy chains (Fig. 3b). As expected, the heavy chain heterogeneity is already discernible in the original IgG2b, the glycosylated component being a minor population (less than 20%). This is evident from the doublet pattern of the heavy chain, the upper band being more resistant to peptic digestion (Fig. 1b).

Precise location of the peptic cleavage site of IgG2b was not determined in this study. The cleavage site may well be inside the hinge region or may reside in the C_H1 domain not very far from the C_H1-hinge boundary, considering the size of the heavy chain fragment (*ca.* 25 K) constituting Fab (Fig. 1b). Therefore, the dramatic loss in antigen-binding capacity of the Fab peptic fragment is rather surprising and may reflect the presence of a critical residue near the hinge that is important to the maintenance of the native conformation of the antigen binding site. Monoclonal IgG2b preparations might be a convenient material for testing this possibility.

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