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Immunology

One disulfide bond in front of the second heavy chain constant region is necessary and sufficient for effector functions of human IgG3 without a genetic hinge

(complement activation/phagocytosis/antibody-dependent cell-mediated cytotoxicity/hinge region/mutagenesis)

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ABSTRACT **We have created four IgG3 mutants without a normal hinge region: (*i*) m0 without a genetic hinge; (*ii*) m0/C131S, where Cys-131 in m0 was mutated to Ser; (*iii*) m0/231C232 (formerly HM-1), where a Cys residue was inserted in m0 between Ala-231 and Pro-232; (*iv*) m0/C131S/231C232, which is a hybrid of m0/231C232 and m0/C131S. The wild-type IgG3 and all mutants bind 5-iodo-4-hydroxy-3-nitrophenacetyl groups. The wild type and mutants, m15 (with 15 aa in the hinge), m0/231C232, and m0/C131S/231C232, were all positive for complement-mediated lysis, antibody-dependent cellular cytotoxicity mediated by peripheral blood leukocytes, and phagocytosis by U937. m0/C131S/231C232 was only weakly positive and sometimes negative for respiratory burst activity mediated by peripheral blood neutrophils (polymorphonuclear leukocytes), whereas m15, m0/231C232, and wild-type IgG3 were strongly positive. The m0 and m0/C131S mutants were mainly negative for complement-mediated lysis, antibody-dependent cell-mediated cytotoxicity, and phagocytosis by U937 and polymorphonuclear leukocytes. The results indicate that a hinge spacer region is not necessary, but the correct alignment of the two second heavy chain constant regions in the IgG3 molecule by a minimum of one disulfide bond is necessary and sufficient for effector functions.**

The four human IgG subclasses are structurally closely related in the homologous domains but vary much in length and structure in the hinge regions (1, 2). The IgG3 subclass has an extremely long hinge region of 62 aa containing 11 disulfide bonds encoded by four exons, while the other IgG subclass genes contain one hinge exon only (2–4). It has a well-ordered secondary and tertiary structure (5), is quite immunogenic (6, 7), and appears as a rod-like structure of ≈40 Å (8) that is disrupted by mild reduction (6, 9) and then loses effector functions (10).

The unusually long IgG3 hinge can be drastically shortened by exon deletion without diminishing complement activation and lysis (11–13). An IgG3 mutant with an IgG4 hinge was also very active in complement-mediated lysis (CML) (14). An IgG3 mutant, m0, without a genetic hinge does not induce CML (12), and IgG1 myeloma proteins without a hinge do not activate complement (15, 16), whereas a mutant, m0/231C232 (formerly HM-1), without a genetic hinge but with a disulfide bond in front of the second heavy chain constant region (C_H2), is active in CML (17). We wanted to do a detailed analysis of the hinge or hinge-related structural requirements for effector functions based on the four mutants m0, m0/C131S, m0/231C232, and m0/C131S/231C232, all without a genetic hinge, and to test these mutants for CML, antibody-dependent cell-mediated cytotoxicity (ADCC), and phagocytosis/respiratory burst. m0/231C232 and m0/C131S/231C232 were positive, whereas m0 and m0/C131S were negative for effector functions.

MATERIALS AND METHODS

Cloning Procedure, *in Vitro* Mutagenesis, and Gene Transfer. The human γ3 constant region gene coding for the G3m(b°) variant was a generous gift from M. P. Lefranc (Laboratoire d'Immunogenetique, Université des Sciences et Techniques du Languedoc, France). The mutant m0 without a hinge region was constructed by exon deletion as described (12), and the mutant m0/231C232 originated from m0 by inserting a Cys between Ala-231 and Pro-232 (18) as described (17). Schematic structures of the mutants are shown in Fig. 1. m0/C131S/231C232 has and m0/C131S does not have a disulfide bond between the H chains. Mutants were assembled by combining a mutant C131S C_H1 *Hin*dIII–*Bgl* II gene fragment (19) inserted onto m0/231C232 and the m0 C_H2–C_H3 gene fragment and verified by DNA sequencing (20). The mutant H-chain gene fragments were further subcloned into the eukaryotic expression vector PSV2gptV_{NP} (21) and transfected by electroporation into the murine myeloma cell line J558L (kindly provided by S. L. Morrison, University of California at Los Angeles), and antibody-producing cells were selected as described (12). wt IgG3 and m15 cells both produce 1–3 μg/ml in outgrowth cell cultures. The m0/C131S cells produced a maximum of 0.5 μg/ml, and m0/231C232 and m0/C131S/231C232 cells produced a maximum of 0.05 μg/ml.

Chimeric Anti-5-iodo-4-hydroxy-3-nitrophenacetyl (NIP) IgG Antibodies. Antibodies were isolated from outgrown cell supernatants by using affinity chromatography with the hapten NIP coupled to Sepharose 4B (Pharmacia) or from ascites fluid by using DE-52 (Whatman) ion-exchange chromatography. Quantification was done in an ELISA assay using NIP–bovine serum albumin-coated microtiter plates (11).

SDS/PAGE and Western Blot Analysis. SDS/PAGE analysis was performed using slab gels electrophoresed in a Minigel apparatus (Bio-Rad) and stained for proteins with Coomassie brilliant blue (Bio-Rad). For Western blot analysis, we used nitrocellulose (162-0115, Bio-Rad) and probed with rat monoclonal antibody (A35-1, PharMingen), anti-

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Abbreviations: NIP, 5-iodo-4-hydroxy-3-nitrophenacetyl; SRBC, sheep erythrocyte; ADCC, antibody-dependent cell-mediated cytotoxicity; CML, complement-mediated lysis; C_H, heavy chain constant region; PMN, polymorphonuclear leukocyte; FcγR, IgG Fc receptor; FITC, fluorescein isothiocyanate; wt, wild type; H, heavy; L, light.

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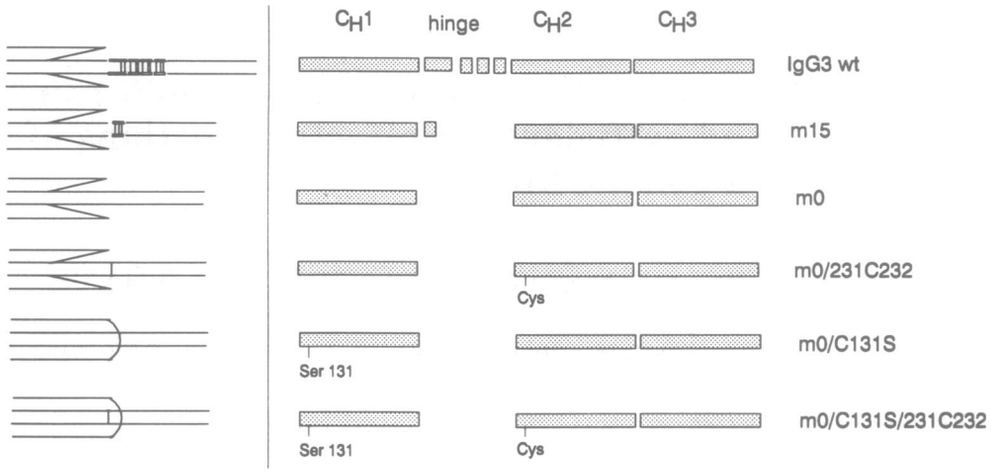

FIG. 1. Schematic drawing of the IgG structure of wild-type (wt) IgG3 and the mutant IgG3 molecules employed in this study (*Left*) and the C_H exons of the various corresponding heavy (H)-chain genes (*Right*). The m0/231C232 was formerly named HM-1 (17).

mouse λ chain, which we biotin-labeled by using biotin-X-NHS (Calbiochem), and a locally produced biotin-labeled monoclonal antibody, 452D12C1, that reacted with a C_H3 epitope present on all human IgG subclasses.

CML and ADCC. The NIP hapten was deposited on the target cell membrane by an indirect method using NIP-labeled rabbit Fab′ antibody fragments against sheep erythrocytes (SRBCs) as described (12, 22). The CML with human complement and ADCC assays were performed using hapten- and ^{51}Cr-labeled SRBCs as described (22, 23). As effector cells for ADCC, we used peripheral blood leukocytes separated by Lymphoprep (Nycomed, Oslo) as described (24).

Phagocytic Assay. U937 monocytoid cell line was used to study IgG Fc receptor (FcγR) I-mediated phagocytosis as described (25). We used fluorescein isothiocyanate (FITC) and NIP double-labeled *Candida albicans* or SRBCs as target cells, and phagocytosis was measured by flow cytometry as described (25). NIP-haptenized FITC-labeled yeast cells or SRBCs were washed twice in Dulbecco's PBS (DPBS) with 0.2% bovine serum albumin and the cell concentration was adjusted to 5×10^7 cells per ml. On U-bottomed microtiter plates, 10 μl of a yeast suspension was mixed with 50 μl of 1:10 dilutions of the various antibodies to be tested and incubated for 30 min at 37°C. After two successive washes, 100 μl of effector cells was added. With the U937, the suspension was centrifuged at $300 \times g$ for 2 min and incubated in humidified atmosphere at 5% CO_2/95% air and 37°C for 30 min. With neutrophils, the incubation was done with continuous agitation at 37°C for 12 min. Phagocytosis was stopped by placing the microtiter plates on an ice bath until phagocytosis was measured with flow cytometry.

Respiratory Burst. Venous blood from normal volunteers was drawn into EDTA vacutainers, and the erythrocytes were lysed with a solution containing NH_4Cl (8.3 mg/ml), $NaHCO_3$ (1 mg/ml), and EDTA (0.08 mg/ml) at pH 6.8. The leukocytes were washed twice with DPBS/0.2% bovine serum albumin and the concentration was adjusted to 5×10^6 cells per ml, and cells were stored at 4°C until use. No additional purification of polymorphonuclear leukocytes (PMNs) was required because all further analysis was done using flow cytometry. Target cells in the respiratory burst assay were *Candida albicans* haptenized with NIP as described above, but without FITC. To detect reactive oxygen intermediates, we used dihydrorhodamine 123 (Molecular Probes) (26) as described (25). Flow cytometry was performed on an EPICS Profile II flow cytometer (Coulter) with a 15-MW argon laser as described (25).

RESULTS

Structural Analysis of the Mutants. The polypeptide structure of the mutants was examined by SDS/PAGE and Western blot analysis. The analysis showed a variable degree of heterogeneity of the preparations. The m15 showed one main protein band at the same position as wt IgG3 that reacted with light (L)-chain and H-chain antibodies and, thus, is secreted as intact disulfide-bonded H_2L_2 molecules (Fig. 2). The m0 gave one protein band that reacted with both antibodies and migrated much faster than m15 and wt IgG3 and, thus, represents HL half molecules (Fig. 2). The m0 mutant was gel-filtered in one peak at 150 kDa under neutral conditions and at one peak corresponding to half molecules under dissociating conditions (data not shown). The m0/231C232 mutant showed one dominating Coomassie blue-staining protein band at 150 kDa that reacted with both L-chain and H-chain antibodies in a Western blot and, thus, represents H_2L_2 disulfide-bonded molecules. In addition there was a fainter protein band reacting with both antibodies in a Western blot that corresponds to HL half molecules. Also, there was a band reacting with λ-chain antibodies only that corresponds to L_2 molecules. While the m15 cell line was a relatively good antibody producer giving ≈1 μg/ml in outgrowth cell culture, the m0/231C232 cell line was a low antibody producer giving 20–50 ng/ml in outgrowth cell cultures. Probably due to the low whole antibody production, this cell line produced an excess of free λ chains; some of these L-chain dimer molecules apparently have NIP binding activity since they could be eluted from a NIP-containing column.

The m0/C131S mutant gave two protein bands close to each other at ≈50 kDa corresponding to H chains and L_2 dimers, verified by Western blot analysis (Fig. 2). Gel filtration of m0/C131S at neural conditions gave one peak at ≈150 kDa under neutral conditions and one main peak at ≈50 kDa under dissociating conditions confirming the presence of a disulfide bond between the L chains (data not shown). Thus, m0/C131S mainly consists, as expected, of molecules made up by noncovalently associated H_2 with disulfide-linked L_2 subunits. The m0/C131S cell line was a medium antibody producer giving ≈500 ng/ml in outgrowth cell cultures with no apparent production of an excess of free λ chains. m0/C131S/231C232 gave a complex pattern showing protein bands at 150, 100, 75, and 50 kDa that corresponded to H_2L_2, H_2, HL, and L_2, respectively in a Western blot. The m0/C131S/231C232 mutant, therefore, consists of a mixture of disulfide-bonded H chains associated with disulfide-bonded L chains that created the intended molecules and also mol-

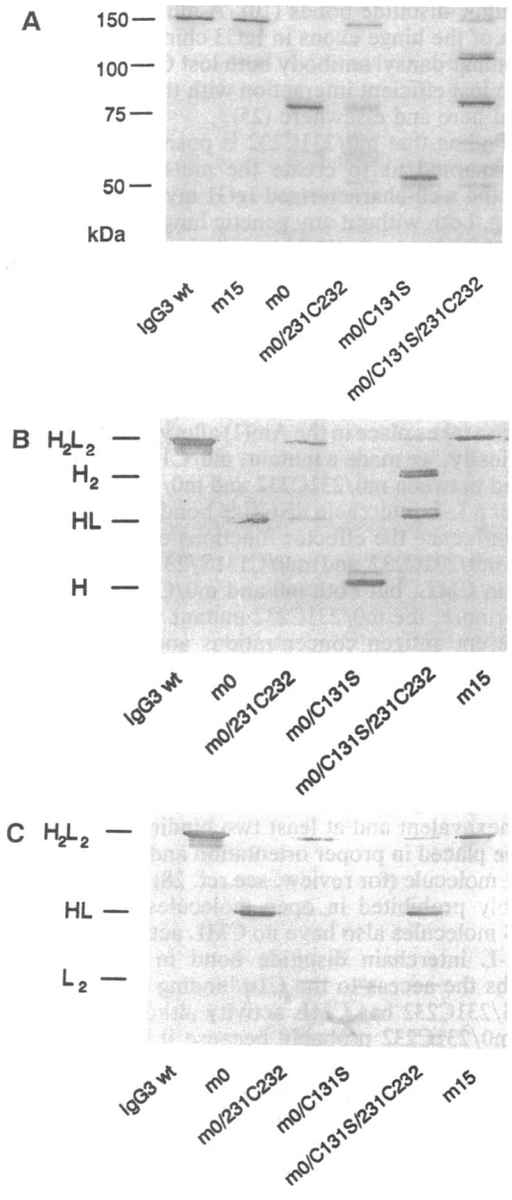

FIG. 2. (*A*) SDS/PAGE (7.5% gel) of the various mutants. (*B*) Western blot of the various mutants probed with biotin-conjugated mouse monoclonal antibody 452D12C1 specific for pan human IgG C_H3. (*C*) Western blot of the various mutants probed with rat monoclonal antibody A35-1 specific for mouse λ chains.

ecules without disulfide bonds between the H chains. The latter molecules dominated as judged by Western blot analysis (Fig. 2). Western blot analysis also showed the presence of some free monomeric L chains that could either originate from excess L chains produced by this cell line or from whole molecules consisting of L chains not forming a disulfide bond to each other. We believe that the desired m0/C131S/231C232 molecule with one disulfide bond between the L chains and one disulfide bond between the H chains is difficult to dissociate to L_2 and H_2 subunits and, therefore, shows up as H_2L_2 molecules in Western blot analysis (Fig. 2). The m0/C131S/231C232 cell line was a low antibody producer, giving ≈100 ng/ml in outgrowth cell cultures and a marked production of an excess of free λ chains, but not as pronounced as for m0/231C232 (Fig. 2).

CML Induced by the Mutants. The capacity of the mutants to induce CML was tested by using SRBCs labeled with NIP-Fab, where the Fab was prepared from rabbit IgGs against SRBCs. The four mutants without a genetic hinge were compared for CML activity in simultaneous experiments employing wt IgG3 and m15, all having the same NIP binding specificity. In experiments at different hapten concentrations and hapten patchiness, the m0 and m0/C131S mutants were negative in CML, whereas mutant m0/231C232 was as effective as m15 and m0/C131S/231C232 was constantly less active (Fig. 3).

We could recover the CML activity of m0/C131S/231C232 at 150 kDa after gel filtration in 4 M guanidine hydrochloride (data not shown), indicating that the formation of a disulfide bond between the L chain did not inhibit CML activity.

ADCC Activity of the Mutants. m0 had a faint ADCC activity that could be higher than shown in Fig. 4*A*, whereas m0/C131S was always completely negative in ADCC. m0/231C232 was equally active as m15 and wt IgG3 and m0/C131S/231C232 was less active (Fig. 4). At low antigen concentration on the target cells, when the sensitivity was lower, m0/C131S/231C232 was less active (Fig. 4*A*).

Phagocytosis Activity of the Mutants. When the U937 cell line was used as effector cells, m0/231C232 and m0/C131S/231C232 were strongly positive, as were wt IgG3 and m15, whereas m0 was very weakly positive, and m0/C131S was negative when *Candida albicans* was used as the target cell (Fig. 5*A*). m0 and m0/C131S were negative in oxidative burst activity, m0/231C232 was as active as m15 and wt IgG3, and m0/C131S/231C232 was slightly positive (Fig. 5*B*). When complement was included, m0 and m0/C131S remained negative, but all the other mutants, including m0/C131S/231C232, were positive (data not shown).

DISCUSSION

The hinge region is necessary for effector functions of naturally occurring antibodies, since IgG1 myeloma proteins

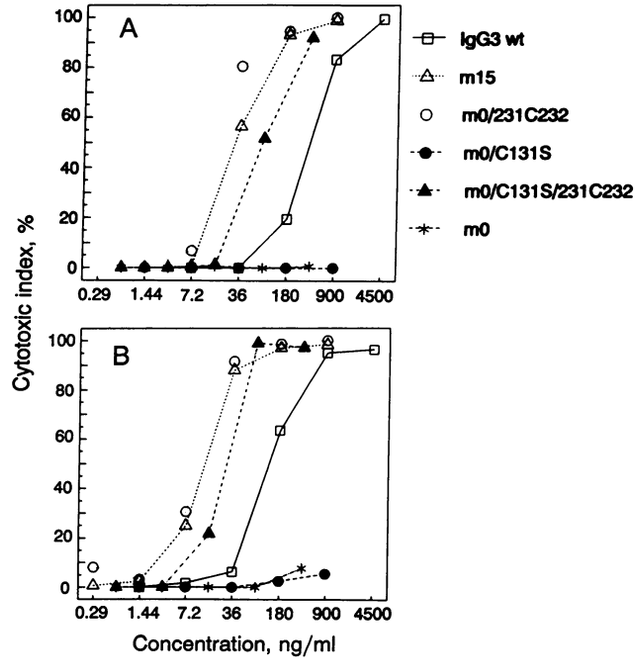

FIG. 3. CML induced by the various chimeric mutant molecules. The target cells, ^{51}Cr-labeled SRBCs, were NIP-sensitized at low patchiness and low antigen concentration, 80 ng of NIP_4-Fab′ (*A*), and at high patchiness and medium antigen concentration, 400 ng of NIP_{60}-Fab′ (*B*). All experiments were performed in parallel, simultaneously. The same dilutions of the antibodies were tested in parallel for anti-NIP binding activity and corrected if necessary as described (12).

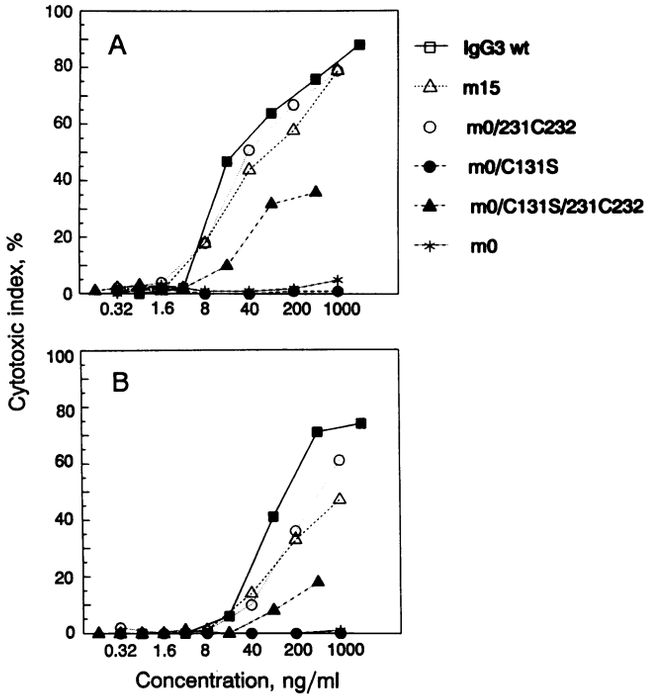

FIG. 4. ADCC activity of the wt IgG3 and mutant variants tested against target cells sensitized at two antigen concentrations and two patchinesses: 80 ng of antigen and NIP_4-Fab' patchiness (*A*) and 400 ng of antigen and NIP_{15}-Fab' patchiness (*B*). The m0 mutant was sometimes more active than shown in *A*.

without a genetic hinge lack such functions (15, 16). Similar loss of effector functions was observed by reductive cleavage

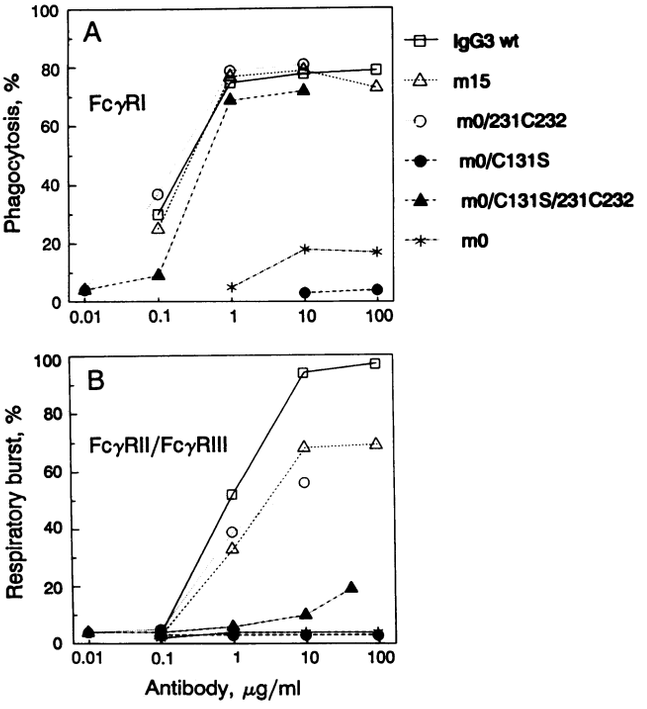

FIG. 5. (*A*) Phagocytic activity of U937 against NIP-sensitized FITC-labeled *Candida albicans* induced by wt IgG3 and the various mutant variants. Phagocytosis was performed without the presence of a complement source. (*B*) Respiratory burst mediated by PMNs against NIP-sensitized SRBCs induced by wt IgG3 and the various mutant variants. The respiratory burst analysis was performed without the presence of a complement source.

of the hinge disulfide bonds (10). A mutant, m0, created by deletion of the hinge exons in IgG3 chimeric NIP antibodies and a similar dansyl antibody both lost CML activity (12, 13) and also lost efficient interaction with the various FcγRs, as reported here and elsewhere (25).

The finding that m0/231C232 is positive in effector functions prompted us to create the mutant, m0/C131S, that mimics the well-characterized IgG1 myeloma proteins, Dob and Mcg, both without any genetic hinge region. In contrast to m0, which is open in the N-terminal part of C_H2, due to lack of noncovalent interaction between the C_H2 regions, the m0/C131S mutant should be closed in this part of the molecule, since the L chains are forced to disulfide bond to each other due to the absence of an available Cys in the H chain. This structure was confirmed by the SDS/PAGE, Western blot, and gel filtration analyses. The same bond formation takes place in the Am(1) allotype in IgA2 molecules (27). Finally, we made a mutant, m0/C131S/231C232, that is a hybrid between m0/231C232 and m0/C131S to investigate whether a L-L interchain disulfide bond, as in Mcg and Dob, could influence the effector functions of m0/231C232.

The m0/231C232 and m0/C131S/231C232 mutants were active in CML, but both m0 and m0/C131S were negative. Furthermore, the m0/231C232 mutant was as active as m15 at different antigen concentrations and, thus, more active than wt IgG3. On the other hand, m0/C131S/231C232 had only slightly more CML activity than wt IgG3. For CML we can, therefore, conclude that the C_H2 domains must be disulfide bonded in the N-terminal end for the molecules to be active. A possible explanation for this could be that the first interacting molecule in the complement cascade, C1q, is itself hexavalent and at least two binding sites on IgG must both be placed in proper orientation and engaged to activate the C1 molecule (for review, see ref. 28). This engagement is probably prohibited in open molecules like m0. The m0/C131S molecules also have no CML activity, indicating that the L-L interchain disulfide bond in this molecule also disturbs the access to the C1q binding site. However, m0/C131S/231C232 has CML activity although it is less active than m0/231C232 probably because it is a mixture of presumably CML-active molecules with H-H and L-L interchain disulfide bonds and CML-inactive molecules with only H-L and no H-H interchain disulfide bonds. As judged from the SDS/PAGE and Western blot analysis, there were about twice as many inactive molecules as active molecules in the m0/C131S/231C232 preparations. Thus the L-L interchain disulfide bonds do not significantly interfere with CML activity when a disulfide bond is present between the C_H2 domains.

Although the molecular flexibility has not been measured for m0/231C232 and m0/C131S/231C232, these molecules probably are very rigid since flexibility depends on the length of the upper hinge (29), which is the polypeptide stretch from the end of C_H1 to the first interchain disulfide bond (28), and neither of these molecules contains an upper hinge. We have reported (12) that hinge flexibility does not play a major role in CML activity and this report also supports this view.

The notion that the hinge creates a necessary distance between the Fab and Fc parts of the molecule to allow molecules like C1q and FcγR to bind to Fc (28) cannot be correct for human IgG3, since both the m0/231C232 and m0/C131S/231C232 molecules have no space between the Fab and Fc region but are active in CML, ADCC, and FcγRI-mediated phagocytosis of U937 cells.

The binding motif on Fc for C1q involves residues 318, 320, and 322 in the C_H2 part of the Fc and is close to the hinge region (30). The reason that m0/231C232 and m0/C131S/231C232 are active may be that the two Fab arms bend away from the Fc or that the Fc region is twisted compared to the plane formed by the two Fab arms. Other explanations might

still be possible, but the answer must await detailed studies by electron microscopy, NMR, or x-ray diffraction analysis.

In this study, we have focused on phagocytosis mediated by the unstimulated U937 myeloid cell line and by unstimulated freshly isolated peripheral blood PMNs from normal donors. Our previous results indicate that U937 mediated phagocytosis solely through the high-avidity FcγRI (25). For phagocytosis by PMNs, both FcγRII and FcγRIIIb seem to be required for full activity (25). All the hinge-truncated variants that were positive in CML were also positive for phagocytosis by U937 and PMNs with the exception of m0/C131S/231C232, which was somewhat lower in ADCC mediated by FcγRIIIa on natural killer cells and very weak in inducing oxidative burst initiated by FcγRII/IIIb on PMNs. Apparently, the L-L interchain disulfide bond makes the molecule less accessible for FcγRII or FcγRIIIb on PMNs than for FcγRI on U937 cells and for FcγRIIIa on natural killer cells.

At the moment, we can only speculate what happens to m0/C131S molecules when the L-L interchain disulfide bond is formed and the molecules become inactive in effector functions. Perhaps the L-L interchain disulfide bond in m0/C131S is forcing the C_H2 domains to approach each other closely as for Mcg resulting in a T-shaped molecule with a structure that obstructs the docking of C1q (31). However, the conformation of the Mcg molecule (31) shows the presence of a theoretical access to the FcγRI motif, involving Leu-235 (32), and Mcg probably interact with FcγRI (J. M. Woof and D. R. Burton as quoted in ref. 33). Our m0/C131S, on the other hand, does not interact with FcγRI, which may indicate a difference in structure between m0/C131S and Mcg. Interestingly, the Dob protein does not interact with FcγRI, so perhaps, m0/C131S, which contains a mouse λ chain, has a structure more similar to the Dob, which contains human κ chain, than to the Mcg, which contains human λ chain, which seems to differ in structure (31). Still, the Mcg and Dob molecules are IgG1, whereas m0/C131S is IgG3, which could, although unlikely, give rise to different conformations of the two sets of molecules.

We can conclude that the H chains must be disulfide-linked in the N-terminal part of the C_H2 domains to create molecules active in effector functions (Fig. 6). The Mcg and Dob molecules are probably inactive because of the lack of such a disulfide bond(s).

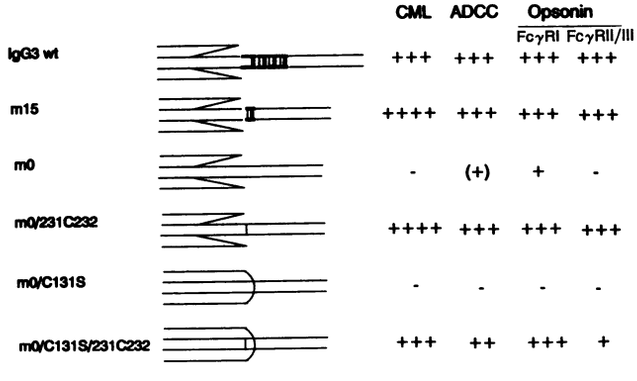

FIG. 6. Summary of the effector activities of chimeric wt IgG3 and IgG3 mutants with identical NIP binding regions. −, negative; (+), borderline positive; +, weakly positive; ++, moderately positive; +++, strongly positive; ++++, very strongly positive.

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