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# Conversion of incomplete antibodies to direct agglutinins by mild reduction: Evidence for segmental flexibility within the Fc fragment of immunoglobulin G

(antiglobulin test/blood-group antigens/hemagglutination/interchain disulfide bonds/protein conformation)

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**ABSTRACT** **Reduction of interchain disulfide bonds converted some IgG incomplete antibodies to direct hemagglutinins. This conversion occurred whether antibody was free in solution or bound to the red-cell surface. Reduced antibody permitted to reoxidize in air no longer behaved as a direct agglutinin; reversion to an incomplete antibody did not occur when reoxidation was prevented by *S*-alkylation. These results suggest that mild reduction of the antibody imparts sufficient freedom to permit bridging between cells and are interpreted as evidence that the interheavy-chain disulfide bonds restrict segmental flexibility within the Fc fragment of IgG.**

IgG alloantibodies with specificity for red-cell antigens often fail to agglutinate red cells suspended in saline and are then termed "incomplete" (1). Studies of cell-surface charge suggested that these antibodies were of insufficient span to bridge between cells kept apart by repelling forces. It had been observed, however, that most examples of incomplete anti-D would agglutinate cells that had an increased number of D sites (2); conversely, some IgG anti-A agglutinins behaved as incomplete antibodies with cells having relatively few A sites (3). The importance of antigen-site number in determining the behavior of IgG antibodies was confirmed by experiments where site number could be varied (4). Antigen mobility may also affect the behavior of these antibodies (5, 6).

In addition to the antiglobulin test (7), many techniques have been used to compensate for the failure of incomplete antibodies to agglutinate red cells suspended in saline: in some, cell-surface properties were altered by using colloid media or enzymes; in others, agglutination was obtained by high-speed centrifugation of the sensitized cells. In 1963, Pirofsky and Cordova (8) described the conversion of incomplete anti-D to a direct saline agglutinin in the presence of 2-mercaptoethanol. Since untreated sera did not agglutinate cells that had been exposed to reducing conditions, these authors concluded that hidden reactive sites in the antibody had been made available by disulfide-bond cleavage. In 1965, however, Mandy *et al.* (9) reinvestigated this finding and concluded that the hemagglutination was a "nonspecific, nonimmunological phenomenon" produced in part by the action of the reducing agent on the red cells. We now present evidence that validates the conclusion of Pirofsky and Cordova, and places their observation in the context of the current understanding of immunoglobulin structure and function.

## MATERIALS AND METHODS

**Immune Sera.** Rh immune globulin (RhIG) was obtained as an aqueous solution of the gamma globulin fraction (Cohn Fraction II) of pooled plasma from donors alloimmunized by pregnancy or by injections of red cells. This RhIG contained approximately 300 μg of anti-D per ml and other blood-group antibodies in low titer. Four sera containing an incomplete antibody of high titer were selected for the isolation and chemical modification of IgG; three (Gue, Les, and Tur) contained anti-D and one (Dai) contained anti-K. Other sera containing incomplete antibodies (anti-D, anti-C, anti-c, anti-E, anti-K, anti-S, anti-Fy$^a$, and anti-Jk$^a$) were tested without isolation of the IgG. The sera had not been heat-inactivated.

**Isolation and Characterization of IgG.** Two methods were used for the isolation of IgG; in both, partial delipidation by centrifugation (20,000 × *g* for 30 min) was performed before isolation. In the first, serum Gue and serum Dai were fractionated by ammonium sulfate precipitation followed by anion-exchange chromatography on Whatman DE-52 cellulose equilibrated in 10 mM Tris·HCl/20 mM NaCl, pH 7.0. Monomeric IgG was obtained from the unadsorbed fraction by gel chromatography on LKB Ultrogel AcA 34 equilibrated in Tris-buffered saline (10 mM Tris·HCl/0.15 M NaCl/0.02% $NaN_3$, pH 7.8). The purity of the isolated IgG fractions was established by immunoelectrophoresis in 1.3% agarose (Kallestad) in barbital buffer (pH 8.6, $\mu = 0.05$, containing 0.85 mM calcium lactate) against a sheep antiserum to whole human serum and class-specific antisera, and by 0.1% sodium dodecyl sulfate/polyacrylamide gel electrophoresis in 7.0% gels (10).

Since in serum Gue anti-D activity was restricted to the fast $\gamma/\beta$ fractions isolated from DE-52 cellulose in the gradient fractions, as observed with another example of anti-D (11), the other two sera (Les and Tur) were fractionated on QAE-Sephadex A-50 (12) followed by gel filtration on Ultrogel AcA 34 in Tris-buffered saline. The IgG fractions were assessed for purity as described above and were found to contain a trace contaminant of $\beta_1$ mobility, identified as transferrin by Ouchterlony analysis. Before testing, all samples were concentrated by dialysis under reduced pressure against phosphate-buffered saline (40 mM in phosphate, pH 7.2, $\mu = 0.15$) containing 0.02% $NaN_3$. The concentration of IgG in whole sera and in the IgG fractions was determined by single radial immunodiffusion and spectrophotometrically from the absorbance at 280 nm using $A^{1\%}_{1\,cm} = 14.0$, respectively.

**Reduction and *S*-Alkylation of IgG.** Each of the IgG samples (about 10 mg/ml) was adjusted to pH 8.6 with 2 M Tris base and mildly reduced with 10 mM dithioerythritol (Sigma Chemical Co.) at room temperature. After 45 min, iodoacetamide (Sigma, twice recrystallized) was added to a final concentration of 25 mM; the samples were left for 1 hr in the dark and then dialyzed into phosphate-buffered saline for testing. RhIG that had been either exposed to alkylating conditions without prior reduction, or reduced and permitted to reoxidize

Abbreviations: $C_L$, $C\gamma1$, $C\gamma2$, and $C\gamma3$, constant homology regions of the light (L) and heavy ($\gamma$) chains of immunoglobulin G (IgG); C1, first component of serum complement; RhIG, Rh immune globulin.

Table 1. Hemagglutinin titers of whole serum and isolated IgG before and after mild reduction

| Antibody* | Red cells | Whole serum† | | IgG fraction†‡ | |
|---|---|---|---|---|---|
| | | Unreduced | Reduced | Unreduced | Reduced and alkylated |
| Anti-D (RhIG) | *DCe/DCe*§ | — | — | 0 | >1000 |
| | *dce/dce* | — | — | 0 | 0 |
| Anti-D (Gue) | *DCe/DCe* | 0 | >100 | — | — |
| | *dce/dce* | 0 | 0 | — | — |
| Anti-D (Les)¶ | *DCe/DCe* | 50 | >100 | 0 | >20 |
| | *dce/dce* | 0 | 0 | 0 | 0 |
| Anti-D (Tur)¶ | *DCe/DCe* | 10 | 50 | 0 | >20 |
| | *dce/dce* | 0 | 0 | 0 | 0 |
| Anti-c (Sea) | *dce/dce* | 5 | 500 | — | — |
| | *DCe/DCe* | 0 | 0 | — | — |
| Anti-c (Hop) | *dce/dce* | 0 | 5 | — | — |
| | *DCe/DCe* | 0 | 0 | — | — |
| Anti-E (Hul) | *DcE/DcE* | 0 | 40 | — | — |
| | *DCe/DCe* | 0 | 0 | — | — |
| Anti-K (Dai) | *K/K* | 0 | 50 | 0 | 20 |
| | *K/k* | 0 | 0 | 0 | 5 |
| | *k/k* | 0 | 0 | 0 | 0 |

— Indicates not tested.
* The indirect antiglobulin titer of each antiserum was 500 or more, except anti-c (Hop), which had a titer of 100.
† Tested in dilutions in 7% albumin.
‡ Adjusted to the concentration of IgG in whole serum (about 10 mg/ml).
§ Probable genotype.
¶ Contained some IgM anti-D.

by dialysis, was prepared as a control. The extent of reduction and alkylation was determined by electrophoresis in 12.5% polyacrylamide sodium dodecyl sulfate gels (10).

**Reduction without Alkylation.** Whole sera and RhIG were serially diluted in 7% bovine-serum albumin in saline, then mildly reduced with an equal volume of 10 mM dithioerythritol in phosphate-buffered saline for 30 min at room temperature. The samples were tested without dialysis. In some experiments 10 mM dithiothreitol or 0.2 M 2-mercaptoethanol was also used as a reducing agent.

**Hemagglutination Tests.** The unreduced and the mildly reduced and alkylated IgG fractions, and the unreduced and reduced sera, were tested for hemagglutination. One volume of red cells (2% suspension in saline) was added to one volume of each sample. After about 2 hr at room temperature, the tubes were spun for 30 sec in a clinical centrifuge and the cells were then examined microscopically. In other experiments red cells, strongly sensitized with incomplete antibodies of known specificity, were washed and then exposed to reducing conditions. After 10 min and after 2 hr, the cells were centrifuged and examined microscopically. As a control, unsensitized red cells were treated similarly.

An assessment of possible nonspecific agglutination due to structural alterations in non-antigen-binding portions of the reduced antibody was made by testing reduced anti-D with mixtures containing D-positive and D-negative cells; in each mixture one population of cells was labeled with fluorescein isothiocyanate (13).

## RESULTS

**Conversion of Incomplete Antibodies to Direct Agglutinins by Mild Reduction.** The effect of mild reduction by dithioerythritol on the incomplete antibodies in whole sera and in IgG fractions is shown in Table 1. Anti-D, anti-c, anti-E, and anti-K were converted to direct agglutinins; anti-S, anti-$Fy^a$, and anti-$Jk^a$ were not converted. The agglutinates were sometimes fragile and were readily dispersed by washing, but the reactions were reproducible and specific. The conversion to a direct agglutinin was most striking with the potent anti-D (RhIG) which, when diluted in 7% albumin, had a titer of more than 1000 after reduction and alkylation, or after reduction alone. Even when the RhIG was diluted in saline, the titer was more than 100 after reduction. Reduction with dithiothreitol or with 2-mercaptoethanol also converted these incomplete antibodies to direct agglutinins. The saline agglutinin titer of RhIG was not increased by the use of cells treated with reducing agent, or by addition of reducing agent to each dilution of the reduced and alkylated antibody. Agglutination could not be attributed to additional antibody present on the cells, since the indirect antiglobulin titer of these antibodies was unaffected by reduction and since the conversion also occurred when sensitization preceded reduction (see below).

Nonspecific reactions occasionally occurred when red cells were exposed to reducing conditions for more than 2 hr or to serum diluted less than 1 in 5 before reduction. The nonspecificity of these reactions was confirmed by the agglutination of autologous cells. RhIG or isolated IgG fractions did not give false-positive reactions after reduction or after reduction and alkylation.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of reduced and alkylated RhIG and isolated IgG fractions showed that reduction of interchain disulfides was virtually complete. Although minor bands corresponding to reduction intermediates were seen, no intact IgG was present. Antibody that had been either exposed to alkylating conditions without prior reduction or reduced and permitted to reoxidize by dialysis behaved both on electrophoresis and in hemagglutination tests as an unreduced antibody. In cell mixtures in which either D-positive or D-negative cells were identified by fluorescence, the agglutinates produced by reduced anti-D did not contain

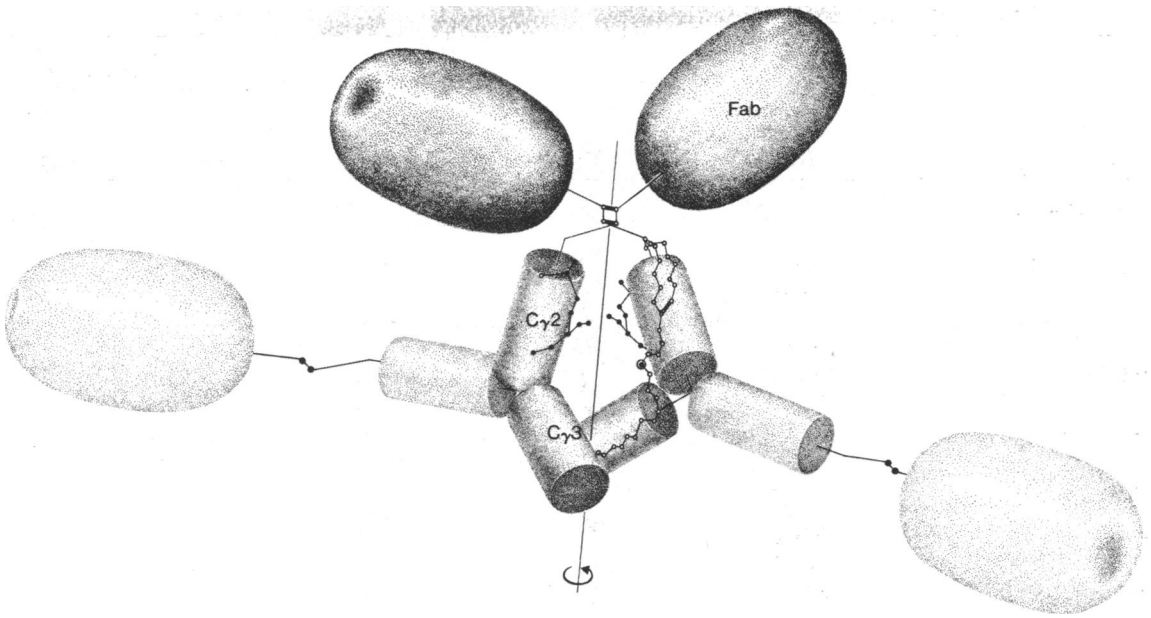


FIG. 1. Diagrammatic representation of the hypothetical extreme in segmental flexibility in an IgG antibody before and after mild reduction. The antibody shown contains two hinge disulfides (Cys 226 and Cys 229) typical of human γ1 and γ4 subclass molecules; γ2 and γ3 antibodies have 4 and 11 hinge disulfides, respectively, and would presumably be equally affected by the reducing conditions used. The antigen-binding regions (Fab) are connected through a flexible hinge region to the Cγ2 and Cγ3 domains, which comprise the Fc fragment. The hinge peptides may be interposed in the solvent space between the Cγ2 domains (see ref. 29). All fragments and domains are shown disposed about a 2-fold axis of rotational symmetry passing through the cystines of the hinge and the interface between the Cγ3 domains. The Cγ2 domains are glycosylated (● - ● - ●); they do not interact. Upon mild reduction interheavy-chain disulfides are cleaved, permitting sufficient flexibility at the Cγ2–Cγ3 contacts to allow the Cγ2 domains and Fab regions to assume positions shown by light stippling. The Cγ3 domains form a strongly interacting dimer; it is assumed that their relative positions are unchanged by reduction. In the x-ray structure of Fc the axes of Cγ2 and Cγ3 do not intersect as shown; these domains are connected by a stretch of chain (O - O - O) passing along the surface of Cγ2 from the second intradomain half-cystine (Cys 321), through the switch sequence (Ser 337 to Gln 342), and disappearing into the inter-Cγ3 contact. The distance from the 2-fold axis to Lys 340 (⊙) is about 15 Å, the length of Cγ2 is about 40 Å, and the additional stretch to the hinge cysteines is probably greater than 15 Å.

D-negative cells; this result excluded the unlikely possibility that the agglutination was due to cell-bridging mediated by antigen-binding and non-antigen-binding (Fc) portions of the reduced antibody.

**Conversion of Cell-Bound Incomplete Antibodies to Direct Agglutinins.** The conversion of an incomplete antibody to a direct agglutinin could also be demonstrated when sensitization preceded reduction. Within 10 min of exposure to reducing conditions, red cells sensitized with incomplete anti-D, anti-c, or anti-E were strongly agglutinated. Within 2 hr of exposure, cells sensitized with anti-C and anti-K were weakly agglutinated; cells sensitized with anti-S, anti-$Fy^a$, or anti-$Jk^a$ were not agglutinated under these conditions.

## DISCUSSION

In many studies of the effect of reducing agents on incomplete blood-group antibodies, direct agglutination after reduction was not recorded (14–19), perhaps because the reduced antibody was allowed to reoxidize by dialysis before testing or perhaps because appropriate tests were not done. The present experiments, however, confirm the observation of Pirofsky and Cordova (8) that some incomplete antibodies that have been mildly reduced are converted to specific hemagglutinins. Furthermore, our results exclude the possibility that such agglutination is only apparently antigen-specific, that is, due to cell-bridging mediated by antigen-binding and non-antigen-binding portions of the antibody. The conversion to a direct agglutinin occurred whether the antibody was free in solution or bound to the red-cell surface. Although physical and immunochemical analyses of IgG have usually failed to show conformational changes accompanying mild reduction (20–22; see ref. 23), such reduction must impart sufficient freedom to permit bridging between cells. These results thus provide indirect evidence that interchain disulfide bonds restrict flexibility in the native IgG molecule.

Conformational motility is a general property of proteins in solution, but few exhibit flexibility of a segmental nature. Previous investigation of segmental flexibility in IgG antibodies has largely been concerned with motion of the Fab regions relative to Fc, and has not usually considered similar motion within the Fc fragment (see ref. 24 for a recent review). Electron micrographs of cyclic complexes of rabbit IgG anti-Dnp antibodies with bis-Dnp haptens have shown that the Fab arms may subtend apparent angles varying from nearly 0 to 180° (25). Since by several criteria the Fab fragments are compact and rigid structures (24) and since the hinge region has a relatively open structure, these angles probably result from flexibility in the hinge. The question of hinge flexibility in solution has been approached by hydrodynamic studies and by experiments using fluorescence depolarization. By the latter technique, Yguerabide *et al.* (26) have calculated that in nanoseconds there is an angular range of rotation of each Fab fragment of about 33°. Flexibility within Fc has been suggested by the decreased resistance to solvent denaturation (27) and by enhanced hydrogen-deuterium exchange (23) in reduced antibody, although in these studies segmental flexibility cannot be distinguished from other kinds of conformational flexibility. Since in our control experiments nonspecific cell bridging by antigen-binding and Fc portions of the reduced antibody was excluded, the conversion of incomplete antibody to a direct agglutinin can most readily be explained by contributions from Fc to an increased separation of combining sites. In view of the compact domain structure of Fc (Fig. 1), these results provide

strong evidence for segmental flexibility within this fragment.

There are insufficient data to allow an accurate determination of the degree of freedom introduced into Fc by mild reduction, but an estimate of the exteme in molecular extension can be made. Since the Cγ3 domains strongly interact, they are unlikely to contribute significant flexibility to the reduced antibody. However, since the Cγ2 domains show no mutual affinity (28), the putative flexibility could extend to the contact region between Cγ2 and Cγ3. The 3.5-Å crystallographic analysis of human Fcγ has shown that this contact is characterized by over 40 $C_\alpha$—$C_\alpha$ distances less than 10 Å; based on structural homology to the V-C switch region contact in the mouse McPC 603 Fab fragment, Huber *et al.* (29) have inferred that this contact must be rigid. While this inference may be valid for the unreduced molecule, it may no longer be valid after reduction of the interheavy-chain disulfides since the paired Cγ2 domains do not interact. Upon reduction sufficient freedom may be gained to permit a fully extended conformation of the switch peptides in Fc (which in the unreduced fragment are irregularly bent between Lys 338 and Gly 341), thus allowing considerable flexibility about Cγ3. If the Cγ2 domains could assume positions at right angles to the molecular 2-fold axis (Fig. 1), such flexibility alone would add about 80 Å (i.e., twice the approximate length of Cγ2) to the distance between antigen-binding sites. The displacement of each switch peptide from the 2-fold axis (about 15 Å) would also contribute to an increased separation of antigen-binding sites and an extended conformation of the hinge region would contribute a further 20–30 Å (minimum). Thus after reduction IgG antibodies might conceivably span about an additional 140 Å (80 + 30 + 30 Å). While these considerations are speculative, the estimate is consistent with an observation by Kunkel *et al.* (30) that cells sensitized with Fab fragments of anti-D were agglutinated by anti-Km(1), since twice the distance from the antigen-binding site to the hinge disulfides, i.e., about 160 Å (31), would then be the maximal *additional* span required by incomplete anti-D to bridge between D sites on adjacent cells. The comparison is valid since, in our experiments, agglutination by reduced antibody occurred under conditions in which the number of antigen sites on the red cells was essentially the same as the number of Km(1) sites provided by the anti-D Fab fragments. It must be stressed, however, that our model of the reduced antibody (Fig. 1) shows a maximal separation of combining sites and that, since some IgG antibodies can bridge between red cells in saline, little added freedom may be needed for direct agglutination. The actual range of conformations after reduction must, of course, be consistent both with reoxidation of the hinge disulfides and with direct agglutination by reduced antibody. A direct assessment of the range of flexibility within Fc in reduced IgG molecules could be made by electron microscopy.

The conversion of an incomplete antibody to a direct agglutinin by mild reduction suggests that the interchain disulfides impart a degree of conformational rigidity to the intact antibody. A number of recent observations have indicated that such restrictions to segmental flexibility in IgG may be of fundamental importance in the expression of the effector functions of the molecule. For example, after mild reduction complement-fixing IgG antibodies no longer bind C1 (32, 33). Isenman *et al.* (33) showed that it was the inter-γ chain disulfides that were essential for complement fixation by whole antibody, but that these bonds were not directly involved in C1-binding. They therefore proposed that in the reduced antibody flexibility might be sufficiently increased to permit modulation by Fab of the C1-binding site. Another biological function, that of binding of whole antibody to membrane receptors specific for Fc on a variety of cells, is also abolished by mild reduction (34–37). The molecular events that underlie these phenomena are not well understood but, since the subunit structure of IgG is independent of interchain disulfide integrity, mild reduction may distort critical relationships between domains. These losses of biological function suggest that the interheavy-chain cystines have been highly conserved during evolution, and that certain IgG proteins that have deletions in the hinge (38) may lack the Fc-dependent functions of the molecule.

The direct agglutination of red cells by some incomplete antibodies shows that mild reduction may compensate for a limited number of antigen sites in hemagglutination. The limit of this compensation is not precisely known although, as shown in Table 1, reduced anti-K agglutinated *K/K* red cells, having about 6000 K sites per cell (39). However, since reduced anti-$Fy^a$ did not agglutinate $Fy^a/Fy^a$ cells having about 12,000 $Fy^a$ sites per cell (40), perhaps $Fy^a$ antigens are relatively restricted in their mobility, as are concanavalin A receptors on adult red cells (41). For the detection of incomplete antibody, direct agglutination after reduction is less sensitive than the antiglobulin test; however, the use of modified sera as typing reagents may be practical and is presently under study (C. A. Laschinger, D. G. Romans, and B. P. L. Moore, unpublished data).

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