

Immunoglobulin Structure and Function as Revealed by Electron Microscopy

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Key Words

Electron microscopy · Immunoglobulin · Antibody · Review · Structure

Abstract

Electron-microscopic (EM) analysis preceded crystallographic analysis [1, 2] of Igs by over a decade and was for a time the only direct way of analyzing their 3-D molecular structure. Once the X-ray structures were deduced, the role of EM gradually shifted from gross structural analysis to the addressing of more sophisticated structural and functional questions. EM remains a vital adjunct to the many physicochemical, biochemical, and serological tools brought to bear on these remarkable molecules as we try to relate form to function. In this review I will highlight some of the many contributions that have been made possible by virtue of being able to 'see' Ig molecules and immune complexes.

EM an Related Techniques

Negative staining is the EM technique most widely applied to the analysis of Ig structure. In its simplest form, target molecules are allowed to adhere to thin carbon films attached to EM grids, then are flooded with an electron-dense stain. The bulk or the stain is removed by capillary action

resulting in a thin film of stain which is allowed to dry. The molecules displace the stain and thus appear light against the darker background when viewed by transmission EM (TEM) as shown in figure 1. Na silicotungstate, uranyl acetate, and uranyl formate are the generally preferred negative stains for viewing the Igs as these give the finest granularity and thus yield the greatest molecular resolution [3]. Detailed protocols using the uranyl formate are given by Roux [4, 5].

Scanning transmission EM (STEM) is occasionally used to image negatively stained Ig molecules. Though images tend be of lower resolution, they can approach TEM in detail [6] (fig. 2).

Although negative stain TEM on carbon membranes has been the standard technique for the EM analysis of Igs, a variety of other approaches have been used with variable success. Uzgiris [7–9] successfully grew 2-D crystals of monoclonal Abs (mAb) by reacting IgG or IgE anti-DNP with haptenated lipid monolayers. 2-D crystals have the potential advantage of facilitating computational image averaging to reveal a level of detail not obtainable by single particle analysis [10]. Unfortunately, these Ig crystals showed considerable local disorder, precluding such analysis. In another study, thin sections of 3-D crystals of a cryoglobulin in complex with serum albumin were stained to show the general crystal packing structure but little molecular detail [11].

In some-studies, Ig has been freeze-dried onto a substrate and unidirectionally or rotary shadowed with heavy

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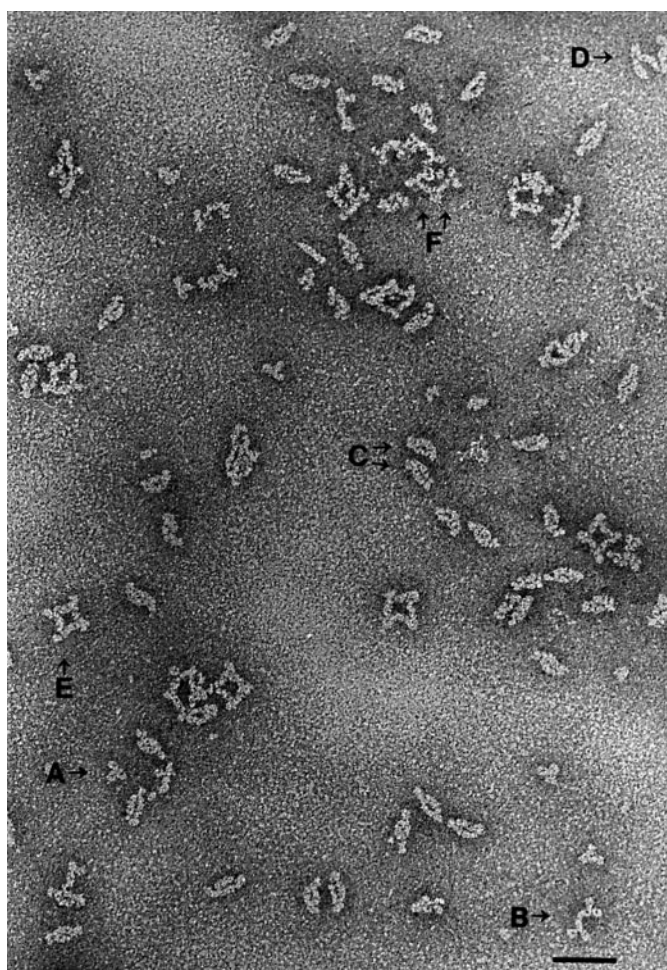


Fig. 1. Negative stain TEM of a field of mAb Id reacting with mAb anti-Id to form complexes of various sizes. A = Monomer; B = monovalent dimer; C = divalent (ring) dimers; D = chain trimer; E = ring tetramer; F = extended chain multimer. Bar = 40 nm. From Roux [4]. With permission from Academic Press.

metal [12–14]. This approach gives the general shape of the molecule and, unlike most negative stain preparations, allows one to measure the shadow cast by the molecule (in unidirectional shadowing), to give some information on the third (z) dimension of the molecules as they protrude above the plane of the underlying support surface. For example, up to 70% of human IgG1 molecules were interpreted to be in a nonplanar ‘tripod’ configuration [13]. Because this technique is more cumbersome and demanding and frequently does not offer the level of resolution as the best negative stain techniques, it has not been widely applied to molecules as small as IgG.

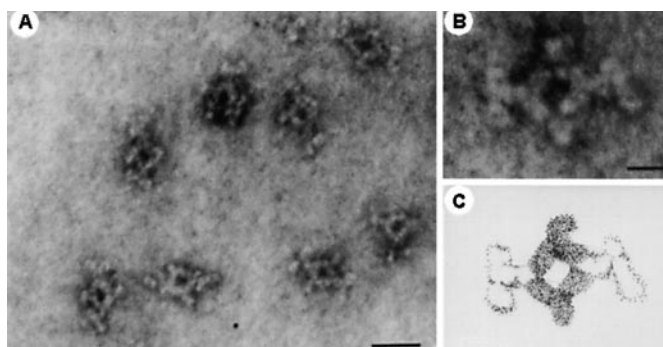


Fig. 2. STEM of negatively stained complexes (A, B) and diagram (C) of mouse IgG1 and rat antimouse IgG1 mAb. Bar = 25 nm in (A) and 10 nm in (B). From Lansdorp et al. [6]. With permission from Wiley-VCH Verlag.

Cryo EM, the analysis of unstained particles suspended in vitreous ice, has the advantage of eliminating the flattening effect of most other applicable EM techniques. Unfortunately, proteins in this state are highly susceptible to electron beam damage. Also, without background stain, the contrast between protein and water is very low so structures can be difficult to identify [15]. Some of these disadvantages can be overcome by analysis of paracrystalline arrays, where computational averaging [16] can give remarkably detailed reconstructions. Such reconstructions are frequently used in conjunction with X-ray crystallography-derived atomic model. Several groups, for example, have imaged the Fab arms of an mAb binding to the capsid proteins on icosahedral virus particles [17–20]. Remarkably, some of these studies show that one of these mAb binds not only through the CDR but makes contact with the antigen in the elbow region (fig. 3).

Atomic force microscopy (AFM) and scanning tunneling microscopy (STM) have been directed to the analysis of Igs but do not generally provide the level of resolution of negative stain TEM or STEM [21, 22]. In these assays, a very small probe is moved over molecules attached to a substrate and the degree of displacement from the surface (AFM) or resistance to the passage of electrons (STM) is measured. Unless molecules are firmly affixed to the substrate, they are subject to distortion or destruction as the probe is moved over their surface [22, 23]. Nevertheless, IgG, dimeric IgA, and serum IgM have been successfully imaged with these techniques [24, 25]. IgM, for example, is seen as a pentameric structure in which each pair of Fab arms often appeared melded together as a broad paddle (fig. 4) whereas some individual arms can be visualized in the IgG and IgA samples [25].

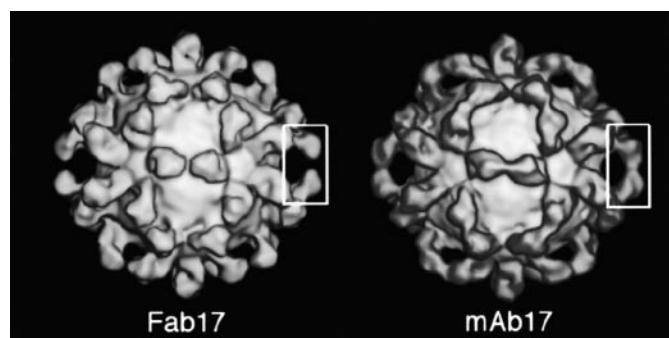


Fig. 3. 3-D reconstruction of cryo TEM images of an IgG virus complex. The upper panel shows the Fab fragments (blue) and Fab arms of intact IgG (red) attached to the surface of a human rhinovirus (gray). The Fc arms are not visible in the reconstruction due to random orientations in the original cryo EMs. Note differences in the details of the Fabs in each case (boxed area). The lower panel shows a molecular model of Fab superimposed on the cryo EM model of Fab and the axis of rotation and angular difference between the two images as diagrammed in figure 11A (angle c). Modified from Smith et al. [80]. With permission from National Academy of Sciences, USA.

Revealing Basic Ig Morphology

IgG

The earliest attempts to characterize Ig suffered from low resolution. For example, IgG was envisioned as a cigar-shaped molecule with reactive sites at each end to account for bivalency. Feinstein and Rowe [26] were the first to suggest a hinged structure based on EM. They perceived unreacted IgG or IgG antibody (Ab) bound to monovalent antigen (Ag) to be 'V' shaped. Upon crosslinking with bi- or polyvalent Ag, a hinge was thought to be 'clicked open' to expose complement-binding sites. They correctly deduced that the disulfide bond(s) were in the hinge region, but they

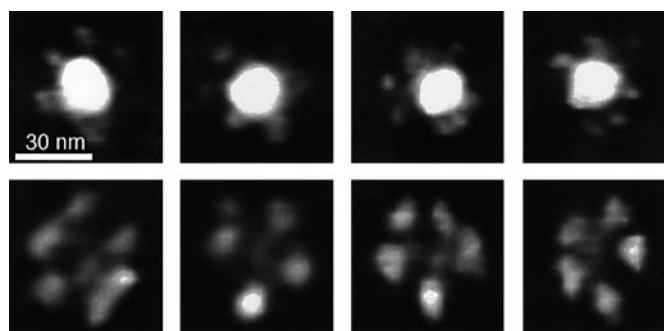


Fig. 4. Cryo-AFM (atomic force micrographs) of murine IgM mAbs attached to mica substrate showing planar subunit structure (bottom row) as diagrammed in figure 7A and molecules which appear to have raised Fc disks (top row) as diagrammed in figure 7E. From Zhang et al. [25]. With permission from Biophysical Society.

failed to discern the Fc region in these early images. It was not until the landmark EM work of Valentine and Green [27] that an accurate picture of IgG emerged. Both uncomplexed IgG and molecules crosslinked by bivalent haptens were viewed, and reasonably precise molecular dimensions noted. The arms of the uncomplexed IgGs were shown to be variably flexible, ranging in configuration from 'T' (180°) to 'Y' (near 0°). Similar angles were observed between bona fide Fab arms of the variously sized and arrayed ring structures in immune complexes that formed upon the addition of bivalent hapten (fig. 5). The Fc regions were clearly seen protruding from the cyclic complexes but were absent from pepsin-digested F(ab')₂ molecules. More recently, EM has been used to determine that the exceptionally long hinge of human IgG3 is a rod-like structure approximately 100 Å in length [28–30].

IgM

Mammalian and cartilaginous fish serum IgM was characterized by EM as being primarily pentameric and radially arrayed (fig. 6) [31–35]. In contrast, *Xenopus* IgM was shown to be hexameric [32, 36] and bony fish IgM tetrameric [37, 38]. For mammalian IgM, the Fab arms were observed to be separated by varying angles, a finding that was cautiously interpreted to indicate either Fab-Fab flexibility or the 2-D projections of more rigid 'V' Fab pairs viewed at various angles as they adhered to their carbon supports. In fact, some IgM molecules show a five-arm 'starfish' configuration as though the Fab 'Vs' were being viewed from the side, rather than the more typical 10 arm 'snow-flake' form (fig. 7) [33, 39–41]. These observations have led to the suggestion that the 'starfish' morphology of

Fig. 5. Negative stain TEM and interpretive diagram of immune complexes composed of polyclonal rabbit IgG antibody crosslinked with bivalent hapten. From Valentine and Green [27]. Magnification $\times 330,000$. With permission from American Society for Biochemistry and Molecular Biology.

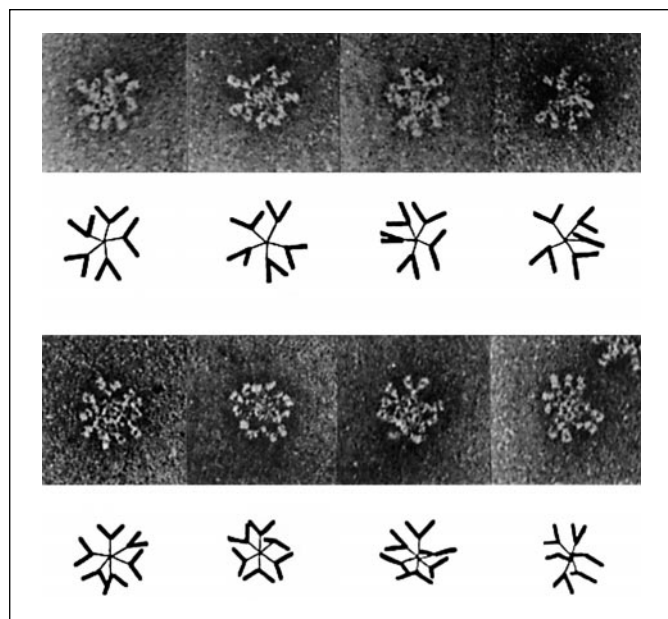
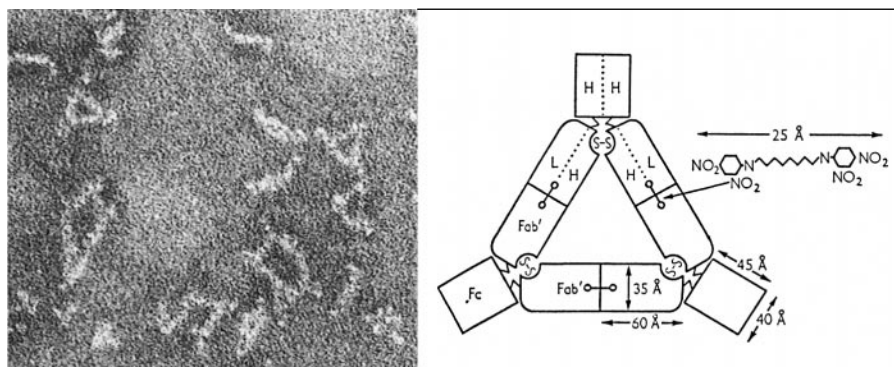


Fig. 6. Negative stain TEMs and diagrams of pentameric (top row) and hexameric murine (bottom row) IgM. From Davis et al. [45]. Magnification $\times 250,000$. With permission from Wiley-VCH Verlag.

IgM is the native form in solution and that the 10 armed radial form is an artifact of the attachment to the carbon membrane during negative stain preparation [42]. This issue is likely to remain unresolved until unreacted IgM can be viewed by cryo EM suspended in vitreous ice.

A related issue has to do with the profound conformational change in IgM as it adheres to some antigenic surfaces. As most dramatically demonstrated by Feinstein et al. [33], the Fab arms and the N-terminal portion of the Fc bend away from the plane of the Fc disc of the molecule to attach at a near right angle to the antigen surface, in their example, bacterial flagella (fig. 8). This configuration is generally de-

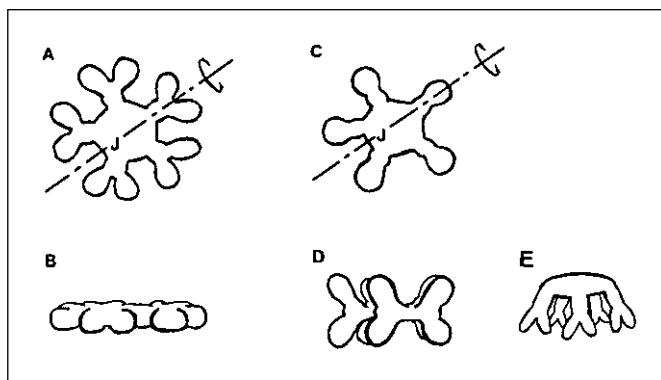


Fig. 7. Snowflake (A, B), starfish (C, D) and staple (E) models of IgM. Modified from Poon et al. [40]. With permission from American Society for Biochemistry and Molecular Biology.

scribed as being 'staple'-like. Thus, the engaged IgM pentamers appear as 10-legged spiders attached to the surface of the flagella. At some IgM-to-flagella ratios, fewer staple configurations are seen and most of the IgM Fab arms are radially extended to crosslink different flagella. Such preparations are less able to fix complement than the 'staple'-rich samples even though there is considerable IgM-antigen interaction. Consequently, the bent nature of the subunits, stabilized by interaction with antigen, is presumed to expose the C1q-reactive sites on $C_{\mu}3$ leading to complement fixation [42, 43]. Interestingly, an AFM analysis of IgM attached to mica shows a majority of the IgM molecules as having a raised central disk [25] (fig. 4). These IgM appear to represent molecules in the staple configuration even though they were nonspecifically attached to antigen-free substrate.

Not all mammalian IgM is pentameric. One of the earliest EM studies [32], for example, noted that some IgM hexamers were present in a mouse myeloma protein (fig. 6). More recently, at least half of the IgM secreted by a trans-

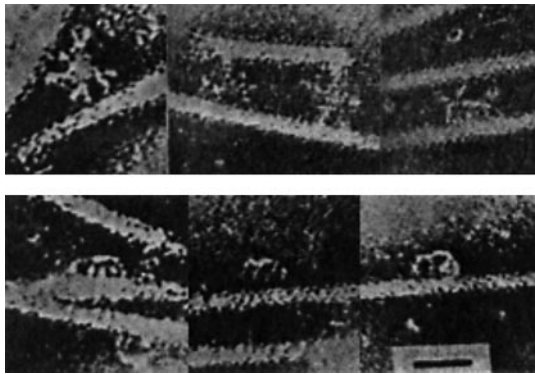


Fig. 8. Negative stain TEM of bacterial flagella with attached IgM. From Feinstein et al. [33]. Bar = 25 nm. With permission from New York Academy of Sciences.

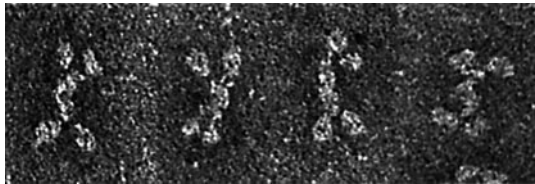


Fig. 9. Negative stain TEM of human IgA dimers [Roux, unpubl. figures]. Magnification $\times 550,000$.

fected cell line in the absence of J chain were shown to be hexameric [44]. In a genetically engineered version of murine IgM, the mutation of one of the three cysteins (to serine; Cys $\rightarrow$ Ser) that are involved in interchain disulfide bond linkage resulted in a failure to incorporate J chain concomitant with hexamer formation [45]. Significantly, the hexamer fractions, as verified by EM, showed a 20-fold greater ability to fix complement. Mutation of Cys 414 $\rightarrow$ Ser yielded pentamers and smaller forms without J chain, and Cys 337 $\rightarrow$ Ser yielded a wild-type mix of J chain-containing pentamers and J chainless hexamers in which the IgM subunits were not covalently associated [41]. In addition to the number of subunits present, differences in the nature of the Fc disk were also noted in EM images. The Fc disks of the Cys $\rightarrow$ Ser414 and Cys $\rightarrow$ Ser337 mutants were more open and disordered, implying they were only covalently associated by the Cys 575 of the C-terminal tail of the μ chain.

IgA

Another Ig molecule subjected to early EM analysis is IgA. IgA from several species were shown to display a wide variety of Fab-Fab angles, suggesting considerable flexibil-

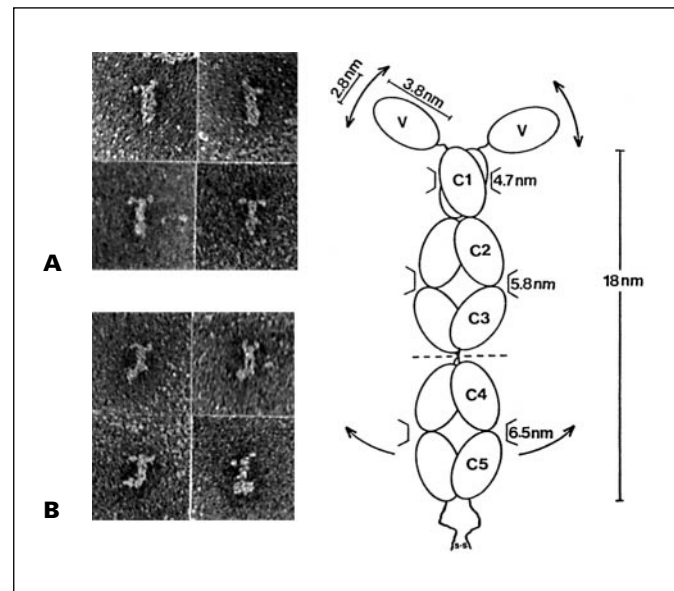


Fig. 10. Negative stain TEM (**A**, **B**) and interpretive diagram of NAR showing the absence of light chains, a pair of single V domain Fabs on a flexible hinge, and an extra long Fc in the straight (**A**) or crooked (**B**) configuration. From Roux et al. [47]. Magnification $\times 280,000$. With permission from National Academy of Sciences, USA.

ity [33] (fig. 9). Dimeric forms of IgA were shown to be joined at or near the distal (C-terminal) ends of the Fc tails, positioning the two sets of Fab arms (as identified by their reaction with visualizable antigen (ferritin)) at opposite ends of the molecule. IgA dimers and larger polymers were shown to be attached to each other with some degree of variability in the angle formed at the Fc-Fc junction, and IgA multimers, to be radially arrayed [46]. Because of its small size, any associated SC could not be identified.

NAR

The morphology of one of the strangest Ig molecules yet described has recently been documented by EM [47]. Sequence analysis has shown that NAR (shark *new* or *nurse* shark *antigen* receptor) is composed of a pair of exceptionally large heavy chains consisting of a V and five C domains. Remarkably, there are no associated light chains. EM analysis shows a molecule with rudimentary Fab arms each of which consists of a single unpaired VH domain attached to an extra long Fc arm by a flexible hinge (fig. 10). Interestingly, an additional flexible segment appears to encompass the lower (C-terminal) 2/5 of the Fc arm, the significance of which is unknown.

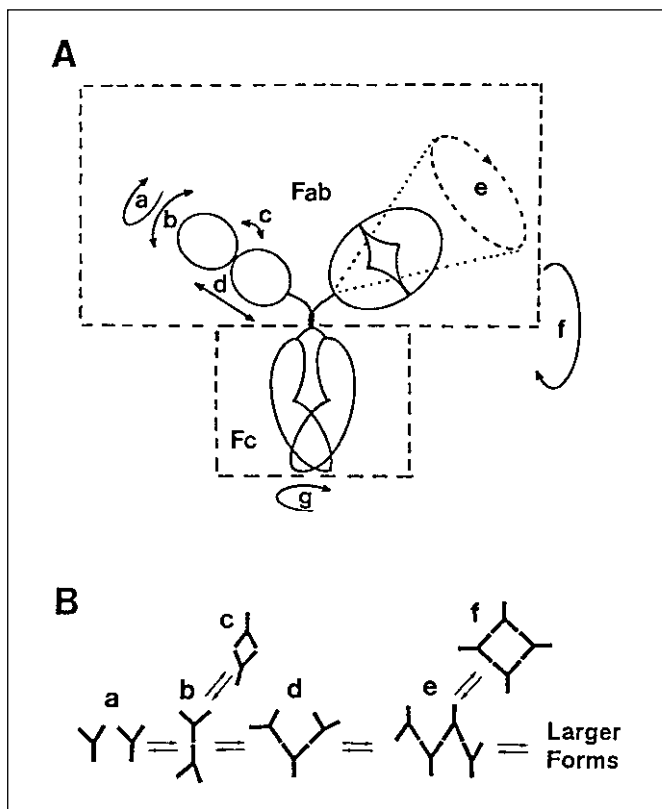


Fig. 11. The modes of Ig flexibility (**A**) and the relationship between Id-anti-Id complexes of defined geometry (**B**). The different modes of flexibility shown in (**A**) include (a) rotation about the long axis; (b) hinge-like folding or wagging; (c) elbow bending; (d) translation (in-and-out motion); (e) conical wagging; (f) Fab-Fc planar folding, and (g) Fc rotation. **B** Progression of immune complex types from unreacted molecules to larger forms and the relationship between linear and cyclic forms. Monomers (a), monovalent dimers (b), divalent 'ring' dimers (c), trimers (d), linear tetramers (e), and ring tetramers (f) are shown. From Roux et al. [30]. With permission from American Association of Immunologists.

Ig Flexibility

A number of physicochemical studies have shown the classes and subclasses Ig molecules to vary considerably in flexibility [48]. Whereas these data define the rate of movement of reporter haptens in the complementarity determining region (CDR) (for example), they do not generally reveal the modes of flexibility. Figure 11 illustrates the variety of motions that can be potentially facilitated by the hinge and elbow (switch) regions of the molecule. Several studies have focused on rotational flexibility. Because of the pseudo twofold axis of symmetry of Fab arms, visual asymmetry was attained by reaction of monoclonal IgG with an asymmetric antigen [49–51] or by reaction of IgG with Fab directed to an allelic marker (allotype) on the lat-

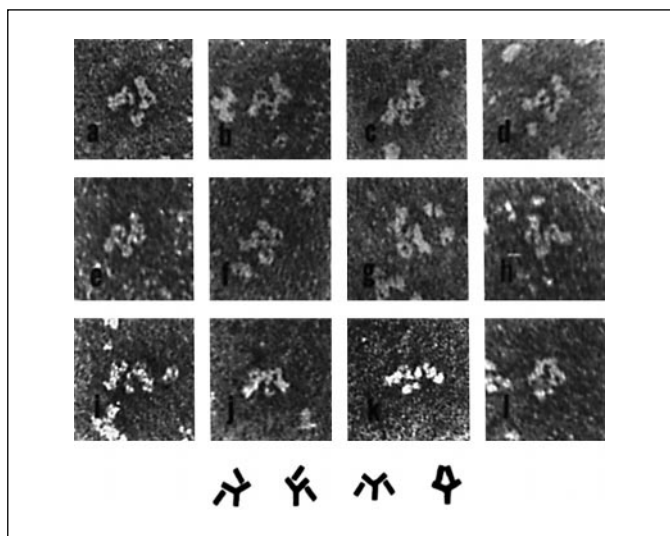


Fig. 12. Negative stain TEMs and interpretive diagrams of rabbit allotype-bearing IgG in complex with Fab anti-allotype mAb showing lateral orientation of the epitopes and rotational flexibility of the Fab arms. From Roux [53]. Magnification $\times 375,000$. With permission from Wiley-VCH Verlag.

eral surface of the Fab arms (fig. 12) [52, 53]. For uncrosslinked complexes, i.e. those in which the complex is composed of a single IgG molecule in which each Fab arm is binding to an isolated indicator molecule, nearly unrestrained rotational flexibility was indicated for rabbit IgG and mouse IgG1 [49, 53]. Because these are the more flexible of the Ig types, it remains to be seen how the various other classes and subclasses behave in similar assays. Irrespective of the relative time each molecule spends in each orientation, certainly these molecules can adopt various rotational orientations to effect antigen crosslinking. Analysis of murine IgG1 and IgG2a engaged in crosslinking of haemagglutinin into chains and rings similarly showed that the Fab arms could rotate at least 180° [50, 51]. The assumption in these assays is that, as the complexes adhere to the carbon membrane support, they rotate to maximize their contact with the membrane. Analysis of crosslinked immune complexes by cryo EM where substrate interactions are minimal will be necessary to determine the degree to which the Fab arms are free to engage in unrestrained full rotational motion in solution.

EM support of flexibility at the VH/VL-CH1/CL elbow region has also been reported [49, 51]. Comparisons were made between uncrosslinked (unconstrained) and crosslinked (cyclic and thus constrained) complexes. Different angles were formed between the Fab and antigen surface in each case, leading to the interpretation that, in order to form

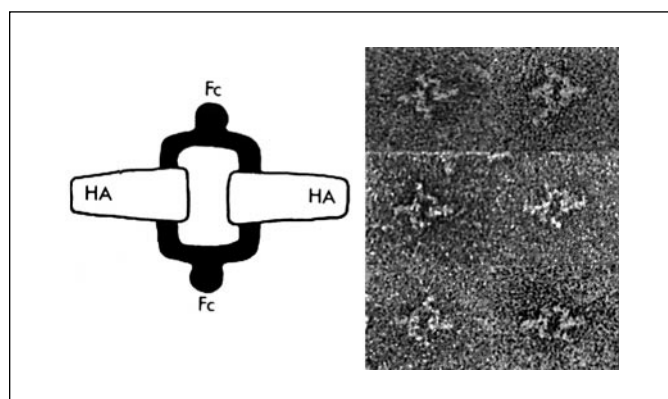


Fig. 13. Negative stain TEM and interpretive diagram of mAb in complex with haemagglutinin showing bent Fab arms. Modified from Wrigley et al. [49]. Magnification $\times 250,000$. With permission from Academic Press.

a cyclic complex, the Fab arms must bend into a strained angle to engage in binding. The individual V and C domains are assumed to be rigid bodies, and the upper hinges of the two Fab arms are tethered by the N-terminal interchain H-H disulfide bond, leaving only bending at the elbow region to accommodate the geometric constraints of cyclic crosslinking. Indeed, some of the electron micrographs appear to show just such bending in the elbow region [49] (fig. 13). These observations are supported by crystallographic data in which Fab can assume different elbow-region angles in different crystal forms [54, 55]. Also, by cryo EM, a $16\text{--}18^\circ$ angular difference was observed between unrestrained Fab and Fc-constrained divalently bound Fab arms of an intact monoclonal antiviral capsid Ab bound to the surface of a virus [18]; i.e. the elbow region must flex to permit divalent binding of IgG to the viral surface (fig. 3). A similar cryo EM analysis of a different mAb-virus combination shows the cognate Fab to be projecting essentially radial to the virus [19]. The significance of this orientation is that, despite potential flexibility of both the hinge and elbow regions, the mAb binds monovalently, being unable to form parallel Fab attachments to neighboring epitopes with both arms on the same surface. The Ab could crosslink two different virus particles but not bind divalently to one. The amount of dynamic flexibility afforded to the typical Fab by elbow region flexibility remains an open question. Certainly, it does not approach that of the hinge region because virtually all X-ray and EM analyses of the crystalline or paracrystalline Ig of Fab binding to antigen show all four domains of Fab at high resolution (implying structural rigidity) but, because of intramolecular motion, rarely show Fc.

Mapping Determinant on Ig Molecules

In the early 1980s, we were interested in a peculiarity of rabbit immunogenetics known as latent allotypes. The allotypes that interested us are genetic markers expressed on the framework region of the VH domain and are inherited codominantly. Remarkably, injection of rabbits with Abs directed against an allotypic marker that was *not* inherited by the rabbit lead to the production of what appeared serologically to be IgG expressing targeted allotypic marker, i.e. a latent allotype had been induced. At about the same time, Id anti-Id networks were being extensively studied by many labs. Because anti-Id Abs are induced in exactly the same manner as latent allotypes, i.e. by injection of specific Ab, such reagents would be expected to behave identically in serological assays. The imperative for our studies was to be able to distinguish between these alternatives. We reasoned that the Ids, being CDR-dependent, should be at the distal ends of the Fab arms, whereas the framework region encoded allotypic markers should be laterally oriented. We first tested the premise by reacting rabbit IgG with bona fide anti-allotype Ab and anti-Id Ab. Both whole IgG Ab and Fab Ab probes were tested, the latter making it easier to distinguish probe from target [52]. As expected, the anti-Id bound to the tips of the Fab arms, and the anti-allotype Ab to the sides (fig. 12). When we similarly assayed rabbits immunized with purified anti-allotype Ab, the resulting IgG was shown to be primarily induced latent allotype-bearing IgG [56]. Remarkably, rabbit VH allotype-like epitopes could also be induced and visualized in mice and guinea pigs by anti-allotype Ab immunization, suggesting that these are evolutionarily conserved epitopes [57, 58]. Such a heretical interpretation would not have been conceived if it had not been for direct EM verification. Using the same approach, we subsequently showed that some rabbit VH allotypes were quite complex and composed of multiple sites on different parts of the VH domain [53] as had been predicted by amino acid sequence analysis [59].

The demonstration that one could visualize the interaction of multiple probes with a single target Ig molecule laid the foundation for more extensive epitope mapping of multiple Ids. In our most comprehensive study, we mapped four Ids and an isotypic determinant [60] on the Fab of a well-characterized anticarbohydrate mAb [61]. Fab probes of each mAb probe were scored, in turn, for angle of interaction, proximal-distal site of interaction, and the rotational orientation of both the target Fab arm and the Fab probe. The position of each Id relative to the other was determined by reaction of the target Id-bearing IgG with pairs of probes, one in the form of Fab and the other as intact IgG

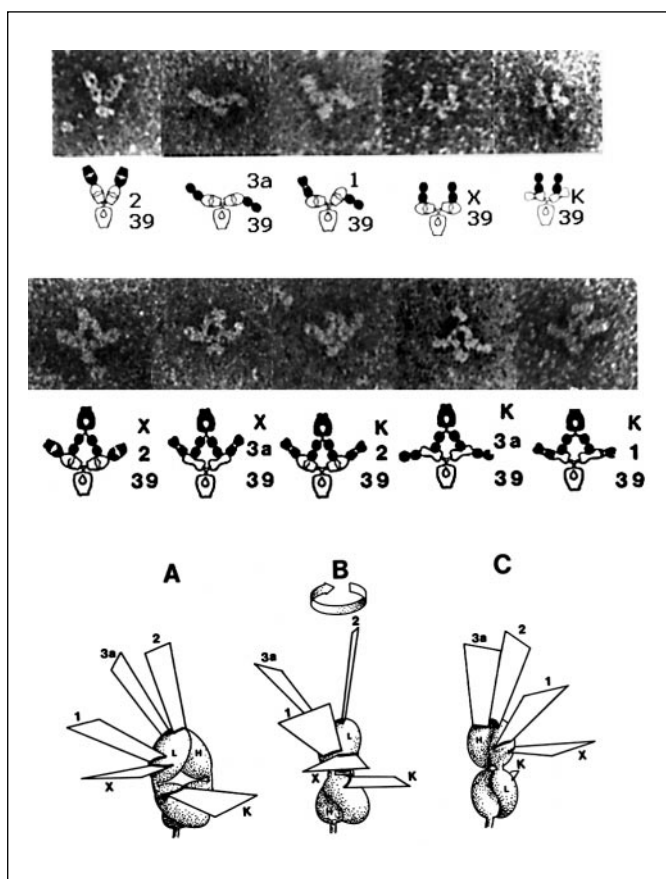


Fig. 14. Negative stain TEMs and 3-D model of Id-bearing mAb IgG probed with mAb anti-Id Fabs (top row) and Fab plus whole IgG molecules (second row). Open figures in diagrams represent Id-bearing IgG. Solid figures represent anti-Id Fab or IgG designated, 1, 2, 3a, and X. Anti-kappa isotype mAb designated K. The bottom row shows a model of Id 39 (stippled figure) depicting the approximate locations and 3-D arrangement of the epitopes recognized by the anti-Id and anti-isotype probes. Each depicted plane passes through the broadest aspect of the Fab probe. From Roux et al. [60]. Magnification $\times 310,000$. With permission from National Academy of Sciences, USA.

(fig. 14). Even though negative stain EM is essentially a 2-D format, by analyzing the geometry of each combination, we were able to generate a 3-D map of the Ids and the anti-kappa chain isotype (fig. 14). One of the more interesting bits of information from this study was that, in contrast to the prevailing view, some idiotopes project laterally from the Fab and can be some distance from the traditional location of the CDR. Also, some of these distant idiotopes are inhibitable by antigen, suggesting the possibility of antigen-induced structural changes in Fab.

Role of Geometry in the Formation of Immune Complexes

Most of the effector functions of Abs involve the crosslinking of receptors and/or ligands. Murphy et al. [62] showed that three different monoclonal anti-BSA Abs generated different types of immune complex arrays under otherwise identical conditions as assessed by EM and light scattering. Differences in the geometry of the elements in such complexes are likely to be an important determining factor in Ab effectiveness. The topological mapping of the reactive sites on the surface of an IgG molecule in our Id-anti-id system presented a unique opportunity to investigate the role that the spatial orientation of the reactive site (in this case idiotopes and the CL isotope) plays in the formation of small immune complexes. Thermodynamic considerations dictate that for any set of divalent reactant (e.g. IgG Id and anti-Id) the smallest complex in which all of the reactive sites are satisfied will be favored. For sets of mutually reactive IgGs, this would be a ring dimer. The degree to which such a complex is inhibited by steric constraints will determine the degree to which the next larger ring form (ring tetramer) will be favored. The interrelationship between immune complexes of various compositions is shown in (fig. 1B). In a temporal sense, the flow of complex formation is from left to right as diagrammed until an equilibrium is reached. By mixing equimolar amounts of reactants of our Ig molecules sampling over time, and scoring the geometric forms of the complexes observed, we were able not only to compare the effect of Abs reacting with different sites on the target IgG but to document the presence of transitional forms of immune complexes [63]. The results show that there is an initial peak of single arm (monovalent) interactions that progress to a predominance of ring forms. Second, the data provide direct evidence confirming the prediction that distally located reactive sites tend not to result in ring dimer formation probably because it is physically difficult for the arms of the reactants to form the parallel orientation necessary for docking of both sets of arms. Conversely, the more lateral epitopes favor ring dimer formation. Several other EM-based studies have also focused attention on the role of epitope location in the formation of immune complexes [29, 62, 64].

Role of Hinge Flexibility in the Formation of Immune Complexes

As stated above, the size and type of immune complexes depend not only on the position of the reactive sites, but also on the flexibility of the molecules involved. For Igs, the hinge region provides most of the flexibility. A number of physicochemical methods have been used in an attempt to quantify Ig flexibility, but most suffer from giving a val-

ue derived from average motion [48]. Though they are useful for comparative purposes, it is difficult to extract meaningful information with regard to specific modes of flexibility. To address this issue Phillips, Schumaker, and colleagues [29, 64] undertook a comparative investigation of the ability of mouse and human IgG subclasses to form specific types of immune complexes as assessed by EM. In their system genetically engineered sets of Ig molecules possessing identical hapten-binding CDRs were reacted with bivalent hapten. For the murine IgG1, IgG2a, and IgG2b subclasses, most of the molecules were observed to form ring dimers. The ring dimers were of two types, an open hinge dimer where the Fab arms of the mAbs were splayed apart and a closed dimer where the Fab arms were near parallel. Because of steric considerations, the latter configuration could only form if the hinge was of sufficient length and flexibility. They found that only the more flexible murine IgG2b subclass was permissive enough for generation of a significant percentage of closed hinge complexes. A similar pattern was observed when comparing a CDR-matched set of human IgG molecules where the rank order of closed (sterically strained) hinges was IgG3 > IgG1 > IgG4 > IgG2 [29]. These data are consistent with the rank order of flexibility obtained by fluorescence depolarization and are correlated with the length of the hinge [65], leading to the conclusion that the length of the upper hinge has the greatest influence on Fab-Fab flexibility [65, 66].

We have also analyzed a matched set of recombinant human IgG molecules with matched CDRs, but rather than induce crosslinking by bivalent hapten, we reacted each subclass with each of two IgG anti-Id mAbs. By reacting the IgGs with Fab probes, we were able to distinguish Fab from Fc arms in the target molecule and take measurements of the hinge-wag component of flexibility, figure 11A, angle b. With these data we were able to determine the average Fab angle for each IgG subclass (IgG3, 136°; IgG4, 128°; IgG2, 127°; IgG1, 117°) (table 1). A comparison of the standard deviation about the average angle was used as a quantitative measure of relative hinge-wag flexibility (IgG3 > IgG1 > IgG4 > IgG2). When equimolar amounts of anti-Id and mAb Id-bearing IgG were mixed, the kinetics of the formation of the various geometric forms of immune complex could be monitored in a functional assay [63]. For these reactions, ring dimers were again the energetically most favored complexes unless inhibited by restrictions on hinge flexibility, whereupon ring tetramers or larger rings were formed (fig. 1). Of the two anti-Ids tested, one gave a rank order for dimer formation (i.e. more flexibility) of IgG3 > IgG1 > IgG4 > IgG2, and the other anti-Id probe,

IgG3 > IgG1 > IgG2 ≈ IgG4. The difference is presumed to reflect differences in topological locations of the two idiotopes and perhaps relative affinities.

To dissect further the role of the upper, middle, and lower hinge regions, we examined genetically engineered versions of human IgG3 in which the native hinge had been modified [67]. Native IgG3 has a long (10 aa) upper hinge, an exceptionally long (50 aa) middle hinge and an average (8 aa) lower hinge). The results of both unrestrained Fab-Fab EM flexibility assay and the immune complex ring dimerization assay confirm that the upper hinge length (and not the sequence) rather than the middle or lower hinge length is correlative with flexibility.

Human IgA2, which has virtually no upper hinge, is consistent with the IgG pattern in that it displays low hinge-fold flexibility. As a probable result, this form of IgA is essentially unable to form ring dimers and even ring tetramers may be somewhat inhibited [67] (table 1).

Perhaps the most surprising results from our EM study related to the hinge fold flexibility and Id-anti-Id complex formation behavior of hingeless Ig molecules. One of the first Ig molecules crystallized was protein Dob, a hingeless IgG in which the light chains, but not the heavy chains, are disulfide bonded to each other [2]. This molecule formed a compact 'T'-shaped structure and has been assumed to be quite inflexible [2, 68, 69]. However, when we analyzed an Id-bearing construct having the same structural features as Dob, it displayed average hinge-fold flexibility and, remarkably, an ability to form ring dimers second only to that of IgG3 (table 1).

IgM and IgE are naturally hingeless having instead an additional C domain in its place. Various evidence has shown these molecules to be rather inflexible presumably for this reason. Indeed IgE was found by our analysis to be about as able to wag its Fab arms as IgG2. Monomeric IgM on the other hand was shown to be able to wag its arms more than any of other Ig molecule, even IgG3. IgM was far more able to form ring dimers than IgG3, at least partly because the average Fab-Fab angle of IgM is closer to that necessary for ring dimer formation (table 1).

Few of these observations on hingeless molecules are predicted by the extensive physicochemical analyses, yet they can be reconciled on the basis of the different parameters being measured. For example, analysis of a model of the Dob-like protein shows that its arms can potentially be rotated and unfolded to produce an open molecule with no obvious steric impediments to Fab arm flexibility [67]. With regard to the naturally hingeless IgM and IgE, their reputation as being rather inflexible comes primarily from the rate of Fab arm spatial movement. EM, as applied in

Table 1. Comparison of Ig flexibility^a

Ig type	IgG1	IgG2	IgG3	IgG4	17-15-15	17-15	15-15-15	15	HM4	HM5	IgA2	IgM	IgE
Upper hinge length (aa)	10 ^b	3	12	7 (5) ^c	12	12	4	4	3	0	1 ^c	NA	NA
% ringe dimer	18	4	47	5	56	41	3	7	2.5	26	0.6	75	26
Mean Fab-Fab angle	117°	127°	136°	128°	132°	133°	123°	119°	125°	106°	111°	106°	141°
Hinge fold flex. funct ^d , Fab-Fab	±43°	±32°	±53°	±39°	±48°	±51°	±42°	±40°	±33°	±39°	±34°	±56°	±34°

^a From Roux and co-worker [30, 67].

^b The upper hinge of IgG1 is disulfide-bonded to the light chain at the forth position leaving five aa between the cysteine and the middle bing.

^c The two C-terminal aa of IgG4 are proline as is the single upper hinge aa of IgA2.

^d The standard deviation of the mean values is taken as a measure of the degree of flexibility (hinge fold flexibility function) about the mean angle.

these studies, not only gives an average angle but samples the entire range of values. Therefore, IgM and IgE with their extra domain may move more slowly than the IgGs but may nevertheless move over as great a distance and more readily make the closed angles necessary for ring dimer formation.

EM as an Interpretive Device

Reduced Hinge Disulfide Bonds

EM is unexcelled as a quick and direct method for unambiguously defining the size, shape, degree of merism, or degree and mode of crosslinking in a preparation. For example, Seegan et al. [3], compared cyclic immune complexes of native with mildly reduced and alkylated rabbit IgG in complex with bifunctional hapten. By direct examination, they determined that the loss of hinge disulfide bonds caused the Fc to splay open in the CH2 region but remain associated through the C-terminal half of CH3, providing a structural basis for the observation that the integrity of the CH2 region was is critical for complement C1q and FcR binding (fig. 15).

Increase Avidity

Lansdorp et al. [6] demonstrated that IgG avidity could be increased if the molecules were rendered functionally tetrameric. They did so by reacting the mAb with anti-Fc mAb IgG to form cyclic tetrameric complexes (fig. 2). Similarly, EM was used to determine the structural basis behind a natural high avidity mutant of a potentially useful anti-streptococcus IgG mAb [70]. The high avidity fraction was about twice as large as native IgG as assessed by HPLC

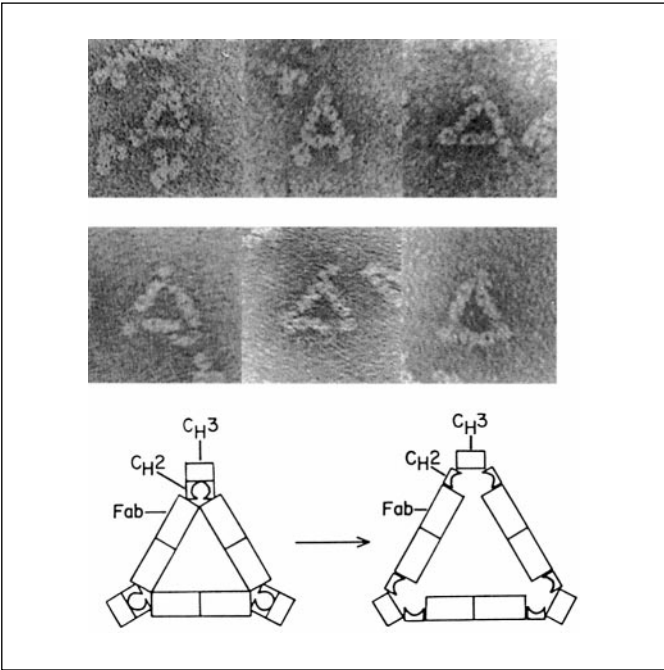


Fig. 15. Negative stain TEMs and interpretive diagrams of trimeric immune complexes composed of native (upper row) or reduced and alkylated rabbit IgG (lower row) crosslinked with bivalent hapten. Note the splayed open Fc in the latter. From Seegan et al. [3]. Magnification $\times 320,000$. With permission from National Academy of Sciences, USA.

analysis as though the molecule had dimerized. The mutant also contained an unusually large light chain composed of a constant region and two tandem VL domains. EM analysis revealed that the basis for the avidity increase was intermolecular crosslinking through pairing of the extra VL do-

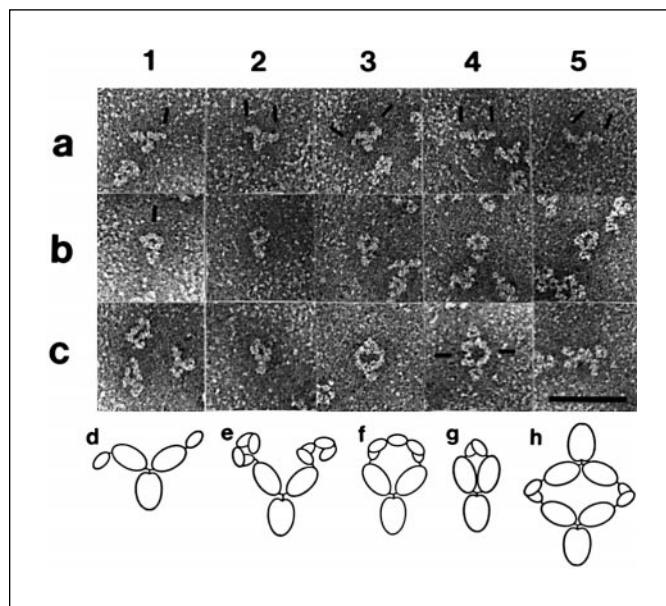


Fig. 16. Negative stain TEMs and diagrams of IgG in which an extra VL domain protrudes from each Fab arm. The extra VL domains can be seen freely protruding from the Fab arms (a1-5 and d), crosslinked with adjacent Fab arms (b1-5, c1, c2, f), or crosslinked with another molecule (c3-5, g). Bar = 50 nm. From Roux et al. [70]. With permission from Elsevier Science.

mains which formed a dimeric ring (fig. 16). Surprisingly, the extra VL domains protruding from the CDR do not interfere with antigen binding. We showed that the Fab arms of some monomeric forms of this molecule auto crosslinked by paired protruding VH domains and that others had unpaired VL domains protruding from the CDR (attesting to the degree of detail that can be visualized by this technique).

Engineered Igs

One of the consequences of being able to genetically engineer mAb is the ability to jettison all unnecessary parts of the molecule. Smaller molecules have better penetrating abilities in tissues and are therefore potentially better therapeutic or diagnostic reagents. The resultant scFv molecules, however, suffer from lower avidity than native Ig because of monovalency. As a result, dimeric and trimeric forms of scFv (diabodies and triabodies) have been created. Lawrence et al. [71] have recently analyzed the structure and geometry of several of these particles by reacting them with Fab anti-Id before EM analysis. They observed that each of the subunits was capable of being tagged by the anti-Id Fab and presumably of antigen binding. Interestingly,

the trimers did not appear to be planar but instead formed tripod-like complexes. Measurements of the arm lengths of both forms suggest some degree of flexibility in the scFv subunits, an advantage when they bind to surface antigen of indeterminate orientation, for example.

Natural Id-Anti-Id Complexes

EM was also instrumental in characterizing the nature of a 'contaminant' that had been a significant concern to the commercial producers of intravenous gammaglobulin (IVIG). IgG pooled from numerous donors contains what appeared to be dimer-sized aggregates of IgG. The proportion of this dimer increased with increased pool size. Among the explanations for the dimer were both aggregates of denatured molecules and immune complexes composed of anti-allotype, rheumatoid factor, cryoglobulin, and anti-Id Ab. Through EM analysis of the dimer fraction, we and others demonstrated that the dimers were Id anti-Id complexes (i.e. CDR-conjugated ring dimers were observed) [72, 73]. We theorized that, the larger the pool size, the greater the chance that any given molecule would find a molecule that by chance had a complementary CDR. This hypothesis suggests that there is a functionally finite number of Ids and anti-Ids in a given species but that any one individual possesses only a small fraction of this total [73]. Pooled bovine and murine IgG similarly dimerized. Although many of the idiotypes were visualized near the tips of the molecules, a significant number were somewhat tangentially oriented and not unlike those seen in the 3-D Id map previously discussed [60]. Another interesting finding was that the most flexible subclass (IgG3) was overrepresented in the dimer fraction and one of the least flexible (IgG2) was underrepresented. In fact, IgG2 purified from these preparations could not self-dimerize. However, when IgG2 was subjected to mild reduction and alkylation that disrupted the inter H-H disulfide bonds and thus increased flexibility, IgG2 could contribute proportionally to the ring dimer fraction and self-dimerize. This observation agreed with our finding that IgG2 was least able to form ring dimers as discussed above [30].

Triggering Cells by Receptor Crosslinking

Through the use of soluble forms of monomeric IgM, anti-isotype reagents directed to the Fc portion have been shown to be good B-cell-triggering agents and have been shown by EM to crosslink IgM molecules readily (fig. 17). In contrast, anti-isotype reagents directed against the CH1 of IgM Fab do not signal B cells and are seen to crosslink the Fab arms of the same molecule preferentially to each other but not to bridge to other molecules [74].

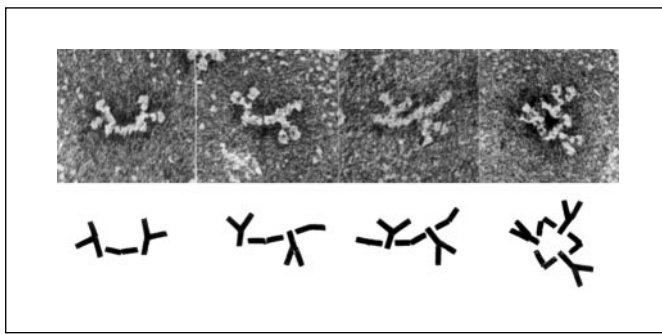


Fig. 17. Negative stain TEMs and interpretive diagrams of monomeric murine IgM in complex with F(ab')₂ mAb anti-C_μ4 showing intermolecular crosslinking. From Rudich et al. [74]. Magnification $\times 300,000$. With permission from The Rockefeller University.

Immune Complexes on Lipid Bilayers

Most EM studies of Igs have been directed either at unreacted soluble Ig molecules or at small soluble immune complexes. An exception is the complexes composed of pentameric IgM and bacterial flagella discussed above [33]. Wharton et al. [75] have demonstrated the feasibility of direct observations on cell surfaces or lipid bilayers by analyzing small antigen (influenza hemagglutinin, HA)-studied liposomes in complex with mAb. Though their primary interest was in epitope mapping of HA, crosslinking mAb is clearly observed to be binding to the antigen on the surface of the liposomes in their EMs (fig. 18).

Fc-Fc Interactions

EM analysis has also been employed in the analysis of Fc-Fc interactions. Moller and Christiansen [76] reacted substrate-bound KLH-anti-KLH immune complexes with soluble ferritin-antiferritin complexes and observed coaggregation. Because F(ab')₂ antiferritin complexes did not coaggregate with intact KLH-anti-KLH aggregates, it was assumed that the aggregating force was via Fc-Fc interaction. In these negative stain preparations, individual Ig molecules were not discernable because of excessive crowding, but the coaggregated KLH (large cylinders) and ferritin (electron-dense core surrounded by a protein shell) were obvious.

Complement Fixation

One of the most important functions of humoral immunity is the initiation of the complement cascade by Ig. The early work of Hyslop et al. [77], using a model system in which cyclic immune complexes are induced by bivalent hapten, showed that fractions rich in ring dimers are poor at comple-

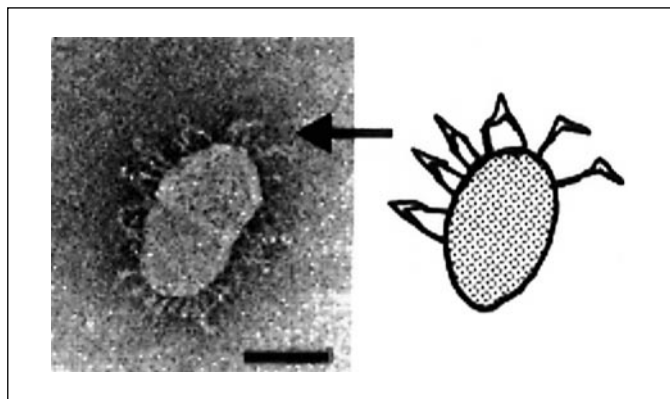


Fig. 18. Negative stain TEMs and interpretive diagrams of anti-haemagglutinin mAb bound to haemagglutinin on the surface of liposomes. Bar = 50 nm. From Wharton et al. [75]. With permission from Oxford University Press.

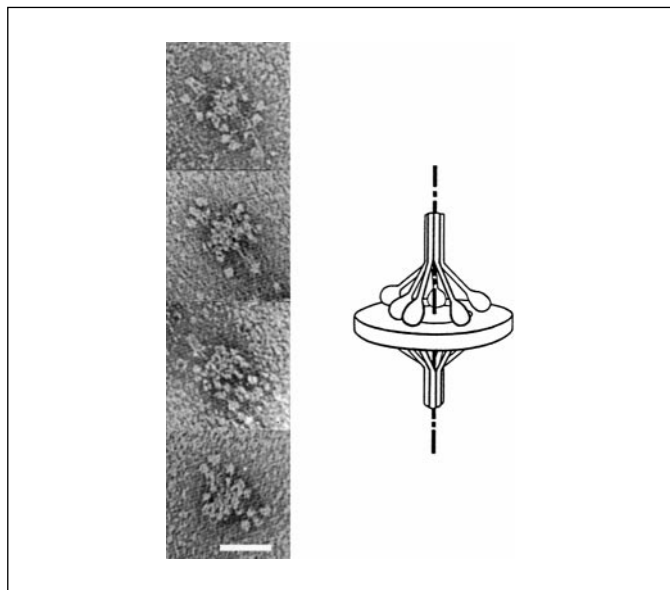


Fig. 19. Negative stain TEMs and schematic diagram of C1q binding above and below the central Fc disk of soluble IgM (uncomplexed with antigen) in low ionic strength buffer. From Poon et al. [40]. Bar = 40 nm. With permission from Journal of Biological Chemistry.

ment fixation in contrast to complement-fixing fractions which contain larger rings forms. For IgM, a single Ag-engaged molecule reacting with one C1q molecule is sufficient for activation. The serologic and biochemical details of this interaction have been extensively studied, but attempts to capture C1q in the act of binding to antigen-bound IgM by EM have been unproductive. Negative stain EM has, however, provided some important clues. Poon et al. [40] have tak-

an advantage of the fact that soluble IgM and C1q will form moderately stable complexes in buffers of low ionic strength. By stabilizing these complexes through chemical crosslinking, they were able to visualize the globular heads of C1q binding to and protruding from one and in some cases both planar surfaces of the IgM molecule (fig. 19). The physiological significance of these observations is open to question because both a low salt concentration and a crosslinking agent were used and the IgM molecule was not antigen bound. Nevertheless, if, as suggested by the authors, this type of interaction represents an enhanced and stabilized version of the physiological process, then it calls into question the widely held assumption that steric changes in the molecule are necessary for exposure of the C1q binding site [78].

Some Unanswered Questions

Perhaps future cryo EM coupled with computer-assisted image analyses of C1q binding to antigen-bound IgM will shed light on sites of segmental flexure in IgM and, for that matter, IgG as the molecules bind to antigen, and we may be

able to visualize these molecules in the act of fixing C1q under physiological conditions. Cryo EM could also be used to capture Ig molecules suspended in solution free of any potentially distorting substrate interactions to better define their native conformational state. We could then determine whether IgM is in the starfish or snowflake configuration, whether IgE has a fixed or flexible hooked Fc [79], the solution conformation of the other Igs, and the degree to which Ig arms are rotationally flexible. A structural basis for the differences between virus-neutralizing and nonneutralizing Ab might also be established. Negative stain EM could be applied to the analysis of Ig bound to recombinant soluble forms of Fc receptor to better define their modes of interaction. The locations of the J chain and secretory component have yet to be structurally mapped on IgA and IgM, and we know little of the subunit organization of the higher polymeric forms of IgA. One of the greatest challenges will be the development of methods for imaging Igs, receptors, and immune complexes on the surfaces of cells and lipid vesicles at the level of detail currently obtained with molecules attached to rigid planar substrates.

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