Structural and Functional Analysis of J Chain-Deficient IgM

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Previous studies have discerned two forms of polymeric mouse IgM: moderately cytolytic (complement-activating) pentamer, which contains J chain, and highly cytolytic hexamer, which lacks J chain. To investigate the relationships among polymeric structure, J chain content, and cytolytic activity, we produced IgM in J chain-deficient and J chain-proficient mouse hybridoma cell lines. Both hexamer and pentamer were produced in the absence as well as the presence of J chain. Hexameric IgM activated (guinea pig) complement approximately 100-fold more efficiently than did J chain-deficient pentamer, which, in turn, was more active than J chain-containing pentamer. These results are consistent with the hypothesis that J chain-containing pentamer cannot activate complement. We also analyzed the structure of IgM-S337, in which the mu-chain bears the C337S substitution. Like normal IgM, IgM-S337 was formed as a hexamer and as both J chain deficient- and J chain-containing pentamers. Unlike normal IgM, IgM-S337 dissociated in SDS into various subunits. For IgM-S337 pentamer, the predominant subunits migrated as mu2x2 and mu4x4, and the subunit distribution was unaltered by J chain. However, J chain was found only in the mu2x2 species, suggesting that some arrangement of inter-mu bonds directs incorporation of J chain. IgM-S337 hexamer also dissociated to mu2x2 and mu4x4, but also yielded several species migrating much more slowly in SDS-PAGE than wild-type mu12x2. To account for these forms, we propose that each mu-chain can interact with three other mu-chains and that some hexameric molecules contain two catenated mu6x6 circles.


J chain is a 15-kDa glycoprotein covalently associated by disulfide bonds with polymeric forms of IgM and IgA (1, 2). The Ig mu heavy chain has three cysteine residues available for intermolecular disulfide bonds. C337, C414, and C575 lie in the Cµ2, Cµ3, and Cµ4/Cµtail domains, respectively, and are thought to join mu-chains via homo-pairs (C337-C337, C414-C414, and C575-C575) (3, 4). Each pentameric IgM molecule contains approximately one J chain attached to the penultimate amino acid of the mu-chain, C575 (2, 5–8). J chain is disulfide bonded via its C14 and C68 to no more than two mu-chains (6, 9). Most or all incorporation of J chain into IgM occurs relatively late in the intracellular assembly process, at a stage when IgM polymers have been formed but are not yet fully linked by disulfide bonds (5, 10).

Two functions have been described for Ig-associated J chain. J chain is necessary for binding of IgM and IgA to the poly-Ig receptor, which functions in epithelial transcytosis of Igs (11–13). Also, J chain modifies polymeric assembly of IgM, in that relatively more pentamer and less hexamer are produced in the presence of J chain. The fraction of IgM that was seen as pentamer in the absence of J chain has varied among different studies, ranging from none up to 50% (14–16). Pentameric IgM differs from hexameric IgM in several ways. First, J chain is found in pentameric, but not hexameric, IgM. Second, pentameric IgM is less cytolytic than hexameric IgM (17, 18). Third, analysis of mutant IgM has suggested that more inter-mu-chain C414-C414 bonds are formed in hexameric IgM than in pentameric IgM (19, 20). These correlations could have multiple explanations. For example, incorporation of J chain into pentamer might affect the disulphide bonding of IgM and in this indirect way depress its cytolytic activity. Alternatively, the hexameric C1q complement component might interact better with hexameric than with pentameric IgM. As well, IgM-bound J chain might sterically hinder the binding of C1q and thereby decrease the cytolytic activity of pentameric IgM.

To extend our understanding of the relationships among disulfide bonding, cytolytic activity, and incorporation of J chain, we have examined the structure and function of wild-type IgM produced in cells expressing or not expressing J chain. We have similarly examined IgM in which one or more mu-chain cysteine residues have been replaced by serine. Our results indicate that pentameric IgM is produced in the absence of J chain and that this J- IgM, like J+ IgM, is much less cytolytic than hexameric IgM. We have not detected any difference in the inter-mu-chain disulfide bonding when comparing J+ and J− pentameric IgM, but incorporation of J chain appears to be restricted in some way by the C414-C414 bond. Our analysis also suggests that each mu-chain in hexameric IgM might be bonded to three other mu-chains rather than to only two mu-chains as is conventionally depicted.

Materials and Methods

Cell lines

Cell lines are based on the Sp6 hybridoma cell line, which secretes IgM specific for DNP and TNP (21). All J+ cell lines producing mutant IgM, except T(µS414C), were obtained by transfecting µ gene constructs into the X10 cell line, which expresses the κ-chain but not the µ-chain (22). The transfectants, T(µS414C), which produces IgM-S414 in the absence of J chain, has been previously described (20). We have used two Sp6-derived mutant cell lines, igm482 and igm43, for size markers and other controls. The cell line igm482 secretes IgM monomer lacking the Cµ4 (domain), and igm43 secretes polymer lacking the Cµ1 domain from which the κ light chain dissociates in SDS (21). IgM10 is derived from the myeloma MOPC-315 and produces IgM bearing λ-chain (24).

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3 Abbreviations used in this paper: TNP, 2,4,6-trinitrophenyl; AU-PAGE, alkaline urea-polyacrylamide gel electrophoresis; SDG, sucrose density gradient; wt, wild type.
Protein analysis

Biosynthetic labeling of cells was performed as described previously (4), except that [35S]methionine (SJ.204, Amersham, Arlington Heights, IL) was used at a concentration of 50 to 100 μCi/ml for labeling.

[35S]Labeled or unlabeled IgM was prepared from supernatants either by affinity purification using DNP-Sepharose (17) or by immunoprecipitation using anti-IgM Abs and protein G-agarose (4).

IgM was fractionated by sucrose density gradient centrifugation at 23,000 rpm for 16.75 h in an SW41 rotor as previously described (17).

Nonreduced IgM was fractionated by SDS-PAGE using a modification of Laemmli’s method (4, 25). To obtain optimal resolution of various IgM species, the concentrations of acrylamide and agarose were varied, as indicated in the figure legends. The sample buffer for SDS-PAGE sometimes contained 25 mM iodoacetic acid to prevent spontaneous reduction, but its inclusion was found not to affect the result. Prestained molecular mass markers (Life Technologies, Grand Island, NY; 14–228 kDa) were included in most experiments. Separated proteins were visualized either by autoradiography and PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA) or using a Western blot procedure (see below).

Radiolabeled J chain was assayed by AU-PAGE (26). To assess individual IgM species, bands were excised from the SDS-PAGE gel, extracted, and then analyzed by AU-PAGE (27). After extraction, IgM was reduced by 50 mM DTT in 0.1 M Tris (pH 8.0), alkylated with an equal volume of 0.5 M iodoacetic acid in 1.5 M Tris-HCl (pH 8.8), and precipitated with 10 vol of acetone at −20°C. Acetone-precipitated samples were dissolved in AU-PAGE sample buffer (0.2 M Tris (pH 8.0), 8.0 M urea, and bromphenol blue) and loaded onto the gel.

J chain was also detected by Western blot analysis (20). Thus, after SDS-PAGE, the nitrocellulose filter was incubated with rabbit anti-human J chain Abs (1/100 dilution; Biogenex, San Ramon, CA), followed by alkaline phosphatase-conjugated mouse anti-rabbit IgG (1/2000 dilution; Jackson ImmunoResearch, West Grove, PA). Buffer for blocking and Ab incubation contained PBS, 0.2% I-Block (Tropix, Bedford, MA), and 0.1% Tween-20. The wash buffer was 0.1% Tween-20 in PBS. The substrate buffer for enhanced chemiluminescence detection was 0.1 M diethanolamine, 1 mL-MgCl2 (pH 10.0), 1% CSPD (Tropix), and 5% N-Block (Tropix).

Complement-mediated hemolysis

Complement-mediated hemolysis of IgM was tested in V-shaped 96-well plates. Samples were diluted in balanced salt solution containing 1% calf serum. Serial dilutions of IgM samples (either from SDG fractions or standard supernatant, from the J chain-deficient hybridoma Sp6 (22, 28)) were incubated with SRBC-TNP at a final concentration of 0.12% for 1 h at 37°C. To remove sucrose, this mixture was centrifuged, and the pelleted IgM-SRBC were resuspended in buffer and re centrifuged. The washed IgM-SRBC were then resuspended at 0.12% in the presence of guinea pig complement at a dilution of 1/250 and incubated at 37°C. After 1 h incubation, unlysed SRBC-TNP were pelleted by centrifugation. Supernatants were transferred to flat-welled microtiter plates, and lysis was measured as absorbance at 405 nm. In some experiments, hemolysis was also tested by the method of Davis et al. (17).

Northern blot analysis

Isolation of cytoplasmic RNA and Northern blot analysis were performed as described previously. Transferred, immobilized RNA was hybridized to end-labeled oligonucleotides specific for the Cα4 exon of the μ gene (4), the V-region of the TNP-specific κ-chain (22), or the J chain (20).

Results

A transfection recipient cell line for production of J chain-deficient IgM

We have previously described an expression system based on the hybridoma Sp6, which produces IgM-k specific for the hapten TNP. Several mutant cell lines lacking the μ gene but retaining the functional κ gene have been derived from Sp6 (22, 28). These μ−, κ+ cell lines serve as recipients for transfected normal and mutant μ genes, which are present as a genomic expression unit in the pSV2neo vector. The mutant μ genes, resulting transfectants, and corresponding IgM are denoted according to the substituted amino acid, e.g., μ-S337, T/μ-S414, and IgM-S414, respectively.

To produce IgM bearing normal and mutant μ-chains in the absence as well as the presence of J chain, we sought to isolate a hybridoma mutant cell line that had ceased to produce J chain but which would nevertheless express the transfected μ genes. In earlier work we fortuitously isolated a transfectoma, T/μ-S414(0), expressing the mutant protein IgM-S414, but not J chain (19, 20). This cell line produces no detectable J chain protein or mRNA. The cause of this J chain deficiency is not known. To obtain a cell line that had lost the μ-S414 pSV2neo vector, the T/μ-S414(0) cell line was subjected to 300 to 400 rad from a 137 Cs source and subsequently irradiated as described previously (4), providing a 35 S-labeled or unlabeled IgM was prepared from supernatants either by affinity purification using DNP-Sepharose (17) or by immunoprecipitation using anti-IgM Abs and protein G-agarose (4).

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Efficient production of IgM pentamer in the absence of J chain

To study the role of J chain in IgM structure and function, we transfected the G10 cell line with plasmids encoding either wild-type μ-chain or μ-S337. From each transfection, two independent transformants were chosen for further study. Their IgM, denoted J− IgM-wt and J− IgM-S337, was compared with IgM produced by previously established (J−) transformants expressing J− IgM-wt or J− IgM-S337 (4). Each transformant produced a level of IgM expression corresponding to 10 to 60% of that of the parental Sp6/HL hybridoma, with overlapping ranges for each pair.

We initially investigated how secreted J− IgM-wt was assembled using sucrose density gradient (SDG) centrifugation in combination with nonreducing SDS-PAGE to assess the state of polymerization, as hexamer is expected to sediment more rapidly than pentamer in SDG centrifugation and to migrate more slowly
than pentamer in SDS-PAGE. For this analysis, J1 IgM-wt and J2 IgM-wt were metabolically labeled with [35S]methionine, and secreted IgM was purified by its affinity to DNP-Sepharose. This IgM was then separated by (nondenaturing) SDG centrifugation into various polymeric and monomeric species. As shown in Figure 2A, J1 IgM-wt had a more prominent monomer peak than J2 IgM-wt. To obtain a more complete separation of the polymeric species, the faster and slower polymeric fractions of the gradient were separately pooled and resedimented (Fig. 2A). Analysis of selected fractions from the secondary gradients indicated that for both J1 IgM-wt and J2 IgM-wt, the faster sedimenting material was greatly enriched for species that have lower mobility in SDS-PAGE, and that, conversely, the slowly sedimenting material had a higher mobility in SDS-PAGE (Fig. 2B). Moreover, the slowly sedimenting J1 IgM polymers have the same mobility in SDS-PAGE as the slowly sedimenting J2 IgM polymers, which have been previously identified as pentamer (17, 18). We conclude from these results that both J1 IgM-wt and J2 IgM-wt include both pentameric and hexameric species. The sequential gradients yielded pentameric material that contained no detectable hexamer band on SDS-PAGE. The hexameric material usually contained detectable pentamer (see below).

In light of previous studies in which hexameric IgM was the predominant species produced in the absence of J chain (14, 15), we were surprised to find that production of hexamer and pentamer did not change much in the absence of J chain. To confirm that secreted J1 IgM-wt protein actually lacked J chain, we compared its J chain content with that of J2 IgM-wt. Individual hexamer, pentamer, and monomer species were extracted from the SDS-PAGE gel as shown in Figure 3A. After reduction, alkylation, and acetone precipitation, the samples were analyzed by AU-PAGE for J chain. To compare the different samples for J chain content, several concentrations of J1 IgM-wt pentamer were loaded onto the gels. As expected from previous reports, J chain was found in J1 IgM-wt pentamer but not in monomer. The preparation of J1 IgM-wt hexamer contained about 2% as much J chain as did the preparation of pentamer, but this is probably due to contaminating pentamer (Fig. 3B). By contrast, we detected no J chain in J1 IgM-wt pentamer or hexamer (<2% of J1 IgM-wt pentamer). This experiment was repeated for IgM-wt and IgM-S337 transformants without prior SDS-PAGE separation of its DNP-purified secreted IgM. In these experiments (not shown), J1 IgM-wt and J2 IgM-S337 proteins also had no detectable J chain (<3% of the content of their J counterparts). These experiments indicated that IgM pentamer was efficiently assembled even in the absence of J chain.

Purification of IgM by binding to DNP-Sepharose and elution with DNP-alanine has the potential to enrich preferentially for some species. Therefore, we have also examined the species present in IgM that was prepared by immunoprecipitation, as this procedure was previously found to better measure the composition of the primary SDG separation, pentamer- and hexamer-enriched fractions were separately pooled as indicated and recentrifuged (open and filled symbols, respectively). Two secondary gradients are shown together for each of the J1 IgM (middle panel) and J2 IgM (bottom panel), where the filled and open symbols represent hexamer and monomer, respectively. B, SDS-PAGE analysis of fractions from secondary SDG. The indicated fractions from pentamer- and hexamer-enriched polymers prepared in A were analyzed following immunoprecipitation. DNP-purified, but unseparated J1 IgM-wt and J2 IgM-wt were included to indicate the multiplicity of species. Samples were separated in the upper phase of a two-phase gel containing 3% acrylamide and 0.4% agarose.

[Diagram and figure legends are not transcribed but refer to the original text]
of secreted IgM (4). 35 S-labeled IgM-wt was prepared from two
μ-wt-J1 and two μ-wt-J2 transformants by immunoprecipitation,
and individual SDS-PAGE bands were quantified by PhosphorIm-
ger analysis. For J2-IgM-wt the following distribution of species
was found (mean ± SD): hexamer, 20 ± 9%; pentamer, 49 ±
10%; and monomer, 31 ± 3%. For J1-IgM-wt the corresponding
values were: hexamer, 16 ± 14%; pentamer, 77 ± 14%; and
monomer, 7 ± 3%. This comparison suggests that J chain in-
creases the production of secreted pentamer at the expense of
monomer. There was approximately threefold more covalent dimer
and trimer in J2-IgM-wt than in J1-IgM-wt; each species consti-
tuted 2% of the total secreted J2-IgM-wt protein.

J chain-deficient IgM pentamer activates complement poorly

Previous reports have shown that pentameric J1-IgM is much less
cytolytic than hexameric J2-IgM, but it has been unclear whether
this difference is due to the presence of J chain in pentamer or to
some other structural difference between the two types of polymer.
To distinguish these possibilities we compared the cytolytic activ-
ity of the pentameric and hexameric species of J1-IgM and J2-IgM
prepared by sequential SDG centrifugations. To gauge whether
cytolytic activity was derived from a single species, we assayed
three fractions from different positions in the pentamer and hex-
amer peaks. As described in Materials and Methods, IgM from the
indicated SDG fractions was serially diluted and adsorbed to TNP-
coupled erythrocytes. The IgM-TNP-erythrocytes were then incu-
bated in the presence of guinea pig serum as a complement source,
and lysis was assessed spectrophotometrically (Fig. 4). As a mea-
sure of cytolytic activity we have calculated the amount of IgM
that lysed 50% of the erythrocytes (Table I). Pentamer from both
J1-IgM-wt and J2-IgM had higher lytic activity than unfractionated IgM-wt,
and this activity increased for the faster sedimenting fractions.
SDS-PAGE analysis of these fractions indicates that the fractions
denoted hexamer contained some pentameric IgM (Fig. 2B). The
level of contaminating pentamer was less in faster migrating frac-
tions, which had greater hemolytic activity. To estimate the activ-
ity of pure hexamer, we assumed that contaminating pentamer did
not significantly contribute to the total lytic activity. Pentamer con-
tamination was calculated from the intensity of the SDS-PAGE

FIGURE 3. Analysis of J chain content. A, Preparation of IgM by SDS-PAGE. 35 S-labeled IgM was purified by immunoprecipitation. The upper gel is
3% acrylamide with 0.4% agarose; the lower gel is 7.5% acrylamide. The indicated IgM species were identified by comparison with the markers (IgM
lacking Cµ1 domain and κ-chain (mutant igm43), 560 kDa; commercial protein marker, 226 kDa). B, Analysis of J chain by AU-PAGE. AU-PAGE was
performed on bands extracted from the SDS-PAGE gel (A). The numbers indicate the amount of radioactivity (counts per minute) loaded in each lane. The
positions of the μ, κ, and J chains are indicated.

FIGURE 4. Hemolytic activities of purified pentamer and hexamer. Pentamer-
and hexamer-enriched fractions from secondary SDG were tested. As indicated, J1 pentamer was either titrated alone or in the pres-
ence of a constant amount of hexamer. Titration of hexamer is indicated by
a filled triangle. The single opposite-pointing triangle corresponds to the
amount (−2 ng/ml) and activity (A405 = −0.2) of hexamer that was mixed
with pentamer.
For simplicity, J chain is not included in these models. Hemolytic activity of polymeric species from J\(^+\) IgM-wt and J\(^-\) IgM-wt

<table>
<thead>
<tr>
<th>Polymer preparation</th>
<th>Fraction Number</th>
<th>Expt.</th>
<th>50% lysis (ng IgM/ml) (or % lysis obtained at 1 μg/ml)</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>J(^+) IgM-wt, pentamer</td>
<td>31</td>
<td>&gt;404 (&lt;15%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>&gt;858 (&lt;15%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>&gt;1000 (&lt;15%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J(^+) IgM-wt, hexamer</td>
<td>36</td>
<td>37.2</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>38</td>
<td>12.9</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>40</td>
<td>7.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J(^+) IgM-wt, pentamer</td>
<td>31</td>
<td>553</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>317</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>35</td>
<td>374</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J(^+) IgM-wt, hexamer</td>
<td>36</td>
<td>18.5</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>38</td>
<td>4.42</td>
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<tr>
<td></td>
<td>40</td>
<td>3.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J(^+) IgM-wt, pentamer</td>
<td>1</td>
<td>&gt;1000 (&lt;15%)</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&gt;1000 (25%)</td>
<td>~1000</td>
<td></td>
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<tr>
<td></td>
<td>3</td>
<td>816</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J(^+) IgM-wt, hexamer</td>
<td>1</td>
<td>6.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9.6</td>
<td>8.1</td>
<td></td>
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<tr>
<td></td>
<td>3</td>
<td>7.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J(^+) IgM-wt, pentamer</td>
<td>1</td>
<td>553</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>55.7</td>
<td>400</td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>589</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J(^+) IgM-wt, hexamer</td>
<td>1</td>
<td>3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.0</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.8</td>
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</table>

\(\text{Fraction Number} \times \text{Expt.} \times \text{50\% lysis (ng IgM/ml) (or % lysis obtained at 1 μg/ml)} \times \text{Mean}\)

In the upper half of the table, the indicated fractions of polymeric IgM prepared in the secondary gradients presented in Figure 2 were assayed for cytolytic activity, and the amount required to lyse 50% of the TNP-SRBC was calculated. The 50% lysis values were determined for three separate preparations of pentameric and hexameric J\(^+\) IgM-wt. When calculating the activity of hexamer in the lower half of the table, the values were adjusted for the amount of contaminating pentamer, as estimated by SDS-PAGE. All hexamer preparations had less than 35% pentamer contamination. In the upper half of the table, which is included here as experiment 1, the values were adjusted for the amount of contaminating pentamer, as estimated by SDS-PAGE. All hexamer preparations had less than 35% pentamer contamination. In cases where 50% hemolysis was not obtained, the number in parenthesis indicates maximal lysis obtained.

Table 1. Hemolytic activity of polymeric species from J\(^+\) IgM-wt and J\(^-\) IgM-wt

A puzzling feature was that the activity of J\(^-\) pentamer compared with that of hexamer (~0.8%) was much lower than the 5% value seen in earlier studies from this and another laboratory (17, 18). We have tested whether this difference reflects changes in the method of assay, with variable results. That is, in repeated testing using the method of Davis et al. (17) we found that J\(^-\) pentamer ranged from 1.4 to 12% of the cytolytic activity of hexamer. We have not identified the source of this variation.

Analysis of disulfide bonding pattern in J chain-deficient IgM-S337 polymers

Inter-\(\mu\)-chain disulfide bonding in polymeric IgM-wt is mediated by three cysteines, C337, C414, and C575, which are believed to form homo-pairs, e.g., C337-C337 and C414-C414 (Fig. 5A). However, the connectivity of the \(\mu\)-chains, i.e., the series and parallel arrangement of disulfide bonds (see Fig. 5 for definitions), is not known. To

FIGURE 5. Models of mutant IgM pentamers. A. Ig domains and cysteines residues available for inter-\(\mu\)-chain bonding. B. Possible arrangement of disulfide bonds and subunits of IgM-S337 pentamer. In the \(\mu\)4ε4 unit (\(\mu\)-chains 5, 6, and 8), the C414-C414 and C575-C575 bonds are in series. In the \(\mu\)8ε2 unit, composed of \(\mu\)-chains 3 and 4, C414-C414 and C575-C575 bonds are in parallel, i.e., the two bonds bridge the same \(\mu\)-chains. C. Model for IgM-Si(337, 414) pentamer. In this model the pentamer is maintained by C575-C575 bonds in series with noncovalent interactions between \(\mu\)-domains. For simplicity, J chain is not included in these models.
were run: DNP-purified IgM-wt (1080-kDa hexamer, 900-kDa pentamer, preparations did not align exactly with corresponding bands from SDG- was included. Due to different preparative techniques, the bands from these cated. For comparison, affinity-purified IgM-S337, not separated by SDG, hexamer or pentamer, respectively. P1 and P2 indicate SDS-PAGE bands associated specifically with either lyzed by SDS-PAGE following immunoprecipitation. H1 through H4 and from secondary SDG. Fractions from the secondary SDG in A

FIGURE 6. Analysis of pentameric and hexameric J Chain-deficient IgM

A. Separation of pentamer- and hexamer-enriched polymers by consecutive SDG. This experiment was performed as described in Figure 3. J IgM-S337 is indicated by circles, and J IgM-S337 is indicated by triangles. Pools of pentamer- and hexamer-enriched polymers, which were used for secondary SDG, are indicated. B. SDS-PAGE analysis of fractions from secondary SDG. Fractions from the secondary SDG in A were analyzed by SDS-PAGE following immunoprecipitation. H1 through H4 and P1 and P2 indicate SDS-PAGE bands associated specifically with either hexamer or pentamer, respectively. μ4κ4 and μ2κ2 units are also indi cated. For comparison, affinity-purified IgM-S337, not separated by SDG, was included. Due to different preparative techniques, the bands from these preparations did not align exactly with corresponding bands from SDG-separated samples. As molecular mass standards, the following samples were run: DNP-purified IgM-wt (1080-kDa hexamer, 900-kDa pentamer, gain information about the connectivity and to ascertain whether it is affected by J chain, we examined IgM-S337. Several observations argue that IgM-S337 closely resembles IgM-wt, except for the absence of the C337-C337 disulfide bond. Thus, IgM-S337 efficiently assembles into pentamer and hexamer that have normal sedimentation rates in SDG. IgM-S337 pentamer and hexamer are comparable to the corresponding wild-type proteins in their cyto lytic activities. Pentameric IgM-S337 contains a normal amount of J chain (19). IgM-S337 polymers are assembled from noncovalent subunits, whose size and composition are determined by the extent to which C414-C414 and C575-C575 join μ-chains in series. In the model presented in Figure 5B, an IgM-S337 pentamer is presented as a structure that would dissociate in SDS to yield one μ4κ4 subunit and three μ2κ2 subunits. In the μ4κ4 subunit, two C414-C414 bonds join the μ-chains in series with one C575-C575 bond. As illustrated, the μ2κ2 subunits are of three types: a subunit bonded by only C414-C414, a subunit bonded by only C575-C575, and a subunit bonded by C414-C414 and C575-C575 in parallel. These examples illustrate how the size of the subunits (here μ2κ2 and μ4κ4) indicates whether C414-C414 and C575-C575 are in series, but not whether they are parallel.

We have tested for differences in the covalent assembly of pentameric and hexameric J+IgM-S337 and J-IgM-S337 by examining the species that are produced from these polymers upon dena turation by SDS. Pentameric and hexameric J+IgM-S337 and J-IgM-S337 were prepared by sequential SDG centrifugations (Fig. 6), as described above for J+IgM-wt and J-IgM-wt. As was seen for IgM-wt, the fraction of IgM-S337 sedimenting as monomer in the primary gradients was about threefold higher in preparations of J-IgM-S337 than in the case of J+IgM-S337, further supporting the idea that IgM-S337 assembles like IgM-wt. As in the analysis of IgM-wt (Fig. 3A), pentamer-enriched IgM-S337 sedimented more slowly than did hexamer-enriched polymers in the secondary gradients. The fact that IgM-S337 polymers had similar sedimentations in secondary and primary gradients and did not release a detectable level of monomer indicates that these poly mers are stable despite their noncovalent structure. In a separate preparation of IgM-S337 (not shown), we found that pentamer was much less cytolytic than hexamer, in agreement with an earlier analysis of IgM-S337 (19); for pentamer from both J+ and J-IgM- S337, 700 ng/ml was required for 50% lysis, while hexamer from J+ and J- transformants gave 50% lysis at 12 and 6.5 ng/ml, respectively.

Selected fractions from the secondary gradients of J+IgM-S337 and J-IgM-S337 were analyzed for subunit composition by SDS-PAGE (Fig. 6B). As reported previously (19) monomer (μ2κ2) and dimer (μ4κ4) species were the predominant subunits for all polymeric fractions tested, each accounting for 30 to 50% of the total IgM, as quantified by PhosphorImager. Extended exposure of the SDS-PAGE gel revealed many bands. Nevertheless, we discerned no major differences in band pattern between J+IgM-S337 and J-IgM-S337 pentamers. As will be considered in Discussion, these results suggest that J chain does not greatly alter the extent to which C414-C414 and C575-C575 link μ-chains in series.

Like hexameric IgM-wt, hexameric IgM-S337 was highly heterogeneous, and here also the J+IgM-S337 and J-IgM-S337 gave comparable patterns (Fig. 6B). As in the case of pentameric IgM-S337, the predominant species were μ2κ2 and μ4κ4. Hexamer

180-kDa monomer), pentameric IgM lacking the C1L domain and κ chain (mutant igm43; 560 kDa) and 226-kDa commercial marker. Samples were separated in the upper phase of a two-phase gel containing 3% acrylamide and 0.4% agarose.
lacked several of the minor species that were present in pentameric IgM-S337 (designated P1 and P2) and that migrated slower than $\mu$4c4. Moreover, unlike pentameric IgM-S337, hexameric IgM-S337 included several bands (H1–H4) that migrated more slowly than either pentameric or hexameric IgM-wt. The identities of these very slowly migrating species are considered further in Discussion.

We were concerned that some of the subunits present in both the pentamer- and hexamer-enriched fractions might truly be associated with only one polymeric species or the other, but may exist in all fractions because SDG centrifugation had yielded incomplete separation. It was not possible to assess the purity of the noncovalent pentamer and hexamer in the same direct manner as we did for IgM-wt. Nevertheless, our data suggest that cross-contamination was not a serious problem. Thus, the fact that pentamer-enriched fractions from IgM-S337 were much less cytolytic than hexamer-enriched fractions indicates that pentamer fractions were not severely contaminated. Also, very pure pentamer was obtained under the same separation conditions used for IgM-wt. IgM-S337 hexamer fractions might have been contaminated to some extent with pentamer, but this also was probably not severe. Thus, IgM-S337 pentamer fractions contained two species with unique SDS-PAGE mobility (P1 and P2 in Fig. 6B). These species could not be detected in the slowly sedimenting hexamer fractions, although other bands that are common to pentamer and hexamer fractions had similar intensities. These observations indicate that hexamer obtained from the faster shoulder of the peak included very little pentamer contamination.

Disposition of $J$ chain within IgM subunits

As shown above, IgM-S337 yielded predominantly species with the composition $\mu$2c2 and $\mu$4c4. The double mutant, IgM-S(337, 414), also assembles efficiently into polymers, but the polymers dissociate in SDS into $\mu$2c2 subunits held together by the C575-C575 disulfide bond (4) (Fig. 5C). As indicated in Figure 7, the overall $J$ chain content of secreted IgM-S337 and IgM-S(337, 414) was similar to that of IgM-wt, consistent with earlier findings that C575, but not C414 or C337, is necessary for $J$ chain incorporation (20). To examine how $J$ chain was distributed among the various substructures present in IgM-S337 and IgM-S(337, 414), $^{35}$S-labeled polymeric IgM was purified by SDG centrifugation (not shown). IgM from the polymer-containing fractions was immunoprecipitated and then analyzed by SDS-PAGE (Fig. 8A). Some of the minor bands shown in Figure 6B were not detected here, probably because of the shorter exposure time. The various bands were extracted from the gel and analyzed by AU-PAGE (not shown). The significance of the restricted distribution of $J$ chain is considered further in Discussion.

FIGURE 7. $J$ chain content of wild-type and mutant IgM. $^{35}$S-labeled, affinity-purified secreted IgM was separated by AU-PAGE, and bands were visualized by autoradiography. IgM-S(414, 575), which is secreted as monomer and half-mer, was included as a negative control for $J$ chain (4, 19).

FIGURE 8. Analysis of subunit structure of polymeric IgM-S337 and IgM-S(337, 414). A, Fractionation of polymeric IgM by SDS-PAGE. $^{35}$S-labeled polymers of IgM-S337 and IgM-S(337, 414) were recovered by immunoprecipitation from SDG. Labeled IgM-wt and IgM lacking C$\mu$4tail (mutant igm482) were prepared from culture supernatants. Samples were separated on a two-phase, nonreducing SDS-PAGE gel (upper phase, 3% acrylamide and 0.4% agarose; lower phase, 4.8% acrylamide and 0.4% agarose). The left panel is an autoradiograph of the gel; the right panel indicates the strong bands (A, B, D, and E) and weak bands (C, F, G, and H) that were extracted for analysis by AU-PAGE. B, AU-PAGE analysis of subunits from IgM-S337 and IgM-S(337, 414) polymer. Species A through H were extracted from the SDS-PAGE gel in A. Half of each extracted sample was loaded onto AU-PAGE (left gel). The gel to the right shows positive and negative controls for $J$ chain content: IgM-wt pentamer and IgM lacking C$\mu$4tail (mutant igm482).
As noted in the introduction, there is direct evidence that an individual \( \mu \)-chain binds via C575 to at most one J chain and that an individual J chain binds via its C14 and/or C68 to at most two \( \mu \)-chains. These observations have left open the possibility that some J chains are bound to \( \mu \) only via C14, while other J chains are bound only via C68. To test whether an individual J chain in fact bridges two \( \mu \)-chains by binding to C575, we have examined the disposition of J chain in IgM-S(337, 414). The major bands migrated approximately as \( \mu 2k2 \) (species D, 21% of the total polymer; species E, 69% of the total polymer) as well as various minor bands of faster mobility. J chain was detected in species D and to a much lesser extent in species E, but not in species F, G, or H. Considering that only C575 was available for intermolecular disulfide bonding in IgM-S(337, 414), the presence of J chain in this \( \mu 2k2 \) unit indicates that J chain bridged the \( \mu \)-chains via a C575-J-C575 linkage.

IgM-S337 and IgM-S(337, 414) polymers yielded \( \mu 2k2 \) subunits (species B, D, and E) that had slightly different mobilities. Nevertheless, under reducing conditions, \( \mu \)-chains from these three species had the same SDS-PAGE mobility, identical with that of \( \mu \)-chain from wild-type polymers (not shown). This suggests that the mobility difference between species D and E seen under nonreducing conditions reflects the presence of J chain, and that differences in intrasubunit disulfide bonding might be responsible for the different mobilities of species B and E.

**Discussion**

This study has addressed four related issues: the effect of J chain on IgM structure, the arrangement of disulfide bonds between \( \mu \)-chain and J chain, the connectivity of the \( \mu \)-chains within polymeric IgM, and the structural features that determine the cytolytic activity of IgM.

Previous analyses of IgM produced in the absence of J chain have led to different conclusions. Cattaneo et al. (16) concluded from their electron microscopic analysis of IgM produced by transfected glial cells that 50% of the IgM made in the absence of J chain was pentameric. This might be an overestimate, as this study did not distinguish hexamer in which one arm was obscured from genuine pentamer. Randall et al. (15) found that the B cell lines WEHI-231 and BCL1 produced some J-deficient pentameric IgM. Niles et al. (14) used transfecteds of a pituitary cell line to produce IgM and found that polymeric IgM secreted in the absence of J chain was exclusively hexameric. In stark contrast, we have found that pentameric IgM is efficiently produced in a J chain-deficient hybridoma cell line. We do not know the reason for these differences, but it is possible that the various cell lines express different intracellular accessory proteins. In fact, Roth and Koshland described a B cell-specific disulfide interchange enzyme (29). Further work, such as the analysis of J chain-deficient mice (12), will be needed to establish which of these systems best mimics the in vivo situation.

To simplify the analysis of inter-\( \mu \) chain disulfide connectivity, we have used the C337S substitution to eliminate the C337-C337 bond. This approach raises the question of whether the C414-C414 and C575-C575 bonds are formed in IgM-S337 as they are formed in IgM-wt. As presented above, this seems to be a valid assumption, as all measurements that we have made, namely the relative distributions of monomer, pentamer, and hexamer; the cytolytic activity; and the J chain content, were similar in IgM-S337 and IgM-wt. What, then, does the subunit composition of IgM-S337, as visualized in SDS-PAGE analysis, tell us about the connectivity of C414-C414 and C575-C575 bonds? In gross terms, IgM-S337 pentamer was heterogeneous and composed predominantly of \( \mu 2k2 \) and \( \mu 4k4 \) subunits; still larger subunits were present in lesser amounts. In \( \mu 4k4 \) and larger subunits, C414-C414 bonds must have joined \( \mu \)-chains in series with C575-C575 bonds. The existence of several subunits migrating approximately as \( \mu 2k2 \) (Fig. 6B) might reflect different bonding arrangements within the \( \mu 2k2 \) units, such as those illustrated in Figure 5A. Our data also indicate that at least some pentameric molecules contained subunits of different sizes. That is, a pentamer that contained \( \mu 4k4 \) must also have contained a different type of subunit, e.g., such a pentamer could have assembled as \((\mu 4k4k)(\mu 2k2)\) or \((\mu 4k4k)(\mu 2k2)_2\). Moreover, not all pentameric molecules were identical, since, if they were, no subunit with a size of \( \mu 2k2 \) or larger could have constituted <20% of the total subunits, and contrary to this prediction, some larger subunits were detected in small amounts.

As mentioned in the introduction, J chain is incorporated into intracellular pentamer before inter-\( \mu \)-chain disulfide bond formation is complete. In principle, this provides the opportunity for J chain to affect inter-\( \mu \)-chain bond formation. For this reason we compared the subunit species derived from J+ and J- IgM-S337. The fact that we did not see an effect of J chain on the SDS-PAGE band pattern indicates that J chain did not alter inter-\( \mu \)-chain bonding, that J chain affected only parallel connecting disulfide bonds, or that J chain content was much less than one per pentamer.

Hexameric IgM-S337 was assembled differently from pentameric IgM-S337. Although, hexameric IgM-S337, like pentameric IgM-S337, had \( \mu 2k2 \) and \( \mu 4k4 \) as major components, hexamer also contained a series of unique subunits with very low mobility in SDS-PAGE. Therefore, at least some pentamer and hexamer must differ in their connectivity. Of the slowly migrating hexameric subunits, H4 migrated approximately with hexameric IgM-wt in SDS-PAGE, and H3, H2, and H1 migrated successively slower. These bands were not heptamer, octamer, and nonamer, because they comigrated with wild-type hexamer in SDG under native conditions. Figure 9 illustrates models for hexameric IgM-S337 that might account for many of the observed subunits. These models make two assumptions: 1) that in hexameric IgM all \( \mu \)-chains are joined by C414-C414 in series with C337-C337 (or, in the case of IgM-S337, C414-C414 in series with noncovalently interacting Cµ2-Cµ2 domains), and 2) that C575-C575 bonds can occur in multiple forms. The first assumption is supported by the observation that IgM-S575 can assemble as covalent hexamer, and in this case the C414-C414 bonds must all be in series with C575-C575 (17). This arrangement might be a hallmark of all hexameric IgM. Figure 9 illustrates the second assumption that there are different modes of C575-C575 bonding. Models A and B are those originally proposed for human IgM by Feinstein and colleagues in 1972. By analogy with catenated DNA we would expect that singly, twofold, and threefold catenated \( \mu 6k6 \) circles would migrate progressively faster in electrophoresis. Thus, the catenated structures might account for the three slowest bands (H1–H3). Species H4 might correspond to model A or D or to a more complex molecule with topologic knots. We suppose that uncatenated \( \mu 6k6 \) circles would migrate like a trimer, a species that was conspicuous by its absence, but the loose structure of such a molecule might have rendered its mobility anomalous. We suppose that hexameric
IgM-wt might also assemble with these different arrangements of C414-C414 and C575-C575 bonds. However, in this case the C337-C337 bonds would be expected even in SDS to maintain the hexameric IgM-wt as a compact structure, which then migrated as a single band regardless of catenation.

We further investigated the relationship between J chain and inter-μ-chain disulfide bonding by examining how J chain is distributed among the subunits in IgM-S337 and IgM-S(337, 414). In IgM-S(337, 414), C575 is the only cysteine available for interchain disulfide bonding, so our finding (Fig. 8) that μ2κ2 subunits contained covalently bonded J chain indicates that the C575 residues were used to link both μ-chains as well as the J chain, i.e., that J chain bridged two μ-chains via C575. Moreover, our results indicate that J chain was present mainly or exclusively in this cross-linked mode. That is, if a significant proportion of J chain were incorporated by a single μ-J disulfide bond, the J chain would also have been present in the less abundant species F through H (Fig. 8), where, in fact, it was not detected. In the simplest case the μ-J-μ disulfide bonds exist in series with Cμ2-Cμ2 interactions. An analogous arrangement has been confirmed for IgA (32). Our data do not show whether the μ-chains were linked by a single J chain (μ-J-μ) or by multiple J chains (μ-J-μ). However, in a study by Frutiger et al. (6), cyanogen bromide digestion of IgM polymer resulted in covalent μ-J chain fragment of 23 kDa, consistent with only one J chain cross-linking μ-chains, (μ-J-μ).

For IgM-S337 polymers, we found that μ2κ2 subunits, but not μ4κ4 subunits, contained J chain. The failure of μ4κ4 subunits to incorporate J chain is intriguing, as these units contained both C575-C575 and C414-C414 bonds. This implies that J-chain incorporation was affected by structural features besides the availability of C575, namely the presence of C414-C414 bonds. Figure 10 illustrates one of many possible models, namely a model in which stereochemical effects prevent J chain from bonding either to μ-chains linked by C414-C414 (μ-chains 8 and 9) or to their neighboring μ-chains (μ-chains 7 and 10). That is, the C414-C414 bond might cause tight packing and exclude J chain from both μ4κ4 and the adjoining two μ2κ2 subunits but allow incorporation into the more distant μ2κ2 (μ-chains 2 and 5). This hypothesis might also explain why a pentamer does not incorporate more than one J chain, as the typical pentamer might contain either a μ4κ4 subunit (bearing C414-C414 in series with C575-C575) or a μ2κ2 subunit (in which C414-C414 is parallel with C575-C575). Similarly, the absence of J chain in hexamer might be explained by its numerous C414-C414 bonds. However, many other models are possible, including structures in which J chain links nonneighboring μ-chains (20).

This analysis also provides insight into why pentameric IgM activates complement less efficiently than does the hexameric species. We have found that J− pentamer, like J+ pentamer, was approximately 100-fold less active than hexameric IgM, indicating that some pentamer-specific feature other than J chain was responsible for the low cytolytic activity of pentamer. One possibility is that the hexameric Clq complement protein is well activated only by a hexameric substrate, although this hypothesis is rendered unlikely by the observation that Clq binds to IgM with only three or four arms (33). Alternatively, the high activity of the hexamer might relate to the arrangement of C414-C414 bonds in hexameric IgM, as the C414S substitution abrogates cytolytic activity (19, 34).

FIGURE 9. Models for IgM-S337 hexamer. In each case, μ-chains are joined by C414-C414 in series with noncovalent Cμ2-Cμ2 interactions. The covalent subunits of each hexamer are indicated by different shadings. In models E and F, the hexamers have different degrees of catenation, as illustrated in the adjoining diagrams.

FIGURE 10. A model for IgM-S337 pentamer. The figure shows one of many possible models to explain why J chain is found only in μ2κ2 subunits.

Covalent dimer: 6-9-8-1
Covalent monomers: 2-5, 3-10, 4-7

FIGURE 9.

FIGURE 10.

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might uniformly and completely join μ-chains in series with C337-C337, while this series arrangement might occur to a much lesser extent in pentameric IgM. There are several reasons why cytolytic activity might require C337-C337 in series with C414-C414. One explanation, based on the observation that the Cα3 domain contributes importantly to the complement activation (3, 34), is that the C414-C414-mediated cross-linking of the Cα3 domains in adjoining subunits generates a better C1 binding site than is present in unlinked Cα3 domains. Another model, suggested by Feinstein and colleagues, is that IgM undergoes a conformational change when it interacts with multivalent Ag and that the rigidity imparted and hexamer; smaller molecules, monomer, dimer, trimer, and tetramer, are also found (36, 37). There is an additional heterogeneity in which heterogeneity is generated by variations in disulfide bonding in assembly can reach equilibrium in which the structure of lowest free energy predominates.

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References