Scand. J. Immunol., Vol. 5, 1976.

Reassembly of Immunoglobulin M Heavy and Light Chains In Vitro

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Schrohenloher, R.E. & Hester, R.B. Reassembly of Immunoglobulin M Heavy and Light Chains In Vitro. *Scand. J. Immunol.* 5, 637–646, 1976.

Reduced and alkylated monoclonal IgM was fractionated into μ and light (L) chains by gel chromatography in 1N acetic acid. Equimolar mixtures of the chains formed a noncovalently bonded structure in 0.01M sodium acetate buffer, pH 4.1, that had the properties of a half subunit. The latter reassociated into a subunit-like structure after transfer into 0.08M sodium phosphate buffer, pH 7.5. The similarity of the reconstituted IgM subunit (IgMs) to that of the native molecule was established by its physicochemical and immunochemical properties. Comparable products were obtained on reassembly of the alkylated μ and L chains from several other monoclonal IgM. The presence of active binding sites for IgG on subunits reconstituted from the chains of proteins with anti-IgG activity further indicated correct assembly of the μ and L chains. High yields of subunit-like products were also obtained by assembly of μ chains from one protein and L chains from another. Evidence was obtained that L chains of appropriate specificity can substitute for the homologous chain in the formation of the active site. Heterogeneous mixtures of high molecular weight products were generated from μ and L chains that were not alkylated. Reduction and alkylation demonstrated that the products represented polymers of reconstituted IgMs. Significant levels of anti-IgG activity were detected in the polymeric IgM generated from the chains of active proteins by precipitation with aggregated IgG.

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Recent studies from this laboratory demonstrated that active products can be regenerated from the isolated polypeptide chains of a monoclonal IgM (protein Po) that binds human IgG (27). Approximately 80% of the materials derived from equimolar quantities of the μ and κ chains from this protein sedimented as a heterogeneous group of polymers that were completely precipitated with heat-aggregated IgG. A large quantity of anti-IgG activity was also detected by indirect hemagglutination. Evidence was obtained that both chains contributed to the formation of the combining site in a specific manner. Thus, this reactive IgM resembled IgG antibodies in this respect (5, 10, 19–22). Recombination of γ and light (L) chains has been shown to give products that closely resembled native IgG with respect to physicochemical and antigenic properties (1, 9, 17, 29). Similarly, recombination of the μ and κ chains from protein Po gave well-defined structural units that closely resembled the subunits of the native protein (23).

In contrast to the results obtained with protein Po, the component polypeptide chains from several other monoclonal IgM proteins failed to give significant yields of active products or subunit-like structures when subjected to identi-

cal conditions for recombination. These conditions were similar to those used for the regeneration of IgG molecules from γ and L chains (1, 9, 17, 29). Briefly summarized, μ and L chains were isolated from reduced protein by gel chromatography in 1N acetic acid. Mixtures of the chains were first dialyzed into 0.08M sodium phosphate buffer, pH 7.5, containing 0.01M dithiothreitol (DTT) in order to re-establish noncovalent interactions. Further reassociation through disulfide bond formation was then effected by prolonged incubation at 2°C after the DTT had been removed by dialysis.

A general approach to the in vitro reassembly of isolated polypeptide chains into IgM subunits and polymeric molecules is described in the present communication. The studies also provide additional information on the interaction of μ and L chains and on the roles of the component chains in the formation of the combining site.

MATERIALS AND METHODS

Isolation of IgM. The IgM proteins used in this study were obtained from patients with Waldenström's macroglobulinemia. The various proteins were precipitated from plasma by the addition of 15 volumes of cold distilled water. Proteins Da and Gr were resuspended in saline and the precipitation procedure repeated twice. Proteins Po, Co, and Le were further purified by gel chromatography on Sephadex G-200 in 0.1M sodium acetate buffer, pH 4.1, as previously described (26, 27). The resulting preparations were essentially free of materials sedimenting in the ultracentrifuge slower than the major macroglobulin component and demonstrated not more than trace amounts of IgG and IgA by immunoelectrophoresis using specific antisera.

Reductive cleavage. The interchain disulfide bonds of the IgM proteins were cleaved by incubation for 3 h with 0.01M DTT at room temperature in 0.08M sodium phosphate buffer, pH 7.5. The protein concentration was 10 mg/ml. Free sulfhydryl groups were alkylated by dialysis against 0.02M iodoacetamide in the pH 7.5 buffer for 4 h at room temperature with stirring (26).

Isolation and recombination of polypeptide chains. Reduced IgM or reduced and alkylated IgM was fractionated into μ and L chains by gel chromatography on Sephadex G-150 in 1N acetic acid as previously described (27). Equimolar quantities of the isolated μ and L chains were recombined and diluted to a protein concentration of 0.1 mg/ml by addition of 0.01M sodium acetate buffer, pH 4.1. The mixture was thoroughly dialyzed against the same pH 4.1 buffer. Further reassociation was achieved by dialysis into 0.01M sodium phosphate buffer, pH 7.5, and, finally, 0.08M sodium phosphate buffer at the same pH. Mercaptoethanol (0.01M) was added to the 0.01M sodium phosphate buffer when polymeric products were prepared from polypeptide chains with free sulfhydryl groups. The products were brought to a protein concentration of 10 mg/ml by ultrafiltration through type PM-10 Diaflo membranes (Amicon Corp., Cambridge, Mass.).

Products were fractionated by gel chromatography on Sephadex G-200 in the 0.08M sodium phosphate buffer. Eluted fractions were concentrated to approximately 10 mg/ml by ultrafiltration as described above.

Analytical procedures. Analytical ultracentrifugal studies were performed in a Beckman Model E ultracentrifuge as previously described (25). Before analysis, samples were thoroughly dialyzed against the 0.08M sodium phosphate or other indicated buffer. Sedimentation rates were corrected to standard conditions ($s_{20,w}$) and refer to a sample concentration of approximately 10 mg/ml unless otherwise stated. Sedimentation rates at infinite dilution ($s^0_{20,w}$) were estimated by linear extrapolation of the $s_{20,w}$ values determined at five sample concentrations, using the method of least squares. The partial specific volumes of the native proteins and reconstituted products were assumed to be 0.725 ml/g (16).

Polypeptide chain composition was determined after dialysis against 1N acetic acid by gel chromatography on Sephadex G-150 in 1N acetic acid (8). Relative yields were calculated from absorbance measured at 280 nm.

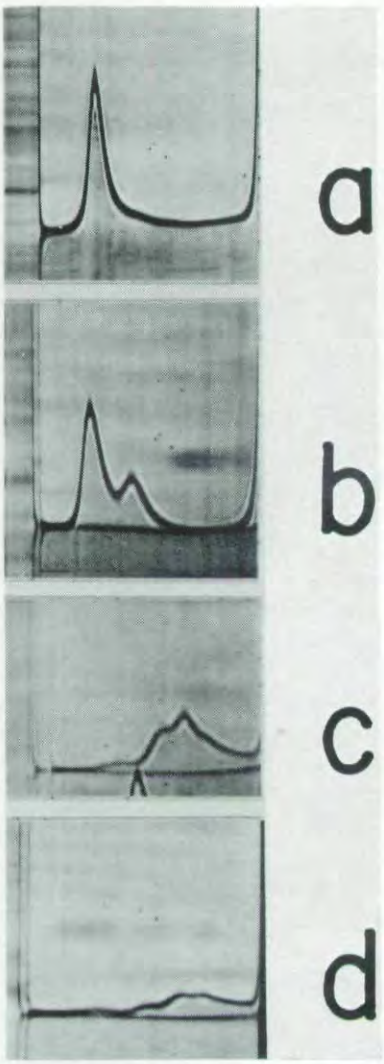

Fig. 1. Ultracentrifugal patterns of μ chains from protein Gr in (*a*) 5mM glycine-HCl buffer, pH 2.5, (*b*) 0.01M sodium acetate buffer, pH 4.1, (*c*) 0.1M sodium acetate buffer, pH 4.1, and (*d*) 4 mM sodium acetate buffer, pH 5.4. The pattern in *a* was recorded after 64 min at 68,000 rpm and that in *b* after 64 min at 60,000 rpm; those in *c* and *d* were recorded after the same interval at 56,000 rpm. The concentrations of the samples were (*a*) 7 mg/ml, (*b*) 9 mg/ml, (*c*) 10 mg/ml, and (*d*) 6 mg/ml. The direction of sedimentation in this and subsequent figures is to the right.

Reactivity with human IgG was determined by indirect hemagglutination of tanned sheep erythrocytes coated with human IgG and precipitation of heat-aggregated human IgG (27). Immunoelectrophoresis was performed as previously described (27). Antisera were obtained from Meloy Laboratories, Springfield, Va. Disc electrophoresis in 5% polyacrylamide was performed in sodium phosphate-sodium docecyl sulfate (SDS)-urea buffer (13).

RESULTS

Interaction of μ and L chains

Application of the previously used recombination conditions (23, 27) to alkylated μ and λ chains isolated from Gr IgM gave only a small yield (18%) of a subunit-like component. Examination of the other products recovered by chromatography on Sephadex G-200 indicated that the μ chains underwent extensive self-association instead of interacting with λ chains to form the four-chain structure characteristic of immunoglobulins. Immunoelectrophoresis and polyacrylamide gel electrophoresis in urea-SDS buffer demonstrated that the remaining μ chains were present as aggregates in the fraction excluded from the column, whereas the remaining λ chains were eluted after the subunits. In contrast, the yield of subunit-like component obtained from the alkylated μ and ϰ chains of Po IgM exceeded 50%.

The self-association of μ chains was further characterized by sedimentation-velocity studies on alkylated Gr μ chains in buffers of different pH and concentration. A single major component was observed in 5mM glycine-HCl buffer, pH 2.5, having an $s^0_{20,w}$ value of 3.0S (Fig. 1a). Asymmetry of the boundary indicated the presence of a small quantity of larger components, which was more apparent in older preparations. When examined in 0.01M sodium acetate buffer, pH 4.1, the $s^0_{20,w}$ value of the major component was 3.6S (Fig. 1b). A second component having an $s^0_{20,w}$ of approximately 8S was also evident. Further increases of either pH or buffer concentration resulted in greater aggregation. In 0.1M sodium acetate buffer, pH 4.1, major components sedimenting at approximately 6S and 7S were apparent, in addition to larger aggregates and a small quantity of 5S material (Fig. 1c). Similarly, most components sedimented at approximately 7.5S and faster in

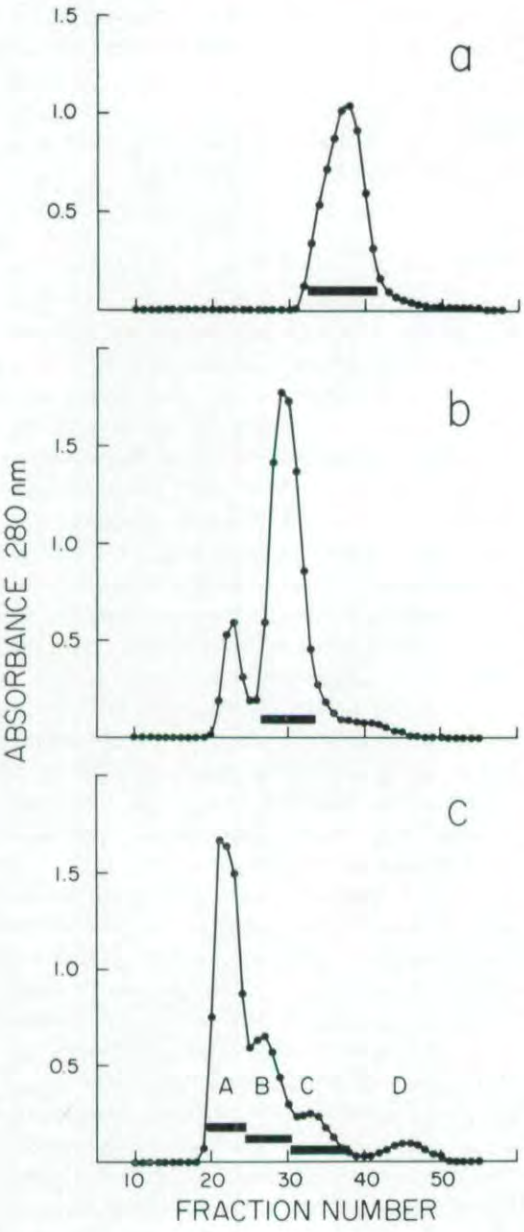

Fig. 2. Chromatography on Sephadex G-200 (2.5 × 100 cm column) of (*a*) an equimolar mixture of μ and λ chains from protein Gr in 0.01M sodium acetate buffer, pH 4.1, (*b*) reconstituted Gr IgM subunits in 0.08M sodium phosphate buffer, pH 7.5, and (*c*) reconstituted Gr IgM in 0.08M sodium phosphate buffer, pH 7.5. The quantities of protein applied to the columns were (*a*) 50 mg, (*b*) 100 mg, and (*c*) 85 mg. The horizontal bars indicate column tubes pooled for further study.

4mM sodium acetate buffer, pH 5.4 (Fig. 1d). Analysis in 4mM sodium phosphate buffer, pH 7.5, demonstrated a broad boundary sedimenting at approximately 11S and only a small quantity of 3S or 4S material.

The above studies suggested that successful reassembly of the IgM subunit might be achieved by conditions that would permit μ/L interactions to be re-established in the absence of extensive μ-chain self-association. Sodium acetate buffer, 0.01M and pH 4.1, was selected for this purpose. Equilibration of an equimolar mixture of the μ and λ chains from reduced and alkylated Gr IgM gave nearly complete recombination. Chromatography on Sephadex G-200 in the pH 4.1 buffer demonstrated that most of the sample eluted as a single component with only a small quantity of lower molecular weight materials present (Fig. 2a). The elution volume indicated that the product was smaller than IgG but larger than either of the component chains. Sedimentation analysis of the isolated product demonstrated a symmetrical boundary having an $s^0_{20,w}$ value of 4.3S (Fig. 3a). Its diffusion coefficient ($D^0_{20,w}$) was 4.7×10^{-7} cm²/sec. The latter was estimated by extrapolation of values obtained by the method of Ehrenberg (7) over the range of 2 to 10 mg/ml. A molecular weight of 80,000 daltons was calculated from these values by the Svedberg equation (31). Dissociation in 1N acetic acid followed by gel chromatography on Sephadex G-150 in the acetic acid indicated the presence of equimolar quantities of the μ and λ chains. These results suggested that the μ and λ chains interacted to form a relatively stable two-chain structure that represented half of the natural four-chain subunit.

Further reassociation of the pH 4.1 product was achieved by dialysis sequentially into 0.01M and 0.08M sodium phosphate buffer, pH 7.5. Chromatography on Sephadex G-200 in the latter buffer revealed a large quantity of a component that closely resembled the subunits of the native IgM (IgMs) (Fig. 2b). Sedimentation analysis of this component gave a single symmetrical boundary having an $s^0_{20,w}$ value of 6.69S (Fig. 3b). Native Gr IgMs gave a similar sedimentation pattern and an $s^0_{20,w}$

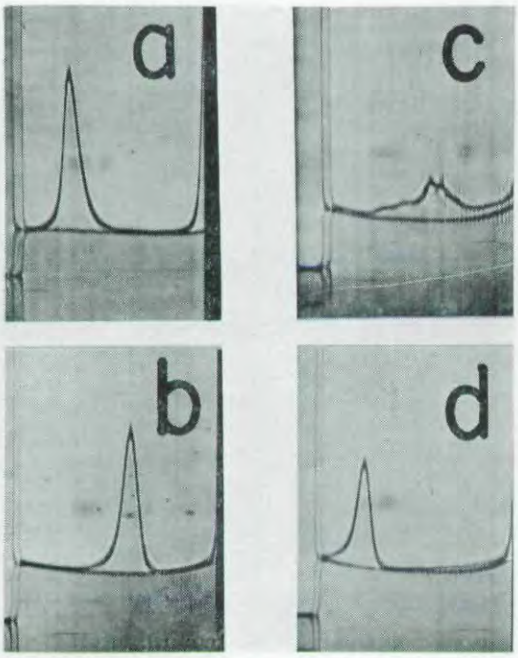

Fig. 3. Ultracentrifugal patterns of (*a*) reconstituted Gr IgM half subunits in 0.01M sodium acetate buffer, pH 4.1, (*b*) reconstituted Gr IgM subunits in 0.08M sodium phosphate buffer, pH 7.5, (*c*) reconstituted Gr IgM in the pH 7.5 buffer, and (*d*) reduced and alkylated reconstituted Gr IgM in the pH 7.5 buffer. Patterns *a* and *b* were recorded after 80 min at 56,000 rpm; those in *c* and *d* were recorded after 32 min at the same speed. The protein concentration of each sample was 10 mg/ml.

value of 6.81S. The distribution of components obtained by gel chromatography of the reconstituted IgMs in 1N acetic acid demonstrated 26% L chain. The yield of L chain from the native IgM was 29%. The reconstituted and native subunits gave similar precipitin lines on immunoelectrophoresis using antiserum specific for IgM or λ-type Bence Jones protein. Each demonstrated a single component that reacted with both antisera. The small quantity of aggregated materials recovered in the void volume (Fig. 2b) appeared to contain only μ chains by immunoelectrophoresis. Both μ and λ determinants were detected in the low molecular weight materials eluted after the subunits.

Comparable results were obtained on reassembly of the μ and L chains of several other monoclonal IgM. The reconstituted and native IgMs of protein Da gave comparable sedimentation patterns having $s^0_{20,w}$ values of 6.30S and 6.57S, respectively. The yield of L chains by gel chromatography of the reconstituted IgMs in 1N acetic acid was 22% and that of the native IgMs was 26%. Well-characterized subunits were also obtained from the μ and κ chains of two monoclonal IgM (Co and Le) that reacted with IgG (Fig. 4a and 4b). The reconstituted IgMs from protein Co (Fig. 4a, upper pattern) sedimented at 6.1S when examined at 8 mg/ml. The sedimentation rate of the subunit derived from protein Le (Fig. 4b, upper pattern) was 6.3S at 7 mg/ml. These values were in agreement with those obtained for the respective native IgMs at similar concentrations. The product obtained from the μ and κ chains of protein Po (Fig. 4c, upper pattern) demonstrated greater heterogeneity than the other reconstituted IgMs. An estimated 65% of the materials detected sedimented at approximately 6S; the rest sedimented at approximately 5S. The subunit prepared from the native protein sedimented as a single component at 6.4S (23).

Although the subunits produced from IgM anti-IgG factors by reduction and alkylation often fail to show activity by serologic procedures, they frequently form soluble complexes with human 7S IgG that can be detected by ultracentrifugal analysis (23, 24, 26). The subunits of proteins Co, Le, and Po each formed such complexes. Similarly the subunits reconstituted from the chains of these proteins reacted with IgG to form faster-sedimenting components not present in either the IgG or the subunit preparations (Fig. 4). The sedimentation characteristics of the complexes formed by reconstituted Co IgMs and reconstituted Le IgMs were similar to those of the native subunits. Those formed by reconstituted Co IgM sedimented at 8.2S and were poorly resolved from the unreacted components (Fig. 4a, lower pattern). As previously observed for subunits of several anti-IgG factors (23, 24, 26), the yield of complexes was dependent on the quantity of IgG present, and the molecular size of the complex, as indicated by its sedimentation rate,

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