Mouse Intracellular Immunoglobulin M

STRUCTURE AND IDENTIFICATION OF A FREE THIOL GROUP

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(Received 27 April 1978)

Monomeric intracellular mouse immunoglobulin M (hereafter designated IgMs) was purified in milligram quantities from the plasma cells of mouse plasmacytoma MOPC 104E after lysis either in the presence or in the absence of iodoacetate. Peptide 'mapping' analysis of the IgMs after partial reduction and carboxy[^{14}C]methylation to label the interchain disulphide bridges showed that the heavy–light bridge and the interheavy bridge present in the $\text{C}\mu 2$ region were already formed at lysis. The cysteine residues in the C-terminal region of the heavy chains, which in pentameric IgM form an intersubunit bridge, had free thiol groups at lysis that were reversibly oxidized during isolation in the absence of iodoacetate, probably forming an intrasubunit inter-heavy-chain disulphide bridge. Isoelectric-focusing studies complemented the above findings, showing that all the intracellular IgMs carried free thiol groups that could be carboxymethylated at lysis, and that in non-alkylated preparations these had reversibly oxidized. On the basis of sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis intracellular μ -chains had a consistently lower apparent molecular weight than did secreted μ -chains, and the estimated difference could be accounted for by the known difference in carbohydrate content. We present evidence that in a position homologous to that of a complex oligosaccharide in the $\text{C}\mu 2$ region of secreted human μ -chains there is a simple oligosaccharide in intracellular mouse μ -chains that becomes complex on secretion. On the basis of the above findings, we present a model for the mouse intracellular IgM subunit and suggest a mechanism for its assembly into secreted IgM pentamers.

Immunoglobulins can be regarded as being derived from a basic structure of two types of polypeptide chains, heavy and light, linked together by the disulphide bridges of cysteine residues [see review by Porter (1973)]. In mammals, the IgM molecule consists of ten heavy (μ) chains and ten light (L) chains [see reviews by Metzger (1970) and Feinstein & Beale (1977)]. The complete amino acid sequence of the human μ -chain (Watanabe *et al.*, 1973; Putnam *et al.*, 1973) has established that the μ -chain is composed of a variable domain, four constant domains and an additional C-terminal stretch of 19 amino acid residues.

Of the five oligosaccharide groups present on the μ -chain of human IgM (Shimizu *et al.*, 1971; Hickman *et al.*, 1972) two have been shown to be simple, containing only mannose and N-acetylglucosamine residues, and the other three to be complex, containing in addition sialic acid, galactose and fucose residues. In each pentameric IgM molecule an additional single J polypeptide chain (Halpern & Koshland, 1970) is disulphide-bridged to the extra-domain C-terminal region of μ -chains (Mestecky *et al.*, 1972)

Abbreviations used: IgM, immunoglobulin M; IgMs, monomeric intracellular immunoglobulin M; SDS, sodium dodecyl sulphate.

and is thought to take part in the mechanism of polymerization of monomeric intracellular IgMs to pentameric IgM. The arrangement of interchain disulphide bridges in human IgM (Beale & Feinstein, 1969; Feinstein *et al.*, 1976; Feinstein & Beale, 1977) is probably identical with that found in mouse IgM (Milstein *et al.*, 1975), except that the disulphide bridge linking the $\text{C}\mu 3$ domains in human μ -chains appears to be absent from mouse μ -chains.

In the mouse the intracellular precursor of pentameric IgM is the disulphide-linked monomeric IgMs (Parkhouse & Askonas, 1969); IgMs is formed within the cell in the absence of significant quantities of pentameric IgM, or of oligomeric forms between monomer and pentamer (Parkhouse, 1971). The polymerization of IgMs occurs very shortly before secretion, at which time the J-chain is incorporated into the molecule (Parkhouse, 1972) and terminal sugars are added to the core sugars already present on the μ -chains (Parkhouse & Melchers, 1971; Melchers, 1972; Parkhouse, 1973).

Stott & Feinstein (1973) demonstrated that all molecules of intracellular IgMs carry free thiol groups. We wished to identify these groups and thus to arrive at the covalent and probable non-covalent interactions in the intracellular IgMs. The present

study describes an investigation into the structure of mouse intracellular IgMs with the mouse plasmacytoma MOPC 104E (McIntire *et al.*, 1965), which secretes pentameric IgM and excess λ -chains. We show that the half-cystine residue in the extra-domain C-terminal region of the μ -chain is present as a free thiol group in intracellular IgMs, which would permit interaction with J chain and result in polymerization of the intracellular units to pentameric IgM.

Experimental

Preparation of mouse IgM

The IgM secreted by mice bearing the plasmacytoma MOPC 104E was purified from serum as reported previously (Milstein *et al.*, 1975).

Preparation of intracellular IgMs

The plasma-cell tumour MOPC 104E (McIntire *et al.*, 1965) was maintained by subcutaneous transplantation in female Balb/C mice. Tumours 2–3 weeks old and transplant generations 21–45 were used. All procedures were carried out at 4°C. Whole tumours were excised and placed in buffer, the constituents of which depended on whether the tumour cells were to be lysed later in the presence of iodoacetate, where it consisted of 0.1M-Tris/HCl buffer, pH 8.0, containing 0.1M-KCl and 5mM-MgCl₂, or whether the tumours were lysed in the absence of alkylation reagent, where the buffer consisted of 0.05M-sodium phosphate buffer, pH 7.2, containing 0.1M-KCl and 5mM-MgCl₂. The volume of tumours was measured by displacement; as a routine 50–100 tumours were used, the average volume per tumour being 1–2 ml. The tumours were rinsed with the appropriate buffer solution and then placed in a Waring blender. A volume of buffer equal to that of the tumours was added and in preparations where proteinase inhibitors were used the following 'cocktail' of inhibitors was added; soyabean trypsin inhibitor (1mg/ml), EDTA (0.01M), phenylmethanesulphonyl fluoride (1mM). When lysis was in the presence of alkylation reagent, iodoacetic acid was added to a final concentration of 0.1M. Finally, a non-ionic detergent, Nonidet P40, was added to a concentration of 1% (v/v) and the mixture was blended at top speed for 1 min. The lysate was centrifuged at 10000g for 30 min (Sorvall SS-34 rotor) and the resulting supernatant was further centrifuged at 100000g for 1 h 30 min (Beckman AH 42 rotor). The supernatant was dialysed for 16 h against HSB buffer (0.02M-sodium phosphate buffer, pH 7.0, containing 0.5M-NaCl and 0.05% NaN₃). The dialysed extract was subjected to stepwise precipitation with (NH₄)₂SO₄ as reported previously (Melchers, 1972) and the 25–43% (w/v) fraction was

suspended in HSB buffer and dialysed for 16 h against the same buffer. The dialysed solution was centrifuged for 30 min at 10000g and the supernatant was applied to a column (5cm×80cm) of Bio-Gel P-300 eluted with HSB buffer. Fractions (3 ml) were monitored for A₂₈₀ and those containing protein were analysed for IgM content by double diffusion in agar gel (Ouchterlony, 1958) with sheep antiserum to mouse IgM, and by polyacrylamide-gel electrophoresis with SDS (Summers *et al.*, 1965). Fractions were pooled and concentrated by ultrafiltration (Amicon Diaflo). The pool containing IgMs was applied to a column (1.6cm×100cm) of Sephadex G-200 eluted with HSB buffer. The fractions were monitored by A₂₈₀ and double-diffusion analysis as described above. Pools were made of the fractions containing IgMs and these were concentrated by ultrafiltration and finally stored in portions at –20°C. Each preparation was examined by polyacrylamide-gel electrophoresis with SDS to determine the banding pattern and purity.

Reduction and alkylation

Portions (0.4 ml) of secreted IgM or intracellular IgMs preparations at 0.012% (w/v) protein concentration in 0.3M-NaCl/0.2M-Tris/HCl buffer, pH 8.0, containing 10mM-EDTA, were flushed with N₂ for 15 min, and dithiothreitol solution in the same buffer flushed with N₂ was added to give a final concentration of 5mM (partial reduction). The samples were incubated at room temperature in stoppered vials for 1 h. Sufficient iodo[2-¹⁴C]acetate at 15mCi/mmol was then added to each to give a 1mM excess over the added thiol, and the samples were alkylated under N₂ for 1 h at room temperature. The concentration of iodoacetate was increased to a total of 33mM by the addition of unlabelled reagent, and the samples were left for a further 1 h at room temperature. Dialysis against phosphate-buffered saline (0.85% NaCl/9mM-sodium phosphate buffer, pH 7.2) was then carried out overnight at 4°C. The samples were further dialysed against 6M-guanidine hydrochloride, pH 8.0. Secreted IgM (1mg) was added to each and the samples were then made 20mM with respect to dithiothreitol for 1 h at room temperature (full reduction) and carboxymethylated with a final concentration of 60mM-iodoacetate for 1 h. Dialysis was then carried out overnight at 4°C against phosphate-buffered saline and a further three times against formic acid/acetic acid/water (1:4:45, by vol.).

Pepsin digestion

Pepsin was added to the samples in formic acid/acetic acid/water (1:4:45, by vol.) to give a protein/enzyme ratio of 50:1 (w/v) and digestion was allowed to proceed at 37°C for 16 h. The peptides were then freeze-dried.

'Mapping' of peptic peptides

Soluble peptides from carboxymethylated preparations were applied as a band to a sheet of 3MM Whatman paper and then subjected to high-voltage paper electrophoresis at pH 3.5 in water/acetic acid/pyridine (189:10:1, by vol.) at 60 V/cm for 1 h 15 min at room temperature. The strip containing the sample was cut out and sewn to a new sheet of paper that was then run at right angles by ascending chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.). Labelled peptides were located by radioautography with Kodirex X-ray film.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

(i) In initial experiments, rod-gel electrophoresis was performed by the technique of Summers *et al.* (1965). Protein dissolved in 0.01 M-sodium phosphate buffer, pH 7.8, containing 0.5 M-urea and 0.1% (w/v) SDS was subjected to electrophoresis in 4.05% (w/v) polyacrylamide gels (acrylamide/bisacrylamide ratio, 30:1.5, w/w) containing 0.1% (w/v) SDS and 0.1 M-sodium phosphate buffer, pH 7.2, at a constant current of 10 mA/gel for 3 h at room temperature. The gels (8 cm × 0.6 cm) were then stained for 1 h at room temperature in 0.1% (w/v) Naphthalene Black in fixer [ethanol/acetic acid/water (25:8:67, by vol.)] and destained by diffusion in fixer at room temperature. In later experiments, rod-gel electrophoresis was performed by the technique of Laemmli (1970) with 7.5% (w/v) polyacrylamide gels.

(ii) Slab-gel electrophoresis was performed in the system developed by Laemmli (1970) with a lower gel of 10% (w/v) polyacrylamide containing 375 mM-Tris/HCl buffer, pH 8.9, and 0.1% SDS. The stacking gel consisted of 3% (w/v) polyacrylamide containing 125 mM-Tris/HCl buffer, pH 6.8, and 0.1% (w/v) SDS. The acrylamide/bisacrylamide ratio in both gels was 30:0.8 and both were polymerized in the presence of 0.0025% ammonium persulphate and 0.00125% *NNN'*-tetramethylethylenediamine. Samples of IgM or IgMs (2 µg) were prepared in 62.5 mM-Tris/HCl buffer, pH 6.8, containing 10% (w/v) sucrose, 2% (w/v) SDS, 0.002% (w/v) Bromophenol Blue and 5% (v/v) 2-mercaptoethanol, in a total volume of 0.1 ml. Each sample was placed in a boiling-water bath for 2 min and when cool was electrophoresed for 6 h at room temperature at a constant voltage of 7 V/cm. The gel slab was stained for 1 h at 37°C in 0.25% (w/v) Coomassie Brilliant Blue R250 in acetic acid/methanol/water (3:2:29, by vol.) and then destained at 37°C in the same mixture.

Isoelectric focusing

Portions of each preparation containing 100 µg of protein in 0.01 M-sodium phosphate buffer, pH 7.2,

were applied to a 2 mm thick (5.1%, w/v) polyacrylamide-gel slab (acrylamide/bisacrylamide ratio, 30:0.8) containing a 3% (w/v) mixture of ampholytes designed to give a pH gradient of 4–8. Isoelectric focusing was carried out at 14°C for 12 h in the LKB Multiphor apparatus. The pH gradient was measured at 14°C directly on the gel with a Pye-Ingold flat membrane electrode. The gel slab was then stained for 1 h at room temperature with 0.1% Coomassie Brilliant Blue R250 in ethanol/acetic acid/water (9:2:9, by vol.) and destained in ethanol/acetic acid/water (6:1:13, by vol.).

Results*Purification of intracellular IgMs*

The detergent lysate of 50–100 tumours was subjected to stepwise precipitation with (NH₄)₂SO₄ by the method of Melchers (1972), and the 25–43% fraction was applied to a column of Bio-Gel P-300 (Fig. 1a). Fractions containing protein were analysed for the presence of IgM by double diffusion in agar gel and by SDS/polyacrylamide-gel electrophoresis (see the Experimental section).

Double-diffusion analysis of the fractions demonstrated the presence of two IgM-positive regions; the first (region A) coincided with the excluded material (0.24 × column volume) and was assumed to be assembled IgM that originated from the intercellular spaces of the tumour, whereas the second IgM-positive region (B) occurred later in the fractionation (0.4 × column volume) and was assumed to be IgMs, as it eluted in the same position as the subunits obtained from partially reduced and alkylated secreted mouse IgM. SDS/polyacrylamide-gel electrophoresis of the fractions confirmed this finding. The fractions were pooled, on the basis of the above analysis, and concentrated by ultrafiltration. Leon *et al.* (1970) established previously that IgM from the MOPC 104E tumour possesses antibody activity against α-(1→3)-linked dextran. We have found (N. E. Richardson & A. Feinstein, unpublished work) that after reduction and alkylation of this IgM, the resulting subunits are retarded with respect to other proteins of the same molecular weight on a column of a dextran derivative (Sephadex G-200). We have taken advantage of this finding when further purifying the crude IgMs preparation from the Bio-Gel P-300 column. On re-fractionation of the crude IgMs on a column of Sephadex G-200 (Fig. 1b), the major protein component was eluted at 0.48 × column volume, whereas the fractions containing IgMs were eluted at approx. 0.9 × column volume (region C). The latter fractions were pooled and concentrated by ultrafiltration. On average a yield of 3–5 mg of intracellular IgMs was obtained from 150 ml of solid tumours. Fig. 2 shows the patterns obtained by 4.05%

Fig. 1. Purification of intracellular mouse IgMs

(a) Chromatography of 25–43% $(\text{NH}_4)_2\text{SO}_4$ fraction of an iodoacetate-treated detergent lysate from 60 ml of MOPC104E tumour tissue on a Bio-Gel P-300 column (bed size $5\text{ cm} \times 76\text{ cm}$; fraction size 3.3 ml) eluted with HSB buffer. The effluent was analysed for protein (A_{280}) and for IgM content by double diffusion in agar gel with sheep antiserum to mouse IgM. Regions A and B were found to contain IgM and IgMs respectively. (b) Chromatography of the pooled and concentrated fractions from region B in (a) on a Sephadex G-200 column (bed size $2.6\text{ cm} \times 100\text{ cm}$; fraction size 2.5 ml) eluted with HSB buffer. The effluent was analysed as in (a). Region C was found to contain IgMs.

Fig. 2. Analysis of intracellular mouse IgMs in SDS/polyacrylamide gels

(a) Electrophoresis was in 4.05% (w/v) polyacrylamide gels, 8 cm long. Gels: A, intracellular IgMs purified from a cell lysate containing iodoacetate; B and C, secreted IgM after selective reduction and alkylation; D, intracellular IgMs purified from a cell lysate made in the absence of iodoacetate. (b) Electrophoresis was in 7.5% (w/v) polyacrylamide gels, 8 cm long; samples were fully reduced before electrophoresis. Gels: A, secreted IgM; B, intracellular IgMs purified from a cell lysate made in the presence of iodoacetate; C, intracellular IgMs purified from a cell lysate made in the absence of iodoacetate. (c) Electrophoresis was in a 10% (w/v) polyacrylamide slab gel; samples were fully reduced before electrophoresis. Tracks: A, secreted IgM; B and C, intracellular IgMs purified from cell lysates made in the absence of iodoacetate; D, intracellular IgMs purified from a cell lysate made in the absence of iodoacetate and proteinase inhibitors; E, intracellular IgMs purified from a cell lysate containing iodoacetate. The extreme left- and right-hand-side tracks show a mixture of marker proteins, consisting of phosphorylase *a* (Pa) (94 000), bovine transferrin (Tf) (75 000), bovine serum albumin (BSA) (68 000) and ovalbumin (Ovalb) (43 000). The two bands across the slab, between bovine serum albumin and ovalbumin, are staining artifacts usually found in our slab gels.

(w/v) SDS/polyacrylamide-gel electrophoresis analysis of purified preparations of IgMs compared with patterns of selectively reduced and alkylated secreted mouse IgM. An IgMs preparation purified from a lysate made in the absence of iodoacetate showed a single component with a mobility similar to that of the $\mu 2\lambda 2$ -band material present in the SDS-containing

gel of the selectively reduced and alkylated IgM (Fig. 2a, gel D). When both preparations were mixed and subjected to SDS/polyacrylamide-gel electrophoresis it was demonstrated that the intracellular IgMs component had a mobility intermediate between those of the $\mu 2\lambda 2$ - and $\mu 2\lambda$ -band components that originated from the selectively reduced and

alkylated secreted IgM. An IgMs preparation purified from a lysate made in the presence of iodoacetate when subjected to SDS/polyacrylamide-gel electrophoresis (Fig. 2a, gel A) showed the presence of a major component with a mobility identical with that of the IgMs prepared from a lysate made in the absence of alkylation reagent; minor bands were also present that had similar mobilities to components $\mu 2\lambda$ and $\mu\lambda$ when compared with the selectively reduced and alkylated secreted IgM. The presence of a $\mu\lambda$ component in the iodoacetate-treated lysate confirmed that alkylation had been effective and agrees with the previous report (Parkhouse, 1971) that the $\mu\lambda$ subunit is the precursor of intracellular IgMs. In addition, this finding demonstrated that the $\mu\lambda$ subunits were co-eluted with intracellular IgMs during column chromatography, suggesting that the $\mu\lambda$ subunits interacted non-covalently to form $\mu 2\lambda 2$ units. The fact that no $\mu\lambda$ units were present in the lysate made in the absence of iodoacetate showed that such units were able to oxidize spontaneously to $\mu 2\lambda 2$ units.

Fig. 2(b) shows a comparison of the patterns obtained on 7.5% (w/v) SDS/polyacrylamide-gel electrophoresis of fully reduced secreted IgM (Fig. 2b, gel A), fully reduced IgMs purified from a lysate made in the presence of iodoacetate and proteinase inhibitors (Fig. 2b, gel B) and fully reduced IgMs purified from a lysate made in the absence of iodoacetate, but in the presence of proteinase inhibitors (Fig. 2b, gel C). The patterns show that the λ -chains had identical apparent molecular weights in both the secreted and intracellular immunoglobulin, but that the intracellular μ -chains had lower apparent molecular weights than secreted μ -chains. This difference in apparent molecular weights of secreted and intracellular μ -chains was also found when we analysed partially reduced preparations.

SDS/polyacrylamide-slab-gel analysis

The patterns obtained when we compared fully reduced secreted IgM with various preparations of fully reduced intracellular IgMs on 10% (w/v) SDS/polyacrylamide-slab-gel electrophoresis (see the Experimental section) are shown in Fig. 2(c). The major components present in each preparation were μ -chains and λ -chains and we found that the intracellular μ -chains always electrophoresed as sharp bands of material with a faster mobility than the μ -chains derived from secreted IgM.

Comparison of the R_F of the secreted μ -chains (Fig. 2c, track A) with those of the marker proteins plotted against $\log(\text{molecular weight})$ gave an apparent molecular weight for the secreted μ -chains of 79400. Fig. 2(c), tracks B and C, show the patterns obtained when fully reduced intracellular IgMs was electrophoresed, which had been prepared in the absence of alkylation reagent, but in the presence of

proteinase inhibitors; the apparent molecular weight of intracellular μ -chains from these preparations was found to be 71600. Fig. 2(c), track D, shows the pattern obtained when fully reduced intracellular IgMs that had been prepared in the absence of both iodoacetate and proteinase inhibitors was electrophoresed. The apparent molecular weight of μ -chains from this material was found to be 69750. Finally Fig. 2(c), track E, shows the electrophoresis pattern obtained after full reduction of intracellular IgMs prepared in the presence of both iodoacetate and proteinase inhibitors; the apparent molecular weight of μ -chains of this material was found to be 71600. The small differences in apparent molecular weights of materials made in the presence or absence of proteinase inhibitors suggested that proteolysis had occurred in preparations made in the absence of proteinase inhibitors. This was confirmed when the preparations were subjected to peptide 'mapping' analysis.

'Mapping' analysis of carboxy[^{14}C]methylated peptides

The limited quantity of intracellular IgMs available was sufficient for the comparison of peptide 'maps' of peptic digests of partially reduced carboxy[^{14}C]methylated intracellular IgMs with those from secreted IgM (see the Experimental section). As controls, an equal amount of each preparation was carboxy[^{14}C]methylated in an identical manner, but without prior reduction. The incorporation of label into control preparations was low in each case, and was between 5.5 and 6% of that incorporated into reduced and carboxymethylated preparations. Thus almost no free thiol groups existed in the non-reduced IgM preparations, even on the IgMs isolated without alkylation. Radioautographs of peptic peptide 'maps' of these control preparations showed no detectable radioactivity associated with the known bridge peptides. Radioautographs of peptide 'maps' of the reduced and carboxy[^{14}C]methylated preparations are shown in Plate 1. The radioactive peptides derived from secreted IgM (Plate 1a) correspond to those we obtained previously (Milstein *et al.*, 1975) and demonstrate that in our procedure, increasing the dithiothreitol/IgM ratio approximately 7-fold has not caused cleavage of other disulphide bridges in the IgM molecule. The 'map' obtained of radioactive peptides from intracellular IgMs prepared in the presence of iodoacetate and proteinase inhibitors is shown in Plate 1(b), whereas the 'map' obtained from intracellular IgMs prepared in the absence of iodoacetate but in the presence of proteinase inhibitors is shown in Plate 1(c). With peptide $\mu 1$ (derived from the μ - λ bridge) as a reference, it was apparent that peptide $\mu 4$ (derived from the C-terminus of the μ -chain) was present in normal yield in intracellular IgMs prepared in the absence of

iodoacetate when compared with the yield of peptide $\mu 4$ in the 'maps' of secreted IgM, but present in only trace amounts in IgMs prepared in the presence of iodoacetate, whereas both intracellular preparations showed the normal yield of peptide $\mu 1$. Peptide $\mu 2$ (derived from the inter-heavy-chain bridge in the $C\mu 2$ region) is heterogeneous in secreted IgM (Milstein *et al.*, 1975) owing to differences in the carbohydrate moiety. Both intracellular IgMs preparations gave rise to a single $\mu 2$ radioactive peptide. This is presumably due to lack of charged sialic acid residues since we find that neuraminidase treatment of secreted IgM alters the $\mu 2$ peptide appearance to resemble the intracellular pattern (N. E. Richardson & A. Feinstein, unpublished work). This fact demonstrates that in mouse, as in man, there is a complex carbohydrate moiety near the $\mu 2$ bridge, which is simpler in the precursor subunit. Peptide 'maps' obtained with intracellular IgMs that had been prepared in the absence of both iodoacetate and proteinase inhibitors demonstrated either an absence or a low yield of peptide $\mu 4$, which together with the low apparent molecular weights of the μ -chains on SDS/polyacrylamide-slab-gel-electrophoresis analysis confirmed that these μ -chains had been proteolytically digested at the C-terminal end of the polypeptide chain.

Analysis of intracellular IgMs by isoelectric focusing

Isoelectric focusing was used by Stott & Feinstein (1973) to demonstrate that free thiol groups on intracellular IgMs can be carboxymethylated on lysis of cells. This technique was used here to confirm that any free thiol groups in the preparations of IgMs from cells lysed in the presence of iodoacetate had been carboxymethylated. The patterns obtained with intracellular IgMs prepared from a lysate made in the presence of iodoacetate and that made in the absence of iodoacetate are shown in Plates 2(a) and 2(c) respectively. The multiple-banding patterns resemble those seen previously with the radioactively labelled intracellular IgMs of the same tumour line (Stott & Feinstein, 1973), and at an earlier time by Awdeh *et al.* (1970), for intracellular IgG. The origin of the multiplicity is unknown. The bands in Plate 2(a) have been shifted towards the acidic end of the pH gradient, indicating that the protein had been carboxymethylated. The pI of the untreated IgMs (Plate 2c) was in the range 5.9–6.2 and that of the alkylated IgMs was in the range 5.6–5.9, i.e. an average shift of 0.30pH unit, which is in agreement with the results of Stott & Feinstein (1973), for radioactively labelled intracellular IgMs from the same tumour line. The actual pI values are systematically lower by 0.2pH unit, but we find this difference is due to the difference in systems used and temperatures at which the gels were run and at which

pH gradients were measured. To ascertain whether there were any remaining free thiol groups present on the untreated IgMs, a sample was treated with 0.1M-iodoacetate at pH8.0 for 1 h, and then dialysed and electrofocused (Plate 2b). The range of pI was identical with that of the untreated IgMs, proving that no free thiol groups were present, and that no other groups had been carboxymethylated. We have found after reduction (N. E. Richardson & A. Feinstein, unpublished work) that the difference in pI between the carboxymethylated and untreated IgMs is reflected in the patterns of the heavy chains but not the light chains.

Discussion

In the present investigation we have prepared mouse intracellular IgMs, from the tumour line MOPC 104E, in milligram quantities. The fact that this immunoglobulin interacts with dextrans (Leon *et al.*, 1970) enabled us to isolate the intracellular IgMs, by using Sephadex-gel chromatography, from other molecules of similar molecular weight. Our finding that intracellular IgM, when isolated, is mainly in the form of disulphide-linked monomers ($\mu 2\lambda 2$) confirms previous studies using the same tumour line (Parkhouse & Askonas, 1969; Melchers, 1972; Stott & Feinstein, 1973), another IgM-secreting mouse tumour line, TEPC 183 (Parkhouse, 1973), and bacterial lipopolysaccharide-stimulated mouse spleen cells (Melchers & Anderson, 1973).

Our isoelectric-focusing results have confirmed the finding of Stott & Feinstein (1973) that the intracellular IgMs from this tumour line carried free thiol groups, and we have shown by peptide 'mapping' that the cysteine residue, $\mu 4$, present at the C-terminus of the intracellular μ -chain, can be carboxymethylated during lysis of the cells, and must therefore be present as a free thiol within the cell. We have shown by our failure to carboxy[^{14}C]-methylate, and by isoelectric focusing, that intracellular IgMs when isolated in the absence of alkylating reagent carries no free thiol groups, whereas on reduction and carboxy[^{14}C]-methylation of such preparations peptide $\mu 4$ is cleaved and radioactively labelled. This finding suggests that during isolation, in the absence of iodoacetate, IgMs formed a stable intrasubunit disulphide bridge between the $\mu 4$ cysteine residues of adjacent μ -chains. Moreover both $\mu 1$ and $\mu 2$ cysteine residues are present as disulphide bridges in the carboxymethylated intracellular IgMs. Thus the disulphide bridge arrangement in the intracellular IgMs is as shown in Fig. 3(a), leading to the probable arrangement of domains seen in Figs. 3(b) and 3(c). This is important to establish, since an alternative possible form of the intracellular unit could exist in which the $\mu 2$ residue is a free thiol group and the $\mu 4$ residue is

EXPLANATION OF PLATE I

Radioautographs of peptide 'maps' of partially reduced carboxy[¹⁴C]methylated secreted mouse IgM and intracellular mouse IgMs

Electrophoresis of peptic digests at pH 3.5 in one dimension was followed by ascending chromatography in butanol/ acetic acid/water/pyridine in the second dimension. (a) Partially reduced secreted IgM; (b) partially reduced intracellular IgMs, purified from a cell lysate containing iodoacetate; (c) partially reduced intracellular IgMs, purified from a cell lysate made in the absence of iodoacetate.

EXPLANATION OF PLATE 2

Analysis of intracellular mouse IgMs by isoelectric focusing

Isoelectric focusing was performed in a 5.1% (w/v) polyacrylamide-gel slab containing ampholytes designed to give a pH gradient of 4–8. (a) Intracellular IgMs purified from a cell lysate made in the presence of iodoacetate; (b) intracellular IgMs purified from a cell lysate made in the absence of iodoacetate, which had been subsequently carboxymethylated; (c) intracellular IgMs purified from a cell lysate made in the absence of iodoacetate.

Fig. 3. Structure of mouse intracellular IgMs

(a) Interchain disulphide bridge arrangement. (b) Proposed domain interactions: ●, interchain disulphide bridge; ◐, cysteine residue; ○, site of attachment of oligosaccharide group. The interactions are those proposed for the IgM pentamer (Feinstein, 1974). (c) Space-filling model.

disulphide bridged within a subunit. Indeed this is the form of subunit that is obtained by gentle reduction with dithiothreitol of pentameric IgM, since $\mu 2$ is the most labile bridge (Milstein *et al.*, 1975). Since IgMs is assembled intracellularly as shown in Fig. 3, it is very likely that this same form of IgMs is found in the B-lymphocyte membrane, although here it is more likely that the $\mu 4$ cysteine residues are bridged within the subunit.

In common with mouse and human IgM subunits prepared by partial reduction and alkylation (Miller & Metzger, 1965; Morris & Inman, 1968; Beale & Feinstein, 1969; Parkhouse, 1975), intracellular IgMs sediments at approximately 7S with no tendency to aggregate into higher-molecular-weight forms (Parkhouse & Askonas, 1969), and thus only weak non-covalent interactions can occur between subunits. Initial results (Askonas & Parkhouse, 1971; Melchers, 1972) with isolated intracellular IgMs from the same tumour line showed that the IgMs molecules lacked the ability to spontaneously polymerize *in vitro* unless they were first treated with reducing agent and in the presence of reduced carrier IgM. Under optimum conditions removal of the reducing agent caused 70% of the intracellular IgMs to be co-polymerized into polymeric IgM (Askonas & Parkhouse, 1971; Melchers, 1972). Askonas & Parkhouse (1971) interpreted this requirement for reduction of the non-alkylated intracellular IgMs for co-polymerization to occur as evidence against the presence of a free thiol on intracellular IgMs. However, we have shown that there are free thiol groups on intracellular IgMs that are lost by oxidation during IgMs isolation to form

a disulphide bridge. When the same authors isolated IgMs after cell lysis in the presence of alkylating reagent, they were able after reduction to co-polymerize the IgMs with an excess of secreted IgM. They interpreted this result as demonstrating that the thiol groups that potentially would form the intersubunit bridge were reversibly blocked within the cell in such a manner as to protect them from irreversible alkylation. However, according to our findings, residue $\mu 4$ must have been alkylated; the degree of reduction used by these authors was sufficient to cleave the inter-heavy-chain disulphide bridge of residue $\mu 2$ (Milstein *et al.*, 1975) in both secreted IgM and intracellular IgMs, which could have allowed the formation of covalently linked polymeric molecules on reoxidation, incorporating intracellular half-subunits through residue- $\mu 2$ bridges only.

Della Corte & Parkhouse (1973) reported the successful polymerization of carrier-free reduced intracellular IgMs *in vitro*, but a necessary requirement was for J-chain and disulphide-interchange enzyme to be present. It was not ruled out, however, that the enzyme was needed merely to reactivate the J-chain. Mestecky *et al.* (1974) have shown that the J-chain is disulphide linked to the half-cysteine residue $\mu 4$ in the μ -chain, and Chapuis & Koshland (1974) presented evidence to show that the J-chain joins the C-terminus of two μ -chains in the pentamer, but it was not clear whether the μ -chains are linked intrasubunit (i.e. parallel to disulphide bridge $\mu 2$) or intersubunit (Koshland, 1975). In Koshland's (1975) model for the assembly of IgM, the J-chain acts as an initiator through disulphide interchange with

Fig. 4. Possible role of J-chain in assembly of IgM
(a) Proposed model of intracellular mouse IgMs oxidized *in vitro* in the absence of J-chain, in which $\mu 4$ has formed an intrasubunit disulphide bridge. (b) Proposed mechanism for initiation of cyclization of pentameric IgM (see the text).

intracellular subunits. However, we find that the subunits carry free $\mu 4$ thiol groups intracellularly, so that disulphide interchange is not required. As we have seen, in the absence of J-chain, the free thiol groups in non-alkylated IgMs become reversibly oxidized (Fig. 4a). We have therefore proposed (Feinstein *et al.*, 1976) that the J-chain effects the intracellular assembly of IgM in the following way. J-chain forms (through oxidation during secretion) an intersubunit link between two IgM subunits, bridging the $\mu 4$ cysteine residues of two μ -chains, one from each (Fig. 4b). This shifts an equilibrium in favour of a specific interaction of each of these units with a further subunit, directing the formation of a disulphide bridge with that subunit, and so on until the cyclic pentamer is completed.

During biosynthesis of intracellular IgMs in this mouse tumour line five simple oligosaccharide moieties comprising mannose and *N*-acetylglucosamine residues are added early to the μ -chain, whereas fucose, galactose and sialic acid residues (terminal sugars) are added at a later stage, just preceding secretion of pentameric IgM (Parkhouse & Melchers, 1971). Hickman *et al.* (1972) have shown that human secreted μ -chains have three complex oligosaccharide moieties, consisting of a core structure and terminal sugar molecules, and two simple oligosaccharide groups made up of a core structure carrying extra mannose residues. The oligosaccharide compositions determined by Melchers (1972) for intracellular and secreted μ -chains confirmed that, like human, mouse IgM has multiple oligosaccharide groups, some of which become complex on the secreted μ -chain, and that the terminal sugar molecules are added on secretion. We have found (J. Clamp, N. E. Richardson & A. Feinstein, unpublished work) that some of the mouse μ -chain oligosaccharide is complex and some

is simple. In particular, we have presented evidence here of an oligosaccharide group attached near the interchain disulphide bridge $\mu 2$, which is converted into a complex oligosaccharide on secretion. The oligosaccharide in the corresponding position in human secreted μ -chains is also complex (Shimizu *et al.*, 1971; Milstein *et al.*, 1975).

Robinson *et al.* (1973) reported a molecular weight for the secreted mouse μ -chain, on gel filtration, of 73000, and quote a value of 71000 obtained by P. A. Small & K. R. McIntire [unpublished work cited in Robinson *et al.* (1973)] from sedimentation-equilibrium studies. These values are similar to that which we calculated for the molecular weight of human μ -chain from the amino acid composition (Watanabe *et al.*, 1973; Putnam *et al.*, 1973) and the composition of the oligosaccharide (Shimizu *et al.*, 1971; Hickman *et al.*, 1972). The calculated total molecular weight is 72200, of which the carbohydrate accounts for 9000. Moreover, we find that human and mouse μ -chains have almost identical behaviour on 10% polyacrylamide gels containing SDS. Our SDS/polyacrylamide-gel-electrophoresis results have shown an apparent mol.wt. of 79400 for secreted mouse μ -chains, which is 7200 higher than the calculated true molecular weight. Thus if the anomalous behaviour of μ -chains in SDS/polyacrylamide gels is attributed entirely to an anomalously high contribution from the carbohydrate groups present on the μ -chain (Segrest *et al.*, 1971), the apparent molecular weight of the carbohydrate moiety is approximately twice its true molecular weight. In secreted human μ -chains the oligosaccharide content is made up of 5400 for the two simple and three core sugar molecules and 3500 for the terminal sugar molecules. We can calculate, applying a factor of two, that the apparent molecular-weight difference owing to the terminal sugar molecules, which we expect to find between secreted and intracellular μ -chains on SDS/polyacrylamide gels, is 7000. This agrees well with the difference of 7800 in our apparent molecular weights between secreted (79400) and non-degraded intracellular mouse μ -chains (71600), which is also seen with intracellular μ -chains from lysates of bacterial lipopolysaccharide-stimulated mouse spleen cells (A. Warley & A. Feinstein, unpublished work). The sharpness of the intracellular μ -chain bands in our SDS/polyacrylamide gels indicates homogeneity and suggests a uniform carbohydrate composition in the intracellular mouse μ -chains, as microheterogeneity in the oligosaccharide moieties present on the μ -chains would be expected to result in a more diffuse banding pattern.

We are grateful to Mr. J. Herbert and Mr. M. Edwards for their technical assistance and thank Dr. D. I. Stott and Mrs. M. E. Percy for their help in the early stages of this investigation.

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