Biosynthesis of Immunoglobulin A (IgA) and Immunoglobulin M (IgM) REQUIREMENT FOR J CHAIN AND A DISULPHIDE-EXCHANGING ENZYME FOR POLYMERIZATION

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Mouse myeloma cells secreting 19S IgM (immunoglobulin M) (MOPC 104E and TEPC 183) or monomer and polymer IgA (immunoglobulin A) (MOPC 315) were incubated with radioactive leucine and the intracellular and secreted immunoglobulins and immunoglobulin subunits were prepared by preparative sucrose-density-gradient centrifugation. Samples were reduced in the presence or absence of isolated J chain, passed over Sephadex G-25 and then incubated at 37°C for 30 min with or without a source of disulphide-interchange enzyme. The extent of reassembly of reduced subunits was then evaluated by electrophoresis in polyacrylamide gels. Provided that J chain and the disulphide-interchange enzyme were supplied, both IgM and IgA could be assembled from their respective subunits, obtained by reductive cleavage of polymeric forms. Under similar conditions, assembly of polymeric forms from intracellular or secreted 7S monomer subunits also occurred. Under these conditions polymerization was total, there being no residue of the monomeric form. Reassembly did not occur in the absence of either J chain or the enzyme. All of the J chain released from IgM by reductive cleavage was incorporated back into the reassembled polymer. The J chain is therefore likely to be an essential structural requirement for polymeric immunoglobulins. A variety of controls ruled out non-specific interactions, and further suggested that the amino acid sequence of polypeptide chains determines the specificity of polymerization. The fact that intracellular IgA and IgM monomer subunits known to be deficient in galactose and fucose can be completely polymerized suggests that the addition of carbohydrate does not control polymerization.

The polymeric immunoglobulins comprise IgA‡ and IgM. In mammals, IgM is almost always secreted as a disulphide-linked uniform pentamer of IgMs (H_2L_2) subunits (Metzger, 1970), whereas secreted IgA is heterogeneous, consisting of monomer and a mixture of polymeric forms (Fahey, 1961, 1963). In both classes of immunoglobulin the polymerization of intracellular H_2L_2 subunits occurs close to the time of secretion (Parkhouse & Askonas, 1969; Bevan, 1971; Parkhouse, 1971*a,b*; Askonas & Parkhouse, 1971; Bargellesi *et al.*, 1972; Della Corte & Parkhouse, 1973). At this time fucose (Parkhouse & Melchers, 1971; Cowan & Robinson, 1972; Della Corte & Parkhouse, 1973) and J chain (Parkhouse, 1972) are incorporated into the polymer molecule. However, it is unlikely that the addition of carbohydrate plays any controlling role in polymerization, since monomer and polymer IgA

appear to be identical in carbohydrate composition, and the addition of fucose to secreted IgA monomer also occurs just before secretion (Della Corte & Parkhouse, 1973).

Although the exact function of J chain has not been demonstrated unequivocally, an obvious role to postulate is in control of polymerization. It is therefore crucial to demonstrate a mandatory requirement for J chain in polymerization and the work described here is directed towards that end.

Before the discovery of J chain (Halpern & Koshland, 1970; Mestecky *et al.*, 1971), we had been able to polymerize IgM from intracellular IgMs monomers as well as subunits obtained from secreted IgM by reduction (Parkhouse *et al.*, 1970; Askonas & Parkhouse, 1971). Reduced secreted IgM reassembled into the pentamer form after removal of the reducing agent by filtration through Sephadex G-25. The success of reassembly depended on two factors, the concentration of protein and reducing agent. A high yield of reassembled IgM molecules occurred when high concentrations of protein (0.5–5.0 mg/ml) were mildly reduced (0.5 mM-dithioerythritol). With either lower protein concentrations

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‡ Abbreviations: IgA, immunoglobulin A; IgM, immunoglobulin M; IgG, immunoglobulin G.

or increased severity of reduction (e.g. 5 mM-dithioerythritol), reassembly was partial. Isolated radioactive intracellular IgMs showed no tendency to polymerize unless first treated with reducing agent; under optimal conditions removal of the reducing agent caused 70% of the subunits to be assembled into IgM, but polymerization only occurred in the presence of reduced carrier IgM. In contrast, we were unable to reassemble polymers from reduced, secreted dimer IgA molecules (Parkhouse *et al.*, 1971). By using a similar methodology we have now demonstrated the specific polymerization of both IgA and IgM molecules from their subunits provided that J chain and a source of a disulphide-exchanging enzyme (Fuchs *et al.*, 1967) are supplied. Under these conditions polymerization will occur at very low protein concentrations and at relatively high concentrations of reducing agent. Thus in, for example, the polymerization of radioactive intracellular IgMs, the J chain replaces the reduced carrier IgM added in previous experiments (Askonas & Parkhouse, 1971).

Materials and Methods

Materials

Plasma-cell tumours. These were kindly sent to us by Dr. M. Potter and maintained in Balb/C mice by subcutaneous transfer of 1 mm³ tumour pieces. We used MOPC 104E (IgM), TEPC 183 (IgM) and MOPC 315 (IgA).

Preparation of serum myeloma proteins. This was as described previously (Parkhouse & Askonas, 1969; Della Corte & Parkhouse, 1973).

Preparation of J chains. The isolated MOPC 315 myeloma protein was adjusted to 10–15 mg/ml and reduced for 1 h at 37°C in 16 mM-Tris–50 mM-glycine buffer, pH 8.7, containing 9 M-urea and 10 mM-dithiothreitol. The sample was loaded directly on to a preparative polyacrylamide gel [4.5% (w/v) acrylamide, 16 mM-Tris, 50 mM-glycine, pH 8.7, containing 7 M-urea] (Brownstone, 1969). The preparative gel measured 90 mm × 50 mm (diam. × length) and electrophoresis was conducted at 220 V and 200 mA. The J chain, which was eluted well ahead of the major protein peak of single heavy and light chains, was dialysed and freeze-dried. The peak containing heavy and light chains did not contain J chain.

Methods

Labelling in vitro of intracellular and extracellular myeloma proteins. Cell suspensions were prepared and incubated with [4,5-³H]leucine (200–500 μ Ci/ml at 29 Ci/mmol) or [U-¹⁴C]leucine (10 μ Ci/ml at 0.2 Ci/mmol) for 4–6 h in a modified serum-free Eagle's medium minus leucine (Vogt & Dulbecco,

1963) and the culture fluid and cell lysate were collected as described previously (Parkhouse & Askonas, 1969). Secreted radioactive IgM, IgMs, IgA monomer and IgA dimer and radioactive intracellular 7S IgA and 7S IgM were purified by sucrose-density-gradient centrifugation of the culture medium and cell lysate. All sucrose solutions were made up in phosphate-buffered saline (130 mM-NaCl–4 mM-KCl–10 mM-sodium phosphate, pH 7.4) containing L-leucine at 0.1 mg/ml, and all sedimentations were at 200 000 g in the Spinco 41 rotor. For preparation of secreted IgM and IgMs the conditions were 5–30% (w/v) linear sucrose gradients, 17 h. The other purifications were with 12–25% (w/v) linear sucrose gradients, 22–24 h. It should be stressed that no carrier protein was added to any of the samples, so that the final product is always high-specific-radioactivity immunoglobulin.

Preparation of smooth- and rough-endoplasmic-reticulum membrane fractions. For this method we are grateful to Dr. S. De Petris. A 10% (w/v) suspension of chopped Balb/C mouse liver in 0.25 M-sucrose was homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle. The suspension was centrifuged at 800 g for 10 min to give a supernatant that was further centrifuged at 10 000 g for 15 min. The resulting supernatant was centrifuged at 105 000 g for 60 min to give a microsomal pellet, which was resuspended in 0.25 M-sucrose and once more pelleted by centrifugation at 105 000 g for 60 min. The final microsomal preparation was resuspended at 12 mg/ml of protein in 0.25 M-sucrose and layered on the top of a discontinuous sucrose gradient consisting of 2.0 M-sucrose (0.5 ml), 1.8 M-sucrose (0.5 ml), 1.6 M-sucrose (1.0 ml), 1.5 M-sucrose (0.5 ml), 1.4 M-sucrose (0.5 ml), 1.3 M-sucrose (0.5 ml), 1.2 M-sucrose (0.5 ml) and 1.0 M-sucrose (0.5 ml). Tubes were centrifuged at 80 000 g for 15 h (Spinco SW 50 rotor), and the separated fractions containing rough (interphase between 1.6 and 1.8 M-sucrose) and smooth (interphase between 1.2 and 1.3 M-sucrose) membranes were diluted to contain 0.25 M-sucrose. The membranes were then pelleted by centrifugation (105 000 g, 60 min). The preparations were examined in the electron microscope and found to conform to morphological predictions (S. De Petris, unpublished work).

Protein. This was determined (Lowry *et al.*, 1951) by using bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as the standard.

Reduction and reconstitution experiments. In the standard system, J chain at 0.2–0.8 mg/ml in 0.02 M-sodium phosphate, pH 7.2, was reduced at room temperature for 60 min with 10 mM-dithiothreitol and then added to a solution of high-specific-radioactivity immunoglobulin for a further 1 h. Unless specifically stated, no carrier immunoglobulin was

Fig. 1. Polymerization of reduced radioactive secreted 19S IgM (from TEPC 183 cells): analyses of samples on sodium dodecyl sulphate-polyacrylamide gels

The IgM was prepared from culture fluids of myeloma cells incubated with [4,5- ^3H]leucine by sucrose-density-gradient centrifugation, reduced with 1 mM-dithiothreitol in the presence or absence of J chain, passed through Sephadex G-25 and then incubated at 37°C for 30 min with or without smooth-endoplasmic-reticulum membranes (3 mg/ml). (a) Isolated 19S IgM; (b) isolated 19S IgM reduced in the absence of J chain and alkylated with iodoacetamide; (c) isolated 19S IgM reduced in the absence of J chain, passed through Sephadex G-25 and incubated with smooth-endoplasmic reticulum membranes; (d) isolated 19S IgM reduced in the presence of J chain,

added and so the concentration of immunoglobulin was very low. During reduction of the radioactive immunoglobulin the J chain was present at 80 $\mu\text{g/ml}$ and the dithiothreitol was 1–5 mM. As controls, J chain was either omitted or replaced by other proteins. Part of the reduction mixture was alkylated with a twofold molar excess of iodoacetamide to assess the extent of reduction of radioactive immunoglobulin; the remainder was separated from the reducing agent by filtration through Sephadex G-25 equilibrated with 0.02 M-sodium phosphate, pH 7.2. The radioactive protein collected from the Sephadex G-25 column was incubated at 37°C for 30 min with or without rough endoplasmic-reticulum membranes (5.0 mg/ml), or smooth endoplasmic-reticulum membranes (0.5–3.5 mg/ml), or total microsomal fraction, prepared as described above (2.0 mg/ml), or liver cell plasma membranes (0.5 mg/ml) (given to us by Dr. Howard Evans, National Institute of Medical Research, London N.W.7), or a purified preparation of the disulphide-exchanging enzyme (2.5 mg/ml). All membrane preparations were of murine origin, and the purified disulphide-exchanging enzyme was prepared from ox liver. When present, membranes were removed by centrifugation (100000g, 60 min), resulting in a loss of <10% of the radioactivity in the pellet. After the incubation at 37°C the samples were adjusted to contain 2% (w/v) sodium dodecyl sulphate and 0.1 M-iodoacetamide, heated for 1–2 min at 100°C and analysed by electrophoresis in sodium dodecyl sulphate-polyacrylamide gels.

Disulphide-interchange enzyme. The enzyme was prepared from an acetone-dried powder of ox liver microsomes by chromatography on Sephadex CM-50 (Gurari, 1969). We are grateful to Dr. R. B. Freedman (Biological Laboratory, University of Kent, Canterbury) for giving us a sample of the purified enzyme.

Polyacrylamide gel analysis. This was done with either the sodium dodecyl sulphate system (Summers *et al.*, 1965) or the alkaline-urea system (Reisfeld & Small, 1966) as previously indicated (Della Corte & Parkhouse, 1973). Results are plotted with the top of the gel to the left-hand side.

Results

Polymerization of IgM subunits

Secreted, radioactive 19S IgM (Fig. 1a) was depolymerized by reduction with dithiothreitol to give mainly 7S IgM (IgMs) and the HL subunit (Fig. 1b).

passed through Sephadex G-25 and incubated at 37°C for 30 min; (e) isolated 19S IgM reduced in the presence of J chain, passed through Sephadex G-25 and incubated with smooth-endoplasmic-reticulum membranes. Details are given in the text and the Materials and Methods section.

In the absence of any carrier protein, removal of the reducing agent by passage through Sephadex G-25 did not result in any significant change in the profile of subunits seen on polyacrylamide-gel electrophoresis, even when the sample was incubated with smooth-endoplasmic-reticulum membranes from mouse liver (Fig. 1c). If isolated J chain was present in the reduction mixture, then polymerization was total, but only occurred when the Sephadex G-25 eluate (Fig. 1d) was incubated with the membrane preparation (Fig. 1e). Thus polymerization of IgM subunits requires both J chain and a cell-membrane preparation. The polymerized material also behaved as 19S on the sucrose gradient.

Provided that J chain was present, the polymerization reaction occurred with smooth membranes at concentrations as low as 0.3 mg/ml. It also occurred with total microsomes (2 mg/ml) and plasma membranes (0.5 mg/ml), but not with rough-endoplasmic-reticulum membranes (5.0 mg/ml).

On the basis of previous findings (Williams *et al.*, 1968) these results suggested that the activity of the membrane fraction was due to the previously described disulphide-exchanging enzyme (Fuchs *et al.*, 1967). Our suspicion was confirmed when a purified sample of the enzyme was found to substitute for smooth-endoplasmic-reticulum membranes in the system. The activity of the enzyme in promoting polymerization of IgM subunits was abolished by heating at 56°C for 30 min.

In the experiments to be described below, there was 80 µg of J chain/ml in the reduction mixtures, and the enzyme was present at 2.5 mg/ml during the incubation period of 30 min at 37°C. However, polymerization did occur at concentrations of J chain and enzyme as low as 20 µg/ml and 0.5 mg/ml respectively. Complete polymerization of subunits was achieved after incubation for 20 min. As indicated in Fig. 1, similar polyacrylamide-gel profiles were consistently obtained with immunoglobulin samples reduced in the presence or absence of J chain, and alkylated or filtered through Sephadex G-25. Polymerization only occurred when J chain and the enzyme sample were provided. For simplicity in presentation these negative controls are therefore omitted from the remaining Figures.

Both MOPC 104E and TEPC 183 cells secrete small amounts of 7S IgM (about 2% of total secreted immunoglobulins). When this was isolated (Fig. 2a), reduced in the presence of J chain (Fig. 2b), filtered through Sephadex G-25 and incubated with the disulphide-exchanging enzyme, complete polymerization to 19S IgM occurred (Fig. 2c).

Similarly, isolated intracellular radioactive 7S IgM from TEPC 183 (and also from MOPC 104E) cells (Fig. 3a) could be totally polymerized after reduction (Fig. 3b) and treatment with the enzyme (Fig. 3c).

Fig. 2. Polymerization of reduced radioactive secreted 7S IgM (from MOPC 104E cells): analyses of samples on sodium dodecyl sulphate-polyacrylamide gels

The 7S IgM was prepared from culture fluids of myeloma cells incubated with [4,5-³H]leucine by sucrose-density-gradient centrifugation, reduced with 2 mM-dithiothreitol in the presence or absence of J chain, passed through Sephadex G-25 and then incubated at 37°C for 30 min with or without the disulphide-interchange enzyme (2.5 mg/ml). (a) Isolated 7S IgM; (b) isolated 7S IgM reduced in the presence of J chain and alkylated with iodoacetamide; (c) isolated 7S IgM reduced in the presence of J chain, passed through Sephadex G-25 and incubated with the disulphide-interchange enzyme (2.5 mg/ml). Details are given in the text and the Materials and Methods section.

In both cases, the presence of J chain and the enzyme were essential for polymerization.

Polymerization of IgA subunits

Secreted radioactive dimer IgA (Fig. 4a) was isolated and reduced (Fig. 4b). After passage over Sephadex G-25 and treatment with the enzyme there was complete polymerization to the dimer form, i.e. α₄ subunits (Fig. 4c).

Similarly, secreted radioactive monomer IgA (Fig. 5a) or radioactive intracellular monomer IgA

(Fig. 6a) yielded entirely dimer IgA after reduction (Figs. 5b and 6b), passage over Sephadex G-25 and enzyme treatment (Figs. 5c and 6c).

Again, in all these experiments both J chain and the enzyme were necessary for polymerization of the reduction mixture.

It was also possible to polymerize free α chains obtained by reduction of IgA isolated from serum. Free heavy and light chains were separated from J chains by preparative polyacrylamide-gel electro-

phoresis as described in the Materials and Methods section (Fig. 7a). Under the standard conditions that we used, a band of α_4 subunits, corresponding to IgA dimer, was obtained when J chain and the enzyme were provided (Fig. 7b). There was no polymerization if J chains (Fig. 7c) or the enzyme (Fig. 7d) were omitted. In this experiment, IgA and J chain were present at concentrations of 1.0 and 0.18 mg/ml respectively. Inspection of the gel (Fig. 7b) reveals that most of the α chain was converted into α_4 subunits. When the concentration of J chain was lowered, formation of α_4 subunits was partial

Fig. 3. Polymerization of reduced radioactive intracellular 7S IgM (from TEPC 183 cells): analyses of samples on sodium dodecyl sulphate-polyacrylamide gels

The sample was prepared from cell lysates of cultures incubated with [4,5- 3 H]leucine by sucrose-gradient centrifugation. After reduction with 2 mM-dithiothreitol in the presence or absence of J chain, samples were passed over Sephadex G-25 and incubated at 37°C for 30 min with or without the disulphide-interchange enzyme (2.5 mg/ml). (a) Isolated 7S intracellular IgM; (b) isolated 7S intracellular IgM reduced in the presence of J chain and alkylated with iodoacetamide; (c) isolated 7S intracellular IgM, reduced in the presence of J chain, passed through Sephadex G-25 and incubated with the disulphide-interchange enzyme. Details are given in the text and in the Materials and Methods section.

Fig. 4. Polymerization of reduced radioactive secreted dimer IgA (from MOPC 315 cells): analyses of samples on sodium dodecyl sulphate-polyacrylamide gels

The dimer IgA was prepared from culture fluids of myeloma cells incubated with [4,5- 3 H]leucine by sucrose-density-gradient centrifugation, reduced with 5 mM-dithiothreitol in the presence or absence of J chain, passed over Sephadex G-25 and then incubated at 37°C for 30 min with or without the disulphide-interchange enzyme (2.5 mg/ml). (a) Isolated dimer IgA; (b) isolated dimer IgA reduced in the presence of J chain and alkylated with iodoacetamide; (c) isolated dimer IgA reduced in the presence of J chain, passed through Sephadex G-25 and incubated with the disulphide-interchange enzyme. Details are given in the text and the Materials and Methods section.

Fig. 5. Polymerization of reduced, radioactive, secreted monomer IgA (from MOPC 315 cells): analyses of sample on sodium dodecyl sulphate-polyacrylamide gels

The monomer IgA was prepared from culture fluids of myeloma cells incubated with $[4,5\text{-}^3\text{H}]$ leucine by sucrose-density-gradient centrifugation, reduced with 5mM-dithiothreitol in the presence or absence of J chain, passed through Sephadex G-25 and then incubated at 37°C for 30 min with or without the disulphide-interchange enzyme (2.5mg/ml). (a) Isolated monomer IgA; (b) isolated monomer IgA reduced in the presence of J chain and alkylated with iodoacetamide; (c) isolated monomer IgA reduced in the presence of J chain, passed through Sephadex G-25 and incubated with the disulphide-interchange enzyme. Details are given in the text and the Materials and Methods section.

(J chain at 0.08mg/ml, Fig. 7e) or absent (J chain at 0.04mg/ml).

Incorporation of J chain into the polymerized molecule

Although we have demonstrated a requirement for J chain in the polymerization of subunits of polymeric immunoglobulins, it is critical to demonstrate that J chain is incorporated into polymer molecules.

To establish this point we took advantage of the fact that subunits of IgM prepared by reductive cleavage can be polymerized at high concentrations

Fig. 6. Polymerization of reduced radioactive intracellular 7S IgA (from MOPC 315 cells): analyses of samples on sodium dodecyl sulphate-polyacrylamide gels

The sample was prepared from cell lysates of cultures incubated with $[4,5\text{-}^3\text{H}]$ leucine by sucrose-gradient-centrifugation. After reduction with 2mM-dithiothreitol in the presence or absence of J chain, samples were passed through Sephadex G-25 and incubated at 37°C for 30 min with or without the disulphide-interchange enzyme (2.5mg/ml). (a) Isolated 7S intracellular IgA; (b) isolated 7S intracellular IgA reduced in the presence of J chain and alkylated with iodoacetamide; (c) isolated 7S intracellular IgA, reduced in the presence of J chain, passed through Sephadex G-25 and incubated with the disulphide-interchange enzyme. Details are given in the text and in the Materials and Methods section.

of carrier IgM in the reduction mixture (Parkhouse *et al.*, 1970; Askonas & Parkhouse, 1971). Under these conditions the endogenous J chain presumably is sufficient for the reassembly. Because the protein was reduced at a relatively high concentration of dithiothreitol (5mM), the disulphide-interchange enzyme was included to ensure complete reassembly.

Secreted radioactive IgM and purified serum IgM were obtained from mouse myeloma MOPC 104E. Radioactive IgM was mixed with carrier IgM at 2mg/ml and reduced with 5mM-dithiothreitol. A portion was alkylated (Fig. 8a), and the remainder was reassembled into IgM by filtration through Sephadex G-25 and incubation with the disulphide-

Fig. 7. Polymerization of free α chains: analyses of samples on sodium dodecyl sulphate-polyacrylamide gels

Isolated MOPC 315 myeloma protein was reduced and J chains were separated from heavy and light chains by preparative polyacrylamide-gel electrophoresis (see the Materials and Methods section). The mixture of single heavy and light chains at 1mg/ml was reduced with 1mM-dithiothreitol in the presence or absence of J chain and then incubated at 37°C for 30 min with the disulphide-interchange enzyme. (a) Single heavy and light chains, free of J chain; (b) single heavy and light chains, reduced in the presence of J chain (0.18mg/ml), passed through Sephadex G-25 and incubated with the disulphide-interchange enzyme; (c) single heavy and light chains, reduced in the absence of J chains, passed through Sephadex G-25 and incubated with the disulphide-interchange enzyme; (d) single heavy and light chains reduced in the presence of J chains (0.18 mg/ml), passed through Sephadex G-25 and incubated without the disulphide-interchange enzyme; (e) as in (b), but with J chains at 0.08 mg/ml. Details are given in the text and the Materials and Methods section.

exchanging enzyme (Fig. 8b). The reduced, alkylated and reassembled samples were electrophoresed in alkaline-urea-polyacrylamide gels to determine the amounts of free J chain. Further, to check for selective loss of J chain in our polymerization system *in vitro*, the reassembled IgM was reduced again with 5mM-dithiothreitol and the released J chain was measured by gel electrophoresis.

It is evident that reduction releases comparable amounts of radioactive J chain from IgM before (0.62% total radioactivity; Fig. 9a) or after (0.66%

Fig. 8. Polymerization of reduced, radioactive, secreted 19S IgM (from MOPC 104E cells): analyses of samples on sodium dodecyl sulphate gels

The IgM was prepared from culture fluids of myeloma cells incubated with [4,5-³H]leucine by sucrose gradient centrifugation, reduced with 5mM-dithiothreitol in the presence of carrier IgM (2 mg/ml), passed through Sephadex G-25 and then incubated at 37°C for 30 min with the disulphide-interchange enzyme (2.5 mg/ml). (a) Isolated 19S IgM reduced in the presence of J chain and alkylated with iodoacetamide; (b) isolated 19S IgM reduced in the presence of J chain, passed through Sephadex G-25 and incubated with the disulphide-interchange enzyme. Details are given in the text and the Materials and Methods section.

total radioactivity; Fig. 9b) reassembly. In the reassembled material (Fig. 9c), J chain could not be detected and must therefore have been entirely incorporated into the polymer molecule.

Specificity of the polymerization system

Neither purified mouse IgG_{2a} (mouse myeloma 5563) or albumin interfered with the polymerization reaction when added to the reduction mixture at 1mg/ml. In addition, reduced isolated radioactive secreted myeloma-5563 IgG_{2a} was not polymerized with J chain under standard conditions. In the latter experiments, the free γ heavy and light chains were only converted into a 7S molecule by the addition of homologous carrier protein (1 mg/ml) and this occurred in the presence or absence of the enzyme preparation.

Fig. 9. Presence of J chain in reduced and alkylated and reduced and reassembled radioactive secreted 19S IgM: analyses of samples on alkaline urea gels

The IgM was prepared, reduced and reassembled as given in the legend to Fig. 8. A portion of the reassembled IgM was reduced with 5mM-dithiothreitol and alkylated with iodoacetamide. (a) Isolated 19S IgM reduced in the presence of J chain and alkylated with iodoacetamide (cf. Fig. 8a); (b) isolated 19S IgM reduced and reassembled to IgM, then reduced again with 5mM-dithiothreitol and alkylated with iodoacetamide; (c) isolated 19S IgM reduced and reassembled (cf. Fig. 8b). Details are given in the text and the Materials and Methods section. The scale given by the left-hand axis (●) is expanded in the region of the J chain peak (○), as indicated by the right-hand axis.

Fig. 10. Simultaneous polymerization of reduced, radioactive 19S IgM (from TEP 183 cells) and IgA (from MOPC 315 cells): analyses of samples on sodium dodecyl sulphate-polyacrylamide gels

The specificity of polymerization was further emphasized by an experiment in which IgM and IgA were mixed, reduced and reassembled without the formation of hybrid molecules. Radioactive secreted 19S IgM was prepared from cells labelled with [^{14}C]leucine, and radioactive secreted IgA (monomer/dimer ratio 2) was prepared from cells labelled with [^3H]leucine. As judged by electrophoretic analysis both the [^{14}C]IgM (Fig. 10a) and [^3H]IgA (Fig. 10c) were depolymerized by reduction, but polymerized entirely specifically to ^{14}C -labelled 19S IgM (Fig. 10b) and ^3H -labelled dimer IgA (Fig. 10d).

The IgM was prepared from culture fluids of myeloma cells incubated with [$\text{U-}^{14}\text{C}$]leucine by sucrose-density-gradient centrifugation. The IgA, consisting of monomer and dimer in a ratio of 2, was prepared from culture fluids of myeloma cells incubated with [$4,5\text{-}^3\text{H}$]leucine. The ^3H -labelled IgA and ^{14}C -labelled IgM were mixed, reduced with 5mM-dithiothreitol in the presence of J chain, passed through Sephadex G-25 and then incubated at 37°C for 30min with the disulphide-interchange enzyme (2.5 mg/ml). The gel profiles have been plotted separately for ^3H and ^{14}C . Two gels were run, one giving profiles

Discussion

Under the conditions of our system *in vitro*, the covalent assembly of polymeric immunoglobulins requires J chains and an enzyme catalysing disulphide exchange. Both IgA and IgM could be polymerized from subunits prepared by reduction of polymer forms (Figs. 1, 4 and 7), and from intracellular (Figs. 3 and 6) or secreted monomer subunits (Figs. 2 and 5). In all cases the polymerization was total, there being no residue of the monomeric form. Reassembly did not occur in the absence of J chain and/or the enzyme. The fact that the J chain used for all the experiments was isolated from IgA supports the contention that the J chains in IgA and IgM are identical (Halpern & Koshland, 1970; Mestecky *et al.*, 1971; Morrison & Koshland, 1972).

In the absence of the disulphide-rearranging enzyme, IgM, but not IgA, can be polymerized from relatively high concentrations of subunits prepared by mild reduction (Parkhouse *et al.*, 1970; Askonas & Parkhouse, 1971; Parkhouse *et al.*, 1971). The importance of the enzyme is therefore underlined by the fact that low concentrations of both IgA and IgM subunits can be polymerized in the presence of the enzyme provided that J chain is supplied.

The J chain is not simply a catalyst for polymerization. All of the J chain released from IgM by reductive cleavage was incorporated back into reassembled polymer (Fig. 9). On the basis of these experiments, the J chain is therefore likely to be an essential structural requirement for polymeric immunoglobulins. In an abstract, however, there is the contrary claim, that human IgM polymer can be formed from reductive subunits in the absence of J chain (Kownatzki, 1972). The reason for this discrepancy may become apparent when more details are available.

A remarkable degree of specificity in polymerization was demonstrated. Not only did reduced albumin or IgG fail to interfere with polymerization, but IgA and IgM subunits could be simultaneously reassembled into specific polymer forms without the formation of hybrid molecules (Fig. 10). Further, reductive subunits of IgG could not be polymerized when J chain and the enzyme were supplied. The specificity for polymerization presumably resides in the amino acid sequence of the polypeptide chains.

In a previous publication evidence was presented to show that the cysteine residues responsible for

inter-7S-subunit linkage are blocked in intracellular 7S subunits of IgM (Askonas & Parkhouse, 1971). To provide further evidence for this point, intracellular 7S subunits were mixed with J chain and the disulphide-interchange enzyme without reduction, or were reduced, passed through Sephadex G-25 and then mixed with J chain and the disulphide-interchange enzyme. In neither case did polymerization result, and we attribute this finding to oxidation of the J chain during preparation and storage. Consequently in all experiments described in the present paper the J chain was reduced before reaction with immunoglobulin.

It is interesting that intracellular and secreted monomer subunits can be completely polymerized. Apart from indicating a mandatory role for J chain in assembly, these findings question a role for carbohydrate in polymerization and demonstrate unequivocally that secreted IgA monomers are certainly capable of further assembly. The similarity in carbohydrate composition between secreted IgA monomers and dimers has already promoted the suggestion that carbohydrate does not control polymerization (Della Corte & Parkhouse, 1973). Perhaps the strongest argument against carbohydrate addition as a controlling factor is the observation that intracellular monomers of IgA and IgM could be completely polymerized. And yet in both cases the intracellular molecules are deficient in fucose and galactose (Parkhouse & Melchers, 1971; Della Corte & Parkhouse, 1973; Parkhouse, 1973). A possible role, as yet untested, for the addition of terminal fucose could be release of immunoglobulin from the cell surface.

Previous studies have led to the conclusion that polymerization of IgM and IgA occurs close to the time of secretion (Parkhouse & Askonas, 1969; Bevan, 1971; Parkhouse, 1971a,b; Askonas & Parkhouse, 1971; Bargellesi *et al.*, 1972; Della Corte & Parkhouse, 1973). At this time, J chain is incorporated into the molecule. Given this, and assuming that the J chain travels from its site of synthesis via the same route as heavy and light chains (i.e. through the membranous elements of the cell), then an important factor in control of polymerization may be the lack of non-covalent interaction between J chain and intracellular 7S subunits (Della Corte & Parkhouse, 1973). The presence of disulphide-interchange activity in the plasma membrane may also be important. Since the Golgi zone contains large amounts of disulphide-interchange enzyme (Williams *et al.*, 1968), the absence of large quantities of intracellular polymer is possibly explained by segregation of subunits (J chain and 7S immunoglobulin) until the assembly site is reached.

In all of the experiments with radioactive immunoglobulin subunits the J chain was the only carrier protein added and at concentrations of <10 µg/ml

(a) and (c) and the other giving profiles (b) and (d). (a) Isolated ^{14}C -labelled IgM, reduced and alkylated with iodoacetamide; (b) Isolated ^{14}C -labelled IgM, reduced and reassembled; (c) isolated ^3H -labelled IgA, reduced and alkylated with iodoacetamide; (d) isolated ^3H -labelled IgA, reduced and reassembled. Details are given in the text and the Materials and Methods section.

polymerization did not occur. At such concentrations we may safely assume that J chain was present in excess of the amounts required for polymerization. However, a certain minimum protein concentration has previously been shown to be essential for polymerization of IgM subunits (Askonas & Parkhouse, 1971). An estimate of the relative concentration of J chain required for polymerization can be made from the experiments with isolated MOPC 315 myeloma polypeptide chains. Taking molecular weights of 54500, 23000 and 16800 for heavy, light (Underdown *et al.*, 1971) and J chains (R. M. E. Parkhouse, unpublished work) of MOPC 315 myeloma protein, the weight ratio of (α_4L_4)/J is 18.5. As shown in Fig. 7, the isolated polypeptide chains could be partially polymerized when the weight ratio of (α_4L_4)/J was 12.5. Polymerization was essentially complete with (α_4L_4)/J at 5.5. Thus under conditions *in vitro*, presumably less efficient than those operating within a cell, polymerization does not require a vast excess of J chain. Further, it is possible that only a fraction of the isolated J chain added in these experiments is in the native form and hence suitable for inclusion in polymer molecules. Finally, the experiment showing quantitative incorporation of released J chain into reassembled polymer (Figs. 8 and 9) demonstrates that an excess of J chain is not required for IgM polymerization.

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References

- Askonas, B. A. & Parkhouse, R. M. E. (1971) *Biochem. J.* **123**, 629–634
- Bargellesi, A., Periman, P. & Scharff, M. D. (1972) *J. Immunol.* **108**, 126–134
- Bevan, M. J. (1971) *Eur. J. Immunol.* **1**, 133–138
- Brownstone, A. D. (1969) *Anal. Biochem.* **27**, 25–46
- Cowan, N. J. & Robinson, G. B. (1972) *Biochem. J.* **126**, 751–754
- Della Corte, E. & Parkhouse, R. M. E. (1973) *Biochem. J.* **136**, 589–596
- Fahey, J. L. (1961) *J. Exp. Med.* **114**, 399–413
- Fahey, J. L. (1963) *J. Clin. Invest.* **42**, 111–123
- Fuchs, S., De Lorenzo, F. & Anfinsen, C. B. (1967) *J. Biol. Chem.* **242**, 398–402
- Gurari, D., (1969) Ph.D. Thesis, University of London, pp. 36–50
- Halpern, M. S. & Koshland, M. E. (1970) *Nature (London)* **228**, 1276–1278
- Kownatzki, E. (1972) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **31**, Abstr. 3043
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mestecky, J., Zikan, J. & Butler, W. T. (1971) *Science* **171**, 1163–1165
- Metzger, H. (1970) *Advan. Immunol.* **12**, 57–116
- Morrison, S. L., Koshland, M. E. (1972) *Proc. Nat. Acad. Sci. U.S.A.* **69**, 124–128
- Parkhouse, R. M. E. (1971a) *Biochem. J.* **123**, 635–641
- Parkhouse, R. M. E. (1971b) *FEBS Lett.* **16**, 71–73
- Parkhouse, R. M. E. (1972) *Nature (London) New Biol.* **236**, 9–11
- Parkhouse, R. M. E. (1973) *Transplant. Rev.* **14**, 131–144
- Parkhouse, R. M. E. & Askonas, B. A. (1969) *Biochem. J.* **115**, 163–169
- Parkhouse, R. M. E. & Melchers, F. (1971) *Biochem. J.* **125**, 235–240
- Parkhouse, R. M. E., Askonas, B. A. & Dourmashkin, R. R. (1970) *Immunology* **18**, 575–584
- Parkhouse, R. M. E., Virella, G. & Dourmashkin, R. R. (1971) *Clin. Exp. Immunol.* **8**, 581–591
- Reisfeld, R. A. & Small, P. A. (1966) *Science* **152**, 1253–1255
- Summers, D. F., Maizel, J. V. & Darnell, J. E. (1965) *Proc. Nat. Acad. Sci. U.S.A.* **54**, 505–513
- Underdown, B. J., Simms, E. S. & Eisen, H. N. (1971) *Biochemistry* **10**, 4359–4368
- Vogt, M. & Dulbecco, R. (1963) *Proc. Nat. Acad. Sci. U.S.A.* **49**, 171–179
- Williams, D. J., Gurari, D. & Rabin, B. R. (1968) *FEBS Lett.* **2**, 133–135