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Immunoglobulin M Biosynthesis

INTRACELLULAR ACCUMULATION OF 7s SUBUNITS

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Immunoglobulin M biosynthesis was studied with mouse plasma cell tumour MOPC 104E as a model system. Cell suspensions prepared from solid tumours were incubated *in vitro* with tritiated leucine; the radioactivity incorporated into intracellular and secreted proteins was analysed by polyacrylamide-gel electrophoresis, sucrose-density-gradient centrifugation and precipitation with rabbit antiserum specific for the macroglobulin. The tumour was found to secrete immunoglobulin M and light chains in a 1:2 weight ratio, with lag periods of 20–30min. Within the cells there was a 7s component precipitable with specific antiserum to the macroglobulin that was shown to consist of heavy and light chains. This 7s subunit of the macroglobulin appeared to accumulate in the intracellular environment, so that even after long periods of incubation (3hr.) no more than trace amounts of fully assembled 19s molecules could be detected in cell lysates. Polymerization of the subunits into the pentamer therefore appears to take place shortly before, or simultaneously with, secretion of the molecules.

Studies on the structure of human IgM* have indicated that the 19s molecule is composed of five subunits, termed IgMs (Miller & Metzger, 1965*a*), with sedimentation coefficients about 7s. Each subunit consists of two heavy chains (μ-chains) and two light chains (κ or λ) (Miller & Metzger, 1965*a*; Lamm & Small, 1966), although the presence of a third light chain has been suggested (Suzuki & Deutsch, 1967). The light chains are common to all immunoglobulin classes, but the μ-chain is unique in its molecular weight, carbohydrate content and antigenic character (see review by Cohen & Milstein, 1967). The basic four-chain structure of IgMs is similar to that suggested for IgG (Fleischman, Porter & Press, 1963) in that all light and heavy chains are covalently linked by disulphide bonds (Miller & Metzger, 1965*a*; Lamm & Small, 1966). The IgM molecule consists of five IgMs subunits linked via disulphide bonds (Miller & Metzger, 1965*b*), which appear to form a circular structure (Svehag, Chesebro & Bloth, 1968). Selective reduction of inter-subunit disulphide bonds yields IgMs (Miller & Metzger, 1965*b*), and the linkage, involving only one cysteine residue per heavy chain (Morris & Inman, 1968), is located in the Fc part of the molecule (Mihaesco & Mihaesco, 1968). One of the two cysteine residues in the Fc part of the heavy chain is situated at, or close to, the *C*-terminus (Doolittle, Singer & Metzger, 1966; Abel & Gray, 1967); which residue is involved in inter-subunit linkage is not yet known.

From the structure, then, the molecule should be decavalent, but reports on the valency of IgM and IgMs have been contradictory, suggesting either one or two antigen-binding sites per subunit (for review see Sela, 1968). However, a human pathological IgM has been found to have ten equal antigen-binding sites for the dinitrophenol group (Ashman & Metzger, 1969).

Our interest lies in the method by which the very large IgM molecule is accurately assembled and efficiently secreted. Mouse plasma cell tumour MOPC 104E (McIntire, Asofsky, Potter & Kuff, 1965), which secretes both IgM and light chains, was used as a model system. Cells in suspension were 'pulsed' with tritiated leucine for various time-intervals in order to study intracellular intermediates and secretion of the final product. The results indicate that IgMs subunits accumulate within the cell and are the biosynthetic intermediates for IgM production, and that the subunits are polymerized very shortly before, or simultaneously with, secretion.

* Abbreviations: IgM, immunoglobulin M; IgG, immunoglobulin G.

MATERIALS AND METHODS

The MOPC 104E plasma cell tumour was kindly sent to us by Dr M. Potter and maintained in Balb/C mice by subcutaneous transfer of 1mm.^{3} pieces from tumours grown 3

weeks after transplantation. Unless otherwise stated, all the experiments described in this paper were done with this tumour line between transplant generations 15 and 30, the cells secreting IgM and light chains of the λ-type (McIntire *et al.* 1965).

The same tumour that had been received in 1966 from Dr M. Potter in generation 22 and carried through 25 transplantation generations in this laboratory was found to secrete much less IgM and mainly light chains, and was only used where stated in the text.

Preparation of serum IgM (*MOPC* 104*E*). The IgM was prepared from serum by a simple two-step procedure. Whole serum (1ml./tube) was fractionated on a linear 10–30% (w/v) sucrose gradient in phosphate-buffered saline (130mM-NaCl–4mM-KCl–10mM-sodium phosphate), pH7·4 (Spinco no. 30 rotor, 27500rev./min. for 16½hr.). The fastest-moving E_{280} peak consisted mainly of IgM and α_2-macroglobulin, and was concentrated by ultrafiltration with dialysis and purified by preparative polyacrylamide-gel electrophoresis [3% (w/v) acrylamide, 16mM-tris–50mM-glycine buffer, pH8·7] (Brownstone, 1969). This yielded 5–7mg. of IgM/ml. of whole serum. Analysis by polyacrylamide-gel electrophoresis showed a single protein band.

Preparation and standardization of antisera. Rabbits were immunized with 1–2mg. of the purified IgM in complete Freund's adjuvant. The final volume of the emulsion was 2·0ml., and 0·5ml. portions were injected into the left and the right thigh on days 1 and 7 respectively. The remaining antigen was injected subcutaneously in several sites after a further 5 weeks. The rabbits were 'boosted' 10 weeks after the final injection of Freund's adjuvant, by five intravenous injections (a total of 2mg. of alum-precipitated IgM over 10 days). The antibody content of the serum was determined by precipitation with various amounts of IgM, and antibody was found to react with IgM and λ-chains.

Labelling in vitro *of intracellular and extracellular protein by plasma-cell tumour MOPC* 104*E*. Mice were killed by cervical dislocation. The tumours were aseptically removed, rinsed in cold Eagle's medium and gently teased into medium with forceps. Care was taken to avoid teasing the necrotic tissue. The larger aggregates were allowed to settle out in a conical centrifuge tube; the cells recovered from the supernatant by centrifugation (600*g* for 5min.) were resuspended in medium at a density of 3×10^7–4×10^7 cells/ml. and incubated at 37° with [4,5-^{3}H]leucine (The Radiochemical Centre, Amersham, Bucks.). As a routine 1·0ml. of the cell suspension was incubated in a plastic Falcon Petri dish (35mm.×10mm.) in an atmosphere of 5% CO_2 in air. Separate dishes were set up for different time-points. At the end of the incubation period, the cell suspension was rapidly collected and cooled in iced water, and the supernatant and cell pellet were harvested by centrifugation (2000*g* for 3min.).

Extracellular protein. The supernatant was adjusted to contain L-leucine at 1·0mg./ml. and IgM at 50–100μg./ml., clarified by centrifugation (19000*g* for 10min.), and the free radioactive leucine was removed by dialysis against 0·05M-sodium phosphate buffer, pH7·4, or by passage over a column of Sephadex G-25 (Pharmacia, Uppsala, Sweden) equal to 10–15 times the sample volume. Separation of the radioactive extracellular IgM and λ-chain was achieved by centrifugation in a linear 10–30% (w/v) sucrose gradient (100000*g* for 16–18gr., Spinco SW50 rotor). Fractions are numbered from the bottom of the tube to the top.

Intracellular protein. The cell pellet was washed once in medium (2·0ml.) and resuspended in phosphate-buffered saline containing 0·25M-sucrose, 1% (v/v) Nonidet P.40 (an alkyl phenyl ethoxylate non-ionic detergent kindly given by Shell Chemicals Ltd., London W.C.2) (Borun, Scharff & Robbins, 1967), 0·2M-iodoacetamide, 5mM-$MgCl_2$, L-leucine at 1mg./ml. and IgM at 50–100μg./ml. The solution was centrifuged at 100000*g* for 90min. The supernatant was freed of radioactive leucine by dialysis or by passage over Sephadex G-25 as described above. About 90% of the radioactive protein was recovered from the Sephadex G-25 column, and no protein was held back preferentially since dialysed material gave identical results.

Removal of the detergent was considered to be essential before further studies, since addition of Nonidet P.40 to extracellular secretions caused reversible aggregation of the light chains as judged by polyacrylamide-gel electrophoresis.

Immunological analysis of intracellular and extracellular proteins. Radioactively labelled proteins were analysed by polyacrylamide-gel electrophoresis (see below) or by sucrose-gradient fractionation followed by immunological precipitation with antiserum to IgM. Maximum separation of IgM and any subunits was achieved on sucrose gradients [5%, 12%, 20% and 30% (w/v) sucrose in 0·15M-NaCl buffered with 25mM-tris–HCl buffer, pH8·0, run at 100000*g* for 16hr. in a Spinco SW39 rotor). Radioactivity in IgM determinants (light and heavy chains) was assayed in the presence of carrier IgM by precipitation with antiserum to IgM, which also reacted with light chain. Alternatively an indirect precipitation procedure was used. After incubation with 10μl. of rabbit anti-IgM, antibody–antigen complexes were precipitated with excess of goat anti-(rabbit IgG). Antibody–antigen precipitates formed by anti-(rabbit IgG) and rabbit IgG in similar proportions served as controls. The antibody–antigen precipitates were washed twice with 0·9% NaCl in the cold. Radioactivity in the total protein was determined by precipitating the protein with 10% (w/v) trichloroacetic acid in the presence of 0·5mg. of bovine serum albumin. When Nonidet P.40 was present, the first acid precipitation was done with 2·5% (w/v) perchloric acid. All radioactive protein samples were then washed by three cycles of 0·15M-NaOH dissolution and trichloroacetic acid precipitation, dissolved in 0·3ml. of formic acid and counted at 10% efficiency in Bray's or Kinard's fluid (Kinard, 1957).

Polyacrylamide-gel electrophoresis. For analytical purposes electrophoresis was carried out in 8cm. gel columns in glass tubes (0·6cm. internal diam.) on 4·25% (w/v) acrylamide, with 2·7% cross-linking, for 4hr. (10mA/tube). The buffer contained 0·1% sodium dodecyl sulphate, 0·5M-urea and 0·1M-sodium phosphate buffer, pH7·2 (Summers, Maizel & Darnell, 1965). The presence of sodium dodecyl sulphate in the gels results in a separation that is primarily a function of the molecular size of the components analysed.

For analysis of radioactive samples electrophoresis was performed for 4hr. (10mA/tube) in 4·25% (w/v) acrylamide in the same buffer as above, with ethylene diacrylate as the cross-linking agent (Choules & Zimm, 1965). The gels were sliced into 1mm. slices, dissolved and counted in Kinard's scintillation fluid (Choules & Zimm, 1965). Fractions are numbered from the negative to the positive electrode.

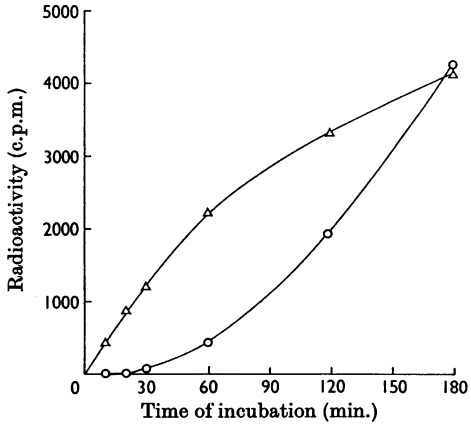

Fig. 1. Kinetics of leucine incorporation. MOPC 104E cells were incubated with [4,5-^{3}H]leucine (25 μc/ml., 1 c/m-mole). Equivalent portions of the total cell suspension and cell-free supernatant were assayed for radioactive protein by acid precipitation. The incorporation of [^{3}H]leucine into the cells was given by the difference between the two values. △, 10^{-1} × Radioactivity of total cellular protein; ○, radioactivity of IgM and light chain secreted into the extracellular fluid and precipitated with antibody to IgM.

RESULTS

Kinetics of leucine incorporation in vitro. Results presented in Fig. 1 show radioactivity of total cellular protein (acid-insoluble fraction) at various time-intervals and radioactivity of secreted IgM plus light chains precipitated with antiserum to the IgM. The rate of incorporation of [^{3}H]leucine into cellular protein is linear for the first hour, after which time it gradually decreases, presumably owing to changes in specific radioactivity of the leucine, limiting amounts of radioactive leucine or suboptimum culture conditions. After the first 20 min. IgM plus light-chain determinants (precipitable with specific antiserum) represent about 25% of the intracellular radioactive protein.

The appearance of detectable radioactive IgM or light chain secreted from the cell was delayed for 20–30 min.; equilibrium labelling of the intracellular IgM pool took about 1 hr. and after that time-interval the rate of secretion of radioactive IgM determinants remained constant for 3–4 hr. During the time-periods studied the cells were therefore actively secreting IgM and light chains.

Analysis of secretion products. Polyacrylamide-gel electrophoresis of radioactive tissue-culture supernatant demonstrated the major radioactive proteins to be IgM and light chains (Fig. 3c). The identity of the components was checked by sucrose-gradient centrifugation and co-precipitation with antisera to IgM (Fig. 6c). Although both IgM and light chains were always present as early as 30 min. after commencement of tissue culture, the light chains/IgM radioactivity ratio was higher at the early time-points than after 2–3 hr. (Table 1). This suggests a slightly longer lag period for secretion of the radioactive large 19s molecules compared with that of the smaller light chains. The ratio at 2 hr. has stayed approximately constant for 15 transplant generations. Assuming a molecular weight of 130 000 for two μ-chains, and 50 000 for two λ-chains, the extracellular fluid contains approximately an eightfold molar excess of light chains. Whether this imbalance in the production of light chains and heavy chains is due to the presence in the cell population of mutant cells forming only light chains (Schubert & Horibata, 1968) or is produced in individual cells is not clear as yet. Significant amounts of incomplete IgM molecules were not detected in the extracellular fluid (Fig. 3c).

Table 1. *Relative amounts of IgM and light chains secreted by plasma cells MOPC 104E at various time-intervals*

The cells were incubated with [^{3}H]leucine (100 μc/ml., 14·7 c/m-mole), and the relative radioactivity of IgM and light chain in the extracellular fluids was analysed by electrophoresis in soluble polyacrylamide gels (see the Materials and Methods section). The radioactivity counts comprising the IgM and light chain peaks were summed; radioactivity of IgM plus light chain represented 100%.

	Relative radioactivity (%)		
Time of secretion (min.) ...	30	60	120
IgM	13	18	37
Light chain	87	82	63

Intracellular IgM determinants. Analysis of radioactive intracellular extracts by sucrose-gradient fractionation demonstrated the presence of light chains and a component that resembled 7s IgG in its sedimentation characteristics. Both components were precipitated by specific antiserum to the IgM (Fig. 2).

To correlate our two methods of analysis, i.e. sucrose-gradient fractionation and analysis of polyacrylamide-gel electrophoresis, radioactive protein from the 7s region of the gradient (tube 8) was analysed by polyacrylamide-gel electrophoresis (Fig. 3a). A large radioactive peak can be observed with mobility similar to that of 7s IgG under these electrophoretic conditions. The pattern of radioactive protein in the intracellular extract also shows such a component (Fig. 3b), but little or no IgM. The position of secreted IgM and the light chains under these conditions of polyacrylamide-gel electrophoresis is also shown (Fig. 3c).

In the experiments described above (Figs. 2 and 3)

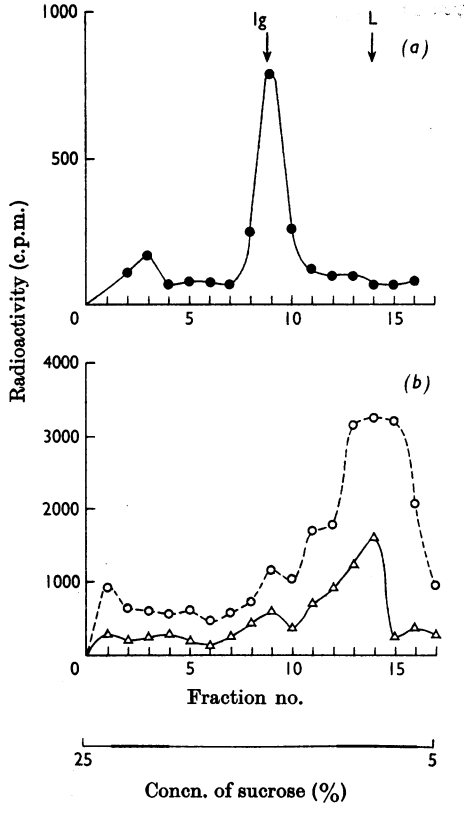

Fig. 2. Sucrose-gradient fractionation of intracellular proteins. MOPC 104E cells were labelled for 10min. with [4,5-^{3}H]leucine (100μc/ml., 14·7c/m-mole) and intracellular proteins were fractionated on a gradient [5%, 12%, 18% and 25% (w/v) sucrose in0·15M-NaCl–30mM-tris–HCl buffer, pH8] at 36000rev./min. for 16hr. in a Spinco SW39 rotor. △, Radioactivity of protein with IgM and light-chain (L) determinants, precipitated with rabbit antibody to IgM and anti-(rabbit IgG); ○, trichloroacetic acid-precipitable protein; ●, ^{3}H-labelled extracellular G-myeloma protein 5563 served as marker in a separate tube.

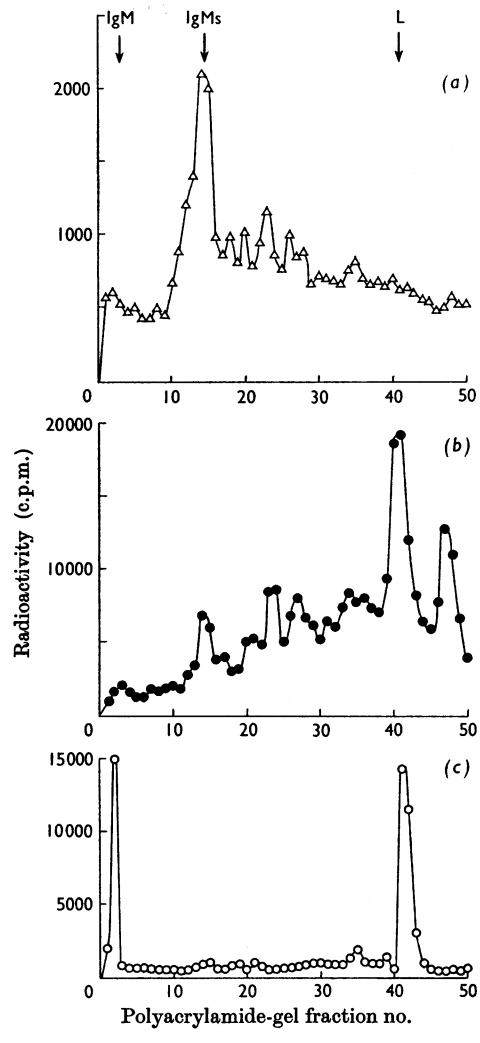

Fig. 3. Polyacrylamide-gel fractionation of intracellular protein. Radioactive proteins were analysed by electrophoresis in 4·25% polyacrylamide gels in sodium dodecyl sulphate–urea, as described in the Materials and Methods section. (*a*) Radioactive protein in the 7s region (tube 8) from sucrose-gradient fractionation of the 10min. intracellular protein (shown in Fig. 2). (*b*) Intracellular extract from a 10min. pulse (see legend to Fig. 2). (*c*) Culture supernatant after a 2hr. incubation of MOPC 104E cells with [4,5-^{3}H]leucine (25μc/ml., 14·7c/m-mole).

the cell extract was prepared from cells lysed in the presence of 0·2M-iodoacetamide. In the absence of iodoacetamide, however, the pattern of radioactivity on polyacrylamide gel or sucrose density gradient was exactly the same. Only trace amounts of the 7s component were observed in lysates of the tumour variant that secreted very small amounts of IgM.

Isolation and identification of IgMs. Plasma tumour cells MOPC 104E were incubated for 60min. at 37° in the presence of [^{3}H]leucine (25μc/ml., 14·7c/m-mole) and intracellular proteins extracted as before. The 7s component with IgM determinants was isolated after electrophoresis on eight polyacrylamide gels. One gel was analysed for location of the 7s component. Slices of the other seven gels corresponding to the IgMs peak were pooled, macerated in 0·1M-sodium phosphate buffer, pH7·4, containing 0·1% of sodium dodecyl sulphate, and protein was eluted overnight in the cold. We looked for heavy-chain and light-chain content of the 7s protein after reduction. Samples were adjusted to pH8·4 with tris buffer of that pH,

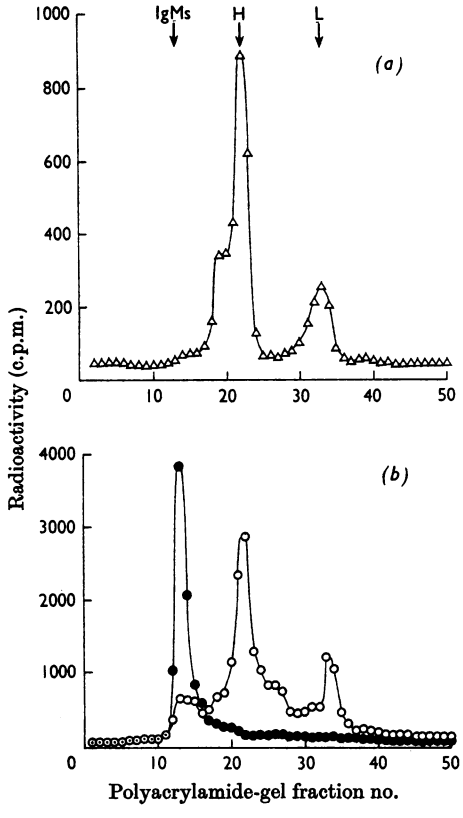

Fig. 4. Comparison of reduction products of extracellular IgM and the 7s intracellular component by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate–urea–phosphate buffer. The 7s radioactive intracellular component was isolated as described in the text. (*a*) [4,5-^{3}H]-Leucine-labelled secreted IgM, prepared by gradient centrifugation, and reduced and alkylated as described in the text (△). (*b*) Intracellular 7s component before (●) and after (○) treatment with β-mercaptoethanol.

reduced with β-mercaptoethanol (final concentration 0·1 M) for 1 hr. at room temperature and then alkylated in the cold with a 50% molar excess of iodoacetamide. A control sample was treated in the same way, except for omission of the β-mercaptoethanol. The control sample showed a homogeneous 7s component (Fig. 4*b*). On reduction the material was converted into two components with the electrophoretic mobilities of heavy chains and light chains obtained by reduction of isolated extracellular radioactive IgM (Figs. 4*a* and 4*b*). The light chains were present in approximately the expected yields of about 25% of radioactivity. The 7s component that is precipitated with antiserum to IgM (Fig. 2) thus appears to be equivalent to IgMs (the IgM subunit containing two heavy chains and two light chains linked by disulphide bonds).

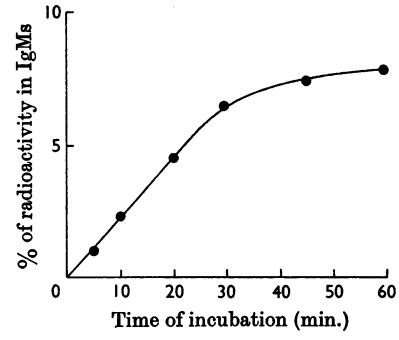

Fig. 5. Proportion of IgMs within cells. MOPC 104E cells were incubated with [4,5-^{3}H]leucine (100 μc/ml., 1·7 c/m-mole). Cell extracts were prepared at various time-intervals and analysed by polyacrylamide-gel electrophoresis. The proportion of IgMs was obtained by expressing the radioactivity in the IgMs peak as a percentage of the total radioactivity recovered from the gel.

Intracellular build-up of IgMs. To estimate the relative amount of IgMs within the cell, intracellular proteins prepared after various pulse times with [^{3}H]leucine (5–60 min.) were analysed by polyacrylamide-gel electrophoresis, and relative radioactivity in IgMs was calculated from the gel patterns. The radioactivity of IgMs relative to that of the total soluble intracellular protein increases for 30 min. (Fig. 5), presumably because leucine in the different cell compartments is not equilibrated. Between 30 and 60 min. it remains at a constant value of about 7% of the total radioactivity. It is noteworthy that up to 1 hr. there was no significant accumulation of IgM within the cell. Once more, only trace amounts of IgM could be detected within the cells after longer periods of incubation, i.e. 3 hr., whereas at such times the culture fluid essentially contains only IgM and light chains (Figs. 6*a*, 6*b*, and 6*c*).

To check that lack of IgM molecules within the cell was not due to loss of the material, recovery of radioactive IgM in the cell extracts was checked by adding radioactive extracellular IgM before lysis of the cell and applying the same methods for preparation and analysis of the intracellular protein extracts. Under these conditions the recovery of IgM was 80% of the added radioactivity; polyacrylamide-gel electrophoresis showed that the material travelled as IgM, demonstrating that the IgMs component observed in cell lysates was not due to degradation of IgM.

DISCUSSION

The mouse plasma cell tumour used for these studies, MOPC 104E, was found to secrete both light chains and IgM in a weight ratio of about 2:1.

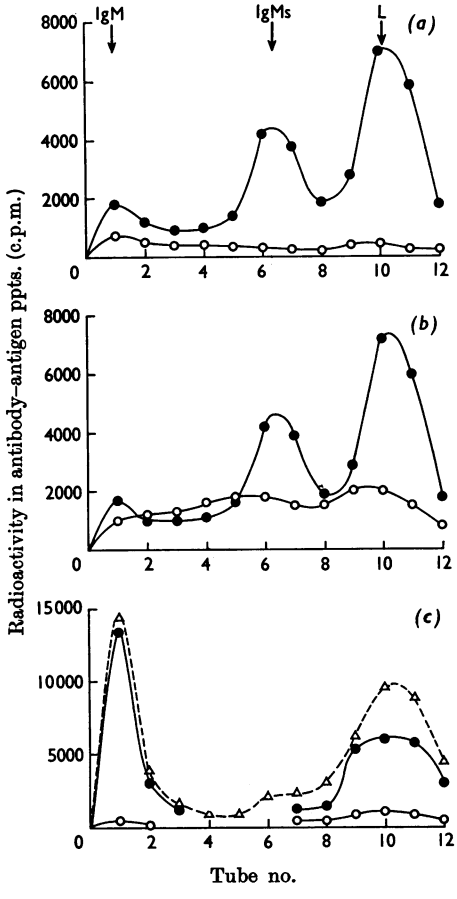

Fig. 6. IgM, IgMs and light chains in intracellular and extracellular fluids after incubation for MOPC 104E for 1 and 3hr. with [4,5-^{3}H]leucine (50μc/ml., 1c/m-mole). Radioactive proteins were fractionated on 5–30% (w/v) sucrose gradients at 36000rev./min. for 16½hr. in a Spinco SW39 rotor. △ Total protein precipitable with trichloroacetic acid (c.p.m.); ●, radioactivity precipitated with specific anti-(mouse IgM) (c.p.m.); ○, radioactivity precipitated with anti-(rabbit IgG) (c.p.m.) (control precipitates). (*a*) Intracellular extract after incubation for 1hr.; (*b*) intracellular extract after incubation for 3hr.; (*c*) extracellular fluid after incubation for 3hr.

This ratio remained unchanged between transplantation generations 15–30, during which time our studies were carried out. On the other hand, changes in this tumour line can occur. A sample of tumour MOPC 104E we received 2 years earlier evolved somewhere between transplantion generations 22–47 into a variant secreting only small amounts of IgM. Schubert & Horibata (1968) have reported a similar conversion of tumour MOPC 104E at transplantation generation 9, and suggested that the tumour contained distinct mutant cell populations, one type secreting IgM and another only light chains. An alternative explanation could be an imbalance in the rate of synthesis of heavy chains and light chains within a given cell. At present we do not yet know which alternative applied to the tumour studied in this work.

The lag period in secretion of radioactive IgM and light chain into the culture supernatant was 20–30min., as previously found for 7s molecules (Askonas & White, 1956; Helmreich, Kern & Eisen, 1961). The higher light chain/IgM radioactivity ratio at earlier times of incubation (30–60min.; Table 1) suggests that secretion of IgM molecules has a slightly longer lag than that of light chains.

In contrast with this extracellular picture (secretion of IgM and light chains), we could find remarkably few completed IgM molecules within the cells incubated for as long as 3hr. Most of the radioactive protein precipitable with antibody to mouse IgM consisted of light chains and a 7s component assayed after sucrose-gradient fractionation. This 7s component could be identified as IgMs since on reduction it yielded light chains and heavy chains. The 7s subunit was not an artifact of the lysed cell preparation; added radioactive IgM was fully recovered as IgM, and did not split into 7s subunits during the extraction of intracellular proteins. In the MOPC 104E tumour line that had partially dedifferentiated and was producing mainly light chain and only trace amounts of IgM only very small amounts of IgMs could be detected within the cells.

The results therefore clearly show that IgMs builds up within the IgM-secreting cell and is an intermediate in IgM assembly. Since only few IgM molecules appear to be present within the cell even after 3hr. incubation (Fig. 6), the polymerization of the subunits to the pentamer and the exit of the molecule to the exterior milieu must be closely linked, if not simultaneous, events. The processes of polymerization and secretion are therefore considered to be intimately related, affording a mechanism for the selective secretion of the large IgM molecule.

The cell compartment in which the polymerization occurs is not yet known, but it is striking that there is no secretion of intermediate forms (dimers, trimers etc.). Although non-covalent interactions between the subunits are not sufficiently strong to maintain a sedimentation coefficient of 19s in solutions of mildly reduced IgM at neutral pH (Miller & Metzger, 1965*a*), under appropriate conditions reconstitution to the intact molecule can occur. As a result, spontaneous formation of polymeric forms of the IgMs might have been expected when the cells were lysed in the absence of iodoacetamide, but this was not observed.

We have no information to suggest exactly what controls the final polymerization of IgMs to IgM, although attachment of carbohydrate or an enzyme catalysing disulphide interchange are obvious candidates for investigation. Whatever the mechanism is, there is good evidence that the polymerization mechanism can be defective, since serum IgMs is found in normal individuals and in a variety of human pathological conditions (Killander, 1963; Rothfield, Frangione & Franklin, 1965; Klein, Mattern, Radema & van Zwet, 1967; Solomon & Kunkel, 1967; Stobo & Tomasi, 1967) and in elasmobranch fishes (Marchalonis & Edelman, 1965; Clem & Small, 1967; Clem, de Boutaud & Sigel, 1967).

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