

PLASMA CELL IMMUNOGLOBULIN M MOLECULES

Their Biosynthesis, Assembly, and Intracellular Transport

A. TARTAKOFF and P. VASSALLI, with the technical assistance of M. DÉTRAZ

From the Department of Pathology, University of Geneva, Faculty of Medicine, 1211 Geneva 4, Switzerland

ABSTRACT

Immunoglobulin M (IgM)-secreting murine plasmablasts have been used to explore the cytologic site(s) of the successive modifications of the polypeptide H and L chains (steps of glycosylation, chain assembly, and polymerization) which occur during intracellular transport (ICT) and the interrelationships between these events. A combination of pulse-chase biosynthetic labeling protocols (using amino acids and sugars), subcellular fractionation, and electron microscope autoradiography was used in conjunction with inhibitors of glycosylation and agents (carboxyl cyanide *m*-chlorophenyl hydrazone [CCCP] and monensin) which block Ig exit from the rough endoplasmic reticulum (RER) or Golgi cisternae. The data are consistent with the following conclusions: (1) Sugar addition and modification occur in three main steps: (a) en bloc addition of core sugars to nascent H chains, (b) partial trimming of these oligosaccharide chains in the RER, (c) quasicordinated addition of terminal sugars (galactose, fucose, and sialic acid) in a very distal compartment between monensin-sensitive Golgi cisternae and the cell surface. (2) H and L chain assembly occurs between nascent H chains and a pool of free light chains present in the RER, followed by interchain disulfide bonding and rapid assembly of monomers into J chain-containing pentamers in the RER. Small amounts of various apparently non-obligatory intermediates in polymerization are also formed. (3) Carbohydrate addition is not required for chain assembly, polymerization, and secretion since completely unglycosylated chains (synthesized in the presence of deoxyglucose or tunicamycin) undergo polymerization and are secreted (although at a reduced rate). (4) Surface 8s IgM molecules do not represent a step in the IgM secretory pathway.

Intracellular transport (ICT)¹ of immunoglobulin (Ig) from its site of synthesis on bound polysomes of the rough endoplasmic reticulum (RER) via the Golgi complex to the point of discharge at the cell

¹ Abbreviations used in this paper are: AsN, asparagine; AR, autoradiography; CCCP, carboxyl cyanide *m*-chlorophenyl hydrazone; CHO, carbohydrate; Con A, concanavalin A; DMEM, Dulbecco's modified Eagle's Medium; EDTA, ethylenediamine N,N,N',N'-tetraacetic acid; endo H, endo- β -N-acetylglucosaminidase H;

GlcNAc, N-acetylglucosamine; ICT, intracellular transport; Ig, immunoglobulin; PAGE, polyacrylamide gel electrophoresis; RER, rough endoplasmic reticulum; R_f, ratio to front; SDS, sodium dodecyl sulfate; [³⁵S]Met, methionine containing ³⁵S.

surface comprises sequential events of maturation and assembly of Ig heavy and light chains. The heavy chains, for example, bear asparagine-linked carbohydrate (CHO) units which mature in a number of stages only some of which have been localized cytologically. It is clear that the core sugars (*N*-acetylglucosamine, mannose, glucose) are added en bloc to nascent chains in the RER (4, 15, 26, 31), but it is not clear at what stage an obligatory trimming of core sugars takes place (13, 25, 34) which precedes Golgi-associated addition of terminal sugars (galactose, fucose, sialic acid). Furthermore, for want of adequate means to discriminate among Golgi subcompartments, terminal sugar addition has simply been ascribed to the Golgi complex as a whole (19, 20, 40, 44).

The assembly of heavy and light chains to form disulfide-bonded H₂L₂ units has previously been shown to take place at the level of the RER (41). However, the final assembly of IgM is less well understood, as it involves the further addition of the J polypeptide chain and pentamerization to (H₂L₂)₅J. The subcellular site of these last events is not known and, in the case of malignant plasma cells (myelomas), the degree to which polymerization takes place before discharge has been reported to vary according to cell line (7).

The present study of nonmalignant IgM plasmablasts identifies the cytological sites of these different stages of chain maturation and assembly and explores their interrelationships, making use of pulse-chase biosynthetic labeling, subcellular fractionation, electron microscope autoradiography (AR), inhibitors of glycosylation, and two agents known to arrest ICT: carboxyl cyanide *m*-chlorophenyl hydrazone (CCCP), which uncouples oxidative phosphorylation and stops Ig exit from the RER (36), and the ionophore monensin, which stops Ig exit from dilated Golgi cisternae (36, 37) apparently without affecting Ig synthesis or other steps of ICT.

MATERIALS AND METHODS

Carboxylcyanide *m*-chlorophenyl hydrazone was obtained from Calbiochem, San Diego, Calif.; pronase type V, bovine albumin, glucose oxidase type V, and dithiothreitol from Sigma Chemical Co., St. Louis, Mo.; Nonidet P-40 from Kolb A. G., Hedingen, Switzerland; lipopolysaccharide W (*Escherichia coli* 0127:B8) from Difco Laboratories, Detroit, Mich.; cycloheximide from Serva Biochemicals, Heidelberg, Germany; Na ¹²⁵I (IMS 300) carrier free and D-1-³H-mannose (TRK 238), 2.7 Ci/mmol were obtained from the Radiochemical Center, Amersham, Bucks, England; D-1-³H(N)-galactose (NET 126) 20 Ci/mmol and L-

5,6-³H-fucose (NET 516) 50 Ci/mmol L-[¹⁴C(U)]leucine (NEC-279) 300 mCi/mmol, L-[3,4,5-³H(N)]leucine (NET-460) 110 Ci/mmol, L-[³⁵S]methionine (NEG-009H) 400 Ci/mmol, and L-[³⁵S]cysteine (NEG-020) 50 Ci/mmol and Protosol from New England Nuclear, Boston, Mass.; monensin (lot 335Ac-2), A23187 (lot 361-V02-252-1), and tunicamycin (lot 361-26E-117) through the courtesy of R. Hamill of the Lilly Research Lab., Eli Lilly and Co., Indianapolis, Ind.; *V. cholerae* neuraminidase from Behringwerke, Marbourg, Germany; endoglucosaminidase H from Seikagaku Kogyo, Tokyo, Japan; P4 from Pharmacia Fine Chemicals, Uppsala, Sweden; and Instagel from Packard, Downers Grove, Ill.

Mouse spleen cells were cultured for 3 d in the presence of lipopolysaccharide according to Andersson et al. (1). Cells were harvested by sedimentation and washing in Hanks' Basic Salt Solution. They were all blasts bearing surface IgM, as judged by immunofluorescence staining with rhodamine-labeled rabbit anti-mouse μ chain antiserum on intact cells, up to 50% being plasmablasts as judged by their cytoplasmic staining with the same antiserum applied to smears of fixed cells. Biosynthetic labeling was performed in methionine-free DMEM for [³⁵S]methionine, in cysteine-free DMEM for [³⁵S]cysteine, in glucose-free Dulbecco's modified Eagle's Medium (DMEM) supplemented with 1 mM pyruvate for ³H-sugars. In each case, media were supplemented with 0.5 mg/ml bovine serum albumin. For 5-min labeling, cells were usually at 10⁶/ml. For longer labeling periods, the cell concentration was usually 10⁷/ml. In pulse-chase experiments, labeling at 0.5 mCi/ml was followed by two washes in DMEM and reincubation in DMEM or its variants (supplemented with 5% fetal calf serum), as indicated, at 10⁷ cells/ml. Incubations were terminated by sedimenting cells for 5 min at 1,000 g. The supernate was saved on ice for immunoprecipitation (14) while the cells were washed, resuspended in 0.5 ml of Hanks' containing 1% NP-40 (and 0.1 M iodoacetamide for samples to be analyzed on borate-SDS gels) and centrifuged for 30 min at 80,000 g. Stock solutions of 1 mM CCCP and 1 mM monensin were prepared in ethanol.

Iodination was performed with 2 × 10⁷ cells in 1 ml of phosphate-buffered saline containing 20 mM glucose, 10 μ g of lactoperoxidase, 1 U of glucose oxidase, and 1 mCi ¹²⁵I, for 30 min at room temperature, and terminated by three washes in phosphate-buffered saline. Before immunoprecipitation, iodinated samples were clarified as described above. Quantitation of radioactive Ig was done as described in (36).

10-15% gradient slab SDS-gels were performed according to Laemmli (18). 2.5-10% SDS-borate slab gels were run according to a modification of a procedure described by Pharmacia Fine Chemicals, Uppsala, Sweden. Both the reservoir buffer and the buffer in the gel were 1.1% Tris base, 0.5% boric acid, 1.1% EDTA, 0.1% SDS. The ratio of acrylamide to bisacrylamide was 30:1.2. Alkaline urea slab gels were run according to Mosmann (22). After electrophoresis, gels were either dried at once or first fixed in 50% methanol-7% acetic acid and stained with Coomassie Brilliant Blue, and then destained and dried or treated for fluorography according to Bonner and Laskey (5). For the SDS gels, immunoprecipitates were dissolved in 1% SDS, 1% beta mercaptoethanol, in one-quarter strength upper gel buffer containing 5% sucrose. For the SDS-borate gels, samples were dissolved in 1% SDS, 0.1 M iodoacetamide in one-quarter strength Tris-borate-EDTA containing 5% sucrose. In both cases, samples were heated for 5 min at 100°C before loading. For the alkaline urea gels, reduction and alkylation of samples was performed as described by Mosmann et al. (22). In all cases, staphylococci were eliminated by centrifugation just before load-

ing. For alkaline-urea second-dimension analysis of samples already fractionated on SDS-borate gels, dried SDS-borate gels were rehydrated by moistening for 30 min with a solution of 30 mM dithiothreitol in 10 M urea, 1% NP-40, 0.2 M pH 8 Tris-HCl and then transferred to the alkaline-urea gel which was overlaid with a minimal volume of 100 mM iodoacetate, 5% glycerol, dissolved in the appropriate reservoir buffer.

For the study of Ig glycopeptides, immunoprecipitates were dissolved in 0.1 ml 1 mg/ml pronase in 0.1 M Tris-HCl, pH 8. After 24 h at 60°C, an additional 0.1 ml of pronase was added and the hydrolysis was continued for 24 h. The incubation was terminated by eliminating the staphylococci by centrifugation and heating the supernate for 5 min at 100°C. Sizing of Ig glycopeptides was performed with a column of P4 measuring 43 × 0.7 cm, operated at 24°C eluted at 1 ml/h with 0.1 M pH 7.3 Tris-HCl. Fractions of 0.2 ml were collected and counted by mixing with Instagel.

For isolation of crude microsomal fractions, aliquots of 10⁸ cells were washed in 10 mM Tris-HCl pH 7.3, 10 mM KCl, 1.5 mM MgCl₂ and swollen for 5 min in 0.5 ml of the same solution. Homogenization was accomplished with 50 strokes of a small Dounce homogenizer (close-fitting pestle), and the homogenate was immediately adjusted to 50 mM Tris-HCl pH 7.3, 100 mM KCl, 5 mM MgCl₂ (TKM), and centrifuged for 1 min at 1,000 g. The resulting supernate was adjusted to 1.2 M sucrose and loaded into a discontinuous gradient with the following layers (37), all containing TKM: 0.5 ml of 2 M sucrose, 0.5 ml of 1.3 M sucrose, the postnuclear supernate, 0.5 ml of 0.5 M sucrose, 0.5 ml of TKM. The gradients were spun in the SW 56 rotor of the Spinco ultracentrifuge for 12 h at 50,000 rpm. Material accumulating at the 0.5 M sucrose/load interface and at the load/1.3 M interface was collected, diluted five times with TKM, and sedimented for 60 min at 50,000 rpm in the same rotor. The pellets were lysed by the addition of 0.5 ml of TKM containing 1% NP-40, sedimented for 30 min at 60,000 g to eliminate ribosomes. The resulting supernate were used for immunoprecipitation.

For study of the oligosaccharide structure of Ig, cell lysates were dialyzed overnight against 0.05% NP-40 50 mM pH 6 Na citrate and then treated for 5 h at 37°C with neuraminidase (0.02 U/ml) or endoglucosaminidase H (0.2 U/ml). Samples were then returned to pH 7.3 by the addition of 1 M Tris-HCl and subjected to standard immunoprecipitation. In the case of glycopeptides, the pH of pronase digests was lowered to 6 with 1 M pH 4 Na citrate for treatment (24 h, 37°C, 0.1 U/ml) with endoglucosaminidase H or, alternatively, the samples were simply adjusted to 0.1 M H₂SO₄ and heated for 30 min at 80°C (9).

Electron microscope autoradiography was performed by standard procedures employing Ilford L-4 emulsion. Exposure was carried out for 1 mo. Photographs were taken with a Philips 300 microscope. Cells were labeled, and grains were counted and scored as described in (36).

RESULTS

Studies of Sugar Addition to μ Chains

These studies were performed on biosynthetically-labeled Ig using both radioactive amino acids and sugars. In the former case, the presence and nature of μ chain or Ig oligosaccharides can be explored because the oligosaccharide content of the molecules modifies their migration in SDS-

PAGE. In the latter case, the study can also be extended to the level of glycopeptides.

STUDIES WITH [³⁵S]METHIONE-LABELED μ CHAINS

In these experiments, the Ig immunoprecipitated with the anti Ig or anti- μ rabbit antibodies has been reduced before SDS-PAGE, so that the mobility of their radioactive μ chains could be directly explored (Fig. 1). The fully glycosylated μ chains present in secreted IgM are more retarded than the μ chains found intracellularly after a 5-min pulse with [³⁵S]Met. When plasma cells are preincubated for 1 h and then pulsed with [³⁵S]-Met in the presence of an agent known to interfere with glycosylation (deoxyglucose or tunicamycin, references 30, 38) the mobility of the μ chains is dramatically increased. The mobility of these three

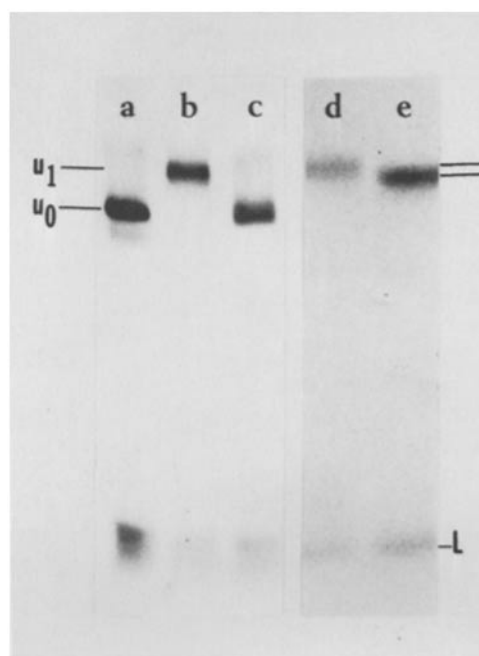


FIGURE 1 SDS-PAGE-AR of reduced [³⁵S]Met-labeled IgM. Cells were labeled for 5 min, washed, and either lysed at once or allowed to secrete for 1 h to provide a sample of extracellular Ig. In track a, labeling was performed after 60-min preincubation with tunicamycin 1 μ g/ml. In track b, a control lysate was immunoprecipitated directly; in track c, the same lysate was treated with endo H before immunoprecipitation. In track d, the extracellular sample was immunoprecipitated directly; in track e, it was treated with neuraminidase before immunoprecipitation.

types of μ chains, which will be called, respectively, μ_2 (extracellular), μ_1 (intracellular), and μ_0 (synthesized after preincubation in the presence of deoxyglucose or tunicamycin), represents different steps of sugar addition as shown by the following experiments: (a) when submitted to the action of the enzyme endoglucosaminidase H (endo H), which cleaves "core" sugars (34, 39), μ_2 chains are entirely insensitive, while μ_1 chains acquire a mobility very close to that of μ_0 (Fig. 1) (the slight difference in mobility can probably be ascribed to residual *N*-acetylglucosamine); (b) when treated with sialidase, μ_2 chains show a slightly increased mobility (Fig. 1) while the mobility of μ_1 chains is unchanged (not shown); (c) μ_2 and μ_1 chains, but not μ_0 , are retained on columns of sepharose-Con A, which bind mannose residues (not shown). Thus, μ_0 lacks sugars, μ_1 contains core sugars, and μ_2 is fully glycosylated. As expected, since L chains lack sugars, their mobility is never affected.

It appears that deoxyglucose does not prevent the transfer of core sugars from preassembled dolichol-oligosaccharides to nascent protein chains, but rather prevents the replenishment of the pool of these intermediates after this transfer has occurred. Thus, when plasma cells are treated with deoxyglucose in conditions where protein synthesis is inhibited by cycloheximide (50 $\mu\text{g}/\text{ml}$), subsequent pulse labeling of the cells (after cycloheximide withdrawal) produces μ_1 chains, presumably because the pool of dolichol-linked sugars has remained intact. The "size" of the pool of dolichol-oligosaccharides, i.e., the period of time during which it can donate sugars to growing polypeptide chains before its exhaustion, has been evaluated either by shortening the period of preincubation with deoxyglucose, or by preincubating with deoxyglucose for 60 min and arresting protein synthesis with cycloheximide at various times during this preincubation. In both cases, it was found (Fig. 2) that protein synthesis can proceed for about 10 min in the presence of deoxyglucose before the preexisting pool of dolichol-sugars is exhausted to such an extent that the predominant species of μ chains subsequently synthesized has the mobility of μ_0 . An interesting point is that such partial depletion generates a family of μ chains of intermediary mobility between that of μ_1 and that of μ_0 , probably corresponding to μ chains bearing different number of oligosaccharide units, as all are converted to μ_0 by endo H (not shown). This suggests that mouse μ chains may bear six oligo-

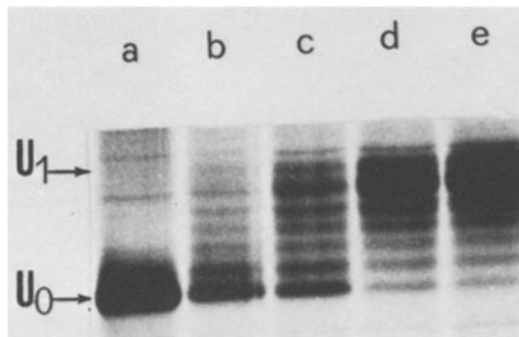


FIGURE 2 SDS-PAGE-AR study of the influence of intermediary deoxyglucose treatments on the mobility of u. Cells were incubated for 1 h in 0.3 mg/ml deoxyglucose prior to 5-min pulse [^{35}S]Met labeling. Cycloheximide 50 $\mu\text{g}/\text{ml}$ was added after 0, 15, 30, 45, 55 min of the deoxyglucose treatment for tracks a-e, respectively. Such intermediary treatments result in the generation of 7 u subspecies of mobility ranging from u_0 to u_1 (arrows).

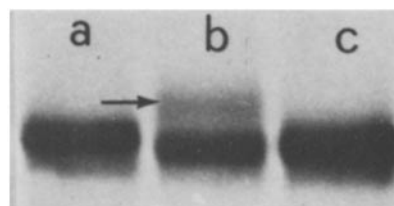


FIGURE 3 SDS-PAGE-AR of u chains immunoprecipitated after pulse or pulse-chase labeling: Cell lysates, (a) after 5 min [^{35}S]Met pulse, (b) 5-min pulse followed by 120-min chase, note the two bands, with the most retarded having a mobility similar to that of the secreted chain (arrow), (c) identical chase but in the presence of monensin, 1 μM .

saccharide side chains. No clear pattern with these sharp intermediates between μ_1 and μ_0 is generated by tunicamycin.

The further steps of intracellular CHO addition and processing have been analyzed as follows: (a) after a 1-min [^{35}S]Met pulse (which is about the time required to synthesize an entire μ chain [29]), only μ_1 chains can be observed in clarified cell lysates; in no case are μ_0 chains found (not shown). Thus, the addition of core sugars appears to occur on growing nascent polypeptide chains, as observed with Ig and other glycoproteins (4, 15, 31). (b) When a 5-min pulse is followed by prolonged period of chase (2 to 12 h), two new forms of μ chains are detectable within the cell (Fig. 3): the predominant species has a mobility slightly

greater than that of μ_1 , is entirely sensitive to endo H (i.e., is transformed into μ_0 by endo H), and probably corresponds to μ_1 chains from which terminal glucose and mannose residues have been trimmed (13, 25, 34);² the minor species has a mobility identical to that of μ_2 , and, like μ_2 , is entirely resistant to endo H (not shown).

The intracellular localization of these two types of chains has been studied by subcellular fractionation both after a 5-min [³⁵S]Met pulse and after a 2-h chase. In the rough microsomal fraction, at the end of the pulse μ_1 is found while "trimmed μ_1 " is detected after the chase. The smooth microsomal fraction after the chase contains both "trimmed μ_1 " and a small amount of μ_2 (not shown). In all these pulse-chase experiments, the bulk of [³⁵S]IgM is always recovered in the rough microsomal fraction. This observation is consistent with previous autoradiographic data (36) in suggesting that the Golgi Ig pool is small and rapidly discharged from the cell. It is only within this limited Golgi Ig pool that apparently completely glycosylated μ chains are detectable. Thus, it is not surprising that, after prolonged periods of continuous amino acid labeling, μ_2 chains cannot be detected in the cell lysates—they simply represent too small a fraction of the intracellular μ chains. It is only after a long chase, i.e., when most labeled μ chains have been secreted, that μ_2 chains constitute a less negligible fraction and therefore can be detected.

The intracellular localization of μ -CHO maturation events has been further investigated by including, during chase incubation, agents which interrupt intracellular transport at defined sites. Incubation in the presence of 10 μ M CCCP (which blocks Ig exit from the RER) or of 1 μ M monensin (which blocks Ig exit from Golgi cisternae) does not block the appearance of "trimmed μ_1 " in whole cell lysates (Fig. 3) and submicrosomal fractions (not shown), although μ_2 does fail to appear. Thus,

² This slight shift in SDS-PAGE mobility is a consistent observation. The degree of acceleration cannot be increased either by using shorter (10, 30 min) or longer (5, 12 h) chase intervals, or by examining, after 60-min chase, Ig recovered from rough or smooth microsomes. A corresponding shift of apparent [³H]mannose-labeled glycopeptide size has not been observed, possibly because of the rapidity of trimming and because at the level of glycopeptides (unlike SDS-PAGE) it is the size of individual (rather than multiple) oligosaccharides which is reflected in mobility.

the observed trimming occurs in the RER, and conversion to μ_2 occurs distal to Golgi cisternae.

In this context, it is also of interest that the inclusion of 5 mM manno lactone (a known inhibitor of liver Golgi mannosidase) (8) or 20 mM deoxyglucose does not alter the normal course of μ maturation after 5-min pulse labeling.

STUDIES WITH ³H-SUGAR-LABELED μ CHAINS

Plasma cells were incubated for various times with one of the following precursors: [³H]mannose, -glucosamine, -galactose, and -fucose. Judging from prior studies (9, 16), only [³H]glucosamine is expected to undergo extensive metabolic conversion to other sugars, in this case to sialic acid. During the period of labeling used, no conversion to amino acids was detected, since light chains were never labeled.

KINETICS OF SUGAR LABELING OF INTRACELLULAR AND SECRETED μ CHAINS AND SECRETION KINETICS OF SUGAR-LABELED IG: After continuous labeling for 30 min to 3 h with [¹⁴C]leucine or the different ³H-sugars, the labeled Ig present in the cells and the culture medium was immunoprecipitated. Analysis of the reduced precipitates by SDS-PAGE thus allowed a quantitative and qualitative comparison of the labeled μ chains (Fig. 4). With [¹⁴C]leucine, only μ_1 chains were detected intracellularly, for the reasons already discussed (vide supra), and their relative amount, when compared to μ_2 chains secreted during the same period of incubation, progressively decreased with time, as expected. Comparable results were observed with [³H]mannose (Fig. 4B), while with [³H]galactose (Fig. 4C) and [³H]fucose (not shown) the picture was entirely different, since the ratio of intracellular to secreted radioactivity was very small, even after only 30 min of labeling, and since intracellular and extracellular chains had μ_2 mobility. Thus, galactose and fucose are added to μ chains very shortly before secretion, and the addition of the terminal sugars, including sialic acid, must occur almost simultaneously.

This conclusion was confirmed in a second type of experiment, in which plasma cells were labeled for 1 h with either [³⁵S]Met, [³H]mannose, [³H]galactose or [³H]fucose, and then chased in non-radioactive medium to follow the time-course of release of radioactive IgM.³ As shown in Fig. 5,

³ In this protocol, unlike the others designed to localize

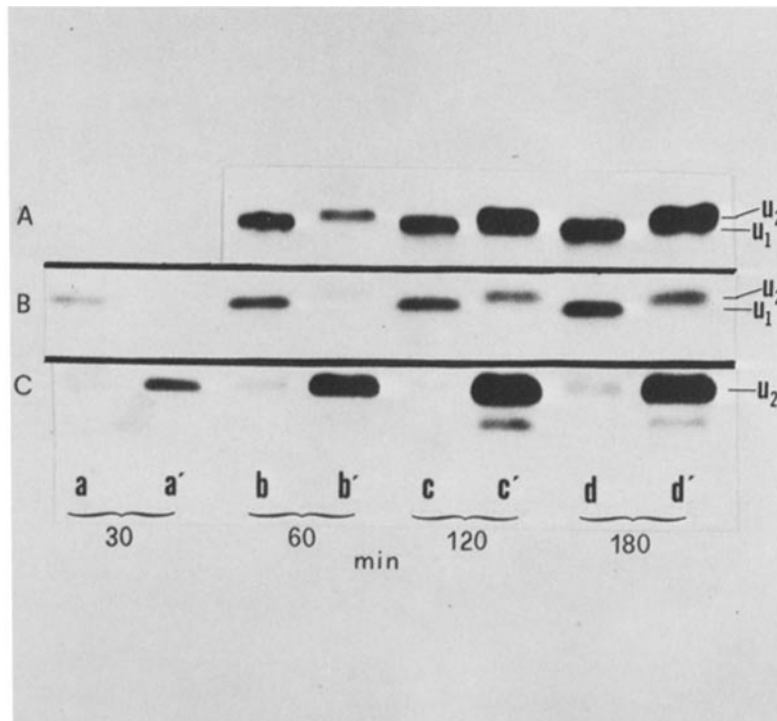


FIGURE 4 SDS-PAGE-AR of the total intra (*a-d*) and extracellular (*a'-d'*) μ chains immunoprecipitated from the cell lysates and culture medium after continuous labeling with [^{14}C]leucine (*A*), [^3H]mannose (*B*), or [^3H]galactose (*C*). (*a, a'*) 30 min, (*b, b'*) 60 min, (*c, c'*) 120 min, (*d, d'*) 180 min. The nature of the minor band with faster mobility observed in *Cc'* and *Cd'* is not known.

the half-time was about 40 min for release of [^{35}S]Met- or [^3H]mannose-labeled IgM and 10 min for [^3H]galactose- or [^3H]fucose-labeled IgM. This type of experiment also offers an additional means of identifying the compartment in which the addition of a given sugar takes place: a monensin-imposed block at the level of Golgi cisternae should interfere with the secretion of chains labeled with a sugar added within or before the Golgi cisternae, but not with the secretion of chains labeled with sugars added in a compartment distal to the Golgi cisternae. Fig. 5 shows that monensin indeed markedly slows the secretion of [^{35}S]Met- or [^3H]mannose-labeled IgM, while the secretion of [^3H]galactose- or [^3H]fucose-labeled IgM is little affected. Using these terminal sugars as label, similar results have been obtained with 10 μM A 23187 in the presence of 1 mM EGTA in calcium-free medium or CCCP, imply-

the site of terminal sugar addition, the results cannot be ascribed to an interference between monensin and the sugar transferases themselves.

ing that Ig exit from the postcisternal Golgi compartment is insensitive to radical reduction of cytoplasmic calcium levels and does not require ongoing respiration (cf. references 36, 37).

EFFECT OF MONENSIN ON SUGAR ADDITION TO μ CHAINS: If the terminal addition of sugars to μ chains occurs in a small compartment distal to the Golgi cisternae, a block in ICT created by monensin should selectively decrease any subsequent addition of terminal sugars to μ chains. To explore this point, cells were preincubated for 15 min with or without 1 μM monensin and then labeled for 60 min with [^3H]mannose, -galactose, or -fucose. The inhibition of incorporation of [^3H]mannose into Ig in the presence of monensin was modest (28%) and close to that of μ chains themselves (detected by [^{35}S]Met-labeling: 20%), while incorporation of [^3H]galactose and [^3H]fucose was much more markedly depressed (86 and 79%, respectively). Incorporation of these four isotopes into proteins other than Ig was inhibited $\sim 20\%$ in all cases in the same protocols.

CHARACTERIZATION OF THE GLYCOPEP-

TIDES OF INTRACELLULAR AND SECRETED μ CHAINS: The elution pattern of the glycopeptides obtained from 2-h ^3H -sugar-labeled IgM has been analyzed by Biogel P4 filtration before and after treatment with endo H, or with H_2SO_4 . Glycopeptides from intracellular μ chains have an elution pattern which depends on the ^3H -sugar used to label the μ chains (Fig. 6). Glycopeptides labeled with [^3H]mannose (Fig. 6A) give a single broad peak (R_f 0.26), are entirely sensitive to endo H (Fig. 6B) which generates a major (R_f 0.52) and a minor product (R_f 0.63), and are insensitive to H_2SO_4 (not shown). With [^3H]glucosamine, a mixture of species is observed (Fig. 6C), and with

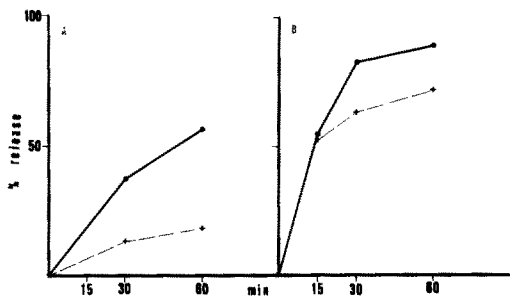


FIGURE 5 Kinetics of secretion of IgM, after labeling the cells for 1 h with [^{35}S]Met (a), or [^3H]galactose (b), washing, and further incubating the cells in chase medium for quantitation of IgM secretion over the ensuing hour. In (a) and (b), the dotted line indicates the results obtained when $1 \mu\text{M}$ monensin is present during 15 min at 0°C just before the chase and during the chase. When cells are labeled with [^3H]mannose, the results are very similar to a, and with [^3H]fucose, the results are indistinguishable from b.

[^3H]galactose a single peak (Fig. 6D) (R_f 0.11), entirely insensitive to endo H, and shifted to an R_f 0.19 by H_2SO_4 treatments (not shown). All glycopeptides recovered from secreted μ chains also show a single peak of R_f 0.11 (Fig. 6E), whatever the precursor ^3H -sugar used, all are insensitive to endo H, and all are shifted to an R_f 0.19 by H_2SO_4 treatment. When labeling is done with [^3H]glucosamine, this shifted peak is associated with a second peak of R_f 0.7, which is the expected elution position of sialic acid (9) (Fig. 6F). These results strongly suggest that: (a) the intracellular [^3H]mannose-glycopeptides contain core sugars, probably trimmed to varying extents; the amount of intracellular [^3H]mannose-labeled "complex" glycopeptides is too small to be detectable (except when the glycopeptides from μ chains present in the smooth microsomal compartment are analyzed separately: see below); (b) the intracellular [^3H]galactose glycopeptides correspond to the small amount of μ chains ready to be secreted, since they appear to be all sialylated; (c) the secreted μ chains bear only "complex" oligosaccharide chains terminating in sialic acid and no chains with highly branched mannose, since, even after [^3H]mannose labeling, a single peak is observed, which shows the same shift after H_2SO_4 treatment as in the case of [^3H]glucosamine.

The above studies show that the detectable glycopeptides of intracellular μ chains differ depending on the precursor sugar. Analysis of the [^3H]mannose-glycopeptides of μ chains recovered after cell fractionation shows that these differences correspond to the sequential passage of IgM through the RER and Golgi complex (Fig. 7). Thus, gly-

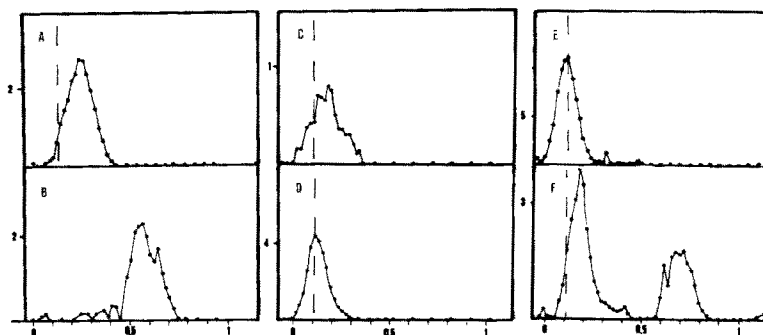


FIGURE 6 Gel filtration on P4 of Ig-derived glycopeptides. Panel A intracellular after 2-h [^3H]mannose label, (B) same, followed by treatment with endo H, (C) intracellular after 2-h [^3H]glucosamine, (D) intracellular after 2-h [^3H]galactose, (E) extracellular after 2-h [^3H]galactose, (F) extracellular after 2-h [^3H]glucosamine, followed by H_2SO_4 treatment. On the abscissa, "O" designates the void volume, and "I" the elution position of [^3H]mannose.

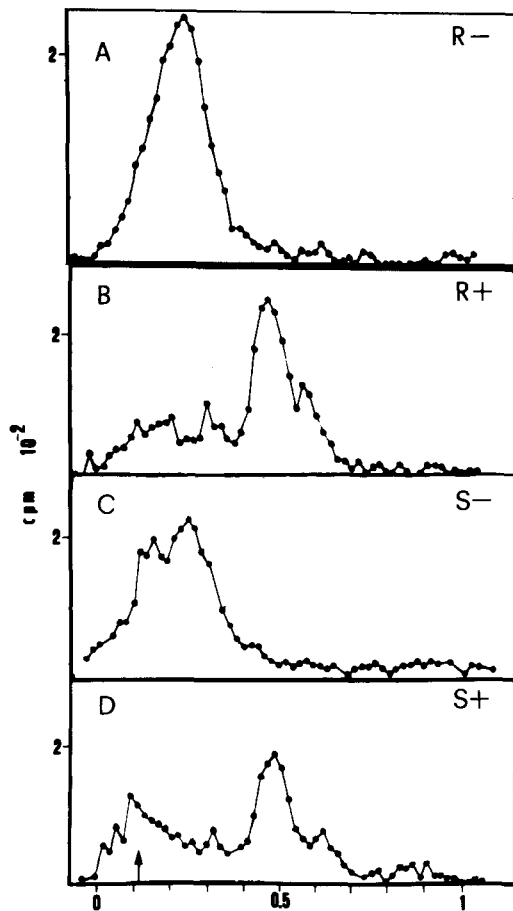


FIGURE 7 Gel filtration on P4 of Ig-derived glycopeptides recovered from rough (*R*) and smooth (*S*) microsomal fractions. In all cases, labeling was carried out for 2 h with [^3H]mannose. (A) rough microsomes (-), (B) same after treatment with endo H (+), (C) smooth microsomes (-), (D) after treatment with endo H (+). The arrow indicates the mobility of extracellular glycopeptides.

copeptides of the rough microsomal fraction (which contains the bulk of the intracellular μ chains) are recovered at an R_f of 0.26 and are almost entirely sensitive to endo H. On the other hand, glycopeptides of the smooth fraction show an overlapping pattern including fast-moving components, and, after endo H treatment, a resistant peak of rapid mobility, similar to that of glycopeptides of extracellular μ chains, becomes visible. Such species are not seen when labeling is done in the presence of monensin (not shown). It is very likely that these fast-moving glycopeptides

correspond to the completely glycosylated chains, which can be detected among total intracellular chains only when [^3H]galactose or [^3H]fucose is used as precursor.

Study of Polypeptide Chain Assembly and Polymerization

The assembly of [^{35}S]Met-labeled μ and L chains has been followed, after various periods of pulse (from 3 min to several hours) and chase (from 10 min to several hours), by precipitating detergent-treated cell lysates with antibodies directed against either mouse Ig or μ chains (to recover all molecules containing μ chains, but not free L chains) and analyzing the immunoprecipitates in nonreducing conditions on borate-SDS 2.5-10% polyacrylamide gradient gels, which allow a resolution of molecules between 20,000 and 1×10^6 daltons.

After a 3-min pulse (Fig. 8A track 1), analysis of the anti- μ immunoprecipitate shows that the plasma cell contains two major Ig species, half monomers (μL) and monomers ($\mu_2\text{L}_2$) of IgM, as well as smaller amounts of $\mu_2\text{L}$ molecules, free μ and free L chains. [The identity of these various molecular forms has been established by: (a) comparison of their migration with known markers (IgG and reduced and alkylated μ and L chains), (b) elution of the gel bands and reelectrophoresis on SDS gels in reducing conditions.] The presence of L chains illustrated in Fig. 8A, 1 indicates the existence of associated but noncovalently joined μ and L chains, as the immunoprecipitate was prepared with an anti- μ antiserum. Since no free μ chains can be detected in these precipitates without the presence of labeled L chains, it appears that μ chains are at all times associated with L chains. In these experiments with short pulse (3-5 min), after immunoprecipitation with anti- μ , the resulting supernate can be reprecipitated with RaMIg. Gel analysis of such precipitates reveals a large amount of free L chains. Quantitation of radioactivity in H and L chains recovered in pulse-chase experiments (see below) shows that this pool of L chains in part gradually associates with subsequently synthesized μ chains. In addition, a portion is secreted without covalent association with other chains (see below), and, finally, a portion must be degraded. Results with ICT block due to CCCP suggest that this degradation occurs in the RER (not shown).

During chase periods, the molecules observed after a brief pulse progressively assemble to pen-

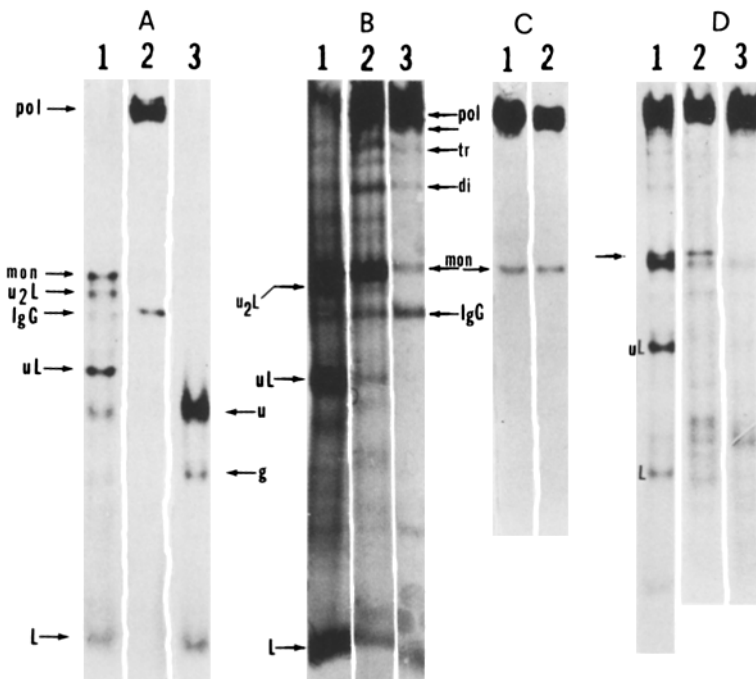


FIGURE 8 SDS-borate-PAGE-AR of Ig (anti-Ig) and IgM (anti-u) immunoprecipitates. [³⁵S]Met label. Frequently, the plasmablast population produces a small amount of IgG which is seen on the gels, even in IgM precipitates, because of the affinity of IgG for the staphylococci used for immunoprecipitation. A1. intracellular IgM after 3-min pulse. A2. extracellular IgM. A3. standards. B1. intracellular Ig after 5 min label. B2. intracellular Ig 5-min pulse followed by 2-h chase. B3. extracellular Ig 5-min pulse followed by 2-h chase. C1. IgM recovered from rough microsomes after 60-min label. C2. IgM recovered from smooth microsomes after 60-min label. D1. intracellular IgM 120-min pulse. D2. intracellular IgM 60-min pulse, 180-min chase. D3. Intracellular IgM 1-h pulse, 3-h chase, followed by pronase treatment before cell lysis (5 mg/ml, 30 min, 37°C). Arrow indicates the 8 s IgM pronase-sensitive band. Abbreviations: *pol*, polymer; *tr*, trimer; *di*, dimer; *mon*, monomer; *g*, gamma chain.

tameric form, which rapidly becomes the predominant form of intracellular IgM (Fig. 8 B, 2). The kinetics of this assembly are such that, after a 5-min pulse, half of the labeled IgM becomes polymeric during a 15-min chase. In addition, minor IgM species between monomers and pentamers become detectable (Fig. 8 B and D; Fig. 9 A, 4) whose mobility on gels is consistent with a composition $(\mu_2L_2)_{1\frac{1}{2}}$, $(\mu_2L_2)_2$, $(\mu_2L_2)_{2\frac{1}{2}}$, $(\mu_2L_2)_3$, $(\mu_2L_2)_{3\frac{1}{2}}$, $(\mu_2L_2)_4$. Extracellular immunoprecipitates contain pentameric IgM and small amounts of L chains; traces of the integral intermediate species are also frequently found (Fig. 8 B, 3; Fig. 9 A).

The intracellular compartment where polymerization occurs was explored by cell fractionation and by following 5-min pulse-labeling with chase incubation in conditions of ICT block. After 60-

min continuous labeling, pentamers were recovered in the rough and smooth microsomal fractions (Fig. 8 C), and their formation in whole cell lysates was not altered by the blocking of ICT with CCCP or monensin (not shown). Thus, polymerization both can and does normally occur already in the RER; furthermore, it does not require ongoing energy production, since the dose of CCCP employed (10 μ M) decreases protein synthesis by >90% over a 1-h period.

Finally, when a relatively long pulse (60 min or more) is followed by a chase of several hours, a second form of monomeric IgM, with a slightly retarded mobility, becomes detectable (Fig. 8 D, 1 and 2). The gel mobility of this species is identical to that of surface 8s IgM observed after ¹²⁵I-labeling of the cell surface with lactoperoxidase (not shown). The possibility that these retarded 8s IgM

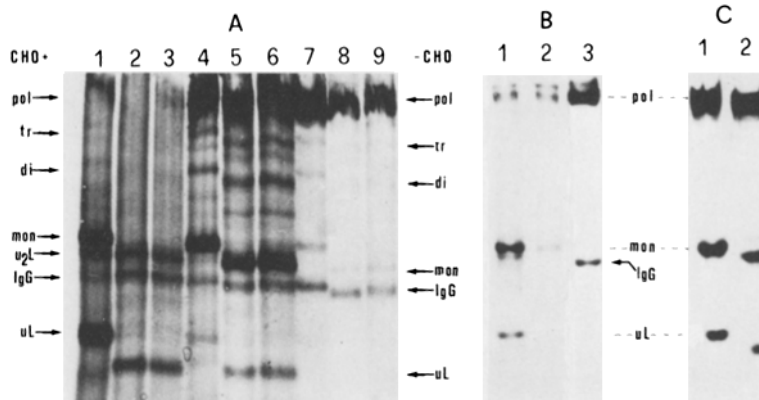


FIGURE 9 SDS-borate-PAGE-AR of Ig immunoprecipitates. A. Tracks 1-3, after 5-min [35 S]Met pulse-labeling. Tracks 4-6, after a subsequent 60-min chase, intracellular. Tracks 7-9, extracellular samples corresponding to tracks 4-6. For tracks 2, 5, 8, cells were incubated for 1 h in 0.3 mg/ml deoxyglucose before labeling. For tracks 3, 6, 9, the preincubation was with 1 μ g/ml tunicamycin. At the left margin are indicated the assignments for intracellular Ig. At the right margin are the assignments for CHO-free intracellular Ig. B: Ig-labeled with 3 H-sugars. (The polymer band often appears as a doublet.) B1. intracellular Ig after 30-min [3 H]mannose label. B2. intracellular Ig after 30-min [3 H]glucosamine label. B3. intracellular Ig after 30-min [3 H]galactose label. The minor band is IgG. C1. intracellular IgM labeled for 1 h with [35 S]Met and chased for 180 min. C2. same after treatment with endo H. Abbreviations as in Fig. 8.

molecules were also located on the cell surface was explored by incubating intact [35 S]Met-labeled cells with pronase, under conditions known, by immunofluorescence, to remove cell surface IgM (see legend of Fig. 8). Analysis of the IgM present in the cell lysate after such treatment showed the selective disappearance of retarded monomeric IgM, indicating that it is on the cell surface, while the pentameric and less retarded monomeric molecules were unmodified. Thus, monomeric surface IgM molecules, which represent only a very small fraction of the total IgM synthesized by these cells, (a) become detectable when most of the labeled intracellular IgM has reached pentameric form, and (b) are different from monomeric intracellular IgM destined for polymerization and secretion.

Relationship between IgM Polymerization, Sugar and J Chain Addition and the Possible Transient Presence of Secretory Ig on the Cell Surface

POLYMERIZATION AND CARBOHYDRATE ADDITION

Since the polymers assemble in the RER, and are the major form of IgM within the cell (Fig. 8 C, 1), while fully glycosylated μ chains represent

only a minority of the μ chains, even after long chase (Fig. 3), the transfer of the terminal sugars must occur on pentameric IgM. This conclusion was confirmed by 30-min labeling with [3 H]mannose, -glucosamine, -galactose and -fucose. As illustrated (Fig. 9 B), [3 H]galactose marked pentameric molecules, while [3 H]mannose was present in greater amounts on monomers than pentamers. [3 H]glucosamine (which labels both glucosamine and sialic acid) gives an intermediate result.

Polymerization of IgM to pentameric form is in fact entirely independent of the presence of sugars on the μ chains, as shown by pulse-chase experiments performed in the presence of deoxyglucose or tunicamycin (Fig. 9 A, tracks 2, 3, 5, 6, 8, 9): intracellular pentameric molecules and the same characteristic intermediate species are observed in amounts comparable to those of cells incubated in normal conditions. Each species has a faster migration than its normal counterpart, as expected from the lack of carbohydrate. It can be shown not only that CHO is not required for polymerization, but that the core sugars of intracellular pentameric IgM, which have been added before polymerization, are sterically accessible to the enzyme endo H. Thus, treatment of cell lysates with the endo H removes core sugars on pentameric and intermediary species, which acquire the same

SDS-PAGE mobility as unglycosylated molecules (Fig. 9C, 1, 2). When analyzed under reduced conditions, the μ chains contained in these species migrate as μ_0 .

POLYMERIZATION AND ADDITION OF J CHAINS

J chain is poor in methionine, and comigrates

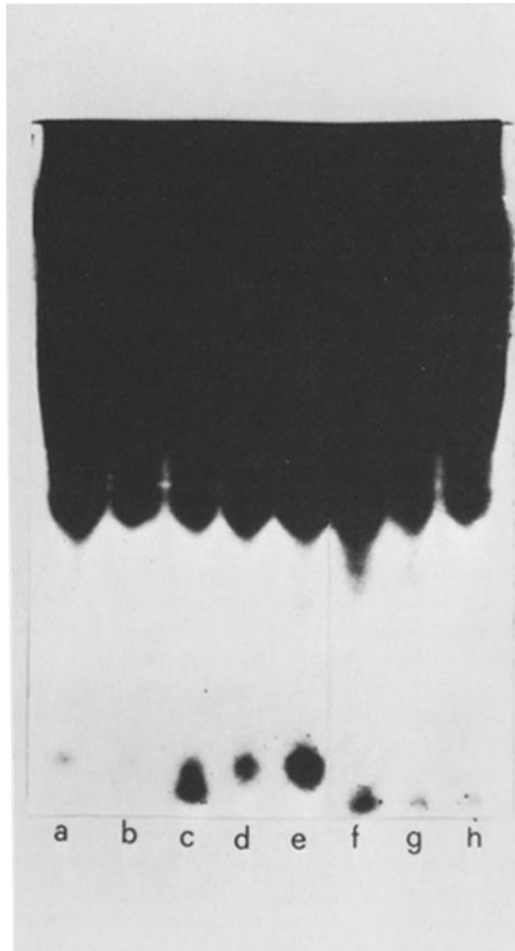


FIGURE 10 Alkaline urea gel fractionation of [^{35}S]cysteine-labeled Ig. Anti-Ig immunoprecipitates derive from cells pulse-labeled for 5 min (*a*) or chased for 2 h to provide intracellular (*b*) and extracellular (*c*) samples. (*d* and *e*) Intracellular samples derived from chase incubations in the presence of $1\ \mu\text{M}$ monensin and $10\ \mu\text{M}$ CCCP, respectively. Samples *f-h* correspond to samples *a-c*, but the cells were pretreated for 1-h with $1\ \mu\text{g}/\text{ml}$ tunicamycin. In all cases, the J chains migrate toward the bottom of the gel more rapidly than the polydisperse polyclonal heavy and light chains which give the smear at the top of the figure.

with light chains in SDS gels, although its molecular weight is 15,000 (17). Thus, to explore J chains, cells were labeled with [^{35}S]cysteine, and anti Ig immunoprecipitates were analyzed in alkaline urea gels after reduction and alkylation. In these conditions, the J chain migrates faster than light chains, fractionation being a function of both charge and size. J chain is a glycoprotein, and the maturation of its oligosaccharide unit can be followed by comparing the migration of intracellular and extracellular J chains, as well as unglycosylated J chains in cells pretreated with tunicamycin. The mobility observed after 5-min pulse labeling (Fig. 10*a*) remains unchanged in all intracellular samples studied, regardless of whether the 120-min chase is in control medium or in the presence of CCCP or monensin. Extracellular J chains, by contrast, have a more rapid mobility, presumably due to the addition of negatively charged sialic acid. When cells are pretreated with tunicamycin, pulse-labeled J chains also have a more advanced mobility, presumably since, as they lack CHO, their hydrodynamic radius is reduced.

All the J chains observed are associated to Ig molecules, since they were detected in the anti-Ig immunoprecipitate. The following further points were established concerning the addition of J chains to Ig molecules: (*a*) subcellular fractionation showed that the J chain is added to Ig at the level of the RER (not shown), (*b*) to explore at what step of polymerization of IgM J chains are added, [^{35}S]cysteine-labeled immunoprecipitates have been fractionated on borate gels, and the gel then placed, after reduction and alkylation, on top of an alkaline urea gel. The J chain was found restricted to the polymeric IgM. Insufficient radioactivity was present to judge whether Ig species between monomer and pentamer contain the J chain; (*c*) the addition of J chain to Ig does not require carbohydrate, since immunoglobulin synthesized in cells preincubated with tunicamycin is associated with unglycosylated J chains (Fig. 10*f*).

DOES THE FINAL ADDITION OF SUGARS TO THE PENTAMERIC IGM MOLECULES OCCUR AT THE CELL SURFACE?

Since the final addition of sugars to the pentameric molecule is such a late event in intracellular transport, and is found by cell fractionation to occur in a smooth membrane fraction which probably includes fragments of the plasma membrane, the possibility that it occurs on the cell surface itself, "at the time of secretion," was explored: (*a*)

by making use of the observation that Ig present on the cell surface can be removed by pronase treatment of intact cells (*vide supra*) and (*b*) by electron microscope radioautography after incubation with [³H]galactose and [³H]fucose.

Cells biosynthetically labeled for 15 min with [³H]galactose or [³H]mannose or surface-labeled with ¹²⁵I were treated with pronase for 60 min at 15°C (to minimize secretion), and the amount of cell-associated radioactive μ was assessed by PAGE-AR (Fig. 11). ¹²⁵I- μ chains were decreased by this treatment, whereas neither [³H]galactose nor [³H]mannose-labeled μ chains was changed. Thus, the small pool of [³H]galactose pentameric

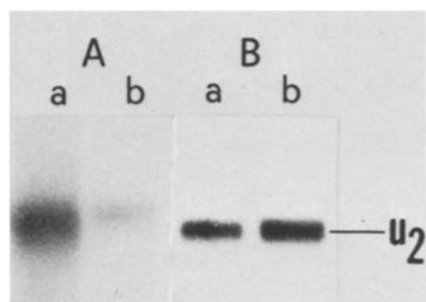


FIGURE 11 SDS-PAGE-AR analysis of pronase-treated cells after surface radioiodination (*A*) or 15-min labeling with [³H]galactose (*B*). Each panel shows the labeling intensity of controls (*a*), and the intensity after 1 h, 15°C incubation with pronase, 1 mg/ml (*b*).

IgM is not located on the cell surface, since it is not altered by conditions which alter surface IgM (assuming that their inherent protease sensitivity is the same). For electron microscope autoradiography, cells were labeled for 15 min with [³H]galactose or [³H]fucose, then fixed and processed. In each case, the ultrastructural localization of several hundred grains was determined. The great majority of the grains were associated with the Golgi complex, and only ~10% with the plasma membrane. Because of the limited resolving power of the method, no attempt was made to distinguish between grains associated with Golgi cisternae and those associated with Golgi vesicles. A representative result is shown in Fig. 12.

ROLE OF CARBOHYDRATE IN IGM SECRETION: Control cells or cells pretreated for 60 min with tunicamycin were pulsed-labeled for 5 min with [³⁵S]Met, washed, and returned to chase medium (lacking tunicamycin) to study the comparative rates of [³⁵S]IgM secretion. In the control case, 16, 44, and 76% of total [³⁵S]Ig are secreted after 30, 60 and 120 min. After tunicamycin, the corresponding figures (for CHO-free [³⁵S]Ig) are 7, 17, and 31%.

Electron microscope autoradiography was employed to judge which steps of ICT were slowed under such conditions. In controls, cells were pulsed for 5 min with [³H]leucine and fixed at once or washed and returned to chase medium for 90 min. Experimental samples were preincubated

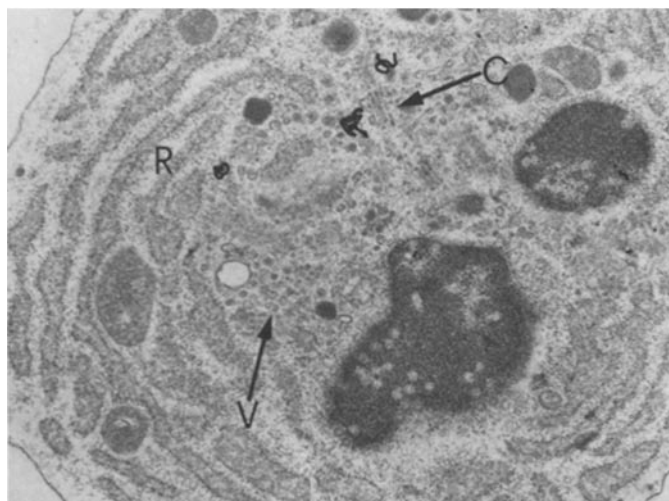


FIGURE 12 Electron microscope autoradiography of cells labeled for 15 min with [³H]fucose. Note association of autoradiographic grains with the Golgi complex. (*RER*) rough endoplasmic reticulum, (*C*) Golgi cisternae, (*V*) Golgi vesicles. $\times 11,000$.

for 60 min with tunicamycin. Several hundred grains were counted for each condition.

The grain distribution was similar for controls and experimental cells. Thus, at the end of the pulse, 84 and 87% of the cytoplasmic grains were associated with the RER and 16 and 13% with the Golgi area. After the chase, the corresponding figures were 64, 72, 36, and 28%.

The simplest interpretation of these data is that both transport from RER to Golgi complex and discharge were slowed. No morphological alterations of the cells were associated with tunicamycin treatment.

DISCUSSION

The study of the intracellular transport (ICT) of Ig molecules and their concomitant glycosylation, assembly, polymerization, and ultimate discharge from the cells, has been attempted principally with myelomas. Using several murine myelomas, it has been observed that the core sugars are added early (i.e., in the RER) and the complex sugars late (i.e., in a smooth membrane compartment) in the intracellular pathway (19, 20, 40, 44). At the time when these studies were conducted, the role of dolichol-linked oligosaccharide intermediates (43) was not yet known. Studies of murine IgM myelomas whose heavy chains are especially rich in carbohydrate and whose mature structure is pentameric show that polymerization is a very late event, occurring apparently "at the time of secretion," since the presence of pentamers was usually not detected within the cell (2, 23). By contrast, studies of a number of human myelomas have detected sizable intracellular pools of polymer (7). The present study uses a cell population containing a high percentage of nonmalignant plasmablasts which secrete primarily IgM. It explores the interrelationship of each of the successive modifications of the polypeptide chains (steps of glycosylation, chain assembly, and polymerization) and their topographic site(s) of occurrence within the cells. The different steps of glycosylation will be discussed first, followed by the assembly and polymerization of the polypeptide chains, then the interrelationship between these events.

Glycosylation

Our data show that the initial glycosylation occurs during a period of time comparable to that required for the synthesis of a complete μ chain (29) and thus are consistent with observations

made with other glycoproteins (4, 15, 31) for which it has been conclusively shown that core sugars are added to nascent polypeptide chains. Present evidence (43) is consistent with the notion that the RER membranes contain a pool of preassembled dolichol-oligosaccharide intermediates which have access to the site where these membranes are crossed by growing nascent chains and therefore can donate core sugars to any suitably situated AsN residue on these chains. A sufficient explanation of the action of deoxyglucose (30) is that it prevents the replenishment of this pool of donor intermediates. Thus, (a) upon prolonged deoxyglucose preincubation, completely unglycosylated μ chains are released within the RER cisternae, and (b) deoxyglucose is without effect, even after a prolonged period of incubation, if protein synthesis is reversibly blocked during the same period by cycloheximide. By varying the length of exposure to deoxyglucose before labeling the cells, or by slowing the rate of labeling with cycloheximide after extensive deoxyglucose treatment (not shown), it is possible to create conditions in which μ chains are synthesized in the presence of limiting amounts of dolichol intermediates. These μ chains constitute a family of species bearing from none to their full normal complement of core sugar side chains (probably six; see Fig. 2). Each member of the family of partially glycosylated μ chains probably represents a collection of positional isomers (for example, chains bearing a single oligosaccharide side chain need not bear it on the same AsN residue). That all the oligosaccharide side chains, whether on partially or completely glycosylated μ chains, result from the en bloc transfer of the same core oligosaccharides is strongly suggested by the observation that treatment of the cell lysate containing these μ chains with endo H (35, 39) converts them to μ_0 . This point is confirmed by the observation that glycopeptides obtained after [^3H]mannose-labeling of the plasma cells, and present in the rough microsomal fraction of the cells, are sensitive to the same enzyme.

Pulse-chase studies employing [^{35}S]Met show that pulse-labeled chains acquire a slightly increased SDS-PAGE mobility during a 10- to 60-min chase interval. Such a shift can be probably attributed to a degree of "trimming" of the core sugars, as has been observed in other systems (13, 25, 34), for instance a loss of terminal glucose or mannose residues. In cells where exit of Ig from the RER has been arrested by CCCP, the occurrence of this shift in mobility is unchanged. In

conjunction with the results of cell fractionation, these observations imply that the first step of trimming occurs in the RER itself.⁴

The addition of complex sugars can be detected by (a) a marked decrease in mobility of the μ chains in SDS-PAGE, (b) a complete resistance of these chains to the action of the endo H, and (c) the labeling of the μ chains with [³H]galactose or [³H]fucose. In confirmation of earlier findings (19, 20, 24, 40) with myeloma cells, this terminal addition of sugars is a very late event in the intracellular pathway, since in [³⁵S]Met-labeling experiments it is not possible to detect intracellular μ chains with the SDS-PAGE mobility of secreted μ chains, except after a long period of chase, where they still represent only a small minority of the total detected μ chains. This late addition of terminal sugars was further documented by (a) cell fractionation studies and (b) analysis of labeling and secretion kinetics of μ chains after incubation with [³⁵S]Met, [¹⁴C]leucine or various ³H-sugars. Cell fractionation experiments confirmed that only the smooth microsomal fraction contains [³H]galactose or -fucose-labeled chains, (not shown) or [³⁵S]Met-labeled chains with a retarded mobility, or μ chains containing [³H]mannose-labeled glycopeptides resistant to endo H. In these last two cases, they represent only a minority of the chains present in the smooth fraction, which constitute themselves only a small part of the total intracellular μ chains, the majority being located in the RER even after long periods of chase. The kinetics of labeling and secretion studied by the quantitative comparison of the labeled chains present within the cells or secreted after various periods of continuous incubation in the presence of [¹⁴C]leucine or ³H-sugars showed a sharp contrast between chains labeled with [¹⁴C]leucine or core sugars on one hand, and chains labeled with terminal sugars on the other. Chains labeled with [³H]galactose or [³H]fucose are present only for a very short time before appearing in the medium: thus, after a 30-min incubation with [³H]galactose or [³H]fucose, most of the labeled chains are present in the medium while with [¹⁴C]leucine or [³H]mannose the converse is true.

The site of terminal sugar addition between the

Golgi complex and the cell surface has been located with greater precision by (a) using monensin to block IgM exit from Golgi cisternae, (b) exploring the presence of fully glycosylated molecules on the cell surface after pulse labeling with terminal sugars, and (c) by electron microscope AR. Monensin prevents the labeling of chains with [³H]galactose or -fucose, but not with [³⁵S]Met or [³H]mannose. Furthermore, in cells labeled with these precursors before monensin addition, the subsequent secretion of [³⁵S]Met- or [³H]mannose-labeled chains is markedly delayed, unlike [³H]galactose- or [³H]fucose-labeled chains. Moreover, monensin prevents the maturation of [³⁵S]Met-labeled chains (as judged by SDS-PAGE) or Ig-derived glycopeptides (as judged by gel filtration) to fully glycosylated chains.

The possibility that the cell surface itself is the site of addition of terminal sugars was ruled out by studying the pronase sensitivity of [³H]galactose- or [³H]fucose-labeled Ig and by autoradiographic studies of cells pulsed with [³H]galactose or [³H]fucose. It is thus possible to conclude that terminal sugars are added either at the moment of exit of Ig from the monensin-sensitive cisternae, or in those smooth-surfaced vesicles which ferry from Golgi cisternae to the cell surface (21). Because of the limited resolving power of the autoradiographic method (27), it is at present not possible to distinguish between these two alternatives.

Previous biochemical studies have identified galactosyl, fucosyl, and sialyl transferase activities in Golgi fractions (28), and there have been attempts to extend these studies to subcompartments of such fractions which have a predominantly cisternal or postcisternal origin (6). The present results do not exclude the possibility that such transferases might be present throughout all Golgi subcompartments (as has been reported [6])—they imply that the principal activities of these enzymes are expressed distal to the monensin-sensitive Golgi cisternae. Strictly speaking, as monensin has not been shown to dilate and block Ig exit from all Golgi cisternae, the present data are also consistent with the notion that monensin acts on the proximal or cis cisternae and that terminal sugar addition normally occurs in distal or trans cisternae, provided they are no longer present after monensin treatment (possibly because of conversion to vesicles).

Addition of the terminal sugars must be a virtually simultaneous event, and if it has to be preceded by any Golgi-associated trimming of

⁴ For unknown reasons, reduction of cytoplasmic calcium levels (using the ionophore A 23187 and EGTA, cf. reference 36) arrests such trimming, as judged by the SDS-PAGE mobility of reduced μ chains (A. Tartakoff, unpublished observation).

sugars, it must rapidly follow such secondary trimming, since, whatever the conditions of labeling and the period of chase, no intermediates were detected. By contrast, in the case of an unusual myeloma secreting glycosylated L chains, some such intermediates have been identified (16).

Chain Assembly and Polymerization

Previous studies of Ig assembly in isolated microsomal systems showed that the association of H and L chains occurs before H chain completion, and involves L chains within the RER and the amino terminal part of nascent H chains still in the process of crossing the RER membrane (41). In the present study, two observations are consistent with this concept: anti- μ antiserum fails to precipitate free μ chains nonassociated with L chains; furthermore, free L chains nonassociated with μ chains are detected in large amounts in the supernate of the anti- μ precipitation.

The covalent disulfide H-L bond appears to occur after noncovalent association. Thus, under appropriate conditions, free L chains can be released from anti- μ immunoprecipitates during SDS-PAGE in the absence of reducing agent. Although molecules with a μ_2 covalent composition were not detected, inter-H chain covalent bonding can occur even before completion of H-L disulfide bonds, as indicated by the observation of a sizable population of μ_2 L molecules after short pulses. (For a further discussion of assembly pathways, see reference 3, 33.) Assembly to monomers (μ_2 L₂) is very rapid and is followed within a few minutes by polymerization to pentamers, so that after appropriate chase periods almost all intracellular IgM is pentameric.

The speed of polymerization suggests that it occurs in the RER, and this has indeed been proven by cell fractionation and by the observation that polymerization is not impaired when ICT of pulse-labeled Ig is blocked by the addition of CCCP, an observation which also indicates a lack of energy requirement for polymerization. As polymerization occurs in the RER, it may be catalyzed by the same disulfide-interchange enzyme considered responsible for establishing correct disulfide bonds in single polypeptide chains (10). In addition to pentamers, minor intermediate oligomeric species are also observed but they represent <1% of the radioactivity recovered in IgM molecules. Since no chase interval has been identified which makes these intermediates predominant,

they appear not to be obligatory intermediates in pentamerization.

Relation between Glycosylation, Polymerization, and J Chain Addition and between Secreted and Surface IgM Molecules

Since polymerization occurs well before terminal sugar addition, polymerization does not restrict the access of the terminal glycosylating enzymes to the core sugars already present. Correspondingly, it has been found that polymerization does not interfere with the accessibility of core sugars to the deglycosylating action of endo H. Hence, the CHO units are located on the "outside" of pentameric IgM.

Ig-associated J chain is recovered exclusively with polymeric IgM, and is present in polymers before exit from the RER; thus, the present data are consistent with its being required for polymerization. J chain is a glycoprotein (17), and the steps of its ICT and glycosylation appear to be similar to those of μ chains, since it is sensitive to tunicamycin, and appears also to acquire terminal sugars just before secretion. Not only do polymerization and J chain addition not interfere with the processing of the oligosaccharide side chains of these molecules, but the presence or absence of sugars appears to play no role in the process of polymerization and J chain addition itself, as shown by the occurrence of pentamers associated with J chains in tunicamycin or deoxyglucose-treated cells. Such unglycosylated pentameric IgM is transported and secreted, although at a reduced rate.

Previous observations of tunicamycin-treated murine myelomas suggest that CHO facilitates IgM, IgA, and IgE secretion (11, 12). However, there is no evidence for a global CHO requirement for protein secretion. A number of secreted proteins lack CHO, and a number of glycoproteins (including IgG [12]) can be secreted with or without their CHO (32). Thus, the role of IgM CHO is unclear, and may be more related to the extracellular fate of these proteins than to their ICT.

Finally, the present observations rule out any model of secretion in which surface IgM is a precursor of secreted IgM, since surface IgM escapes polymerization and is present on the cell surface in monomeric form (42), while polymerization of secreted IgM occurs within the cells well before their secretion. The cells used in the present study all bore IgM on their surface as seen by

immunofluorescence. These IgM molecules can be biosynthetically labeled and detected, although they are present in only very small amounts relative to secretory IgM. The identification of these molecules and their μ chains, both of which have a retarded SDS-PAGE mobility compared to intracellular secretory IgM, relies on their susceptibility to iodination and their sensitivity to pronase treatment of intact cells. Evidence that surface μ chains have a polypeptide structure different from that of secretory μ chains, and that, like secretory μ chains, they are glycosylated in two steps, has been presented elsewhere (42).

We thank Mr E. Denking for his photographic assistance.

This work was supported by grant 3.324.78 from the Swiss National Science Foundation.

Received for publication 23 April 1979, and in revised form 16 July 1979.

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