THE SYNTHESIS, ASSEMBLY, AND SECRETION OF GAMMA GLOBULIN BY MOUSE MYELOMA CELLS

VI. ASSEMBLY OF IgM PROTEINS*

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The synthesis, assembly, and secretion of immunoglobulin G, and to a lesser extent of IgA, have been described (1-5). However, only one mouse myeloma producing IgM has been studied in detail (6-8). Since some human IgM-producing tumors contain large amounts of intracellular 19S polymer, while others do not, the present experiments were performed in order to determine if similar heterogeneity exists in a number of mouse myeloma tumors producing IgM.

Materials and Methods

Tumors were obtained from Doctors K. R. McIntire and Michael Potter of the National Cancer Institute (MOPC 104E, McPC 471, McPC 1748, TEPC 183, McPC 774) or from Doctors Melvin Cohn and Judith Hirst at the Salk Institute (Y 5781, W 3469). The sera of mice bearing these tumors contained homogenous 19S IgM proteins as studied by paper strip electrophoresis, immunoelectrophoresis, and ultracentrifugation.

All of the tumors were carried subcutaneously in BALB/c mice except McPC 471 and McPC 1748. These grew exclusively in the spleen whether they were injected subcutaneously or intraperitoneally. McPC 1748 was subsequently conditioned to grow subcutaneously, and comparison of immunoglobulin synthesis by cells from both sites revealed no differences. The preparation of tumor cells for labeling has been described earlier (1).

Experiments were performed in two ways. For continuous labeling the single-cell suspensions were washed twice in Eagle's minimal essential medium (MEM) containing 1/80 the

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Abbreviations used in this paper: MEM, Eagle's minimal essential medium; SDS, sodium dodecyl sulfate; VTL, valine, threonine, leucine.
normal concentrations of valine, threonine, and leucine (1/50 VTL). They were resuspended in the same medium and incubated at 37°C. 10 μCi each of prewarmed 14C-labeled valine, threonine, and leucine were added to the culture. Aliquots were then removed for analysis at various times.

Pulse chase experiments were performed after washing the single-cell suspensions twice in MEM containing only 1/50 the normal concentrations of valine, threonine, and leucine (1/50 VTL). Cells were then resuspended in this medium at a concentration of 1-2 × 10^7 cells/ml. 20 μCi each of prewarmed 14C-labeled valine, threonine, and leucine were added to the medium and the cells allowed to incubate for 21/2 min. At that time prewarmed MEM, containing excess unlabeled valine, threonine, and leucine, was added to the incubation vessel to yield a final concentration of 5 × 10^6 cells/ml and ten times the concentration of VTL. Samples were then removed at various times after chase. The adequacy of the chase was assessed by the determination of radioactivity precipitable with 5% trichloroacetic acid (TCA) in aliquots of the total culture removed at each time point.

As each sample was removed from the incubation mixture it was chilled to 4°C and made 0.06 M in iodoacetamide. At the end of the incubation all samples were spun in the cold and the supernatant medium was removed for analysis of secreted immunoglobulin. The cells were washed with cold medium, spun, and then resuspended in cold buffer. 0.1 vol of 5% Nonidet P-40 (Shell Chemical Corp., New York) was added and the cells were allowed to stand in the cold for 15 min and then spun at 100,000 g for 30 min. The nuclear-ribosomal pellet was discarded. The supernatant fluid was then analyzed as a cytoplasmic extract.

The secreted material and the cytoplasm were subsequently handled identically. Aliquots were made 2% in sodium dodecyl sulfate (SDS), boiled for 1 min, and dialyzed against 0.1% SDS in 0.01 M phosphate buffer before electrophoresis. Other aliquots were precipitated directly with antisera specific for either μ or light chain determinants in antibody excess. The immune precipitations were carried out in the presence of 0.06 M iodoacetamide at 4°C. They were allowed to stand for 24 to 48 h, spun, washed twice with ice cold 0.02 M phosphate-buffered saline, then dissolved in 0.4-0.5 ml of 2% SDS in 0.1 M phosphate. These were then electrophoresed on 20-cm SDS-containing polyacrylamide gels, the top 2 cm of which were 3.5% acrylamide, the remainder 5% for 16 h. Most gels were run with an aliquot of 3H-labeled secreted material obtained from the murine IgG2b-producing tumor MPC-11 as a marker. The gels were crushed onto planchettes (single-labeled experiments) for counting in a gas-flow counter or into vials for scintillation counting.

Some preparations of secreted material and cytoplasm were centrifuged on 5-20% sucrose gradients at 35,000 rpm for 16 h. The 19S and 7S fractions were pooled, dissociated with SDS, dialyzed, and analyzed on SDS gels. Reductions and alkylations of dissociated material were performed as previously described (3).

**RESULTS**

Cells from seven IgM-producing mouse myelomas were incubated with radioactive amino acids for 1 h and the labeled intracellular proteins were precipitated with antiserum specific for mu chains. The amount of immunoglobulin relative to total intracellular protein varied considerably among the different tumors (Table I). In six of the tumors the major intracellular protein precipitable with anti-mu antiserum had a mol wt of 185–195,000 and was presumably the 8S μL2 IgM monomer (Fig. 1 a). When tumors were labeled in vivo by injecting isotope directly into the tumor and sacrificing the animal after a suitable period, a similar profile of intracellular molecules was noted (Fig. 1 b).
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TABLE I

Analysis of Cytoplasm after 45 min of Incubation with Labeled Amino Acids*

| Tumor      | Cell protein as Ig | Intracellular immunoglobulin as | µL2 | µL  | L2 | L  |
|------------|--------------------|---------------------------------|-----|-----|----|----|
|            | %                  | µL2                             | µL  |     |    |    |
| MOPC 104   | 30                 | 31                              | 8   | 60  |
| MCPC 774   | 10                 | 62                              | 16  | 21  |
| MCPC 471   | 5                  | 16                              | 76  |     |
| MCPC 1748  | 4                  | 49                              | 26  | 20  |
| TEPC 183   | 20                 | 48                              | 24  | 12  |
| W 3469†    | 1-2                | 75                              | 24  |     |
| Y 5781‡    | 12                 | 66                              | 22  | 12  |

* Column 2 represents the amount of material precipitable with anti-immunoglobulin antiserum compared with that precipitable with 5% ice-cold trichloroacetic acid. The data in columns 3 to 7 were derived from the relative amounts of radioactivity found in each of these molecules by gel electrophoresis.

‡ These data represent the fraction of anti-µ-precipitable material found in each molecular species indicated. Light chains were not considered in these two tumors.

Fig. 1. SDS-polyacrylamide gel electropherograms of immunologic precipitates obtained from the TEPC 183 tumor. Migration is from left to right with the smallest molecules furthest to the right. a and b compare anti-µ precipitates (ppt) of cytoplasm (cyto) obtained from in vivo and in vitro labeling of the same tumor. The major anti-µ-precipitable molecule in both preparations was the 8S subunit (IgM). c and d compare an anti-µ precipitate of serum obtained after 3½ h of in vivo labeling with that seen after 3 h of in vitro secretion. Both samples show 19S IgM as the major extracellular protein.

Two other peaks precipitable with anti-µ and anti-light chain antisera were found in the cytoplasm of all the tumors after 45–60 min of incubation. One of these did not enter the 5% acrylamide gel, was usually seen in very small amounts, and probably represented partially or fully assembled 19S IgM.
(Fig. 1 c and d). This polymer was the predominant molecule secreted by the cells either into the medium (Fig. 1 d) or into the serum of the animal bearing the in vivo-labeled tumor (Fig. 1 c). The other had a mol wt of 95–100,000 daltons (Fig. 2), contained mu and light (L) chains upon reduction, and represents μL. Finally, when antiserum against light chain was used, an additional peak with mol wt of approximately 22,000 daltons was detected and seemed to represent free L chains (Fig. 2 b).

Detailed kinetic studies of the assembly of the subunit were carried out with four tumors. In all the tumors, free light chains served as a precursor to the 8S molecule. MOPC 104E produced a large excess of free light chain that was found intra- and extracellularly as light chain monomer. Several of the other tumors produced excess light chains but none to the extent seen in this tumor (Table I).

Fig. 2. a and b show the electropherograms of two aliquots of cytoplasm of the McPC 1748 tumor. a shows an anti-mu precipitate and b an anti-kappa precipitate of cytoplasm. Both were dissociated in SDS before electrophoresis. c indicates the molecular weights of the immunologically precipitable species according to their migration on the gels.
Two additional peaks, which were precipitable with anti-mu but not with anti-light chain antiserum, were seen in early time points of pulse chase experiments (Fig. 3 a, 2½ and 5 min). They had mol wt of 70–75,000 and 55–60,000. The smaller, designated \( \mu_o \), appeared to be a precursor of the larger, \( \mu_L \).

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**Fig. 3.** Shows the electropherograms of anti-mu precipitate of cytoplasmic samples of McPC 774 obtained after a 2½ min pulse with \(^{14}\)C-labeled amino acids followed by a chase with a large excess of cold amino acids. The times indicated represent total time after introduction of label. The position of the MPC-11 marker proteins are indicated by the arrows. b shows a kinetic plot of the same data indicating the precursor roles of \( \mu_o \), \( \mu_L \), and \( \mu_c \) and the end-product nature of \( \mu-L_2 \). The light chain data is omitted from the figures.
Molecules designated $\mu L$ were precipitable with both anti-heavy and anti-light chain antisera and had a mol wt of 95–100,000. As previously noted, $\mu L$ was often seen in the presence of large amounts of $\mu \alpha L_2$, but the kinetic experiments indicated a probable precursor role for this molecule (Fig. 3b).

In six of the seven tumors fully assembled 19S IgM represented 15% (or less) of the total intracellular immunoglobulin. In the seventh tumor (Y 5781), after 3 h of chase, there is more intracellular 19S than 8S material (Fig. 4). This is distinctly different from the other tumors and very similar to the majority of human IgM-producing tumors studied in our laboratory. The cytoplasm of Y 5781 also contains anti-mu-precipitable peaks of intermediate size between the 8S and 19S molecules. These are seldom intracellularly in the other tumors.

Cells that do not contain covalently bound 19S material have been examined for the presence of noncovalently bound polymer. The cytoplasm was centrifuged through a 5–20% sucrose gradient. The 19S and 7S fractions were pooled, dissociated in SDS, and electrophoresed. The 19S fractions contained only fully assembled material, indicating that there was no 8S material that sedimented as noncovalently bound polymer.

The predominant secreted molecule in all the tumors was the fully assembled 19S polymer. In some, pulse chase experiments intermediates similar to those seen intracellularly in Y 5781 were also noted. Some 8S IgMs were noted extracellularly in all the tumors studied. In several experiments it was possible to demonstrate a progressive shift of radioactive material from 8S to intermediates to 19S molecules extracellularly, indicating that the 19S material was covalently assembled outside the cell (Fig. 5).

The kinetics of secretion were similar to those reported for IgG- and IgA-producing tumors. There was a 20 min lag before the appearance of labeled L chain in the medium. Heavy chain containing material could be seen after 60 min in most experiments.

When reduced and alkylated samples of immunologically precipitated cytoplasm and secreted material obtained from the same tumor were examined on the same gel, the peaks could be superimposed, indicating no differences in electrophoretic mobility detectable in this system (Fig. 6).

The ratio of heavy to light chain found in reduced and alkylated anti-mu precipitates obtained from cells continuously labeled with multiple amino acids ranged between three and four to one and corresponded to an estimated mol wt ratio of $7 \times 10^4$ to $2.3 \times 10^4$ daltons for mu and light chains.

**DISCUSSION**

Several conclusions can be drawn from these experiments. In all tumors studied $\mu L$ is the major precursor of the $\mu \alpha L_2$ subunit. This is analogous to IgG2b-producing mouse tumors and rabbit lymph node cells in which $\gamma L$ is designated $\mu L_2$, and was no longer detectable after 41/2 min of chase (Fig. 3a and b). The larger chased into higher molecular weight molecules.
Fig. 4. Electropherograms of anti-mu-precipitated cytoplasm obtained after various pulse periods of the Y 5781 tumor. There is a relative increase in radioactivity of the 8S subunit up to 1–2 h with a subsequent increase in 19S material. Between 3 and 4 h there is a relative decrease in 19S material related to its more rapid rate of secretion. Peaks intermediate in size between 8S and 19S are readily noted. During this period the secreted material contain both 19S and 8S material with a large preponderance of 19S.
Fig. 5. Electropherograms of anti-mu-precipitated molecules obtained from secreted material of TEPC 183 after pulse chase labeling. The early time point contains 8S material and a larger peak with a broad leading shoulder. At 2 h the intermediate peaks are more discrete. By 180 min the bulk of the counts have shifted to the largest molecular species.

the major precursor of γ2Lc (1, 9). μL also served as the precursor in most human IgM-producing tumors (10).

Free light chains have been noted as precursors in all mouse and human tumors in which kinetic studies have been performed. Most of the tumors
produce excess light chains. These observations led some observers to hypothesize that the light chains were necessary to release the heavy chains from the polyribosome (9, 11). Later studies in cells producing IgG and IgA demonstrated the presence of free cytoplasmic heavy chains, indicating that covalent binding of light chain is not necessary for ribosomal release (1, 2, 4, 5). The present experiments demonstrate that free cytoplasmic mu chain is an early detectable precursor of $\mu L$.

It has previously been reported that in the course of short-term pulse-labeling experiments a polypeptide was identified that was serologically precipitable with anti-mu antiserum, but was smaller in size and lacked carbohydrate (12). The present studies confirm that observation and extend it to several other tumors. In addition it seems that the smaller mu chain is the precursor of the larger. The difference in size may be more apparent than real, since the attachment of carbohydrate to a polypeptide has an effect on the electrophoretic mobility in SDS out of proportion to its molecular weight.

The demonstration of different modes of assembly in human IgM-producing tumors made it seem likely that a similar heterogeneity would exist in the murine system. However, there are differences. The majority of the mouse tumors contain little intracellular polymer larger than 8S while most human tumors contained intracellular polymer. It is possible that examination of a larger number of tumors of both species would indicate a similar quantitative distribution of tumors containing intracellular 19S IgM. The presence of little 19S IgM in the cells of the actively immunized mouse spleen suggests that the
average assembly behavior of the available murine IgM-producing tumors approximates that of a population of normal spleen cells synthesizing IgM (13).

The heterogeneity raises two additional questions. First, is it a function of the primary structure of the protein or is it a property of some cellular mechanism? Second, what is the relationship between polymerization and secretion? Other investigators have stated that assembly must take place at, or close to, the time of secretion (6, 7). The pulse chase experiments in several of the mouse tumors reveal extracellular 8S IgM and a family of polymers intermediate in size between 8S and 19S. It also appears that these subunits and intermediates chase into 19S material. If this is an accurate representation of in vivo events then it is surprising that hybrid 19S molecules are not found in the serum. Three factors could be responsible: (a) Different IgM-secreting clones may be sufficiently separated in space so that subunits secreted from different cells are some distance from each other. (b) There may be a greater relative affinity of like subunits for each other than for other dissimilar subunits. In vivo studies have shown that heavy and light chains derived from the same antibody molecule or myeloma protein preferentially recombine after reduction. This has not yet been definitively established for IgM subunits. (c) The newly secreted subunits may be associated by noncovalent bonds. Such a mechanism would effectively inhibit any significant degree of disulfide bond formation among heterogeneous subunits.

The kinetics of chase of 8S subunits into 19S IgM indicate that the formation of the intersubunit disulfides may not be as rapid as that between mu and light chains nor as that between \( \mu \)L dimers. The apparent difference could also be explained by a substantial intracellular pool of 8S subunits. This would produce a situation in which 19S molecules could be seen with an indeterminate proportion of labeled and unlabeled subunits. The resultant mixing of these subunits makes clear-cut kinetic data more difficult to obtain.

Recent data has indicated that subunits produced by partial reduction of extracellular IgM differ in carbohydrate composition from those found intracellularly (14). The same study suggested conformational differences between the intra- and extracellularly derived subunits. The role of these differences in the assembly and secretory processes is not yet clear. If the present results demonstrating extracellular assembly are correct, the transition between the intracellular and extracellular form of the IgM subunit must take place at the time of secretion.

SUMMARY

The study of the synthesis, assembly, and secretion of IgM by seven murine myeloma tumors has revealed that free mu chain can be detected intracellularly after release from the ribosome. It combines with light chains to form \( \mu \)L. The major intracellular protein in six of the seven tumors was the 8S subunit. One tumor contained considerable amounts of 19S material intracellularly.
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larly. Those tumors that did not contain $19\text{S}$ IgM intracellularly appeared to assemble the subunits outside the cell.

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