SYNTHESIS AND ASSEMBLY OF IMMUNOGLOBULINS BY MALIGNANT HUMAN PLASMACYTES AND LYMPHOCYTES

II. Heterogeneity of Assembly in Cells Producing IgM Proteins*

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Malignant plasma cells and lymphocytes which produce large amounts of homogeneous immunoglobulins are particularly well suited for the investigation of protein synthesis in mammalian cells. The synthesis of heavy and light chains and their subsequent assembly into fully assembled IgG (H_2L_2) has been studied extensively in cells obtained from both murine and human myelomas (1, 2). The structure of IgM is more complex and its assembly deserves special attention since the H_2L_2 monomer must undergo further assembly into the $(\mu_2L_2)_5$ pentamer (3).

The present report describes the assembly and secretion of IgM immunoglobulin by cells obtained from the bone marrow and lymph nodes of patients with Waldenström's macroglobulinemia whose sera contained large amounts of monoclonal IgM proteins. The 8S (μ_2L_2) subunit was the major intracellular precursor of the 19S $(\mu_2L_2)_5$ polymer in cells obtained from all the patients. In one group of patients however, 19S protein appeared to accumulate intracellularly before secretion, while in the other group little 19S polymer was detectable inside the cell. Cells of both groups secreted predominantly 19S polymer under the experimental culture conditions.

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Materials and Methods

Bone marrow cells were aspirated from the sternum or iliac crest of 10 patients. The samples were collected in heparinized syringes and immediately placed in sterile tubes containing 10 ml of Eagle's minimal essential medium with $\frac{1}{20}$ the usual concentrations of valine, threonine, and leucine, and 100 units of heparin ($\frac{1}{20}$ medium). In two patients enlarged lymph nodes were removed intact at the time of laparotomy and placed in the same medium. After the adventitial fat and connective tissue were removed, the lymph nodes were minced into small fragments with scalpel blades, and placed in a 25 ml Erlenmeyer flask containing 10 ml of the same medium. The fragments were shaken vigorously in a rotary shaker for 5 min at 37°C and the supernatant fluid containing loose cells was passed through a stainless steel wire mesh to remove debris and provide a single cell suspension. The suspension was spun at 600 *g* for 5 min and the supernatant fluid removed. The marrow cells were spun at 600 *g* for 10 min, washed in $\frac{1}{20}$ medium, then suspended in distilled water for 60 sec to lyse the red cells. Isotonicity was restored by adding a concentrated balanced salt solution. Cells from either source were then washed twice with $\frac{1}{20}$ medium, or medium totally depleted of valine, threonine, and leucine, and resuspended in 1 ml of medium. A fraction was removed, diluted with erythrosin B dye, and the viable nucleated cells counted in a hemocytometer (4). The cells were resuspended at a final concentration of 5×10^6 or 1×10^7 cells/milliliter.

15 μCi each of ^{14}C-labeled valine, theronine, and leucine were added to the cell suspensions for continuous labeling experiments. "Pulse-chase" experiments were performed by incubating the cells with 20 μCi of each of these amino acids, then adding an excess of unlabeled valine, threonine, and leucine to bring the final concentration of these amino acids to 10 times that found in Eagle's medium. This chase stops incorporation of the labeled amino acids but allows protein synthesis to continue. Because the human marrow samples were a mixed cell population containing a limited number of immunoglobulin-producing cells, a relatively long incubation with label was necessary to obtain sufficient incorporation for analysis. 1 hr pulses were adequate, however, to study the assembly of monomers into polymers.

The labeled cells were treated as described in reference 2. Cytoplasmic lysates or secreted material were precipitated with antiserum specific for mu or light chain determinants. The labeled mu chains present were quantitatively coprecipitated with goat anti-mu chain antiserum and with 15 μg of carrier human macroglobulin. Light chains were precipitated directly by the addition of rabbit anti-human light chain antiserum. Preliminary precipitin curves were carried out to insure that all precipitations were performed in slight antibody excess. Self-assembly of chains into subunits or subunits into polymer, during the course of immunologic precipitation, was prevented by carrying out the precipitations in the cold in the presence of 0.03 M recrystallized iodoacetamide. The washed precipitates were dissolved in 2% sodium dodecyl sulfate (SDS)[1] with 0.03 M iodoacetamide and 0.01 M sodium phosphate buffer and placed in a boiling water bath for 1 min. In some experiments the dissociated material, the precipitated material, or both, were reduced and alkylated. In these experiments the samples were mixed with tritium-labeled secreted material from the murine plasmacytoma MPC-11 and made 0.15 M with 2-mercaptoethanol. They were allowed to stand for 30 min at 37°C, then were dialyzed against 0.015 M 2-mercaptoethanol overnight. The samples were then made 0.02 M with iodoacetamide, incubated at 37°C for 30 min, and then redialyzed against 0.01 M phosphate buffer containing 0.1% SDS.

The SDS-dissociated samples, the dissociated immune precipitates, and the reduced and alkylated samples were subjected to electrophoresis on 20 cm acrylamide gels containing 0.1% SDS. The top 2 cm of each gel was 3.5% acrylamide while the remainder was 5%. The exclusion limit of the 5% gel is between 350,000 and 400,000 daltons. The tritium-labeled secreted material from MPC-11 provided internal molecular weight markers for each gel and served as

[1] *Abbreviation used in this paper:* SDS, sodium dodecyl sulfate.

a control for proteolytic activity in the cytoplasmic samples (5). In this gel system the rate of migration of a protein down the gel is inversely related to the logarithm of its molecular weight (6).

Gels were ground with a Maizel gel grinder (Savant Instruments, Inc., Hicksville, N.Y.) and extruded either onto planchettes for single ^{14}C-labeled experiments or directly into scintillation vials for double-labeled experiments. The planchettes were dried and counted in a Nuclear-Chicago gas flow counter (Nuclear Chicago Corp., Des Plaines, Ill.). The vials were allowed to stand overnight in the cold before addition of 10 ml of toluene-triton solution after which they were counted in a Beckman liquid scintillation counter.

Secreted material and cytoplasm were sedimented through 12 ml 5–20% linear sucrose gradients made up in 0.02 M phosphate-buffered isotonic saline, pH 7.2, in an SW-41 swinging bucket rotor at 35,000 rpm for 15 hr. 1 mg each of purified unlabeled IgG and IgM proteins were added to each gradient to serve as optical density markers. Gradients were analyzed by scanning the fractions at 280 mμ and subsequently measuring the amount of radioactivity precipitable with 5% trichloroacetic acid in each of the collected fractions.

Individual studies could only be compared qualitatively, since the degree of incorporation of labeled precursor into immunoglobulin was affected by several variables. Although all experiments were carried out at similar concentrations of nucleated cells, the number of immunoglobulin-producing cells varied. Alterations in the synthesis of immunoglobulin with the cell cycle (7) and the effect of effete cells which excluded erythrosin B but did not synthesize immunoglobulin could not be controlled. In spite of this, the rate of incorporation of labeled precursor into immunoglobulin expressed as counts per cell per minute roughly corresponded to the proportion of potential immunoglobulin-forming cells in the sample, i.e., lymphocytes and plasma cells seen on smear and stain of the marrow.

Serum and urine samples obtained from these patients were analyzed by standard electrophoretic and immunoelectrophoretic techniques and analytical centrifugation in a Spinco Model E ultracentrifuge. Proteins were usually purified by euglobulin precipitation followed by Sephadex G-200 filtration. Proteins which were not euglobulins were initially fractionated by starch block electrophoresis and then subjected to gel filtration. Light chain and mu chain types of most proteins were determined serologically either in whole serum or after isolation and purification. The mu chain types of some of the samples were determined by peptide mapping (8).

RESULTS

Bone marrow and lymph node cells from patients producing large amounts of macroglobulins were incubated with radioactive amino acids and the intracellular and secreted proteins were analyzed on SDS containing acrylamide gels. When the total soluble cystoplasmic proteins from the cultured cells of one such patient were examined, three well-defined but relatively small peaks of radioactivity were noted in fractions 8, 18, and 30 (Fig. 1 *A*). In addition a large amount of low molecular weight material was seen in fractions 45–70. When the cytoplasm was immunologically precipitated with antiserum specific for the IgM heavy chains (mu chain), and the specifically precipitated labeled protein examined, three distinct peaks representing about 10% of the total labeled cytoplasmic protein were seen. (Fig. 1 *B*). The same pattern was obtained when the cytoplasm was specifically precipitated with antiserum reactive with light chain antigenic determinants (not shown). Based on their migration relative to the mouse immunoglobulin markers (indicated by arrows in Fig. 1 *B*), the molecular

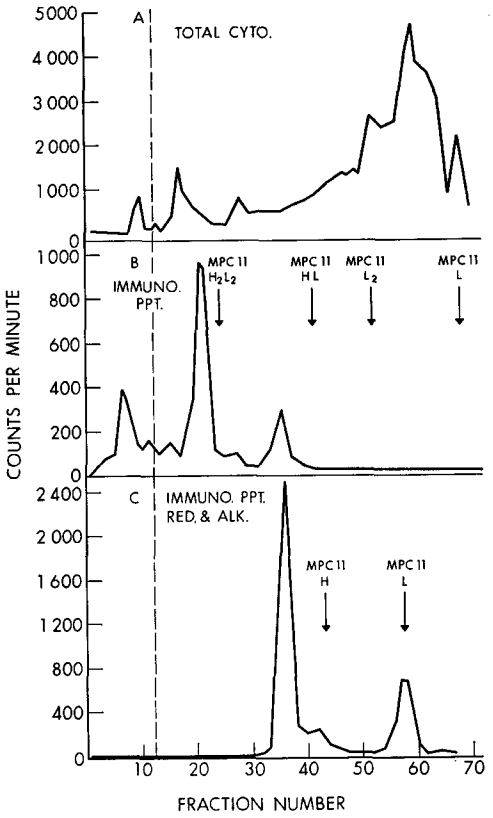

FIG. 1. (*A*) Electrophoresis of SDS-dissociated cytoplasm prepared from cells after a 2 hr incubation with ^{14}C-labeled amino acids. The broken vertical line represents the junction between the 3.5 and the 5% gel. (*B*) The ^{14}C-labeled cytoplasm shown in *A* was precipitated with an antiserum directed against mu antigenic determinants. The precipitate was dissociated in SDS and mixed with ^{3}H-labeled secreted material obtained from the murine plasmacytoma MPC-11. The arrows indicate the positions of the ^{3}H-markers. (*C*) An additional fraction of the same cytoplasm shown in *A* and *B* was immunologically precipitated with an anti-mu antiserum. The precipitate was dissociated, mixed with a sample of ^{3}H-labeled MPC-11 secretion, and the mixture reduced with 2-mercaptoethanol and alkylated with iodoacetamide. The positions of the mouse heavy and light chains are indicated by the arrows.

weights of these proteins were greater than 360,000 (fraction 7), approximately 198,000 (fraction 22), and 98,000 (fraction 34) (Fig. 2). Upon reduction and alkylation almost all of the specifically precipitated material was converted into two radioactive peaks which co-electrophoresed with the heavy and light chains obtained by reduction and alkylation of the isolated serum IgM of the same patient. The cytoplasmic light chains co-electrophoresed with the mouse marker light chains (Fig. 1 *C*) while the mu chains obtained from cytoplasm, secreted

material, or isolated serum protein were larger than the mouse heavy chain marker (molecular weight 55,000) and had an estimated molecular weight of 70,000. This estimate is consistent with that obtained for mu chains by other workers using different techniques (3, 9).

Because of their size (Fig. 2), precipitability with anti-mu and anti-light chain antisera and the ratio of light to heavy chains in the reduced and carboxymethylated immunological precipitates (Fig. 1 *C*), the protein migrating in fraction 34 of Fig. 1 *B* is thought to be μL half molecules, that found in fraction 22 is μ_2L_2 monomer, and the protein in fraction 7 is a higher μ_2L_2 polymer, presumably $(\mu_2L_2)_5$. Other experiments have demonstrated that electrophoretic migration of 8S monomer obtained by mild reduction and alkylation of the isolated serum protein was similar to that of the intracellular μ_2L_2 subunit. The low

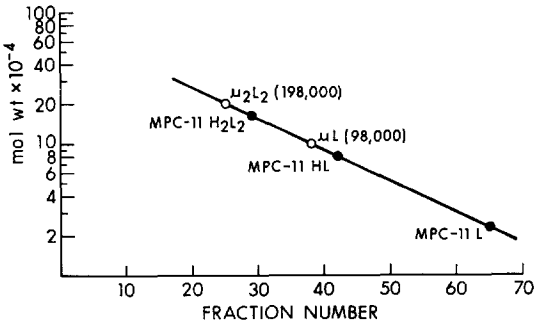

FIG. 2. The migration of marker proteins of known molecular weight and that of the molecular species migrating into the 5% gel in Fig. 1 *B* are plotted against the logarithm of the molecular weight.

molecular weight material seen in fractions 45–70 of Fig. 1 *A* was not precipitable with a polyvalent anti-immunoglobulin antiserum and probably represents nonimmunoglobulin cell protein.

After 1 hr of incubation the major intracellular immunoglobulin in the cells of all of the patients examined was the μ_2L_2 subunit (Fig. 3 *A* and *B*, bottom panel). Since little or no 19S polymer was seen after 60 min, further experiments were carried out using longer labeling times. These studies revealed that there were at least two discrete patterns of assembly and secretion of completed IgM proteins. Experiments typical of each of the groups are illustrated in Fig. 3, which shows the electropherograms of cytoplasm obtained from cells of patients SN and BE after precipitation with antiserum specific for mu chains. The cells were labeled for 1 hr with ^{14}C amino acids and then chased with a large excess of nonradioactive amino acids; after 60 min of label the major intracellular immunoglobulin was the 8S subunit in both groups. However, at later times after

the chase, cells from six patients resembled patient SN and accumulated increasing amounts of fully assembled IgM intracellularly (Fig. 3 *B*). Cells from two other patients, as in BE, never contained more than 15% of their total intracellular IgM as the 19S polymer. Fig. 4 shows the relative proportions of

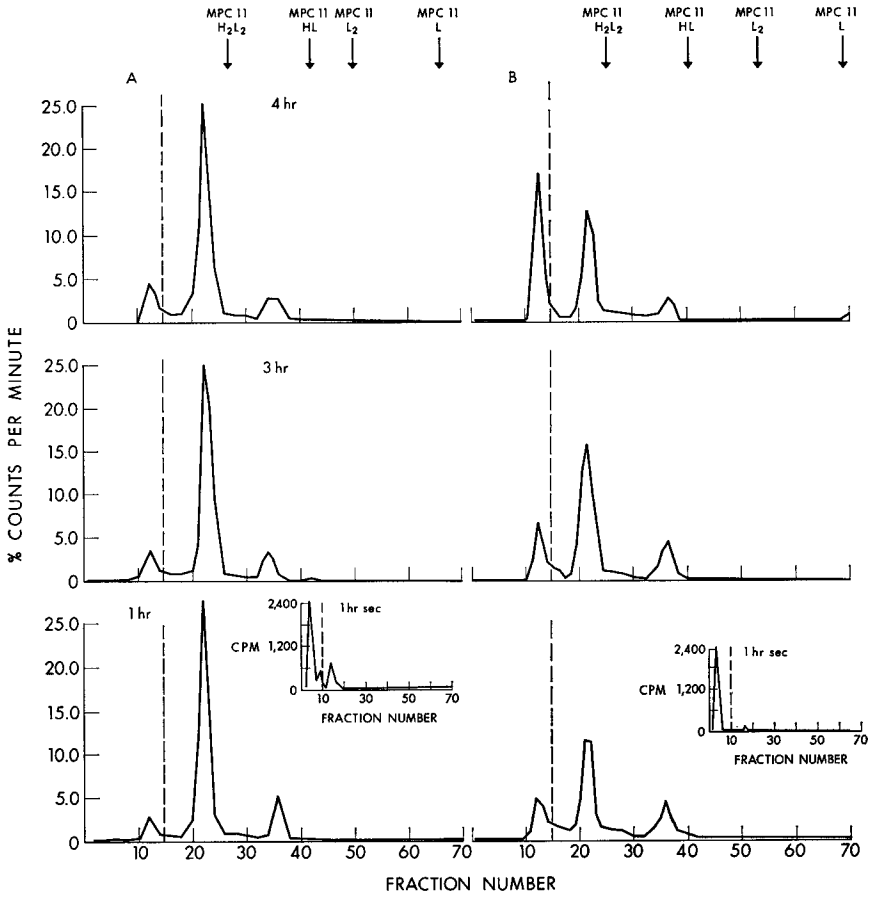

FIG. 3. Cells from two patients BE(*A*) and SN(*B*) were pulsed for 1 hr and then chased as described in Materials and Methods. The cytoplasms were immunologically precipitated with antiserum directed against mu antigenic determinants. In order to compare the two, the results are expressed as the percentage of total cytoplasmic counts precipitable with the anti-mu antiserum. A plot of 1 hr secreted material is shown in the inserts.

8S and 19S proteins which were present in cells of both groups at various times after chase. The kinetics were blurred somewhat by the long label and the effects of secretion of the fully assembled polymer, but in SN, by 3 hr after chase, the amount of 19S material in the cytoplasm exceeded the amount of 8S monomer.

At later time points, the ratio of 19S to 8S continued to increase. In the cytoplasm of BE there was little or no increase in the proportion of IgM found as the fully assembled polymer. We have chosen to designate the two groups accumulator and nonaccumulator. Mixing experiments were performed to determine whether intracellular material was contaminated with assembled IgM that had already been secreted and was nonspecifically absorbed to the cells during culture. Incubation of labeled 19S secreted material with unlabeled cells revealed no conversion of the 19S material to the 8S monomer, nor did any radioactive polymer remain with the cells during the preparation of unlabeled cytoplasm.

Both groups contained detectable amounts of μL. Its precursor role in mono-

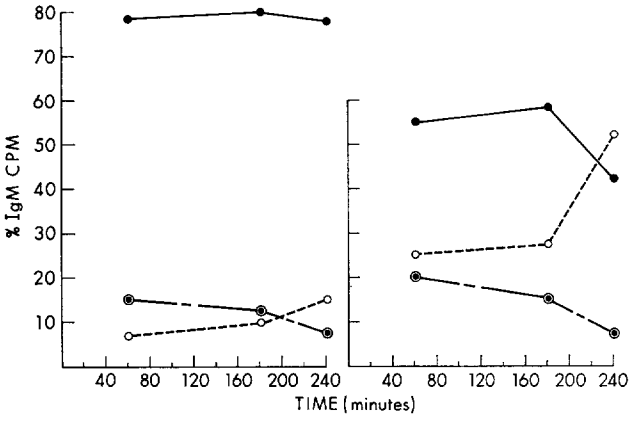

FIG. 4. The data from Fig. 3 is plotted against time. ●——● μ_2L_2 monomer; ○---○ μ_2L_2 polymer; ◉——◉ μL half molecules.

mer assembly has not yet been established. However, the data plotted in Fig. 4 suggests such a role. Molecules of intermediate size between 8S and 19S were not seen intracellularly in the pulse-chase experiments. However, in several experiments in which continuous labeling techniques were used, peaks of radioactivity which were precipitable with anti-mu antiserum and which migrated between 8S and 19S were observed (Fig. 5). The exact size of these proteins could not be accurately estimated because well-characterized markers of sufficiently high molecular weight were not available.

When secreted material obtained from both groups was examined after 1 hr of label, the major protein was found to be the 19S polymer (inserts, Fig. 3 *A* and *B*). Small amounts of 8S monomer were seen at all time points in both groups but the peak was more prominent in the secretions obtained from nonaccumulator cells. In addition, the 1 hr secretion from nonaccumulator cells appeared to contain one or more peaks migrating between 8S and 19S (insert,

Fig. 3 *A*). These were precipitable with anti-mu chain antiserum and were not seen in the cytoplasm of the same cells.

Experiments were performed to ascertain the degree of noncovalent polymerization of the 8S subunits intra- and extracellularly in both groups. Undissociated cytoplasm or secreted material was sedimented through 5–20% sucrose gradients with known 19S and 7S markers (Fig. 6). The gradient fractions corresponding to the 19S and 7S peaks and the intermediate valley were pooled,

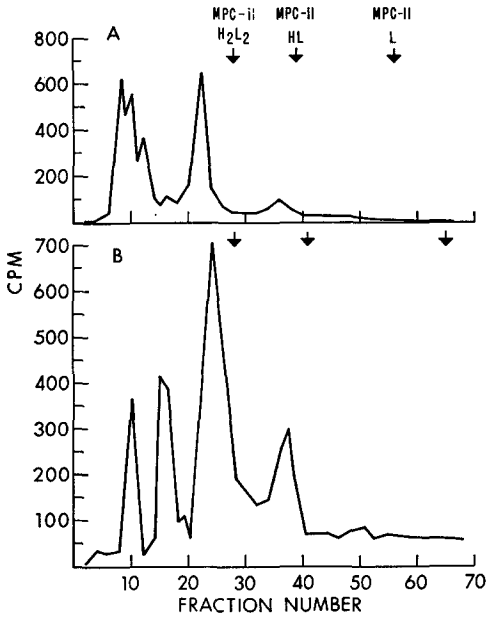

FIG. 5. Electropherograms of the cytoplasm from cells labeled continuously for 3 hr from patients ST and LE and immunologically precipitated with antiserum directed toward mu determinants. The junction between the 3.5 and 5% gel is at fraction 9 in panel *A* and fraction 11 in *B*. The MPC 11 markers are indicated by the arrows.

dissociated in SDS, and gelled as above (Fig. 7). In the accumulator cells studied, the material which sedimented with the 19S IgM marker was covalently bonded IgM since no material migrating as μ_2L_2 was found on electrophoresis of proteins isolated from this region of the gradient. The material which sedimented with, or close to, the 7S IgG marker was 8S monomer and did not dissociate in SDS. When similar studies were carried out with material obtained from nonaccumulator cells, the cytoplasm was found to contain 1–2% of its immunoglobulin as noncovalently bound 19S IgM. The secretion contained no material which sedimented with the assembled 19S polymer in sucrose and migrated as 8S monomer on the SDS gels.

Pulse-chase or continuous labeling experiments were carried out with cells from 10 patients; however, the studies were carried beyond 1 hr in only 8. Fig. 3 demonstrates that 1 hr samples are not adequate to differentiate between the two groups.

In two instances it was possible to compare synthesis and assembly in the same individual in two distinct anatomic sites. In both patients, labeling of

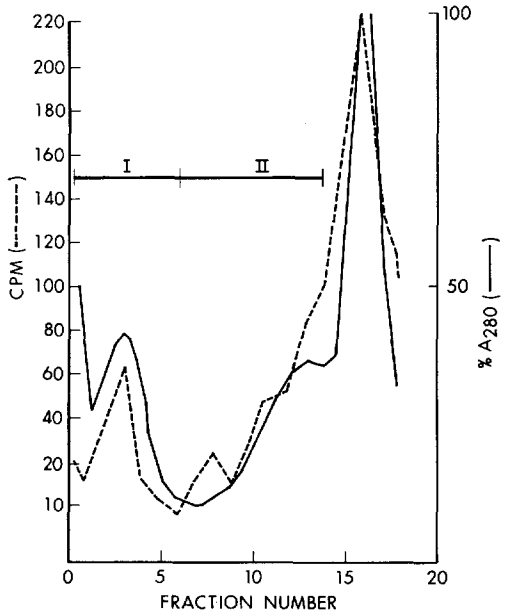

FIG. 6. Cytoplasm was prepared from cells of patient ST after 60 min of label with ^{14}C-amino acids and analyzed on a sucrose gradient. The bottom of the gradient is at fraction 1, the top at fraction 18. The optical density peak at fraction 3 represents the added human 19S marker, that at fraction 13 the 7S marker, and that at fraction 16 the low molecular weight cytoplasmic proteins. I and II represent the pools selected for gel electrophoresis as shown in Fig. 7.

marrow and lymph node cells carried out simultaneously revealed identical patterns of synthesis, assembly, and secretion by cells obtained from both sites (Fig. 8). Studies carried out on peripheral lymphocytes from individuals whose marrow produced large amounts of IgM, demonstrated both a relatively low rate of total protein synthesis and incorporation of labeled amino acid into secreted IgM.

Sequential studies were performed on one patient who required an additional bone marrow examination to assess the effects of chlorambucil therapy. Although the number of lymphocytes in the marrow had decreased considerably,

and the degree of incorporation of radioactive amino acids was markedly reduced the second time, the pattern of assembly was the same.

DISCUSSION

All the human IgM-producing cells studied utilized the 8S μ_2L_2 subunits as the quantitative major precursor of the fully assembled 19S molecule. This finding is consistent with observations in the murine macroglobulin-producing

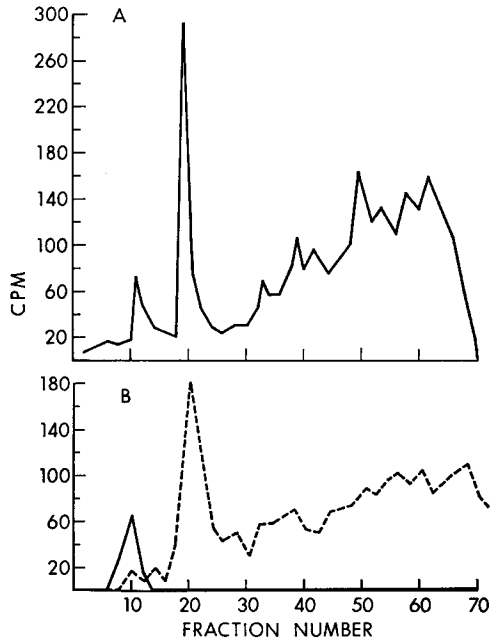

FIG. 7. (*A*) An additional portion of the cytoplasm analyzed in Fig. 6 was dissociated and electrophoresed without immunologic precipitation. The junction between the 3.5 and 5% gels is at fraction 12. (*B*) The solid line (——) represents the electropherogram of the labeled material obtained from pool I shown in Fig. 6. The dashed line (----), the material obtained from pool II. The junction between the 3.5 and 5% gel is at fraction 11.

tumors MPC 104E (10) and MPC 471.[2] It also reinforces the contention that the serum 8S IgM seen in some hyperglobulinemic patients and in some individuals with Waldenström's macroglobulinemia is a synthetic rather than a catabolic product (11, 12).

The 8S protein is probably assembled in a manner similar to IgG with either HL, H_2L, or both serving as intermediates. It has been demonstrated that murine tumors producing IgG_1 and IgG_{2a} proteins assemble their immuno-

[2] Buxbaum, J., and M. D. Scharff. Unpublished observations.

globulins primarily via H_2 and H_2L, while those assembling IgG_{2b} employ both pathways (13). Our studies were not designed to determine the sequence of the early steps in assembly, but the data illustrated in Figs. 3 and 4 and short-term labeling experiments in MPC 104E suggest that in the tumors studied, μL is the major precursor of the μ_2L_2 subunit. Further studies employing shorter incubation periods with a larger number of human tumors may reveal a pattern of heterogeneity similar to that seen in IgG production in the mouse.

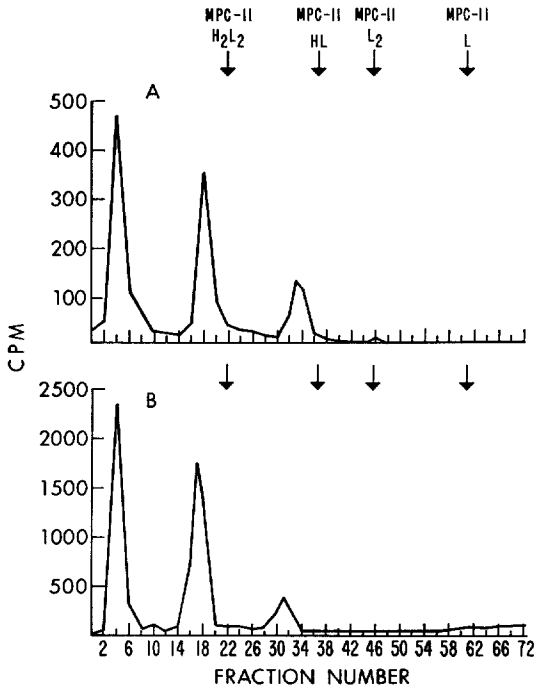

FIG. 8. Cytoplasms were prepared from cells obtained from two different anatomic sites (*A* marrow, *B* lymph node) of the same patient. After 4 hr of incubation with label, immunologic precipitates were prepared with anti-mu antiserum and gelled.

The large amount of labeled 8S material seen intracellularly and the absence of large amounts of intermediate polymers in the pulse-chase experiments suggests that a pool of monomers exists inside the cell, and that these polymerize to form the 19S protein. It seemed possible that the 8S monomers would first be joined by noncovalent interactions with subsequent formation of the intersubunit disulfides. However, we were unable to demonstrate any intra- or extracellular noncovalently assembled 19S protein which could be dissociated to monomer or intermediates by SDS.

The discovery of two distinct modes of assembly of IgM proteins was unex-

pected and further investigation of the basis of the differences may provide insight into the process of polymer formation. The presence of 8S IgM in the sera of normal individuals and those with polyclonal hypermacroglobulinemia, and the rare occurrence of individuals with macroglobulinemia whose sera contain large amounts of subunits suggest that a third variant exists in which unlinked monomers are secreted in substantial quantities. The differences in assembly may reflect structural differences between mu chains. We have not examined the serum proteins of these patients for possible differences in amino acid sequence, in the number or position of inter mu chain disulfides, or in their carbohydrate moieties (14) to determine whether any of these correlate with the accumulator property. On the other hand, the different groups could reflect functional differences in the cells synthesizing the proteins. The cells may vary in their enzymatic constitution so that the quantitative or temporal relationships between assembly and secretion are not the same in cells from different patients.

The demonstration of heterogeneity in immunoglobulin synthesis and secretion by malignant plasma cells and lymphocytes raises questions relevant to the production of these proteins in the course of the normal immune response. If myeloma proteins are structurally representative of normal anitbodies then it may also be true that the various assembly pathways of the different tumors are representative of subpopulations of normal IgM-producing cells and should be demonstrable in an IgM anti-body-producing cell population. These studies, and those of others, suggest that in humans there may be at least three distinct populations, accumulators, nonaccumulators, and a less frequently occurring third group of monomer secretors, all of which are capable of synthesizing IgM antibodies.

SUMMARY

Bone marrow or lymph node cells from 10 patients whose sera contained large amounts of monoclonal IgM proteins were incubated with radioactive amino acids in short-term tissue culture. Samples of soluble cytoplasmic extracts and secreted material were examined by immunologic precipitation with specific antisera, acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and sucrose gradient centrifugation. In all samples studied, 8S IgM was the major intracellular precursor of the fully assembled 19S protein. Cells obtained from some patients contained little or no fully assembled 19S protein intracellularly; however, cells from most patients seemed to accumulate fully assembled 19S molecules intracellularly before secretion. Secreted material from both groups contained large amounts of 19S IgM. The differentiation between accumulating and nonaccumulating cells did not correlate with heavy or light chain antigenic type. Synthesis and assembly appeared to be identical in cells obtained from different anatomic sites in the same patient Studies carried out

in one patient before and after therapy revealed no qualitative differences in the pathway of assembly and reflected only a decrease in the total number of immunoglobulin-producing cells.

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