IgM-PRODUCING TUMORS IN THE BALB/c MOUSE

A Model For B-Cell Maturation*

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The steps involved in the maturation of normal bone marrow-derived (B) lymphocytes into immunoglobulin (Ig)-secreting plasma cells after antigenic or mitogenic stimulation have been described according to cellular morphology in morphology in electron micrographs (1, 2), immunofluorescent analysis of surface-bound and intracytoplasmic Ig (3), and biochemical studies of IgM synthesis, surface representation, and secretion (4–6). In the present studies the state of maturation of cells obtained from five murine IgM-producing lymphocytomas has been characterized by a variety of morphological and biochemical criteria. The degree of maturity of each of these cell populations has been assessed by comparison to normal mitogen-stimulated B lymphocytes.

Materials and Methods

Tumors.—Mouse tumors transplantable in BALB/c-mice were obtained from Doctors K. R. McIntire and M. Potter, National Cancer Institute, NIH, Bethesda, Md. (McPc 1748, TEPC 183, and McPc 774) or from Doctors Melvin Cohn and Judith Hirst at the Salk Institute, La Jolla, Calif. (Y 5781, W 3469). Tumors were grown subcutaneously.

Morphology.—Animals were sacrificed by exsanguination under light ether anaesthesia. Sera were collected and stored for serologic studies. Organs were examined in situ or removed aseptically for further studies. Subcutaneous tumors were prepared for morphologic studies by four methods. Imprints were made from freshly cut tumor surfaces and stained with Wright's and Giemsa stains. Blocks 30–50 mm on a side were fixed in 10% formalin, embedded, cut, and stained with hematoxylin and eosin. Cell suspensions were spun in a cytocentrifuge and stained with Wright's and Giemsa stains. Additional fresh tumor was cut into 1–2 mm cubes, placed in 1.5% glutaraldehyde in 0.1 ml sodium cacodylate, coded, and prepared for electron microscopy (7).

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Immunofluorescence.—

Antisera: A specific antimouse μ-chain antiserum was raised in rabbits by injection of purified IgM mouse myeloma protein MOPC 104, followed by absorption of the serum with a mixture of purified IgG_κ and IgA myeloma proteins, made insoluble by cross-linking with ethylchloroformate (8). The specificity of the antiserum was assessed by immunodiffusion in agarose gel. A further control for specificity was performed after conjugation with fluorochrome by staining of IgM and IgG plasma cells. The antiserum did not stain an IgG1-producing myeloma cell line, which was stained both on the membrane and in the cytoplasm by a specific anti-IgG1 antiserum. The IgG fraction of the antiserum, prepared by ammonium sulfate precipitation and DEAE chromatography, was labeled with fluorescein or rhodamine isothiocyanate (Baltimore Biological Labs., Baltimore, Md.) according to Cebra and Goldstein (9).

Staining of cells: Cell suspensions were prepared from the solid tumors by teasing on stainless steel gauze. After allowing spontaneous sedimentation of cell clumps, the suspensions were washed twice in the cold in Hank's balanced salt solution + 10% fetal calf serum.

Double staining of cells for surface and intracytoplasmic immunoglobulins was performed as described by Pernis et al. (3). Briefly, the cell suspensions were incubated in the cold with an equal vol of rhodamine-conjugated antiserum for 30 min in an ice bath, and washed three times with cold medium. The pellet was then resuspended in 3% bovine serum albumin in phosphate-buffered saline (PBS)[1] and smeared on slides by means of a Shandon-Elliot cytocentrifuge (Shandon Scientific Co., Inc., Sewickley, Pa.). The cell smears were fixed for 10 min in 95% ethanol, rehydrated in several changes of PBS, and exposed to the fluorescein-conjugated antiserum for 45 min at room temperature in a moist chamber. After thorough washing in PBS, the slides were mounted in phosphate-buffered glycerol.

Microscopy: The preparations were observed under a Leitz Orthoplan microscope equipped with an Opak-Fluor vertical Illuminator (Leitz GmbH, Wetzlar, Germany), using selective illumination for fluorescein or rhodamine (10). Photographs were recorded on Kodak-Tri Pan 23 DIN black-white film.

Biosynthetic Studies.—Preparation of cell suspensions, cell viability determinations, labeling of cells with L-[4,5-^{3}H]leucine (50 Ci/mmol, 66 μCi/ml, batch 40), D-[1-^{3}H]mannose (3.3 Ci/mmol, 50 μCi/ml, batch 13), D-[1-^{3}H]galactose (5.7 Ci/mmol, 50 μCi/ml, batch 13) and L-[1-^{3}H]fucose (2.82 Ci/mmol, 50 μCi/ml, batch 5), all from the Radiochemical Centre, Amersham, England), separation of the labeled cells from their supernatant medium, lysis of cells with Nonidet P40 (NP-40, Shell Chemical Co., Zürich, Switzerland), nonionic detergent, dialysis of cell lysates and supernatant media, centrifugation of the dialyzed lysates at 22,000 *g* for 2 h, determinations of total radioactivity incorporated into macromolecular, nondialyzable material, and into serologically precipitable material have been described elsewhere (4, 5). Serological precipitations were done by reacting soluble complexes of radioactive mouse myeloma IgM and excess rabbit (antimouse Ig) antiserum with pig (antirabbit Ig) globulin at equivalence point (sandwich technique). We are grateful to Dr. F. Franek, Czechoslovak Academy of Sciences, Prague, Czechoslovakia, for supplies of the pig serum. Values obtained with specific (antimouse Ig) precipitations were corrected by subtracting from values obtained with nonspecific (anti-*E. coli* β-galactosidase) precipitations. All serological determinations were done at least in duplicate. The IgM-specific antisera were raised in rabbits by repeated injections of 1 mg each of purified (11) extracellular 19S MOPC 104E IgM (λ, μ). The specificity of these antisera has been described (6, 12). Size and polypeptide chain composition of the radioactive IgM was determined in intracellular lysates or extracellular fluids by sucrose gradient sedimentation followed by serological analysis of the sucrose gradient fractions (6, 11), or was determined in serological precipitates from the intracell-

[1] *Abbreviations used in this paper:* EM, electron microscopy; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; RER, rough endoplasmic reticulum.

ular lysates and the extracellular fluids containing the radioactive mouse IgM by polyacrylamide gel electrophoreses. Composite gels (13) of 2.5% cross-linked polyacrylamide in 0.5% agarose were used for the dissociated serological precipitates for size analysis of the radioactive IgM, while 7.5% cross-linked polyacrylamide gels (14) were used for the dissociated, reduced, and alkylated serological precipitates for analysis of the polypeptide chain composition of radioactive mouse IgM. Both gel procedures were adapted to the soluble gel procedure of Choules and Zimm (15). Quantitative determinations of radioactivity in different neutral hexoses and hexosamines were done by column chromatography on Dowex 2 (× 8) (neutral hexoses [16]) (Dowex Ion Exchange Resins, Dow Chemical Co., Midland, Mich.), and on Amberlite CG-120 (hexosamines [17]) (Amberlite Cation & Anion Exchange Resins, Rohm and Haas Co., Philadelphia) with hydrolysates and serological precipitates of radioactive myeloma protein as described (18). Actinomycin D was obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Biologic Behavior of Tumors.—McPc 1748 was obtained as a splenic tumor. The tumor grew only in the spleen whether it was inoculated subcutaneously or intraperitoneally. After the fourth passage in our laboratory a small nodule was noted at the site of injection of the tumor cells. The nodule was excised and passed subcutaneously. After three additional passages the tumor grew as well subcutaneously as it did in the spleen. Studies of IgM synthesis with cells obtained from both sites in the same animal yielded virtually identical results with respect to the amount of IgM synthesized relative to total cell protein and the pattern of assembly and secretion. All animals with subcutaneous tumors had enlarged, grossly infiltrated spleens at the time of sacrifice.

W 3469 and Y 5781 grew both subcutaneously and in the spleen while animals with the TEPC 183 and McPc 774 tumors had some splenic enlargement but no gross infiltration. When minced spleens from mice bearing the latter two tumors were injected into new animals, either intraperitoneally or subcutaneously, tumors appeared in 20–50% of the animals. Hence the degree of splenic colonization achieved by cells from these two tumors appeared to be somewhat less than that of the others. Mean survival times were ascertained by the subcutaneous injection of 10^7 viable tumor cells per animal into groups of six–ten animals per tumor and observing the animals daily until death (Table I).

TABLE I

Characterization of Five IgM-Producing Mouse Tumors

Tumor	Group	Med. surv. time (days)	Splenic homing	Immunofluorescence		Electron microscopy		
				Cytoplasmic IgM	Membrane-bound IgM	Free polysomes	Bound polysomes	A-particles
1748	I	30	+++	+	+++	+++	−	−
3469	I	21	+++	+	++++	+++	−	−
5781	II	28	++	+++	+	++	++	+
774	II	<50	+	++++	−	+	++++	+++
183	II	23	+	+++	−	+	++++	++++

Immunofluorescence.—All tumors gave suspensions of large cells, with the nucleus of considerable size. However, the average cell diameter was smaller in tumors McPc 1748 and W 3469 than in TEPC 183, McPc 774, and Y 5781. The first two tumors had in addition relatively less cytoplasm and on the whole the appearance of these cells was more that of intermediate lymphocytes, whereas TEPC 183, McPc 774, and Y 5781 tumor cells had the appearance of large "blast" cells with a relative increase of the proportion of cytoplasm over nucleus.

Striking differences between the tumors were obvious with regard to the surface and intracytoplasmic immunoglobulins. Here again two groups of tumors were apparent: McPc 1748 and W 3469 showed abundant membrane-bound and scanty intracytoplasmic immunogloblins (see Fig. 1 *a* and *b*). It should be remembered that restaining of membrane immunoglobulins in the second step in this staining procedure may generate an increased intensity of the second staining not due to intracytoplasmic immunoglobulins.

On the other hand the membrane immunofluorescence showed only a few spots on the cells of tumors TEPC 183 and McPc 774, and none at all on Y 5781 cells; these last three tumors, by contrast, had abundant intracytoplasmic immunoglobulins, so that the pattern of intracytoplasmic staining was similar to that of plasmablasts or young plasma cells (Fig. 1 *c–e*).

Electron Microscopy (EM).—At least 400–500 cell profiles of each tumor were examined on three occasions with the microscopist unaware of the identity of the individual tumors. The results for each tumor were identical on each occasion.

Using cytoarchitectural features the tumors could be divided into two major groups (Fig. 2). The majority of cells in group I tumors (McPc 1748 and W 3469) contained numerous cytoplasmic polysome clusters. There was little rough endoplasmic reticulum and the golgi zones were quite small. Many mitochondria were present. The nuclei had large areas of electron dense, clumped heterochromatin, a feature similar to that seen in the resting small lymphocyte (Fig. 2 *a* and *b*). About 10% of cell profiles were lymphoblastoid or mitotic cells.

Group II tumors, of which McPc 774 and TEPC 183 were typical, were comprised predominantly of larger cells with much smaller nuclear to cytoplasmic ratios. Although many polysome aggregates were noted free in the cytoplasm, an extensively developed rough endoplasmic reticulum (RER) with various states of cisternal dilatation was prominent. The golgi was extremely well developed and again many mitochondria were present. Many of the cells showed prominent nucleoli and large areas of adielectronic euchromatin (Fig. 3 *a* and *b*). 10–20% of the cell profiles had the electron microscopic features of small lymphocytes, lymphoblasts, mature plasma cells, and mitotic cells.

Y 5781 contained cells with moderately developed RER and moderate numbers of cytoplasmic polysome aggregates. Y 5781 cells appeared to be

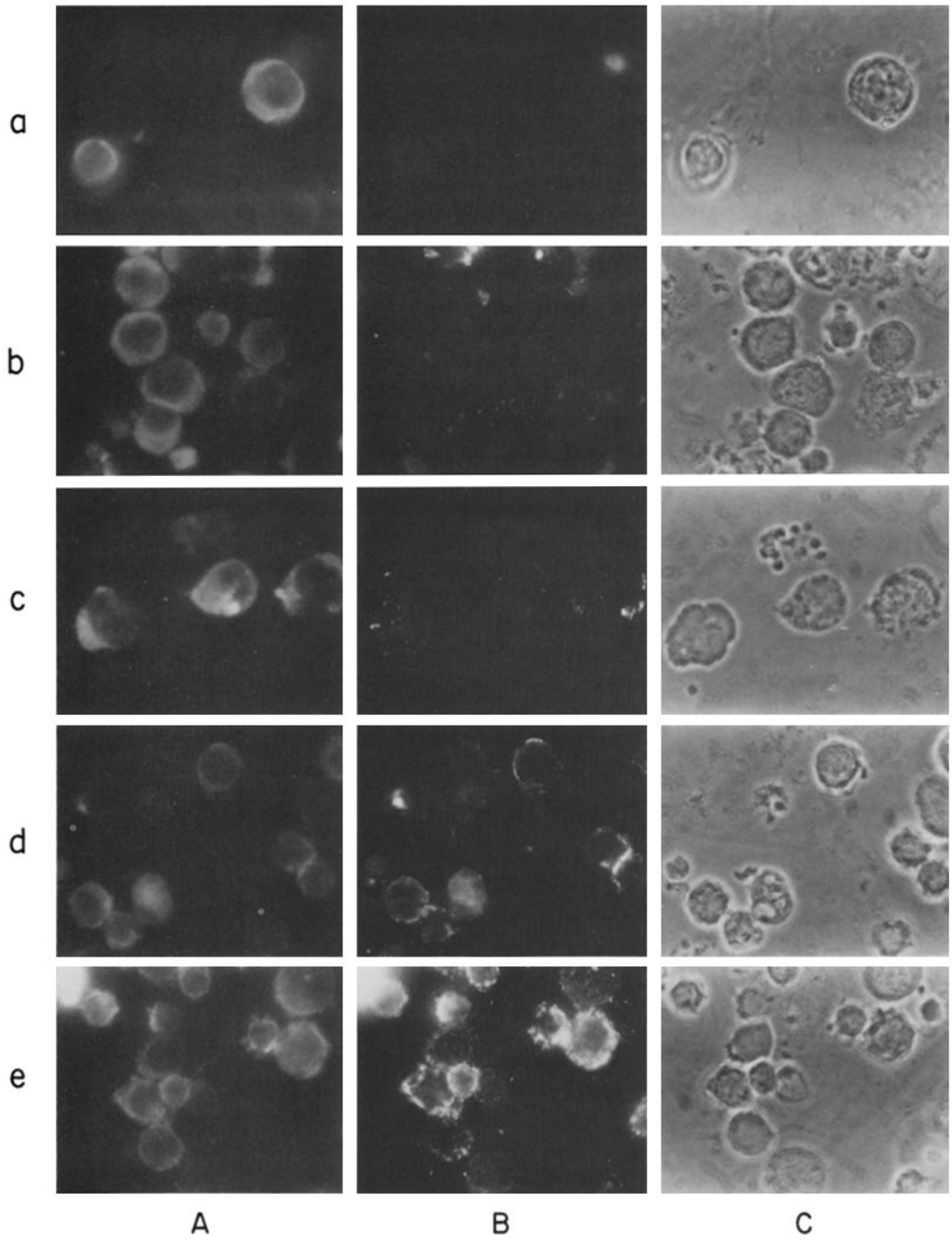

FIG. 1. Mouse tumor cells, stained in suspension with a rhodamine-conjugated antimouse μ-chain antiserum, flattened on slides, and stained after fixation with the same antiserum conjugated with fluorescein. (A) Phase contrast; (B) Specific visualization of rhodamine (surface immunoglobulins only); (C) Specific visualization of fluorescein (intracytoplasmic immunoglobulins plus some restaining of surface immunoglobulins). (*a*) Tumor McPc 1748: bright staining of surface immunoglobulins (B) and weak staining after fixation (C), mostly due to restaining of surface immunoglobulins; (*b*) Tumor W 3469: the cells show definite staining of surface immunoglobulins, and some staining of the cytoplasm; (*c*) Tumor TEPC 183: the surface immunoglobulins are very scanty, but still detectable as few spots, whereas intracytoplasmic immunoglobulins are clearly detectable; (*d*) Tumor McPc 774: surface immunoglobulins are barely detectable on a few cells, that by contrast contain appreciable amounts of immunoglobulins in the cytoplasm; (*e*) Tumor Y 5781: the cells appear devoid of surface immunoglobulins, as detectable by immunofluorescent staining, and contain considerable amounts of intracytoplasmic immunoglobulins.

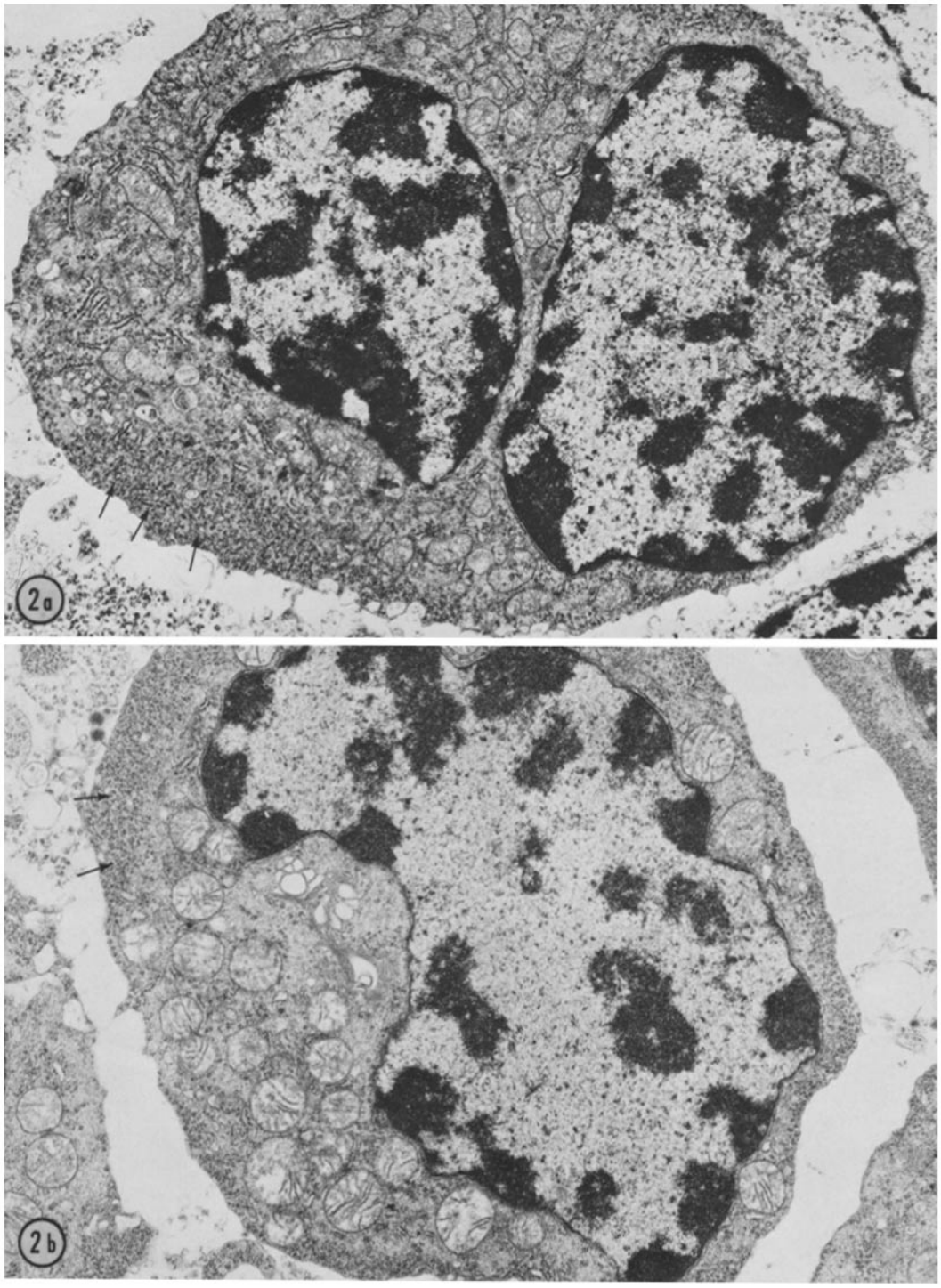

FIG. 2. (*a*) Cell from McPc 1748. Note the numerous cytoplasmic polysome clusters (arrows) and occasional strands of rough surfaced endoplasmic reticulum. × 10,000. (*b*) Cell from W 3469 showing similar EM features to McPc 1748. Note the prominent clusters of polysomes (arrows). × 10,000.

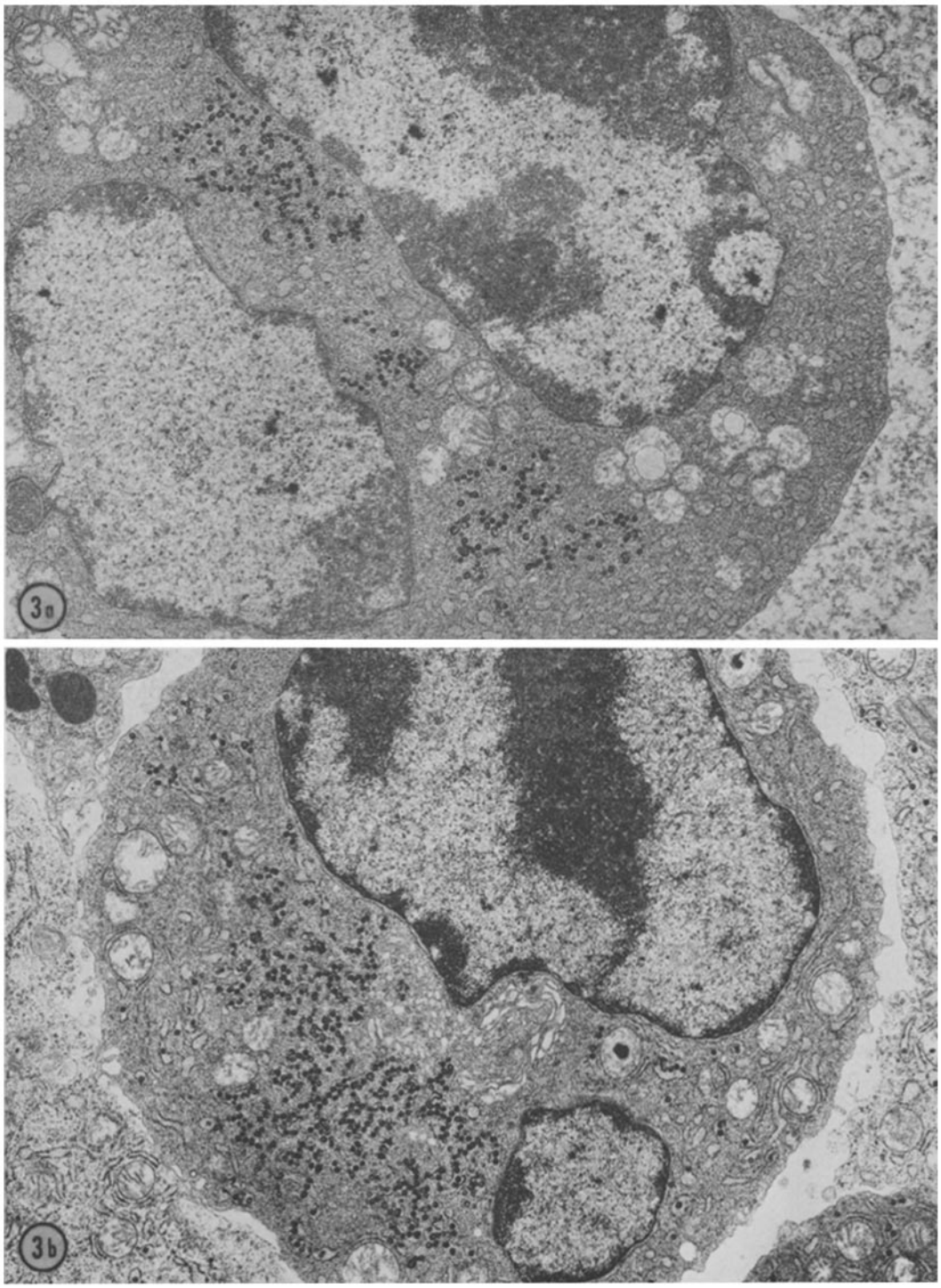

FIG. 3. (*a*) Cell from TEPC 183. Typical plasmacytoid cell with extensively developed rough surfaced endoplasmic reticulum and numerous intercisternal A particles. × 10,000. (*b*) Cell from McPc 774 with well developed rough surfaced endoplasmic reticulum and prominent intracisternal A type particles. × 10,000.

morphologically intermediate between the prototypes of groups I and II cells, although somewhat closer to those of group II (Fig. 4 *a* and *b*).

An additional feature noted by EM was the variability of expression of the frequently described murine myeloma associated intracisternal A particles (19). In an extensive examination of McPc 1748 and W 3469 cells no particles of this type were noted. In striking contrast, the majority of TEPC 183 and McPc 774 cell profiles contained large numbers of particles in their characteristic intracellular distribution (Fig. 5 *a*, *b*). These were often observed in metaphase cells.

Biochemical Studies: Synthesis of of IgM—

Polypeptide chains: Cell suspensions of the mouse tumors McPc 1748, W 3469, TEPC 183, McPc 774, and Y 5781 incorporated radioactive leucine linearly up to 6 h into total intracellular plus secreted protein and into immunologically precipitable IgM. Two distinct patterns of labeling of intracellular and secreted IgM were observed with the five tumor cell suspensions (Fig. 6).

With three group II tumor cell suspensions—TEPC 183, McPc 774, and Y 5781—equilibration of the intracellular pool of IgM with newly synthesized radioactive IgM molecules was reached after 4 h. Consequently a steady state in the rate of secretion of radioactive IgM was reached at the same time (Fig. 6 *c–e*). All three tumors produced labeled μ- and L-chains in the expected molar ratios as judged by polyacrylamide gel electrophoresis of the reduced and alkylated radioactive IgM.

With the two group I tumor cell suspensions, McPc 1748 and W 3469, equilibration of the intracellular pool of IgM with radioactive molecules was not achieved within the 6-h labeling period (Fig. 6 *a* and *b*). Very little radioactive IgM was secreted. This is particularly evident from the amount of radioactive IgM secreted at 4 h of labeling by these two tumor cells, when compared to the three actively secreting tumor cells McPc 774, TEPC 183, and Y 5781 (Table II). The tumors produced μ- and L-chains in the expected molar ratios. Any possible production of excess L chain could again not be detected due to the λ-L-chain specificity of our anti-IgM antiserum (see above).

Carbohydrate moieties: In close similarity to the results of the leucine-labeling experiments, two distinct patterns of labeling of intracellular and secreted IgM were observed with incorporation of radioactive mannose into the five tumor cell suspensions (Fig. 7).

With the three group II tumor cells equilibration of the intracellular pool of IgM with newly synthesized radioactive molecules was reached after 4–5 h. Consequently a steady state in the rate of secretion of IgM molecules was reached at the same time (Fig. 7 *c–e*).

With the other two tumor cells, McPc 1748 and W 3469, no equilibration of the intracellular pool of IgM with radioactive IgM molecules was reached within the 6-h labeling period. Very little IgM was secreted (Fig. 6, *a* and *b*). The incorporated label was contained in the "core" sugars, glucosamines and

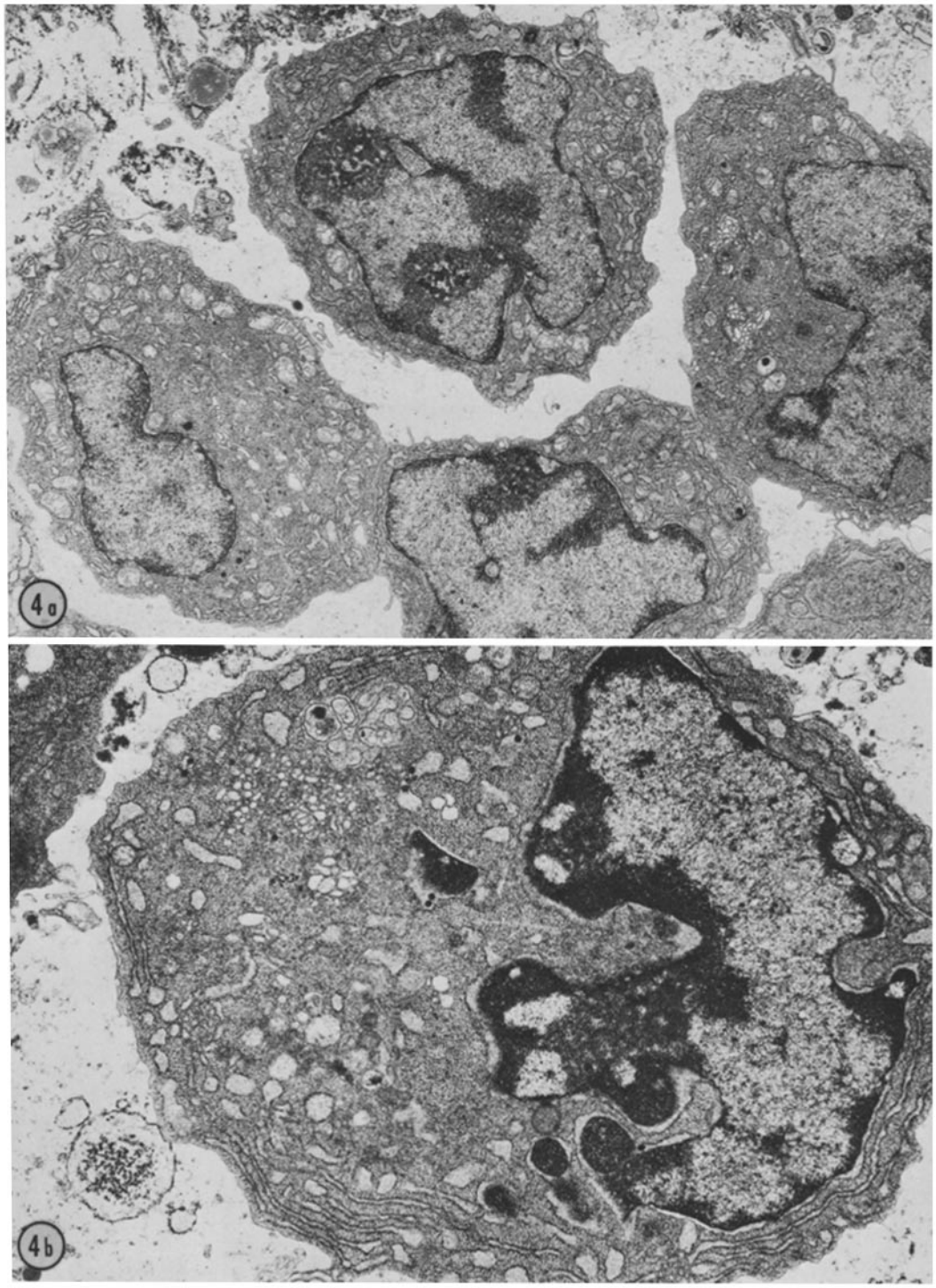

FIG. 4. (*a*) Low power micrograph of cells from Y 5781 showing moderately developed rough surface endoplasmic reticulum and occasional intracisternal A particles. × 5,250. (*b*) Cell from Y 5781. × 10,000.

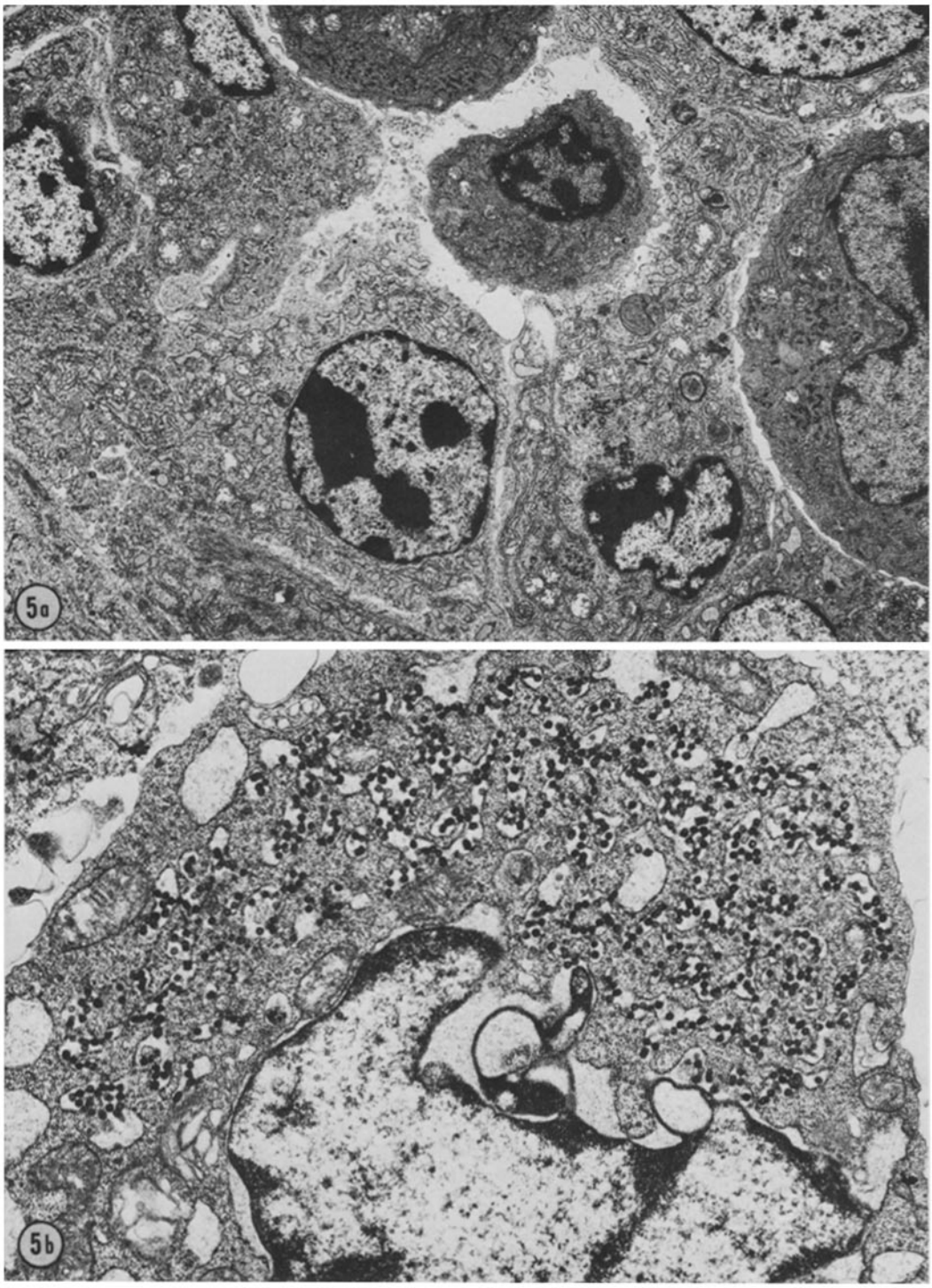

FIG. 5. (*a*) McPc 774. × 5,000 showing distribution of A particles in all cells of field. (*b*) TEPC 183. × 14,000. Higher power view of particle distribution and morphology.

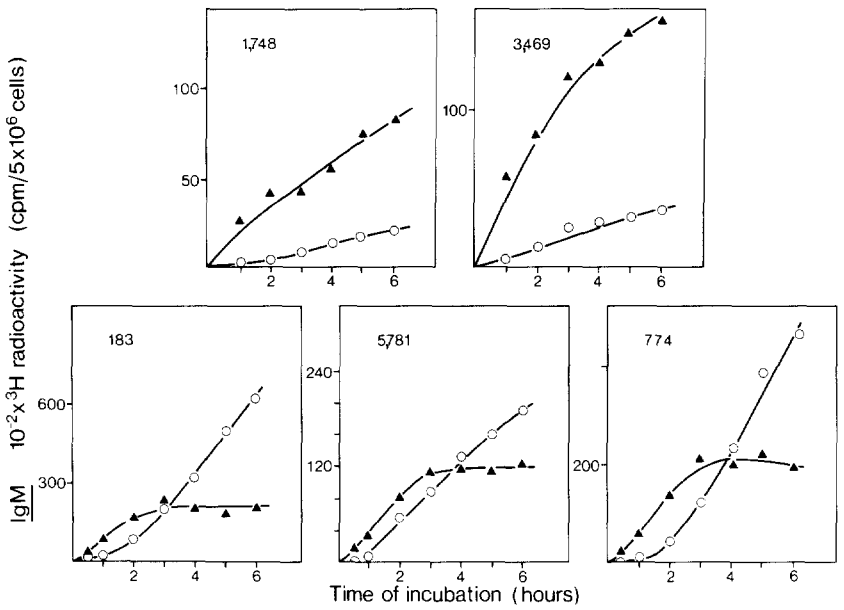

FIG. 6. Time-course of incorporation of [^{3}H]leucine into intracellular (▲) and extracellular (O) IgM of five IgM-producing tumor cells. For details see the Materials and Methods.

mannoses as analyzed after acid hydrolysis of the labeled, serologically precipitated IgM as free-labeled sugars (see Materials and Methods). All five tumor IgMs carried similar amounts of labeled mannose, hence of "core" sugars. This could be judged from a comparison of the amount of leucine-label in the polypeptide portions with the amount of mannose-label in the carbohydrate portions of the five intracellular IgM molecules (Table III). Polyacrylamide gel electrophoresis of the reduced and alkylated radioactive intracellular IgM showed that the μ chains but not the L chains carried the label, and thus the carbohydrate moieties.

All five tumor cells, labeled for up to 6 h, contained very little galactose- or fucose-labeled IgM (Fig. 8). The data in Table II show that the amount of galactose and fucose radioactivity in 4-h-labeled intracellular IgM is very small and approximately the same for all five tumor IgMs. Also, in comparison to leucine label, intracellular IgM from all five tumor cells carried very little galactose or fucose label (Table III). We conclude, that as in MOPC 104E tumor cells (20) and mitogen-stimulated B cells (12), fucose and galactose residues are added just before, or at the time, IgM is secreted from the cells.

The three group II tumor cells—TEPC 183, McPc 774, and Y 5781—secreted galactose- and fucose-labeled IgM from within the first 30 min of labelings linearly up to 6 h. These tumor cells secrete large numbers of IgM molecules

TABLE II

Rates of IgM Synthesis and Secretion and the Ratio of the Rates of Synthesis and Secretion of IgM Over Those of all Proteins in the Cell of Five Mouse Tumors as Compared to Small, Resting, Unstimulated B cells taken from (4) and 72-h Mitogen-Stimulated B Cells taken from (5) in Labeling Experiments with [^{3}H]*Leucine,* [^{3}H]*Mannose,* [^{3}H]*Galactose,* [^{3}H]*Fucose*

Cells	IgM $10^2 \times$ ^{3}H radioactivity (cpm/5 $\times$ 10^6 cells incorporated in 4 h)		Ratio of rates (IgM/total protein)	
	Secreted	Intracellular	Secreted	Intracellular
[^{3}H]Leucine-labeling				
Small, resting unstimulated B cells	13	15	0.050	0.015
McPc 1748	19	65	0.058	0.020
W 3469	35	130	0.090	0.040
TEPC 183	330	210	0.33	0.060
McPc 774	210	200	0.56	0.140
Y 5781	125	120	0.41	0.160
72-h mitogen-stimulated B cells	300	130	0.35	0.090
[^{3}H]Mannose-labeling				
Small, resting, unstimulated B cells	2	20	0.020	0.025
McPc 1748	4	52	0.030	0.045
W 3469	8	50	0.045	0.045
TEPC 183	52	54	0.185	0.10
McPc 774	100	160	0.455	0.225
Y 5781	70	72	0.35	0.18
72-h mitogen-stimulated B cells	60	80	0.20	0.15
[^{3}H]Galactose-labeling				
Small, resting, unstimulated B cells	ND	ND	—	—
McPc 1748	7	4	0.016	0.003
W 3469	12	8	0.030	0.004
TEPC 183	28	3	0.175	0.012
McPc 774	92	8	0.260	0.006
Y 5781	92	4	0.305	0.013
72-h mitogen-stimulated B cells	20	2	0.30	0.04
[^{3}H]Fucose-labeling				
Small, resting, unstimulated B cells	ND	ND	—	—
McPc 1748	2	1	0.006	0.020
W 3469	2	2	0.050	0.050
TEPC 183	64	8	0.540	0.050
McPc 774	13	1.7	0.210	0.036
Y 5781	28	1	0.140	0.050
72-h mitogen-stimulated B cells	20	2	0.30	0.040

* Data also shown in Figs. 6–8.

ND, not detectable.

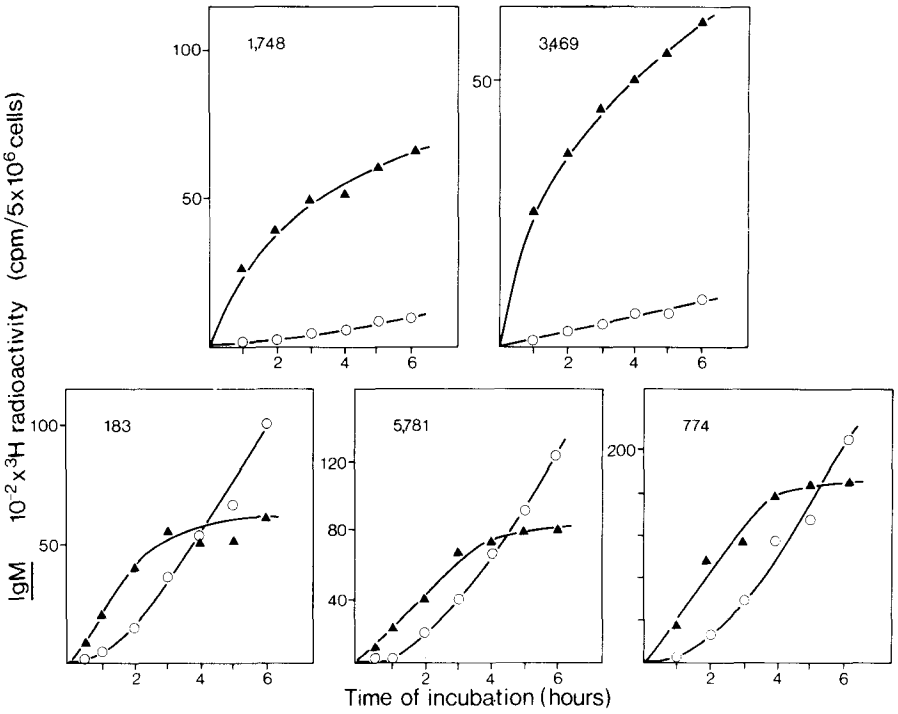

FIG. 7. Time-course of incorporation of [^{3}H]mannose into intracellular (▲) and extracellular (○) IgM of five IgM-producing tumor cells. For details see the Materials and Methods.

TABLE III

Ratios of Radioactivities Incorporated into Carbohydrate Moieties of IgM ([^{3}H]*Mannose-*, [^{3}H]*Galactose-*, [^{3}H]*Fucose-Labeling*) *over those Incorporated into Polypeptide Portions of IgM* ([^{3}H]*Leucine-Labeling*)*

Tumor	Mannose/leucine		Galactose/leucine		Fucose/leucine	
	Secreted	Intracellular	Secreted	Intracellular	Secreted	Intracellular
McPc 1748	0.21	0.80	0.37	0.06	0.10	0.015
W 3469	0.23	0.39	0.34	0.06	0.06	0.015
TEPC 183	0.17	0.49	0.09	0.03	0.19	0.073
McPc 774	0.71	0.80	0.65	0.04	0.09	0.009
Y 5781	0.56	0.60	0.74	0.03	0.22	0.008

* Data taken from Table II, Fig. 6 *a–e*, Fig. 7 *a–e*, and Fig. 8 *a–e*.

which contain the "terminal" sugars. Polyacrylamide gel electrophoresis of the reduced and alkylated galactose- and fucose-labeled IgM molecules secreted by the three tumor cells showed that the μ-chains, but not the L chains, carried the labels and therefore contained these "branch" sugars.

The amount of galactose- and fucose-labeled IgM found in the secreted

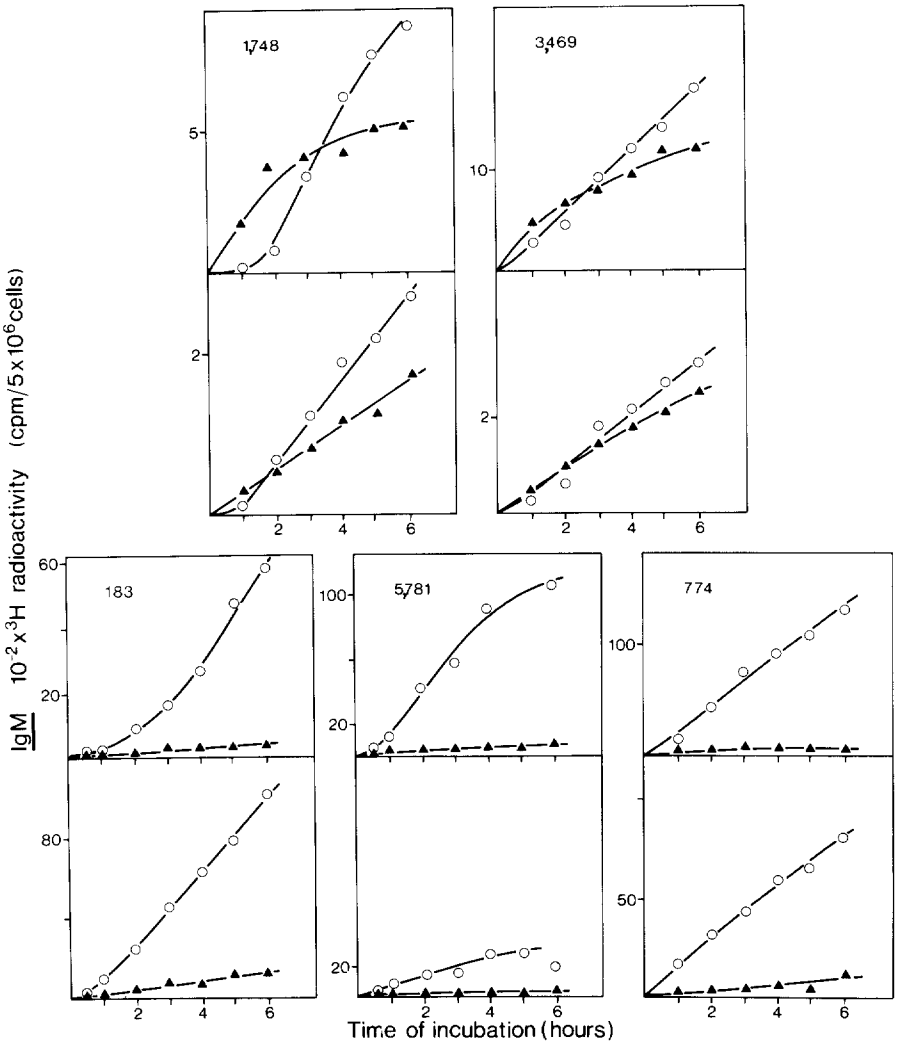

FIG. 8. Time-course of incorporation of [^{3}H]galactose (*top*) and [^{3}H]fucose (*bottom*) into intracellular (▲) and extracellular (○) IgM of five IgM-producing tumor cells. For details see the Materials and Methods.

material of the McPc 1748 and W 3469 cells was very small. However, when the ratios of galactose to leucine and fucose to leucine incorporated into secreted IgM were examined, they did not differ significantly from those seen in the group II cells (Table III). The similarity in these ratios seen in the two groups suggests that the relatively low incorporation of branch sugars into released IgM is consistent with the small amount of IgM activity secreted during the period of observation.

It was apparent that there was more radioactivity in galactose-labeled,

secreted McPc 774 and Y 5781 IgM than in TEPC 183 IgM (Table III). It was also evident that radioactive fucose labels TEPC 183 IgM more than McPc 774 and Y 5781 IgM (Table III). Analysis of the radioactivity contained in 5 h-fucose- or galactose-labeled secreted TEPC 183, McPc 774, and Y 5781 IgM showed that galactose was mainly incorporated into galactose positions and fucose mainly into fucose positions of the carbohydrate moieties of IgM. Hence these differences may reflect quantitative differences in the amount of certain sugar residues in carbohydrate moieties of IgM. Heterogeneity in carbohydrate moieties of glycoproteins is a well-documented phenomemon (21).

Ratios of rates of synthesis and secretion of IgM over the rates of synthesis and secretion of all cellular macromolecules: In Table II the ratios of rates of synthesis and secretion of IgM over those of all labeled cellular macromolecules are calculated from data obtained in a 4-h labeling period with radioactive leucine, mannose, galactose, and fucose. The five tumor cells are compared to unstimulated and to 72-h stimulated B cells (4, 5). It is evident that in group I tumor cells (McPc 1748, W 3469) IgM synthesis and secretion comprises a much smaller part of the synthesis and secretion of all cellular macromolecules (between 2 and 4% of the intracellular and 6 and 9% of the secreted cpm in the four labeling experiments) than in group II tumor cells (between 6 and 16% intracellular and 35 and 56% secreted). This was confirmed by the carbohydrate incorporation studies.

Turnover of newly synthesized IgM: Fig. 9 shows the disappearance of labeled IgM from the five tumor cells when the five tumor cells were labeled for 4 h with radioactive leucine, then transferred into nonradioactive chase medium and the disappearance of labeled IgM from the cells and its appearance in the supernatant medium measured after various periods of chase time.

In group I cells (McPc 1748 and W 3469) most IgM disappears with a relatively long median disappearance time (between 8 and 15 h). In group II cells two populations of IgM molecules are easily distinguishable. One disappears with a median time of 1.5–3 h. These rapidly disappearing IgM molecules represent between 65 and 75% of all intracellular labeled IgM molecules in TEPC 183 and McPc 774 cells and 90% in Y 5781 cells. The remainder of the IgM molecules disappear slowly, in McPc 774 cells with a $T_{1/2}$ between 8 and 15 h. In TEPC 183 and Y 5781 cells the $T_{1/2}$ of intracellular IgM in the second phase is 15–25 h.

Stability of IgM synthesis in the presence of Actinomycin D: The five tumor cells were tested for the resistance of their IgM synthesis to inhibition of actinomycin D. Biosynthetic incorporation of radioactive leucine into IgM was measured in the presence and absence of 0.5 μg/ml actinomycin D. The data in Fig. 10 are expressed as ratios of labeled amino acid incorporated into IgM to that incorporated into total cell protein during the same incubation period. The resistance of IgM synthesis to actinomycin D-inhibition relative to that

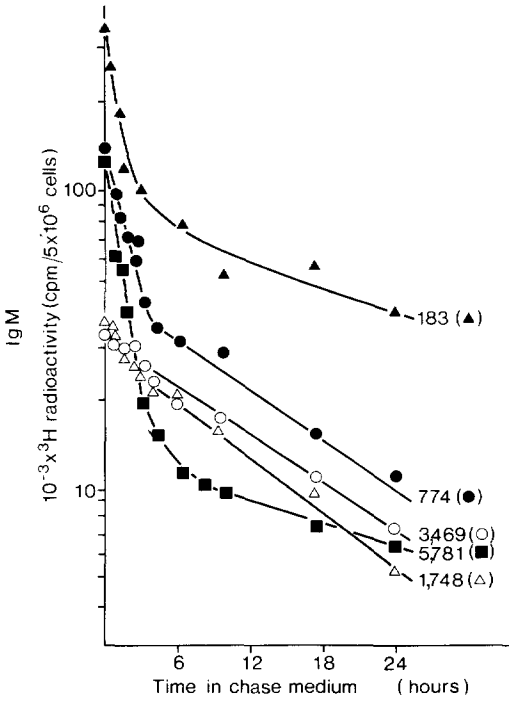

FIG. 9. Release of radioactive IgM from five tumor cells labeled for 4 h with [^{3}H]leucine, thereafter placed in nonradioactive medium. Release (turnover) of labeled IgM was followed in aliquots of the labeled cells at different time periods of chase as described in detail in the Materials and Methods.

of all cellular proteins is slight in group I cells but marked in TEPC 183, Y 5781, and McPc 774 cells.

DISCUSSION

Myeloma cells and their protein products have played an important role in the analysis of antibody synthesis and structure. It has been assumed that myeloma tumors represent clones of antibody-forming cells which have become malignant but retain their differentiated function. Hence, they can serve as useful models for the study of processes which take place in normal Ig-synthesizing cells.

The IgM-producing BALB/c tumors described here could be separated into two groups using a number of morphologic and functional criteria. Group I cells had strong surface immunofluorescence with anti-μ antiserum, few cytoplasmic membrane-bound polysomes, and synthesized only a small portion of their total cell protein as IgM. IgM synthesis was actinomycin D-sensitive. Its turnover was slow and intracellular equilibration of labeled precursors was

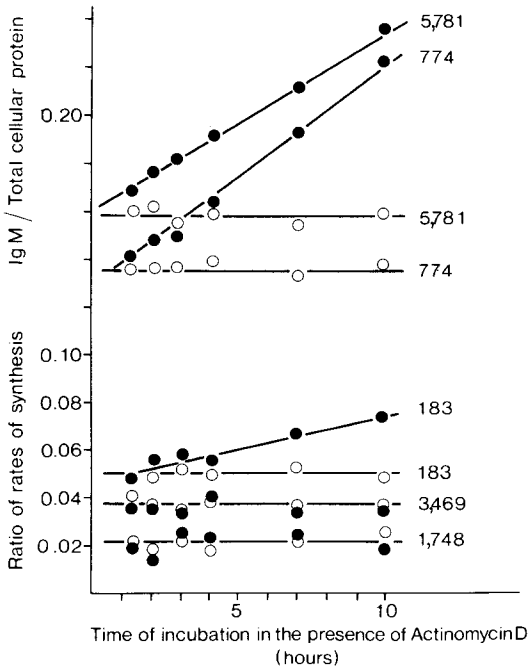

FIG. 10. The effect of actinomycin D (0.5 μg/ml) on the ratio of IgM synthesis in five tumor cells producing IgM. Ratios are given of the rates of IgM synthesis over the rates of synthesis of all cellular proteins at different time periods of incubation in the presence (●) or absence (○) of actinomycin D. Synthesis comprises IgM found inside the cells and secreted from the cells during the time period of incubation (2 h). Details are given in the Materials and Methods (see also ref. 4 and 5).

difficult to attain. Although some 19S IgM was secreted, the amount was very small. Intracisternal A particles were not detectable in numerous electron microscopic sections of these cells.

Group II cells had little surface Ig but intense intracellular fluorescence when cytoplasmic staining was performed with the same anti-μ antiserum. Electron microscopy demonstrated an extensively developed RER and prominent Golgi zones. The synthetic ratio of IgM to total protein was high, and IgM turnover was rapid. IgM secretion was copious and its synthesis appeared to be relatively resistant to actinomycin D. The cells obtained from these tumors contained substantial numbers of intracisternal A particles.

Abundant intracytoplasmic Ig and the lack of surface-bound Ig is strictly correlated with the development of the RER in group II cells. Although the development of the amount of rough endoplasmic reticulum in maturing B lymphocytes may be continuous the initiation of the development of it may be a discernible event during B cells maturation. In fact, stimulation of normal B lymphocytes by mitogens in the absence of DNA synthesis leads to the

development of immature plasmablasts with numerous intracytoplasmic polyribosomes, but no visible RER (22). These cells resemble the group I tumor cells. Upon release of the DNA synthesis inhibition, cells develop, which resemble group II tumor cells. It appears likely that the analysis of more IgM-producing tumor cells will more clearly distinguish two groups (I and II), which are discontinuous with each other.

The clear distinction between these two groups of IgM-synthesizing tumor cells prompted a comparison of their morphologic and functional features with those of lipopolysaccharide (LPS)-stimulated B cells (5, 6). Extensive studies had indicated that when normal murine B cells were exposed to this B-cell mitogen, they increased their cytoplasmic volume, developed an extensive RER with its associated polyribosomes and became more plasmacytoid (1, 2). Analysis of protein synthesis in these cells has demonstrated that the amount of Ig synthesis (as IgM) relative to total cell protein synthesis increased markedly. The turnover of the cell-associated IgM became more rapid as the major portion of the newly synthesized molecules was secreted without delay. Actinomycin D appeared to have little effect on IgM synthesis later in the response (5).

Fig. 11 demonstrates the relationship between each of the tumors and LPS-stimulated B cells. The data obtained from the amino acid and carbohydrate

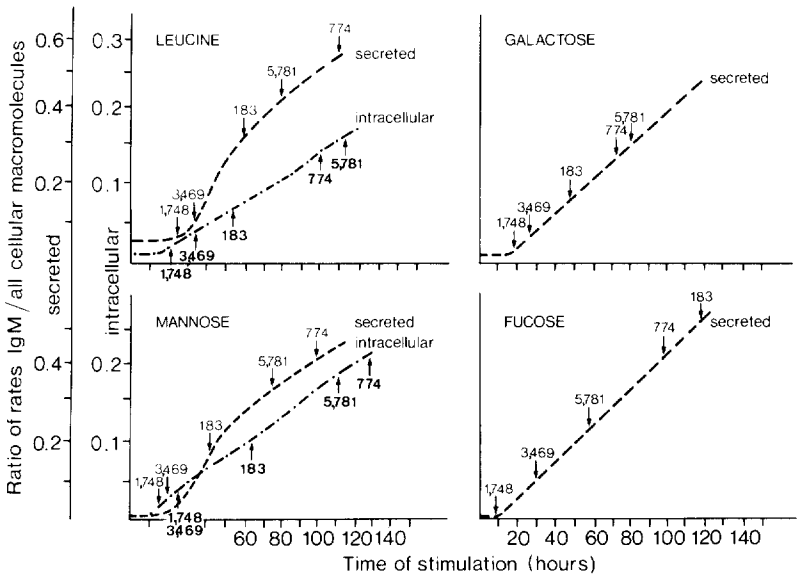

FIG. 11. Relative maturity of five IgM-producing tumor cells in comparison to normal mitogen-stimulated B cells. Ratios of rates of intracellular synthesis and of secretion of IgM over those of total protein synthesis and secretion at various times after mitogenic stimulation of normal B cells (5) are given by dashed lines. Data obtained with the five tumor cells (McPc 1748,W 3469, TEPC 183, Y 5781, and McPc 774) (Table II) are indicated by arrows.

labeling experiments with each tumor have been superimposed on curves derived from identical experiments performed with stimulated B cells (5). McPc 1748 is analogous to a 10–20 h LPS-stimulated spleen cell suspension while McPc 774 closely resembles a 100–120 h stimulated cell suspension. None of the tumors studied in this group appear to represent an unstimulated B cell.

Group I tumor cells, like early B cells, had most of their cell-associated IgM at the surface. Initially they released IgM at a slower rate than group II cells which secrete their immunoglobulin with kinetics similar to mature plasma cells. After 6 h, the rates became similar as the pace of release was slowed in the late B-cell tumors. This biphasic release of cell-associated IgM could be explained either by the presence of two distinct populations of IgM molecules, maybe in two different populations of cells, or by the equilibration of labeled and unlabeled subunits in the face of a random assembly and secretion process, in which the 8S subunits are not assembled into polymers in the order in which they are synthesized.

The possibility that two separate populations of IgM molecules could result from a separate regulation of the biosynthesis of membrane-bound and of secreted Ig is also supported by the differences observed in synthesis and turnover of IgM in resting and in mitogen-activated B lymphocytes (4, 5), by the difference in size and carbohydrate composition of these different types of IgM (4, 5), and by studies with another mouse myeloma cell producing membrane-bound and secreted IgG (23). The problem of the intracellular segregation of Ig chains and their direction to either a surface or a secretory pool may be approached using the tumor cells as models.

Comparison of the kinetics of incorporation of various labeled sugars into the IgM of the group I and II tumor cells indicated that molecules bearing the terminal sugars may have been associated with the cells for a longer period of time in group I cells. Although the carbohydrate composition of the cell-associated IgM was not chemically determined, the sugar to leucine ratio of the IgM secreted by each of the tumors revealed no systematic differences between group I and group II cells In both groups the sugar residues were associated exclusively with the μ-chains, however the labeling pattern suggested that there may be variations in the carbohydrate composition within groups I and II.

The apparent dichotomy of effect of actinomycin D in the two groups of tumors was quite striking. The lack of sensitivity in group II argues for a longer period of utilization of preformed mRNA by these cells. Recent studies in Hela and L cells have indicated that this is probably not a function of messenger degradation but may represent a failure of re-initiation along the same mRNA molecules. This may be a function of the number or nature of the cytoplasmic ribosomes or some other, as yet undetermined, factor (24, 25).

It is difficult to interpret the biologic behavior of the tumors with respect to the relative maturity of the cells as a B lymphocyte. Animals bearing group

I tumors have enormously enlarged infiltrated spleens. When these spleens are passed into new animals 100% of the recipients develop tumors. Spleens from group II-bearing animals produce tumors in a minority of recipients. This may reflect some variation in the surface or migratory properties of B cells at different stages of maturation.

The final striking disparity between group I and group II cells was in the presence of intracisternal A particles. We have been unable to detect these particles in many examinations of group I cells, while they are abundant in in group II cells. The particles do not bear a direct relationship to the proliferative capacity of the cells, since both tumor types were fatal. Previous workers have attempted to correlate the presence of the particles with Ig production (26); however, EM studies of non-Ig-producing lines cloned from the MPC 11 tumor revealed large numbers of particles (27). The ability to synthesize Ig is not an absolute requirement for their expression. This difference between group I and II cells may not be qualitative, but even if it represents a quantitative difference, there may be some process involved in B-cell maturation which allows the flagrant expression of these presumably viral elements only by the more mature numbers of the series. In conclusion, it appears that these IgM-producing BALB/c tumors represent various stages of B-cell maturation which have been retained despite the malignant state. They should serve as a useful model for examining the process of B-cell maturation which results in the IgM antibody-secreting cell.

SUMMARY

Five adjuvant induced BALB/c tumors producing IgM—McPc 1748, W 3469, TEPC 183, McPc 774, and Y 5781—were characterized morphologically by electron microscopy, analysis of the distribution of surface-bound and intracytoplasmic IgM using immunofluorescence, and by biochemical study of IgM synthesis, turnover, and secretion. The cells of different tumors appear to represent different stages in B-cell maturation when compared to normal, lipopolysaccharide-stimulated B cells. Thus, McPc 1748 tumor cells resemble 10–25-h stimulated normal B cells, 3469 cells resemble 20–35-h stimulated B cells, TEPC 183 cells resemble 45–65-h stimulated B cells, Y 5781 cells resemble 80–110-h stimulated B cells, and McPc 774 cells resemble 100–130-h stimulated B cells.

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