THE INTRACELLULAR DISTRIBUTION OF GAMMA GLOBULIN IN A MOUSE PLASMA CELL TUMOR (X5563) AS REVEALED BY FLUORESCENCE AND ELECTRON MICROSCOPY*

By RICHARD A. RIFKIND,‡ M.D., ELLIOTT F. OSSERMAN, M.D., KONRAD C. HSU, Ph.D. and COUNCILMAN MORGAN, M.D.

(From the Departments of Medicine and Microbiology, Columbia University, College of Physicians and Surgeons, New York)

Plates 57 to 66

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Considerable evidence implicates the plasma cell as the major, if not the sole, source of the serum gamma globulins (1). The configuration of the plasma cell, as revealed by electron microscopy (2, 3), is typical of cells actively engaged in protein synthesis and secretion (4–8). Fluorescent antibody staining has revealed the presence of gamma globulin within the cytoplasm of plasma cells (9–11). In the course of a cytologic study of antibody synthesis, observations have been made upon the intracellular distribution of gamma globulin in an experimental plasmacytoma (X5563) of the C_3H mouse. This tumor has been shown to synthesize and liberate into the serum a protein (12–14) with the immunoelectrophoretic properties of a gamma 2 globulin (15). This protein will be referred to as the "myeloma globulin." It is the purpose of this paper to report light and electron microscopic studies of the X5563 plasma cells stained with fluorescein- and ferritin-conjugated antibodies to the myeloma globulin product of these cells.

Materials and Methods

The Tumor.—The X5563 plasma cell tumor of the Heston subline of the C_3H mouse was originally obtained in its 16th transplant generation in 1957 through the generosity of Dr. M. Potter of the National Cancer Institute, Bethesda. The present studies were performed on tumors of the 56th through 60th transplant generations, and it is noteworthy that the biological and biochemical properties of the tumor (12-15) have remained stable up to the present time. It is a slow growing, rarely metastasizing, well differentiated tumor which is carried by serial subcutaneous transplantation in the axillary region.

The Antisera.—The globulin fraction of serum from tumor-bearing mice was prepared by ammonium sulfate (1.64 M, pH 7.2) precipitation. Rabbits were immunized with three weekly

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injections of this globulin fraction in Freund's complete adjuvant and were bled 10 days after the last injection. Immunoelectrophoretic analysis of this antiserum demonstrated a very strong precipitin arc against the abnormal myeloma globulin in addition to minor precipitin arcs against beta and alpha globulin constituents.

The gamma globulin fraction from the immune rabbit serum was obtained by repeated precipitation with sodium sulfate (1.436 M) and then conjugated either with fluorescein isothiocyanate, or with ferritin (16, 17) employing xylylene 2,4-diisocyanate as the coupling reagent. The fluorescein-conjugated antiserum was serially absorbed with acetone-extracted rat and mouse liver powder. The ferritin-conjugated preparation was freed of unconjugated globulin by a series of three 2 hour centrifugations at 100,000 G as suggested by Singer and Schick (18). Immunoelectrophoretic studies of the partially purified ferritin-conjugates revealed the presence of free ferritin and the ferritin-globulin complex.

Fluorescent Antibody Staining.—Frozen sections of the X5563 tumor were fixed for 15 minutes in cold acetone and stained for 30 minutes with the fluorescein-conjugated anti-mouse myeloma globulin. As a control, specific inhibition of fluorescence was produced by prior exposure of the tumor sections to unconjugated antiserum.

Ferritin-conjugated Antibody Staining.—Tumor tissue was excised and teased into suspension in phosphate-buffered saline at pH 7.2. A pellet of tumor cells, obtained by slow speed centrifugation, was resuspended in cold 5 per cent phosphate-buffered formalin for 10 minutes following which the cells were washed 3 times in cold buffered saline. The final pellet of cells was drained of excess buffer, frozen in a CO_2-ethanol mixture, thawed, and suspended in ferritin-conjugated rabbit anti-mouse myeloma globulin for 15 minutes at room temperature. The cells were then washed 3 times in cold buffered saline, fixed in osmium tetroxide, and embedded in epoxy resin according to the method of Luft (19). Sections, stained for 1 hour with 1 per cent uranyl acetate in 50 per cent ethanol, were examined in an RCA EMU 2B electron microscope.

Control preparations consisted of tumor cells exposed in a similar manner to ferritin-conjugated rabbit globulin lacking immunologic specificity for mouse myeloma globulin. Cells so treated revealed no staining with the ferritin label.

RESULTS

Frozen sections of the X5563 tissue stained with fluorescein-conjugated rabbit anti-mouse myeloma globulin are illustrated in Figs. 1 to 5. Fig. 1 demonstrates the over-all distribution of globulin-containing cells as seen at low magnification. Several clusters with bright, specific cytoplasmic fluorescence are evident. The remainder of the cells, though faintly visible by virtue of their autofluorescence, fail to display appreciable immunologically specific staining.

The distribution of globulin within individual cells is illustrated in Figs. 2 to 5. Fig. 2 shows a cell with a large, possibly multilobed, nucleus and scanty cytoplasm. Several discrete fluorescent cytoplasmic aggregates are seen. Adjacent cells lack specific fluorescence. Another cell with more extensive cytoplasmic fluorescence is illustrated in Fig. 3. Neighboring cells display specific but less intense fluorescence. In Fig. 4 the cytoplasm is distended with innumerable fluorescent aggregates while the cell illustrated in Fig. 5 displays an almost uniformly fluorescent cytoplasm.

Although it is clear from the preceding photographs that myeloma globulin is restricted to the cytoplasm, where it appears either in aggregated or diffuse form, precise localization requires the resolution obtained by electron microscopy. Fig. 6 shows an electron micrograph of an X5563 myeloma cell stained with ferritin-con-

jugated anti-mouse myeloma globulin. The nucleus is fairly large and exhibits margination of chromatin. The nuclear membrane is infolded at several points and where it is sectioned tangentially the annular profiles of nuclear pores (*np*) may be seen. The space between the inner and outer nuclear membranes, as well as within the remainder of the rough-surfaced endoplasmic reticulum (*er*), contains finely granular, irregularly distributed electron-dense material. Virus-like particles (*v*), previously described in this and related mouse tumors (20–22), are found within the endoplasmic reticulum. Mitochondria and free ribonucleoprotein granules fill the remainder of the cytoplasm. The Golgi apparatus is seen in this section as an aggregate of small vesicles (*g*). At this low magnification it is difficult to identify the ferritin-conjugated antibody.

Fig. 7 illustrates a portion of the cytoplasm of another myeloma cell at higher magnification. Several cisternal elements of the endoplasmic reticulum are apparent (*er*). Within three of these the attachment of ferritin molecules to aggregates of granular material indicates the presence of myeloma globulin. A viral particle (*v*) with an electron-dense double membrane appears to be budding into another element of the endoplasmic reticulum which is free of the ferritin label. The surface of this cistern exhibits very few ribonucleoprotein (RNP) particles in the vicinity of the virus. Numerous RNP particles and a few ferritin molecules are scattered in the cytoplasm. Ferritin molecules are also evident on the cellular surface at the right-hand margin of the figure.

In Fig. 8 the nucleus (*N*), Golgi membranes (*gm*) and vesicles (*gv*) in the lower third of the micrograph are untagged by the ferritin-labeled antibody. Numerous mitochondria, free-lying RNP particles, and distended cisternae of the endoplasmic reticulum occupy the upper two-thirds of the figure. A portion of this field is seen at higher magnification in Fig. 9. Four mitochondria, devoid of ferritin, lie next to a swollen element of the endoplasmic reticulum. The finely granular contents of this cistern are heavily tagged with the ferritin-antibody complex. The discontinuities of structure in this and succeeding micrographs are probably artifacts resulting from ice-crystal formation.

The nucleus and adjacent cytoplasm of a myeloma cell are seen in Fig. 10. The inner nuclear membrane appears to have been split into two lamellae, perhaps as a consequence of freezing. Ferritin is found in the dilated perinuclear space (*ps*) as well as within cisternae of the endoplasmic reticulum. Furthermore, close inspection suggests the presence of ferritin on many free RNP particles.

Fig. 11 illustrates, at relatively low magnification, a section of a cell at the level of the Golgi apparatus. Cisternae of the endoplasmic reticulum are seen either in parallel arrays (upper quarter of the figure) or randomly distributed. Those which contain the virus-like particles exhibit few ferritin granules. The remainder are filled with variable amounts of a finely granular material which has been tagged with the ferritin-antibody conjugate. The smooth-surfaced vesicles (*s*) and lamellae (*g*) are essentially free of labeled antibody. Ferritin is present in the cellular ground substance, intermixed with the free RNP particles which occupy most of the cytoplasm. The inset to the figure is a light micrograph of a cell which, by virtue of considerable diffuse cytoplasmic fluorescence and a relatively unstained Golgi region (*g*), appears similar to that illustrated by the electron micrograph. The resemblance of this fluorescein-

stained cell to typical mature, non-neoplastic plasma cells (23) suggests that it represents a relatively advanced or differentiated stage of myeloma cell development.

The cytoplasm of another cell sectioned through the Golgi zone is displayed in Fig. 12. A small vesicle of the endoplasmic reticulum (*er*) and three aggregates of agranular membranes, constituting the lamellar portion of the Golgi apparatus (*g*), are labeled with ferritin. Mitochondria (*m*) and the remainder of the cytoplasm are unlabeled. Cells with ferritin-tagging of the Golgi apparatus were infrequently encountered. Two areas from the preceding figure are shown at higher magnification in Figs. 13 and 14. In Fig. 13 the Golgi membranes are aligned along the border of a vacuole. Particles of the ferritin-antibody complex are seen in parallel arrays between the Golgi membranes (indicated by an arrow). Several ferritin molecules have tagged the intracisternal material at the point of transition (marked *j*) between the agranular Golgi system (below) and a rough surfaced element of the endoplasmic reticulum (above). In Fig. 14 two groups of Golgi membranes are separated by a congeries of small vesicles (*gv*). In the upper right the ferritin label is visible within cisternae formed by the agranular membranes. Near the left margin the membranes are apparently oblique to the plane of section making it difficult to ascertain the sites of ferritin deposition.

Fig. 15 illustrates a fragment of cytoplasm containing a large cisternal element of the endoplasmic reticulum, the contents of which are heavily tagged with ferritin-conjugated antibody. Although there are numerous RNP particles on the membranes of this cistern, there are few in the cytoplasmic matrix, which is composed of fine granules and fibrils. The latter, resembling the filamentous material encountered in the cytoplasmic ground substance of a variety of cell types, do not appear to have affinity for the labeled antibody. The ferritin-conjugated antibody appears to be more closely associated with areas containing large quantities of the granular component. The cell membrane is disrupted and aggregates of ferritin-tagged granular material (arrows) have been extruded into the extracellular space. The inset is a fluorescence micrograph of a cell with a ragged cell margin and large aggregates of fluorescein-conjugated antibody in the cytoplasm. It seems likely that this cell was at a stage of cytoplasmic disruption similar to that observed in the accompanying electron micrograph.

A portion of this preceding electron micrograph is seen at higher magnification in Fig. 16. The degree of ferritin tagging of the intracisternal contents is clearly greater than that of the cytoplasmic matrix. Both within and outside the endoplasmic reticulum the ferritin label is found in association with a finely granular material, presumably composed of myeloma globulin.

DISCUSSION

As a model system for the cytologic study of protein synthesis the plasma cell tumors present several advantages. They provide an accessible and localized population of cells producing a protein product which is antigenically related to components of normal mouse serum (15). Cells in all phases of the developmental and secretory process can be observed. The neoplastic plasma cells resemble their normal counterpart in exhibiting a highly developed endoplasmic

reticulum and extensive Golgi apparatus, but it must be recognized that the protein product of the myeloma cell has no demonstrable antibody activity. Accordingly, extrapolation to normal plasma cells of data obtained from these tumor cells should be made with caution.

The over-all distribution of myeloma globulin is best evaluated by the fluorescent antibody technique. Over half the cells contain no demonstrable globulin (Fig. 1) and the number and distribution of such cells conforms closely to those of the most immature plasma cells as judged in comparable, routinely stained preparations. Small amounts of specific fluorescence (Fig. 2) are seen as bright aggregates in cells which exhibit a high nucleocytoplasmic size ratio and resemble the immature plasma cells of normal lymphoid tissue (11, 24). Accumulation of the secretory product is manifested by increasing numbers of these discrete fluorescent aggregates. The most intensely stained, and presumably most mature, myeloma cells show both diffuse and aggregated fluorescence (Figs. 4, 5, and the inset to Fig. 11).

The light microscope can provide only limited information as to the intracellular loci involved in the process of protein synthesis and secretion. Clarification of the more intimate details of structure as it pertains to this cellular function requires the resolution achievable by electron microscopy. The ferritin-conjugated antibody technique provides an electron-dense, immunologically specific staining reagent which may be employed to localize the myeloma globulin by electron microscopy (16–18). A means for correlation of light and electron microscopic observations is thereby provided.

In the electron microscope a highly developed endoplasmic reticulum is the most striking feature not only of the myeloma cell (Figs. 6 and 8) but of plasma cells in general (3). The evidence implicating this structure in protein synthesis, which derives both from cytological and biochemical investigations, has been extensively reviewed (4, 6–8). It is generally accepted that the endoplasmic reticulum (as represented by its derivative, the microsome fraction) participates in globulin and antibody synthesis (14, 25–27). The distribution of myeloma globulin, as revealed by tagging with the ferritin-antibody complex, provides additional cytologic evidence relating the endoplasmic reticulum to protein secretion. Many cells display heavy ferritin labeling of the contents of dispersed or clustered cisternae (Fig. 7), including the perinuclear space (Fig. 10). Accumulation of myeloma globulin is manifested by an increase in the number and size of ferritin-tagged, rough-surfaced vesicles (Figs. 8 and 9). Presumably it is these accumulations which appear as fluorescent aggregates in the light microscope (Figs. 2 to 4).

Characteristically, in cells which contain myeloma globuln as demonstrated by the ferritin-antibody conjugate, many cisternae remain untagged (Fig. 7). It is unlikely that this is an artifact due to inadequate penetration of the cell by the labeled antibody because tagged and untagged cisternae are often closely

approximated. Rather, the endoplasmic reticulum appears to constitute a functionally heterogeneous population. Consistent with this hypothesis is the inverse correlation between the degree of staining with ferritin-conjugated antibody and the number of virus-like particles within the endoplasmic reticulum. The absence of ribonucleoprotein (RNP) particles from the membranes of the endoplasmic reticulum in the vicinity of the virus-like particles (Fig. 7 and see references 21 and 22) may be associated with a decreased production of globulin in such regions.

In addition to tagging of the cisternal contents, ferritin molecules are occasionally scattered among the RNP particles which fill the extracisternal cytoplasmic matrix (Figs. 10 and 11). Although RNP particles are currently considered to be active sites of protein synthesis in general (7, 28) and of globulin synthesis in particular (29), and therefore might be expected to be tagged with the ferritin-conjugate, two factors preclude definitive identification of ribosome-bound globulin in these sections. First, the high concentration of nucleoprotein particles makes it difficult to distinguish pairing of label and antigen, and second, the electron density of the RNP particles often obscures the image of overlying ferritin molecules.

Two hypotheses have been proposed regarding the manner in which the secretory product is released. The first is predicated upon the commonly observed association between protein secretion and a highly developed Golgi apparatus in a variety of different cell types (5, 30–42). Moreover, a communication between this system and the endoplasmic reticulum has been reported (43). Recently, by means of electron microscopic radioautography, Caro (44) demonstrated the accumulation of newly synthesized protein in the Golgi vacuoles of pancreatic acinar cells. From these and similar observations the concept has been advanced (5) that the Golgi membrane system is involved in packaging proteins into vesicles. It is assumed that these vesicles transport the protein to the cellular surface where it is discharged. The distribution of Golgi vesicles containing electron-dense material both in normal (45) and neoplastic (21, 22) plasma cells, suggests that such a mechanism may be active in these cells as well (46).

Another possible mechanism for the secretion of globulin by plasma cells has been advanced by Ortega and Mellors (11) on the basis of observations made with the fluorescent antibody technique. They proposed that the cell disrupts, thereby extruding its contents. Thiéry (47) and Bessis (46) also described this process of cytoplasmic fragmentation, termed clasmatosis, in plasma cells of immunized animals and in myeloma, by means of both phase contrast and electron microscopy. The extreme distension of the endoplasmic reticulum in plasma cells containing Russell bodies (48, 49) or protein crystals (47–49) likewise suggests that cellular disintegration may be one mode of release for plasma globulin.

Our observations are consistent with both of the foregoing hypotheses. On the one hand, small amounts of globulin were identified not only at the point of transition between the endoplasmic reticulum and the Golgi apparatus but also

within the latter structure itself (Fig. 13). On the other, fragments of myeloma cell cytoplasm containing ferritin-conjugated antibody within extremely distended cisternae of the endoplasmic reticulum were encountered (Fig. 15). The presence of ferritin in the extracisternal cytoplasm, on the disrupted cellular surface and in the adjacent extracellular space presumably reveals globulin in process of release as a consequence of cellular disintegration.

SUMMARY

Ferritin- and fluorescein-conjugated antibody staining has been applied to a study of a mouse plasma cell tumor. The presence of myeloma globulin within cisternae of the endoplasmic reticulum was observed at a stage of the secretory process when the remainder of the cytoplasm was essentially free of labeled globulin. The distribution of ferritin suggested a functional heterogeneity among units of the endoplasmic reticulum. Apparently, progressive accumulation of globulin results in distension of the endoplasmic reticulum and, occasionally, in the appearance of considerable quantities of this secretory protein in the extracisternal cytoplasmic matrix. Participation of the Golgi apparatus in the packaging and release of small quantitites of globulin seems likely. In addition, however, fragmentation of the peripheral cytoplasm with rupture of distended ergastoplasmic vesicles appeared to be another pathway whereby globulin is secreted.

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EXPLANATION OF PLATES

PLATE 57

FIGS. 1 to 5 represent micrographs of myeloma tissue stained with a fluorescein-conjugated rabbit anti-mouse myeloma globulin antiserum.

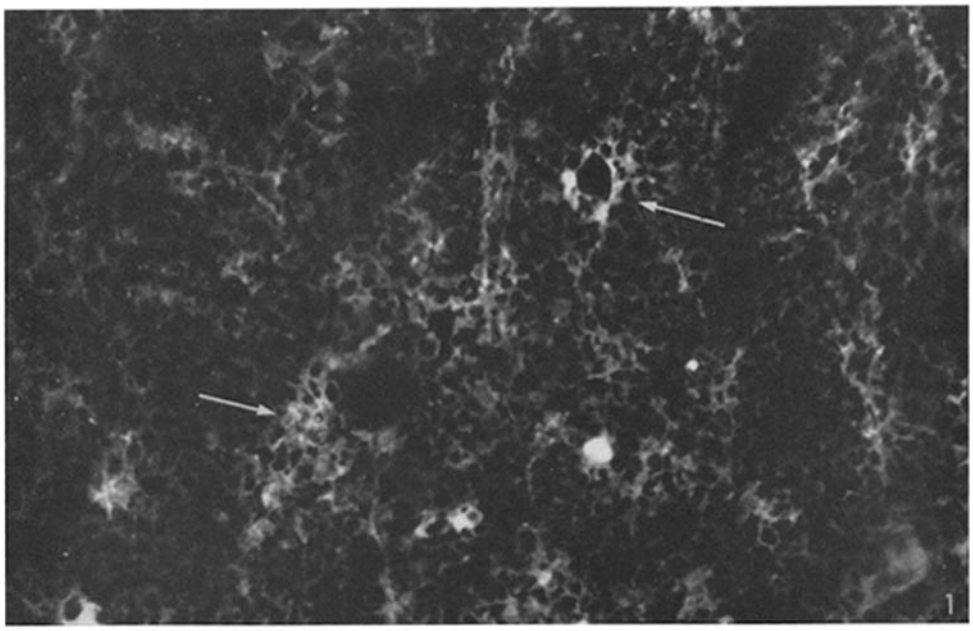
FIG. 1. A low magnification micrograph displaying clustered and occasionally isolated tumor cells with specific cytoplasmic fluorescence, indicated by arrows. The faintly stained cells, constituting the majority in the field, are seen only because of their low-grade autofluorescence. × 200.

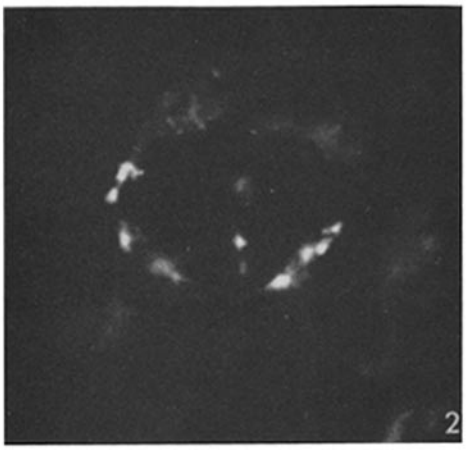
FIG. 2. An early stage of protein accumulation in an immature myeloma cell. Aggregates of fluorescing myeloma globulin are confined to the narrow cytoplasmic rim. × 1500.

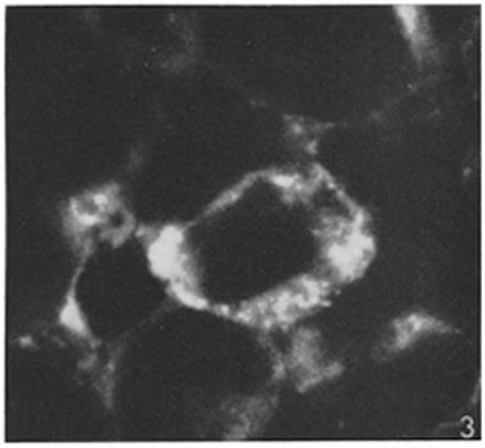
FIG. 3. An increased number of fluorescent aggregates in the cytoplasm of a more mature-appearing myeloma cell. × 1500.

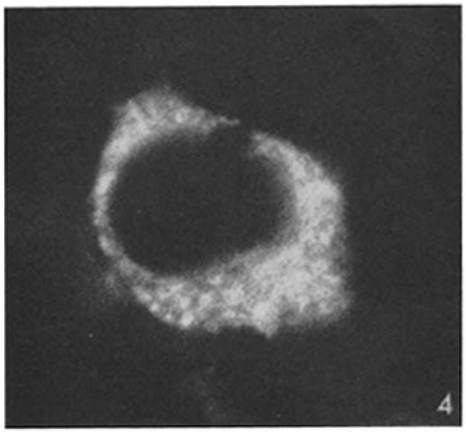
FIG. 4. Further accumulation of fluorescein-labeled aggregates of myeloma globulin within the expanded cytoplasmic mass of another cell. × 1500.

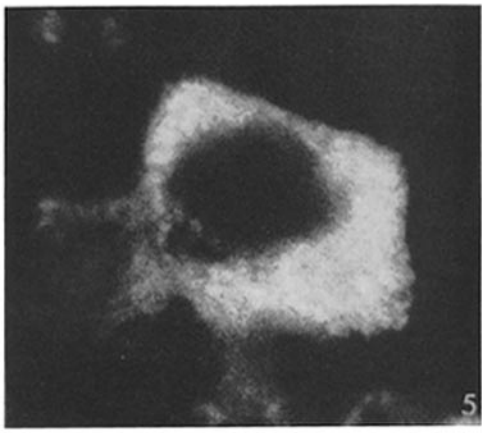
FIG. 5. A cell with intense but more diffuse cytoplasmic staining with fluorescent antibody. × 1500.







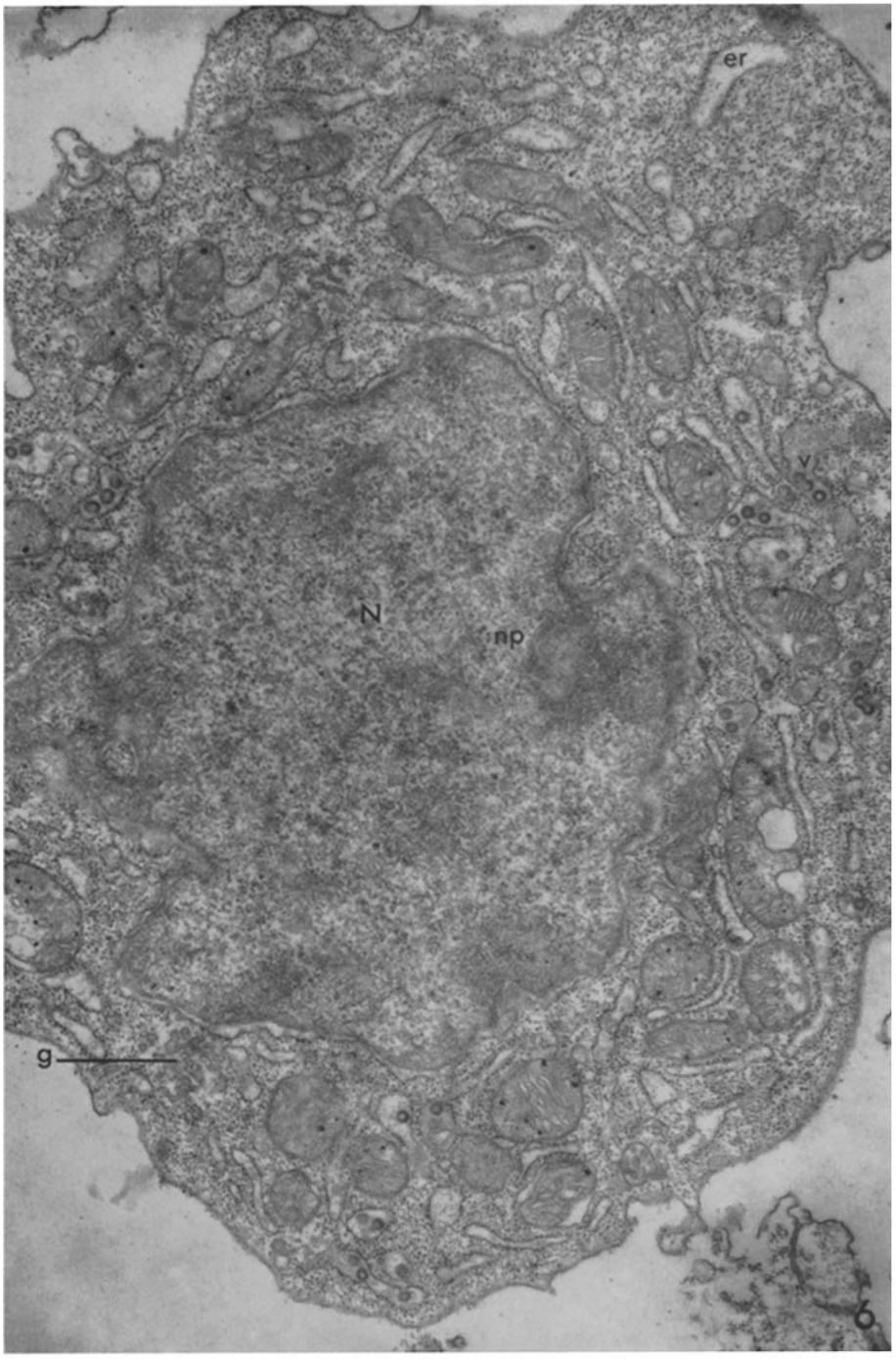




(Rifkind *et al.*: Gamma globulin in mouse plasma cell tumor)

PLATE 58

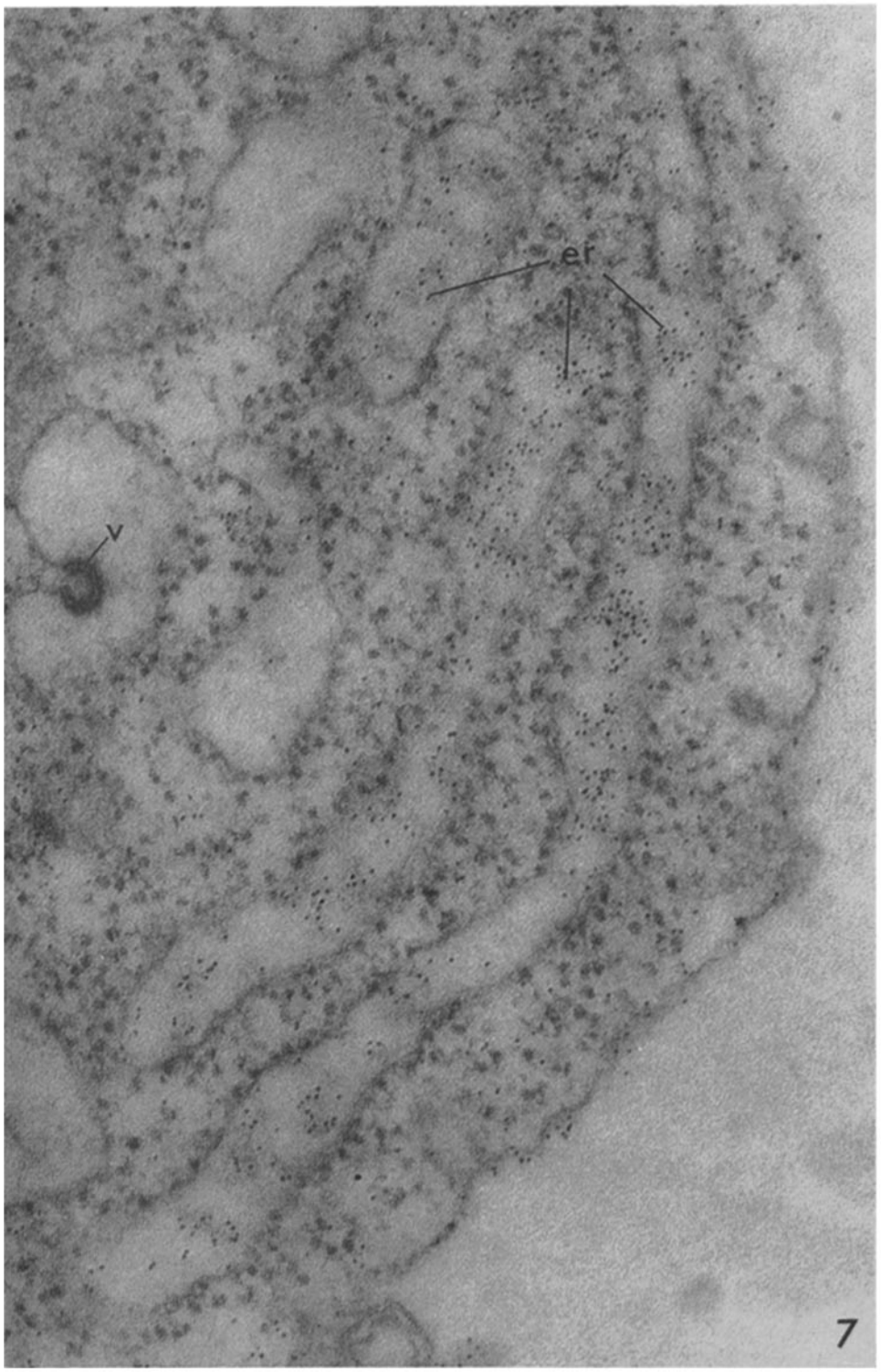
FIG. 6. A low power electron micrograph of a tumor cell treated with ferritin-conjugated antibody. The nucleus (*N*), nuclear pores (*np*), Golgi apparatus (represented in this section only by its vesicular component, labeled *g*), a highly elaborated endoplasmic reticulum (*er*) and virus-like particles (*v*) are illustrated. An abundance of free-lying ribonucleoprotein particles fills the cytoplasm. Ferritin is not clearly visible at this magnification. × 23,000.



(Rifkind *et al.*: Gamma globulin in mouse plasma cell tumor)

PLATE 59

FIG. 7. Another cell seen at higher magnification. Some ergastoplasmic cisternae (*er*) contain ferritin, whereas others exhibit little or no labeled antibody. One of these contains a virus-like particle (*v*). The surface of this cisterna bears comparatively few ribonucleoprotein particles, especially in the vicinity of the virus. Tagging of the cell surface is seen along the right-hand margin. × 109,000.

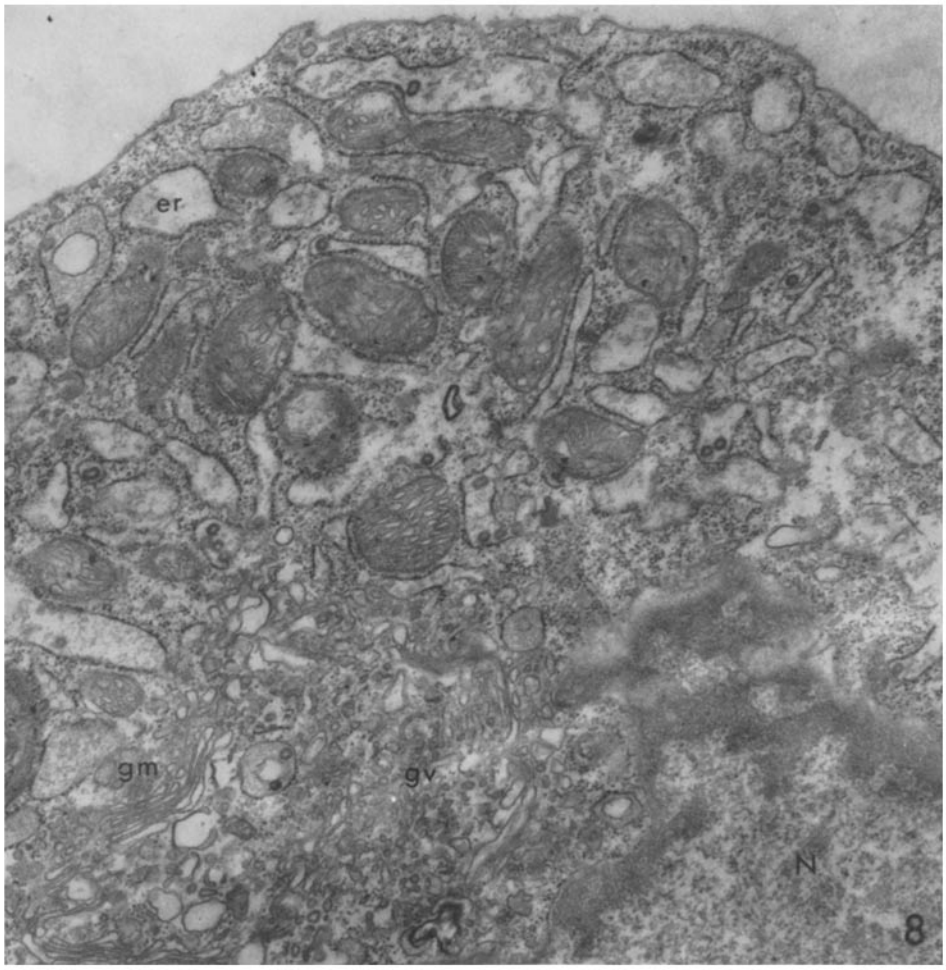


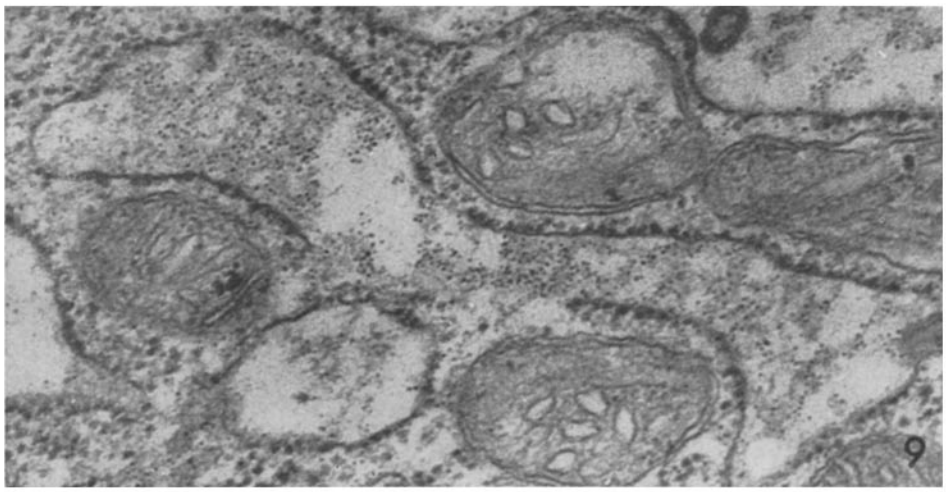
(Rifkind *et al.*: Gamma globulin in mouse plasma cell tumor)

PLATE 60

FIG. 8. A section through the Golgi zone of a tumor cell. Golgi membranes (*gm*) and vesicles (*gv*) are not labeled with ferritin. Mitochondria, free ribonucleoprotein granules, and distended cisternae of the endoplasmic reticulum (*er*) fill the cytoplasm. The nucleus is visible at the lower right. × 23,000.

FIG. 9. Part of the preceding figure shown at higher magnification. The contents of the cisternae are labeled with ferritin. Mitochondria and the surrounding cytoplasm are unlabeled. × 70,000.

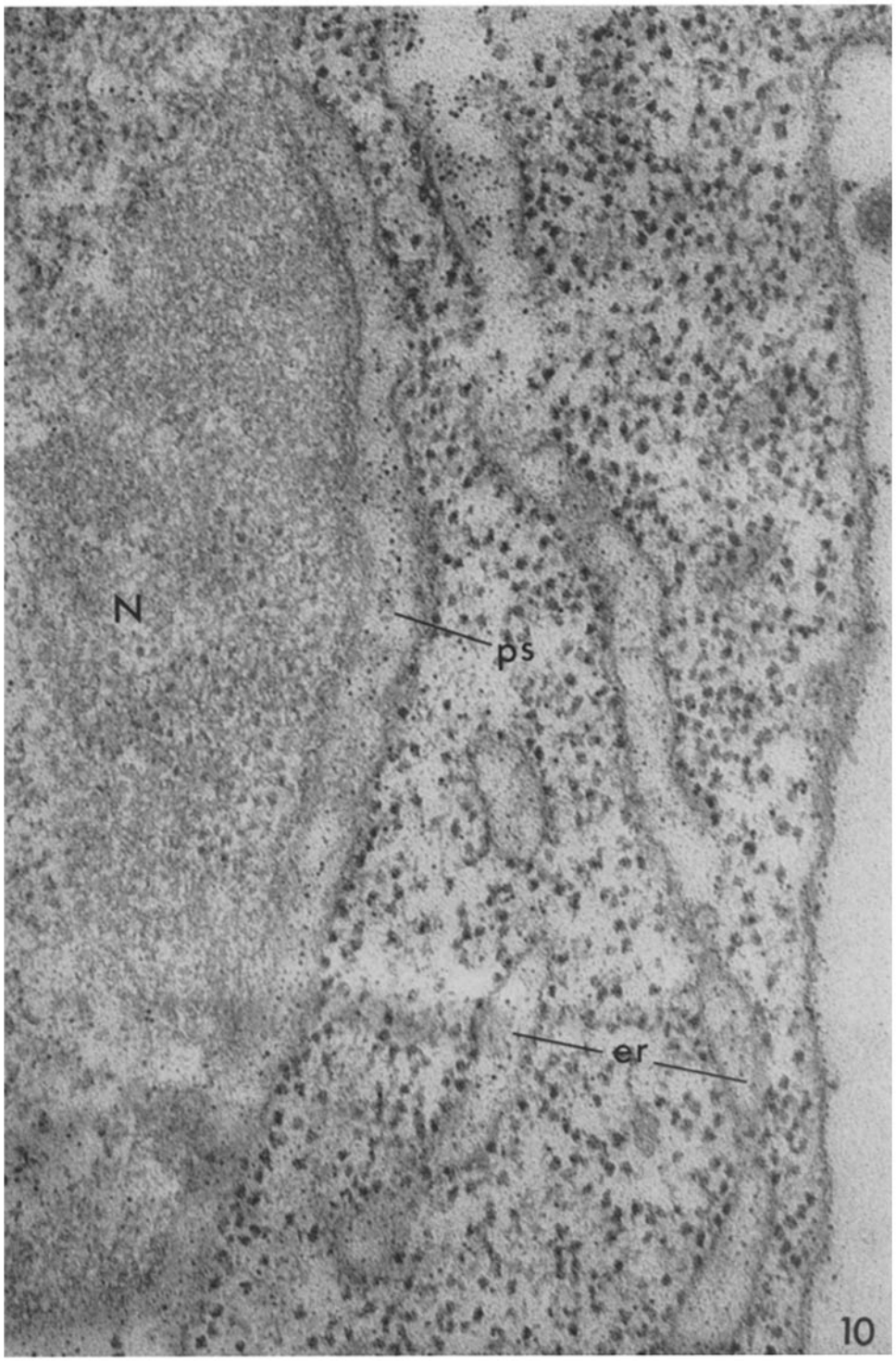




(Rifkind *et al.*: Gamma globulin in mouse plasma cell tumor)

PLATE 61

FIG. 10. Ferritin-conjugated antibody concentrated in the perinuclear space (*ps*) and other units of the endoplasmic reticulum (*er*). Ribonucleoprotein particles are scattered through the cytoplasm. The cell surface, at the right-hand margin of the figure, is tagged with ferritin. × 100,000.



(Rifkind *et al.*: Gamma globulin in mouse plasma cell tumor)

PLATE 62

FIG. 11. Ferritin within the endoplasmic reticulum (*er*). Virus-containing cisternae (*v*) display minimal amounts of the labeled antibody. Vesicular (*s*) and lamellar (*g*) elements of the Golgi apparatus show little tagging although there is a moderate amount of ferritin dispersed between the free-lying ribonucleoprotein of the cytoplasmic matrix. × 37,000.

INSET: A tumor cell with diffuse cytoplasmic fluorescent staining. A few focal concentrations of globulin are visible near the nucleus (*gr*). The Golgi zone (*g*) is less intensely stained than the surrounding cytoplasm. × 1800.

PLATE 63

FIG. 12. The cytoplasm of another myeloma cell. The margin of the nucleus is seen at the upper right. Cisternae of the endoplasmic reticulum (*er*), mitochondria (*m*), and ribonucleoprotein granules occupy most of the cytoplasm. Three clusters of agranular membranes (*g*), constituting the Golgi apparatus, are seen at higher magnification in the next two figures. × 48,000.

PLATE 64

FIG. 13. A portion of the cell illustrated in the preceding micrograph. Parallel Golgi membranes are separated by cisternal spaces which contain ferritin-labeled antibody (arrow). A vesicle, partially damaged by freezing, occupies the center of the field. At its right-hand margin a junction between rough-surfaced endoplasmic reticulum (above) and the Golgi system (below) is indicated (*j*). At this point the cisternal contents are labeled with ferritin. × 90,000.

FIG. 14. Another detail from Fig. 12. Two aggregates of lamellar Golgi membranes are seen. On the right the membranes clearly form flat cisternal structures, several of which contain ferritin-conjugated anti-myeloma globulin. At the left-hand margin of the figure the membranes are tangentially sectioned and hence the disposition of labeled antibody is not clearly revealed. Small Golgi vesicles (*gv*) appear free of ferritin. × 90,000.

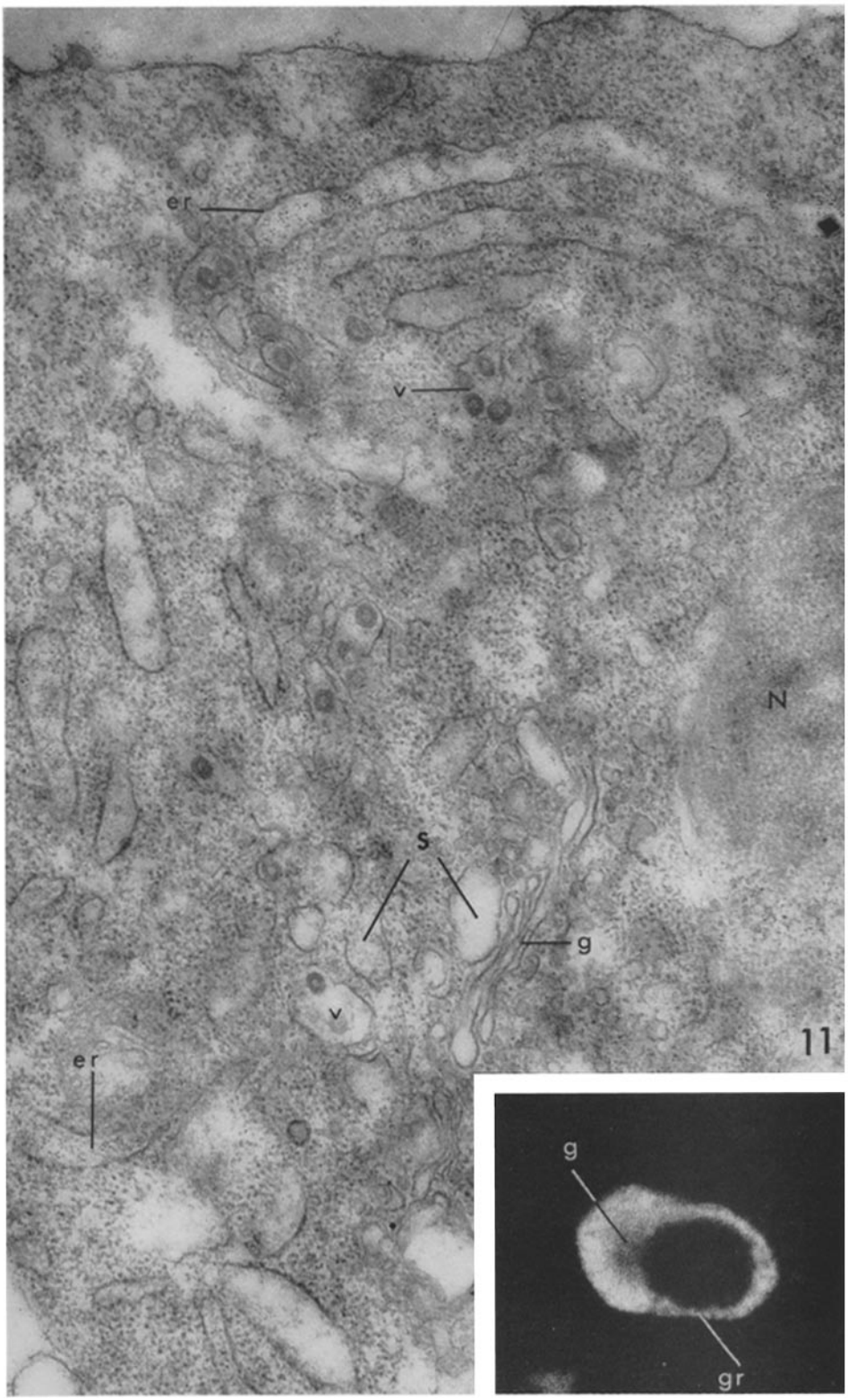
PLATE 65

FIG. 15. A fragment of myeloma cell cytoplasm containing a dilated cisterna of the endoplasmic reticulum which is filled with ferritin-tagged granular material. Lesser amounts of a similar tagged material are found in the extracisternal cytoplasm and the adjacent extracellular space (arrows). × 20,000.

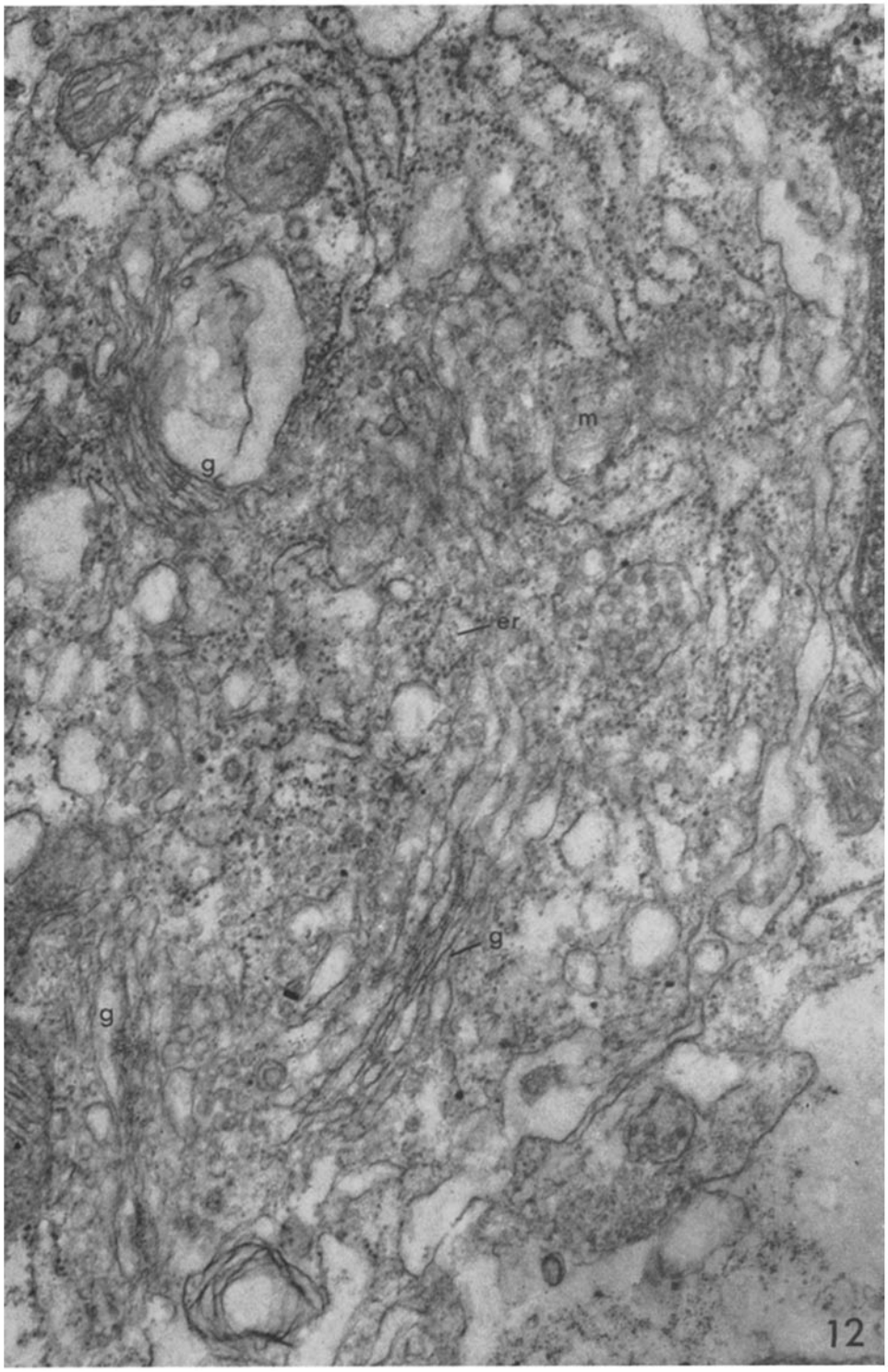
INSET: A fluorescence micrograph of a cell presumed to be at a comparable stage of disintegration. The cytoplasmic fluorescence is disposed in large aggregates and the cell margin is very uneven. × 1500.

PLATE 66

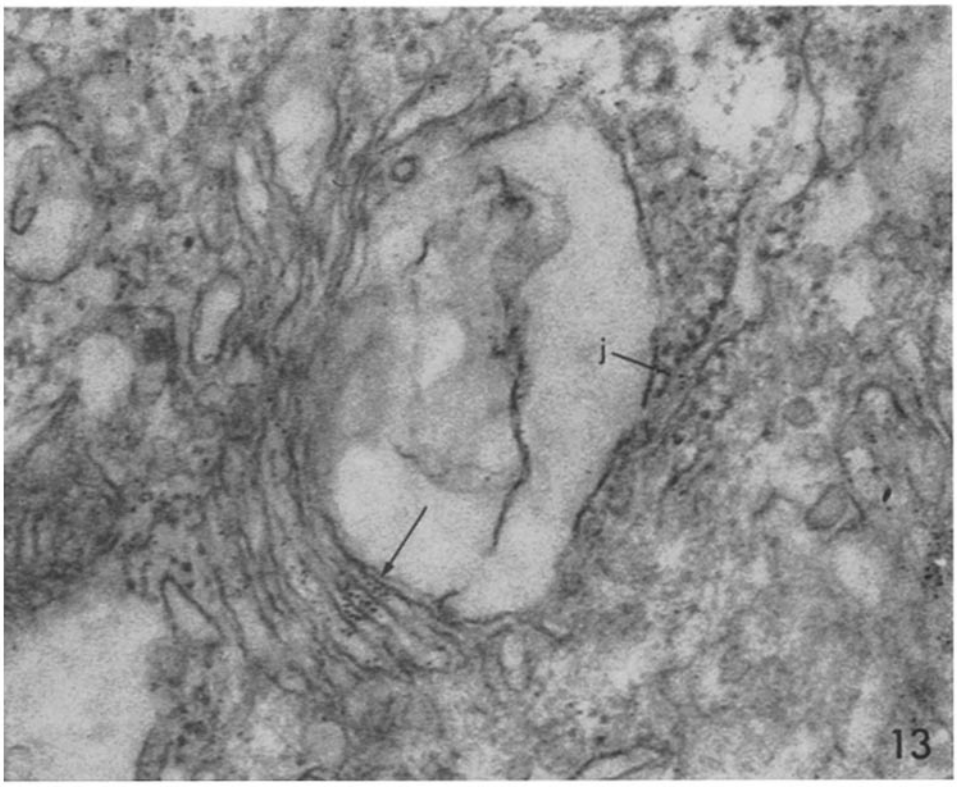
FIG. 16. A portion of the preceding figure at higher magnification. The granular cisternal contents (*er*) are more heavily labeled than the surrounding cytoplasm. Ribonucleoprotein particles (*rp*) are found only upon the endoplasmic reticulum. × 120,000.

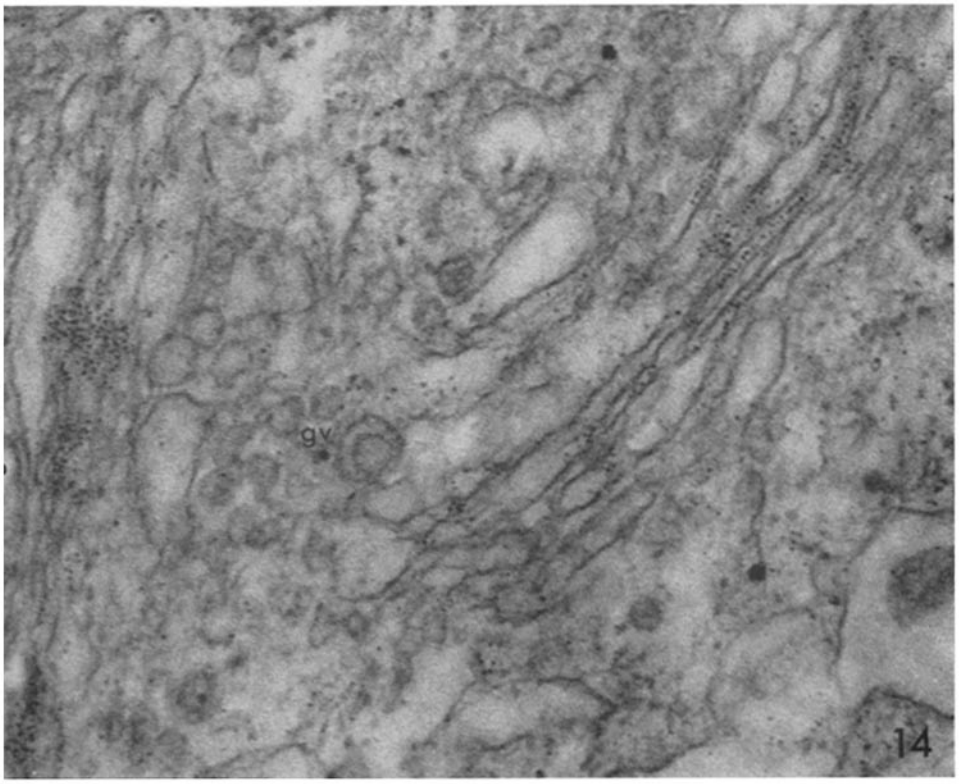


(Rifkind *et al.*: Gamma globulin in mouse plasma cell tumor)

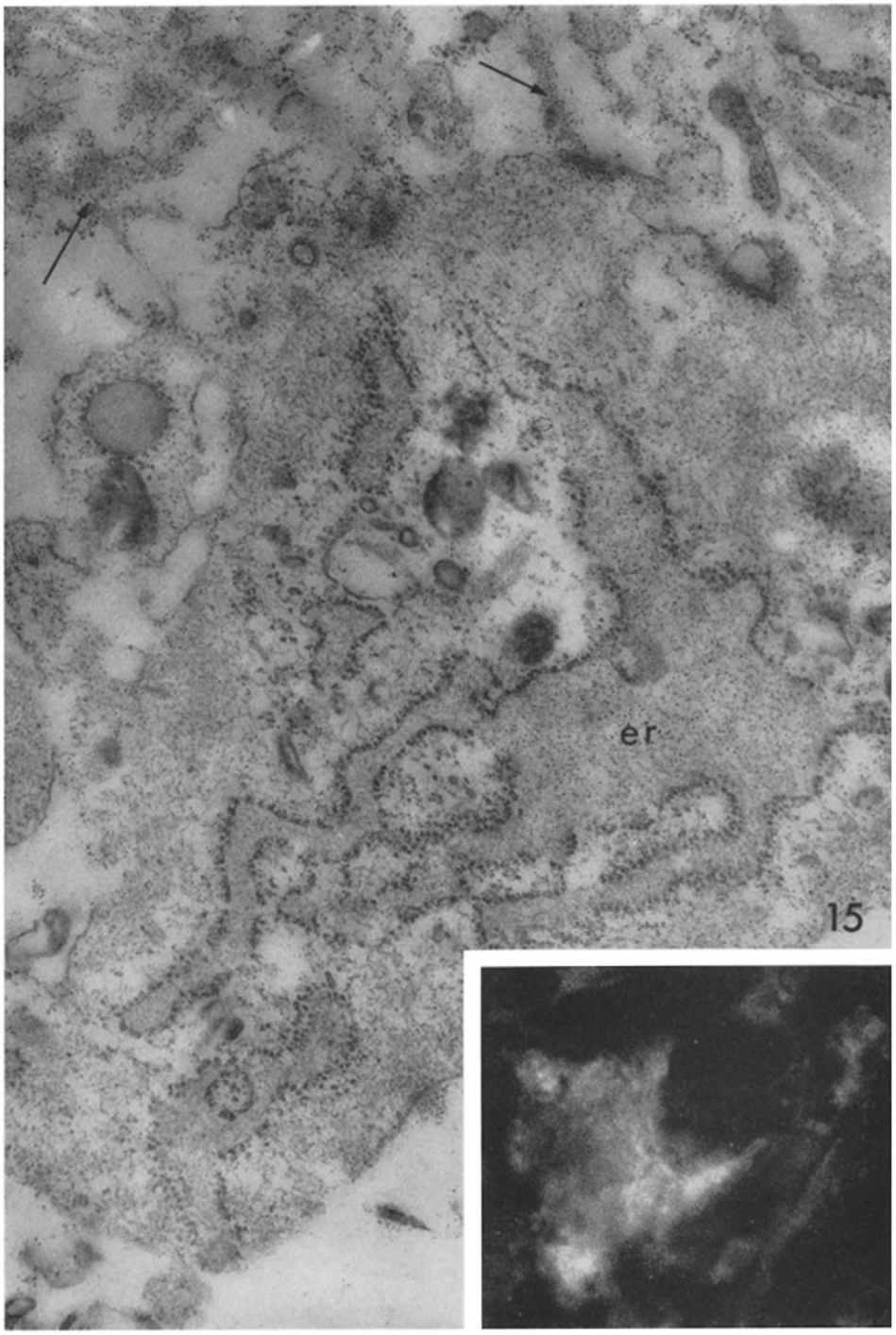


(Rifkind *et al.*: Gamma globulin in mouse plasma cell tumor)

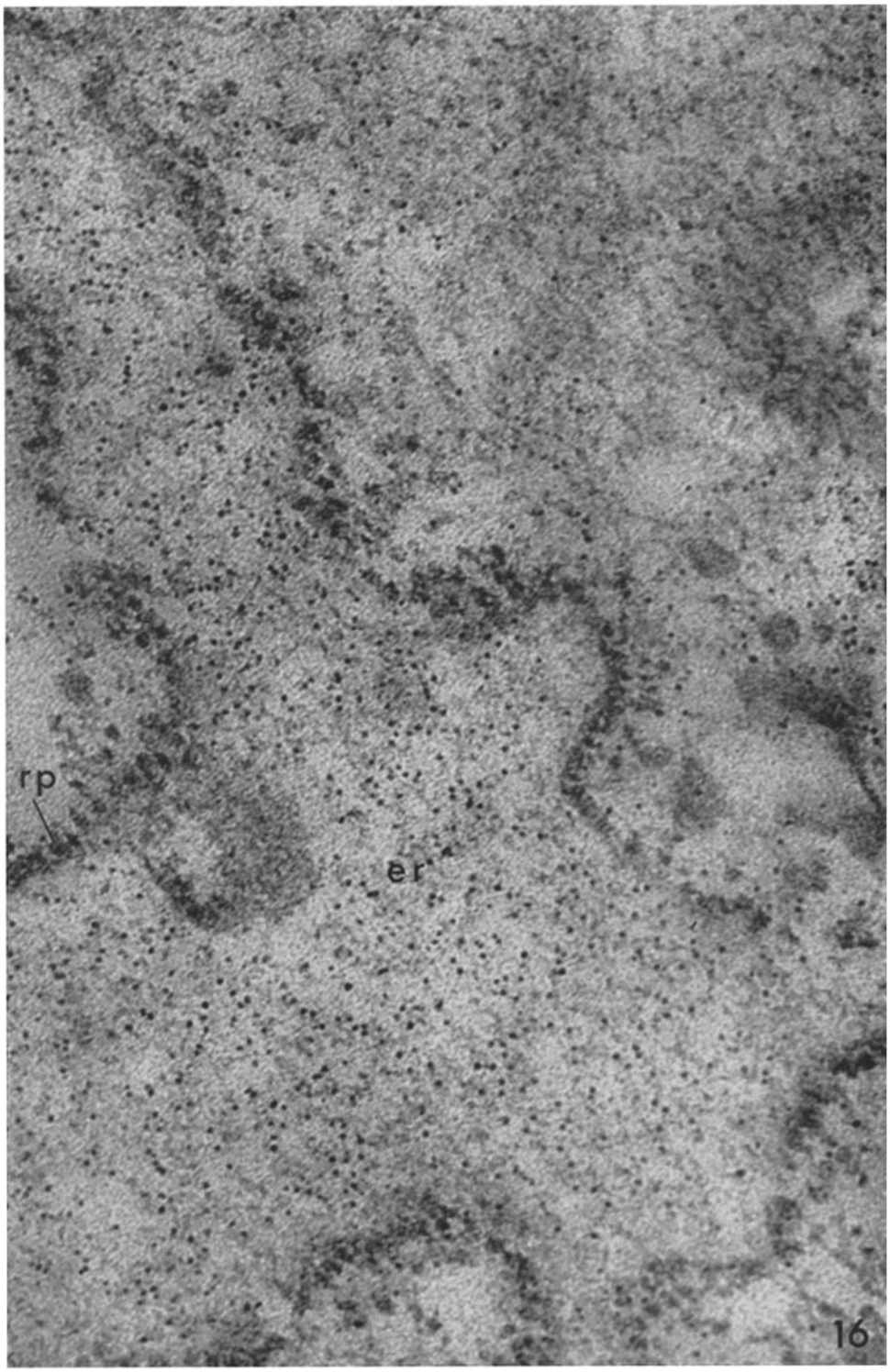




(Rifkind *et al.*: Gamma globulin in mouse plasma cell tumor)



(Rifkind *et al.*: Gamma globulin in mouse plasma cell tumor)



(Rifkind *et al.*: Gamma globulin in mouse plasma cell tumor)