LOCALIZATION OF ANTIBODIES BY ELECTRON MICROSCOPY IN DEVELOPING ANTIBODY-PRODUCING CELLS

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ABSTRACT

The development of antibody-producing cells in the early stages of the secondary or hyperimmune response has been studied with the electron microscope in lymph nodes of adult chinchilla rabbits immunized with ferritin or apoferritin. The intracellular distribution of antiferritin antibodies was determined in the lymph node cells at 1 to 5 days after a booster injection, employing the labelling technique previously used by the authors (12) to demonstrate the localization of antibodies in mature plasma cells. Antibodies were first detected at 48 hours in blasts; *i.e.*, cells which have a poorly developed endoplasmic reticulum and a cytoplasm filled with many ribosomes grouped in clusters. The label was subsequently found in forms intermediate between blasts and plasma cells (plasmoblasts, immature plasma cells), in which the endoplasmic reticulum appeared progressively more developed. Antiferritin antibodies were also found in cells in mitosis. In all the above cell types, antigen-antibody precipitates were consistently found in the perinuclear space and in the cisternae of the granular endoplasmic reticulum, from an early stage in the development of the latter. Evidence was also obtained for the presence of antibody in the Golgi area. The results are discussed in relation to the possible cellular sites of antibody synthesis.

In a previous paper (12) de Petris, Karlsbad, and Pernis described the subcellular distribution of antiferritin antibodies in the typical plasma cells present in lymph nodes of hyperimmunized rabbits on the 4th and 5th day after a booster injection of antigen. The antibodies (marked with ferritin) appeared to be localized in the perinuclear space and cisternae of the endoplasmic reticulum, and possibly also in the Golgi region.

We have now extended this research to the early stages of the immune response, namely to the study of the cells present in the lymph nodes at 24 to 72 hours after restimulation. These early stages are characterized by active cellular division, accompanied by a series of cellular transformations entailing marked ultrastructural changes. In an effort to throw light on the cellular aspects of antibody synthesis, we have tried to correlate the beginning and further development of the antibody-synthesizing activity, as deduced from the initial and subsequent intracellular distribution of labelled antibodies, with the morphological changes which occur in the antibody-producing cells and eventually lead to their full differentiation into plasma cells.

The technique employed for labelling the antibodies was essentially the same as that used in the previous investigation (12): it consisted in incubating ferritin with lymph node cells of animals immunized with either ferritin or apoferritin, after suitable treatment aimed at allowing the penetration of the marker (ferritin) into the cells. The localization of antibodies was deduced from the distribution of ferritin-antiferritin microprecipitates.

Preliminary communications on this subject

were presented at the Padua and Prague meetings of electron microscopy (10).

MATERIALS AND METHODS

ANTIGENS: Horse ferritin (FT) was purchased from Mann Research Laboratories Inc. (New York) and was used after further purification by recrystallization. Apoferritin (AFT) was prepared from FT by the method of Granick and Michaelis (19). Human gamma globulin (Cohn fraction II) (HGG) was obtained from Mann Research Laboratories Inc. (New York).

IMMUNIZATION: Adult chinchilla rabbits were immunized by one or several weekly injections of FT or AFT (about 2 mg protein in each hindfoot-pad). A booster injection of the same amount of antigen was given 4 or more weeks after the last injection and the animals were sacrificed by exsanguination 1, 2, 2½, 3, 4, and 5 days later. Serum antibodies were determined only qualitatively by the ring capillary test.

PREPARATION OF THE TISSUES FOR LIGHT AND ELECTRON MICROSCOPY: The popliteal lymph nodes were rapidly removed and prepared for light and electron microscopy.

1. For light microscopy, slices of the lymph nodes were fixed in Carnoy's solution and further treated by conventional methods. Sections were stained with methyl green-pyronin (Unna-Pappenheim stain).

2. For electron microscopy, some thin tissue slices were fixed in 1 per cent osmium tetroxide buffered according to Palade (36) or Millonig (28), dehydrated in a graded series of alcohols and embedded in Araldite according to the standard techniques (27). Most of the lymph node tissue was used instead for labelling the intracellular antibodies with FT. A detailed description of the technique employed is given in our previous paper (12). Most of the present experiments were carried out on isolated lymph node cells. The lymph nodes were teased with needles in cold, buffered saline, and the isolated cells were passed through a stainless steel gauze, centrifuged, washed in buffer, and then fixed for 15 minutes in 4 per cent formaldehyde buffered according to Millonig (28). The cells were then washed and centrifuged three times, frozen in an isopentane–dry ice mixture, thawed and incubated for about 15 minutes in a solution of FT in buffered saline (about 3 to 6 mg protein/ml; throughout this paper the concentration of FT will be expressed in milligrams of protein per milliliters, independently of the iron content: the actual concentration, taking into account the iron content, would therefore be 15 to 20 per cent higher). In control experiments, aimed at detecting non-specific labelling, higher concentrations of FT were used (up to about 40 mg/ml). The cells were then centrifuged, washed three times with buffered saline, and the final pellet was fixed in 1 per cent osmium tetroxide (28, 36), dehydrated, and embedded in Araldite as above. In some experiments the labelling technique was applied to whole lymph node sections as well as to isolated cells (12): in this case, slices of lymph node were fixed for 1 hour with 4 per cent buffered formaldehyde, infiltrated with 10% dimethyl sulfoxide (for about 1 hour), and slowly frozen in a "cold box." Sections 15 to 20 μ thick were cut in a cryostat, incubated with FT, washed, and prepared for electron microscopy as described previously (12). The labelling technique applied to sections is rather cumbersome and the penetration of the label is more limited than in the case of isolated cells.

Although usually the cells prepared by the above methods did not show evidence of gross damage, in different experiments there were noticeable differences in the preservation of fine cell structure. These differences were perhaps connected to variations hard to control and inherent in the freezing and thawing process. Compared with the same cell types present in control sections, isolated cells often appeared slightly shrunken and with nuclear and cellular outlines more irregular and ruffled.

Thin sections were cut with a Servall Porter-Blum ultramicrotome and examined with a Siemens Elmiskop I. The tissues were usually stained with 2 per cent uranyl acetate during dehydration; most sections were also poststained with 5 per cent uranyl acetate in 50 per cent alcohol to obtain added contrast.

RESULTS

Cytological Observations

LIGHT MICROSCOPY: The popliteal lymph nodes of hyperimmunized rabbits examined 24 and 48 hours after a booster injection of FT were characterized by the presence of many large rounded cells having big vesiculated nuclei, with prominent nucleoli, and pyroninophilic cytoplasms. Such cells have been given different names by different authors: transitional cells (14), large lymphocytes (32, 48), activated reticular cells (25), large pyroninophilic cells (18), hemocytoblasts (15, 54), blasts (34), etc.; we shall refer to them hereafter by the generic term "blasts." At 3 days many of the blasts apparently underwent a reduction in volume involving both nucleus and cytoplasm: the nuclei took up a more eccentric position and their chromatin appeared more condensed. These changes were further accentuated at 4 and 5 days, the number of blasts declining concurrently with a progressive increase in the number of typical mature plasma cells with eccentric nuclei and strongly

pyroninophilic cytoplasms. Several mitoses were observed at 2 to 3 days.

ELECTRON MICROSCOPY: With the electron microscope, blasts were detected consistently at 24 hours and in large numbers at 48 hours; they were easily recognized by their large size and big clear nuclei, with loosely arranged chromatin condensed into a thin peripheral rim, and with one or more large reticular nucleoli, often extremely well developed (Fig. 1). The cytoplasm of these cells presented a well developed Golgi apparatus with several parallel stacks of membranes, but few vesicles and vacuoles, often surrounding one or two centrioles. The mitochondria were large and irregularly shaped, with a clear matrix and few irregular cristae. The ground cytoplasm was almost completely filled by large numbers of ribosomes, mostly grouped in small clusters, usually of 4 to 10 units (Fig. 2). The rough endoplasmic reticulum (ER) was poorly developed and in most blasts it consisted of only one or few slender profiles, sometimes connected with the perinuclear space. Smooth vacuoles and vesicles were also very scanty. Mitoses were frequently observed in these cells.

Some blasts at 48 hours and most of them at 72 hours appeared to undergo structural changes (Figs. 3 and 4). Their nuclei became smaller and contained more condensed chromatin; the cytoplasm maintained its characteristics, but showed increasing numbers of cisternae of the ER, containing a fine flocculent material. Many cisternae were flattened, with more or less parallel membranes, but several of them appeared somewhat dilated. The membranes of the cisternae were studied with ribosomal particles which, when favourably oriented, *i.e.* lying in the plane of the section, appeared regularly arranged in distinct rows or spirals, consisting of variable numbers of ribosomes (up to 13 to 15) (Fig. 4). The remaining cytoplasm still contained many free[1] ribosomes mainly grouped in clusters. Such cells may be referred to as plasmoblasts or immature plasma cells, according to their stage of differentiation. (However, since there is a whole series of intermediate stages between blasts and plasma cells, it is difficult and perhaps not very significant to classify rigidly the different transitional forms, especially because in thin sections the true nuclear and cellular dimensions are hard to determine. Unless otherwise specified, we shall therefore use the term immature plasma cell for all the intermediate stages.)

At 72 hours some typical plasma cells (*e.g.* 3, 4, 5, 30, 47) appeared in the lymph nodes. In subsequent days they increased in number, while there was a marked decrease in the number of blasts. The plasma cells would appear to derive from plasmoblasts and immature plasma cells by increasing development of the ER (which comes to occupy a substantial part of the cytoplasm); by reduction in nuclear volume with condensation of the chromatin into coarse clumps; by reduction in the size of mitochondria which become more regularly shaped (round or elongated) and denser; and by extensive development of the Golgi complex which eventually consists of a large number of vesicles, stacks of parallel membranes, and vacuoles, often containing a material comparable in density to that present in the cisternae of the ER. Some young plasma cells undergoing mitosis were observed.

A more detailed description of the cells present in the lymph nodes of hyperimmunized rabbits will be published elsewhere (11).

Detection of the Antigen in Blasts and Immature Plasma Cells

Since the intact molecule of the antigen (FT) used in most of these experiments is clearly visible in thin sections owing to its high iron content, a survey of the micrographs of blasts and immature plasma cells was made to ascertain the possible presence of *antigen* molecules in these cells. No appreciable amounts of FT were ever found in blasts or immature plasma cells (or in small lymphocytes). The presence of *a few* molecules of FT in the ground cytoplasm or in the nucleus could not be excluded in some of these cells, owing to the difficulty of identifying isolated molecules, but most of the cells appeared quite free of FT (*cf.* reference 12). On the contrary, a fairly large number of FT molecules, either scattered in the cytoplasm or enclosed within vacuoles, was found in several reticular cells and macrophages. In some cases, however, these molecules are likely to represent autologous (rabbit) FT, indistinguishable from horse FT with the electron microscope: autologous FT is indeed often found in macrophages which, for example, have phagocytosed red blood cells.

Detection of Anti-FT Antibodies

The search for FT molecules labelling intracellular antibodies presupposes that all FT found in

[1] In the present context we shall use the term "free" ribosomes to refer to ribosomes (either isolated or grouped in clusters) *not bound* to membranes.

the cells entered them at the time of incubation and was not previously present in the cells. The observations referred to in the previous paragraph, on lymph nodes of animals immunized with FT, had already proved that apart from macrophages and reticular cells no other lymph node cells contained FT in amounts capable of interfering with the incubation experiments. However, to rule out completely the possibility that any FT present in the cells might represent residual antigen from previous immunizing injections, some rabbits were immunized with AFT, while FT was used *only* for the incubation. AFT was used because it is antigenically identical with FT (26), but since it lacks the iron core of the FT molecule it cannot be detected in thin sections. All the FT found after incubation in blasts and plasma cells of animals immunized with AFT must therefore have entered the cells during incubation. The results described below refer to the labelling of lymph node cells from animals immunized either with FT or AFT. As expected, the results in the two cases were exactly superimposable (Figs. 5 to 11).

In the isolated cell preparations obtained 24 hours after restimulation we did not observe any definite labelling of the cells, including blasts which were relatively few at this stage; the only exception was an occasional mature plasma cell, presumably remaining from previous immunizing injections. On the contrary, the marker was detected in several blasts and immature plasma cells of lymph nodes taken 48 to 72 hours after restimulation. In accordance with our previous observations, large quantities of FT were also found in the plasma cells present from the 3rd day onwards.

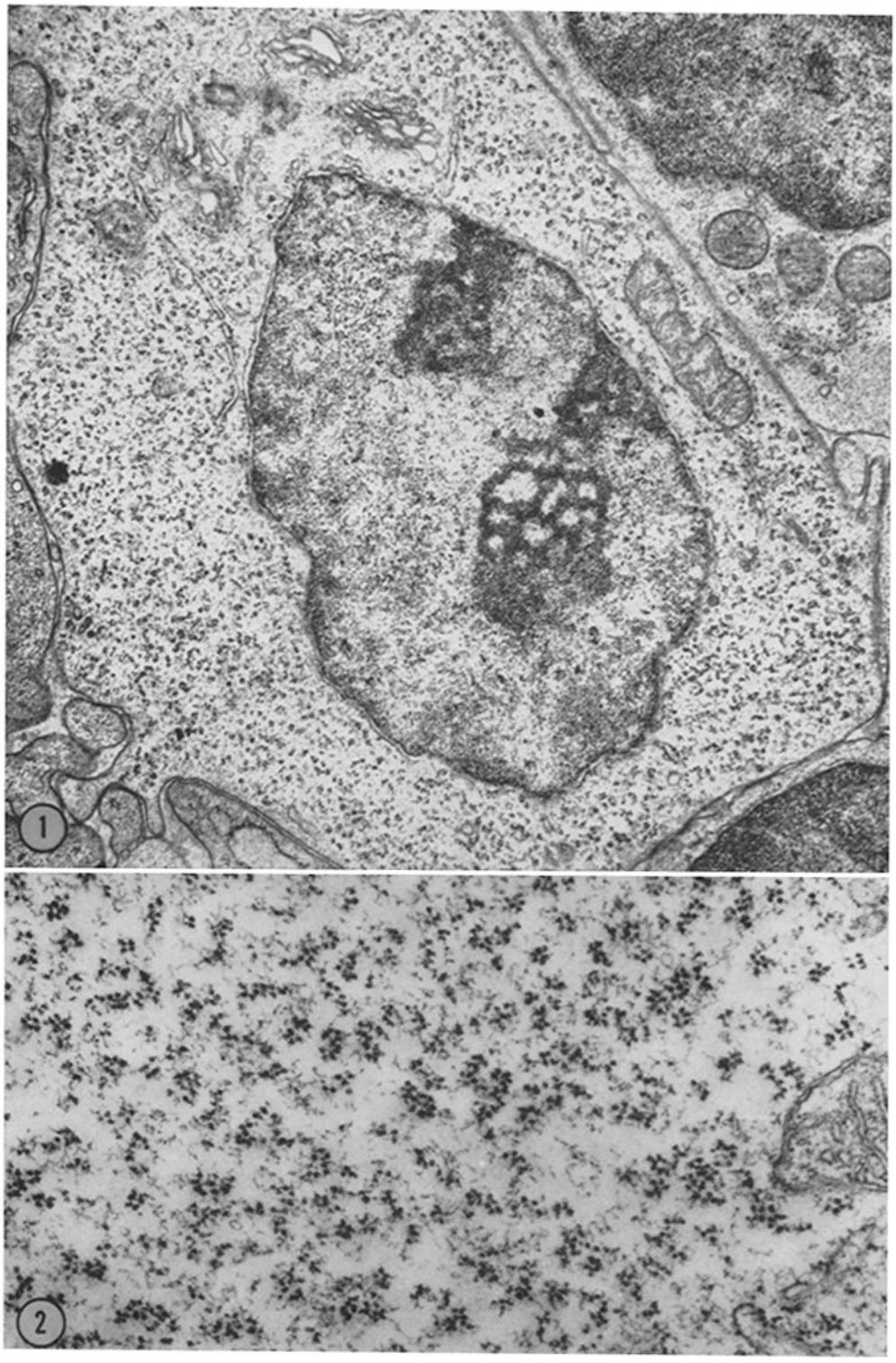
The FT molecules, distributed more or less uniformly or grouped in small clusters, were localized almost exclusively within the cisternae of the granular ER and in the perinuclear space, as shown in Figs. 5 to 8. The dense FT cores usually appeared surrounded by a less electron-opaque, flocculent material, the aggregates having the appearance of FT–anti–FT microprecipitates (*cf.* reference 12). This strict pattern of localization was even more striking in the very immature blasts having only a few profiles of ER present in their cytoplasm (Fig. 7): the remaining extensive area of their ground cytoplasm, where the characteristic ribosomal clusters were often still visible, was devoid of FT as a rule. (In the formalin-fixed and subsequently incubated cells the arrangement of ribosomes in characteristic clusters was not always well preserved (*e.g.* Figs. 6, 8, and 10), probably owing to damage during the freezing and thawing process.)

The intracisternal concentration of FT molecules (*i.e.*, of FT–anti–FT microprecipitates) was already quite high in several blasts in which the cisternae were few and still flattened (Figs. 6 and 7): it was of the same order, but somewhat lower, than that found in plasma cells. In a series of parallel counts made on blasts and plasma cells it ranged from 500 to 1000 molecules/μ^2 in blasts, as compared with 700 to 2600 molecules/μ^2 in plasma cells. The higher values were usually found in the mature plasma cells with dilated cisternae. The total quantity of FT (and therefore presumably of anti-FT antibodies) in a single blast is, of course, lower than in a plasma cell because the

Figs. 1 to 4 and 10 to 12 refer to animals immunized with FT, and Figs. 5 to 9 to animals immunized with AFT. Figs. 5 to 12 are micrographs from isolated cell preparations.

FIGURE 1 Rabbit lymph node 48 hours after restimulation: a typical blast. Note the large clear nucleus with loosely arranged chromatin and two large, reticular nucleoli. The cytoplasm is almost completely filled by ribosomes grouped in small clusters. Two centrioles and a Golgi complex are clearly visible: the latter consists of several groups of parallel membranes, somewhat dilated, and of relatively few vesicles and vacuoles. The granular endoplasmic reticulum is practically absent (only one or two flattened profiles are recognizable in this section). Apart from having a large mitochondrion and a few vesicles, the cytoplasm appears completely devoid of organelles. × 18,000.

FIGURE 2 Higher magnification of the cytoplasm of a 48-hour blast, showing the grouping of ribosomes in clusters. × 50,000.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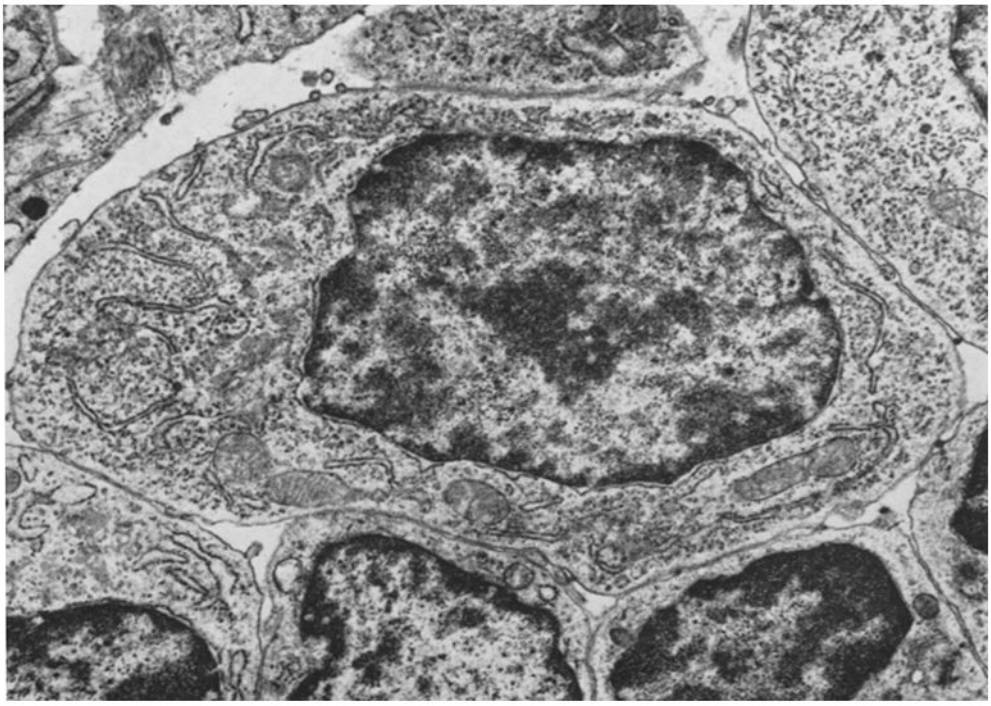


FIGURE 3 72-hour blast in an intermediate stage of transformation into a plasma cell (plasmoblast). The nuclear chromatin is slightly more condensed than in the blast in Fig. 1, especially near the nuclear surface. The cytoplasm is reminiscent of that in Fig. 1, but is crossed by several slender profiles of the rough endoplasmic reticulum. At the top right, part of the cytoplasm of a blast in the same stage of development as the one in Fig. 1 is visible. × 11,500.

total space occupied by the cisternae in the former is less than in the latter.

Cisternae containing FT were often found to be continuous with the perinuclear space, in both blasts and immature plasma cells (Fig. 8). Serial sections indicated that one and the same cisterna could be connected to the perinuclear space at more than one point; the frequent interconnections between different cisternae and between cisternae and perinuclear space probably facilitate the diffusion throughout the ER of FT molecules penetrating it through one or more discontinuities in the cell membranes.

FT molecules, either free or in aggregates, were not found as a rule in any other cell region (*e.g.*, nuclei) or organelle (*e.g.*, mitochondria). A few molecules, free or in small clusters, were occasionally present in the ground cytoplasm (*e.g.*, Fig. 7) or in the nucleus of some cells, but without any characteristic distribution: they are believed, therefore, to be molecules escaped from the cisternae or perinuclear space through random discontinuities in the membranes caused by freezing (Fig. 6), or perhaps, when isolated, to be molecules displaced by the microtome knife (7). The only possible exception to the above could be the Golgi area which in most cells was free of label but in several instances contained fairly large amounts of FT, generally in aggregates. In blasts FT was found only occasionally and in very small amounts in some vacuoles of the Golgi area. On the contrary, in immature and , more frequently, in mature plasma cells several large aggregates were found in this region; the probability of finding FT in the Golgi region of cells of the plasmocytic line would thus seem to be correlated to the degree of development of the Golgi system itself. FT aggregates were occasionally enclosed in recognizable vacuoles delimited by a smooth membrane (as in Fig. 11), but they were more commonly free, adjacent to disrupted vacuoles or vesicles (as in Fig. 10), or lying in regions of the

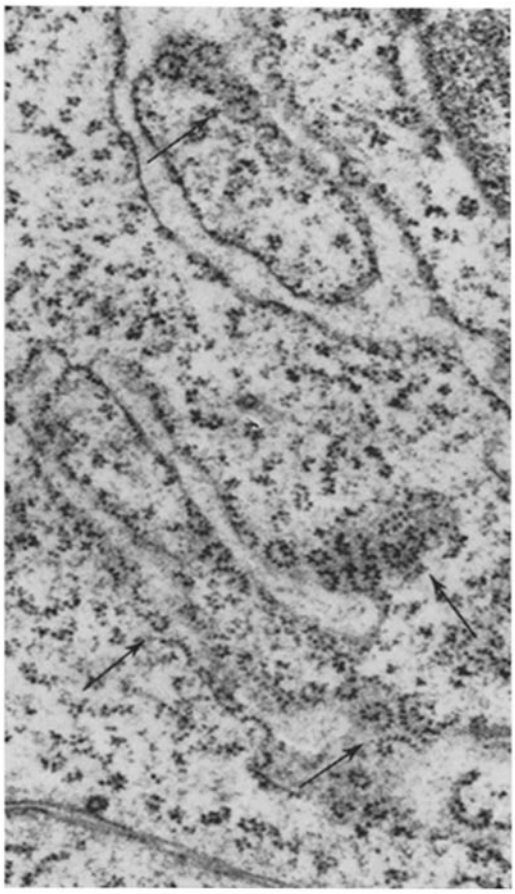


FIGURE 4 Higher magnification of the cytoplasm of an immature plasma cell at 72 hours, showing some tangentially cut cisternae. The ribosomes in the ground cytoplasm are grouped in small clusters, while those associated with the surface of the cistermae of the ER are lined up in distinct rows or spirals (arrows). × 40,000.

Golgi area which appeared most severely damaged by the freezing and thawing process.

FT–anti-FT microprecipitates were also found in some cells undergoing mitosis; from the dimensions of these cells and the characteristics of their cytoplasm (in particular the development of the ER), the cells were classified as blasts and immature plasma cells. Fig. 9 shows a blast in early telophase: the two nuclei are far apart, and the perinuclear space has reformed along most of the periphery of each nucleus but is still incomplete on the sides of the nuclei facing the interior of the cell. A few profiles of ER and some mitochondria are arranged at the periphery of the cell. FT molecules are clearly visible both in the cisternae of the ER and in the perinuclear space, still only partially surrounding the nucleus, while the remaining cytoplasm is free of label.

As regards the other cell types present in the lymph nodes, after incubation we have never found FT in typical small lymphocytes (which have a very scanty ER); FT was sometimes found, either free or in vacuoles, in the cytoplasm of reticular cells or macrophages, but without a clearly specific localization. In these cells it may, however, be residual antigen from the immunizing injections or, in some cases, autologous FT.

In agreement with our previous observations on mature plasma cells (12), we did not observe a specific localization of FT molecules on the plasma membrane of antibody-containing cells (or of any other type of lymph node cell). Only occasional clusters composed of few FT molecules (Fig. 10) were observed adherent to the plasma membrane, but they looked like small antigen–antibody precipitates, similar to the fairly large precipitates found at times extracellularly, especially in isolated cell preparations.

Controls

Suspensions of lymph node cells from animals immunized with an antigen unrelated to FT, namely HGG, were incubated with FT to determine the extent of unspecific labelling. Even with concentrations of FT in the incubation fluid up to three times higher than those routinely used (9 to 15 mg/ml), labelling was limited only to a few cells in which only a few molecules of FT were found, without any specific localization. This indicates that in the present experiment nonspecific labelling does not occur to any significant extent and confirms our previous conclusion (12) that the labelling of cells with FT by the present technique reveals the distribution of true antigen–antibody precipitates.

Another set of experiments was carried out to ascertain the extent of penetration of the label into the various cellular regions. For these experiments, lymph node cells of animals immunized with FT or with an unrelated antigen were treated by the usual method, but were fixed with osmium tetroxide immediately after incubation with FT, without being previously washed to remove the label not specifically combined. The concentration of FT routinely used in the incubation fluid (*i.e.*, 3 to 6

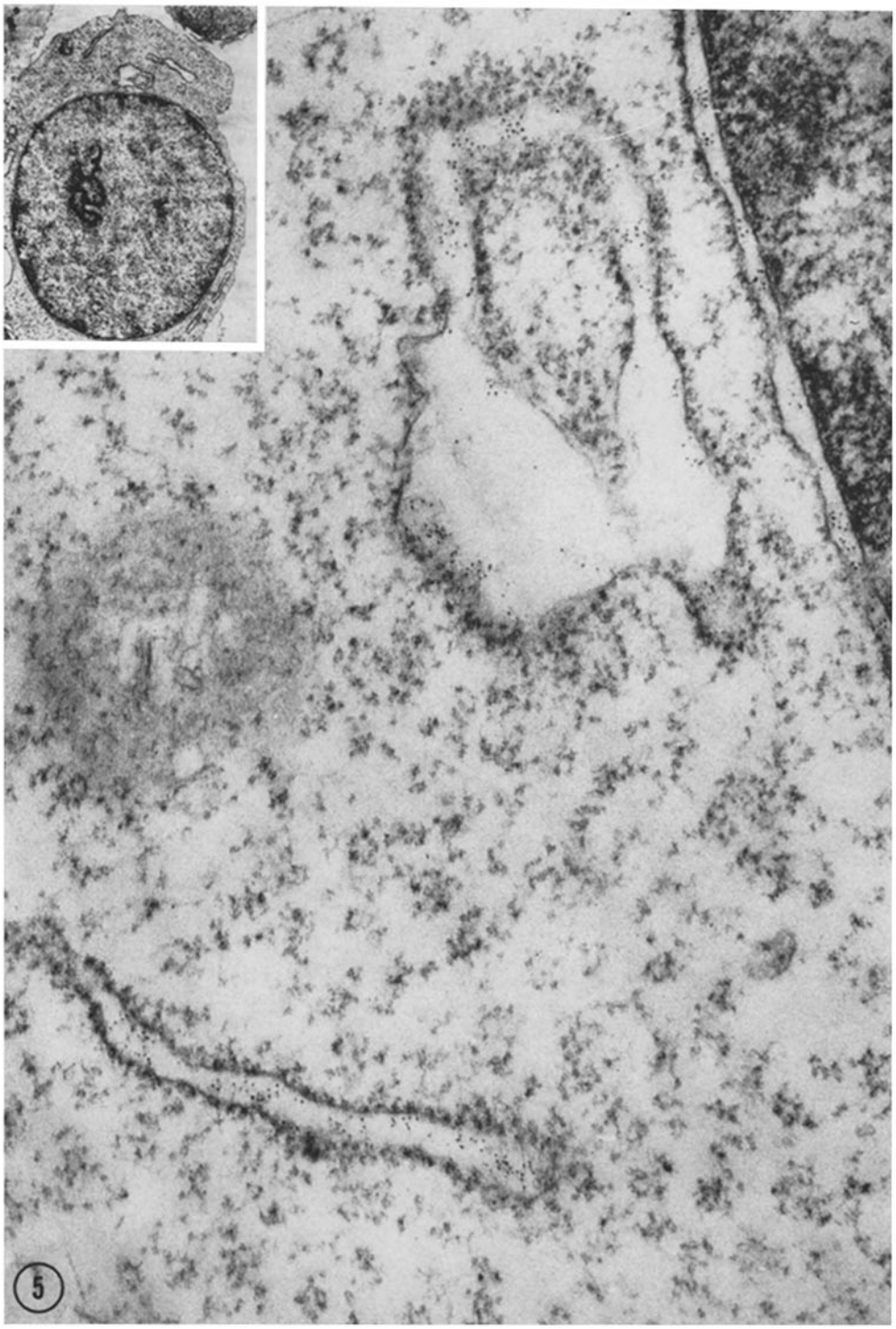
mg/ml) was found to be too low to yield an appreciable degree of unspecific labelling; the experiment was therefore repeated using higher concentrations (30 to 40 mg/ml). Under these conditions the cytoplasm of practically all the cells (blasts, plasma cells, lymphocytes, reticular cells) was labelled with more or less uniformly dispersed FT molecules, in a concentration of 100 to 300 molecules/μ^2 (Fig. 12); in several cisternae of plasma cells from animals immunized with FT the labelling was markedly more intense (about 1000 to 2000 molecules/μ^2), but in these cases it presumably corresponded to specific antigen–antibody precipitates. FT was absent from most mitochondria and other small organelles (vacuoles, granules) delimited by a membrane; for example, relatively few molecules were present in the vacuoles of the Golgi area. As a rule the nuclei of blasts, immature plasma cells, and reticular cells were labelled, the concentration of FT being of the same order as in the cytoplasm; however, while the labelling was fairly uniform in the clear areas of the nucleus (and of the nucleolar region) which in blasts occupy most of the nuclear volume, the FT apparently failed to penetrate into the regions of condensed chromatin and into the condensed reticular strands of the nucleolus. The label was generally absent from the nuclei of cells having extensive areas of condensed chromatin (*i.e.* plasma cells and small lymphocytes); FT molecules were present in the clear areas of some of these nuclei (Fig. 12), but usually in lower concentrations than in the cytoplasm of the same cells. From the above results we conclude that the label can penetrate easily into the large nuclei of the more immature cells which have a loosely arranged chromatin, but it tends to be excluded from the condensed nuclei of the more mature or inactive forms. In turn this indicates that the absence of nuclear labelling, at least in blasts and immature plasma cells, observed in our preparations, cannot be attributed to a failure in the penetration of the label, but rather to the absence of specific antibody in the nuclear region.

When FT concentrations higher than 30 mg/ml were used, a large number of FT molecules were found attached to the cell membrane (Fig. 12). When cells incubated with the same concentration of label were washed three times, as usual, prior to fixation, all the FT was removed from the membranes; at the same time, the background of FT in the cytoplasm and nuclei was markedly reduced, but not completely abolished. Since under these conditions the specific labelling of the cisternae did not appear to be more intense than that obtained with the lower concentrations of label (3 to 6 mg/ml), with which the background is practically nil, the latter concentrations appeared more suitable for the labelling experiments.

DISCUSSION

The results of the present investigation, namely the detection and ultrastructural localization of antibodies in primitive blasts and subsequently in intermediate forms (plasmoblasts, immature plasma cells) with an increasingly more developed ER, confirm and extend previous light (references 9, 14, 23, 25, etc.) and electron microscopic data (*e.g.* references 3, 5, 16, 29) on the origin and differentiation of plasma cells. In particular the plasma cell precursor is identified with the blast, which is a cell with definite ultrastructural characteristics (see above, and references 11, 16, 29), which differentiate it clearly from the other lymph node cells and in particular from reticular cells. There is no evidence therefore in this study for a *direct* derivation of plasma cells from reticular cells. The statement on the role of blasts as plasma cell precursors does not necessarily imply that all lymph node cells morphologically recognizable as blasts evolve directly into plasma cells. Thus in the primary contact hypersensitivity reaction to 2-phenyl-4-ethoxy methylene-5-oxazolone, many blasts apparently identical with those described in the present experiments appear in the involved regional lymph nodes (13); there is evidence that

FIGURE 5 Detail of a 48-hour blast incubated with FT. The label is concentrated in the perinuclear space (upper right) and in two cisternae of the ER. The top cisterna is cut somewhat obliquely and has been partly deformed by an ice crystal. The remaining cytoplasm, filled with ribosomes grouped in clusters, is free of FT. Part of a tangentially cut mitochondrion is visible in the center left of the figure. Inset, low magnification of the cell from which the detail was taken. × 76,000; inset, × 5,500.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only a small proportion of these develop into plasma cells, while most of them transform into small lymphocytes (13, 49). In the secondary humoral antibody reaction with which we are dealing, however, the vast majority of blasts probably do evolve into plasma cells.

From their first appearance in the most immature cells of the plasmocytic line, antibody molecules are predominantly and perhaps exclusively concentrated inside the cisternae of the ER. These results are in agreement with the previously described localization of antibodies in the cisternae of the ER of fully developed plasma cells (12). Antibodies were first detected in 48-hour blasts in which the ER was represented only by few cisternal profiles. Although a certain number of blasts was already present in the 24-hour preparations, we did not observe a clear labelling of these cells. We do not know whether this failure is to be ascribed to the fact that the number of antibody molecules in the perinuclear space and cisternae (when present) was insufficient to produce insoluble antigen–antibody precipitates, or whether antibody synthesis had not in fact yet started at this stage. It is quite possible that blasts, which have a generation time of 10 to 12 hours (32), divide a couple of times before beginning to synthesize antibodies. In any case, all the evidence points to the fact that antibody synthesis begins *de novo* in cells having the ultrastructural characteristics of blasts. Moreover, the presence of antibodies in 48-hour blasts, which appear still quite primitive in the sense that their ER is represented only by a few cisternal profiles, indicates that the ER does not develop to a considerable extent before antibodies appear in it, but rather that antibody synthesis begins at an early stage in the development of the ER and proceeds parallel to its further extension.

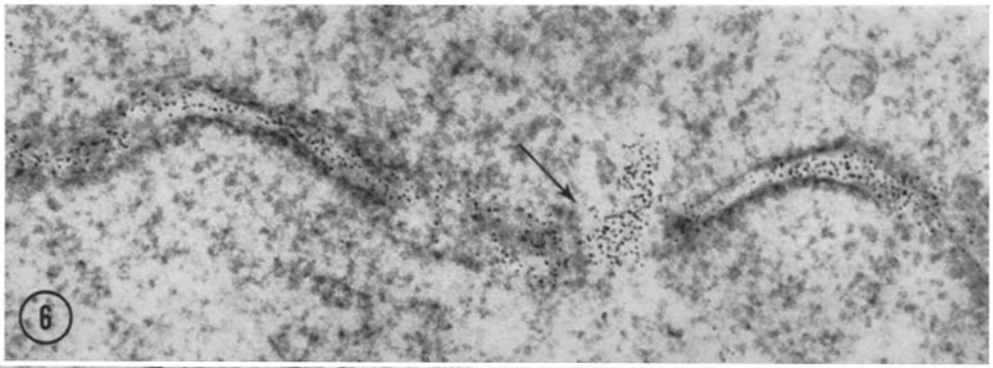
The concentration of intracisternal FT does not seem to vary markedly in the different stages of development of cells of the plasmocytic line (although it is higher in the more mature plasma cells with dilated cisternae). It is likely, therefore, that after an initial rise the intracisternal concentration of antibody also remains fairly constant, or increases slowly during the progressive development of the ER; it must be noted, however, that under out experimental conditions it is only possible to derive a lower limit for the antibody concentration and not the actual value (12). Probably a balance is eventually set up in the cells between antibody synthesis, antibody secretion, and development of the ER, resulting in a net increase in total antibody content, while the intracisternal concentration of antibody remains fairly constant.

The presence of antibodies in blasts and immature plasma cells, which are actively dividing cells, indicates that the characteristic function of the fully differentiated plasma cell, namely the production of antibodies (33, 34), is already present in cells which are not only poorly differentiated morphologically, but are probably still capable of division. This is confirmed by the direct detection of antibodies in cells in mitosis. In this particular cellular system, therefore, apparently no clear-cut separation exists between the stage of cell proliferation and that of cytodifferentiation. Of course we cannot state whether antibody synthesis is actually occurring during mitosis or only inbe-

FIGURE 6 Detail of the cytoplasm of a 60-hour blast incubated with FT. A long slender profile of the ER is labelled with FT. Note the high concentration of the label. The arrow points to a region of discontinuity in the cisternal membranes (probably produced by freezing), from which the antibody molecules have apparently diffused out. × 74,000.

FIGURE 7 Detail of a 48-hour blast incubated with FT. The cytoplasm is filled with ribosomes grouped in clusters. A flat cisterna runs parallel to the perinuclear space (and to the cell membrane not shown in the micrograph). FT is concentrated in the lumen of the cisterna and of the perinuclear space. A small cluster of FT molecules is visible in the ground cytoplasm (probably an artifact due to the escape of antibody molecules from disrupted membranes of the ER). × 63,000.

FIGURE 8 Detail of a 60-hour immature plasma cell, incubated with FT, showing a cisterna of the ER continuous with the perinuclear space. Antigen–antibody precipitate is localized exclusively within the cisterna and perinuclear space. × 69,000.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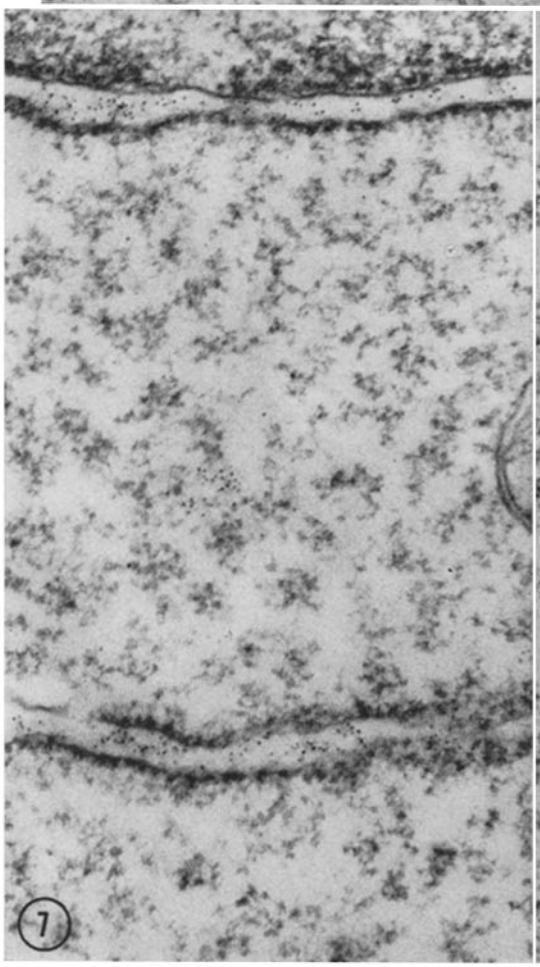
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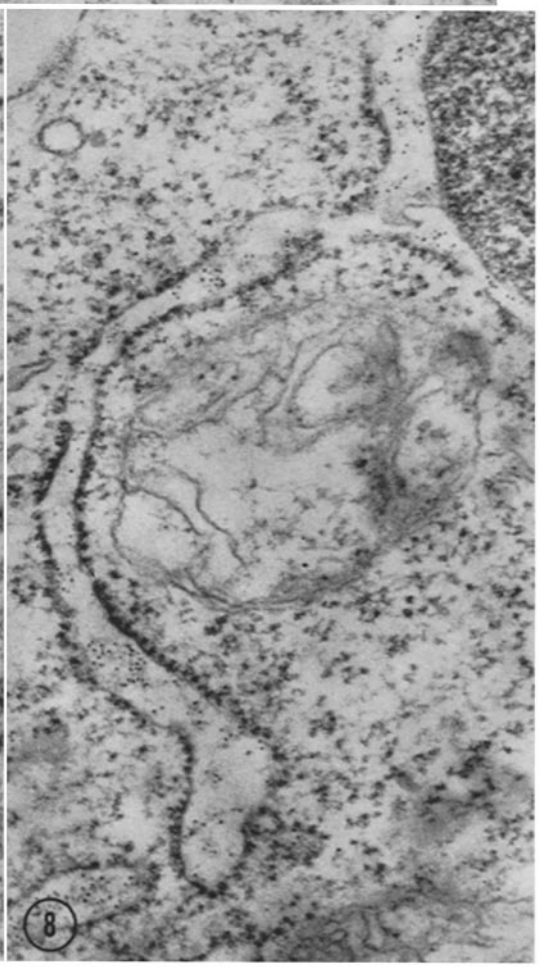
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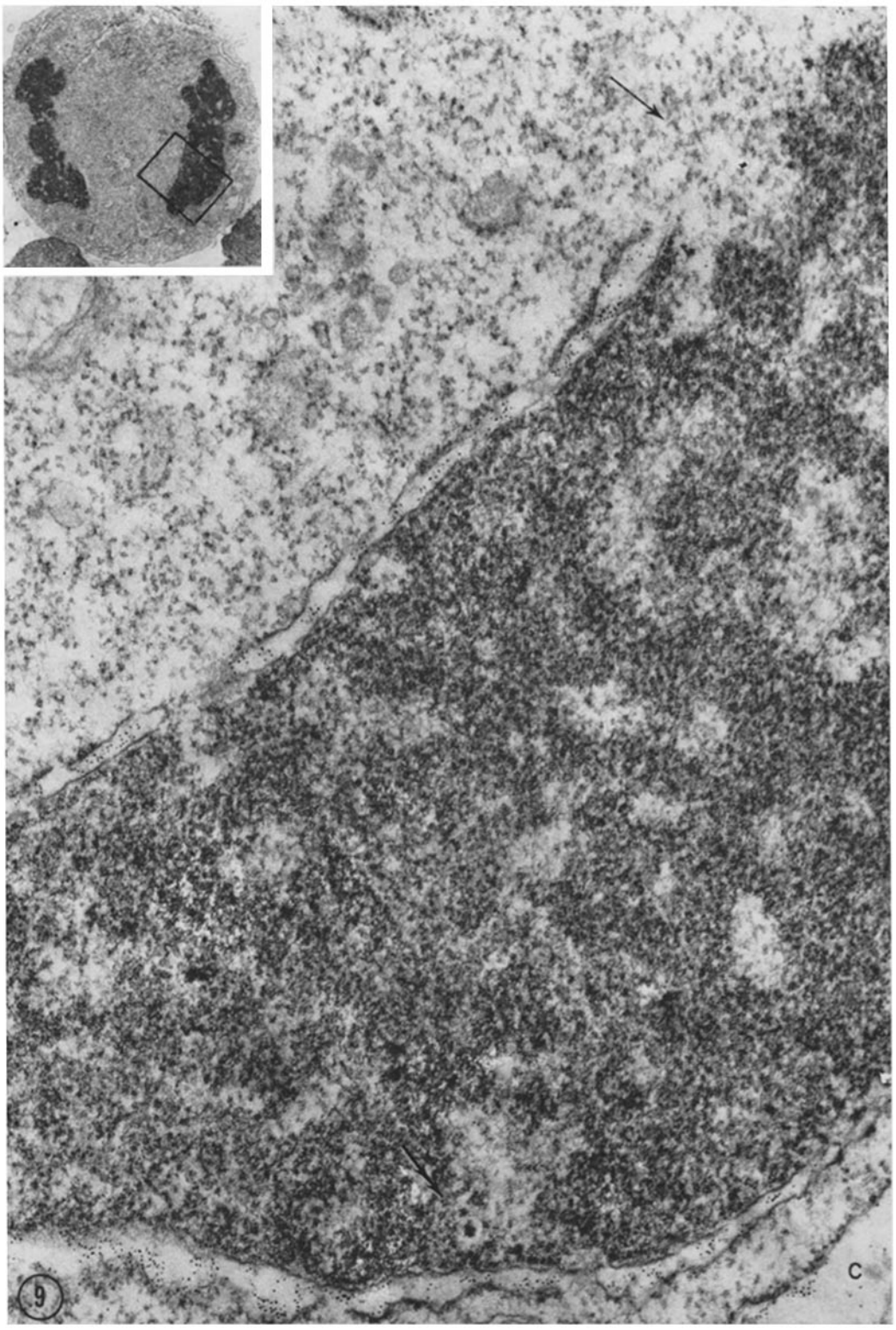
tween cell divisions. As in interphase cells, the antibodies in mitotic cells are always confined in the cisternae of the ER which probably derive both from the ER itself and from the original nuclear envelope (38). At the end of mitosis some of the antibody-containing cisternae apparently adhere once more to the nucleus, reforming the perinuclear space. These findings are in keeping with immunofluorescence investigations which have demonstrated the presence of antibodies in dividing cells (2, 44, 50, 51).

Where are antibodies actually synthesized? Unfortunately, no definite answer is available at present because our technique allows us to detect only complete (precipitating) molecules of antibody; we may therefore advance only some conjectures.

The association between protein synthesis and ribonucleic acid (RNA) (ribosomal, messenger, and transfer) is now well established. Both blasts and plasma cells are very rich in ribosomes: in blasts the ribosomes are for the greater part free in the cytoplasm where they are mostly grouped in clusters (usually of 4 to 10 ribosomes); some of the ribosomes, however, are associated with elements of the ER, and in this case they appear arranged on the membranes in distinct rows or spirals. In passing from blasts to immature and then mature plasma cells, the fraction of ribosomes associated with the membranes increases parallel to the increase in amount of the ER, and in the mature forms most ribosomes are membrane-bounded. Both the clusters of free ribosomes and the rows and spirals of the membrane-associated ones are believed to correspond to those groups of linearly arranged ribosomes, termed polyribosomes (polysomes), which appear to be the sites of protein synthesis (17, 52, 55). The different appearance in the two cases is probably only due to the presence of a supporting membrane which maintains the regular linear configuration of the membrane-bounded ribosomes, while such a support is lacking for the free ribosomes. Besides this, the more flexible configuration of the latter perhaps favours further aggregation during fixation and subsequent treatment. In any case, the grouping of ribosomes into clumps is not a mere technical artifact, because in this extreme form it is characteristic of blasts, while in other kinds of cells (*e.g.* small lymphocytes) most of the ribosomes, often present in comparable numbers per unit area, remain uniformly dispersed in the cytoplasm. These observations are difficult to reconcile with the density gradient analysis results of Stenzel *et al.* (46), who suggested that in antibody-synthesizing cells the ribosomes are present as monomers or dimers. Since the electron microscopic observations leave no doubt that the vast majority of ribosomes in the cells actually engaged in antibody synthesis are grouped in clusters of more than two ribosomes, analogous in appearance to the polyribosomes of reticulocytes and other cells (41, 43), further experiments on the isolation and characterization of antibody-synthesizing units seem to be desirable.

Nossal and Mitchell (34) have shown by radioactive labelling that blasts synthesize RNA actively: most of this newly synthesized RNA is relatively stable, but there is some evidence for the presence also of a fraction with a high turnover rate. On the contrary, in mature and immature plasma cells the synthesis of RNA is very limited: the bulk of the RNA in these cells would therefore seem to be practically stable. These data are in good agreement with the electron microscope observations. The high level of RNA synthesis in blasts may be correlated with the large amount of ribonucleoprotein both in the cytoplasm (polyribosomes) and in the nucleus (*e.g.* prominent nucleolus) of these cells. Even supposing that most of these ribonucleoproteins are relatively stable, it seems reasonable to suppose that considerable synthesis of these substances occurs in blasts, as they

FIGURE 9 Detail of a 60-hour blast in mitosis (telophase), incubated with FT, showing part of a reforming nucleus. The perinuclear space is still only partially surrounding the newly formed nucleus (in the top right a region of the nucleus is not yet enclosed by the nuclear membrane, arrow). Label is present in the incomplete perinuclear space and in a cisterna (*c*) of the ER just visible in the bottom right corner. A double arrow marks a "perichromatin granule" (53). Inset, low magnification of the whole cell. × 61,500; inset, × 4,000.


9
C

are actively dividing cells.[2] Moreover, since recent experiments (24) have shown that nuclear RNA synthesis takes place in the clear regions of loosely arranged chromatin, and not in regions of condensed chromatin, the general loose appearance of the nuclei of blasts and also of immature plasma cells would also be compatible with an active nuclear RNA synthesis in these cells. On the other hand, it seems reasonable to identify the stable RNA of plasma cells with the membrane-bounded ribosomes (and possibly with the corresponding messenger RNA), which increase in number in the more immature stages and apparently persist in differentiated plasma cells. Although both the free and the bound polyribosomes are probably associated with protein synthesis, it is likely that the stable RNA, namely the ribosomes associated with the membranes of the ER, are those actually involved in antibody (gamma globulin) synthesis. In fact the predominant ribosomal fraction in plasma cells, in which antibody synthesis is particularly active (33, 34), is the membrane-bounded fraction. On the contrary, in blasts, which are very rich in free polysomes, antibody synthesis is not very developed, as deduced from the fact that the total antibody content per cell is very low or nil in the most primitive forms of these cells (*i.e.* those with very scanty ER). The free polyribosomes of the actively dividing blasts may conceivably be associated with the synthesis of structural proteins, or proteins (*e.g.* enzymes) connected with the further differentiation of these antibody-producing cells.

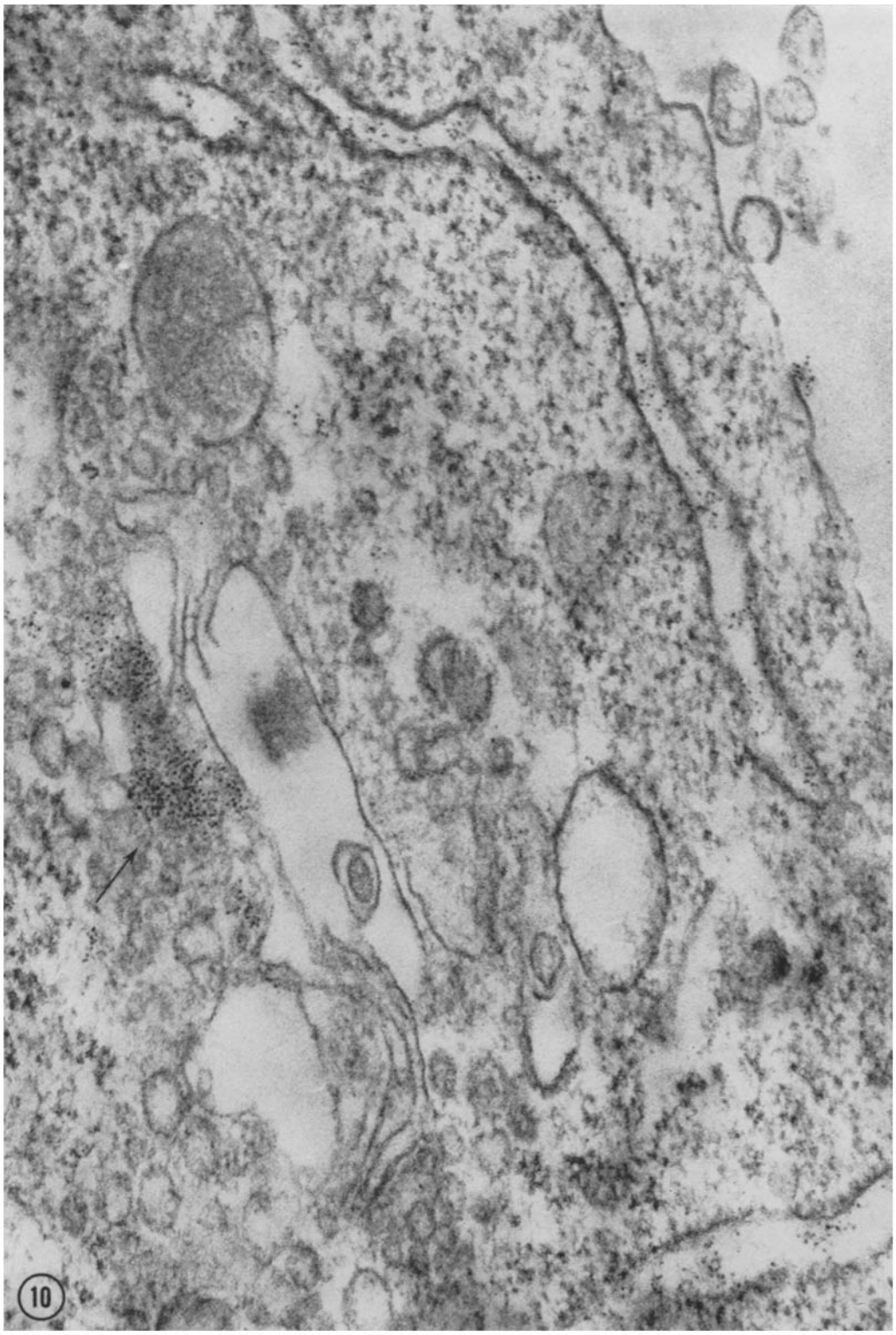
It may be noted that the hypothesis, that the antibody-synthesizing sites correspond to membrane-bounded ribosomes, accounts very well for the increase in such sites per cell suggested by some authors to explain some of their experimental results (*e.g.* references 21, 50). One cannot exclude, of course, the possibility that gamma globulin polypeptide chains (8) may be synthesized in the ground cytoplasm on the polyribosomes not bound to membranes and then transferred to the interior of the cisternae and here assembled into the complete molecules, capable of reacting with the antigen. We think it unlikely, however, that macromolecular polypeptide chains synthesized *at a distance* from the cisternae, and which have presumably already assumed a folded configuration, may be capable of penetrating the intact membranes of the ER. The present experiments, unfortunately, have yielded no definite evidence on this point, since FT molecules failed to label ribosomes; several possible explanations for this failure were advanced in the previous paper (12).

It is interesting to note that the maximum number of ribosomes in the polysomes associated with the membranes is at least 13 to 15. Shorter polysomes either may represent polysomal chains, which are actually shorter, or may be parts of larger polysomes divided by the plane of the section. If the correlation between number of ribosomes in single polysomes and length of synthesized polypeptide chains is valid also in this case (45, 52), at least some of the proteins synthesized by the membrane-bounded polysomes would have a molecular weight of up to about 50,000. These proteins could correspond to one of the polypeptide chains of the gamma globulin molecule (*e.g.* A chain, reference 8) or, alternatively, a single polysome could direct the synthesis of two or more shorter chains, as reported in other polysomal systems (40).

The present results confirm and extend the previous immunofluorescence data on the localization of and the progressive increase in concentration of antibody or gamma globulin in blasts and plasma cells (23, 35, 50, 51). Immunofluorescence observations cannot, however, yield sufficiently

[2] On the basis of morphological evidence it is difficult to judge the degree of stability of the messenger RNA of the polysomes: the observed increase in number of free, isolated ribosomes (as compared with ribosomes associated in polysomes) in blasts undergoing mitosis (11) may depend partly on the instability of this messenger RNA.

FIGURE 10 Detail of an immature plasma cell at 72 hours (plasmoblast, corresponding to the stage depicted in Fig. 3), incubated with FT, showing the Golgi area and part of two flattened cisternae marked with FT. A large FT-containing precipitate is visible in the Golgi area (arrow): it is not enclosed within recognizable membranes, but is situated in a zone apparently damaged by freezing (perhaps corresponding to disrupted vesicles) and adjacent to damaged vacuoles. × 89,000.


10

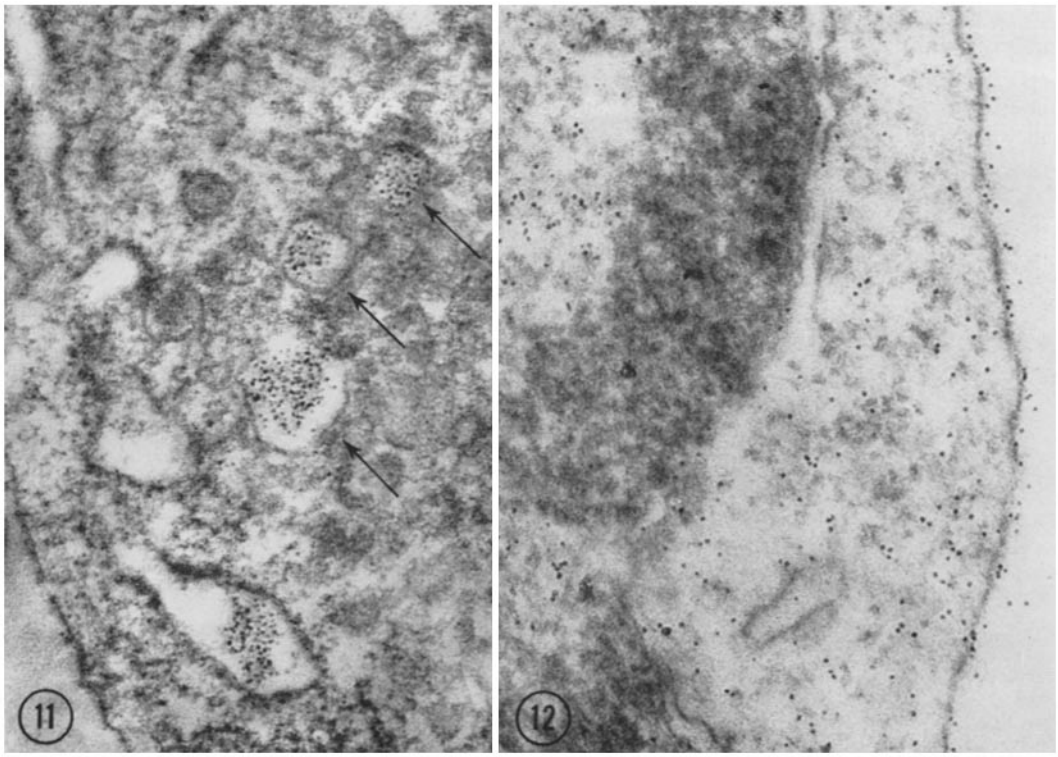


FIGURE 11 Detail of an immature plasma cell incubated with FT, showing a cisterna of the granular ER and part of the Golgi area. FT molecules are contained in the cisterna and in three vacuoles (arrows) of the Golgi complex, delimited by smooth membranes. × 77,000.

FIGURE 12 Detail of a control cell (small lymphocyte), incubated with about 40 mg/ml of FT and fixed without previous washings. FT molecules are fairly uniformly distributed throughout the cytoplasm and nucleoplasm (except for the areas of condensed chromatin). Several FT molecules are attached to the cell membrane. This section was not poststained. Compare, for example, the distribution of FT with that in Fig. 11, which refers to a cell, from an animal immunized with FT, labelled in the same experiment with only 3 mg/ml of FT and washed prior to fixation. × 95,000.

detailed information on the subcellular localization of the antibodies, because the appearance and distribution of fluorescent materials seem to be considerably affected by technical factors (20, 51). Gamma globulins are probably redistributed in the cell, especially if the tissues are frozen and thawed as is usually done. The redistribution of antibodies, the overlapping of images (due to the fact that the cisternae are not tubular elements but large flat structures), and the limited resolving power of the light microscope may account for the more or less uniform distribution of fluorescence observed, by several authors, not only in plasma cells but also in blasts. It should be noted, however, that Urso and Makinodan (50) have reported that in the early stages of the immune response the fluorescence is distributed in many of the cells in discrete "islands," while subsequently it becomes increasingly more diffuse. At any rate, in keeping with our observations, which have offered no evidence for a specific localization of FT in any particular nuclear region, the fluorescence has usually been found to be concentrated in the cytoplasm. Several authors did not find any fluorescence in the nucleus (35, 51), while others did find some (50, 56). Nuclear staining was attributed, by some workers, to technical artifacts (35, 51) (*e.g.*, redistribution and diffusion of gamma globulin molecules). Supporting evidence for this comes also from the recent experiments of

Hamashima, Harter, and Coons (20) on the localization of albumin and fibrinogen in liver cells, in which nuclear staining was present or absent depending on the previous treatment of the tissues. The detection of fluorescence in the nucleus need not always be an artifact: since, as indicated by our experiments, antibodies are always present in the perinuclear space, overlapping of images in the relatively thick sections used for immunofluorescence microscopy could well account for the nuclear staining. This is likely to occur especially in immature cells which present frequent nuclear indentations; in suitable sections, and even more so in whole cells observed in smears (50), the antibodies contained in the perinuclear space of the indented region could appear as discrete bright spots or islands on a dark background. Sainte Marie and Coons have recently reported the presence of a "fine bright fluorescent line" surrounding the nucleus of "large and medium immature plasmacytes" (see plate 89: Figs. 9, 10, and 12, reference 44); this may correspond to the perinuclear localization of antibodies observed by us in immature antibody-producing cells.

Another point having a bearing on our data, on which conflicting results were obtained by immunofluorescence, is the presence of antibodies (or gamma globulins) in the Golgi area of blasts and plasma cells. Most of the early authors reported an absence of staining in the perinuclear "halo" corresponding to this region (*e.g.* references 23, 35). Two recent studies have, however, demonstrated the presence of substantial amounts of gamma globulin in this area (44, 50), and in some cases, the fluorescence was even brighter than in the remaining cytoplasm. Once more the contradictory results probably arise from technical artifacts, but this time the critical factors are probably related to the greater or lesser degree of accessibility of the fluorescent molecules to the closed compartments of the Golgi region. If so, one would tend to accept as more reliable the more recent results, which suggest that antibody molecules *are* present in the Golgi area of immature and mature plasma cells. This conclusion is in agreement with the finding of abundant FT–anti–FT aggregates in the Golgi region of some cells of the plasmocytic line in the present investigation. These aggregates are believed to be true antigen–antibody precipitates since they were never present in the Golgi area of blasts or plasma cells of control sections and since they were found after incubation also in animals immunized with AFT. The fact that not all the cells with labelled ER contained FT also in their Golgi region may be attributed to technical factors, which in electron microscope experiments are probably even more critical than in immunofluorescence ones. The penetration of FT into the membrane-bounded compartments of a cell can only be accomplished if some discontinuities occur in the cell membranes (12). While this requirement is easily met in a largely interconnected system like the ER, it is more difficult to fulfill in the complex Golgi system, which consists of several distinct vacuoles. Breaks in the membranes may easily lead to extensive destruction of the vacuoles, thus increasing the difficulty of identifying the structures involved. Moreover, since the penetration of FT requires some time (however short it may be), some displacement of the antibody molecules is likely to occur (as is probably the case in the cell depicted in Fig. 10), although it is probably partially hindered by the existence of cross-linkages formed, during formalin fixation, between some of the antibody molecules (unpublished results). Nevertheless, in some cases FT molecules were found inside distinct vacuoles. For the above reasons, we believe that the conspicuous FT aggregates found in the Golgi region of some immature and mature plasma cells are not mere artifacts, but witness the presence of anti-FT antibodies in this region. This conclusion (although yet to be definitely proved) would be in line with the suggestion that the Golgi area is involved in the secretion of antibodies, in analogy to the role performed by this system in other secretory cells; *e.g.*, pancreatic cells (6) and fibroblasts (39). Purely morphological observations, which, carried out on control sections, demonstrate the presence in the Golgi area of vacuoles and vesicles containing flocculent material comparable in density to that present in the cisternae of the ER, and which provide evidence for exchanges of material between the cisternae and the Golgi system (11), would also support a secretory role for the Golgi complex.

The examination of control (not incubated) sections failed to reveal any substantial amount of *antigen* (FT) molecules in blasts and immature plasma cells, in agreement with previous observations on mature plasma cells (12, 37). In only an occasional cell of the plasmocytic line did we find a few small dots interpretable as FT molecules,

while in most of the cells the presence of the antigen could be excluded. These findings appear at variance with the electron microscopic observations of Wellensiek and Coons (54) and the radioautographic data of Roberts and Haurowitz (42). They agree, on the contrary, with the reports of Nossal and coworkers (1, 31), who found no evident labelling of blasts and plasma cells by radioactive antigens. It is not known whether heavier labelling with FT could be obtained using more massive amounts of antigen for immunization. Dilution of antigen by cell division could account for its absence from plasma cells, but hardly from 24- and 48-hour blasts. To evaluate the significance of these negative findings, one should bear in mind that the identification of the isolated FT molecules may be uncertain and also that the contents of a single cell are distributed over 100 to 200 thin sections. We estimated that at least 3 to 5 molecules of FT must be visible per section to ensure the detection of FT in the cell; this means that the lower limit for the detection of FT, supposedly uniformly distributed, is about 1000 molecules per cell. The present data indicate, therefore, that in most blasts and plasma cells there were probably less than 1000 *intact* FT molecules. On account of the large number of random sections photographed and examined, it is quite likely that the actual number was substantially lower (compare with the values of Ada and Nossal, reference 1). The presence of *fragments* of FT molecules, invisible with the electron microscope, cannot however be excluded. Unlike blasts and plasma cells, the reticular cells and macrophages of our preparations often contained relatively large amounts of FT, either enclosed within vacuoles or free in the cytoplasm (11, 37). All the above findings favour the view, supported also by other authors (5, 22, 23), that blasts (and hence plasma cells) are not derived from those cells which have taken up *most* of the antigen.

We are greatly indebted to Dr. Luisa Amante for preparing the apoferritin and purified ferritin. We also wish to express our appreciation to Professor Pernis for many helpful discussions.

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Note added in proof: Since the manuscript was submitted for publication, M. D. Scharf and J. W. Uhr, *Science*, 1965, **148**, 646, have reported the results of density gradient experiments demonstrating the presence of polyribosomes composed of eight and more ribosomes in lymph node cells of immunized rabbits. These results are at variance with those of Stenzel *et al.* (46), but quite in agreement with our electron microscope observations.

BIBLIOGRAPHY

1. ADA, G. L., and NOSSAL, G. J. V., Biochemical aspects of the role of antigen in the induction of antibody formation, *in* 6th. International Congress of Biochemistry, Abstracts, New York, 1964, **32**, II-1.
2. BANEY, R. N., VAZQUEZ, J. J., and DIXON, F. J., Cellular proliferation in relation to antibody synthesis, *Proc. Soc. Exp. Biol. and Med.*, 1962, **109**, 1.
3. BERNHARD, W., and GRANBOULAN, N., Ultrastructure of immunologically competent cells, *Ciba Found. Symp., Cellular Aspects Immunity*, 1960, 92.
4. BRAUNSTEINER, H., FELLINGER, K., and PAKESCH, F., Demonstration of a cytoplasmic structure in plasma cells, *Blood*, 1953, **8**, 916.
5. BRAUNSTEINER, H., and PAKESCH, F., Electron microscopy and the functional significance of a new cellular structure in plasmocytes. A review, *Blood*, 1955, **10**, 650.
6. CARO, L. G., and PALADE, G. E., Protein synthesis, storage and discharge in the pancreatic exocrine cell. An autoradiographic study, *J. Cell. Biol.* 1964, **20**, 473.
7. CASLEY-SMITH, J. R., The displacement of ferritin molecules by the microtome knife, *J. Micr.*, 1962, **1**, 335.
8. COHEN, S., and PORTER, R. R., Structure and biological activity of immunoglobulins, *Adv. Immunol.*, 1964, **4**, 287.
9. CONGDON, C. C., and MAKINODAN, T., Splenic white pulp alteration after antigenic injection: relation to time of serum antibody production, *Am. J. Path.* 1961, **39**, 697.
10. DE PETRIS, S., and KARLSBAD, G., Localization of antibodies in developing plasma cells, *in* Electron Microscopy, Proceedings of the 3rd European Regional Conference, Prague, 1964, (M. Titlbach, editor), Prague, Publishing House of the Czechoslovak Academy of

Sciences, 1964, **B,** 219. Ulteriori osservazioni sulle cellule produttrici di anticorpi, *in* Atti del 4th Congresso Italiano di Microscopia Elettronica, Padova, 1963, (G. Lanzavecchia and V. Meneghelli, editors), Società Italiana di Microscopic Elettronica, 1964, 33.

11. DE PETRIS, S., and KARLSBAD, G., material in preparation.
12. DE PETRIS, S., KARLSBAD, G., and PERNIS, B., Localization of antibodies in plasma cells by electron microscopy, *J. Exp. Med.*, 1963, **117,** 849.
13. DE PETRIS, S., KARLSBAD, G., PERNIS, B., and TURK, J. L., Ultrastructure of cells present in lymph nodes during the development of contract sensitivity, *Internat. Arch. Allergy Appl. Immunol.*, in press.
14. FAGRAEUS, A., Antibody production in relation to the development of plasma cells: in vivo and in vitro experiments, *Acta med. Scand., Suppl.*, 1948, **204,** 1.
15. FAGRAEUS, A., Nomenclature of immunologically competent cells, *Ciba Found. Symp., Cellular Aspects Immunity*, 1960, 3.
16. FERNANDO, V. P., and MOVAT, H. Z., Maturation of plasma cells after antigenic stimulation, *in* Proceedings of the 5th International Congress for Electron Microscopy, Philadelphia, 1962, (S. S. Breese, Jr., editor), New York, Academic Press, Inc., 1962, **2,** TT-10.
17. GIERER, A., Function of aggregated reticulocyte ribosomes in protein synthesis, *J. Mol. Biol.*, 1963, **6,** 148.
18. GOWANS, J. L., MCGREGOR, D. D., COWEN, D. M., and FORD, C. E., Initiation of immune responses by small lymphocytes, *Nature*, 1962, **196,** 651.
19. GRANICK, S., and MICHAELIS, L. J., Ferritin. II. Apoferritin of horse spleen, *J. Biol. Chem.*, 1943, **147,** 91.
20. HAMASHIMA, Y., HARTER, J. G., and COONS, A. H., The localization of albumin and fibrinogen in human liver cells, *J. Cell. Biol.*, 1964, **20,** 271.
21. INGRAHAM, J. S., Dynamic aspects of the formation of serum antibody in rabbits. Exponential and arithmetic phases in the rise of titre following a reinjection of sulphanilazo bovine-γ-globulin, *J. Immunol.*, 1964, **92,** 208.
22. LA VIA, M. F., FITCH, F. W., GUNDERSON, C. H., and WISSLER, R. W., The relation of antibody formation to reticuloendothelial structure and function, *in* Reticuloendothelial Structure and Function (J. H. Heller, editor), New York, The Ronald Press Company, 1960, 45.
23. LEDUC, E. H., COONS, A. H., and CONNOLLY, J. M., Studies on antibody production. II. The primary and secondary responses in the popliteal lymph node of the rabbit, *J. Exp. Med.*, 1955, **102,** 61.
24. LITTAU, V. C., ALLFREY, V. G., FRENSTER, J. H., and MIRSKY, A. E., Active and inactive regions of nuclear chromatin as revealed by electron microscope autoradiography, *Proc. Nat. Acad. Sc.*, 1964, **52,** 93.
25. MARSHALL, A. H. E., and WHITE, R. G., Reactions of the reticular tissues to antigens, *Brit. J. Exp. Path.*, 1950, **31,** 157.
26. MAZUR, A., and SHORR, E., A quantitative immunochemical study of ferritin and its relation to the hepatic vasodepressor material, *J. Biol. Chem.*, 1950, **182,** 607.
27. MERCER, E. H., and BIRBECK, M. S. C., Electron Microscopy, Oxford, Blackwell Scientific Publications, 1961, 63.
28. MILLONIG, G., The advantages of a phosphate buffer for OsO_4 solutions in fixation, *J. Appl. Physics*, 1961, **32,** 1637.
29. MOVAT, H. Z., Discussion, *in* Immunopathology, 3rd. International Symposium, La Jolla, 1963, (P. Grabar and P. A. Miescher, editors), Basel/Stuttgart, Schwabe and Co., 1963, 144.
30. MOVAT, H. Z., and FERNANDO, V. P., The fine structure of connective tissue. II. The plasma cell, *Exptl. and Mol. Path.*, 1962, **1,** 535.
31. NOSSAL, G. J. V., ADA, G. L., and AUSTIN, C. M., Behaviour of active bacterial antigens during the induction of the immune response. II. Cellular distribution of flagellar antigens labelled with Iodine-131, *Nature*, 1963, **199,** 125.
32. NOSSAL, G. J. V., and MÄKELÄ, O., Autoradiographic studies on the immune response. I. The kinetics of plasma cell proliferation, *J. Exp. Med.*, 1962, **115,** 209.
33. NOSSAL, G. J. V., and MÄKELÄ, O., Elaboration of antibodies by single cells, *Ann. Rev. Microbiol.*, 1962, **16,** 53.
34. NOSSAL, G. J. V., and MITCHELL, J., The nature of RNA synthesis in immune induction, *in* Immunopathology, 3rd International Symposium, La Jolla, 1963, (P. Grabar and P. A. Miescher, editors), Basel/Stuttgart, Schwabe and Co., 1963, 113.
35. ORTEGA, L. G., and MELLORS, R. C., Cellular sites of formation of gamma-globulin, *J. Exp. Med.*, 1957, **106,** 627.
36. PALADE, G. E., A study of fixation for electron microscopy, *J. Exp. Med.*, 1952, **95,** 285.
37. PERNIS, B., DE PETRIS, S., KARLSBAD, G., and BAIRATI, A. JR., unpublished results.
38. PORTER, K. R., and MACHADO, R. D., Studies on the endoplasmic reticulum. IV. Its form and distribution during mitosis in cells of onion root tip, *J. Biophysic. and Biochem. Cytol.*, 1960, **7,** 167.

39. REVEL, J. P., and HAY, E. L., An autoradiographic and electron microscopic study of collagen synthesis in differentiating cartilage, *Z. Zellforsch. u. Mikr. Anat.*, 1963, **61,** 110.
40. RICH, A., PENMAN, S., BECKER, Y., DARNELL, J. E., and HALL, C. E., Polyribosomes: size in normal and polio-infected HeLa cells, *Science*, 1963, **142,** 1658.
41. RIFKIND, R. A., DANON, D., and MARKS, P. A., Alterations in polyribosomes during erythroid cell maturation, *J. Cell. Biol.*, 1964, **22,** 599.
42. ROBERTS, A. N., and HAUROWITZ, F., Intracellular localization and quantitation of tritiated antigens in reticuloendothelial tissues of mice during secondary and hyperimmune responses, *J. Med. Exp.*, 1962, **116,** 407.
43. ROSS, R., and BENDITT, E. P., Wound healing and collagen formation. IV. Distortion of ribosomal patterns of fibroblasts in scurvy, *J. Cell Biol.*, 1964, **22,** 365.
44. SAINTE MARIE, G., and COONS, A. H., Studies on antibody production. X. Mode of formation of plasmocytes in cell transfer experiments, *J. Exp. Med.*, 1964, **119,** 743.
45. STAEHELIN, T., WETTSTEIN, F. O., OURA, H., and NOLL, H., Determination of the coding ratio based on molecular weight of messenger ribonucleic acid associated with ergosomes of different aggregate size, *Nature*, 1964, **201,** 264.
46. STENZEL, K. H., PHILLIPS, W. D., THOMSON, D. D., and RUBIN, A. L., Functional ribosomes in antibody producing cells, *Proc. Nat. Acad. Sc.*, 1964, **51,** 636.
47. THIERY, J. P., Microcinematographic contributions to the study of plasma cells, *Ciba Found. Symp., Cellular Aspects Immunity*, 1960, 59.
48. TROWELL, O. A., The lymphocyte, *Internat. Rev. Cytol.*, 1958, **7,** 235.
49. TURK, J. L., and STONE, S. H., Implications of the cellular changes in lymph nodes during the development and inhibition of delayed type hypersensitivity, *in* Cell-bound Antibodies, (B. Amos and H. Koprowski, editors), Philadelphia, Wistar Institute Press, 1963, 15.
50. URSO, P., and MAKINODAN, T., The roles of cellular division and maturation in the formation of precipitating antibody, *J. Immunol.*, 1963, **90,** 897.
51. VAZQUEZ, J. J., Antibody- or gamma globulin-forming cells, as observed by the fluorescent antibody technic, *Lab. Inv.*, 1961, **10,** 1110.
52. WARNER, J. N., KNOPF, P. M., and RICH, A., A A multiple ribosomal structure in protein synthesis, *Proc. Nat. Acad. Sc.*, 1963, **49,** 122.
53. WATSON, M. L., Observations on a granule associated with chromatin in the nuclei of cells of rat and mouse, *J. Cell. Biol.*, 1962, **13,** 162.
54. WELLENSIEK, H.-J., and COONS, A. H., Studies on antibody production, IX. The cellular localization of antigen molecules (ferritin) in the secondary response, *J. Exp. Med.*, 1964, **119,** 685.
55. WETTSTEIN, O., STAEHELIN, T., and NOLL, H., Ribosomal aggregate engaged in protein synthesis: characterization of the ergosome, *Nature*, 1963, **197,** 430.
56. WHITE, R. G., COONS, A. H., and CONNOLLY, J. M., Studies on antibody production. III. The alum granuloma, *J. Exp. Med.*, 1955, **102,** 73.