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Cellular Differentiation and Antibody Localization During the Primary Immune Response in Peroxidase Stimulated Lymph Nodes of Rat

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The development of antihorseradish peroxidase-producing cells was studied by electron microscopic immunocytochemistry in rat popliteal lymph node after a single immunization of antigen with complete Freund's adjuvant. Specific antibody synthesis could be observed during the maturation stages of two morphologically distinct cell types from the blast cell to the ergastoplasm-rich cell stage of either lymphocyte-like or reticulum-like cells. The first antihorseradish peroxidase antibodies were localized on Day 5 after immunization in some ergastoplasmic cisternae of mature plasma cells but not in blast cells. Specific antibody could be localized in typical blast cells from Day 6. In earlier days of the immune response blast cells occurred in increased numbers; however, antihorseradish peroxidase antibodies could not be localized with the methods employed.

INTRODUCTION

Many investigations on the cellular basis of humoral antibody production at both light and electron microscopic levels have been published. Reconstructions of events from the time of immunization to the recognition of specific antibody within the cells at the ultrastructural level have been undertaken by several authors utilizing either the ferritin–antiferritin method (1, 2), the plaque forming and rosette-forming tests (3–6) or the enzyme–antienzyme techniques (7–12). However, from where plasma cells arise and develop is still an uncertainty. In two previous communications (9, 12) we have shown that plasma cells may develop from both tissue fixed and not, circulating cells of the lymphoreticular system.

The present work, utilizing the procedures for visualizing specific intracellular antibodies by an improved immunoenzyme technique (13), was initiated to explore the fine structure and cytologic development of antibody-producing cells in the popliteal lymph nodes of rat during a long period after a single antigen injection, i.e., horseradish peroxidase.

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MATERIALS AND METHODS

Animals. Five-month-old female WAG rats were used.

Antigens. Horseradish peroxidase RZ 3 was purchased from Sigma Chemical Co., St. Louis, MO; and from C. F. Boehringer, Mannheim, Germany. Bovine serum albumin was obtained from Povite, Amsterdam.

Immunization schedule. Peroxidase, 1 mg, dissolved in 0.2 ml saline, was dispersed in 0.2 ml complete Freund's adjuvant (Difco Lab., Detroit, USA) and injected into both hind footpads of each rat. The animals were bled and killed 3 (8),[3] 4 (8), 5 (8), 6 (10), 7 (9), 8 (9), 10 (10), 12 (8), 13 (8), 15 (12), 18 (7), 21 (8), 30 (7), 35 (6) days and 8 (3) months after a single injection of antigen. Thus, a total of 121 rats was used for revealing antibody to HRP. Six rats immunized with BSA and six rats immunized with glucose oxidase served as controls.

Circulating antibodies. Characterization and measurement of circulating antibodies was carried out by enzymo-immunoelectrophoresis and passive hemagglutination, respectively (9). Rat antiperioxidase antibody was specifically isolated by passage of the antiserum on an insoluble peroxidase immunoadsorbent (14).

Antisera or specifically isolated antibodies were examined for their capacity to inhibit the catalytic activity of peroxidase. Increasing amounts of antisera or antibodies were added to a standard amount of peroxidase (2 μg). After incubation at room temperature for 2 hr, the reaction mixture was measured for remaining peroxidase activity, using *o*-dianisidine as the chromogenic substance, according to a published procedure (15).

Light microscopy. Cell suspensions were prepared and treated in two ways: (1) cells were smeared on slides, fixed, incubated in peroxidase, stained for peroxidase activity and then positive cells were counted, as previously described (9). (2) suspensions were centrifuged for 5 min at 800*g*. The cell pellet was resuspended and fixed in 4% freshly prepared formaldehyde (Merck, Germany) in 0.2 *M* cacodylate buffer pH 7.2 for 30 min at 4°. The cells were then repeatedly washed in the same buffer for 1 hr, incubated with 100 μg/ml peroxidase for 2 hr at 4°, washed and finally stained for peroxidase in Graham and Karnovsky's medium (16). The suspended cells were deposited on a microscope slide, covered with a cover slip, examined and counted in the light microscope.

Electron microscopy. The following procedures for tissue conservation and for immunocytochemical reactions, were adopted after a study to define their optimal conditions (13). Both popliteal lymph nodes of each rat were taken at a given day and immediately fixed in 4% formaldehyde solution, freshly prepared from paraformaldehyde, in 0.2 *M* cacodylate buffer at pH 7.2. The tissues were cut with razor blades either into blocks of 1.5–2 mm or into slides 2–3-mm thick in a drop of fixative, then transferred to a vial with fixative and kept at 4° for 24 hr on an agitator. Subsequently, the fixative was removed by several changes of buffer for at least 24 hr. In some cases, tissue blocks of 1 mm^3 were fixed in 2.5% glutaraldehyde in cacodylate buffer for 1.5 hr at 4°. Two rats (10 days after the antigen injection) were fixed by cardial perfusion (17) with 4% formaldehyde for 1 hr.

[3] Number in parentheses denotes the number of animals examined on each day.

Then, the popliteal lymph nodes were removed, cut into slices and fixed for a further 23 hr in the same fixative. Prior to treatment with antigen, the tissue blocks were reduced to small fragments with a razor blade. From the larger slices, frozen sections were cut at 40-μ in a cryostat (Dittes—Duspiva) as described elsewhere (13). All immunocytochemical reactions were carried out at room temperature: (a) incubation with horseradish peroxidase, 1 mg/ml cacodylate buffer for 24 hr; (b) washing, 3 × 10 min in buffer; (c) revealing of peroxidase activity by incubation for 25 min in the substrate medium of Graham and Karnovsky (16), followed by two washes of 2 min in buffer; (d) postfixation in 2% OsO_4 in cacodylate buffer for 1 hr.

All control tissues were prefixed as described above, incubated in the respective reagent media, then postfixed in OsO_4. The specificities of the reactions were examined on lymph nodes, stimulated with peroxidase, by staining with the substrate medium, but without prior incubation of the tissue with peroxidase. Furthermore, lymph nodes, stimulated by glucose oxidase or BSA, were incubated with peroxidase and then revealed with the medium of Graham and Karnovsky. Some tissue fragments were directly postfixed in OsO_4 without any other incubation procedure.

After postfixation, the tissues were dehydrated in ascending alcohol and embedded in Epon (18).

The flat embedded 40-μ frozen sections were examined by light microscopy in order to select appropriate areas of medulla or cortex for ultrastructural examination. The chosen regions were traced with a needle and the corresponding surfaces were trimmed using a Leitz stereoscope at × 160 magnification. For each day of the immune response a minimal number of ten different regions containing specific antibody-producing cells were processed for electron microscopy. Up to Day 7, when the determination of positive cells was difficult by light microscopy, 15–20 different foci of the medullary regions and equal numbers of the cortex were examined for each day.

Embedded tissue fragments and 40-μ frozen sections were cut with a Sorvall MT-1 ultramicrotome and mounted on Formvar and carbon-coated 200 and 300 mesh copper grids. Sufficient sections were cut in order to examine grids: (a) without staining, (b) after staining with lead citrate (19) for 30 sec, and (c) after double staining with uranyl acetate and lead citrate.

To examine the cells, present in lymph node suspensions, six popliteal lymph nodes (15 days after antigen injection) were dissected and cell suspensions prepared as previously described (9), fixed in 4% formaldehyde for 6 hr, washed, incubated with peroxidase, rewashed and subsequently incubated with the appropriate substrate followed by postfixation in buffered OsO_4 as described above. During all steps, the cells were resuspended and centrifuged at 1000 rpm for 3–5 min. From the centrifuged and embedded cell suspensions semithin and ultrathin sections were cut alternatively in order to examine the cells from the whole processed lymphoid samples.

The sections were examined with a Siemens Elmiskop I, operating at 80 kV with 50-μ objective apertures. Semithin sections from all blocks were cut and stained by the methylene blue–Azure II method (20) and examined with the light microscope.

RESULTS

Circulating Antibodies

Traces of circulating antibodies were observed on Day 8 after antigen injection which increased exponentially until Day 21. From Day 21 up to Day 35 no further significant increase of the antibody titer was observed. Enzymo-immunoelectrophoresis revealed in all antisera the presence of antibodies of the Ig_G class (Ig_{G1} and Ig_{G2}). In some sera the presence of small amounts of Ig_M antibodies were also detected.

The inhibition tests for the catalytic activity of peroxidase have shown that in order to have a 10% inhibition of the catalytic activity, the presence of 15 molecules of antibody was needed for 1 molecule of peroxidase, and that even at a very high antibody excess (60 molecules of antibody) the inhibition was never greater than 20%.

Light Microscopy

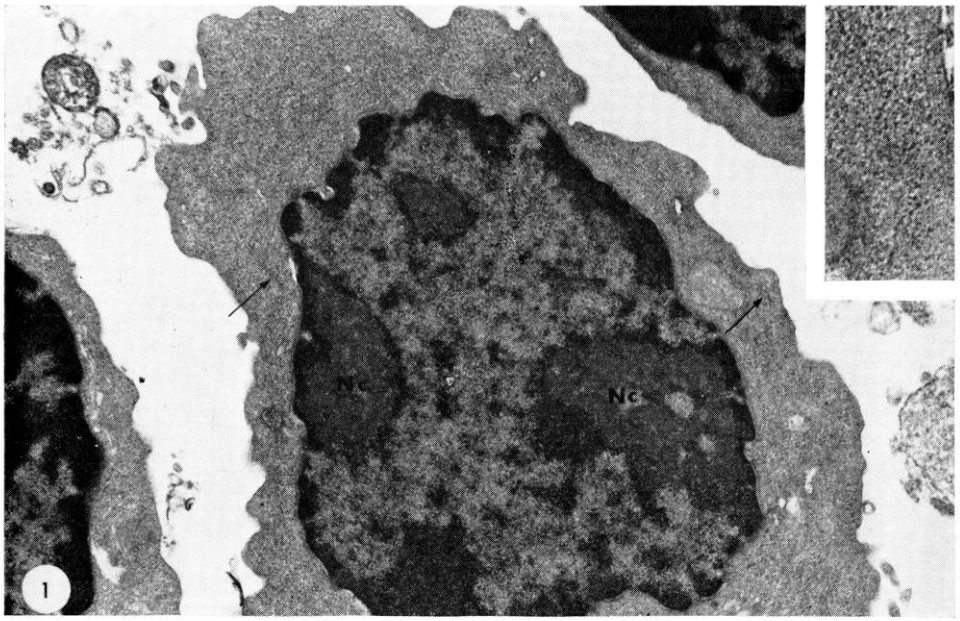
Five days after the antigen administration, 4/per thousand positive cells were found, and their number increased exponentially until Day 16 when 26/per thousand positive cells were counted. From Day 16 to Day 35 the number of positive cells remained unchanged.

Electron Microscopy

Cytological observations on lymph node cells. The classification of lymphoid cells as small or large lymphocyte, blast cell, plasma cell and reticulum cell used in the present paper is based on the morphological criteria described by Bernhard and Leplus (21) and Mori and Lennert (22) in their respective atlases on lymphoid tissues.

In the present study all cells with a lymphocytic aspect were called *dense cell-type*, and those of the reticulum cell-type were called *clear cell-type*. Throughout differentiation we observed continuous pathways of maturation for both cell lines. Cellular filiations of the dense cell-types could always be established without difficulty because of the natural contrast of the cytoplasm and the nucleus and of the good preservation of the cytoplasmic membranes. Furthermore, the cytoplasm always contained many ribosomes, typical of the lymphocytic series. In contrary to the dense cell-type, reticulum cells and their differentiation products showed poor fixation of cytoplasmic membranes, thus cellular filiations were often difficult to appreciate whether after fixation in formaldehyde by immersion or perfusion, or after fixation in glutaraldehyde. In cell suspensions we failed to demonstrate intact clear cell-types. The natural contrast of reticulum cell-types was very weak and during all maturation stages we observed, as a typical feature, grouped ribosomes which became membrane bound, thus "clearing" the cytoplasm.

(a) *The dense cell-type.* After antigenic stimulation we observed, apart from *small, medium* and *large lymphocytes* (Fig. 1), lymphocytic cells of increased size, with the chromatin reduced to irregular patches and one or two reticular nucleoli. At this stage, formation of long, flat ergastoplasmic lamellae could be seen. Thus, the large lymphocyte already exhibited a blastoid character (Fig. 2). Further formation of the ergastoplasmic system indicated the *blast cell* stage (Fig. 3). Between plasmoblasts, young plasma cells and mature *plasma cells* (Figs. 4–6) a



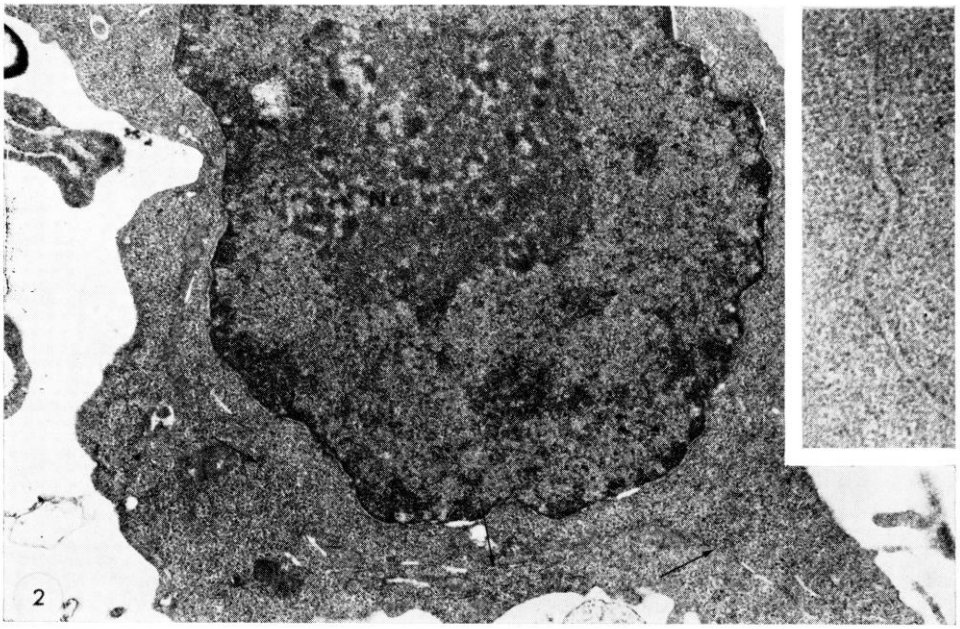


FIG. 1. Large dense lymphocyte in progressive development. Enlarged nucleoli (Nc) can be seen in the cleared interchromatinic area. The well-developed cytoplasm contains few scanty ergastoplasmic lamellae (→) and many free ribosomes. Five days after immunization, formaldehyde fixation, 40-μ frozen section. Uranyl acetate and lead citrate staining. ×12,650. Inset: High power view of free cytoplasmic ribosomes. ×40,000.

FIG. 2. Large dense lymphocyte in blast transformation. Small rim of chromatin along the nuclear membrane. In the clearer interchromatinic area a very large nucleolus (Nc) can be seen. The large cytoplasm contains somewhat more, but still flat ergastoplasmic lamellae (→). Five days after immunization, formaldehyde fixation, 40-μ frozen section. Uranyl acetate and lead citrate staining. ×10,700. Inset: High magnification of a flat ergastoplasmic lamella with ribosomes bound on its membranes. ×34,000.

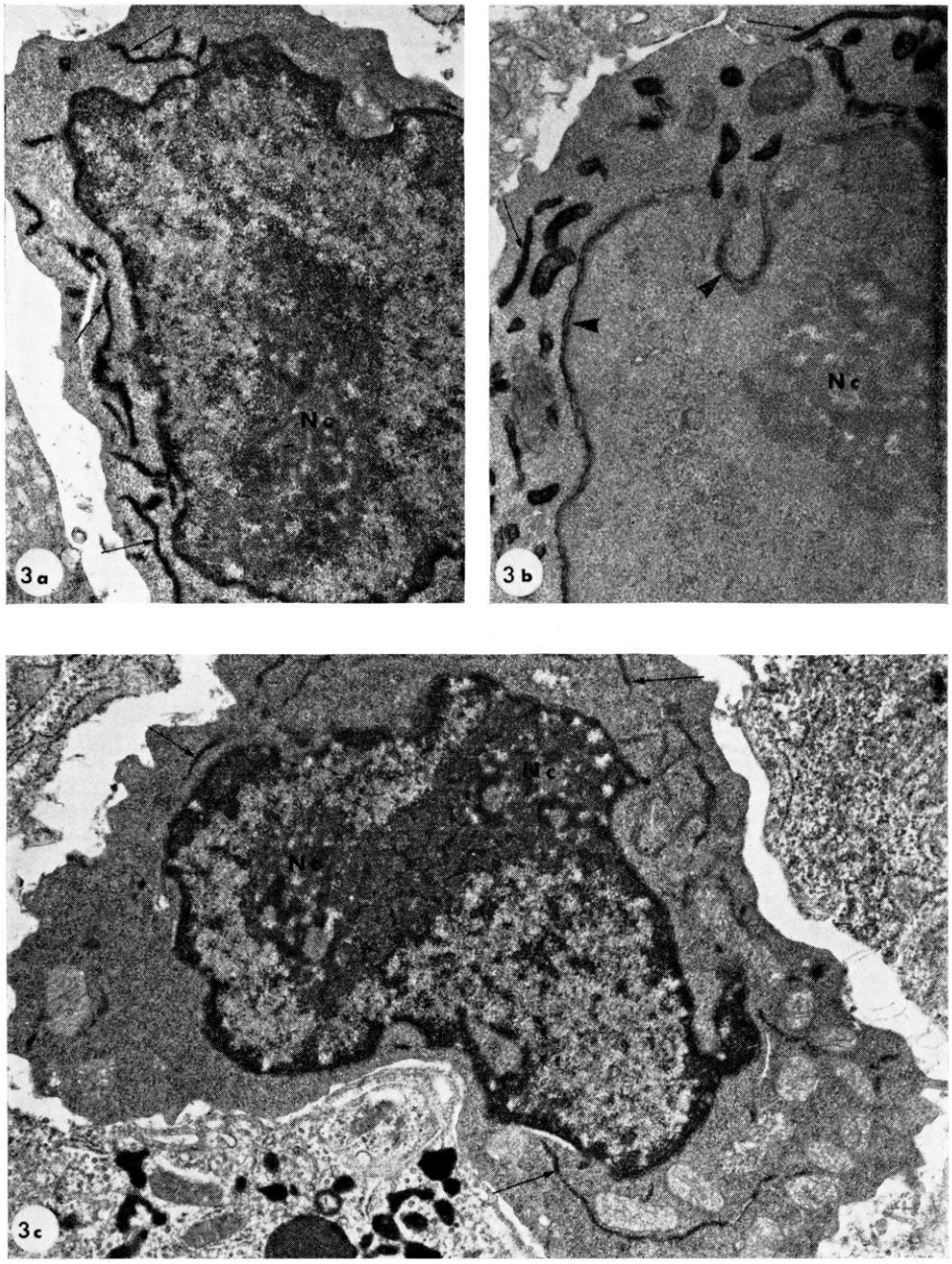


FIG. 3. Typical lymphocytic blast cells (dense type) synthesizing antihorseradish peroxidase antibody in the few flat ergastoplasmic lamellae (→) and the PNS (►). The nuclei are very irregularly shaped and contain well developed nucleoli (Nc). Condensed chromatin can be seen along the nuclear envelope. (a) 6 days after immunization, formaldehyde fixation, 40-μ frozen section. Uranyl acetate and lead citrate staining. ×11,700. (b) 10 days after immunization, fixation by perfusion (1 hr) and immersion (23 hr) in formaldehyde, 40-μ frozen section. Lead citrate 30 sec. ×17,300. (c) 15 days after immunization, glutaraldehyde fixation, 40-μ frozen section. Uranyl acetate and lead citrate staining. ×10,400.

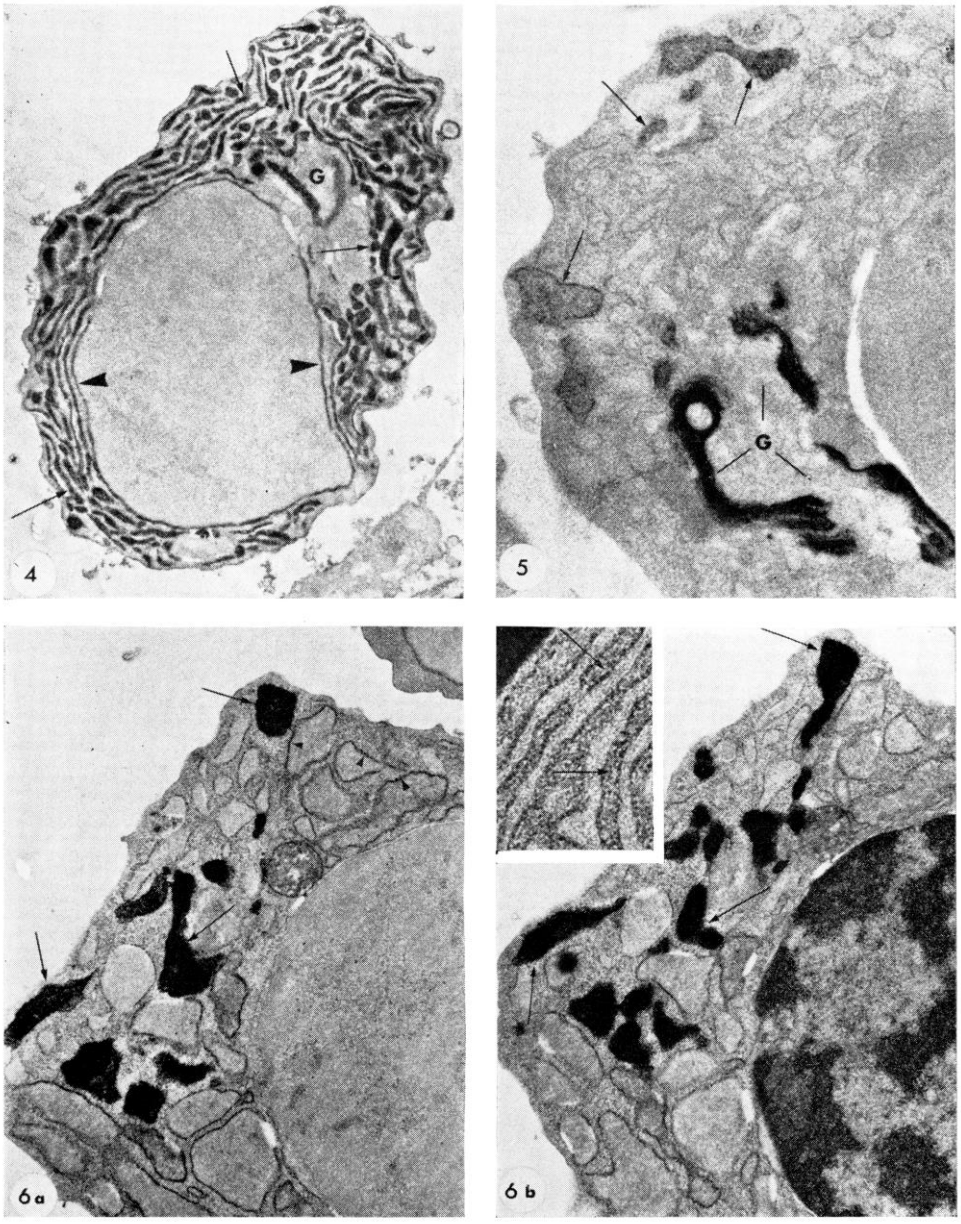


FIG. 4. Mature plasma cell (dense type). Note specific antibody within RER (→) and PNS (►); the Golgi complex (G) contains also anti-HRP antibody. Eight months after immunization, formaldehyde fixation, 40-μ frozen section. Not counterstained. ×9,450.

FIG. 5. Mature plasma cell (dense type) containing specific antibody within some ergastoplasmic cisternae (→) and the Golgi complexe (G). 5 days after immunization, formaldehyde fixation, 40-μ frozen section. Not counterstained. ×16,000.

FIG. 6. Mature plasma cell (dense type) which contain in some slightly distended cisternae (→) reaction product. Some cisternal membranes and ribosomes are strongly contrasted (►). Note fine granular, dense aspect of the cytoplasmic matrix as caused by numerous free ribosomes. Five days after antigen injection, formaldehyde fixation, 40-μ frozen section. (a) Lead citrate 30 sec. ×13,150. (b) Same cell in another ultrathin section, doubly stained. Note that no further details can be distinguished in the cytoplasm, but nuclear fine structure is better visible. ×13,150. Inset: High power view of free cytoplasmic ribosomes (→). ×38,600.

great variety of transitional cell forms occurred. At the final maturation stage, the cisternae of the RER appeared extremely distended and filled with flocculous or condensed material, characteristic for *MOTT* cells (Fig. 7). Some plasma cells contained large spherical *RUSSELL's* bodies (Fig. 8).

(b) *The clear cell-type.* From the described lymphocytic cells, we distinguished reticulum cells and pathways of differentiation where the reticulum cell character was still apparent. Activated cells had well-developed nucleoli of reticular appearance. During further maturation, numerous ribosomes were seen in the cytoplasm to form typical rosettes and narrow channels of ergastoplasmic lamellae. We termed these cells as *reticulum blast cells* (Fig. 9). Between blast cells and ergastoplasm-rich cells the same maturation steps were observed (Fig. 10) as described above.

Localization of antihorseradish peroxidase antibody. Antibody to HRP was first localized on Day 5, before circulating antibodies were found and when anti-HRP antibody-producing cells were not precisely determined by light microscopy. The cells, containing intracellular anti-HRP, which were found at the ultrastructural level, were very few in number. All these cells were dense type mature plasma cells showing the specific reaction in only some ergastoplasmic compartments. The well-developed Golgi complex was regularly stained (Figs. 5, 6). In the perinuclear space anti-HRP could not be detected. Most of those cells were seen in the medullary cords; but even in the cortex, which contained mostly unstained lymphocytes

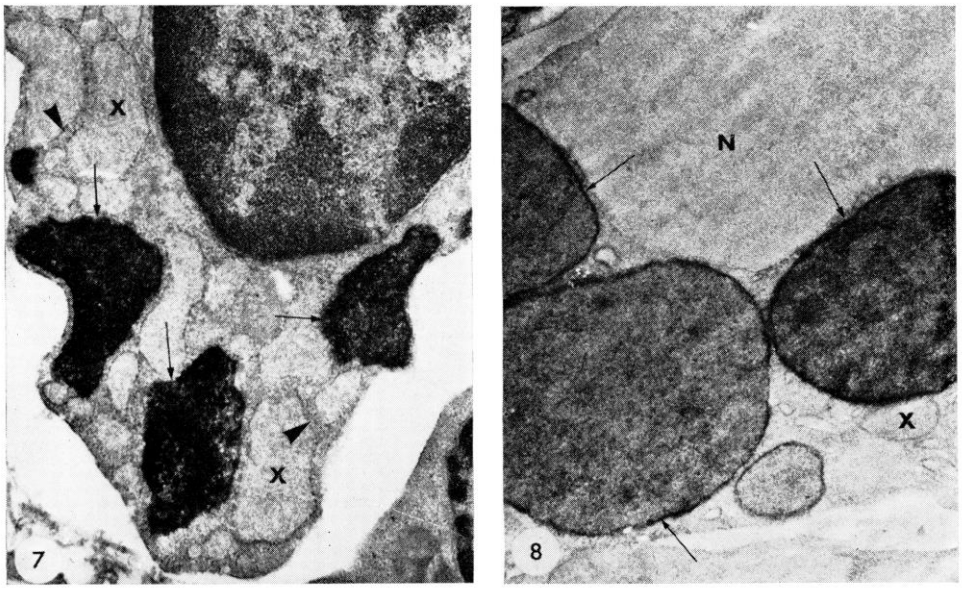
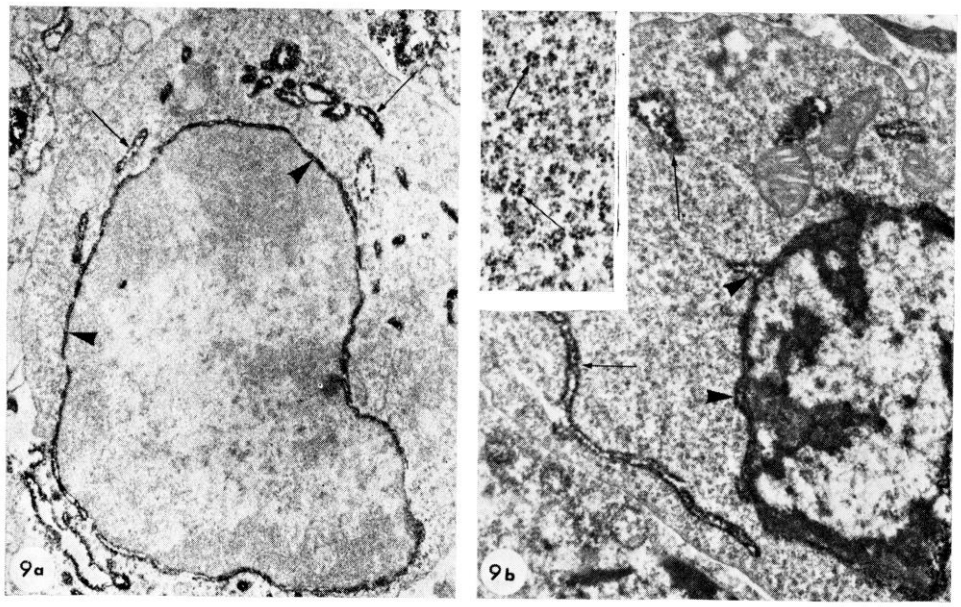


FIG. 7. Mature plasma cell (dense type, MOTT cell-type) with specific reaction product in some heavily distended cisternae (→). Note fine granular, dense aspect of the cytoplasmic matrix (►). Other cisternae are negative (x). Ten days after antigenic stimulation, 40-μ frozen section. Uranyl acetate and lead citrate staining. ×16,700.

FIG. 8. Mature plasma cell (dense type) containing specific antibody in form of RUSSELL's bodies (N = nucleus; x = negative cisternae). Ten days after antigen injection, formaldehyde fixation, 40-μ frozen section. Not counterstained. ×16,000.



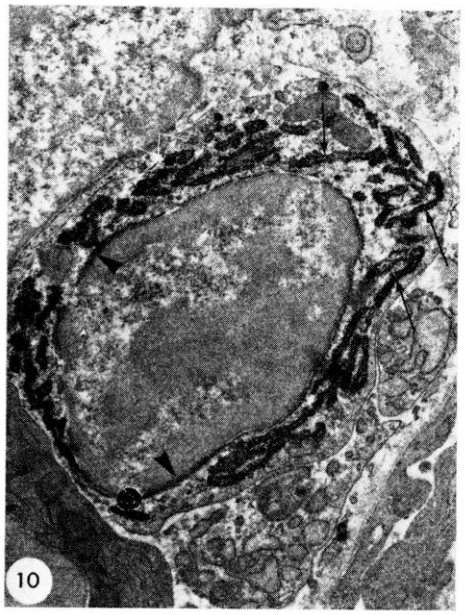


FIG. 9. Blast cells (clear cell-type) synthesizing specific antibody in the few ergastoplasmic lamellae (→) and the PNS (►). Fifteen days after HRP injection. (a) Formaldehyde fixation, 40-μ frozen section. Not counterstained. ×12,150. (b) Glutaraldehyde fixation, 40-μ frozen section. Uranyl acetate and lead citrate staining. ×17,900. Inset: Ribosomes rosettes (→) at high magnification. ×42,800.

FIG. 10. Young plasma cell (clear type) synthesizing specific antibody in the PNS (►) and the ergastoplasmic cisternae (→). Note the electron lucid cytoplasmic matrix. Seven days after antigen injection, formaldehyde fixation, small tissue fragment. Lead citrate 30 sec. ×11,650.

and blast cells, some of these cells, synthesizing anti-HRP, were present. Although antibody was found for the first time on Day 5 in mature plasma cells, we observed, even on the third day after stimulation, all maturation steps of the clear and dense cell-types in which, however, antibody could not be detected. Occasionally mitotic figures of the dense cell-type were noted, while in clear cell-types no mitoses were observed. On Day 6 the medulla presented the same features as on Day 5. In the cortex were seen dense type mature plasma cells containing anti-HRP in either some or all of their ergastoplasmic cisternae, but rarely was the perinuclear space also stained. At this time, Day 6, in the cortex some dense type, blast cells, whose RER and PNS had specific reactions, could be observed (Fig. 3a).

Seven days after the antigen injection there was an increase of specific antibody-producing cells. The medulla contained mostly mature cells of the dense type, but some dense immature plasma cells also occurred. The antibody was distributed either in some ergastoplasmic lamellae or in the whole RER including the PNS; the Golgi apparatus was often stained too. Occasionally some clear type plasmablasts and plasma cells were seen with the reaction product in the ergastoplasm and in the PNS (Fig. 10). The positive cells in the cortical regions, always fewer in number consisted of immature and mature dense type plasma cells, but an increase in dense blast cells was observed. Apparently clear cell-types were absent from the cortical regions. The intracellular distribution of antibody was the same as in the cells of the medullary cords.

Eight days after the challenge, no further alteration of appearance within the lymphoid tissue cells could be stated.

On Day 10, the anti-HRP-producing cells became strikingly numerous. In the medulla were now seen great numbers of dense type mature and immature plasma cells, plasmablasts and blast cells in approximately equal numbers (Fig. 3b). Some cisternae within the mature cell stages were heavily distended and contained the specific peroxidase reaction product (Fig. 7). Occasionally, we observed plasma cells with highly distended and rounded cisternae, where antibody had accumulated to form Russell's bodies; the PNS and the nondistended RER were negative (Fig. 8). Clear type plasmablasts and plasma cells could be regularly found, but still fewer in number than dense type cells. 12 and 13 days after the injection, the same positive cells as on Day 10 were observed in the medulla. Cortical regions contained numerous immature and mature dense type plasma cells; their blast cell precursors still occurred. The mature cells had often distended cisternae throughout the ergastoplasmic system whether containing positive reaction or not. The perinuclear space of those cells was often positively stained as was also the case for the Golgi complex.

From Day 15 to 35 all maturation stages of the dense type cells were present, but blast cells (Fig. 3c) and plasmablasts became fewer in number.

Between Days 13 and 18 we could count an increased number of clear type cells of all maturation steps. From then on, up to Day 35 both clear type and dense type cells were numerous (Figs. 9, 11).

Eight months after HRP injection we still found mature plasma cells, mainly of

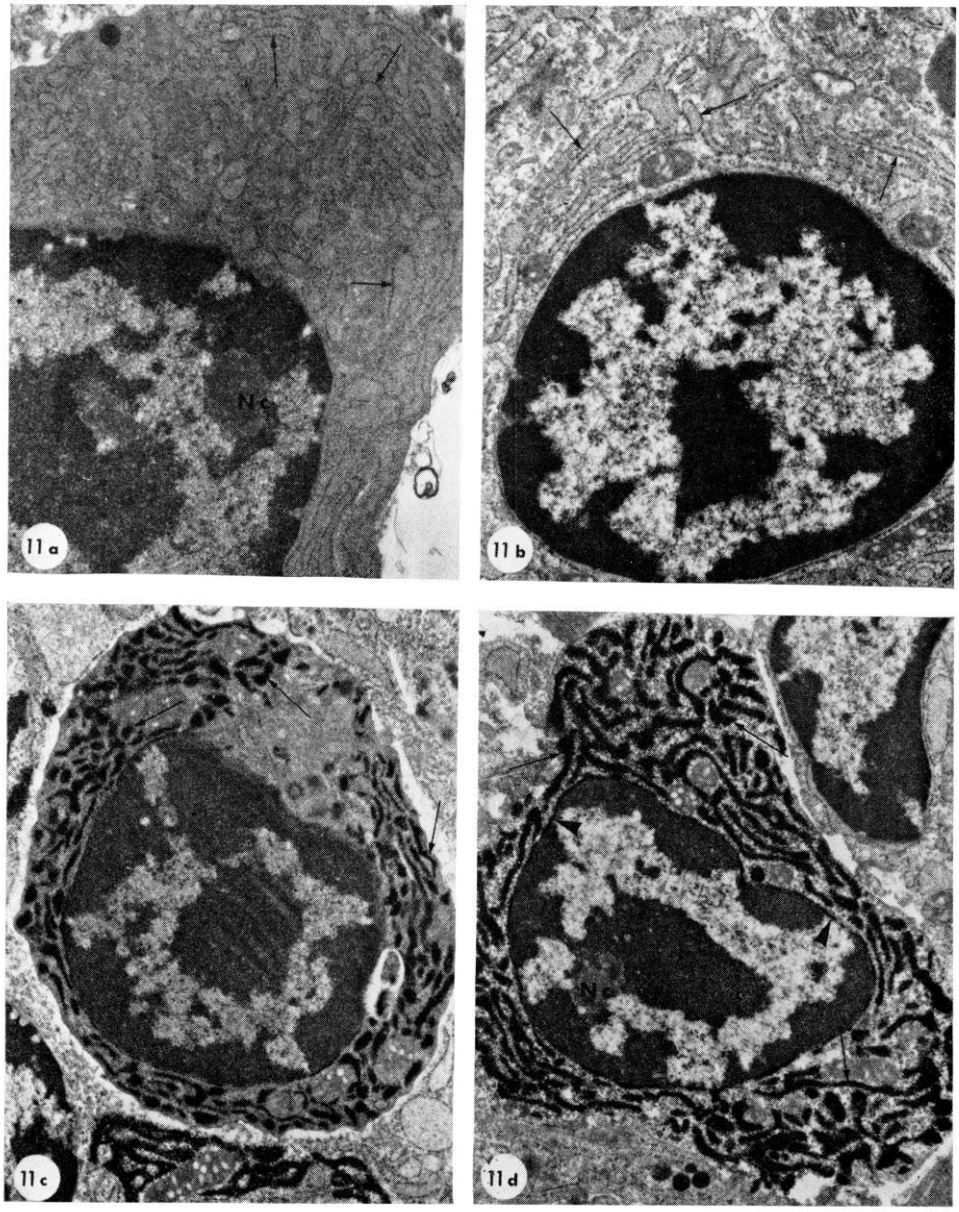


FIG. 11. Mature plasma cells; (dense and clear types) 15 days after immunization, glutaraldehyde fixation, 40-μ frozen section. Uranyl acetate and lead citrate staining. (a) Typical aspect of a lymphocytic plasma cell (dense type). No reaction product within the RER (→), note small sized nucleolus (Nc). ×13,700. (b) Typical aspect of a reticulum cell-type plasma cell (clear type). No reaction product within RER (→). ×12,500. (c) Lymphocytic plasma cell (dense type) with specific antibody reaction in the RER (→). ×9,450. (d) Reticulum cell-type plasma cell (clear type) with a HRP antibody in the RER (→) and the PNS (►). ×10,000.

the dense type, containing the specific reaction product in more than half of the counted cells within the whole ergastoplasmic system including the PNS. The Golgi complex was usually stained too (Fig. 4). Heavily distended cisternae in those cells were rarely seen. In very few cells did we observe staining only in the PNS and some peripheral ergastoplasmic cisternae, which were rather flattened.

Specificity of the immunocytochemical reaction and fate of injected material. Peroxidase stimulated lymph nodes, when incubated in Graham and Karnovsky's medium alone, showed only the known and already described endogenous peroxidase activity to certain cells: eosinophiles, red blood cells and occasionally macrophages (7). The same reaction occurred when glucose oxidase and BSA immunized lymph nodes were treated with DAB and hydrogen peroxide or were incubated with peroxidase prior to this staining procedure. During all days studied homogenous, electron-dense material could be observed extracellularly, but usually engulfed by phagocytic cells. Sometimes, especially late in the immune response, we observed phagocytized plasma cells in which anti-HRP could still be visualized (Fig. 12). Eight months after injection, heavily charged macrophages were found and electron-dense material could still be observed in the extracellular space. In all controls at each day studied, such material could be seen in direct contact with the cytoplasmic membranes of lymphocytic and plasmocytic cells. Occasionally, droplet-like, dense substances without membranes were seen in lymphocytes either free in the cytoplasm, attached on the mitochondria or in the perinuclear space; no such material could be observed within the nucleus (Fig. 13). We did not prove whether these substances consisted of HRP still emulsified with Freund's adjuvant, or only of Freund's adjuvant alone; since in BSA and in glucose oxidase stimulated control tissues the same electron-dense material also appeared either after DAB plus H_2O_2 treatment or after a single postfixation with OsO_4 without any previous cytochemical reaction.

DISCUSSION

The purpose of the present paper was to establish the differentiation pathways of immunocompetent cells in rat popliteal lymph nodes synthesizing antihorseradish peroxidase antibodies and to follow the localization of those antibodies over a long period after a single injection of the enzyme antigen.

Three main points arose from our study: (a) *two different cell-types* were involved in antibody synthesis; (b) the *first specific antibody* was found on *Day 5* after the antigen challenge; (c) antibody was first observed in some RER of few *mature plasma cells* and not in blast cells.

(a) *Cell-types involved in antibody synthesis.* At the electron microscopic level, two morphologically distinct types of immunocytes were reported to be involved in specific antibody synthesis (9, 12). In the present paper we established continuous maturation steps and distinguished between lymphocytic and reticulum-like cells. Cells of the lymphocytic line are tentatively referred to as *dense cell-types* and those of the reticulum like line as *clear cell-types*. Both cells at the final stage of maturation result in ergastoplasm-rich cells, i.e., plasma cells.

The fragility of reticulum cells is a well-known phenomenon, and indeed, we noted poor fixation of the plasma membranes of all reticulum cell-types. Better fixa-

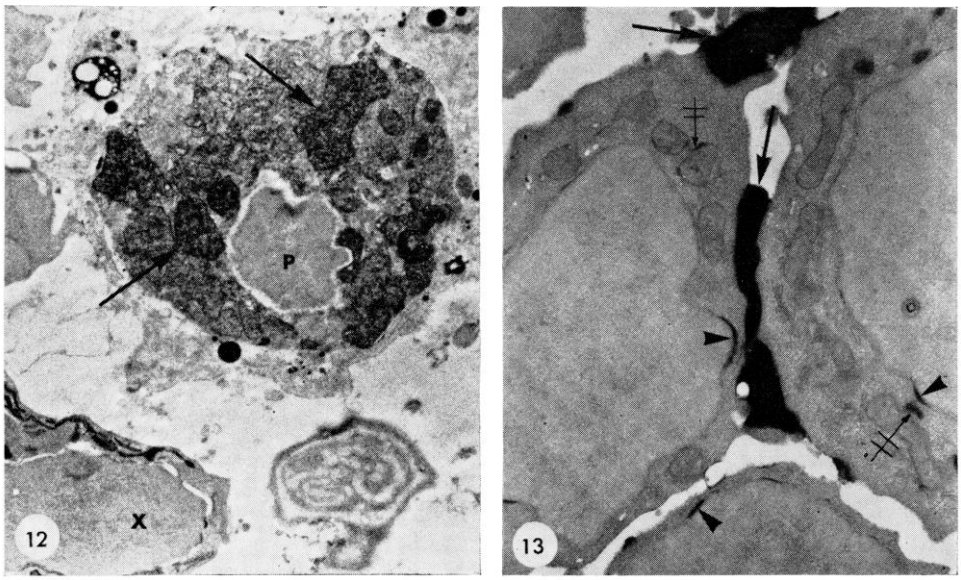


FIG. 12. Phagocytized mature plasma cell (P) in which specific antibody still can be visualized (→). In the lower left part of the picture a dense type plasma cell (x) containing anti-HRP antibody in the RER. Twenty-two days after immunization, formaldehyde fixation, small tissue fragment. Not counterstained. ×8,950.

FIG. 13. Three lymphocytic cells with electron-dense material attached on their cytoplasmic membranes (→). Note electron dense substances also in the cytoplasm, on mitochondria (→) and in the PNS (►). Twelve days after antigen injection, formaldehyde fixation, 40-μ frozen section. Lead citrate 30 sec. ×13,150.

tion was observed for all differentiation and maturation steps of the lymphocytic series. Similar results were obtained with formaldehyde perfused animals, or when lymph nodes were fixed in glutaraldehyde.

Further evidence of the existence of two distinct cell lines was found in sections which were not counterstained. Dense cell-types of all maturation steps showed generally a marked natural contrast of the nucleus and the cytoplasm as compared with the clear cell-types. Consistently, the cellular filiations of dense cell-types could be easily distinguished from all neighbouring cells, but this was rarely the case for the clear cell-types. A striking point was the constant compact appearance of the cytoplasm of dense cell-types even in the very mature state, i.e., in cells containing a well-developed ergastoplasm with distended cisternae where we found in the small remaining cytoplasmic matrix many free ribosomes, thus resulting in an enhanced contrast. Dense cell-types at either blast or mature stage did not seem to be bound to the tissue network. Furthermore, cell suspensions, prepared from the lymph nodes, contained only dense cell-types, morphologically intact. So, it seems likely that dense cells may be part of a more or less circulating population of cells. Similar cells have been described to be present in efferent peripheral lymph, thoracic duct lymph and peripheral blood (4, 23–25).

When reticulum-like cells were involved in progressive differentiation, we often observed that the cells could be found grouped together. The clear cell-types may

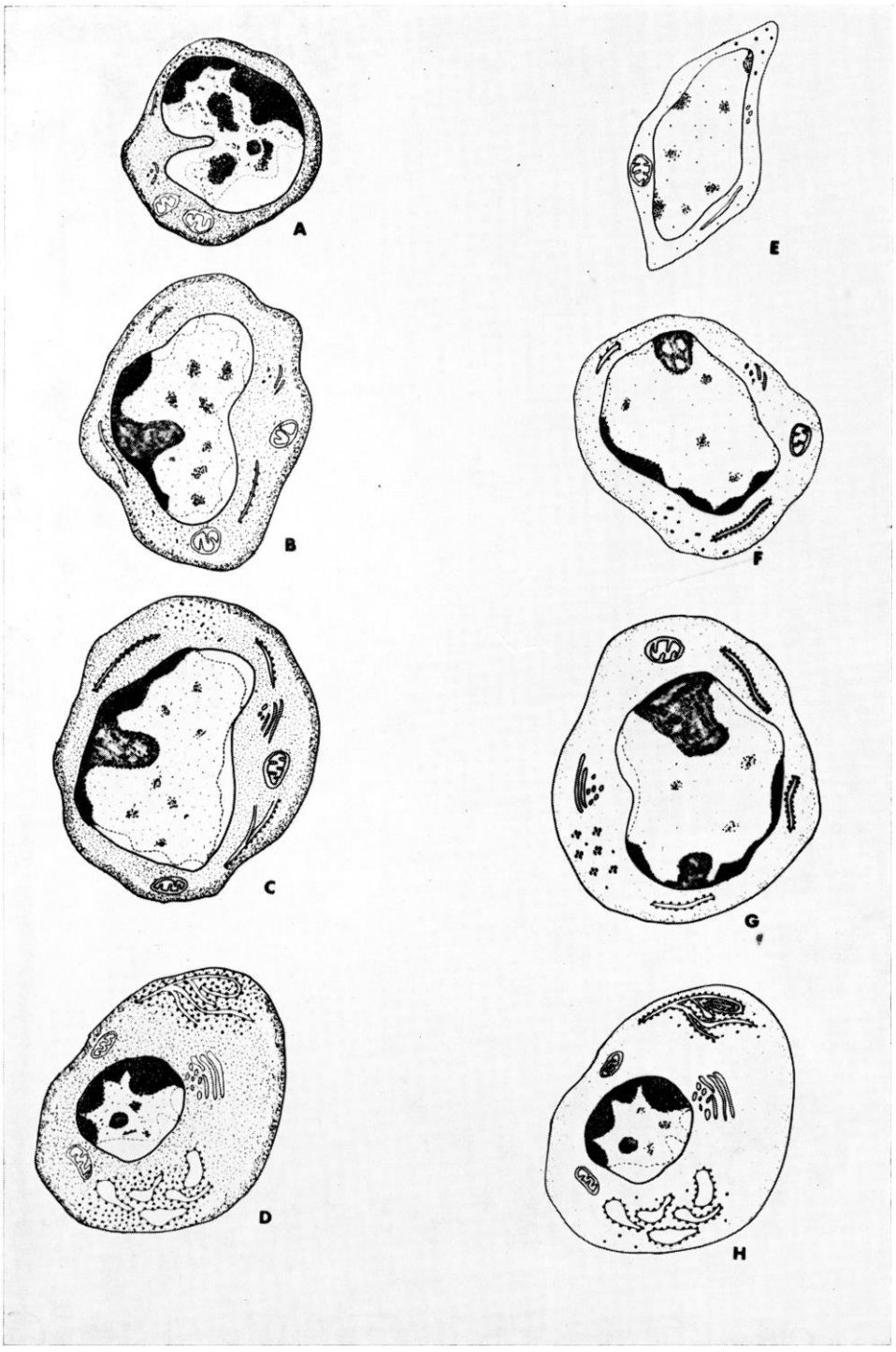


FIG. 14. Schematic representation of the developmental steps of the two cell-types involved in antibody synthesis. *Dense cell-types (A–D)*: small lymphocyte (A), medium/large lympho-

be directly derived from tissue-bound cells. This hypothesis is strengthened by our observation that clear cell-types are absent in lymph node suspensions. It is therefore to be stressed that experiments on antibody synthesis, performed with cell suspensions, may reveal only a part of the immunological phenomena.

From all the above evidence, it might appear that dense and clear cell-types arise from different precursors, one from circulating lymphocytes, and the other from tissue reticulum cells. The origin of the plasmocyte is still a matter of controversy. Several authors have stated that plasmocytes may arise from reticulum cells (26–28), while others have claimed that small circulating lymphocytes are the precursors of the plasmocytes (29, 30). The present work and other studies (9, 12) tend to indicate that indeed two cell-types, reticulum cells and lymphocytes, can generate morphologically distinct plasmocytes (31). The suggested developmental steps of the two cell-types involved in antibody synthesis are represented in Fig. 14.

(b) *Timing of specific antibody.* The first specific anti-HRP antibodies could be detected in a few cells on Day 5 after the antigen administration. In the electron microscope we observed the immunocytochemical reactions only in a few cisternae of the RER and this will be discussed below. At the *light microscopic level* we found on Day 5 faintly positive cells only when cells were fixed in suspensions with formaldehyde, but never in the classical smear preparations. However, the intracellular antibody staining was difficult to localize. During the subsequent days there was an exponential raise of positive cells up to Day 16, followed by a plateau. Three days after the first intracellular visualization of antibody, i.e., on Day 8, we detected traces of circulating antibodies, which raised exponentially up to Day 21, followed by a plateau, also. Thus, it could be deduced that the number of positive cells and the titer of circulating antibodies are in close relation. However, as detected by electron microscopy, clear cell-types are absent in cell suspensions. It seems to us that the amount of circulating antibody might indicate the ability of immunocytes to secrete rather than to synthesize antibody.

(c) *Cellular response to HRP stimulation.* The response of dense cell-types to a single antigen injection seemed to be quite different from that of the clear types. In our system, the first cells to synthesize specific antibody were mature plasma cells of the dense type, where the reaction product could only be localized in some cisternae and in the Golgi apparatus. Later, positive clear blast cells appeared, together with the present dense blast cells, with a following shift to their mature stages; immature cells were also always present. Then, the antibody distribution could be either in the whole RER, including the PNS, or only in some cisternae. Sometimes only parts of membranes of the RER and their ribosomes were stained. MOVAT *et al.* (32), studying stimulated rabbit popliteal lymph nodes by morphological criteria, concluded that the typical feature of the immunological response is the appearance of blast cells with a following shift to mature stages. In the present work, combining morphological criteria with cytochemical detection of specific antibody, we found that indeed blast cells appear soon after antigenic stimulation,

cyte (B), dense type blast cell (C), dense type plasma cell (D). *Clear cell-types (E–H)*: undifferentiated reticulum cell (E), activated reticulum cell (F), reticulum blast cell (G), clear type plasma cell (H).

but with our methods antibody could not be detected in these cells, first specific reaction being visible on Day 5 in mature plasma cells.

It could be argued that the localization of anti-HRP antibodies by our method is due to difficulties of penetration of the incubation media or to inhibition of the enzyme antigen by its homologous antibody, but there is evidence that this cannot be the case. First, up to Day 4 neither blast cells nor plasma cells were stained, but on Day 5 a number of mature plasma cells were positive; later in the immune response a wide-range of cisternal marking occurred. Eight months after the stimulation, more than half of the positive cells were mature plasma cells with specific antibody reaction in the whole ergastoplasmic system. Second, by preparing cell suspensions, where one expects to have optimal conditions for penetration of macromolecules, we observed the same features as in the tissues described above. Third, DePetris *et al.* (2) demonstrated good penetration of ferritin into the intracellular space by using high concentrations of antigen. However, these concentrations did not enhance the formation of specific antigen–antibody complexes. Finally, inhibition studies of HRP by homologous antibodies have shown that 15 molecules of antibody per molecule of peroxidase decrease the catalytic activity by only 10%. Thus, cells synthesizing antibodies which inhibit the enzymatic activity of peroxidase are quite low and can be neglected. With the procedures described in this paper, nonspecific staining at the intracellular level was excluded by several control reactions, where lymph nodes, stimulated by nonrelated antigens, had been incubated in peroxidase and the corresponding substrate.

The observation that antibody was found for the first time 5 days after antigen administration in some ergastoplasmic cisternae of dense type mature plasma cells, is at variance with the reported observations in the secondary immune response to HRP (7) and ferritin (2) in rabbit, where antibody was first found in the perinuclear space of blast cells and other immature stages. Preliminary experiments in restimulated rat lymph nodes seemed to indicate that antibody also appeared within the perinuclear space. It might be then possible that the primary and secondary immune responses to peroxidase are different not only quantitatively but also qualitatively, and this might be true for other antigen–antibody systems. Furthermore, as antibody first appeared in plasma cells and not in blast cells it seems probable that immunocyte differentiation and antibody synthesis are not necessarily linked as cellular functions. Since antibody appeared for the first time in only a few ergastoplasmic cisternae of mature dense-type plasma cells, the question arises as to what the other cisternae contained. It could be that antiperoxidase antibody was present in these cisternae, but at quantitites too low to be detected with the method employed. Alternatively, mature plasma cells synthesizing immunoglobulins might be able to respond to a secondary antigenic stimulation, thus giving anti-HRP reaction in some cisternae on Day 5.

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