Lymphocyte Differentiation and Antibody Synthesis in the Secondary Immune Response of Peroxidase Stimulated Lymph Nodes of Rat

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Cell Tissue Res. 156, 391-402, 1975

Summary. Lymphocyte differentiation and specific antibody synthesis were studied in the course of the secondary immune response of horseradish peroxidase immunized rat popliteal lymph nodes by electron microscopic immunocytochemistry. From primary immunization, mature plasma cells containing specific antibody remained as long as 10 months. Antihorseradish peroxidase antibodies were localized in lymphocytic blast cells on Day 3 after restimulation with antigen. In the subsequent days blast cells were still present, and shifting to mature stages occured. During immunocyte differentiation and maturation vesicles and tubules of the Golgi complex became filled with specific antibody suggesting occasional secretion of antibody via the Golgi apparatus. At very mature cell stages, antibody was accumulated in distended cisternae.

Key words: Immune response - Cellular differentiation - Antibody synthesis.

Introduction

Antigenic and catalytic properties make enzymes useful tools for studying the cellular basis of antibody synthesis. Thus, enzyme antigens have been employed to follow intracellular events in immunocytes at both the light and the ultrastructural level (Leduc *et al.,* 1968; Avrameas and Leduc, 1970; Bosman and Feldman, 1970; Sordat *et al.,* 1970; Straus, 1970; Kuhlmann and Avrameas, 1972).

In experiments using various markers, immunoglobulins were localized on membrane bounded ribosomes, in the perinuclear space (PNS), in the cisternae of the rough surfaced endoplasmic reticulum (RER), and in the Golgi apparatus (DePetris *et al*., 1963; Melchers and Knopf, 1967; Leduc *et al*., 1968; Kuhlmann and Avrameas, 1972).

In a previous communication we demonstrated cellular differentiation and specific antibody production of the lymphoreticular system during the primary immune response in peroxidase stimulated lymph nodes of rat (Kuhlmann and Avrameas, 1972). In the present paper, cytologic development of lymphoid cells and specific antibody synthesis during the secondary immune response after horseradish peroxidase injection were studied.

Material and Methods

Five month old rats (Wistar substrain AG) were used. Horseradish peroxidase RZ 3 (HRP) was purchased from C. F. Boehringer-Mannheim, Germany.

Immunization Schedule. 1 mg of peroxidase, dispersed in Freund's complete adjuvant, was injected into both hind footpads of each rat. 8-10 months later, a booster injection was done with the same quantity of antigen dissolved in phosphate buffered saline. The animals were bled and killed 1 (6)[1], 2 (6), 3 (6), 4 (4), 5 (4), and 6 (4) days after the second antigen administration. Also, 5 rats were examined 8 and 10 months after a single injection of peroxidase.

12 rats immunized with either bovine serum albumin or glucose oxidase served as controls (Kuhlmann and Avrameas, 1972).

Electron Microscopy. Both popliteal lymph nodes of each rat were dissected out from animals at a given day and immediately put in a drop of fixative chopped with razor blades into small blocks and slices. Formaldehyde, freshly prepared from paraformaldehyde (Merck, Germany), and purified glutaraldehyde (TAAB Laboratories, England) in 0.2 M cacodylate buffer at pH 7.2 were used: (a) 4% formaldehyde for 24 hours at 4°C; (b) 2.5% glutaraldehyde for 1.5 hours at 4°C.

Fixation was carried out in 20 ml vials under continuous agitation. The tissues were washed at 4°C for at least 24 hours during which the cacodylate buffer was changed several times.

Prior to immunocytochemical incubations, the tissue blocks were reduced with razor blades to extremely small fragments. Frozen sections from the larger slices were cut at 40 µ in a Dittes-Duspiva cryostat as described elsewhere (Kuhlmann and Miller, 1971). Subsequent incubation procedures were carried out at room temperature: (a) incubation with 1 mg HRP/ml cacodylate buffer for 24 hours; (b) washing 3 x 10 min in buffer; (c) revealing of peroxidase activity by incubation for 20 min in 3,3'-diaminobenzidine and H_2O_2 (Graham and Karnovsky, 1966) followed by two washes of 2 min in buffer; (d) postfixation in 2% OsO_4 in cacodylate buffer for 1 hour.

Control tissues were treated as already described (Kuhlmann and Avrameas, 1972). Briefly, tissues were incubated with or without peroxidase antigen followed by the appropriate enzyme substrate and postfixation in OsO_4.

Tissue blocks and cryostat sections were dehydrated in ascending alcohol and embedded in Epon (Luft, 1961). From the flat embedded 40 µ. frozen sections, areas of medulla and cortex were selected (Kuhlmann and Avrameas, 1972). For each day of the immune response 10-15 different regions containing specific antibody synthesizing cells were processed for electron microscopic examination.

[1] Number in parantheses denotes the number of animals examined on each day.

Ultrathin sections were cut with a LKB Ultrotome III and mounted on Formvar and carbon coated 200 mesh copper grids. Sufficient sections were cut in order to study unstained, lead citrate stained (Reynolds, 1963), and double stained (uranyl acetate and lead citrate) grids. In some cases 1 μ thick sections were also examined without being counterstained. A Siemens Elmiskop I A was used operating at 80 and 100 kV with 50 μ objective apertures.

Results

Classification of lymphocytes and their differentiation products during immune response has been described elsewhere (Kuhlmann and Avrameas, 1972).

Specificity of the Immunocytochemical Reactions

Control tissues, i.e. lymph nodes immunized with bovine serum albumin or with glucose oxidase, when incubated with or without HRP followed by Graham and Karnovsky's medium, showed only the known and described endogenous peroxidase activity to certain cells. These cells, however, are not immunocytes and do not synthesize immunoglobulins (Leduc *et al*., 1968).

Timing of Antihorseradish Peroxidase Antibody

Popliteal lymph nodes, examined 8-10 months after a single HRP injection, still contained immunocytes in which specific antibody was present. These cells were observed in both medullary and cortical regions but mainly in the cords of the medulla. All these cells were typical plasma cells having specific staining in the PNS (perinuclear space)and throughout the RER (rough surfaced endoplasmic reticulum). In most plasmocytes the cisternae of the endoplasmic reticulum were not distended (Fig. 1). In some cases, however, the antibody reaction was seen in few distended cisternae and in the PNS. In numerous immunocytes, the Golgi apparatus could also be stained, but its vesicles and lamellae were small and flat. MOTT cells were rare.

On Day 1 after restimulation with peroxidase antigen no significant differences were observed, and lymph nodes presented the same features as described above. Also, the number of positive cells was judged to be equal. On Day 2 after the challenge no further alteration of appearance within the lymphoid tissue cells could be found.

On Day 3 after antigen injection the following characteristic events were found: antihorseradish peroxidase producing cells became strikingly numerous and, most important, positive lymphocytic blast cells were present for the first time in cortical and also in medullary regions. The specific reactions occurred in the PNS, in the few lamellae of the developing endoplasmic reticulum and, if present in the section, in the Golgi apparatus (Figs. 2, 3). Furthermore, mature plasma cells were still found with features described above. At this time numerous plasma cells could be seen to contain heavily stained concentric and distended cisternae throughout the cytoplasm. Sometimes Russel bodies were also found in these cellular stages. The Golgi complex was always well developed and its cisternae and vesicles heavily filled with anti HRP antibody (Figs. 4-6).

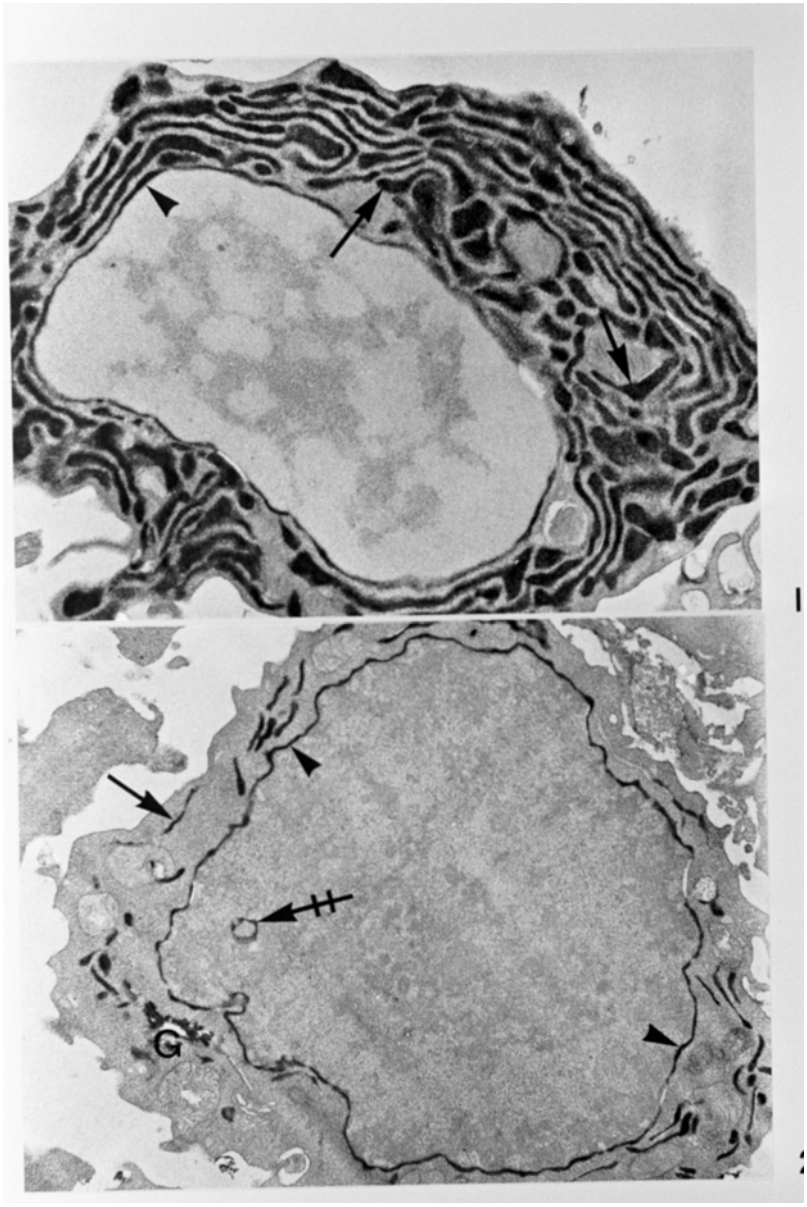

Fig. 1. Mature plasma cell containing anti HRP antibody in PNS and RER cisternae (→). 8 months after immunization. Formaldehyde fixation. Lead citrate, 30 se-conds. x 16200.

Fig. 2. Lymphocytic blast cell synthesizing specific antibody in PNS and RER lamellae (→); the Golgi apparatus (G) is also stained. Note tangentially cut invaginations of PNS in the nucleus. 3 days after HRP restimulation. Formaldehyde fixation. Lead citrate stai-ning. x 13800

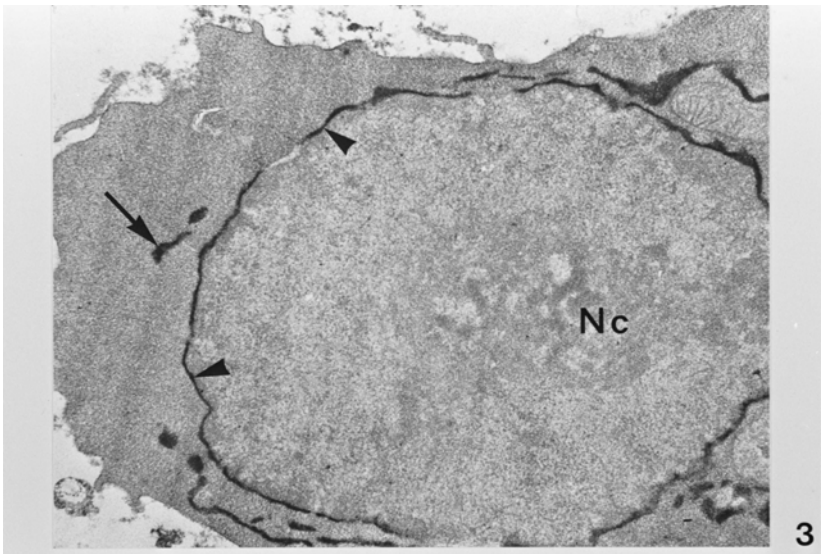

Fig. 3. Blast cell as in Fig. 2. Glutaraldehyde fixation; nucleolus (*NC*). Lead citrate, 30 seconds. x 16200

From Day 4 to Day 6 all maturation stages of immunocytes were present and the number of positive cells increased (Figs. 7, 8). In cortex and medulla, blast cells were still numerous.

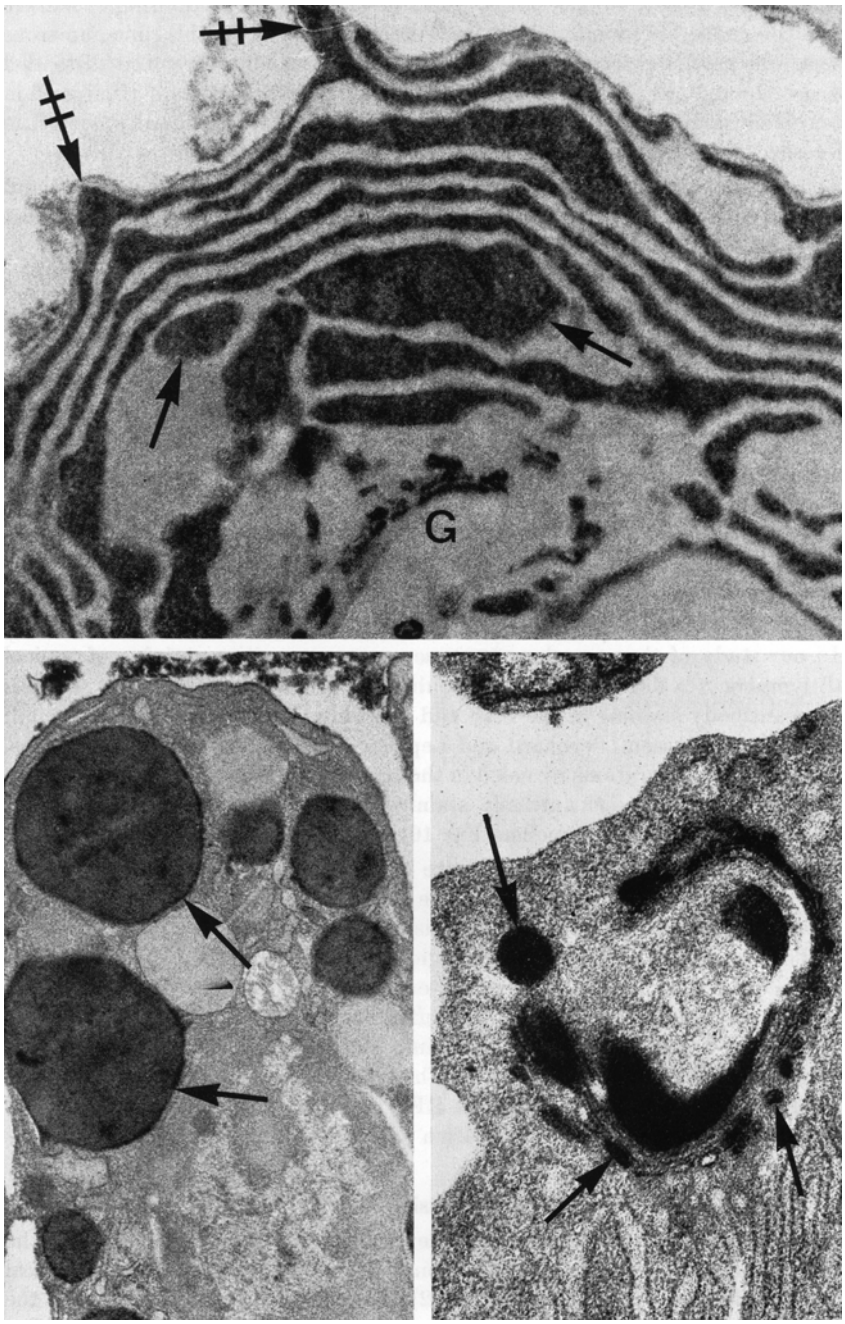

Fig. 4. Mature plasma cell with positive antibody staining in distended RER cisternae (→) and Golgi apparatus (G). Note protrusions of RER cisternae at the cell periphery. 3 days after restimulation. Glutaraldehyde fixation. Not counterstained. x 20500

Fig. 5. Positive Russel bodies (→) in a mature plasma cell. Day 3 of the secondary response. Glutaraldehyde fixation. Lead citrate staining. x 15000

Fig. 6. Golgi complex of a plasma cell. Note heavy and specific antibody staining in its lamellar and vesicular system (→). 3 days after antigenic restimulation. Formaldehyde fixation. Lead citrate, 30 seconds. x 10000

During differentiation and maturation of immunocompetent cells, specific antibody has been localized in sites corresponding to the perinuclear space, the cisternae of the endoplasmic reticulum including membrane bounded polysomes, and the Golgi apparatus.

At immature stages of developing immunocytes, antihorseradish peroxidase antibody was found in the PNS and throughout RER lamellae. Blast cells and immature plasma cells both possessed deep indentations of their nuclei. Often the PNS was cut tangentially so that staining reactions were located in the nucleus by which nuclear pores were readily seen (Figs. 2, 9). Vesicles and tubules within the Golgi complex became filled with specific antibody (Figs. 2, 7, 8).

In our study of the secondary immune response, positive staining of typical small lymphocytes did not occur. In doubtful cases, when in ultrathin sections positive antibody staining in the PNS and concomitantly characteristics of small lymphocytes were seen (Bernhard and Leplus, 1964; Kuhlmann and Avrameas, 1972), 1 μ thick preparations revealed in the 100 kV operating electron microscope features of blast cells, *i.e.* antibody staining within the developing and interconnected system of RER lamellae (Fig. 10).

During gradual maturation, the entire cytoplasm became filled with heavily stained RER cisternae (Fig. 4) and at the height of maturity, specific antibody was found to accumulate in few RER cisternae which have become more and more distended (Fig. 5). In the Golgi region small vesicles were clustered along the convex surface of the lamellar system which became also distended and positive vesicles appeared to be pinched off at their edges (Figs. 6, 11).

During the developmental stages of immunocytes, RER cisternae could be observed at the extreme periphery of the cytoplasm and also protrusions of parts of it were seen (Fig. 4). Opened RER cisternae toward the extracellular space have been noticed in mature plasma cells (Fig. 12).

Discussion

In a previous paper, morphological development of plasma cells during the primary immune response in rats has been described using immunocytochemical methods (Kuhlmann and Avrameas, 1972). This communication reports on the differentiation of lymphocytes in the course of specific antibody synthesis during the secondary immune response of peroxidase-stimulated lymph nodes of rat.

Three main points arose from our study: (a) 10 months after a single antigen injection mature plasma cells containing specific antibody were still found; (b) typical changes after a second antigen stimulation occurred on Day 3; (c) specific antibody was observed in lymphocytic blast cells and in immature cells shifting to mature stages. Positive staining of typical small lymphocytes was not observed.

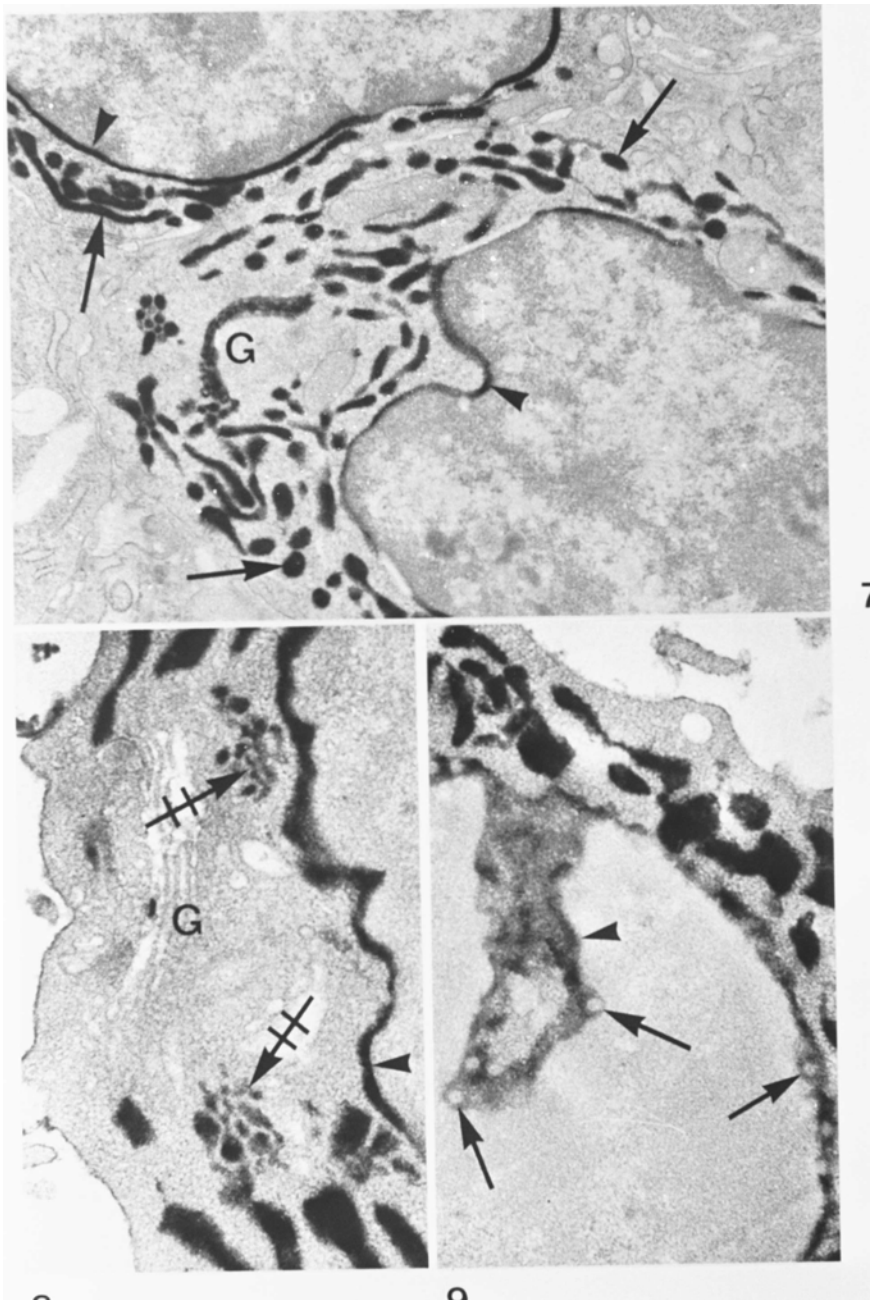

Fig. 7. Two plasma cells containing anti HRP antibody in PNS and RER cisternae (→). The Golgi apparatus (G) is also stained. 6 days after restimulation. Glutaraldehyde fixation. Lead citrate, 30 seconds. x 16000

Fig. 8. Immunocyte synthesizing specific antibody in PNS and RER cisternae (→). Note transport of antibody toward the Golgi apparatus (G). Day 6 of the secondary immune response. Glutaraldehyde fixation. Lead citrate staining. x 38000.

Fig. 9. Same tissue as in Fig. 8. An invagination of the positive PNS is cut tangentially. Note nuclear pores (→). Lead citrate, 30 seconds. x 30000

Cells Remaining from Primary Immunization

As long as 8-10 months after a single peroxidase injection, antibody containing immunocytes were found in popliteal lymph nodes. These immunocytes were typical mature plasma cells, and the questions arise as to how they are generated and what their life span

is. For primary immunization with peroxidase antigen, we used complete Freund's adjuvant which is known to release quantities of antigen over a long period of time (Freund, 1956). Thus, the immune response to a single injection may persist for a very long time. The fate of the generated plasma cells in our experimental work was not clear. Also, we do not know if plasma cells stop synthesis of antibody when PNS and RER cisternae are replete, nor do we have evidence if plasma cells are capable of storing antibody for a certain period of time.

Experimental data on the natural life span of mature plasma cells could not be elaborated. In the course of the secondary immune response, anti HRP positive mature plasma cells which remained from the primary immunization stained more heavily and progressively for specific antibody. This observation may indicate that upon a second antigenic stimulation differentiated immunocytes are capable of either a second cycle or enhanced antibody synthesis.

Secondary Immune Response

The typical event after antigenic restimulation of the lymph nodes was observed on Day 3 when lymphocytic blast cells were detected which synthesized antiperoxidase antibody. Then, throughout the secondary immune response, positive blast cells were found. Together with these cells, immature immunocytes shifting to their mature stages were seen.

The characteristic feature of the secondary immune response that antibody was found 3 days after antigen challenge in PNS and ergastoplasmic lamellae of blast cells, is at variance with the reported observations in the primary immune response to HRP (Kuhlmann and Avrameas, 1972), where antibody was first found in some RER cisternae of mature plasma cells. In this connection it might be interesting to note that studies on the secondary immune response to HRP (Leduc *et al*., 1968) and ferritin (DePetris and Karlsbad, 1965) in rabbits have indicated that antibody was first found in the PNS of blast cells and other immature stages.

It seems then that primary response is not only quantitatively but also qualitatively different from the secondary response. The results which have been obtained after a single antigen administration suggest that cellular differentiation and antibody synthesis are not two functions of the immunocyte necessarily linked. On the contrary, in the secondary response, the immunocyte differentiation and the antibody synthesis seem to be interdependent.

Probably, blast cells are the result of immunologic memory linked to lymphoid organs (Gowans and Uhr, 1966). The finding of antibody synthesizing blast cells and immature plasma cells during the immune response and the observation that these cells were in mitoses, suggest a recruitment from the committed lymphocyte pool.

Cellular Response to HRP Restimulation

Apart from the mature immunocytes remaining after primary immunization, the second challenge with antigen in physiological solution led to a characteristic event, i.e. the appearance of typical blast cells on the third day, synthesizing specific anti HRP antibody in the PNS and the developing RER system. During subsequent days blast cells were further recruited, and shifting to mature stages could be readily observed. At the final

stage of maturation, antibody could be distributed either in the whole RER or only in few cisternae.

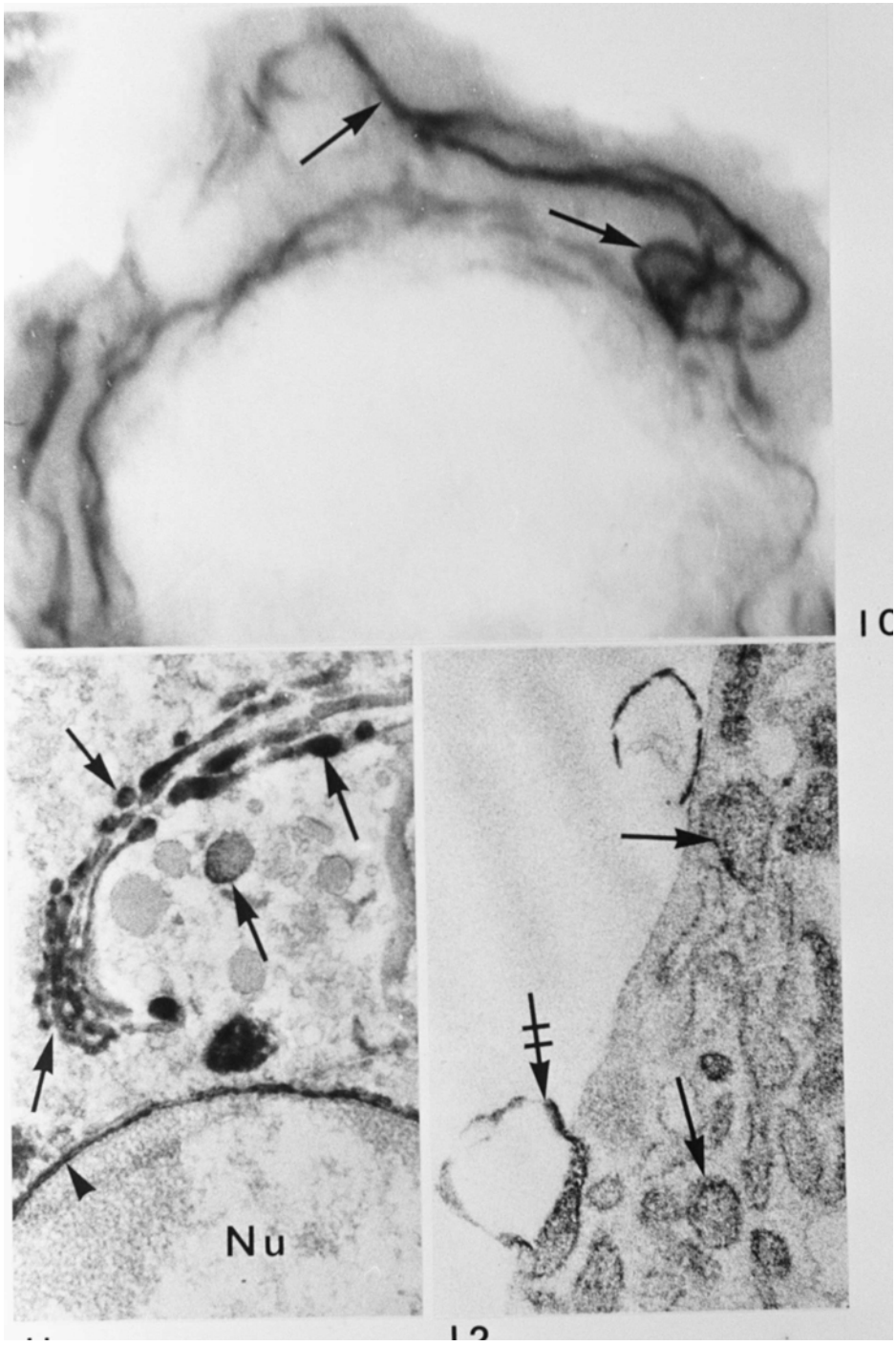

Fig. 10. Positive blast cell in a 1 μ thick Epon section. Note interconnected system of antibody containing RER lamellae (→). 3 days after restimulation. Glutaraldehyde fixation. Not conterstained. x 19200

Fig. 11. Golgi complex of a plasma cell containing anti HRP antibody in its lamellae and vesicles (→). The PNS of this cell is also stained.; nucleus (*Nu*). 4 days after restimulation. Formaldehyde fixation. Lead citrate, 30 seconds. x 30500

Fig. 12. Part of a plasma cell with specific antibody staining in RER cisternae (→). Note broken RER cistern at cell periphery. Day 4 of the secondary immune response. Formaldehyde fixation. Not counterstained. x 33600

In our material, we did not localize positive staining in typical small lymphocytes; staining occurred concomitantly with developing rough surfaced endoplasmic reticulum which is the characteristic sign of differentiating blast cells. Using 1 μ thick sections in the electron microscope operating at 100 kV the youngest immunocytes, containing specific antibody, were classified as typical blast cells where the interconnecting RER consisted of flat lamellae.

During the secondary immune response of HRP stimulated rabbits, new formation of specific antibody was also found to occur in the PNS, while remnants of antibody from the primary immune response were still to be found in RER cisternae at the cell periphery (Avrameas and Leduc, 1970). The same authors also reported on pinching off of parts of the cytoplasm containing endoplasmic reticulum. Furthermore, clasmatosis, originally described by Thiery (1959) based on cinematographic observations, was suggested to be a possible way for excretion of cellular products. In our study, indeed, mature plasma cells were also found to contain distended RER cisternae which were located extremely near the cell surface. Breaking off was also observed, but the importance of this observation is difficult to assess. In the course of immunocyte maturation, secretion of specific antibody via the Golgi apparatus was observed to be a typical pathway; progressive development of the Golgi complex was seen denoting heavy and specific antibody staining within its lamellar and vesicular parts. Evidence of clasmatotic processes were only found at very mature cell stages. At the height of maturation when plasma cells attained their final stage and antibody had accumulated in heavily distended cisternae, clasmatosis might be an important mechanism in order to release cell products.

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