

COMPARATIVE STUDY FOR ULTRASTRUCTURAL LOCALIZATION OF INTRACELLULAR IMMUNOGLOBULINS USING PEROXIDASE CONJUGATES

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J. Immunol. Methods 5, 33-48, 1974

SUMMARY

Various experimental conditions were tried to stain intracellular IgG globulins within popliteal lymph node cells of rats and rabbits by the use of specific antibodies and their Fab fragments labelled with peroxidase following two different procedures.

Fixation with 4% formaldehyde/24 hours, 1% formaldehyde and 1% glutaraldehyde/1-1.5 hours and 1-1.5% glutaraldehyde/1-1.5 hours provided satisfactory ultrastructural conservation of the tissues. None of these fixatives appeared to denature IgG globulins, but formation of cross-linkages prevents conjugates from reaching antigenic sites. Weak aldehyde fixation may enhance penetration of conjugates; however, ultrastructural detail is poorly preserved and proteins will leak out from insufficiently fixed tissue, thus increasing the possibility of staining artifacts.

Under our conditions, best results were obtained by using frozen sections prepared from well-fixed specimens. Apparently, the most important points are tissue fixation and sampling, above all other experimental steps. Conjugates with molar ratios of antibody or Fab fragment to peroxidase being one, seem more effective, although no significant differences were noted between peroxidase conjugates of whole antibody molecules and Fab fragments.

1. INTRODUCTION

There is a great interest in biological and medical research for localizing cellular components at the ultrastructural level using various markers (Rifkind et al., 1960; Pierce, Jr., et al., 1964; Nakane and Pierce, Jr., 1967; Sternberger, 1967; Hämmerling et al., 1969; Avrameas, 1970; Karnovsky et al., 1972). However, many difficulties for the intracellular localization of antigenic substances still exist.

In studies which combine immunological and electron microscopic methods both satisfactory conservation of the tissue and demonstration of the immunological reaction must be re-

* Supported by grants of the Deutsche Forschungsgemeinschaft, Bad Godesberg, Germany.

conciled. Our aim was to obtain procedures with which intracellular proteins can be reproducibly stained at the fine-structural level.

In this study immunoglobulins were identified within immunocompetent cells of immunized rats and rabbits by use of sheep antibodies conjugated to horseradish peroxidase. The influence of various fixations, antibody preparations and tissue sampling procedures upon the quality of intracellular tissue staining was evaluated.

2. MATERIAL AND METHODS

2.1. *Animals and immunization*

Adult rabbits (Giant Flemish or New Zealand) weighing approximately 4 kg each were injected into the hind footpads with an emulsion of complete Freund's adjuvant containing 5 mg of bovine serum albumin (BSA; Povite, Amsterdam) or *E. coli* alkaline phosphatase (Worthington-Freehold, N.J.). Three to five-months old WAG rats (derived from Wistar strain and raised at the Institut de Recherches Scientifiques sur le Cancer, Villejuif) were immunized in the same way, but using only 1 mg of protein.

In order to obtain the maximum number of immunocompetent cells, animals were killed 15-20 days after primary injection, or 3 days after a booster injection into the same hind footpads with the same quantity of protein in physiological buffer.

2.2. *Labelled antibodies*

Sheep anti-rabbit and anti-rat IgG *antibodies* or their *Fab fragments* were prepared and coupled with *horseradish peroxidase* (HRP, RZ 3; Boehringer-Mannheim, Germany) as described previously. Briefly, pure antibodies were isolated from whole immune sera using immunoabsorbents of copolymers of bovine serum albumin and rabbit or rat IgG respectively (Avrameas and Ternynck, 1969). Fab fragments were obtained from isolated antibody by papain digestion according to Porter (1959). Pure antibodies and their Fab fragments were concentrated (5 mg/ml and 2.5 mg/ml respectively) and then conjugated with peroxidase using glutaraldehyde; (a) *one-step procedure*: antibodies or Fab fragments were mixed with HRP and then glutaraldehyde was added; (b) *two-step procedure*: first, HRP was treated with an excess of glutaraldehyde and then allowed to react with the preparation of antibody or Fab. The procedures followed have been described in detail (Avrameas, 1969; Avrameas and Ternynck, 1971). Peroxidase-labelled antibody and Fab were separated from uncoupled peroxidase and antibody by gel filtration on Sephadex G-200. For controls, normal sheep IgG and its Fab fragments were conjugated with peroxidase.

2.3. *Tissues*

Animals were bled and the popliteal lymph nodes were immediately dissected out and placed in chilled Eagle's medium. A part of the tissues were sliced with razor blades into blocks and fixed. The other part was teased at 4°C in Eagle's medium and cell suspensions were washed twice by centrifugation at 1200 rpm.

2.4. Fixation and processing of tissue blocks

Formaldehyde freshly prepared from paraformaldehyde (Merck, Germany) and purified glutaraldehyde (TAAB, England) were used in 0.2 M cacodylate buffer at pH 7.2. All fixation procedures were carried out at 3-4°C under continuous agitation: (a) 4% formaldehyde for 24 hr; (b) 1% formaldehyde and 1% glutaraldehyde for 1-1.5 hr; (c) 1-1.5% glutaraldehyde for 1-1.5 hr. After fixation, the tissues were washed at 3-4°C for at least 24 hr with several changes of the buffer solution.

Prior to treatment with antibody, the tissue blocks were reduced to *small fragments* with a razor blade. From the larger slices, *frozen sections* were either cut at 10-40 μ in a Dittes-Duspiva cryostat (Kuhlmann and Miller, 1971) or at 5-10 μ in the cryo-ultramicrotome (Reichert, Austria) after BSA encapsulation as described in detail elsewhere (Kuhlmann and Viron, 1972).

Ultrathin frozen sections were cut from prefixed lymph node blocks, collected with Marinuzzi rings and floated on the surface of the incubating solutions (Kuhlmann and Viron, 1972).

2.5. Treatment and fixation of cell suspensions

Cell suspensions were either fixed immediately like tissue blocks or fixed after digestion of cell surface components by various enzyme preparations.

Cells (2×10^8) were suspended at 37°C for 10 min in 6 ml of Eagle's medium containing 0.2% *trypsin* (Choay, France). The reaction was stopped by adding 3 ml of calf serum and cells were immediately centrifuged and washed twice with Eagle's medium containing 30% calf serum. Similarly, 2×10^8 cells in 10 ml of Eagle's medium were treated with 50 units of *neuraminidase* from *Vibrio cholerae* (Behringwerke-Marburg, Germany) or with 5 mg of *hyaluronidase* (Sigma Co., St. Louis) for 60 min at 37°C. For treatment with *papain* (Worthington-Freehold, N.J.), 2×10^8 cells were suspended in 10 ml of Hank's solution containing 0.01 M EDTA and 0.01 M cysteine, mixed with 43 units of papain, and allowed to incubate for 60 min at 37°C. Control cells were suspended in 10 ml of Eagle's medium for 60 min either at 37°C or at 4°C. Cell suspensions were centrifuged and washed twice with Eagle's medium and fixed in (a) 4% formaldehyde for 24 hr or (b) 1.5% glutaraldehyde for 1 hr. Cells then were washed for 12-24 hr with several changes of cacodylate buffer.

In some cases cell suspensions were transferred into aqueous solutions of 15% bovine serum albumin and then insolubilized with glutaraldehyde (Kuhlmann and Viron, 1972). Ten-micron cryostat sections were cut, incubated in conjugates and subsequently in 3,3'-diaminobenzidine (DAB) and H₂O₂ medium (Graham and Karnovsky, 1966) and finally processed for light- and electron-microscopic examination.

2.6. Incubation procedures with labelled antibodies

After fixation of the preparations, free aldehyde groups were blocked using buffered 0.1 M lysine at pH 7.5 for 20-30 min.

Tissue blocks, frozen sections and cell suspensions were incubated in the different antibody-peroxidase conjugates for 1, 3 or 24 hr at 3-4°C or at room temperature. After incubation, blocks and sections were rinsed 3 times in buffer and subsequently washed with several changes of the buffer solution in 20 ml vials under gentle swirling according to 4 schedules: (a) 3 times in 10-15 min; (b) 6 times in 2hr; (c) 24 hr with 10 buffer changes; (d) 48 hr with 20 buffer changes. Cell suspensions were washed and centrifuged 4 times in buffer.

Peroxidase activity was revealed by incubation in the medium of Graham and Karnovsky (1966) for 20 min for blocks and thick sections, 10 min for cell suspensions and 3 min for ultrathin frozen sections. After washings in cacodylate buffer, blocks and thick sections were postfixed for 1 hr, cell suspensions for 20 min and ultrathin frozen sections for 2 min in cacodylate-buffered 2% OsO₄.

2.7. *Controls*

The specificity of the reactions was examined on tissue incubated with HRP alone and with normal IgG or their Fab fragments labelled with HRP, and then stained. Furthermore, lymph nodes were incubated with conjugates of sheep antibody anti-bovine chymotrypsin or their Fab fragments.

2.8. *Electron microscopy*

After post-fixation, the tissues were dehydrated in ascending alcohol and embedded in Epon (Luft, 1961) as described in detail elsewhere (Kuhlmann and Miller, 1971).

Epon-embedded tissues and cell suspensions were cut with a Sorvall MT 1 ultramicrotome and mounted on Formvar and carbon coated 200 mesh copper grids. Sufficient sections were cut in order to examine grids stained and unstained for 30 sec with lead citrate (Reynolds, 1965) or not. The sections were examined with a Siemens Elmiskop I operating at 80 kV and with a 50 μ objective aperture.

3. RESULTS AND DISCUSSION

The present paper was initiated to explore procedures with which intracellular antigens can be detected at the fine-structural level. We have chosen lymphoid tissue as material because the experimental conditions were thought to be most reliable: antigen as well as specific antibody were available in a highly pure form. Furthermore, the chosen cell system was of great advantage since solid tissues and cell suspensions could be compared in parallel.

Only lymph nodes in which a high proportion of immunoglobulin (Ig) -containing cells were observed by light microscopy were used for subsequent ultrastructural examination. The intracellular localization of Ig globulins in immunocompetent cells corresponded to what has been described as the perinuclear space (PNS), the ergastoplasmic reticulum (RER) and the Golgi apparatus using either anti-enzyme antibody techniques (Leduc et al., 1968; Kuhlmann and Avrameas, 1972) or specific antibodies coupled with peroxidase (Leduc et al., 1969). In addition, Ig globulins were observed in the extracellular space and on cell surface membranes.

3.1. Specificity and control reactions

Specificity of the immunological and histochemical reactions was controlled by incubation in HRP alone or in conjugates of normal sheep IgG or sheep antibody to unrelated antigen-like bovine chymotrypsin, followed by Graham and Karnovsky's medium. Only the known and already described endogenous peroxidase activity in certain cells, which do not synthesize Ig, was seen (Leduc et al., 1968).

Artifactual staining of damaged and pycnotic cells occurred and enhanced contrast of portions of cell surface membranes including the adjacent cytoplasmic matrix was seen (Fig. 1). This type of staining, however, was quite different from the typical and specific immunocytochemical reactions, where only PNS, RER and Golgi complex were tagged.

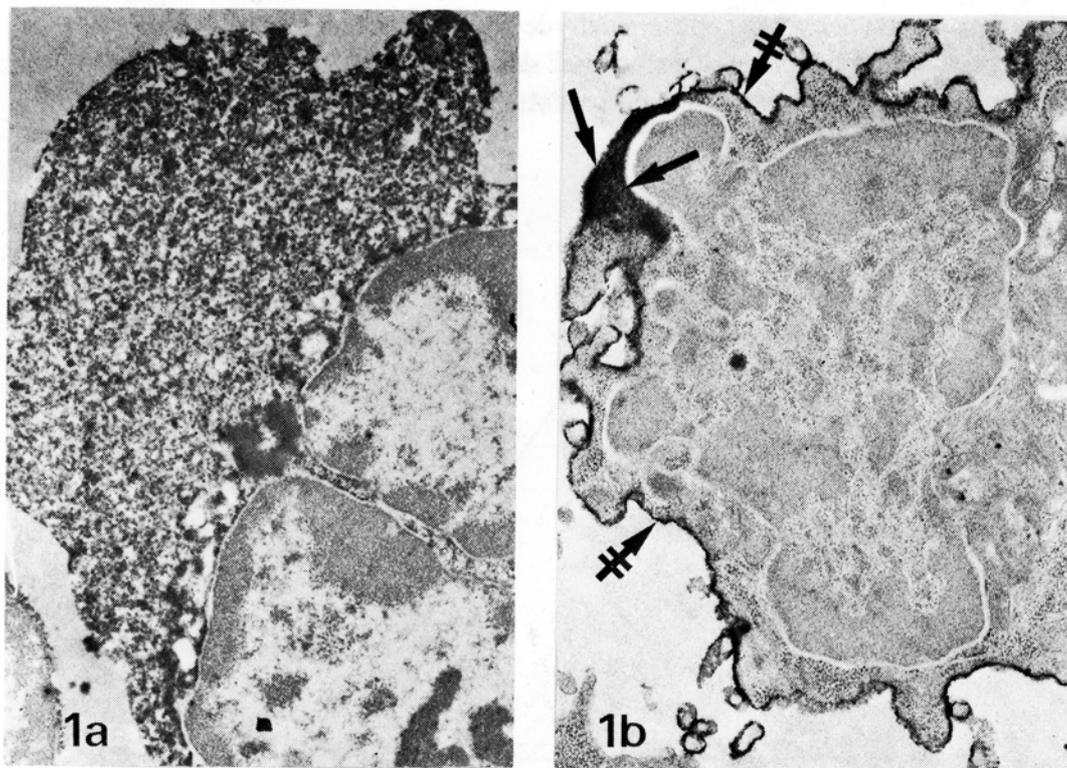


Fig. 1. Artifactual staining of cells: (a) Formaldehyde-fixed tissue; incubation in normal sheep IgG globulins conjugated with HRP. Note damaged cell, where staining occurs throughout the cytoplasm. Lead citrate 30 sec. \times 12,000; (b) Tissue as in fig. 1a, but incubated in specific antibody conjugate. Note staining of the cell surface membrane (\leftarrow +) of a lymphocyte, where parts of the cytoplasm (\leftarrow) have been stained too. Lead citrate 30 sec. \times 14,000.

After incubation in conjugates, the specimens were usually rinsed 3 times with buffer followed by 3 washings of 10-15 min each. The specificity of immunocytochemical staining was not affected when the washing procedures were prolonged up to 48 hr.

When, in the washing solutions after conjugate incubation of constant numbers of frozen sections, HRP activity was measured by photometrical methods, a high release of peroxidative activities from formaldehyde-fixed tissue was observed. In contrast, release of HRP activity from glutaraldehyde or glutaraldehyde-formaldehyde-prefixed tissue sections was too low to be measured (Fig. 2). When sections of either formaldehyde or glutaraldehyde-formaldehyde or glutaraldehyde-fixed lymph nodes were incubated in normal sheep IgG or Fab fragment

coupled with HRP, only negligible amounts of peroxidative activities were found in the washings.

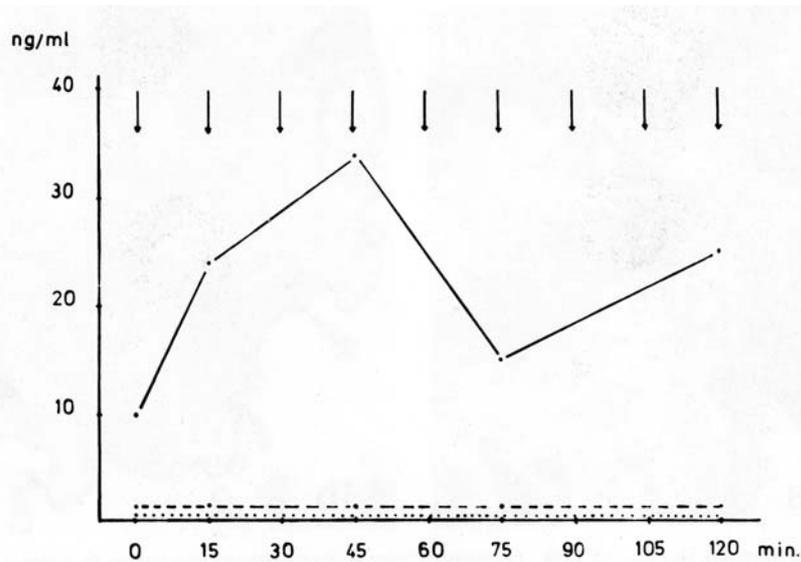


Fig. 2. Peroxidase (ng/ml) in washing solutions after incubation in Fab conjugates of either formaldehyde- (—) or glutaraldehyde- (---) fixed tissue. Formaldehyde-fixed tissue was also incubated in conjugate of normal sheep IgG (...). After initial rinsing (3 times) of tissue sections, buffer was changed every 15 min (↓).

The different behaviour of glutaraldehyde- and formaldehyde-fixed tissues during the washing procedures could be due to the different cross-linking qualities of the two fixatives; that being a continuous release of small amounts of conjugate-antigen complexes occurring in formaldehyde-fixed organ preparations.

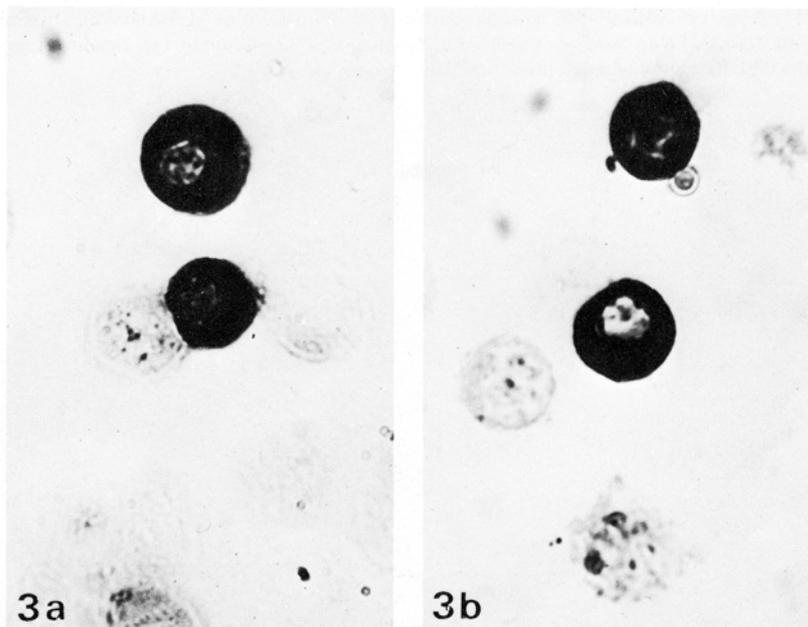


Fig. 3. Detection of immunoglobulins in the cytoplasm of immunocytes; smears of lymph node cells were fixed in alcohol-ether: (a) Incubation in whole antibody molecules conjugated with HRP. Original $\times 1,000$; (b) Incubation in Fab fragments coupled with HRP. Original $\times 1,000$.

3.2. Immunocytochemical staining under different experimental conditions

As already reported (Avrameas and Ternynck, 1971), the different conjugates when tested on fixed smears of lymph node cells showed good specificity and excellent staining in the cytoplasm of immunocompetent cells (Fig. 3). No labelled cells were seen when we used normal IgG or their Fab fragments coupled with HRP.

Peroxidase antibody conjugates prepared by the *one-step* procedure have been shown by SDS-acrylamide electrophoresis and by molecular filtration on Sephadex G-200 column to possess molecular weights higher than 210,000 while those prepared by the *two-step* technique were mainly composed of a homogenous fraction of a molecular weight of 210,000. Fab conjugates prepared by the one-step method have molecular weights ranging from 80,000 to 160,000 while those obtained by the two-step procedure give a homogenous peak at 80,000.

At the electron-microscopic level, conjugates prepared by the one-step procedure stained well (Fig. 4), but not as effectively as conjugates obtained by the two-step procedure (Figs. 5-7). This difference might be explained by the highly heterogenous populations of antibody conjugates after one-step coupling. Yet, nonspecific deposition of immunocytochemical products as described by Kraehenbuhl et al. (1971) did not occur with any of the conjugates used in the present work.

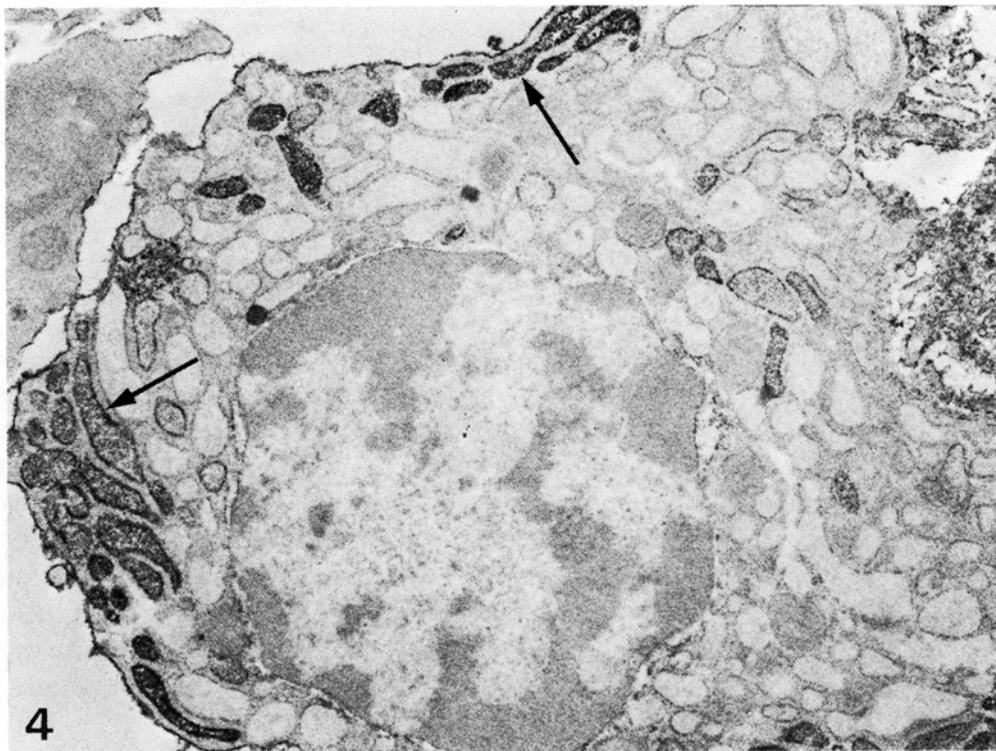


Fig. 4. Plasma cell with specific antigen-antibody reaction in some ergastoplasmic cisternae (←); 4% formaldehyde fixation, small tissue fragment and incubation in Fab fragment conjugated with HRP by the one-step procedure. No counterstain. $\times 13,200$.

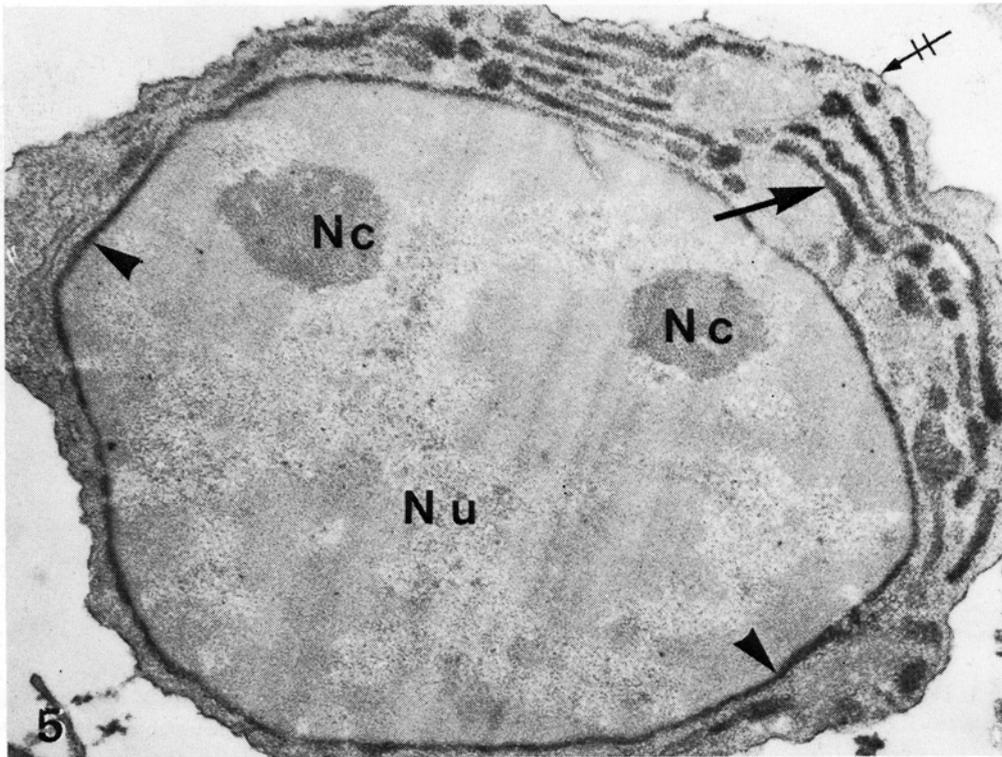


Fig. 5. Specific antigen staining within ergastoplasmic cisternae (←) and PNS (↔) in a plasma cell. Lymph node fixed in 1% formaldehyde and 1% glutaraldehyde for 1.5 hr; 40 μ -frozen section. Incubation was in Fab conjugated with HRP by the two-step technique. Note staining reaction of the cell surface membrane (↔); nucleus (Nu), nucleolus (Nc). Lead citrate staining. $\times 15,000$.

Conjugates where the molar ratio of antibody or Fab fragment to HRP is one (*two-step coupling*) proved to be very effective. Staining of antigenic determinants of Ig with those conjugates seemed to be more reliable because purification of the active complexes was readily obtained. No difference in staining capacity of whole antibody or Fab fragment conjugates were observed when thick frozen sections were used (Figs. 6, 7). Because intracellular penetration of conjugates in small, hand-cut tissue fragments was uncontrollable (see 3.4.), a valuable comparison of the different staining qualities of either whole antibody or Fab conjugates could not be elaborated.

For incubation purposes the concentration of antibody molecules and Fab fragments could be as low as 0.5 mg/ml without any effect upon antigen staining. Also, no differences were encountered when incubation times varied from 1 to 24 hr either at 3°C or at room temperature.

3.3. Effect of aldehyde fixations on the immunocytochemical staining

Ultrastructural detail for immunocytochemical work was well preserved with the employed fixation procedures, which have already been described in a previous paper (Kuhlmann and Miller, 1971). To examine the effect of aldehyde fixation on the immunological reactivity of Ig antigenic determinants, cell suspensions were fixed (see 2.4.), encapsulated by insolubilization into BSA and cut in the cryostat. Following peroxidase immunocytochemical reactions, intracellular antigen could be demonstrated at both the light- and the electron-microscopic

levels (Fig. 8). Thus, antigen-antibody reactions were still possible after fixation in 4% formaldehyde for 24 hr or 1.5% glutaraldehyde for 1.5 hr.

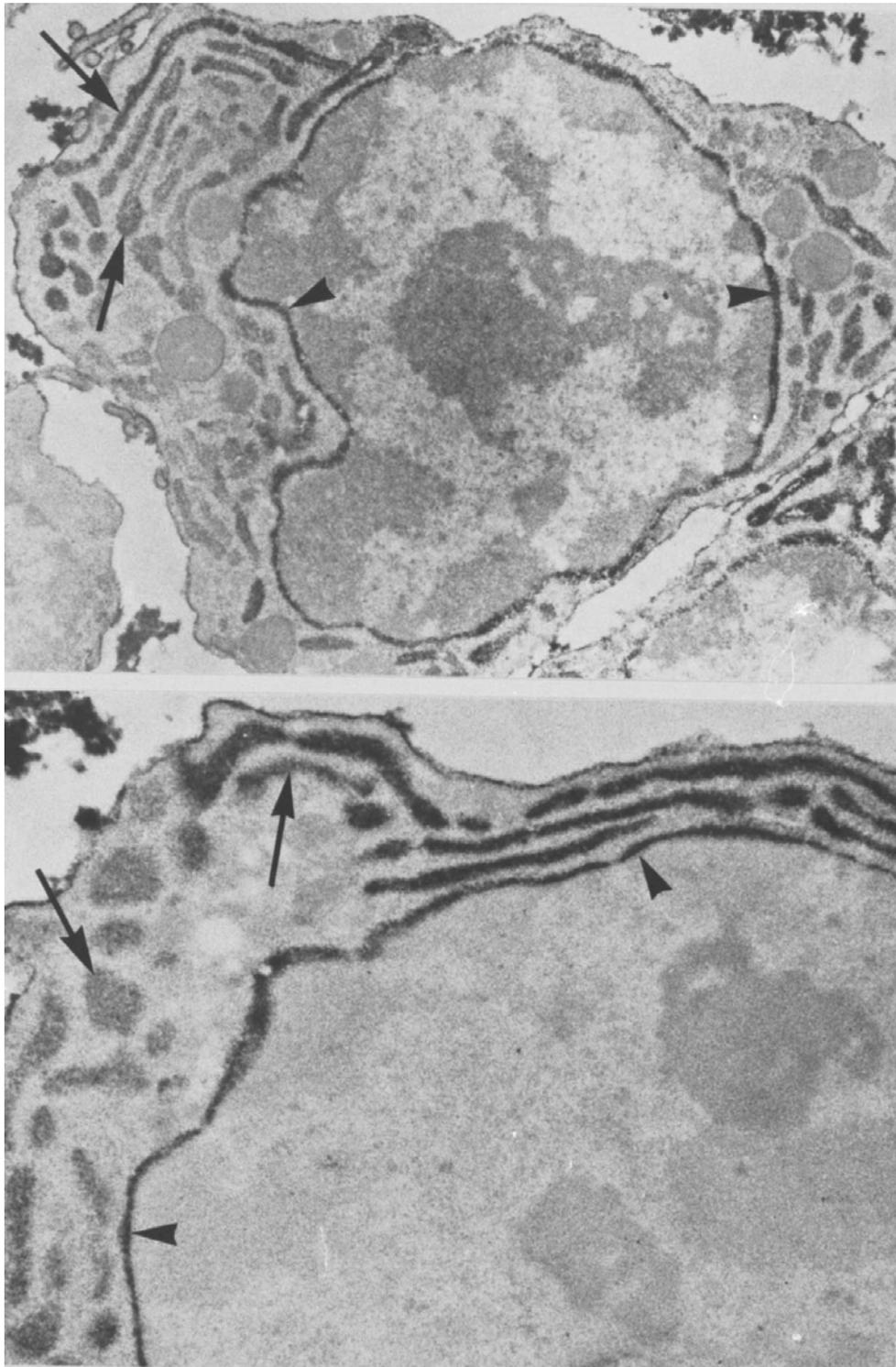


Fig. 6. Plasma cell synthesizing Ig in RER cisternae and PNS. Lymph node fixed in formaldehyde; 15 μ m thick frozen section incubated in whole antibody conjugate after two-step coupling. Lead citrate. x 11,900.

Fig. 7: Same lymph node as in Fig. 4; 15 μ m thick frozen section incubated in Fab fragment coupled with HRP by the two-step procedure. Specific reaction in RER cisternae and the PNS. No counterstain. x 22,500.

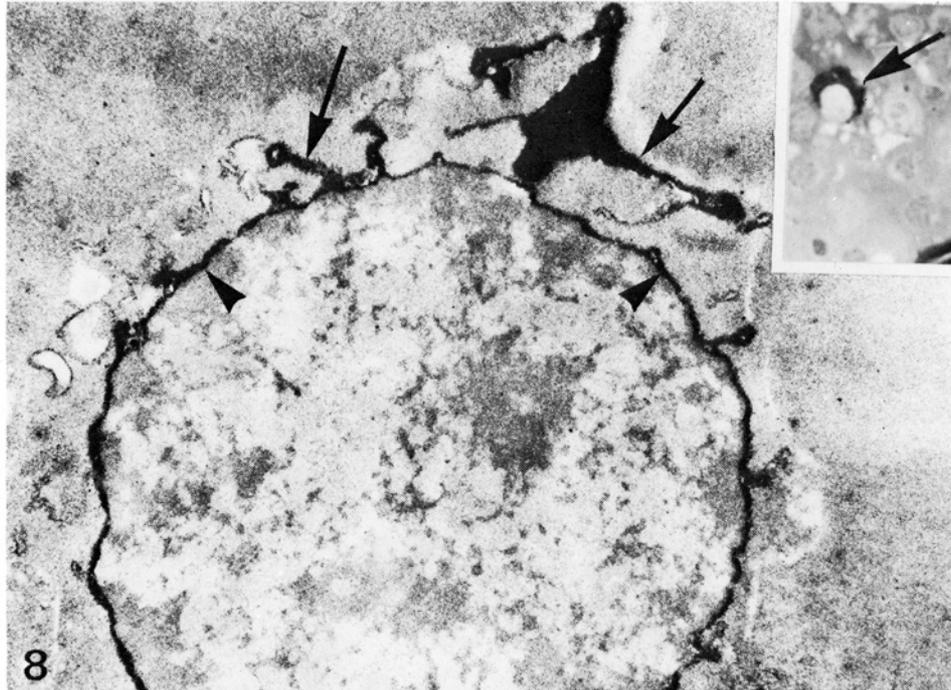


Fig. 8. Electron micrograph of an Ig synthesizing cell; lymph node cell suspension fixed in 1.5% glutaraldehyde for 1 hr, then insolubilized in BSA using glutaraldehyde as cross-linking reagent and cut at $10\ \mu$ in a cryostat. Specific staining in the PNS (\blacktriangleleft) and in RER cisternae (\leftarrow); lead citrate. $\times 13,800$; Inset: semi-thin section at the light microscopic level; note positive plasma cell (\leftarrow). Counterstained with Toluidine Blue. Original $\times 400$.

Furthermore, studies on the ultrastructural localization of anti-enzyme antibodies in immunocompetent cells have demonstrated that none of the employed fixatives, even the stronger ones, inhibited the subsequent immunocytological reactions (Kuhlmann and Miller, 1971). From all the above it may be deduced that appropriate fixation procedures must be elaborated independently for each biological material.

Yet, it must be kept in mind that certain fixatives, especially glutaraldehyde, may hinder the accessibility of antigenic determinants by labelled antibodies because of cross-linkages. The extent of this phenomenon is extremely difficult to assess, and negative results are consequently difficult to interpret. They do not exclusively mean either a deficient penetration of conjugates or a lack of staining because antigenic determinants were no longer available.

3.4. Intracellular staining of Ig in solid tissue preparations

The many series of experiments with different fixation procedures have demonstrated that the staining of intracellular Ig with anti-Ig antibody conjugates was mainly governed by the penetration of the incubation media and not by the aldehyde fixation used.

When examining the thickness of organ preparations which could be penetrated by the enzyme-antibody conjugates, we observed that in the case of small hand-cut tissue fragments, conjugates penetrated poorly and most often only border-lining cells came into contact with the incubation media. Generally, these failed to penetrate reproducibly into the cells and staining was obtained preferentially in the extracellular space of only superficial cell layers.

Even if the edges of the tissue blocks were examined in the electron microscope, positive cells were rarely found (Fig. 4).

Consequently, our next approach was the use of cryostat sections. Conjugates of either whole antibody molecules or Fab fragments diffused completely in 40 μ frozen sections, as verified by Epon-embedded transverse sections (Figs. 9,10). Thus, all cells of the organ preparations have come into contact with the antibody conjugate. At the fine-structural level, heavy staining of the extracellular space and of cell surface membranes was observed, but in the depth of the 40 μ sections intracellular antigen tagging was usually not seen. Cells lining the section surface exhibited intracellular staining (figs. 5-7).

From the above evidence it is clear that cell membranes may act as barriers and thus techniques which disrupt them would be advantageous. Therefore, consistent results were obtained with 10-15 μ -thick frozen sections (Figs. 6, 7), for which strong fixatives, as those in the present study, must be employed. Otherwise, poor results are to be expected and were obtained using weak fixatives (Leduc et al., 1969). Thinner sections in the range of 5-10 μ were also efficient, but the fragility of such preparations made them difficult to manipulate.

We conclude from our experiments that, using strong fixatives and appropriate sampling techniques, frozen sections are preferable to insufficiently prefixed tissue blocks.

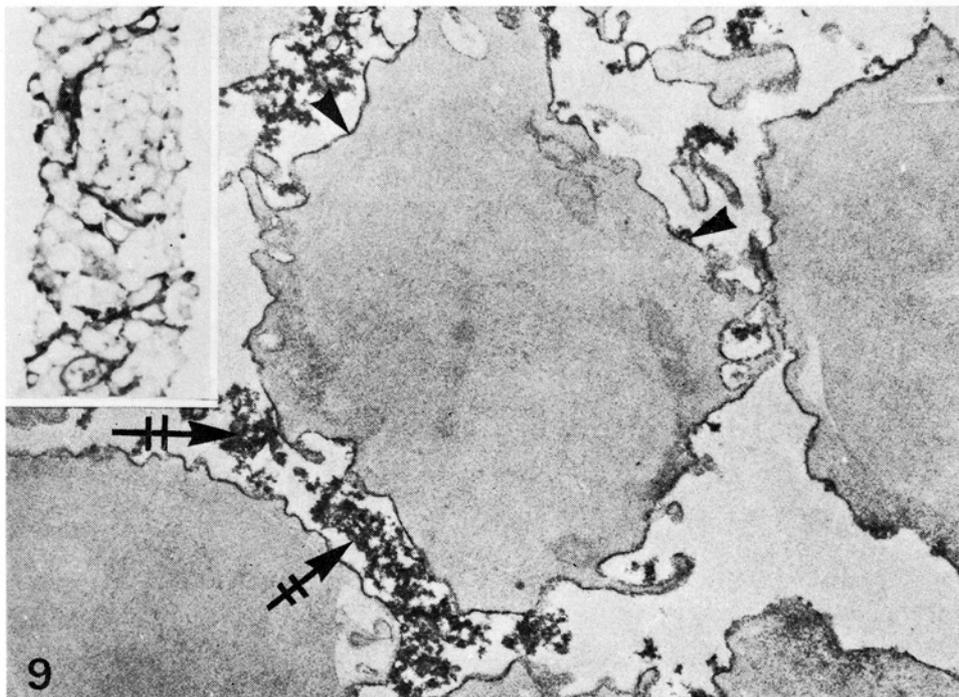


Fig. 9. Staining of extracellular spaces (+) and cell surface membranes (◄). Formaldehyde fixation; 40 μ -frozen section incubated in Fab conjugate. No counterstain. $\times 10,800$; Inset: transverse section after Epon embedding at the light-microscopic level. Note good penetration of the conjugate across the tissue preparation. Original $\times 400$.

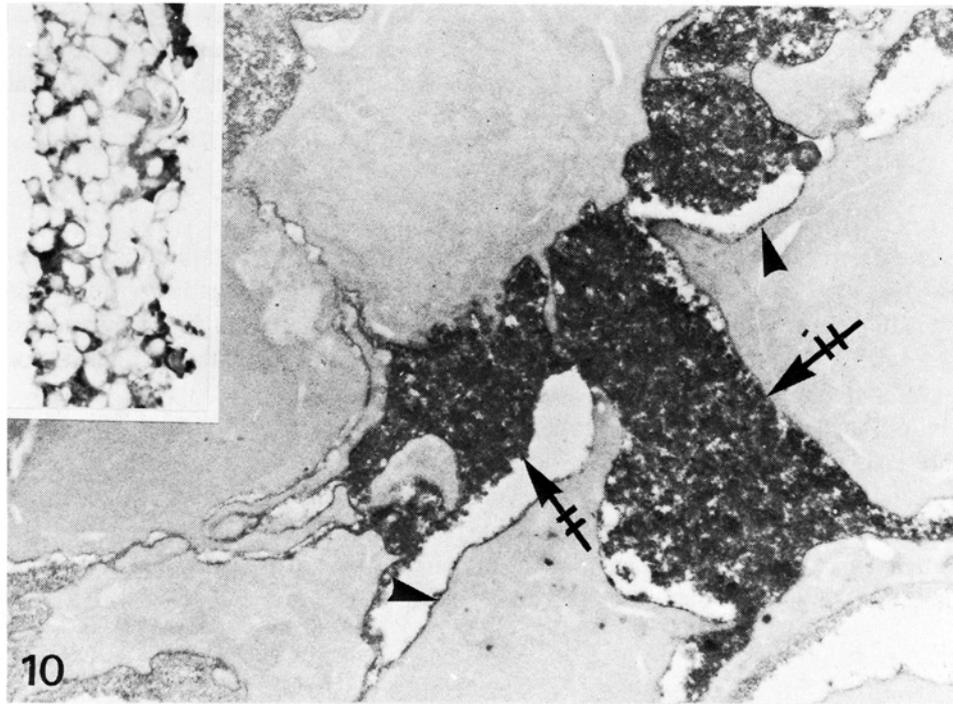


Fig. 10. Extracellular spaces (++) and cell surface membranes (◄) are heavily tagged. Fixation in 1.5% glutaraldehyde for 1.5 hr; 40 μ -frozen section incubated in Fab conjugate. No counter-stain. $\times 11,000$; Inset: semi-thin section; good penetration of the conjugate across the 40 μ -frozen section. Original $\times 400$.

3.5. *The approach of ultra-thin sections*

Ultra-thin sectioning of embedded material has been employed for the localization of antigens by several authors (Sternberger et al., 1965; Striker et al., 1966; Thomson et al., 1967; Kawarai and Nakane, 1970; McLean and Singer, 1970; Sharabadi and Yamamoto, 1971) and penetration of conjugates was observed although negative results were also reported (Leduc et al., 1969). In our system, staining of antigen on ultra-thin sections after glycol methacrylate (GMA) or methyl and butyl methacrylate embedding was without success. Thus, we did not present these techniques in detail. It may be that in our experiments the remaining antigenic determinants in the sections were either masked or were too few to be stained histochemically.

In order to avoid plastic embedment and dehydration, ultra-thin frozen sections from prefixed lymph nodes were tried. Sufficiently thin sections (Bernhard and Leduc, 1967) to be examined in the electron microscope were not stained by the HRP-conjugated antibodies. Thicker preparations were stained, but fine-structural localization of Ig globulins could not be judged because the dense and heavy reaction product of the marker substance obscured any detail. It may be possible that ultra-thin sections of our material do not contain sufficient determinants to interact with the conjugates or that the oxidized DAB does not accumulate in the usual manner.

3.6. *Localization of Ig in cell suspensions*

Apart from positive reactions on plasma membranes of certain numbers of lymphocytes, blast and plasma cells, these cells were most often negative within their intracellular compartments,

i.e. in the PNS, the RER and the Golgi apparatus. These results underline the necessity of altering the limiting plasma membranes for intracellular staining of Ig antigenic determinants using conjugates.

In order to overcome this problem, enzymatic digestions of the cell-coat of viable cells were performed. After subsequent fixation, washing and incubation in the usual way, enhanced penetration of conjugates was obtained, but the total number of stained cells was still moderate, and many cells remained completely negative (Figs. 11, 12). However, quantitative data on intracellular staining after neuraminidase, hyaluronidase, or papain digestion were impossible to obtain. During trypsin treatment many cells were lost, and those remaining often suffered with respect to their fine structure. Staining reaction in positive cells was restricted most often only to some RER and PNS compartments, and never was the whole ergastoplasmic system tagged. In all these cases, the plasma membranes could be stained or not. We conclude that enzymatic treatments under our conditions did not guarantee optimal penetration.

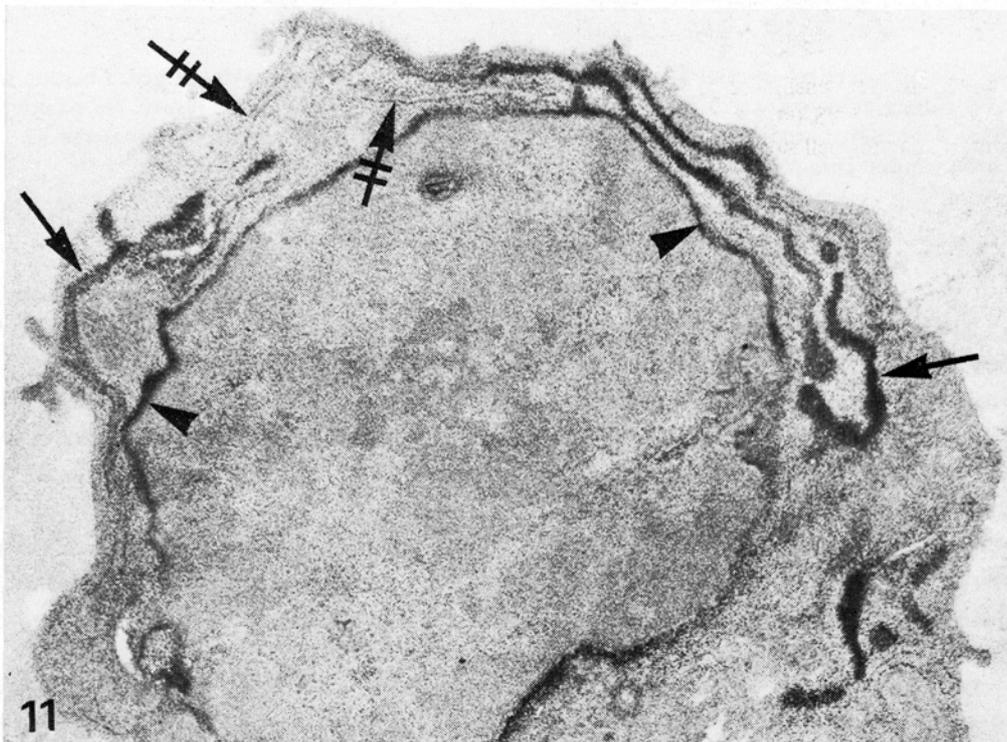


Fig. 11. Cell suspension treated with neuraminidase, then fixed in formaldehyde and incubated in Fab conjugate. Staining reaction in the PNS (◄) and in some RER cisternae (←), others are negative (⇧). No counterstain. $\times 15,000$.

3.7. Observations on extracellular Ig

Ig excreted from immunocompetent cells was responsible for the regularly observed extracellular staining. The more excreted Ig was fixed in the extracellular space, the more intracellular penetration of specific conjugates was impeded. This, could be either by blocking the diffusion of the reactants across the extracellular space or by covering cell surface membranes with the secreted Ig. Both phenomena depended on the cross-linking capacity of the aldehyde used as fixative. Thus, in glutaraldehyde-fixed lymph nodes extracellular staining was much

heavier than in formaldehyde-fixed ones (Figs.9, 10), but penetration of conjugates through the tissues was facilitated by the use of frozen sections.

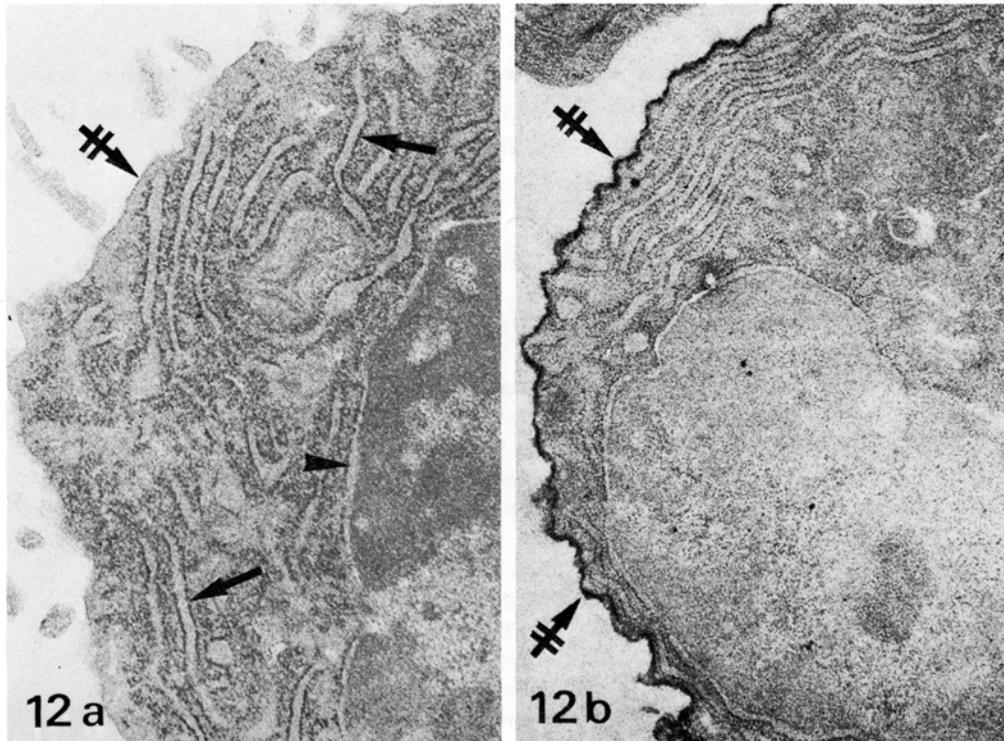


Fig. 12. Cell suspension as in fig. 11; lead citrate counterstained: (a) No staining at all in the RER (←), the PNS (◄) and on the cell surface membrane (⇐). × 20,000; (b) No intracellular staining, but the cell surface membrane (⇐) is tagged. × 15,000.

ACKNOWLEDGEMENT

This work was begun when W.D.K. was at the Institut de Recherches Scientifiques sur le Cancer in Villejuif. We thank Dr. W. Bernhard for facilities in his laboratory and Mrs. J. Burglen for excellent assistance.

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