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# Applications of monoclonal antibodies in morphological, structural and functional studies in electron microscopy

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**The massive advances in our understanding of the structure of cells and tissues in the last decades have depended on the increased resolution achieved with the electron microscopy. Electron microscopy is now a standard technique with wide applications in all branches of science and technology, and every year several papers are available describing new and sophisticated techniques that allow to obtain direct evidence for understand by electron micrographs of cells, subcellular particulates or macromolecules. In particular, the request of new immunocytochemical methods is focused to delucidate many problems in biological and medical sciences. This paper delineates the role of different immunocytochemical techniques used at ultrastructural level and focalizes the practical and theoretical interrelations between these methodologies.**

KEY WORDS: Antibodies monoclonal - Immunocytochemistry - Electron microscopy.

Immunocytochemistry (ICC) is a technique based on the recognition of an antigen by a specific labelled antibody.[1-4] ICC is employed to analyse the location and the identification of intra or extracellular antigens. Immunological staining contributes new highly specific data on the localization of documented antigens or occasionally of particular chemical residue. Certain dynamic experiments are possible and sometimes staining can be quantitated and its pattern analysed. The identification of these substances can occur at light microscopy, including also confocal microscopy, as well at the electron microscopy level. The latter can be employed at transmission electron microscope (TEM) or at scanning electron microscope (SEM) level. ICC is based on the use of antibodies (Ab) labelled with visual markers such as fluorochrome, ferritin, radioiodine, gold particles or with enzymes such peroxidase or phosphatase. At electron microscope level these last tracers act via the deposition of electron-dense precipitate. The use of opportune markers and probes records the reaction between antibody and antigen. Anyway for any research project that requires the use of labelled antibodies it is important to carefully evaluate the label to be used since each has its advantage and disadvantages. It is generally the case in all branches of science, the use of any particular technique or methodology is largely dependent on prior knowledge and expertise of the operator. In electron microscope methods it is essential to have moreness than casual understanding of light microscopy results. Moreover, a study using fluorescent or peroxidase-labelled antibody at the LM level is highly recommended before proceeding to EM immunological staining. These preliminary preparation are absolutely vital to achieve the full potential of the immunocytochemical method. But what must be remembered always, is that the results obtained at light and electron microscopy must be congruent.

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*Antibody*

Antibodies (Ab) are multichain glycoproteins with an equal number of heavy chains and light chains. The molecule is made up of four polypeptide chains and made up of two equal pairs linked together by a disulfide bond and non-covalent interactions. The basic symmetrical units are symmetrical and each half contains one heavy (H) chains and one light (L) chains making up a complex Y shape with each of its three tips formed by two roughly compact and globular structures joined by laxer segments. The two tips of the Y present the "combinatory sites" each of which are mainly made up by hypervariable regions of an H chain and an L chain forced to interwine repeatedly to allow the six hypervariable segments to form a superficial furrow to combine with hydrophilic antigens, or a deep receptacle to collect the hydrophobic antigens. The hinge section then, enables the two branches to effect significant oscillations in the three spatial dimensions easing the backup of two "antigenic determinants", always identical one to the other. The antibodies may be polyclonal or monoclonal. The polyclonal antibodies (pAb) are obtained by the serum of laboratory animals (rabbits, goats, horses) after immunization through an immunogenic determinant. They are characterized by a variable affinity for the antigen and can identify additional antigenic determinants (or epitopes) present in the same Ab molecule (Ab in low or medium specificity). The monoclonal antibodies (mAb) are produced by a single mouse or rat cellular clone obtained through the hybridization of a myelome cell with a plasmacell capable of synthesizing a highly specific Ab. The principal advantages of monoclonal antibodies consist in the monospecificity and in the possibility to make available in time, of a quantity theorically infinite, of the same Ab that would ease an elevated reproduction of the reaction. The principal disadvantage consists in the possibility that the only antigenic determinant recognized by the Ab is casually present even in a different molecule from the one intended to localize (cross reaction). The antibodies that are most frequently used in immunocytochemistry belong to the IgG class. Anyway antibodies that may be used in immunocytochemical reactions sharply, if possible, satisfy the following requisite:

— they must show a definite affinity with the substrate,

— they must possess an intense linking attribute,

— they must finally possess an adequate concentration.

The dilution of the primary antiserum varied, however, in relation both to the type of substrate employed and to the method, directly or indirectly, used for the immunocytochemical activity.

Immunological staining may be applied in either of two ways. First, the antibody may be conjugated to the label and used to stain the antigen directly (the direct method). Alternatively, a "primary" unconjugated antibody may be applied to the antigen and the antibody thus bound stained with a labelled secondary antibody against the "primary" antibody (the indirect method). Either of these approaches may be used in the immunocytochemistry techniques at light and electron microscopy.

*Antibody labels*

The first great majority of localization of antigens at the ultrastructural level involved the use of antibodies labelled chemically to ferritin an electron-dense marker.[5,6] Ferritin is an iron storage protein with a high electron density. It consists in an almost roundish peptide that has a molecular weight of about 445,000 containing up to 5,000 iron atoms. Ferritin present some disadvantages, such as the large molecular dimensions due to apoprotein, even if electrodense core is concentrated into a roughly cubical central region about 7 nm in diameter and the possibility of the formation of aggregates which renders difficult the penetration in the tissues.

Radioactive iodine ($^{125}I$) has been used as labelling for antibody.[7-9] It is used for morphological study in both light and ultrastructural evaluation and its presence can be detected after radioautographic as electron-opaque silver grains. At the ultrastructural level $^{125}I$ label has the advantage that the label can be observed also at low magnification and that the scintillation counting can be carried out on antibody-labelled cell population to determine the total antibody uptake. The disadvantage of ultrastructural radioautography is in the use of radioactive materials and the low resolution results

in part because the nuclear emulsion over the cells contains silver halide crystal that can be reduced to metallic silver at some distance from the radiation emitting source. Thus, a silver grain does not represent the exact point where radiolabelled antibody is located. Nakane and Pierce [10 11] introduced a new approach for the localization of antigens, when they prepared enzyme-antibody conjugates. The binding of these reagents to antigen is detected by means of an appropriate enzyme cytochemical reaction. The main advantages of these methods are first, in the production of a "stain" visible with the light microscope and, secondly, in the possibility of obtain, using a specific substrate, an electron-dense precipitate easy to observe at electron microscope level.[12 13] Enzyme-antibody conjugated are much lower molecular weights than ferritin-antibody conjugates improving the penetration properties of labelled antibodies stain into the tissue or the cells. Initially has been suggest two possible labelling enzymes horseradish peroxidase and wheat-germ acid phosphatase. The latter anyway have proved too unstable for general use and have recently been substituted labelling the antibody with alkaline phosphates. Horseradish peroxidase (HRP), a substance rich in enzymatic activity, is a glycoprotein with a molecular weight of about 40,000, of small dimensions (4.4 nm in diameter) and thus equipped with an acceptable penetration in the intracellular compartments. The evidence for the enzymatic reaction catalysed by the HRP is based on the formation of an electron-dense reaction with the appropriate chromatic characteristics and an electronic density, generally represented by a benzidine derivate, 3-3' diaminobenzidine (DAB). In particular, the reaction induced by HRP causes a release of oxygen in the presence of hydrogen peroxide and the oxidation of the DAB that immediately reaches the area of the reaction as an insoluble precipitate that appears to have a brownish colour under a light microscope. Thus, the product caused by the reaction, reacted with osmium tetroxide forming an insoluble product highly electron-dense, allowing the localization of the antigenic sites.

Recently, there has been a great deal of progress in the field of immunocytochemistry mainly because of the introduction of gold particles and to the application of the complex protein A-gold for the localization of intracellular antigens on ultrathin sections.[14-16] The physical-chemical characteristics of gold particles make them the most widely-used markers for electron microscopy post-embedding immunocytochemical analysis. In fact, besides having a high electronic density and being a marker with a well-defined shape, it allows an ideal visualization and localization of the antigen-antibody reaction and offers the possibility to affect quantitative analysis. It may be attached, through electrostatic absorption, to various molecules without affecting its binding capacity. Attached to the Protein-A, it may be used for a variety of studies, guaranteeing also, a highly accurate performance. The gold particles may be prepared in the desidered size starting from less than 1 nm. The possibility to determine its diameter, makes it possible to mark different antigens on the same section. It may be used simultaneously either scanning or transmission electron microscopy.

*Fixation of tissue, cells and cellular fractions*

Although some applications of immunological stains are made to antigens on live cells and to freshly isolated subcellular fractions, most have the necessity to mark antigens in or on fixed cellular material.[17 18] To obtain a correct immunocytochemical localization of the antigenic sites, experiments on the survival of antigenicity is necessary in all projects where fixed material is employed. It has been calculated that 40-50% of receptor sites may be lost due to fixation. Each antigenic determinant will be affected differently by the fixative via direct modification or conformational changes or both.

These effects will be modified by several factors including accessibility of the molecules to the fixative vehicle and the lenght of fixation. At present it is necessary for all experiments to proceed with great caution and every effort should be made to gain information on fixative-induced loss of antigenicity in each system studied. Fixatives are capable of stabilizing the cellular structures to prevent dislocation phenomenon and the extraction of the substances contained in them and thus also the antigens. The fixative should also be able to preserve the antigenicity since it is the most important factor for success in ICC. The goal of fixation is to

avoid structural decomposition, to hinder diffusion of soluble component and to fortify the cell. It becomes quite evident, therefore, that the maintenance of a satisfactory morphology and sufficient antigenicity are phenomena inversely proportional and thus the choice of an ideal fixative for electron immunomicroscopy must be effected by finding a compromise between these two requisites. Generally, a minor degree of "cross linking" results in a better preservation of antigenicity enabling, at the same time, the reagents to reach the antigenic reactive sites.

The fixative that are still more widely used are those with a base of glutaraldehyde and formaldehyde in different concentrations regarding the characteristics of the antigen under examination.[19] For those antigens that would be completely changed by even a bland fixation in glutaraldehyde, it is recommended the use of formaldehyde only. Often, in cases involving more fragile antigens a less than 4% formaldehyde solution can be used or fixatives specifically introduced for immunocytochemistry such PLP (periodate-lysine-paraformaldehyde)[20] or picric acid-formaldehyde.[21]

Despite the fixative used, fixation must be done in a way that is as rapid and uniform as possible in every part of the sample.

Anyway after an aldehyde-fixation it is strongly recommended to treat the tissue with a glycine 1% to prevent the formation of eventual clusters of free aldehyde groups that could react somehow with aminogroups of the primary antibody. Regarding the formaldehyde fixative the commercially available products are often unsuitable for electron microscopy since they contain a significant concentration of methanol. Formaldehyde fixatives have to be prepared from powdered paraformaldehyde.

### *Cell permeabilization*

The low penetration rate of antibodies through cell membranes may constitute a main obstacle in showing intracellular antigens, particularly in EM. The optimal fixation conditions necessary for the preservation of the morphological features without the loss of diffusible antigens involve a rapid and complete fixation, whereas antigenicity preservation usually requires the lowest degree of fixation. Therefore, a compromise between the opposing criteria of fixation and permeabilization should be established always. It remains very difficult, however, to establish and to guarantee the effect of fixation on the penetration of the antibody. Various procedures have been perfected to increase the penetration of the reagents in well fixed tissue or isolated cell population.

Cells or small pieces of tissue can be permeabilized either by chemical agents or by some detergent substances. These last may be applied during different phases of the process to improve the antibody penetration, but should be accompanied by a fixation to prevent both loss of internal structure ad loss, or diffusion, of antigen. Triton X-100 may be added to the dilution buffer of the primary antibody in variable concentration between 0.04 to 0.4%. The Triton X-100 treatment causes, however, a certain amount of destruction of the membranes causing a damage of cell morphology. The other agent NP-40 or digitonin presents the same problems observed using Triton X-100. Saponine, a glycoside of vegetable origins capable of forming complexes with cholesterol of plasma membranes in concentrations less than 0.03 mg/ml, seems to have a less drastic effect compared to Triton X-100 and sometimes it is unquestionably preferable and if used correctly can be used also for intracellular studies.[22-26]

### *Pre- and post-embedding techniques*

The essential difference between light and electron microscopy, it is essential to have a better preservation of the ultrastructure. This requirement creates, as previously explained, an obstacle for the demonstration of intracellular antigens since the penetration of labelled antibodies through membranes decreases considerably upon improved preservation. Several approaches have been tried to avoid these difficulties.

One general way is to improve permeability of membranes and to detect the antigens before embedding ("pre-embedding staining methods"), the other is the detection of antigens on ultrathin sections after embedding and sectioning ("post-embedding staining methods"). The technique of pre-embedding consists in bringing about an immunological reaction before the sample is submitted to the inclusion process. This technique for long time has been principally used for the local-

ization of surface antigens in isolated cells. It may be also used in the localization of tissue antigens (extra or intracellular) though this use has some drawbacks. For example, the antibodies and markers have a limited penetration and this causes an obstacle for the diffusion of immunological reactives. The cross-linking introduced by the fixative inhibit the penetration of the antibody in the tissue and this is a particularly evident with bifunctional fixatives such as glutaraldehyde. Therefore, all samples must necessarily be submitted to a preliminary bland digestion to make the plasma membrane permeable. The last procedure also causes a partial destruction of the submicroscopic morphology. Anyway the main advantage of the pre-embedding technique is found mainly in the maintenance of antigenicity, and as will be shown, the pre-embedding technique may be the method preferred in several immunocytochemical reactions. The antibody marker most widely used in immune-electron-microscopy pre-embedding techniques is represented by peroxidase, especially when the immune-marker must be executed to analyse intracellular antigens; other markers such as ferritin and gold particles have the decided disadvantage of limited penetration due to their large size. In the post-embedding, all immunological reactions are made directly on ultrathin sections mounted on grids. In immunocytochemical reactions in post-embedding, higher concentrations of glutaraldeyde may be used with respect to those normally used in pre-embedding and this suggests that the effect of the fixative is to be found not so much on the antigenicity itself but rather on the possibility of the antibody's penetration. However, several problems may arise: (1) the processes of inclusion, the dehydration and embedding may all be detrimental for antigenicity especially in the case when the antigen is present in low concentrations or is especially prone to deconstruction or given the impossibility of marking; (2) plastic embedding materials absorb nonspecifically proteins; (3) epitopes are covered by plastic; (4) staining products spread out more on the surface than intracellularly, giving relatively poor resolution. Samples may be fixed, processed and immunostained by standard procedure but, in case of unsatisfactory results loss of antigenicity must be suspected. Nevertheless, the post-embedding methods are the most popular for ICC.

*Control observation in immunocytochemistry*

Each immunocytochemical analysis is subjected to a series or rigid controls of specificity to determine the exact interpretation of the obtained results. The simplest, but also the most useful control consist in the use of substrate that are certainly positive or negative.

Anyway, to study an antigen, one should make use at least of the following controls relating to the method employed.

1. Application of a homologous normal serum in the site of the primary antiserum; 2. blockage of the reactive site for the marked antiserum with a homologous non-marked serum; 3. removal of primary antiserum to observe eventually non specific reactions between the samples and secondary antibody; 4. the use of normal serum in the site of the secondary antiserum or omission of the same; 5. if the tracers are enzymes-labelled antibodies, it is fundamental to inactivate the endogenous activity eventually contained in the target cells.

## Our approach

*Pre-embedding immunocytochemistry*

Our experiences in immunocytochemistry have been focused on the use of pre-embedding techniques since this method gives us the possibility to identify antigens present at cytoplasmic or at nuclear levels and to recognize certain intracellular dynamic events related to cell activation and cell differentiation.[22-26] In the following sections, we demonstrate that this method is easy to use and contributes significantly to morphological study of cell biology. All experiments have been made both at light and electron microscopy using a differently labelled secondary antibody. As previously described the immunocytochemical analysis has been carried out at the first at light microscopy level using cytospun-fixed cells.

*Surface antigens*

Many papers describe the localization of surface antigens and include a considerable variety of experimental details. In general the extracellular antigens can be easy to detect using also a pre-

embedding technique. In our experience, good results are obtained using fixed cells since live cells are capable of taking up proteins by pynocitosis. The choice of the fixative depends on the activity of antibody used and how morphological features need to retain.

*Analyses of intracellular antigens*

The development of methods using labelled antibodies for EM intracellular studies gives new views because into the cells the antigens exist under two different forms, first linked to a specific intracellular structure giving then a stable antigen; secondly the antigens are not associated and move free into the cells giving dynamic antigens.

*Analyses of steady antigens*

For this purpose we prove that by a pre-embedding technique it is possible to localize antigens present in different cellular compartments. For the analysis of nuclear enzymes as terminal deoxynucleotidyl transferase (TdT) we used cells fixed in suspension with 2% paraformaldehyde and permeabilized with saponin to improve penetration of the antibody reagents. The distribution of the product of immunoperoxidase reaction appears exclusively confined to interchromatinic regions as a fine immunoprecipitate (Fig. 1A). This result is the same observed at light microscope level.

*Staining sub-cellular fractions*

The isolation of particular organelles, membrane or nuclear components is a highly sophisticated process. Many such fractions are well suited to the application of pre-embedding immune microscopy methods. Usually the obtained purified fractions can be processed as described above since the antigenic determinants are easily reached by the specific antibody. A preparation of nuclear matrix obtained from lymphoblastoid cells has been used to identify the intranuclear sites of TdT. The distribution of the product of immune-peroxidase reaction is shown in Figure 1D as an immunoprecipitate which appears homogeneously distributed in the residual nuclear framework. The procedure used for the isolation of the nuclear matrix does not seem to affect the localization and chemical structure of the enzyme since as compared to the in situ features. A different pattern of TdT localization has been obtained analyzing the cells during the different phases of cell cycle. Figure 1B shows a methaphase with the immune precipitate homogeneously distributed at cytoplasmic level. A cortical rat thymocyte (Fig. 1C) displays the TdT localized at nuclear and at cytoplasmic level. A modulation of TdT can be observed during the thymocytes differentiation. The results obtained by pre-embedding ICC prove that the localization of the TdT is under the control of cell growth and cell differentiation. During the interphase the TdT is localized at nuclear level, linked to nuclear matrix while during the mitosis or during genes rearrangement moves to cytoplasmic level without a specific ultrastructural localization.

*Analyses of dynamic antigens*

Different enzymes involved in signal trasduction as PKC and PLC or intracellular movement of certain surface receptors can be revealed by immunocytochemical analyses. Using a human lymphoblastoid leukemic cell line (Fig. 2A) it is possible to show the complete absence of PKC at the nuclear level, while the cytoplasm appears diffusely stained (Fig. 2B). After a specific activation, an intracellular movement of PKC can be detected from the cytoplasmic compartment into the nucleus (Fig. 2C). Detailed ultrastructural analysis proves that PKC penetrates inside the nucleus through the nuclear pores (Fig. 2D). Same results can be visualized by confocal microscopy analysis. PKC is stored at cytoplasmic level without specific localization or in activated cells it is possible to observe an intranuclear movement of the enzyme. At this level the specific immunoprecipitate is localized at interchromatinic regions. Pre-embedding immunocytochemical analysis of glucocorticoid-treated thymocytes displays during the induced-apoptosis an increase and an intranuclear movement of PKC, which is evenly distributed in the cytoplasm while, at the nuclear level, it appears restricted to peculiar areas. The intranuclear localization of immunoreactivity, more evident at higher magnification (Fig. 3), suggests that PKC translocated to the nucleus is bound to the residual nuclear matrix.

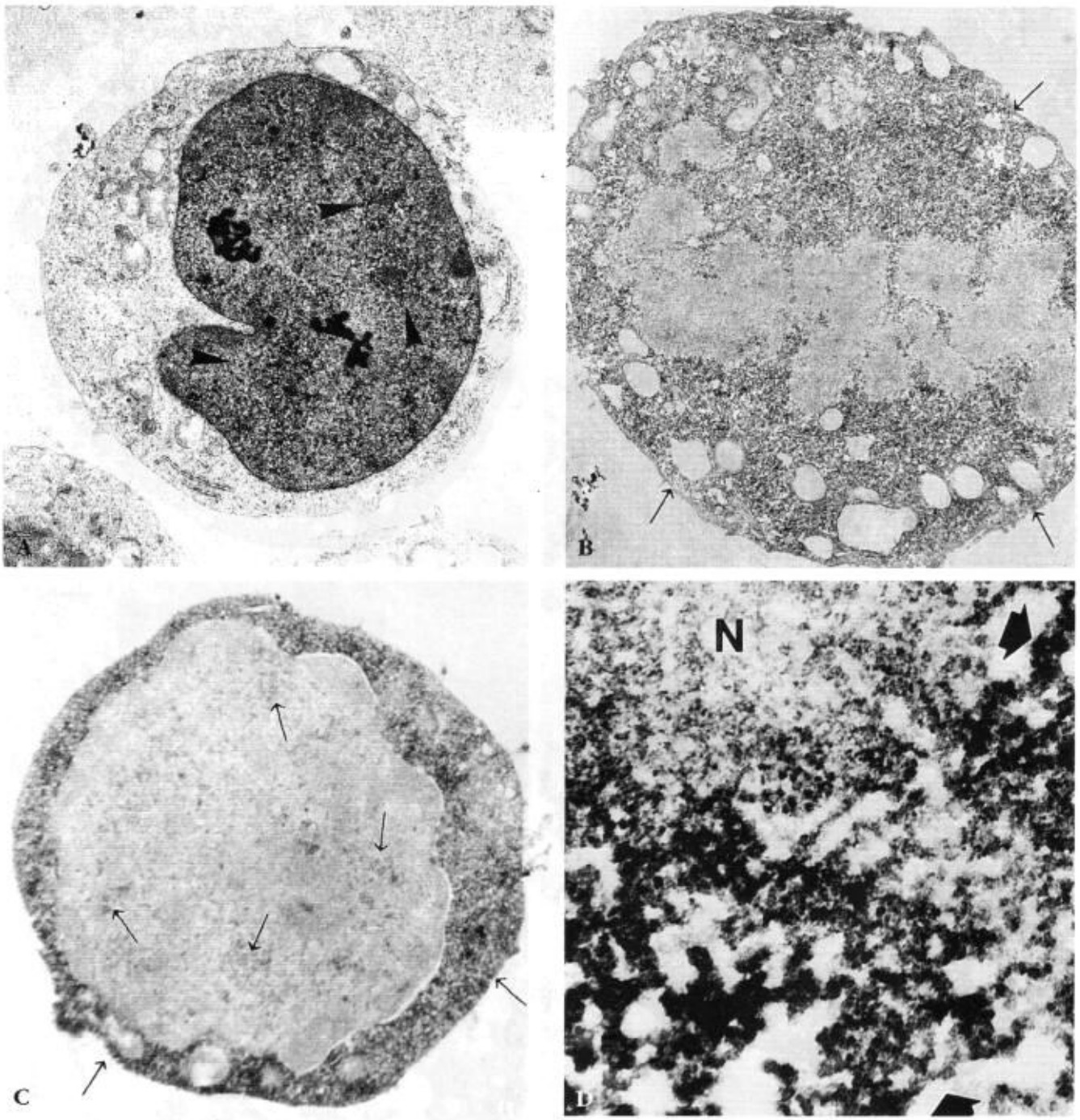


Fig. 1.—Electron microscopy observation of peroxidase-stained sample. At this level it is possible to localize the TdT, as a fine dark immuneprecipitate, at interchromatinic region (A). During the mitosis (B), in this picture is reported a methaphase, it is possible to documentate an intracytoplamic movement of the TdT as well has been observed at light microscopy. Section C shows immature rat thymocyte with the TdT colocalized at nuclear and at cytoplasmic level. Section D demonstrates a nuclear matrix preparation reporting the exact site-binding of nuclear TdT. The nucleolus (N) appears unstained. In these pictures we prove that by pre-embedding ICC it is possible to analyse the diverse localization of the TdT during cell cycle or differentiation processes.

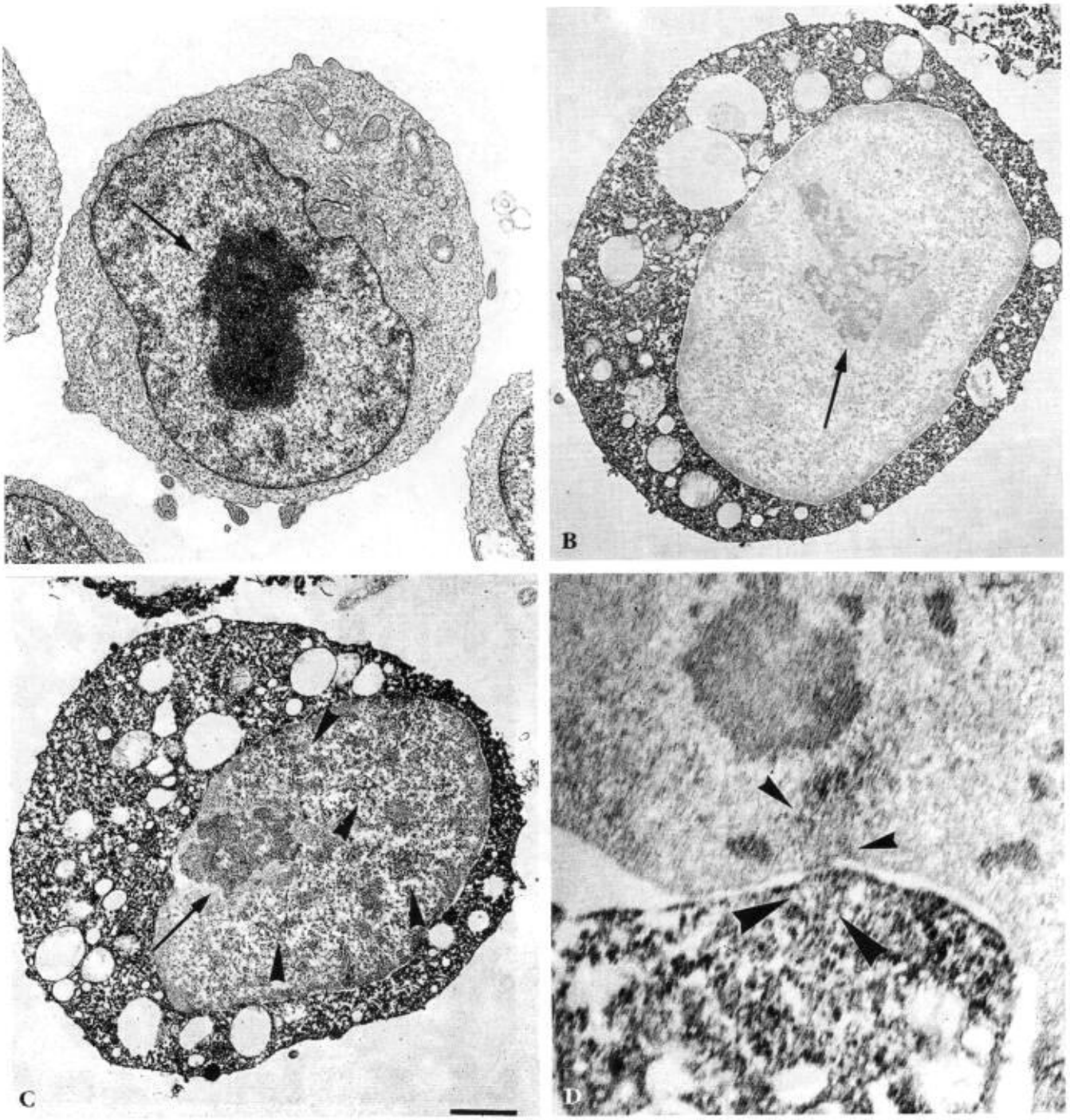


Fig. 2.—ICC analysis of an unlinked antigen, here the PKC, carried out as described. Section A show the ultrastructure of unstimulated cell. In this condition the PCK (B) appears omogeneously distributed at the cytoplasmic level, while if the cells are stimulated by a specific PKC-activator it is possible to observe an intranuclear translocation of PKC (C) through a nuclear pore (D). ICC methods give to us the possibility to display that in stimulated cells different changes occur as morphological features and intracellular movement of particular enzymes. Head arrows: immunoprecipitate. Arrows: Nucleolus. Bar 2.5 μm.

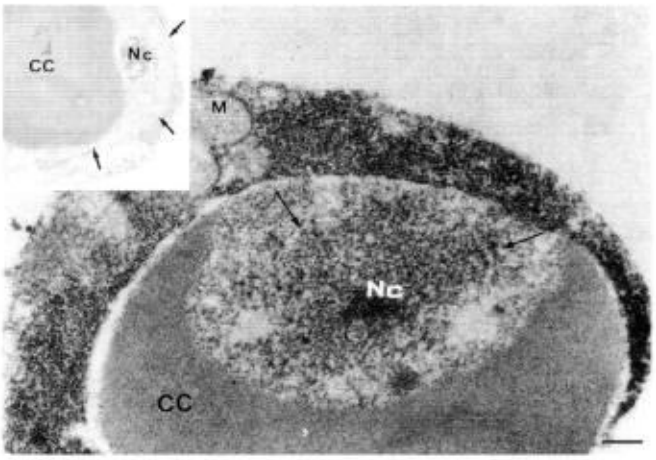


Fig. 3.—Electron microscope analysis of apoptotic rat thymocytes. The insert shows lead citrate-stained thymocyte. Higher magnification of apoptotic thymocyte immunostained with anti-PKC antibody. The immunoprecipitate appears localized in the cytoplasm and at nuclear level, where it colocalize with the residual nuclear matrix. CC: condensed chromatin. Nc: nucleolus. In the insert arrows indicate the intact nuclear membrane, while at higher magnification indicate the immunoprecipitate. M: mitochondrion. Bar 1 µm.

## Conclusions

The development of new ICC techniques originates from the necessity to acquire ever more interesting information from cytomorphological research. Anyway, the fate of ICC should not be only limited to identify the cellular framework but indeed to visualize cell dynamism.

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