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Use of periodate-lysine-paraformaldehyde for the fixation of multiple antigens in human skin biopsies

Pieri L., Sassoli C., Romagnoli P., and Domenici L.

Dipartimento di Anatomia, Istologia e Medicina Legale, University of Florence, Florence, Italy

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SUMMARY

Periodate-lysine-paraformaldehyde (PLP) has been proposed as a fixative for glycoprotein antigens which should stabilize periodate oxidized polysaccharide chains through lysine mediated crosslinks, either directly or by the intermediation of formaldehyde. In spite of premises and attempts reported in the literature, this fixative has never become popular for the study of membrane antigens of immune system cells, which leads to doubts on its real efficacy. We have addressed this issue in biopsies of human skin and found that PLP followed by cryoprotection with 30% sucrose and cryosectioning, or PLP fixation of isolated epidermal sheets, consistently provided for good preservation of morphology and intense labeling of major histocompatibility complex class II molecules, CD1a, CD4, CD8, E-cadherin, cytokeratins in general, cytokeratin-18 in particular, and bromo-deoxyuridine, incorporated by cycling cells in vitro, and for the demonstration of tyrosinase enzyme activity. PLP-fixed, osmicated and epon-embedded epidermal sheets proved as good as sheets fixed with a mixture of formaldehyde and glutaraldehyde for electron microscopic morphological analysis. Also, these sheets were amenable to immunoperoxidase staining of Langerhans cell membrane antigen CD1a and keratinocyte membrane antigen E-cadherin before being osmicated and prepared for electron microscopy. In a parallel paper, we had also shown that oral mucosa biopsies fixed in PLP showed good morphology and immunolabeling of CD54, CD80, CD83 and CD86. Therefore, we conclude that PLP can be proposed as a multi-task fixative for light and electron microscopic analysis of membrane, cytoplasmic and nuclear antigens of immune system cells and keratinocytes.

INTRODUCTION

The skin is a barrier between the body interior and the external environment and represents a first line of defense against possible pathogens.

It embodies a local immune system which includes several cell types: CD4- and CD8-positive T lym-

Key to abbreviations: MHC-II: major histocompatibility complex class II antigens; PLP: periodate-lysine-paraformaldehyde PBS: phosphate buffered saline; BrdU: bromo-deoxyuridine

Correspondence to: P. Romagnoli - Viale Pieraccini 6 - I-50139 Firenze (Italia)
E-mail: paolo.romagnoli@unifi.it

phocytes, mastocytes, macrophages and epidermal and dermal dendritic cells (Streilein, 1983, 1993). The epidermis hosts a peculiar type of dendritic cell, i.e. Langerhans cells, characterized by specific inclusions named Birbeck granules (Birbeck *et al*., 1961). In the epithelia of humans, only Langerhans cells express CD1a antigens (Melian *et al*., 1996). These cells can play different roles to generate and maintain immunological and inflammatory reactions (Streilein, 1983; Bos *et al*., 1987; Abbas, 1994). During inflammation, the members of the local immune system increase in number, and many of them express antigens correlated with activation.

Morphological analysis is mandatory to study these cells in their spatial relationships. Electron microscopy allows to identify the cells on the basis of their morphological characteristics and to analyze their exact localization and relations to other cells and extracellular structures, e.g. basement membranes. The analysis of organelles also gives indications about the functional activity of cells. Immunohistochemistry allows to identify many kinds of local immune system cells and their differentiation stage on the basis of membrane antigens specifically expressed. Some of these molecules are correlated with the functional activity of the immune system, since they stabilize the adhesion between dendritic cells and lymphocytes, and stimulate these latter cells during immune responses. All membrane antigens relevant to immune system cell characterization and function are glycoproteins (Abbas, 1994).

Keratinocytes are the principal cell population of epidermis; they interact with local immune system cells to control the differentiation state and function of the latter (Pieri *et al*., 2001). Keratinocytes are generated by local stem cells (Jones *et al*., 1997), from which also Merkel cells derive. The latter express cytokeratins and are connected with keratinocytes by desmosomes, but do not became engaged in the differentiation process leading to keratinization (Moll *et al*., 1984; Lewis *et al*., 1994).

Simultaneous analysis of the morphological and immunohistochemical characteristics of all cell populations of skin, and of local immune system cells in particular, is hampered by technical difficulties; granting for a nice preservation of both morphological and immune reactivity at the moment of fixation is an especially hard task. Fixation is a crucial step during histological preparation because it stabilizes and preserves the tissue structures as faithfully as possible, but reaches this goal through denaturation of proteins that represent most of the tissue antigens and can lose their antigenicity by denaturation. This explains why many efforts have been devoted to devising new fixation methods able to preserve localization and antigenicity of molecules together with good tissue and cell morphology.

An interesting proposal has been the PLP fixative, which is based on a mixture of paraformaldehyde, lysine and periodate (McLean and Nakane, 1974) and is considered to be especially efficient for the fixation of glycoconjugate molecules. Paraformaldehyde is not active as such, but once transformed into formaldehyde it can cross-link amino groups (Gabe, 1976); transformation into formaldehyde is obtained by heating and alkalinization before use. Lysine and periodate, on the contrary, do not need to be modified to be active. To explain the action of this mixture, it has been proposed that carbohydrate moieties in tissues are oxidized by periodate to form aldheyde groups; lysine, a divalent amine, then cross-links carbohydrate-containing molecules by reacting with these aldehyde groups (McLean and Nakane, 1974). According to some authors, formaldehyde is added to achieve stabilization of proteins and lipids (Kallen and Jenecks, 1966), while according to other authors, there is an interaction between formaldehyde and lysine linked to oxidized carbohydrate molecules that explains the fixative efficiency of this mixture (Hixson *et al*., 1981).

A Medline search (paraformaldehyde - periodate - lysine from 1973 to January 2002) has brought out that PLP has been used as a fixative in eleven papers from 1974 to 1980, in 98 from 1981 to 1990, in 62 from 1991 to 2000, and in 4 papers in 2001. The vast majority of papers deals with immunohistochemistry of cell and interstitial antigens; a few papers (8) deal with lectin histochemistry, enzyme histochemistry (12 papers) and nucleic acid hybridization histochemistry (6 papers). Nineteen papers are aimed at detecting surface antigens of immune system cells by light or electron microscopy. In these latter studies, PLP has been credited for preservation of several cell surface antigens of the mouse (Parr *et al*., 1980; van Ewijk *et al*., 1980; Gendelman *et al*., 1983; Whiteland *et al*., 1995), rat (Ruan *et al*., 1994; Whiteland *et al*., 1995;

Allaerts *et al*., 1996) and humans (Hancock *et al*., 1982; Collings *et al*., 1984; Dienes *et al*., 1984; van Duinen *et al*., 1984; Chiba *et al*., 1986; Holgate *et al*., 1986; Hall *et al*., 1987; Pollard *et al*., 1987; Kadin *et al*., 1988; Agnarsson and Kadin, 1989; Drach *et al*., 1989; Yano *et al*., 1990; Oka *et al*., 1993; Ahmed *et al*., 2001), following different preparatory techniques: dissociated cells, cryosections, paraffin sections, pre-embedding immunoelectron microscopy and flow cytometry. It is noteworthy that all but one studies on human cells date back to 1990 or earlier; taking also animal studies into account, only five papers on membrane antigens of immune system cells upon PLP fixation seem to have been published after 1990.

Therefore, in spite of apparently excellent credentials and application to many studies during the 1980s, PLP has not enjoyed generalized use, and this raises the suspicion that getting good results regularly may be more difficult than what can be imagined from published reports. To address this issue, we have evaluated PLP as a fixative for both detailed morphological and immunohistochemical analysis of cell populations of the skin, with special regard to immune system cells, keratinocytes and related (i.e., Merkel) cells.

MATERIAL AND METHODS

Reagents

Albumin (bovine), 5-bromo-2'-deoxyuridine (BrdU), dihydroxyphenylalanine (DOPA), glucose, L-lysine, poly-L-lysine, potassium chloride, sodium chloride and tablets containing diaminobenzidine plus urea stabilized hydrogen peroxide plus Tris buffer, penicillin and streptomycin were from Sigma (Milano, Italia); disodium hydrogen phosphate, dihydrate, was from Riedel de Haen (Seelze, Germany); EDTA, disodium salt, and paraformaldehyde were from BDH (Poole, UK); Dulbecco's Modified Eagle's Medium (D-MEM) and fetal calf serum (FCS) were from Seromed (Berlin, Germany); collagen disks were from ICN (Costa Mesa, California); sodium dihydrogen phosphate, monohydrate, and sodium metaperiodate were from Farmitalia/Carlo Erba; glutaraldehyde and osmium tetroxide were from Electron Microscopy Sciences (Ft. Washington, PA); sodium cacodylate was from Fluka (Buchs, Switzerland). Embedding medium for cryosectioning was from Bio-Optica (Milano, Italia); Gel/Mount for mounting fluorescent slides was from Biomeda (Foster City, CA). Phosphate buffered saline, 0.1 mol/L, pH 7.4, (PBS) was made up of 136.9 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L $Na_2HPO_4.2H_2O$, 1.7 mmol/L $NaH_2PO_4.H_2O$. Antibody dilutions and sources were as follows: monoclonal against major histocompatibility complex - class II molecules (MHC-II; 1:150) was from Ancell (Bayport, MN); against CD1a (1:50), from Harlan/Sera-Lab (Crawley Down, UK); against CD4 (1:50) and CD8 (1:50), from Sigma; a monoclonal against all cytokeratin molecules (1:25) was from Chemicon (Temecula, California); one against cytokeratin-18 (Clone RGE 53; 1:10) was from ICN (Milano, Italia); one against E-cadherin (1:1000) was from Takara (Otsu, Shiga, Japan); one against BrdU (1:200) was from Janssen-Cilag (Beerse, Belgium). Unlabeled and fluorescein isothiocyanate-labeled goat antimouse secondary antibodies (1:50) and mouse peroxidase-antiperoxidase complex (monoclonal; 1:500) were from Sigma.

Paraformaldehyde-lysine-periodate (PLP) fixative was prepared by mixing immediately before use equal volumes of 8% (w/v) paraformaldehyde in bidistilled water and 0.04 mol/L lysine in twice concentrated (i.e., 0.2 mol/L) PBS and adding 0.55 g/L $NaIO_4$.

Tissue sampling and fixation

Biopsies of clinically normal abdominal and breast skin were obtained at plastic surgery, in accordance with the Italian law and the principles of the Helsinki declaration. Part of the specimens was cut into small blocks (less than 2 mm as smallest diameter) which were either immediately embedded in medium for cryosectioning, frozen at -80°C and cryosectioned, or fixed with PLP for 30, 60 or 120 min at room temperature, soaked in 30% (w/v) glucose at 4°C as cryoprotectant, embedded in medium for cryosectioning, frozen at -80°C and cryosectioned. Cryosections were placed on polylysine-coated slides and dried in air; those from unfixed frozen specimens were fixed with acetone for 5 min at 4°C or with PLP for 5, 10, 20, 60, 120 or 240 min at room temperature. All cryosections were stored at -20°C until labeled for immunohistochemistry. Alternatively, thin slices were cut from skin biopsies with a keratome and either cultured to label cycling cells, as specifed

below, or directly incubated one to two hours in 20 mmol/L EDTA in PBS, at 37°C. The epidermis was then separated from the dermis by gentle traction and immediately divided into two portions that were immersion-fixed with acetone and PLP, respectively. Fixation in acetone was protracted for 5 min at 4°C, fixation in PLP was protracted for 5, 10 or 20 min at room temperature. Epidermal sheets were then stored in PBS at 4°C until labeled for immunohistochemistry. Skin sections and some epidermal sheets were labeled by indirect immunofluorescence for MHC-II and CD1a (characteristic of Langerhans cells in the epidermis), all cytokeratins together and E-cadherin (characteristic of keratinocytes), cytokeratin-18 (characteristic of Merkel cells), CD4 (characteristic of helper T lymphocytes) and CD8 (characteristic of cytotoxic-suppressor T lymphocytes). Other sheets were labeled by pre-embedding indirect immunoperoxidase for CD1a and E-cadherin and analyzed by electron microscopy (see below). On the basis of the results obtained by fluorescence microscopy (see results section), the sheets used for pre-embedding immunoelectron microscopy were fixed in PLP 20 min. To visualise melanocytes**,** an enzymatic test for DOPA-oxidase (Gomori, 1952) was used on epidermal sheets fixed in PLP for 20 min, as detailed below.

Labeling of cycling cells

Some skin slices, obtained from biopsies with a keratome, were placed on collagen disks that were soaked with D-MEM (with 1.0 g/L D-Glucose, 3.7 g/L $NaHCO_3$ and 1.0289 g/L N-acetyl-L-alanyl-L-glutamine), supplemented with penicillin (100 IU/mL) and streptomycin (100 μg/mL), and incubated in a humidified chamber at 37°C, in air with 5% CO_2 (Forma Scientific, INC incubator, Ohio, USA). After 42 h, the medium was replaced with a fresh one containing 3 μg/mL BrdU. Control specimens were incubated in medium without BrdU. After 6 h, the skin slices were washed with PBS and transfered to EDTA solution to isolate the epidermis: epidermal sheets were isolated, fixed and stored as detailed above.

To tag with specific antibodies the cells that had incorporated BrdU, epidermal sheets were first subjected to controlled proteolysis with 0.1% (w/v) trypsin in 50 mmol/L Tris-HCl buffer, pH 7.4, containing 0.1% (w/v) $CaCl_2$, for 30 min at 37°C. After washing in PBS, histones were removed by 0.1 mol/L HCl for 10 min at room temperature, then DNA was denatured and purines were removed with 2 mol/L HCl for 30 min at 37°C. Hydrolysis was blocked with 100 mmol/L Tris-HCl buffer, pH 8.7, for 10 min at room temperature, followed by 100 mmol/L Tris-HCl buffer, pH 7.4, for 10 min at room temperature (adapted from Lacy *et al.*, 1991). The slices were then processed as described below for immunohistochemistry.

Immunohistochemistry

Thawed sections and epidermal sheets were soaked in PBS, blocked with 1% (w/v) albumin in PBS. Triton X-100 (3%) was added to the blocking bath of sheets to be labelled for cytokeratins. The sheets were then labeled with primary antibodies for 90 min at 37°C, followed by either fluorescent secondary antibodies for 60 min at 37°C, or by unlabeled secondary antibodies and mouse peroxidase-antiperoxidase complex, each for 60 min at 37°C. Antibodies were diluted as indicated in the reagents section with 0.1% (w/v) albumin in PBS. All preparations for fluorescent microscopy were mounted wet with Gel/Mount and observed in a Zeiss (Oberkochen, Germany) Axioskop microscope equipped for epifluorescence; epidermal sheets were also observed in an MRC-1024 laser scanning fluorescence microscope (Bio-Rad, Hemel Hempstead, UK). Epidermal sheets labeled with peroxidase were subjected to revelation of the enzyme with diaminobenzidine (0.7 mg/mL) and urea hydrogen peroxide (1.6 mg/mL) in 0.06 mol/L Tris buffer, pH 7.6, and then prepared for electron microscopy. Omission of primary antibody was used as control for immunohistochemical reactions.

Detection of tyrosinase activity

Some epidermal sheets were fixed in PLP for 20 min and incubated in a freshly prepared solution of 0.05% DOPA in PBS with 2.5% (w/v) sucrose, overnight at 4°C followed by 2 h at 37°C, washed in PBS and mounted wet with Gel/Mount.

Electron microscopy

Some PLP-fixed epidermal sheets, either unlabeled or immunolabeled for CD1a and E-cadherin as described above, were osmicated and embedded in Epon 812 for electron microscopic analy-

sis. By comparison, some sheets were fixed with 2% (w/v) formaldehyde and 2.5% (w/v) glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.4, osmicated and embedded in Epon. One micron-thick sections were observed unstaind by light microscopy and about 70 nm-thick sections were observed in a Jeol (Tokyo, Japan) 1010 electron microscope, at 80 kV, either unstained or upon staining with uranium and lead salts.

RESULTS

Immunohistochemistry of acetone fixed specimens

This method, which is used as the gold standard for immunohistochemistry, allowed for good labeling of reactive cells with all antibodies tested, as expected. Tissue morphology was good in cases of epidermal sheets, not so good in cases of skin and gingival sections, where distortion and fissuring of tissues were sometimes observed. At laser scanning microscopy of epidermal sheets, labeling for MHC-II and CD1a was evident not only at the surface of epidermal dendritic cells (i.e., Langerhans cells), but also in intracytoplasmic vesicles, which tended to fuse together where they were more numerous, i.e. in the paranuclear region of the cytoplasm.

Immuno– and enzyme histochemistry of PLP fixed specimens

Fixation of tissue blocks of skin in PLP at room temperature, followed by cryoprotection and cryosectioning, gave good results for both immunohistochemistry and morphology. All antigens tested could be revealed; immunostaining was intense upon 30 and 60 min of fixation, but faint upon 120 min of fixation. Tissue morphology was far better in these specimens than in cryosections of previously unfixed specimens; preservation was better with 60 or 120 min than with 30 min of fixation (Fig. 1). All control specimens resulted unlabeled.

Fixation in cold PLP of cryosections from previously unfixed skin specimens provided results similar to acetone fixation if PLP was used for 5 min. For longer fixation times, the immunostaining was progressively less intense and was not present at all upon 4 h of fixation. The use of PLP on cryosections gave no advantage on acetone in regard to morphology.

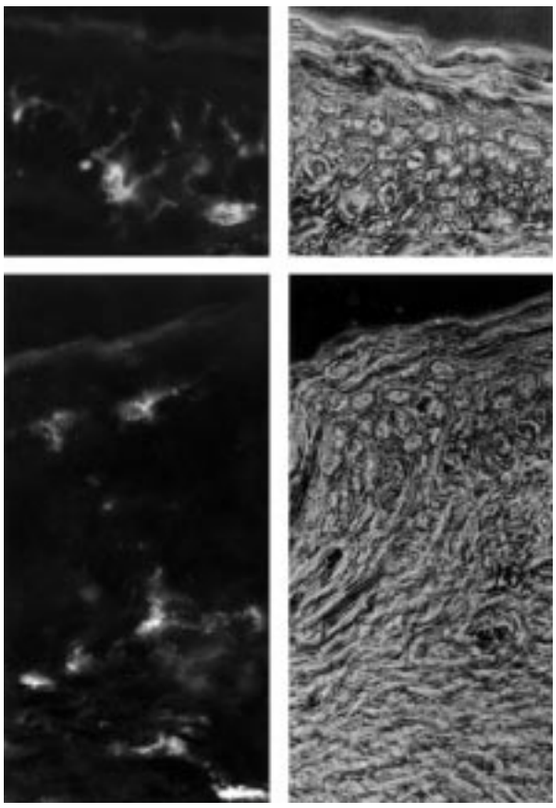

Fig. 1 - Cryosections of whole skin fixed with PLP for 1 h and cryoprotected before freezing allowed for intense labeling of membrane antigens of immune system cells and good preservation of tissue morphology. Upper and lower panels on the left show, respectively, immunfluorescence for CD1a (specific of Langerhans cells) and MHC-II (specific of Langerhans cells in the epidermis and of dendritic macrophages in the dermis). Panels on the right show the same fields by phase contrast microscopy. x 350.

Epidermal sheets fixed for 20 min were best preserved. In these specimens, the results matched those in acetone-fixed sheets, with intense staining with all antibodies tested. Langerhans cells observed at fluorescence microscopy were stained intensely for MHC-II and CD1a, and at laser scanning microscopy, these cells showed labeling both at the cell surface and intracytoplasmatic vesicles (Fig. 2), which appeared better discriminated then upon acetone fixation. Lymphocytes could be immunolabeled for either CD4 or CD8 (Fig. 3); these cells were few, as expected in normal epidermis. Keratinocytes fixed in PLP and labeled for E-cadherin and for cytokeratin with a pan-cytokeratin antibody

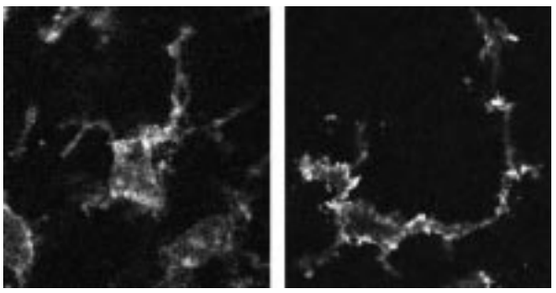

Fig. 2 - The labeling of Langerhans cells for CD1a (left panel) and MHC-II (right panel) in sheets fixed with PLP could be analyzed in detail by laser scanning fluorescence microscopy. Besides membrane labeling, small, antigen-loaded, cytoplasmic vesicles could be resolved near the nucleus and in dendrites. x 800.

(Fig. 4), showed intense labeling at fluorescence microscopy in all cell layers . Acetone fixation did not allow to label adequately the basal cell layer with pan-cytokeratin antibody. Analysis by laser scanning fluorescence microscopy allowed to recognize E-cadherin labeling all around suprabasal keratinocytes and, on the contrary, only on those surfaces of basal keratinocytes that faced neighboring cells. Upon labeling for cytokeratin-18 and analysis by laser scanning fluorescence microscopy, Merkel cells appeared as roundish cells, some with short cell projections (Fig. 4).

The nuclei of cycling cells that had incorporated BrdU were labelled intensely by specific antibodies; chromosomes were neatly shown in cells that had already entered mitosis at the moment of fixation (Fig. 5).

The cytochemical detection of tyrosinase allowed to label melanocytes for light microscopy in limited areas of sheets, where melanocytes appeared evenly scattered within epidermis (Fig. 5).

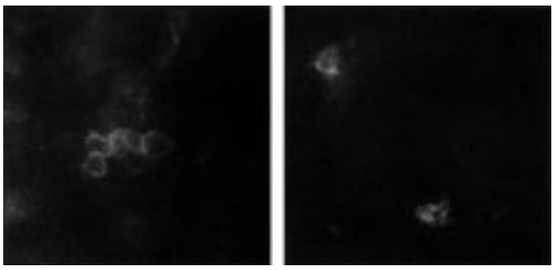

Fig. 3 - A few CD4 (left panel) and CD8 (right panel) positive lymphocytes were labeled by immunofluorescence whithin epidermal sheets upon PLP fixation. x350.

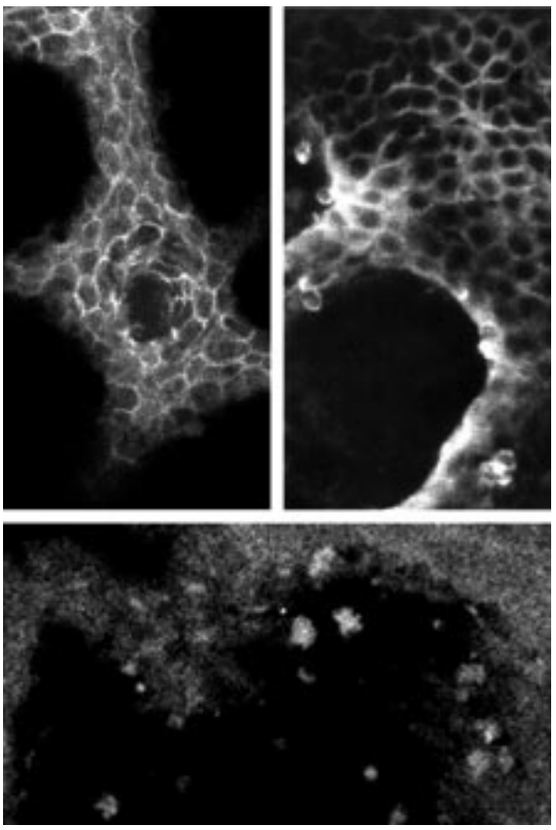

Fig. 4 - Keratinocytes within PLP-fixed epidermal sheets could be labeled by a monoclonal to E-cadherin (upper left panel; laser scanning microscopy) and by one recognizing a common epitope of all cytokeratin molecules (upper right panel; conventional fluorescence microscopy). E-cadherin was visible on cell surfaces facing other keratinocytes but not basement membrane. Merkel cells could be labeled by a monoclonal antibody against cytokeratin 18 within basal cell layer of PLP-fixed epidermal sheets (lower panel**;** laser scanning microscopy). In all panels epidermal ridges are shown including in part their lateral borders. Upper panels: x400; lower panel: x800.

Electron microscopy and immunoelectron microscopy

Fixation of epidermal sheets in PLP without osmication gave very poor results. The cells appeared shrunken, the surface and inner membranes were not preserved, only tonofilaments could be recognized easily. On the contrary, fixation of sheets in PLP for 20 min followed by osmication provided results comparable with those of Karnovsky's fixation and osmication for both the general shape and inner details of the different cell layers, including

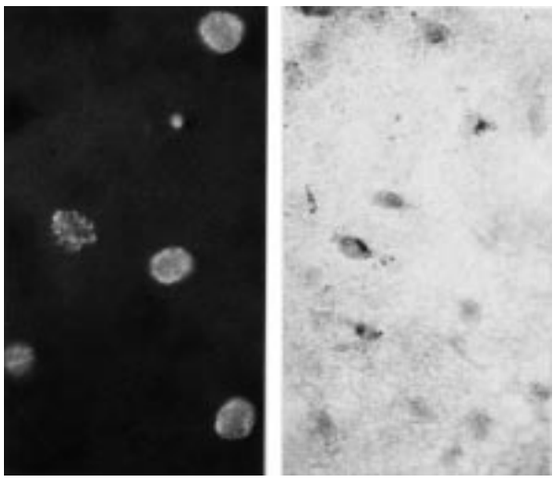

Fig. 5 - Left panel: cycling cells incorporating bromodeoxyuridine were immunolabeled by a specific antibody within PLP-fixed epidermal sheets. Labeled chromosomes can be recognized in a cell that had entered mitosis at the moment of fixation. Right panel: melanocytes were shown by tyrosinase activity diffuse to the cytoplasm within PLP-fixed epidermal sheets. Light field microscopy. x 350.

surface and inner membranes (Fig. 6). Morphological preservation was less good upon fixation for 5 min: many cells appeared with interrupted membranes and the cytoplasm devoid of organelles.

Indirect immunoperoxidase for CD1a provided strong and specific labeling of Langerhans cells, which were labeled all over their surface membrane and were shown to contain Birbeck granules as well (Fig. 7). Indirect immunoperoxidase staining for E-cadherin labeled keratinocyte surface membrane, with most intense labeling of desmosomes (Fig. 8).

DISCUSSION

The results of this study show that PLP fixation provides for good preservation of cell and tissue morphology at both light and electron microscopy, as well as for strong immunoreactivity of many surface membrane antigens of human leukocytes, keratinocytes and Merkel cells with routine anti-

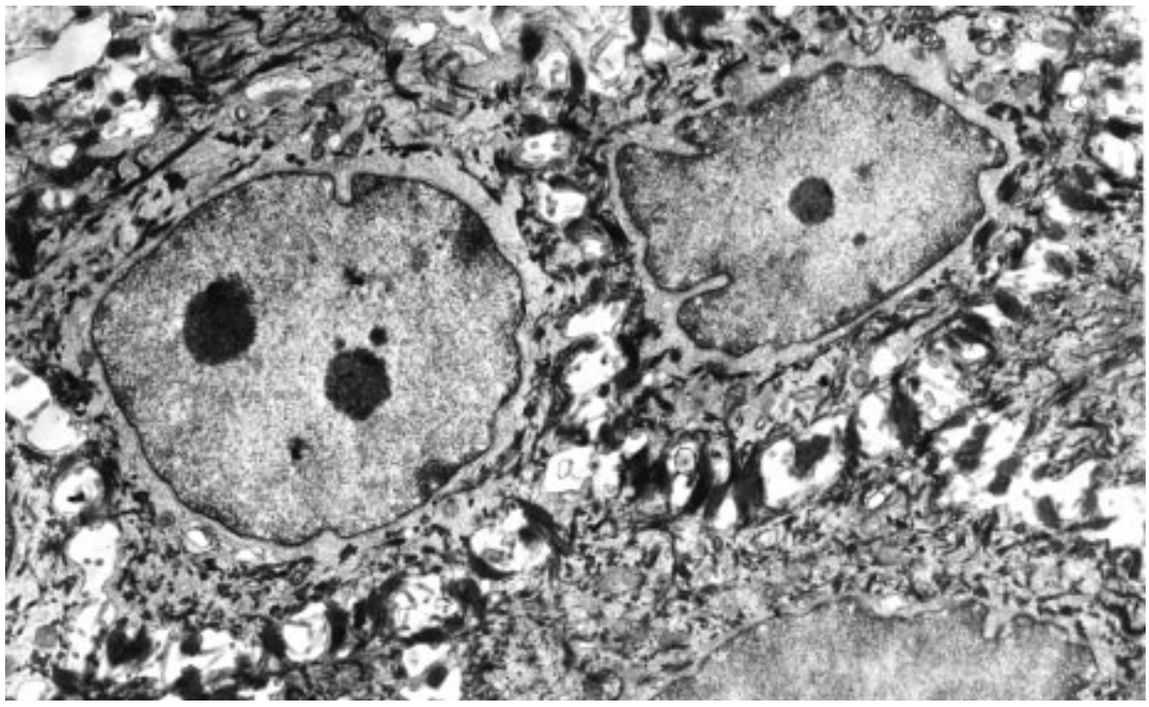

Fig. 6 - Epidermal cells were well preserved for electron microscopy in sheets fixed with PLP, osmicated and epon embedded. The photomicrograph shows the prickle cell layer, with intact cell surface membrane, intercellular junctions and intracellular details, including membrane bound compartments and cytoskeleton. x 12000.

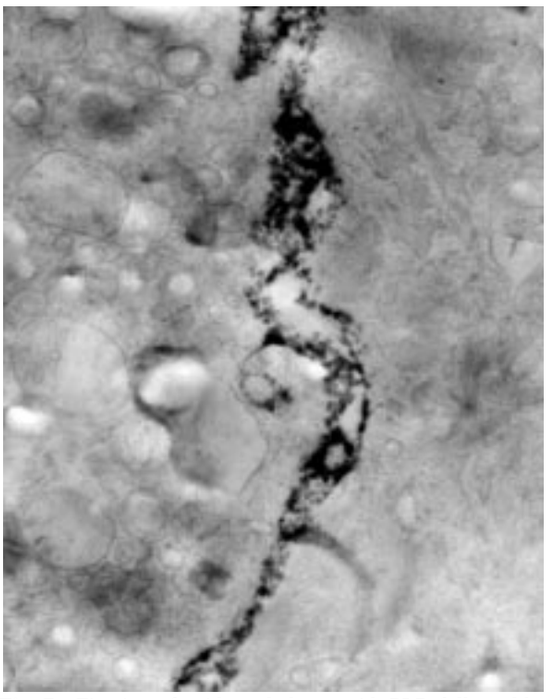

Fig. 7 - Fixation of epidermal sheets with PLP, followed by indirect immunoperoxidase labeling of CD1a, osmication and epon embedding allowed for demonstration of antigen at the cell surface and identification of well preserved Birbeck granules within cytoplasm of Langerhans cells at electron microscopy. x 37000.

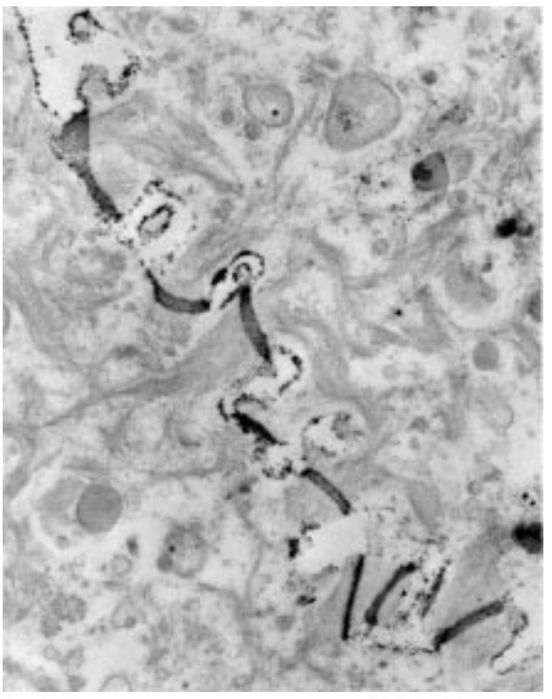

Fig. 8 - Fixation of epidermal sheets with PLP for 20 min. followed by indirect immunoperoxidase labeling of E-cadherin, osmication and epon embedding allowed for electron microscopical demonstration of antigen at the cell surface of keratinocytes, with especially sharp labeling of desmosomes. x 30000.

bodies, i.e. with antibodies not specifically designed for formalin-fixed specimens**,** and as a rule not efficient after formaldehyde fixation. The numbers of Merkel cells seen in our preparations were not estimated exactly, because this was beyond the aims of this study, they were anyway few, in agreement with the data in the literature which indicate that in human abdominal skin the density of these cells is low (Lacour *et al*., 1991). The preservation of these cells allowed to show in many instances the cell projections typical of Merkel cells (Lacour *et al*., 1991). Also, it was possible to detail the cell surfaces where E-cadherin was localized and to show that this molecule is absent from the basal surface of cells of the basal layer, as expected (Jensen *et al*.,1997).

The good morphological preservation of cryosections of PLP-fixed, frozen tissue, as compared with cryosections of fresh frozen tissue, can be interpreted as depending on two properties. Firstly, fixation itself stabilizes tissue structure against shrinkage during freezing and against mechanical and autolytical artefacts of sections during thawing that occurs before completing air drying and proceeding to definitive, chemical fixation. Secondly, chemical fixation with PLP allows for the use of a cryoprotectant before freezing and this in turn prevents mechanical artefacts during freezing and thawing of sections. Specimens cannot be soaked in cryoprotectants if not previously fixed, unless they undergo at least partial autolysis before freezing.

The good results of PLP and cryosectioning lead to propose this method as a valuable alternative to conventional acetone fixation of cryosections of fresh frozen tissue, for the immunohistochemical detection of membrane antigens of the immune system cells. The use of routinely adopted antibodies in these conditions allows for easy comparison of the results with those obtained with the same anti-

bodies in other laboratories or in previous experiments of the same laboratory; a further advantage may be the lower cost of these antibodies than those designed for formalin fixed specimens.

Fixation of fresh frozen sections with PLP confirmed the efficacy of this mixture for the preservation of membrane antigens and showed that fixation cannot be prolonged indefinitely. From the practical standpoint, this method gave no advantage over acetone fixation of similar sections.

The results obtained with PLP at electron microscopy were comparable with those of Karnovsky's (1965) mixture from the morphological standpoint. Fixation in PLP provided the additional advantage of preserving membrane antigen immunoreactivity, as shown by pre-embedding labeling for CD1a and E-cadherin. Formaldehyde alone does not provide for enough good morphology at electron microscopy and is usually admixed with at least minimum amounts of glutaraldehyde, making the preservation of membrane antigens hardly predictable. On the contrary, the use of lysine and periodate together with formaldehyde should not pose problems for the preservation of antigenicity during fixation. The deleterious effect of glutaraldehyde on membrane antigen immunoreactivity is widespread knowledge (Pearse, 1980), but its cause has not been analyzed in detail. It may depend on the quick formation of stable intra- and interchain crosslinks (Reale and Luciano, 1970; Pearse, 1980) or on the stabilization of heterophasic lipid moieties (Wigglesworth, 1971), or both; a role for lipid preservation in masking cell membrane antigen reactivity can be inferred by the fact that the gold standard fixative for those antigens, i. e. acetone, can extract lipids in part while coagulating proteins.

All membrane antigens labeled here are glycoproteins. The efficacy of PLP may therefore be explained by the selective interaction of this fixative with glycoproteins (McLean and Nakane, 1974; Hixson *et al*., 1981) and immunolabeling indicates that the kind of crosslinks that form does not inhibit access of antibodies to their targets. Labeling Langerhans cells within epidermal sheets - a type of preparation that allows to study whole, unsectioned cells by conventional and laser scanning fluorescence microscopy - has shown a finer resolution of labeling within the cell body upon PLP than upon acetone fixation. This result confirms, at this level of analysis, the good preservation of cell inner structure, including membranes, that was also shown by electron microscopy, and that is obviously not attainable with acetone.

Cytoplasmic antigens, such as cytokeratins, were also well preserved by PLP, which was expected on the basis of the known efficacy of formaldehyde for intracellular antigens. Fixation with PLP was also effective for analyzing cycling cells by BrdU incorporation and immunolabeling. The procedure requires quite harsh treatment of fixed tissue with acid and enzymes to expose BrdU on DNA strands, and it is, therefore, remarkable that high quality images could be obtained of both interphase nuclei and chromosomes during mitosis. Fixation with PLP also allowed for the demonstration of tyrosinase activity in melanocytes, although only in part of the sheet surface. We did not explore further the reasons of the variability of results among different areas of epidermal sheets, because this was not the primary target the study. However, the results indicate that enzyme activites can be preserved by PLP, which adds value to this fixative for histological and cytological studies on skin.

Another recent paper of this laboratory has shown that PLP is a valuable fixative for leukocyte antigens in human gengival biopsies (Cirrincione *et al*., 2002), in line with the results shown here. In that paper, we could also show sharp immunolabeling of CD54, CD80, CD83, and CD86, besides MHC-II, CD1a, CD4 and CD8. We should like to conclude that PLP proved to be a valuable fixative mixture for different types of microscopic analyses and, therefore, can be proposed as a first choice solution in all cases where good preservation of morphology must be coupled with immunohistochemical labeling of membrane antigens, and where several types of preparative techniques (such as light microscopy, immunohistochemistry, enzyme cytochemistry and electron microscopy) should be applied to one single specimen. In the latter case, the possibility of trimming and subdividing tissue blocks upon fixation, instead of before it, can grant better preservation of tissue structure, easier selection of portions to be destined to different preparatory lines and, last but not least, safer working conditions, since fixation inactivates most infectious agents possibly affecting human tissues.

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