Technical Advancement

Microwave-Stimulated Antigen Retrieval—An Update

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Since the development of antigen labelling in the early 1940s there have been tremendous technological developments that now allow the detection of a wide range of diagnostic antigens in routinely fixed, paraffin-embedded tissue sections. Much of these developments have come about as a result of antigen retrieval. Proteolytic digestion was the most widely used method of antigen retrieval until the introduction of microwave irradiation in 1991. This marked a major milestone in immunolabelling, and has been mostly responsible for the rapid acceptance of immunohistology and its irreplaceable role in diagnosis. While other methods of generating heat were employed for retrieval, microwave irradiation has been widely employed not only

for paraffin sections but also for cytological preparations, cryostat sections, plastic-embedded sections, for immunoelectron microscopy, *in situ* hybridization and for the demonstration of DNA fragmentation in apoptosis. The precise mechanism of action of microwaves remains speculative but it is evident that several factors influence its effectiveness. These include temperature and time of exposure to irradiation, pH and osmolality of retrieval solution and the nature and duration of fixative. To achieve optimal immunolabelling, particularly of the more capricious antigens, further work is required to fully understand the influence that these factors have on immunolabelling.

Key words: Microwaves, Antigen retrieval, Immunohistochemistry, *In situ* hybridization, Electron microscopy

I. Introduction

Microwaves (MWs) are a form of non-ionizing radiation with a typically standard frequency of 2.45 GHz, a wavelength of 12.2 cm and photon energy of 10^{-5} electron volts. When dipolar molecules such as water or the polar side chains of proteins are exposed to the rapidly alternating electromagnetic fields, they oscillate through 180° at the rate of 2.45 billion cycles per second. The molecular movement or kinetics so induced results in the generation of instantaneous heat that is proportional to the energy flux and continues until radiation ceases. It was only recently recognized that molecules other than water and the polar side chains of

proteins may oscillate in the electromagnetic field generated by MWs. Molecules with an uneven distribution of electrical charge such as inorganic material and copper oxides could also be rotated.

II. Antigen Preservation and Antigen Retrieval

The fluorescent antibody method was first applied to tissue sections in 1941 by Coons *et al.* [6] but although this method of demonstrating tissue antigens was successfully applied to several areas in pathology such as renal and skin diseases, it did not attain widespread appeal among surgical pathologists. This stemmed from the requirement for fresh frozen tissue, impermanence of the preparations and poor cytomorphologic details in such material. The substitution of horseradish peroxidase for fluorescein-isothiocyanate as

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the label of the specific antibody allowed more permanent staining [30] but it was the eventual development of the peroxidase-antiperoxidase technique by Sternberger in 1970 [40] that provided sufficient sensitivity for antigen detection to be performed on fixed, paraffin-embedded tissue sections. With the development of hybridoma technology by Kohler and Milstein in 1975 [13] an increasing range of sensitive and specific antibodies could be produced, giving great impetus to diagnostic immunohistochemistry. While more sensitive detection methods continued to be developed in subsequent years, the ability to reliably and consistently demonstrate a wide range of antigens in routinely processed fixed tissue sections remained a pre-requisite for diagnostic application of the technique.

Immunohistochemistry or "immunohistology", as we prefer to call it so as not to detract from the fact that this is a histology-based procedure [24] has rapidly become established as an important and often indispensable adjunct to microscopic examination. However, the preservation of cellular antigens in fixed tissues is variable and fixation dependent. Leong and Gilham [16] clearly demonstrated that tissues fixed in formaldehyde displayed a distinct and progressive loss of staining of many antigens, frequently proportional to the duration of exposure to the fixative. There was an appreciable loss of staining of some antigens after three days; many antigens were lost after seven days and most were not demonstrable after 14 days of fixation. Interestingly, enzymatic predigestion, which was the prevalent method of antigen retrieval, per se, was useful for the unmasking of only some antigens, ineffective in many and even deleterious in others. Fixation in alcohol and alcohol-based mixtures such as methacarn were advocated as substitutes to 10% buffered formalin but morphologic preservation was suboptimal.

Leong *et al.* [25] clearly showed that tissues immersed in normal saline and fixed by exposure to MWs displayed uniformly superior antigen preservation compared to those tissues fixed by formaldehyde, and some antigens which were not demonstrable in the latter preparations were readily labelled in MW-fixed sections. A major breakthrough in antigen retrieval came about when Shi *et al.* [36] found that MW heating of paraffin-embedded tissue sections in the presence of heavy metal solutions, such as lead thiocyanate, up to temperatures of 100°C "unmasked" a wide range of antigens in fixed tissue sections. It was subsequently shown that MW-irradiation of deparaffinised-rehydrated sections in 10 mmol citrate buffer solution at pH 6.0, produced, with few exceptions, increased intensity and extent of immunostaining of a wide variety of tissue antigens [7, 8, 18]. This has proven to be one of the most important applications of MWs as antigen retrieval has made it possible for the optimization of immunostaining in fixed tissue sections [20]. The use of citrate buffer eliminated the need to employ heavy metal solutions which, when heated, generate toxic fumes. Several commercial antigen retrieval reagents are available but they mostly do not produce any better results than that obtained with citrate buffer, which can be prepared in the

laboratory at very low cost [22].

Besides the method of fixation as we have shown with formalin and MWs [25], there is evidence to show that a variety of other factors influence the efficacy of antigen retrieval. Among these are the osmolality, pH and chemical composition of the reagent, and the nature of the antigen of interest. It has been suggested that immunoreactivity is generally increased when the sections are irradiated in reagents of higher pH (pH 8–9). With variation of retrieval solution pH, most antigens fell into one of three patterns of reaction [37]. Some antigens such as CD45 (leukocyte common antigen), PCNA (proliferating cell nuclear antigen), AE1, EMA (epithelial membrane antigen) and NSE (neuron specific enolase) showed excellent retrieval throughout the pH range. Others such as ER (estrogen receptor) and MIB1 showed strong intensity of staining at very low pH and at neutral to high pH, but a dramatic decrease at moderately acidic pH (pH 3–6). The third group of antigens such as CD43 and HMB45 showed increasing intensity of immunostaining with increasing pH, but only weak staining at low pH. It was therefore suggested that a high pH is most likely to produce optimal retrieval for the majority of antigens. When working out the optimal pH for a new antigen, it is best to start with a high pH.

The applications of MWs in immunohistology extend into all aspects of the staining procedure. Leong and Milios [15] demonstrated that exposure of cryostat sections briefly to MWs before of commencement of immunolabelling produced better quality cytomorphology and staining. MWs were shown to accelerate antibody-antigen reactions in the staining of labile lymphocyte membrane antigens in cryostat sections [15]. The same authors similarly demonstrated the use of irradiation to accelerate immunolabelling in paraffin-embedded sections [17] and the same technique has been applied for immunofluorescence labelling [4]. A similar procedure has been adopted for freshly frozen brain sections with notable enhancement of immunostaining, without affecting the integrity of cytomorphology.

MWs have been applied between sequential rounds of a three-layer immunoenzyme staining (mouse Mab, goat anti-mouse IgG and mouse PAP or mouse APAAP) and color development technique for multiple antigen detection [14]. The MWs denatured bound antibody molecules resulting in the blocking of crossreactivity between the sequential staining steps, allowing the use of primary and other antibodies raised in the same species. Besides serving a role in antigen retrieval, MWs also inactivate peroxidase and alkaline phosphatase enzymes present in PAP and APAAP complexes, which would otherwise lead to inappropriate color development.

III. Application to Cytological Preparations

A major problem in immunocytochemistry is the high background staining that occurs in cytological preparations. This is the result of both proteinaceous material and red blood cells so that the sometimes-intolerable level of back-

Fig. 1. Immunostaining of air-dried smears fixed in 0.1% formal saline and following MW-antigen retrieval. Note distinct staining for desmin in the left panel, which is a fine needle aspirate from the inguinal node from a patient with metastatic rhabdomyosarcoma. The right panel represents bronchial washings and shows low molecular cytokeratins in respiratory cells with virtually no background staining. Both ciliated and squamous cells are present in the smear.

ground staining makes the interpretation of such preparations difficult so that many laboratories have resorted to the use of cell blocks for immunocytochemistry, an alternative which is not always available [19, 27]. In a study of 22 different fixatives and fixation protocols previously described for cell preparations, we found that 0.1% formal saline (van der Griendt's fixative) served the dual purpose of rehydrating air-dried smears as well as washing off the proteinaceous background and causing lysis of red blood cells. This resulted in a much cleaner background with less noise to signal ratio [41]. A wide range of antigens was preserved by this method and when coupled with MW-antigen retrieval, optimal demonstration of cellular antigens was achieved with virtually no background staining (Fig. 1). Smear previously fixed in alcohol or other fixatives may be post fixed in 1% formal saline with some reduction in background staining. The breast prognostic markers including estrogen and progesterone receptors, Ki-67 and c-erbB-2 were clearly demonstrable following fixation in 10% buffered formalin and with MW antigen retrieval [42].

IV. Antigen Retrieval and Superheating

A variety of antigen retrieval techniques to generate heat for antigen retrieval have been developed and include the use of microwaves, pressure cookers, steamers, autoclaves and even the Swedish sauna. It has been suggested that heating above 100°C may be the most effective although this has not been substantiated. While the pressure cooker is claimed to develop temperature above boiling point, it was not until the development of the Mega T/T that accurate control of time and temperature became available. The glass pressure generator developed by Milestone allows temperatures of up to 120°C to be attained and accurately maintained over required durations (Fig. 2). We have demonstrated that this instrument produces definite enhancement of most diagnostic antigens. Importantly, it was

possible to obtain consistent and strong immunolabelling of antigens, which are difficult to achieve with previous methods of retrieval [28].

V. Applications in Molecular Techniques

The use of MWs in molecular analyses is a more recent development. Initial applications of MWs in molecular studies were for the achievement of the high temperatures necessary to denature probe and tissue DNA; MWs providing a method with ease of control and rapidity of heat generation. This application has also been adapted for the accelerated detection of mRNA [3].

Formaldehyde-fixed tissues remain the most common source of material for molecular studies and protease digestion is an essential procedure for unmasking the cross-linking effects of this fixative before *in situ* hybridization (ISH) can be performed. Recent studies demonstrate that the exposure of the formalin-fixed, paraffin-embedded sections to MWs in citrate buffer in a manner similar to that applied for antigen retrieval produces enhanced signal detection for both DNA [1] and mRNA. Microwaving for 15–20 min in 10 mmol citrate buffer at pH 6.0 was shown to be more effective than heating at 70°C for 30 min, in sodium chloride-sodium citrate. The procedure was performed in combination with proteinase K digestion (at reduced digestion time) which was applied before irradiation [39]. The same retrieval method was equally effective for mRNA as shown with Epstein-Barr virus EBER RNA with quantitative confirmation of the increased sensitivity rendered by MW pretreatment [32]. Importantly, it renders RNA-ISH a more consistent and reliable procedure (Fig. 3). The technique has also been successfully applied for terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) for apoptotic cells, proving more sensitive than proteolytic digestion and detergent treatment [31].

Fig. 2. Milestone Mega T/T instrument. The left picture shows the microwave oven with computer control panel on the right of the oven. Within the oven is the glass pressure cooker shown better in the right photograph. This instrument allows accurate computerized control of time and temperature with capabilities of attaining superheating antigen retrieval at 120°C.

Fig. 3. *In situ* hybridization (ISH) for EBER RNA. The left panel shows ISH without MW retrieval. Note the clearly stronger staining in the right panel following MW retrieval.

VI. Ultrastructural Antigen and Enzyme Preservation in MW-fixed Tissues

Numerous reports have described better ultrastructural preservation of various tissue enzymes and antigens in MW irradiated tissues compared to conventional fixation procedures. This is not unexpected considering the convincing documentation of this observation in paraffin-embedded tissues. MW irradiation in aldehyde fixatives result in drastically shortened durations of exposure to the fixative so that various lipolytic enzymes such as lipase and sphingomyeli-

nase display much better ultrastructural definition and localization. Using rapid MW fixation, it has been possible to demonstrate, at ultrastructural level, various proteins, which are generally not demonstrable by conventional fixation protocols. Such molecules include tumor necrosis factor [2], nuclear matrix proteins, glutathione peroxidase, various neuropeptides and myelinated fibers, and Ca^{2+} ions. In general, MW fixation improves antigenicity of glutaraldehyde-sensitive antigens while producing excellent preservation of ultrastructural details [12].

VII. Antigen Retrieval in Thick Sections and Immuno-electron Microscopy

The use of MWs for antigen retrieval in paraffin-embedded tissue sections has been hailed as a revolution in immunohistochemistry. Despite the now extensive use of MWs for antigen retrieval in paraffin sections, the technique has rarely been reported in resin embedded sections. Although there are numerous reports of immunolabelling in resin-embedded sections, the method has been generally regarded as capricious and difficult and is not routinely employed in diagnostic situations. Recently, three studies showed that MW-stimulated antigen retrieval could be successfully applied to plastic-embedded tissues including acrylic glycol methacrylate (GMA) [43], epoxy Polarbed 812 [29], and methyl methacrylate (MMA) [9].

All three studies employed 10 mmol citrate buffer at pH 6.0 as the retrieval solution and demonstrated, with few exceptions, that immunogenicity was improved following MW antigen retrieval. We have also shown that MW-stimulated antigen retrieval can also be effectively employed on grids for immuno-electron microscopy [38]. Briefly, thin sections cut on formvar-coated nickel grids were placed on plastic grid plates (Hiraoka Staining Kit, Fort Washington, PA, USA) and immersed in a glass beaker of 50 ml 10 mmol citrate buffer. Another beaker with 350 ml of water was placed in the oven cavity to absorb some of the MW radiation. The buffer was heated to 85–90°C and maintained at

the same temperature for 5 min. The grids were allowed to remain in the buffer for a further 20 min then transferred to TBS-buffer before immunolabelling. The immunolabelling of most antigens studied was clearly enhanced. These included vimentin, cytokeratin, type IV collagen, IgA and IgG (Fig. 4). There was clear staining for β -catenin, an antigen that is notoriously difficult to demonstrate by immunoelectron microscopy even in cryo-microtomy sections.

Besides enhancement of labeling, it is notable that background staining in irradiated specimens both for light and electron microscopy was reduced. Morphology of the tissue was assessed to be about the same in quality to that of non-irradiated specimens and interestingly, staining of the endoplasmic reticulum appeared to be stronger and sharper following exposure to MWs.

VIII. Discussion

There have been attempts to classify the role of MWs in tissue fixation [23, 26] but they have not added significantly to our understanding of the action of MWs as a primary fixation agent. Its use with conventional chemical fixatives most likely relates to the acceleration of diffusion of the fixative throughout the tissue. The depth of penetration of a fixative is the product of the coefficient of diffusibility of the fixative and the square root of time [21]. In the case of 4% formaldehyde, this coefficient constant is 0.78, although it may be less in some types of human tissues. The coefficient

Fig. 4. Methyl methacrylate-embedded sections of glomeruli in membranous nephropathy. The left panel shows immuno-gold labelling for IgG. Note the clear deposits of IgG in the glomerular basement membranes. The right panel of the same glomerulus has been immuno-labelled for type IV collagen. It shows a clearly fragmented glomerular basal lamina representing the focal deposits of IgG.

of diffusibility of 1–4% glutaraldehyde at 4°C is even lower at 0.33–0.5. While heat may increase the diffusibility of the fixative, high temperatures can also increase enzymatic activity and hasten the process of autolysis. For example, heating of 4% formaldehyde to 60–70°C can hasten the fixation process but the risk of tissue distortion is also increased. It is thought that formaldehyde and other aldehydes have the property of forming cross-links between proteins, thereby forming a gel that retains cellular constituents in their *in vivo* relationships to one another. Soluble proteins are fixed to structural proteins and rendered insoluble, so that the entire structure is given some mechanical strength, allowing it to withstand the rigours of tissue processing. The cross-linking between protein molecules is mostly formed by a reaction with the basic amino acid lysine, although other groups such as imino, amido, peptide, guanidyl, hydroxyl, carboxyl, sulphhydryl groups and aromatic rings may also participate. Only those lysine residues on the exterior of the protein molecule react and they usually account for 40–60% of the total lysine residues. Although the extent of protein denaturation which results from the fixation process is not of importance in light microscopy, denaturation is of particular relevance in immunolabelling both by immunofluorescence and immunoenzyme techniques, as well as in molecular analysis and high resolution electron microscopy. Similarly, the shape of molecules should not be changed significantly if they are to be recognized by biochemical analysis. Glutaraldehyde causes a loss of up to 30% of the alpha helix structure of protein, depending on the type of protein and fixation with osmium tetroxide or post-osmification of glutaraldehyde-fixed material causes the complete denaturation of protein. As such, for immuno-electron microscopy, frozen sections should be employed or fixatives should be used in low concentrations and exposure restricted to short durations [21].

When dipolar molecules such as water, the polar side chains of proteins and other asymmetrical molecules are exposed to the oscillating electromagnetic field of MWs, they rotate through 180° at frequency of 2.45 billion cycles per second. The rapid oscillation of molecules generates heat, which is currently thought to be the primary factor responsible for many of the applications of MWs in tissue preparation. However, as with many laboratory stains and procedures, much of our knowledge concerning the actions of MWs is empirical. The staining of tissue sections, for example, is based on at least two important factors, namely the diffusion of the dye or antibody into the cells and its binding to the substrate or antigen. Diffusion is a physical process that can be greatly accelerated by irradiation, but the influence of MWs on binding mechanisms is more complicated and less well understood.

The rapidly oscillating electromagnetic field of MWs may itself have an effect on chemical reactions and proteins. While heat or thermal energy will increase molecular kinetics and hasten chemical reactions, the rapid rotation of molecules directly induced by the MWs will give rise to greatly increased collision of molecules, which will in turn acceler-

ate chemical reactions. The heat generated may represent an epiphenomenon secondary only to the kinetics. Hjerpe *et al.* [10] examined MW stimulation of CEA/anti-CEA reaction in an enzyme-linked immunosorbent assay system. Despite continuous cooling by ice, MW stimulation increased reaction rates by a factor of 1000, allowing the investigators to conclude that such rate increases were far too large to be explained solely by the modest increase in temperature. Choi *et al.* [5] went further to demonstrate the existence of a “microwave effect.” They showed that the rate of droplet temperature increase obtained in a thermal cycler was similar to that achieved by MW irradiation. However, the immunostaining obtained from a 3-minute incubation at 37°C in the thermal cycler followed by 2-minute incubation without heating was much weaker than that seen with MWs. Takes *et al.* [44] similarly demonstrated that 7-s MW irradiation followed by 5-min room temperature incubation for each step of the avidin-biotin peroxidase complex procedure produced good immunolabelling. The droplet temperature will rise no more than 5°C following 7-s irradiation at 100% power in a 850-watt oven so that temperature was not a significant component of the accelerated reaction. Others have argued that there is no significant microwave effect and the accelerated reactions are a function of heat. Hopwood *et al.* [11] concluded that MW irradiation did not produce cleavage or polymerization of proteins and irradiation resulted in an electrophoretic pattern that was similar to that obtained when lysozyme and hemoglobin was heated in formaldehyde to 60°C for 30 min. Interestingly, Porcelli *et al.* [33], more recently, showed results to the contrary. In a study of S-adenosylhomocysteine hydrolase and 5'-methylthioadenosine phosphorylase, two thermophilic and thermostable enzymes, they found that exposure to MWs caused a non-thermal, irreversible and time-dependent inactivation of both enzymes. Conformational changes of S-adenosylhomocysteine hydrolase, detected by fluorescence and circular dichroism techniques, suggested that MWs induced protein structural rearrangements not related to temperature. In a study of cross linking of collagen induced by glutaraldehyde as reflected by the shrinkage of porcine collagen, Ruijgrok *et al.* [35] concluded that MWs did not induce a “substantial nonthermal effect on enhancement of glutaraldehyde cross-linking of collagen” in the range of 4–20°C. Interestingly, one of the authors of this paper also co-authored the earlier work with CEA/anti-CEA described above [10].

The recent demonstration that exposure to ultrasound can significantly increase antibody-antigen reaction in immunostaining lends further support to the relevance of molecular movement as an important factor in the acceleration of chemical reaction as the amount of heat generated by this physical modality is negligible [34]. A number of other hypothetical physical mechanisms may also play a role in the actions of MWs. Although the proton energy generated in MW fields is too small to alter covalent bonding, they may readily affect the integrity of non-covalent secondary bonding, including hydrophobic interactions, hydrogen bonds and van der Waal's interactions that make

up the precise steric interactions at the cell membrane.

Common chemical fixative exists in solution as oligomers and become degraded to monomers and dimers. An aqueous solution of glutaraldehyde (glutaric dialdehyde) is a complex mixture at room temperature, consisting approximately of 4% free aldehyde, 16% monohydrate, 9% dihydrate and 70% hemiacetal. The free glutaraldehyde may form polymers or may form a monohydrate and a dihydrate which can cyclise to form a hemiacetal that in turn can polymerize. The polymer has been favored as the reactive species but some authors suggest that pure monomeric glutaraldehyde is the best fixative and much less inhibitory to enzymes than the mixed monomeric-polymeric product. It has also been suggested that the success of glutaraldehyde as a fixative is dependent on the range of different molecules present simultaneously in the aqueous solution [26]. The formation and diffusion of monomers and dimers in various aldehyde fixatives is enhanced by MW irradiation. This enhancement may be effected both by the indirect heat generated by MWs and directly by the irradiation itself, offering another mechanism of acceleration of chemical fixatives.

IX. References

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