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R. von Wasielewski · M. Werner · Martina Nolte
L. Wilkens · A. Georgii

Effects of antigen retrieval by microwave heating in formalin-fixed tissue sections on a broad panel of antibodies

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Abstract Formaldehyde fixation of biopsy specimens for routine purposes has often been held responsible for the poor reproducibility of immunohistochemical studies. Recently, antigen retrieval (AGR) using microwave irradiation was described as a potential tool to enhance immunostaining. A comparison of conventional staining and staining after microwave heating was performed for 52 markers, using tissues fixed in formaldehyde for 24 h, 1 to 6 weeks and 3 years respectively, as well as consultant case material. After adequate duration of fixation (24 h), only a few markers (17%) showed better results after AGR, but this percentage was increased to 50% when tissues were fixed for longer periods. Maximal enhancement was obtained in the group of consultant cases (58% of tested markers demonstrated better staining results), in which the period of fixation and tissue processing was unknown. To achieve reliable enhancement with AGR, continuous heating (100° C) should not be shorter than 20 min. In conclusion, AGR may become the most important tool to simplify and equalize immunohistochemical techniques, if critically evaluated.

Introduction

The possibility to immunophenotype various formalin-fixed tissues using monoclonal antibodies has become an important part of histopathology over the last decade. However, ". . . the most common cause of poor reproducibility in immunohistologic studies is the unpredictable alteration of antigenic sites that is introduced by tissue fixation and processing" (Battifora 1991). Prolonged formalin fixation results in what has been called a masking effect with weak or false negative immunostaining. Moreover, uneven staining patterns within a specimen may occur if inadequate duration of fixation causes a combination of alcohol- (during tissue processing and rehydration) and formaldehyde fixation (Fox et al. 1985; Elias et al. 1989; Battifora 1991). In cases of heterogeneous formalin fixation, a refixation after deblocking of the tissue often completes the preservation and considerably improves immunostaining.

After overfixation, pretreatment with proteases has, for many years, been the limited approach to overcome the problem of antigen-masking (Battifora and Kopinski 1986). Recently, however, new techniques of antigen retrieval (AGR) have been described, which are useful not only for samples after prolonged formalin fixation but also to facilitate immunostaining with antibodies that previously were not suitable for routinely fixed and processed material (Shi et al. 1991; Cattoretti et al. 1992, 1993; McCormick et al. 1993; Munakata and Hendricks 1993; van den Berg et al. 1993; von Wasielewski et al. 1993). Besides the new technique of AGR, microwaves have proven to be useful for several purposes in pathology, e.g. tissue fixation or acceleration of conventional staining and immunostaining (for introduction and review see: Boon and Kok 1992; Login and Dvorak 1994).

In this study we present the results of a wide range of antibodies with respect to their behaviour after AGR in a microwave oven, systematically tested with various periods of fixation and heating times and simultaneously compared with conventional immunohistochemistry using serial sections. In contrast to other published reports, we investigated not only tissues fixed under controlled conditions but also routine cases of unknown fixation, which had shown unsatisfactory results using conventional immunohistochemistry.

Materials and methods

Tissues and fixation

Samples of normal and malignant tissues obtained either directly after surgical removal or from autopsy cases were subdivided into four groups (A–D) according to different fixation procedures.

R. von Wasielewski · M. Werner · M. Nolte · L. Wilkens
A. Georgii (✉)
Pathologisches Institut der Medizinischen Hochschule Hannover, Konstanty-Gutschow-Strasse 8, D-30625 Hannover, Germany
Tel. 0049-511-532-4500; Fax 0049-511-532-5799

Table 1 Tissues and fixation periods used (*s* surgical specimen, *a* autopsy tissue)

	Group A	Group B	Group C	Group D
Fixation	Controlled fixation 24 h (10% buffered formalin)	Controlled fixation 7, 14 and 42 days (10% buffered formalin)	Unknown fixation procedure (consultant case material)	Prolonged formalin fixation for ≥3 years (initially 10% buffered formalin, no further monitoring of fixation fluid)
Tissues/ diagnosis	Normal tonsil, skin, nerve, lymph node, liver, pancreas, kidney, small intestine, Hodgkin's lymphoma, malignant melanoma	Normal tonsil, skin, nerve, lymph node, liver, pancreas, kidney, small intestine, Hodgkin's lymphoma, malignant melanoma	Non Hodgkin's lymphoma, Hodgkin's lymphoma, adenocarcinoma of different origin	Normal skin, small intestine, pancreas, peripheral nerve, liver, kidney, Hodgkin's lymphoma, malignant melanoma
Number of samples	44 (s)	44 (s)	45 (s)	10 (s) 18 (a)

Table 2 Markers used in this study (*M* monoclonal, *P* polyclonal, *C* cytochemical, *L* lectin)

Marker in alphabetical order	Type	Dilution/ digestion	Source[a]/ catalogue no.	Clone
a-1-Antitrypsin	P	1:300/–	DA/A012	–
a-1-Antichymotrypsin	P	1:100/–	DA/A022	–
BCL-2("B-cell leukaemia")	M	1:40/–	DA/M887	124
BerH2 (CD30)	M	1:20/D	DA/M751	Ber-H2
Bauhinea purpurea	L	1:200/–	EY/BA2501	–
CAE (chloracetate esterase)	C	–/–	SI/91C	–
CD 3	P	1:100/D	DA/A452	–
CBE 77	M	1:8/–	1/–	–
Chromogranin	M	1:1/–	CA/E001	LK2H10
Collagen IV	M	1:40/D	DA/M785	CIV22
Elastase	M	1:1000/D	DA/M752	NP57
F VIII (von Willebrand factor)	M	1:200/D	DA/M616	F8/86
GP IIIa (platelet glycoprotein IIIa; CD 61)	M	1:50/D	DA/M753	Y2/51
HMB 45 (melanoma)	M	1:500/–	DA/M634	HMB45
IgA	P	1:100/D	DA/A408	–
IgG	P	1:500/–	DA/A423	–
IgM	P	1:500/–	DA/A426	–
Insulin	M	1:2/–	AM/RPN708	–
Kappa	P	1:5000/–	DA/A191	–
Keratin Pan (KL1)	M	1:400/–	DI/0128	KL1
Keratin Pan (AE1/AE3)	M	1:1000/D	BM/1124161	AE1/AE3
Keratin Pan (MN 116)	M	1:1/D	1/–	–
Keratin 1–8	M	1:10/D	PG/709108	Ks pan1-8
Keratin 18	M	1:10/D	BM/814385	CK2
Keratin 19	M	1:10/D	PG/621088	Ks19.1
Keratin 20	M	1:20/D	DA/M7019	Ks20.8
Keratin, high molecular weight	M	1:200/D	DA/M630	34βE12
KP 1 (CD 68)	M	1:100/–	DA/M814	KP1
L26 (CD 20)	M	1:100/–	DA/M755	L26
Lambda	P	1:5000/–	DA/A193	–
LCA (CD 45; leukocyte common antigen)	M	1:200/–	DA/M701	2B11+PD7/26
LN 1 (CDW 75)	M	1:4/–	BT/812225	LN1
LN 2 (CD 74)	M	1:2/–	BT/812230	LN2
Lysozyme	P	1:100/–	DA/A099	–
M 1 (CD 15)	M	1:30/D	DA/M733	C3D-1
MAC 387	M	1:300/–	DA/M747	MAC387
MB 1 (CD 45RA)	M	1:8/–	BT/812265	MB1
MB 2	M	1:1/–	BT/812270	MB2
MIB 1	M	1:40/**	DI/505	MIB-1
MT 1 (CD 43)	M	1:80/–	BT/812255	MT1
Neurofilament	M	1:1/–	CA/E010	DP5.43.12
NKI-C3 (melanoma)	M	1:20/–	LS/MC3	NKI-C3
NSE (neuron-specific enolase)	M	1:100/–	DA/M783	BBS/NC/VI-H14
Oestrogen receptor	M	1:1/**	DI/1344	ER1D5.26
PCNA (proliferating cell nuclear antigen)	M	1:40/–	DI/NA03	PC10
Progesterone receptor	M	1:40/–	DI/PR1808	mPR1
p53	M	1:40/D	DA/M7001	DO7
S-100	P	1:1000/–	DA/Z311	–
Synaptophysin	M	1:15/–	DA/M776	SY38
UCHL 1	M	1:200/–	DA/M742	UCHL1
Vimentin	M	1:40/–	DA/M725	V9
4 KB 5 (CD 45R)	M	1:50/–	DA/M754	4KB5

Sources: AM, Amersham, Braunschweig; BM, Boehringer Mannheim, Mannheim; BT, Biotest, Dreieich; CA, Camon, Wiesbaden; DA, DAKO, Hamburg; DI, Dianova, Hamburg; EY, E-Y Laboratories, Santa Mateo, Calif., USA; LS, Laboserv, Gießen; PG, Progen, Heidelberg; SI, Sigma, Deisenhofen, Germany; 1, Prof. Delsol, University of Toulouse. All suppliers from Germany if not otherwise stated

** Microwave pretreatment recommended by the supplier

Specimens of groups A and B (n=44) were freshly obtained, divided into four parts and fixed in 10% buffered formalin for 24 h (group A) or 7, 14 and 42 days (group B). After fixation, tissues were immediately embedded in paraffin using a Hypercenter2 automatic processor (Shandon, Frankfurt am Main, Germany). Group C consisted of 45 consultant cases which had previously shown insufficient immunostaining without microwave pretreatment in our laboratory. Tissues of group D (n=28) were collected from cases which had been stored in formalin for 3 years (Table 1).

Preparation

Sections were cut of 2 µm in thickness, mounted on poly-L-lysine coated slides (Sigma) and air-dried overnight at 54° C. Slides were dewaxed in xylene, rehydrated and transferred to *TRIS*-buffered saline (TBS).

Antigen retrieval

AGR was performed with a Panasonic microwave oven (model 4540, 900 W). Specimens were placed horizontally into a plastic jar containing 800 ml monohydrated citric buffer (pH 6.0, 0.01 *M*; Sigma) and a maximum of 40 slides. Using two plastic containers, boiling (100° C) was accomplished after 8 min of irradiation (±30 s) and specimens were then heated continuously for 30 min. Sections were cooled down slowly to room temperature over 15 min, rinsed in bidistilled water and subsequently transferred to TBS. Preliminary comparative studies were performed using different irradiation fluids [bidistilled water, citric acid monohydrate pH 6.0, Antigen Retrieval Solution (BioGenex)], an electric hot plate as heating source and variable cooking times up to 120 min. If irradiation exceeded 30 min, the buffer was substituted to keep the slides submerged.

Immunohistochemical methods

Various monoclonal and polyclonal antibodies as well as one lectin and one histochemical reaction were tested (Table 2). Enzymatic digestion was omitted after AGR, compared to conventional immunostaining where enzymatic pretreatment was performed as recommended by the manufacturer (Table 2). Visualization of the immunohistochemical reaction was achieved by a modified alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique (Werner et al. 1990). Staining reactions with and without microwave pretreatment were performed simultaneously to exclude day-to-day variations in the detection system. Between 4 and 40 different cases were tested for each marker and group in order to achieve reliable results.

Evaluation

The staining intensity was evaluated on a point score as follows-3, strong; 2, moderate; 1, weak; or 0 absent. Within each of the four groups (A–D), points for each marker were added together and divided by the number of respective cases. This average score of the staining intensity was compared between staining reactions with, versus those without, AGR. If the mean scores between both methods differed by more than one point, the reaction was graded as better (+) or worse (–) than staining without AGR; otherwise the comparison was regarded as equal (o)

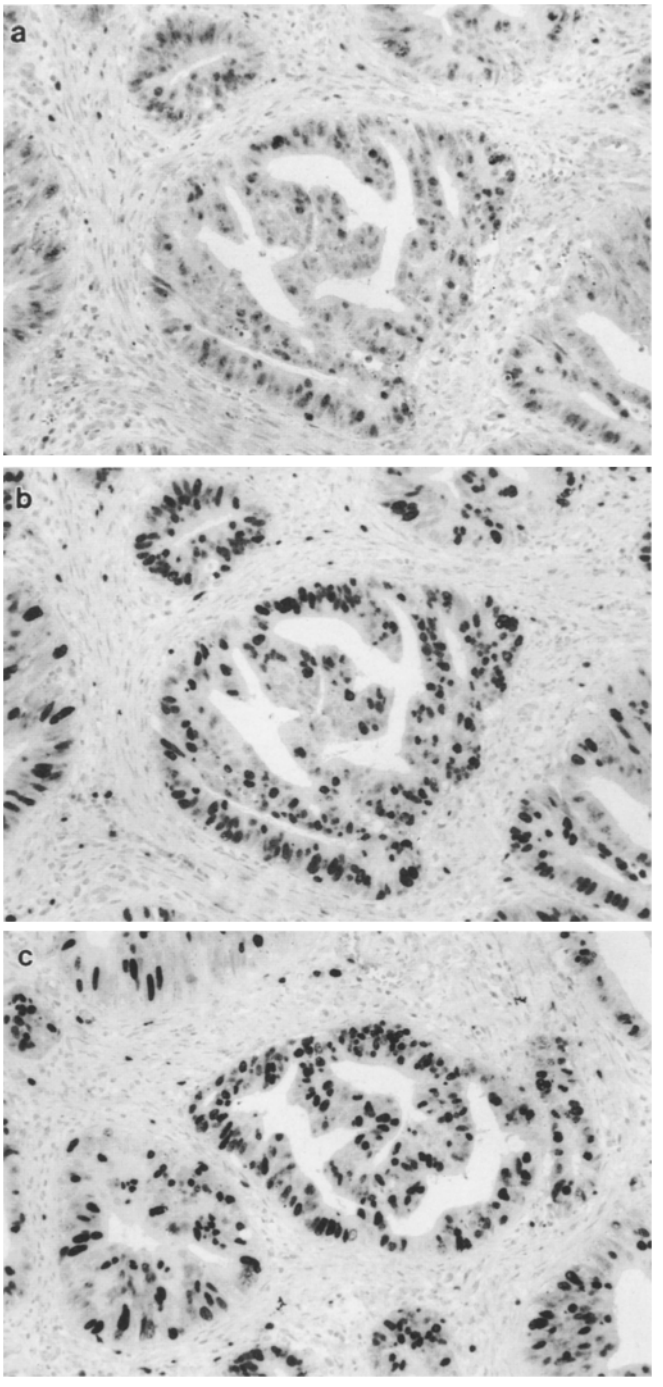

Fig. 1a–c MIB 1 (Ki-67 antigen) staining after (**a**) 15/(7)min; **b** 30/(22)min; **c** 120/(112)min of microwave heating in serial sections of a colon carcinoma (net boiling time at 100° C in parenthesis). Note the increase in number and intensity of positive cells after a sufficient length of heating (cf. **b** and **a**). After extensive boiling (**c**), the staining quality is the same as that obtained at 30 min. The excellent tissue preservation after such extensive treatment is remarkable

Results

No difference was observed between boiling the slides on a conventional hot plate or in a microwave oven (20 min at 100° C), but the latter procedure was more convenient to perform. Maximal enhancement of the staining intensity by AGR was achieved by continuous boiling (100° C) of the slides for 20–35 min (net boiling time) in a citric acid monohydrate buffer (pH 6.0) and was therefore used in all subsequent experiments. After prolonged boiling for up to 120 min, no further increase of the

Table 3 Percentage ratings among the different groups of formaldehyde fixation. The gradation into better (+), equal (o) and worse (−) refers to the scoring system described in the Materials and methods

Group[a]	Better (+)		Equal (o)		Worse (−)	
	ratio	%	ratio	%	ratio	%
A	9/52	17%	37/52	71%	6/52	12%
B	26/52	50%	19/52	37%	7/52	13%
C	25/43	58%	12/43	28%	6/43	14%
D	23/45	51%	11/45	24%	6/45	13%

[a] Fixation period: Group A, 24 h; group B, 1, 2 and 6 weeks; group C, unknown, consultant cases; group D, ≥3 years

staining intensity could be observed, but the enhancement obtained with some of the markers appeared to be less intensive. A reduced net boiling time (100° C) of 7 min led to suboptimal enhancement. With antibody MIB 1 (Ki-67 antigen), not only was a less intense staining reaction obtained but also a decrease in the number of positive cells (Fig. 1a–c). In some of the sections tissue morphology was impaired, affecting mostly connective tissue. In bone marrow biopsies, osteous trabeculae constantly shrank or were lost, but the cellular compartment remained unaffected by AGR.

The results of the comparative immunostaining within the differently processed tissue groups are summarized in Table 3 and the detailed scoring shown in Table 4. The majority of the markers tested (71%) scored equally when a controlled fixation of 24 h was performed (group A). Only nine markers showed constantly better staining (+) with microwave irradiation (Table 4), of which two are already recommended to be suitable only after non-enzymatic antigen retrieval (MIB 1 and anti-oestrogen antibody). Among these nine antibodies, especially the keratin antibody of high molecular weight and anti-BCL-2, staining was dramatically enhanced after AGR (Fig. 2a–d). Immunostaining with six (groups A, C, D) and seven (group B) of the markers respectively resulted in a remarkable decrease or even loss of reactivity (e.g. GP IIIa) when specimens were boiled prior to immunostaining.

If formalin fixation was extended up to 6 weeks (group B), two subgroups could be distinguished by different staining results after conventional immunohistochemistry (Table 5). The one subgroup (B1; n=21) displayed almost constant staining intensities after all three fixation periods (1, 2 and 6 weeks). The beneficial effect of antigen retrieval seen here in five of the markers tested also stayed constant in these cases: the relative increase in staining intensity after AGR was essentially the same after 7, 14 and 42 days of fixation, respectively (see Fig. 3a).

Within the other subgroup (B2; n=29), prolongation of fixation resulted in a decrease of staining intensity without AGR. Here, the beneficial effect of AGR (19/29 markers from group B2) was either a maximal enhancement over the whole investigation period or the negative effects of advance in fixation were at least reduced (Fig. 3b, c). The antibodies against oestrogen-receptor and Ki67 (MIB 1) were not included for this comparison because they stain after AGR only. Overall, half of the markers tested (26/52) scored better (+) after microwave retrieval within group B.

Table 4 Results of the 52 markers tested. The gradation into better (+), equal (o) and worse (−) refers to the scoring system described in the Material and methods. (+, Improvement after antigen retrieval; o, no difference of staining intensity; −, decrease or losses of staining intensity after antigen retrieval; #, no staining, neither with or without microwave irradiation) (*n.t.* not tested)

Markers in alphabetical order	Group			
	A	B	C	D
a-1-Antitrypsin	o	o	o	o
a-1-Antichymotrypsin	o	o	o	o
BCL-2	+	+	+	#
Ber H2	o	+	+	+
Bauhinea purpurea	o	o	o	o
CAE	−	−	−	−
CD3	o	o	+	+
CBE 77	+	+	+	#
Chromogranin	o	o	n.t.	+
Collagen IV	o	+	+	o
Elastase	−	−	−	−
F VIII	−	−	−	−
GP IIIa	−	−	−	−
HMB 45 (melanoma)	o	o	n.t.	o
IgA	o	o	o	n.t.
IgG	−	−	−	−
IgM	o	−	o	n.t.
Insulin	o	+	n.t.	+
Kappa	o	o	o	o
Keratin-Pan (KL 1)	o	+	+	+
Keratin-Pan (AE1/AE3)	o	+	+	+
Keratin-Pan MN 116	o	+	+	+
Keratin 1–8	o	+	+	+
Keratin 18	o	o	o	+
Keratin 19	o	+	n.t.	+
Keratin 20	+	+	n.t.	+
Keratin, high molecular weight	+	+	+	+
KP 1	o	+	+	+
L 26	o	+	+	o
LN 1	o	o	o	n.t.
LN 2	o	+	+	+
Lambda	o	o	o	o
LCA	o	o	+	o
Lysozyme	o	+	+	n.t.
M 1 (C3D-1)	o	+	+	+
MAC 387	−	−	−	−
MB 1	o	o	o	o
MB 2	o	o	+	+
MIB 1[a]	+	+	+	+
MT 1	o	+	+	#
Neurofilament	o	+	n.t.	+
NKI-C3	o	o	n.t.	o
NSE	o	+	n.t.	+
Oestrogen receptor[a]	+	+	+	n.t.
PCNA (PC10)	+	+	+	+
Progesterone	+	+	+	n.t.
p 53	+	+	+	n.t.
S-100	o	o	o	+
Synaptophysin	o	o	n.t.	+
UCHL 1	o	o	+	#
Vimentin	o	+	+	+
4 KB 5	o	o	o	#

[a] Microwave irradiation already recommended by the manufacturer

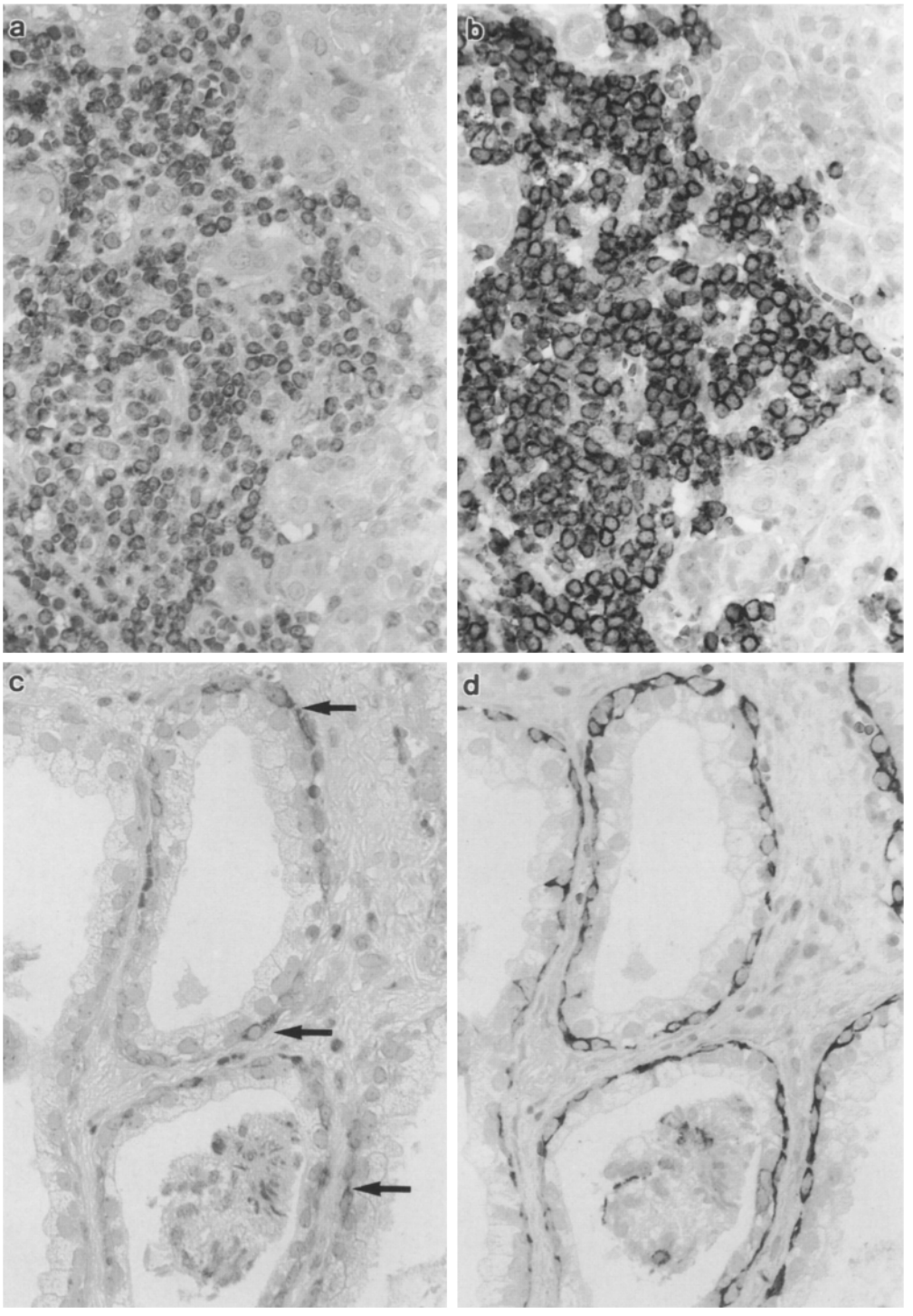

Fig. 2a–d Comparison of serial sections between conventional (**a, c**) and microwave pretreated (**b, d**) staining. **a, b** BCL-2 immunoreaction showing lymphoma infiltration of the pancreas is strongly enhanced after antigen retrieval (AGR). **c, d** High molecular weight keratin, marking basal cell layers in prostate. The *arrows* show only weak positivity in conventional immunostaining (after enzymatic digestion). After heating, a strong and complete reaction was obtained without enzymatic digestion (**d**)

The highest percentage of markers to benefit from AGR was found within the group of consultant cases, displaying weak or absent immunoreactivity if stained conventionally (group C). The results of group D are comparable to the findings of group B. Only 2 of the 22 markers (9%), which had been scored as better (+) in group B displayed an equal (o) score within group D. None scored worse (–) and three were completely unreactive (#) whichever method was chosen (Table 4).

False positive staining after microwave heating was not detectable with the exception of one of the tested reactions. CD3 exhibited in some cases cytoplasmic positivity in blastic cells of high malignant B-non Hodgkin's lymphomas. This effect, which was observed after AGR only, could be constantly repeated and was seen using even a tenfold higher dilution of primary antibody than was recommended.

Table 5 Scoring of Group B with respect to fixation periods. Group B was split into subgroups B1 and B2 according to the staining results after 1, 2 or 6 weeks of formaldehyde fixation and conventional immunostaining. A prolongation of the fixation period from 1 to 6 weeks did not considerably alter the staining results of markers listed in subgroup B1. The markers of subgroup B2 showed decreased staining intensity when duration of fixation was extended to 2 and 6 weeks compared to staining performed after 1 week of formaldehyde fixation

Subgroup B1 (constant results after 1, 2 and 6 weeks of fixation; n=21)	Subgroup B2 (decreasing staining results after 1, 2 and 6 weeks of fixation; n=29)
CBE 77[a]	BerH2[a]
Lysozyme[a]	BCL-2[a]
L26[a]	Collagen IV[a]
M 1[a]	Insulin[a]
Neurofilament[a]	Keratins (n=7)[a]
	KP 1[a]
a-1-Antitrypsin	LN 2[a]
a-1-Antichymotrypsin	MT 1[a]
Bauhinea purpurea	NSE[a]
CAE	PCNA[a]
CD 3	Progesterone receptor[a]
Chromogranin	p53[a]
HMB 45	Vimentin[a]
IgA	
IgG	Elastase
Kappa	F VIII
Lambda	GP IIIa
LCA	IgM
MAC 387	Keratin 18
MB 2	LN 1
NKI-C3	MB 1
S-100	Synaptophysin
	UCHL 1
	4 KB 5

[a] Markers scoring better (+) after antigen retrieval

Discussion

Lack of standardization still hampers immunohistochemical methods (Elias et al. 1989; Wick 1989; Battifora 1991; Miettinen and Kovatich 1991; Swanson 1991; Swanson and Ackerman 1991). Although promising suggestions have been made (e.g. Biological Stain Commission, see Elias et al. 1989), the problem is still acute for pathologists using immunohistochemistry with consultant case material.

Recently, non-enzymatic antigen retrieval has been shown in several reports to enhance immunostaining (Shi et al. 1991; Cattoretti et al. 1992, 1993). In general, our results with tissues that have been fixed under controlled conditions in formalin (groups A, B, D) are in accordance with those findings, indicating that immunohistochemical staining reactions with many antibodies may benefit from this new technique. For example vimentin staining, which is known to be very sensitive to prolonged formaldehyde fixation (Battifora 1991), is extremely enhanced after microwave irradiation (Table 4; Shi et al. 1991; Suurmeijer and Boon 1993), but is unaffected by enzymatic digestion (Leong and Gilham 1989). Even in cases where the results of optimized enzymatic

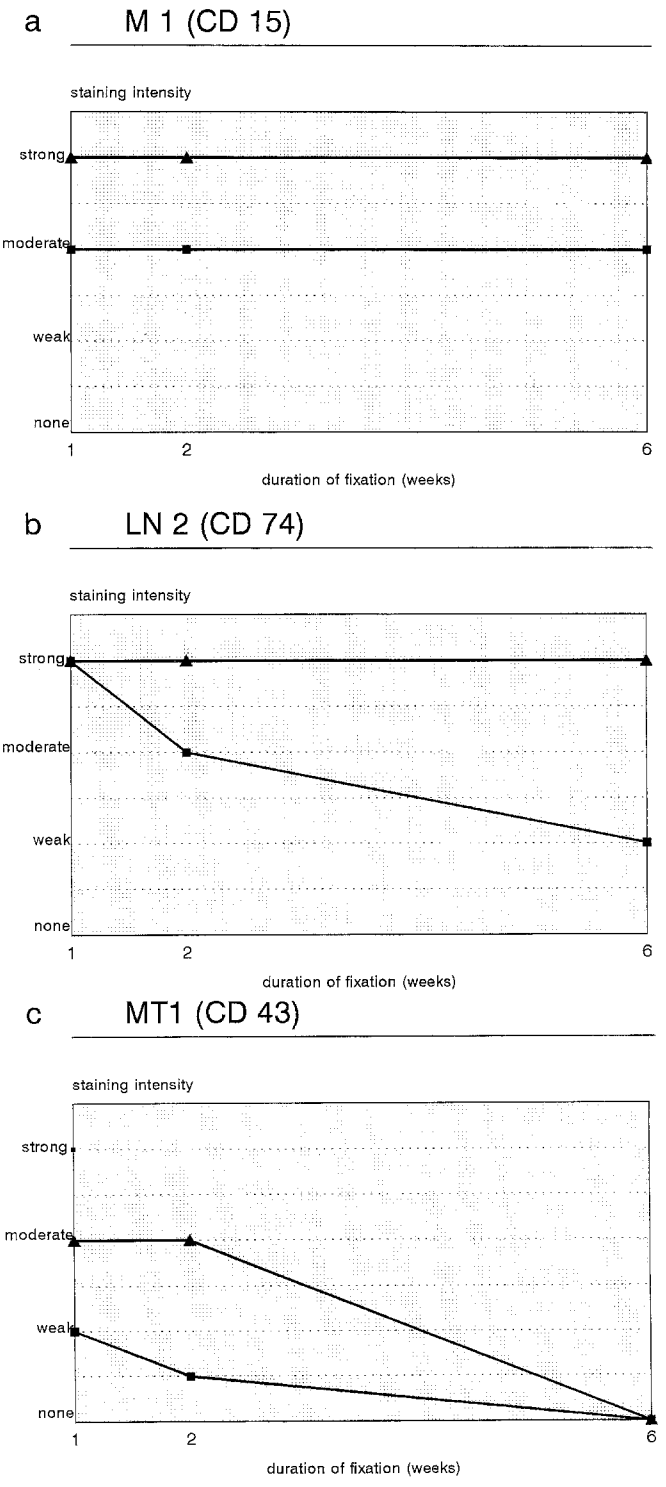

Fig. 3a–c Staining intensity as a function of the fixation period (only group B). **a** Dako M1 (CD 15) as an example of constant staining results after 1, 2 and 6 weeks of formaldehyde fixation (subgroup B1). Although conventional immunohistochemistry achieves reliable and constant staining intensity, the marker benefits additionally from AGR in all stages of fixation period. **b** LN 2 (CD 74), an example for a decrease of staining intensity with prolongation of fixation (subgroup B2). AGR, in contrast, results in strong staining intensity even after 6 weeks of fixation. **c** MT1 (CD 43): decrease of staining intensity occurrs with both methods, but AGR reduces the negative effects of prolonged fixation to a certain extent. *Squares,* Conventional immunohistochemistry; *triangles,* immunohistochemistry after AGR

treatment compare well with those of non-enzymatic microwave pretreatment, the latter procedure seems preferable because of its simplicity.

The usefulness of AGR for cases with insufficient immunostaining without microwave pretreatment has not yet been investigated. With 58% of the tested markers showing better results after microwave heating (group C), our results suggest that AGR offers especially for those cases an advantageous method for enhanced immunohistochemistry. However, because it is highly unlikely that the majority of our consultant cases have been fixed for longer periods than those examined in group B, reasons other than a pure overfixation have to be considered as causing unsatisfactory immunostaining without AGR. Among these, the temperature of fixation and other processing procedures have been suggested to influence the preservation of antigens (Leong and Gilham 1989; Battifora 1991). Our data sustain the possibility of such effects and indicate that these variables to the results of immunohistochemical staining should not be underestimated; however, AGR in many cases is able to overcome even those limitations.

Different explanations have been given for the possible mechanisms of AGR in a microwave oven (Shi et al. 1991; Cattoretti et al. 1993; Suurmeijer et Boon 1993). Among these, the breaking up by heating of cross-links between proteins caused by formalin fixation seems the most likely from our results. Since tissues fixed in a coagulating fixative, such as ethanol, do not benefit from microwave AGR (Shi et al. 1991; Suurmeijer et al. 1993), the causal effect should be assumed to the nature of formaldehyde fixation. Recently, Cattoretti et al. (1993) have suggested that further protein denaturation caused by boiling might be the major cause of non-enzymatic AGR. This suggestion does not explain why microwave heating becomes more effective after prolonged formalin fixation than after adequate fixation periods (24 h). Therefore, further studies are required to clarify the role of denaturation and the causes of AGR by microwave heating.

Only a few studies have been published so far concerning the microwave pretreatment, but results (e.g. keratin immunoreactivity), using different retrieval solutions and methods, have already been conflicting (Shi et al. 1991; Battifora 1991; Cattoretti et al. 1993; Dookhan et al. 1993). It it likely that the results of staining after AGR depend much on the protocol used (irradiation buffer, duration, etc.; von Wasielewski et al. 1994). Therefore, standardization of the protocol seems advisable. The example of MIB-1 staining after different irradiation times shown here indicates that an adequate duration is needed to achieve the true proliferation index. With a shorter microwave pretreatment, the number of positive nuclei may be smaller than the actual proliferating compartment. From our results it seems that the commonly used 2×5 min treatment (Shi et al. 1991; Cattoretti et al. 1992; Dookhan et al. 1993; Merz et al. 1993; van den Berg et al. 1993) is too short. This is of special importance when the number of positive cells is essential for an exact result (e.g. receptor positivity or proliferation indices).

The observation that false positive cytoplasmic staining was seen with CD3 in some of the cases underlines the need to investigate AGR effects for every individual antibody. Although we investigated a considerable number of cases (n=40), no unexpected binding patterns, such as staining of nucleoli with L26 or loss of cytoplasmic staining with BerH2 could be observed as reported by others (Charalambous et al. 1993; Norton 1993). As was also mentioned by Cattoretti et al. (1993), the outcome of immunostaining after AGR is not predictable, and its seems essential to evaluate the benefits and disadvantages very carefully.

In conclusion, our results seen against the background of the literature show that AGR is a simple method to overcome the pitfalls often encountered with immunostaining. However, AGR should be critically evaluated, as different antibodies react in different ways to microwave pretreatment. Nevertheless, when applied after careful investigation, AGR may become the most important tool to simplify and standardize immunohistochemical techniques.

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