Immunoreactivity to MIB-1 in breast cancer: methodological assessment and comparison with other proliferation indices

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Abstract. The recent availability of the monoclonal antibody MIB-1 (which is able to detect the human nuclear cell proliferation-associated antigen Ki-67 even on formalin-fixed, paraffin-embedded sections, microwave-treated and routinely processed for immunohistochemistry) could open new avenues for validation of the clinical role of tumour cell proliferation on large, consecutive and unselected series of human tumours. However, the routine use of such a marker requires a methodological standardization as well as the comparative assessment of some technical and biological aspects. The MIB-1 index was determined in parallel samples from 50 consecutive invasive breast carcinomas processed with different fixatives for different times. The median values of MIB-1 indices following 2, 6 and 24 h of formalin fixation were similar (29.4%, 30.6% and 29.7%, respectively) and consistent with those reported in the literature; squared linear regression coefficients were 0.99. The median values of MIB-1 indices were markedly lower in Bouin-fixed, paraffin-embedded, and in frozen samples (20.0% and 19.8%, respectively), with a poor correlation coefficient with the values detected following formalin fixation ($R^2=0.456$). Moderate and poor correlations were observed between Ki-67 index and MIB-1 detected on frozen (r_s , 0.78) or formalin-fixed, paraffin-embedded samples (r_s , 0.47) and a minimal concordance was observed between TLI and MIB-1 or Ki-67 (r_s , 0.25 and 0.22, respectively). Our results indicate interference of the fixative type on immunoreactivity to MIB-1 and also suggest that Ki-67 and MIB-1 reacted with different epitopes of the same antigen.

Assessment of the proliferative rate of tumour cells is becoming an integral part of the biofunctional assessment of human cancers (Silvestrini 1994). In breast cancer in particular, it is already used as a complement to pathological staging to identify patients suitable for different therapeutic approaches (Amadori *et al.* 1994). More recently, cell kinetics has been shown to have a promising role also as an indicator of response to chemical and hormonal treatments (Silvestrini & Daidone 1995).

Most cell kinetic studies have been directed towards quantifying the S-phase cell fraction by analysing functional (active incorporation of ^3H -thymidine) or phenomenological

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(quantification of DNA content) aspects. Approaches have also been proposed to analyse the whole cell proliferating fraction (Quinn & Wright 1990). Among these, detection of the proliferation-associated antigen Ki-67 has been used in pathology and proposed as a complement or as a possible alternative to grading, but the monoclonal antibody (MAb) available since the early 1990s, extensively characterized by immunobiochemical and molecular studies (Gerdes *et al.* 1991), was applicable only to frozen material. The recent availability of a novel Ki-67-equivalent MAb (MIB-1) (Cattoretti *et al.* 1992) and the development of methodological approaches for antigen retrieval from paraffin-embedded sections by microwave processing (Shi, Key & Kalra 1991) have renewed interest in the antigen and its prognostic validation.

Several studies have reported comparisons among different proliferation indices, evaluated on the same (paraffin-embedded material) and/or on different (fresh or frozen) pathological material (Kubbutat *et al.* 1994, Gee *et al.* 1995, Leong *et al.* 1995). In particular, a number of studies have compared the immunodetection of Ki-67 protein by different reagents and in different methodological conditions (Gerdes *et al.* 1991, Kubbutat *et al.* 1994, Barbareschi *et al.* 1994, Leong *et al.* 1995). However, only some of them have been performed on a substantial series of human tumours or in comparison with other proliferation indices, and no comparative assessment of technical aspects was made in any of them.

In the present study, we determined on parallel samples from a consecutive series of 50 invasive breast carcinomas MIB-1 immunoreactivity following different fixatives and fixation times. Additionally, MIB-1 immunoreactivity was compared to Ki-67 immunoreactivity, and both were compared to the S-phase cell fraction evaluated as ^3H -thymidine labelling index.

MATERIAL AND METHODS

The study was performed on 50 consecutive breast cancers from previously untreated patients who underwent surgery at the Istituto Nazionale Tumori di Milan. The series included 38 infiltrating ductal carcinomas, three infiltrating lobular carcinomas and seven cases diagnosed as mixed infiltrating ductal and lobular carcinomas. In all the cases, tumours were larger than 2.5 cm in diameter at pathological examination.

Immediately after surgery, part of the tumour material was snap-frozen in liquid nitrogen and processed for determination of MIB-1 and Ki-67 immunoreactivity and part was fixed at room temperature in 10% buffered formaldehyde for 2, 6 and 24 h and in Bouin's solution for 2 h, then routinely paraffin embedded and processed for determination of MIB-1 immunoreactivity. For each sample, parallel specimens were processed for determination of the ^3H -thymidine labelling index (TLI).

Determination of the TLI

Tumour material, sampled by the pathologist from different areas, was cleansed of fat and necrotic tissues and minced into fragments of a few mm³. Samples containing 10–15 pooled fragments were incubated for 1 h at 37°C with ^3H -thymidine using a commercial kit (Ribbon, Milan, Italy), as previously described (Benini *et al.* 1995). Concordance among batches of the proliferation kit was assessed by verifying ^3H -thymidine specific activity by liquid scintillation counting and by repeating TLI measurements on the same subset of tumours. At the end of the incubation period, the specimens were fixed for 6 h in 4% buffered formaldehyde and subjected to conventional histoautoradiographical procedures (Silvestrini 1991).

Determination of proliferation indices by immunocytochemistry

For each specimen, frozen and paraffin-embedded tissues were previously examined on haematoxylin and eosin-stained sections and then immunostained.

Frozen tissues

Two 2- μ m serial consecutive sections of each frozen sample were cut, mounted onto poly-L-lysine-coated slides (Sigma, Milan, Italy), air dried overnight, and then fixed in cold acetone (4°C) for 10 min. Subsequently, the slides were immunostained according to a standard protocol and incubated, one with a 1:50 dilution of the Ki-67 MAb (Dako, Milan, Italy) and the other with a 1:100 dilution of the MIB-1 MAb (Immunotech, Marseille, France).

Paraffin-embedded tissues

After fixation in 10% buffered formaldehyde, or Bouin's solution, the specimens were embedded in paraffin. Sections of 2 μ m were cut and mounted onto poly-L-lysine-coated slides, dried overnight at 37°C, deparaffined in xylene, rehydrated in graded alcohol and, after three rinses in phosphate-buffered saline (PBS), immersed in 10 mM citrate (in distilled water, pH 6.0). Sections were then submitted to microwave oven processing (Shi *et al.* 1991, Cattoretti *et al.* 1992), as previously described, for two cycles of 8 and 5 min at 700 W. Subsequently, the slides were immunostained according to a standard protocol and incubated with a 1:100 dilution of the MIB-1 MAb.

Immunocytochemical procedure

After washing with PBS for 15 min (three cycles), all sections were treated with 3% H₂O₂ in methanol for 1 h to block endogenous peroxidase. After rinses, normal goat serum (Dako) was used as a suppressor serum for 1 h at a 1:20 dilution. The excess serum was removed, and the sections were incubated overnight with primary antibodies at 4°C. After 1 h at room temperature to avoid thermal shock and several brief washes, sections were overlaid with a 1:100 dilution of biotinylated goat anti-mouse IgG (Dako) and then incubated for 30 min with a 1:350 dilution of streptavidin conjugated with horseradish peroxidase (Dako), followed by rinses. Colour was routinely developed using 3-amino-9-ethyl carbazole for 10 min followed by a light haematoxylin counterstaining (Carazi's hematoxylin). Slides were mounted with glycerine gel.

All dilutions of normal sera, antibody staining and streptavidin–horseradish peroxidase were performed using PBS containing 5% bovine serum albumin. All steps in the staining procedure were performed the same way for all antibodies on all sections. Negative controls involved a substitution of the primary antibody with non-immune serum from the same species in which the antibody was produced. Tissues with known positive immunostaining for MIB-1 or Ki-67 were used as positive controls. Reproducibility of staining and concordance between batches of MIB-1 and Ki-67 monoclonal antibodies was tested on positive controls.

Data analysis

The fractions of cells immunostained with MIB-1 or Ki-67 MAbs or incorporating ³H-thymidine were quantified independently by two observers by scoring 1000 (for MIB-1 and Ki-67) and 3000 (for TLI) cells from consecutive fields. The interobserver concordance

was satisfactory, and correlation coefficients between observers were always greater than 0.85. Quantification of Ki-67, MIB-1 or TLI was expressed as the percentage of labelled cells over the total number of tumour cells. The concordance between the different methodological procedures used for determining MIB-1 immunoreactivity was evaluated by linear regression analysis with estimation of the squared Pearson correlation coefficient (R^2). Additionally, data were compared by a descriptive method, as reported by Altman & Bland (1983). In this analysis, the average of MIB-1 values, obtained on each tumour by two procedures differing in terms of fixative fixation time, was plotted against the difference between the two MIB-1 values. Such an analysis made it possible to evaluate the disagreement magnitude between methodological procedures as a function of the average values and to spot outliers and possible trends. The concordance between the different proliferation indices was evaluated by Spearman's rank correlation coefficient (r_s).

RESULTS

Reactivity of MAb MIB-1 on frozen and variously fixed paraffin-embedded sections

In our series of infiltrating breast carcinomas, immunoreactivity to MIB-1 was always nuclear in frozen and paraffin-embedded specimens submitted to various types and times of fixation. However, on visual inspection, formalin-fixed, paraffin-embedded specimens showed more reactive nuclei than frozen or Bouin-fixed specimens. Such a finding clearly emerged by considering the median MIB-1 labelling index observed for each set of samples (Table 1). In particular, for the tumour specimens submitted to formalin fixation, median values and ranges were similar, regardless of the time of fixation, and higher than those reported for samples from the same tumour specimens frozen or fixed in Bouin's solution. Such a difference was also reflected in the pattern of the frequency distribution of MIB-1 labelling indices, which exhibited a bell-shaped behaviour for formalin-fixed samples and was negatively skewed for frozen or Bouin-fixed samples. In fact, only about 15% of formalin-fixed samples exhibited less than 15% immunoreacting cells compared to 40% of frozen or Bouin-fixed samples.

When the immunoreactivity in the different methodological conditions was analysed on individual tumours, and samples fixed for 24 h in buffered formalin were used as reference standard, the concordance was maximal among specimens fixed in formalin for different times ($R^2=0.0996$, $P<0.0001$), and the estimates of MIB-1 were almost superimposable in specimens fixed for 2 or 24 h (Figure 1a) as well as in specimens fixed for 6 h (data not shown). Conversely, a moderate concordance was observed between formalin- and Bouin-fixed specimens (Figure 1b) as well as between formalin-fixed and frozen specimens (Figure 1c). However, although the same value was observed for the linear regression coefficients

Table 1. Immunostaining of breast cancers with MIB-1 in frozen or variously fixed specimens

	Median (%)	Range (%)
Formalin, 2 h	29.4	8.6–98.9
Formalin, 6 h	30.6	13.1–76.2
Formalin, 24 h	29.7	9.2–93.6
Bouin, 2 h	20.0	3.0–82.7
Freezing	19.8	4.8–70.0

Figure 1. Linear regression analysis to compare immunostaining values obtained with the MIB-1 antibody on paraffin-embedded breast cancer specimens fixed in 10% buffered formalin for 24 h to those obtained on specimens fixed in formalin for 2 h (a), fixed in Bouin (b) or frozen (c).

($R^2=0.45$), the patterns of disagreement were markedly different. In fact, MIB-1 values estimated in formalin-fixed specimens were generally higher than those estimated in Bouin-fixed specimens from the same tumours (Figure 2a). Conversely, such a defined pattern was not present when formalin-fixed and frozen samples were matched (Figure 2b), since the disagreement was equally due to an under, or an overestimation of MIB-1-positive cells in frozen compared to paraffin-embedded specimens. Additionally, by using as a cut-off the median values of immunostained cells following the different technical procedures (i.e. 30% for formalin-fixed and 20% for Bouin-fixed or frozen samples) and samples formalin-fixed for 24 h as a reference set, an underestimation of MIB-1 immunoreactivity was observed with a similar frequency (six cases) of Bouin-fixed or frozen samples, whereas an overestimation was slightly more frequent in the latter (Table 2).

Comparison between the different proliferation indices

At first, immunoreactivity to MIB-1 was compared to that detected against MAb Ki-67, which is a reagent developed against the same proliferation-related antigen. Overall, in this case series the median Ki-67 labelling index evaluated on frozen samples was 9.4% (range, 3.0–62.0%), which is two to three times lower than the median MIB-1 detected on formalin-fixed or frozen material. On individual tumours, the degree of correlation between Ki-67 and MIB-1 indices was partial and dependent even on methodological conditions. In fact, the concordance, expressed in terms of the Spearman correlation coefficient, was moderate when the MIB-1 labelling index was determined on 24 h formalin-fixed, paraffin-embedded specimens (r_s , 0.47; $P < 0.001$) and increased when it was determined on frozen specimens (r_s ,

Figure 2. Disagreement magnitude between MIB-1 estimates in formalin- and Bouin-fixed samples (a) or formalin-fixed and frozen samples (b).

Table 2. Concordance* between MIB-1 immunostaining following the different methodological procedures

	Bouin-fixed (% of + cells)		Frozen (% of + cells)	
	≤20	>20	≤20	>20
Formalin-fixed (% of + cells)				
≤30	22	3	20	5
>30	6	19	6	19

*Number of cases.

0.78; $P < 0.0001$), in particular on cryostatic sections consecutive to those used for Ki-67 determination.

Successively, MIB-1 and Ki-67, as putative indicators of the fraction of proliferating cells, were matched on individual tumours with the TLI, an indicator of the S-phase cell fraction which represents a standard reference for proliferation studies. For these analyses, MIB-1 immunoreactivity determined on samples fixed for 24 h in buffered formalin was considered. Overall, the median TLI was 3.6% (range, 0.7–13.8%), in agreement with the median value detected on a large series of breast cancers (Silvestrini, Daidone & Gentili 1981) and three and 10 times lower than the median Ki-67 or MIB-1 indices, respectively. The concordance between these proliferation markers was generally poor, as indicated by the low correlation coefficients (TLI vs. MIB-1: r_s , 0.25; TLI vs. Ki-67: r_s , 0.22). Only a trend in favour of a direct relation between MIB-1 and TLI was observed ($P = 0.08$).

A quantification of the disagreement between proliferation indices was obtained by considering the distribution of cases in four different proliferation classes, defined for each proliferation index by using the quartiles of the frequency distribution (Table 3). Again, the concordance with TLI was more frequently observed for MIB-1 (in 42% of the cases) than for Ki-67 (in 27% of the cases). Disagreements observed in about 60% of the cases were similarly due to an overestimation or an underestimation by indicators of the proliferating fraction with respect to the indicator of the S-phase cell fraction.

Table 3. Comparison between proliferation indices: case distribution (%) in the proliferation classes

TLI: quartiles‡	MIB-1: quartiles*				Ki-67: quartiles†			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th
1st	7	7	4	4	4	9	2	7
2nd	7	11	4	7	9	9	6	4
3rd	7	4	11	2	4	4	7	9
4th	4	4	4	13	2	8	9	7

*Quartiles for MIB-1: first, ≤20%; second, 20–30%; third, 30–45%; fourth, >45%. MIB-1 immunoreactivity was detected on 24 h formalin-fixed specimens.

†Quartiles for Ki-67: first, <6%; second, 6–9.5%; third, 9.5–19%; fourth, ≥19%. Ki-67 immunoreactivity was detected on frozen specimens.

‡Quartiles for TLI: first, ≤2.2%; second, 2.3–3.6%; third, 3.7–6.5%; fourth, ≥6.6%.

Diagonal line indicates a comparable proliferative activity as evaluated by the different proliferation indices.

DISCUSSION

Immunoreactivity to MIB-1 following different methodological conditions was investigated in a set of invasive breast carcinomas. In this limited series of tumours, median values and ranges of ^3H -thymidine and Ki-67 labelling indices were in accord with published data on larger and unselected series of breast cancers (Silvestrini *et al.* 1981, Silvestrini 1991, Leonardi *et al.* 1992). Our data, in accord with results of other authors (Cattoretti *et al.* 1992, Barbareschi *et al.* 1994, Kubbutat *et al.* 1994), confirmed that formalin fixation followed by paraffin embedding and microwave irradiation was a procedure capable of exposing a large number of Ki-67 epitopes to MIB-1 and showed that MIB-1 immunoreactivity was consistent over a fixation time of 2 to 24 h. Conversely, freezing or fixation in Bouin solution led to a lower fraction of immunostained nuclei possibly by altering the Ki-67 epitope, which reacts with MIB-1 MAb. In fact, at least following Bouin fixation, a lower immunoreactivity was consistently observed compared to that seen with formalin fixation. Although some disagreement could also be ascribed to intra-tumour heterogeneity, the systematic lower estimates of MIB-1 immunoreactivity following Bouin than formalin fixation seemed to be due to fixation artefacts rather than to biological reasons.

The finding of an incomplete correlation between MIB-1 and Ki-67 was in keeping with the hypothesis that the two MAbs react with different epitopes of the Ki-67 antigen (Barbareschi *et al.* 1994). Evidence of the higher concordance between Ki-67 and MIB-1 determined on frozen rather than paraffin-embedded material emphasized the importance of tumour heterogeneity. In fact, the former comparison was performed on consecutive sections and the latter only on contiguous tumour samples due to the different fixation procedures.

Neither MIB-1 nor Ki-67 was related to TLI in the overall series, and such a finding was consistent when subclasses according to quartiles of the frequency distributions (defined for each individual proliferation index) or individual tumours were considered. This lack of a relation may not be due, as expected, to a different degree of overestimation by MIB-1 or Ki-67 indices (which detect cells in the different phases of the cell cycle) in comparison to TLI (which detects only cells in a specific cell cycle phase), but to an underestimation to a similar degree in an overall two-thirds of cases. Such findings confirm that there are biological differences among proliferation indices (MIB-1, Ki-67 and TLI), the first two based on a phenotypical approach and the latter one based on an active DNA-precursor incorporation and associated to a specific cell-cycle phase.

In conclusion, Ki-67, MIB-1 or TLI cannot be considered as alternative proliferation indices, as already emerged in the only published comparative analysis of the clinical role of the former two markers (Rudas *et al.* 1994). However, a possible complementarity of the different information provided by the proliferation markers, which already emerged for example for TLI and flow-cytometric S-phase cell fraction in non-Hodgkin's lymphomas (Costa *et al.* 1992), needs to be investigated. Although the biological significance of the exposure of different or of an excess of antigens to microwave treatment of paraffin-embedded material remains to be defined, the availability of MIB-1 should allow such comparative studies even on human solid tumours from archival material following formalin fixation, as well as prospective studies on routine material, once the fixation procedures are accurately standardized.

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