Conclusion

Present data show that all the revelation systems can be used usefully with high Ab dilutions but also show that there are differences in sensitivity among the systems. Such differences must be kept into consideration when standardization of procedures or a immunohistochemical quality control must be defined.

These data suggest again that if the results of immunohistochemical stains are not satisfying, it is probable that the problem is not related to immunostaining itself but more probably to a factor before immunostaining, *i.e.,* in fixation or antigen retrieval.

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Standardization of reporting and quantitation

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Introduction

Most discrepancies among published reports of immunohistochemcal assays are attributable to methodological divergence. However, many others are due to variance in the interpretation of the immunostains.

Often, the interpretation of immunohistochemical stains is qualitative and subjective, quantification of the reaction being of little import. A diagnostic decision is based upon whether a certain molecule is expressed or not by cells. For example, any amount of expression of leukocyte common antigen by neoplastic cells suffices to tilt the diagnosis in favor of lymphoma.

Proper interpretation of most immunostains, however, depends to some extent on a semiquantitative estimation of the antigen content. This may require the setting of cutoff levels between what is to be considered positive and negative results. Reasonable reproducibility of immunostains is essential for these cutoff levels to be applicable. For many reasons, achieving reproducible thresholds applicable across laboratories, is one of the most difficult challenges of modern immunohistochemistry.

As quantitative tissue-based biochemical assays are being replaced by immunohistochemistry, interest in accurately measuring immunostains is on the rise. The development of novel cancer therapies will fuel the demand for accurate measurement of a growing number of target molecules as a means to select patients. For many reasons, to be discussed, quantitative immunohistochemistry, when applicable, is the tool of choice for this purpose.

Numerous methods for visual scoring of immunohistochemical assays have been proposed to improve quantitation. These have been shown to be more reproducible than visual estimates, and often to be of clinical relevance. However, these scoring systems suffer from their own interobserver reproducibility problems (1, 2). Clearly, standardization of most of the factors responsible for variation in the intensity of immunostaining is essential for any of these scoring systems to perform adequately.

Computer-assisted image analysis has proven superior to visual estimates in providing quantitative immunohistocheinical assays, particularly when applied to frozen sections or fine needle aspirations (3). These systems, particularly if automated, hold promise of improving the accuracy and reproducibility of quantitative immunohistochemistry as well as to become tools for more accurate inter- and intralaboratory quality control.

Causes of discreDancy in the interpretation of immunostains

Many conflicting results in the literature are due to technical reasons, particularly variations in the choice of fixatives and duration of fixation. More recently, whether epitope retrieval was employed – and by which means – has added another source of discord. An increasingly common cause of contradictory reports nowadays, is the lack of well-defined standards about what constitutes a positive result. Examples of such a loose state of affairs abound in the literature and some of these will be discussed with suggestions for improvement.

Another source of problems is that a quantitative approach to distinguish positive from negative immunostains is often used by authors. Unfortunately, the authors' cutoff levels are not easily exported to other laboratories and worse, their intralaboratory reproducibility has frequently not been validated.

Lamentably, at the present time, there is no consensus as to what constitutes an adequate threshold of interpretation for most immunostains. For example, some laboratories include as positive estrogen receptor, any case in which even a single tumor cell shows any degree of detectable reactivity. Other laboratories require at least 20% of the cells to immunoreact. To make things worse, many times these cutoffs have been arbitrarily set without the validation of a dinicopathological study. These variable cutoffs reflect variable methodological sensitivities among laboratories.

Moreover, it is important to keep in perspective that when a retrospective study is carried out for such clinicopathological validation, it is usually done on selected case material from a single institution, with relatively uniform fixation and processing. Additionally, in these studies, to minimize dailyvariation, slides are immunostained together, in a single run (4). A cutoff level is then sought, usually with aid of statistical analysis. This procedure is perfectly reasonable to minimize variations due to method, and is particularly applicable when comparing different antibodies or methods. Unfortunately, this approach has little bearing on real life immunohistochemistry. Unpublished studies in our laboratory, for example, have shown daily variation in optical density of as much as 30% in immunostains for estrogen receptor when the same block of tissue was used as a daily control. Clearly, the variation was methodological, despite the fact that an automated processor was used. Add to this divergence of fixation and processing – as in the case of specimens handled by a reference laboratory – and the complexity of the problem comes into focus.

As these thresholds are intimately related to tissue fixation, processing and other methodological variants, the development of interlaboratory control materials containing defined amounts of the

target and comprehensively controlling all steps of the procedure is necessary to achieve true quantitative immunohistochemistry.

Quantitative immunohistochemistry

Whether the ultimate goal is to find clinically meaningful cutoff levels or the accurate measurement of antigen molecules per cell, interlaboratory reproducibility of immunostains is fundamental.

At least two separate forms of quantitative immunohistochemistry are readily evident. In its simplest form, events are merely measured, with no attempt to assay for the quantity of analyte expressed. Examples of this type are counting micrometastases in bone marrow samples or measuring peritumoral blood vessels. In these examples, minor variations in the intensity of the immunoreactivity, attributable to the method of staining or fixation procedure, have little impact in the quantification itself. With good control of specimen preparation and staining procedures, these simple quantitative procedures are currently attainable by most laboratories.

Accurate conversion of immunoreactivity into levels of analyte per sample, is much more complex, requires special equipment and, given the vagaries of fixation and processing, is not currently possible on archival paraffin-embedded material. However, prospective studies may be possible with the use of specially designed control materials, as discussed below.

Controls in quantitative immunohistochemistry

Sections of tissue expressing the target molecule, as well as tissues known not to express it are currently used routinely in immunohistochemical procedures in virtually every laboratory. The problem with such control tissues is that, at best, they only serve to control the immunostaining procedure itself. Other sources of variation such as fixation and processing are not controlled. Moreover, the amount of target molecule present in the control tissues is, more often than not, unknown.

The ideal control for quantitative immunohistochemistry should apply to the entire procedure, from fixation to interpretation of results. A suggested possible approach is to suspend in a solid matrix, cultured cells – expressing known, and independently measured, quantities of the target molecule – and to place such artificial tissues within the tissue cassette alongside the specimen (5). By this means, both specimen and control are simultaneously subjected to fixation, processing, antigen retrieval, staining and interpretation.

This would be particularly useful in cases where the need for quantitative immunohistochemical assays is anticipated, as in the case of breast biopsies. Some progress along these lines has been recently reported using breast cancer cell lines that express known amount of hormone receptors (6). The addition of automated, computer-assisted microspectrophotometry, will facilitate conversion of intensity of immunoreactivity into actual expression of molecules. This then can lead to reports of quantitative immunostains in terms familiar to clinicians, with the added accuracy that immunohistological methods offer (4).

Perhaps of more immediate importance, such artificial tissues could provide a means of assuring comprehensive interlaboratory standardization of immunohistological methods, as convenient and reproducible check samples, readily scored by automated computerized microspectrophotometry.

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Automation in immunohistochemistry

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The purpose of this conference is to briefly discuss to the advantages and the disadvantages of the recent advances in automation of immunochemistry. The term "automation" must be understood as the set of precesses involved in the preparation of stains in immunohistochemistry, including all the procedures, as well as the generation of the results and their interpretation, all requiring no direct manual intervention. Automation in immunohistochemistry offers the opportunity to improve our levels of sensitivity, reproducibility and standardization, while the working time is reduced and reagents are saved. Immunohistochemistry is a repetitive technique that consists of a cycle of washing with buffer, application of the reagent, then rewashing with buffer, which makes this a procedure which can be easily automated.

Problems of immunohistochemistry without automation

Three critical points in immunohistochemistry that depend directly on the human intervention can be delineated as follows:

i) Appropriate handling of the specimen, its suitable fixation, its later inclusion in paraffin, as occurs with most of the samples, and the preparation of the sections are all vital in the final result of the stains.

ii) Another critical step includes the preparation of reagents, antibodies and application of solutions, times of incubation, suitable dried washing and crystals. These activities are done manually and repetitively. To present, these activities have been object of automation and will be discussed in further detail.

iii) The interpretation of the results is always the responsibility of an expert pathologist who is familiar with each one of the antibodies, its diagnostic possibilities and limitations. This it is the most difficult aspect at the present time. An attempt is now being made to remove the human intervention from this process, but many years remain before automatic interpretation and quantification of stains can be applied to diagnostic routines.

A immunohistochemistry technician is able to handle 40-50 slides every 4 h, excluding the time taken for the previous process