Immunostaining of formalin-fixed, paraffin-embedded tissues for malignant lymphomas

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Abstract

With the advent of new monoclonal antibodies that are applicable to formalin-fixed, paraffin embedded sections, immunophenotyping is becoming increasingly important in the diagnosis and classification of lymphomas. However, multiple factors such as fixation, trypsinization and even type of antibodies used have certain effects on the final outcome of the staining procedure. In this paper we report our experience and the problems encountered in our laboratory when we first tried to establish a workable immunostaining protocol for formalin-fixed, paraffin embedded tissue sections using the immunoalkaline phosphatase technique.

Key words: Immunophenotype, malignant lymphoma, Hodgkin's disease, APAAP technique, non-Hodgkin's lymphoma.

INTRODUCTION

Immuno-enzymatic methods were first introduced in the early 1970's for studying human haematological neoplasms. However, up until the late 1970's the majority of these studies of cell surface markers in lymphoid tissues and bone marrow samples were performed on cell suspensions only. This limitation presented several disadvantages.

When a cell suspension is prepared from a tissue sample, there is not only loss of topographical relationships between the different elements in the tissue; very often there is also the risk of selective depletion of one cell population relative to another leading to the possibility of a low yield of the cells in question. Furthermore, the surface labelling of the cells must be carried out within a short period of obtaining the sample.

The availability of cryostat sections of human tissues for immuno-enzymatic procedures has more or less eradicated the above shortcomings. In fact, most of the antibodies produced then worked only on fresh tissues that were mildly fixed in acetone just prior to staining. [1,2] However, this advancement is not without its own problems. While most routine histology laboratories will have a functioning microtome to prepare paraffin sections, cryostats are still considered a luxury by many. Moreover, more often than not, even in laboratories equipped with cryostats, most samples arrive in formalin.

In the late 1980's with improved technologies in the production of monoclonal antibodies, many new antibodies suitable for formalin-fixed, paraffin-embedded tissues began to emerge in the market.[3-7] The advent of this new technology was most welcome as then, for the first time, detailed immunostaining studies could be carried out on most samples regardless of whether fresh tissues were available or not. Retrospective as well as prospective studies could also be carried out.

Unlike fresh tissues, formalin-fixed, paraffin-embedded samples have undergone some very harsh treatment in the tissue processing procedure. Many of the antigens present have been inactivated. Very often, proteolytic enzyme digestion has to be employed to unmask the antigens present and the type of fixative as well as the duration of fixation affect the final outcome of staining.[8-12]

We began to experiment with immuno-enzymatic procedures in March 1989 and, until May 1991, have immunophenotyped a total of 130 cases of various types of malignant lymphomas. We now recognise that multiple factors, such as fixation, trypsinization, type of antibody used, indicator system employed and even the quality of the tissue sections obtained have varying effects on the final outcome of staining. The aim of this paper is to share our experience in this exercise and to highlight some of the common problems that any laboratory will encounter when it first begins to establish its technical procedures in this field.

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MATERIALS AND METHODS

Tissues

Samples used were routine surgical samples received by the Histopathology Laboratory, UKM that were diagnosed as lymphomas based on morphology. They had been fixed in 10% neutral buffered formalin and processed routinely. Paraffin sections were 4 um thick and were allowed to incubate at 60°C for at least 4 hours or overnight before use to encourage adherement to the glass slide.

Just prior to staining, the paraffin sections were dewaxed and hydrated to 70% alcohol in the normal manner.

Antibodies

The panel of primary antibodies used is listed in Table 1. Initially, rabbit anti-mouse IgG (RAM) and the conjugate, mouse-APAAP (alkaline phosphatase anti-alkaline phosphatase) were used as secondary antibodies. However this was later changed to biotinylated/RAM (BRAM) and the avidin-biotin/alkaline phospahtase complex (ABC-AP).

All the above primary and secondary antibodies were obtained from Dakopatts, Denmark. Dilutions of primary antibodies and BRAM were with 0.05M TRIS buffer saline (TBS) pH 7.6 while that for ABC-AP was with 0.05M TRIS buffer pH 7.60.

TABLE 1: Panel of antibodies

Antibody	CD*	Source
UCHL 1	45R0	Dakopatts
L26	20	Dakopatts
T3	3	Dakopatts
MT 1	43	Clonab/Dakopatts
MB 1	37	Clonab
MB 2		Clonab
Leu M1	15	Dakopatts/Becton Dickinson
Ber H2 (Ki-l)	30	Dakopatts
Lysozyme		Dakopatts
a-1-antitrypsin		Dakopatts
Mac 387		Dakopatts
KP 1	68	Dakopatts
Elastase		Dakopatts
LCA	45	Dakopatts
CK		Dakopatts
EMA		Dakopatts

*CD = Cluster of differentiation

Trypsinization

Hydrated tissue sections that were to be trypsinized were first washed in cold, then 37°C 0.05M TBS/2.5% sucrose pH 7.8 before incubating in a solution of 0.1% typsin in 0.05M TBS pH 7.8 for 15 minutes in a 37°C shaking water-bath.

The treated sections were then washed in 3 changes of cold TBS/sucrose to terminate the reaction. They were kept moist in 0.05M TBS pH 7.6 until use.

Immunoalkaline phosphatase staining technique

Briefly, the sections were first stained with the diluted primary antibody for 1 hour, BRAM for 30 minutes and ABC-AP for 30 minutes. All incubations were carried out at room temperature with adequate washings in TBS pH 7.6 between each step. Enhancement of sensitivity may be carried out by repeating the BRAM and ABC-AP steps for further 15 minutes each.

The colour of the positive reactions was then developed in a solution containing Naphthol AS-B1 phospate as substrate with 5% New Fuchsin as chromogen for 30 - 45 minutes. The sections were then washed in running tap water, counterstained in Harris hematoxylin, dehydrated, cleared and mounted in DPX.

Staining strategy

The staining strategy is illustrated in Fig. 1.

RESULTS

Positive cells appear red (either cytoplasmic or membraneous) while unstained cells remain colourless against a background of blue nuclei.

It is interesting to point out that in many cases the tissues may present as a mixture of normal and neoplastic cells. It is not uncommon to see cases of T-cells-rich B-lymphomas or the presence of normal B cells in T-lymphomas. In general, the malignant cells stained less strongly than the reactive elements.

Table 2 shows the phenotype of cases of lymphomas seen in this department from March 1989 to May 1991.

Six cases were unclassified because they were negative for panel 1 and 2, but positive for only anti-LCA of panel 3.

Four cases which were negative for panel 1 and 2 turned out to be positive for anti-cytokeratin and/or anti-epithelial-membrane-antigen and negative for anti-LCA. These cases were metastasis mistakenly diagnosed as lymphoma.
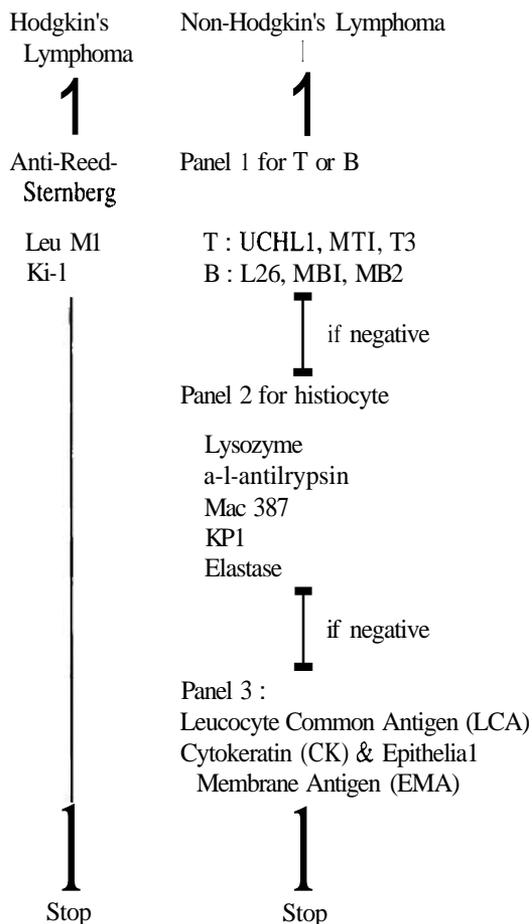
Hodgkin's Non-Hodgkin's Lymphoma
Lymphoma

$\downarrow$ $\downarrow$

Anti-Reed- Panel 1 for T or B
Sternberg

Leu M1 T : UCHL1, MTI, T3
Ki-1   B : L26, MBI, MB2

     if negative

Panel 2 for histiocyte

 Lysozyme
 a-l-antilrypsin
 Mac 387
 KP1
 Elastase

    if negative

Panel 3 :
Leucocyte Common Antigen (LCA)
Cytokeratin (CK) & Epithelial
 Membrane Antigen (EMA)

Stop   Stop

FIG. 1: Immunostaining strategy

The results show a failure rate of 14% (181 126) in identifying a marker for malignant lymphoma, despite working controls with sections of tonsils.

TABLE 2: Immunophenotype of 130 cases of lymphoma seen in the Histopathology Division, UKM

Immunophenotype	No. of cases
T	36
B	58
Hodgkin's	7
Histiocytic	2
Unclassified	6
Inconclusive	17
Non-lymphoma	4

DISCUSSION

There are a number of immuno-enzymatic methods developed for the detection of human leukocyte antigens. Among them are the immunofluorescence method which may be considered as the oldest established technique, the immunoperoxidase method which, until the last few years, was the most commonly used, and, more recently, the immunoalkaline phosphatase method which was developed and popularised by Mason and his colleagues.[2,13]

We chose to use the immuno-alkaline phosphatase or APAAP technique in this study because, unlike immunoperoxidase methods which frequently encounter interference from cells that are rich in endogeneous peroxidase, the APAAP procedure is suitable for labelling tissues that are heavily infiltrated with neutrophils and/or eosinophils as often seen in samples of bone marrow tissues.[13]

We did not carry out a comparison between the sensitivities of the immunoperoxidase and the immunoalkaline phosphatase techniques. We realized as we proceeded, that there was an increase in sensitivity if the avidin-biotin complex of the alkaline phosphatase method was used instead of the APAAP similar to that reported in study of immunoperoxidase techniques.[14]

Other reagent components such as the chromogen used in the development of the positive reaction also affected the final staining effect of the cells. Initially Fast Violet B salt was used but this was later changed to hexazotized New Fuchsin as the latter's staining is less diffuse and its end product insoluble in organic solvent, thus making DPX a suitable mounting medium. Together with the ABC-AP system they gave a sharper and more crisp staining to the positive cells.

As mentioned earlier, types of fixative and the length of fixation time will also affect the immunostaining reaction. All of our routine specimens were fixed in 10% buffered formalin but total fixation time varied from sample to sample. Contrary to common belief that cell surface markers are less labile than enzymes, we have many times obtained 'blank' results from under-fixed or poorly fixed samples. The poorly fixed cells just refused to take up any of the antibodies resulting in a very clean unstained section. On other occasions strong reactions were seen only on the well fixed parts of a very large piece of lymph node leaving the poorly fixed centre portion unstained.

Although Bouin's Solution has been listed as a mild fixative that is able to preserve the antigens better than formalin in many cases, bone marrow biopsies that were fixed and decalcified overnight in Bouin's fixative were not suitable for immuno-enzymatic analysis. Staining with UCHLl tended to give a false strong positive reaction while false negative results were obtained when other antibodies like L26 and MT1 were used. This was most probably due to the extremely acidic nature of the fixative which, over a long period of fixation time, had completely destroyed the antigens present on the cells. However this did not apply to short fixation times, as bone marrow cryostat sections fixed for 5 minutes in Bouin's fixative just prior to staining exhibit excellent antigen preservation.[15]

Badly cut sections may also contribute to some degree of false positive reactions and heavier background. Antibodies seemed to have certain affinity to jagged irregular edges and no amount of washings can remove them completely. Enzymatic colour reactions developed at these 'sites' can at times look so real that they appear to be formed on the surface of the cells. Thick sections, on the other hand, having tolerated multiple incubations with different layers of antibodies have the tendency to float off towards the end of the whole staining procedure just prior to the final step. Similarly, paraffin sections that have spent some time in the 60ºC oven were less likely to dislodge from the glass slide than those that were just dried on the hot plate.

Proteolytic enzyme digestion has always been described as the practical yet little understood step before immunostaining that will unmask the antigens which may have been cross-linked by conventional formalin fixation and routine paraffin embedment, thus making them 'unrecognisable' by the antibodies that were raised in animals against their natural states. It is believed that this enzyme treatment breaks the cross-linkage thus revealing the antigenic site. There have already been many reports on the effectiveness as well as the many pitfalls of proteolytic enzyme digestion.[8,16-18]

Since the precise mechanism of proteolytic digestion is little understood, it is very important that we do not include this procedure indiscriminately in the immunostaining routine in over-eagerness to improve the staining reaction. Some antigens may be destroyed by the enzyme digestion, while other larger molecules of proteins may be cleaved to smaller molecules which

contain antigenic sites that were not initially present, giving rise to a false positive reaction.

We have tried various incubation times and concentrations of trypsin hoping to establish 'optimal' treatment times for the various types of antigens involved. We found that the recommended incubation of rehydrated sections in 0.1% trypsin solution of pH 7.8 for 15 minutes at 37°C sufficed in most cases of routine T/B immunophenotyping. However, a longer incubation time of up to 45 minutes at a concentration of 0.2 - 0.3% trypsin was more effective for the demonstration of immunoglobulins and the cytokeratin (CK) antigen. On the other hand, trypsinization in many cases tended to reduce the intensity of the reaction of the leucocyte common antigen (LCA) in the abnormal cells rather than enhance it. Likewise trypsinization with antibodies like the T-cell marker UCHLl will result in a false positive reaction.

In the cases of Hodgkin's lymphoma, we initially had very little success with the use of the Reed-Stenberg (RS) cell markers Leu M1 (CD 15) and Ki-l (CD 30). Even under extreme conditions of typsinization of 30 minutes at 0.4% trypsin concentration, coupled with an extended incubation time of the primary antibody to 3 hours at 37 ºC; the RS cells in 5 out of the 7 cases remained unstained. Of the remaining 2 cases, 1 was positive with the Ki-l marker only while the other was positive for both markers. Even so, the positive cells were only weakly stained in both cases.

However, when the same 7 cases were repeated using 0.4% pepsin in 2N HCl for 15 minutes at 37 ºC, 6 were positive with intense reaction for both markers. Since then, this has become the standard procedure for the diagnosis of Hodgkin's lymphoma cases in our laboratory.

Our panel of antibodies consisted of UCHLl, MT1 and CD3 for T-cell markers, L26 and MB1 or MB2 for the B-cell. Except for both UCHLl and L26 which have been consistantly reliable from the start, all the other markers have performed unpredictablely. More often than not a positive UCHLl or L26 reaction was not reflected by their respective partner. In other times, the positive reaction produced by them were comparable to each other.

In our search for a useful histiocytic marker, we have experimented with various antibodies namely a-1-antitrypsin (AT), lysozyme, MAC 387, elastase and KP-l. As realised by many, both a-1-AT and lysozyme are not specific for histiocytes, while MAC 387, anti-elastase and KP-1 are more specific.

Immunophenotyping is rather costly and time consuming. An interpretable result is a final combination of the multiple factors mentioned above. We feel that any institution that wishes to embark in this must be fully committed from the start. Not only are the antibodies expensive, the respective laboratory must also be able to afford the expertise to work out the optimum staining procedures necessary for each antibody under local conditions. Even so, there can still be times when the unpredictable will occur and the results uninterpretable.

ACKNOWLEDGEMENT

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