Immunohistochemical analysis of Hodgkin's disease using microwave heating

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Abstract

Aims—To assess the effect of microwave heating on immunohistochemical staining of CD15 and CD30 antigens in Hodgkin's disease tissue samples.

Methods—Formalin fixed, paraffin wax embedded sections from 20 cases of Hodgkin's disease (six mixed cellularity, 14 nodular sclerosis) were immunostained for CD15, using two antibodies (DAKO-M1 and Leu-M1) and for CD30 using the antibody Ber-H2. The staining was carried out by conventional techniques which included pretreatment of sections with trypsin and on untreated sections following heating with microwaves. With antibody Leu-M1 an additional method, using a specific anti-mouse IgM bridge both with and without microwave heating, was also included. The results for each method were compared by counting positively stained Reed-Sternberg cells and estimating the staining intensity.

Results—Microwave heating resulted in a substantial increase in the number of cells stained with antibodies to CD15 and also in the staining intensity. The best results were obtained using Leu-M1 with specific rabbit anti-mouse IgM bridge and microwave heating. Dramatic enhancement of the staining of Reed-Sternberg cells for CD30 was achieved following microwave heating, together with disappearance of the non-specific staining of plasma cells.

Conclusion—Microwave heating is strongly recommended for the immunohistochemical staining of CD15 and CD30 expressed by Reed-Sternberg cells in Hodgkin's disease.

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In contrast to non-Hodgkin's lymphomas, the diagnosis of Hodgkin's disease, depending as it does on the presence of the characteristic Hodgkin and Reed-Sternberg cells, has historically posed less of a problem to pathologists. With increasing application of immunohistochemical techniques to lymphoma diagnosis, however, the distinction between Hodgkin's disease and non-Hodgkin's lymphoma has become steadily more blurred.¹ Classic Hodgkin and Reed-Sternberg cells may be difficult to find in the syncytial² and grade 2³ variant of nodular

sclerosing Hodgkin's disease and also in lymphocyte depleted Hodgkin's disease.¹ Conversely, a number of newly identified categories of non-Hodgkin's lymphoma contain cells that closely resemble Reed-Sternberg cells: these include certain peripheral T cell lymphomas,^{4,5} CD30 positive anaplastic large cell lymphoma,^{6,7} and so-called T cell rich B cell lymphoma.^{8,9} This has led to attempts to define the immunophenotype of Hodgkin and Reed-Sternberg cells. The two most useful antibodies for this, both of which are suitable for the study of paraffin wax sections, are CD15¹⁰ and CD30.^{11,12} Neither CD15 nor CD30 antigen expression alone is in any way specific for Hodgkin and Reed-Sternberg cells, but the expression of both, in the correct histological context, is strongly suggestive of Hodgkin's disease. Even in the best hands, however, it has proved difficult to obtain consistent results using these antibodies.^{13,14} In the light of recent reports of improved immunostaining following microwave heating,^{15,16} we investigated the use of microwaves as a means of increasing the sensitivity of immunostaining for CD15 and CD30 antigens in Hodgkin's disease.

Methods

Paraffin wax blocks from 20 cases of Hodgkin's disease (six mixed cellularity, 14 nodular sclerosis (12 grade 1, two grade 2)) were retrieved from the surgical pathology files of the Department of Histopathology, University College London Medical School. Sections (5 µm thick) were cut on to Vectabond coated slides (Vector laboratories, Peterborough, England) and stained with two different antibodies to CD15 antigen and a single antibody to CD30 antigen using the following three methods.

1 Sections were dewaxed and taken through industrial methylated spirit to distilled water. After 10 minutes of treatment with a 1 in 1000 solution of porcine pancreas type 2 crude trypsin in 1 in 1000 calcium chloride at 37°C sections were stained using the streptavidin peroxidase method with two CD15 antibodies (Dako M1; Dako Ltd, High Wycombe, Bucks, and Leu M1; Becton Dickinson, Oxford, England) at a 1 in 20 dilution and CD30 (Ber H2, Dako Ltd, High Wycombe, Bucks) at 1 in 10.

2 Sections were stained with Leu M1 (CD15) at 1 in 20 using method 1 modified by the substitution of biotinylated goat anti-

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Hodgkin's disease tissue, nodular sclerosis subtype, showing the effects of microwaves on the staining of Reed-Sternberg cells with anti CD-15 (Leu-M1), using a specific goat anti-mouse IgM bridge (A-D) and anti CD-30 (Ber-H2) (E-H). Reed-Sternberg cells are stained weakly (A and C). This is greatly enhanced in the same fields (B and D) after microwave treatment. Weak staining of CD-30 seen in (E) and (G) again shows dramatic enhancement when matching fields (F and H) are examined after microwave treatment. Plasma cell reactivity with anti-CD30 seen in G is not present following microwave treatment (H).

mouse IgM at 1 in 100 for the biotinylated rabbit anti-mouse Ig.

3 After dewaxing sections were placed in a glass Coplin jar containing a 0.01M sodium citrate buffer at pH 6.0. The jar, covered with clingfilm, was heated in a Toshiba 600W microwave oven for two 5 minute cycles, ensuring that there had been no loss of buffer during the first cycle. The sections were then rinsed in distilled water and stained as in methods 1 and 2 but without treatment with trypsin.

For each antibody and each method the percentage of positive staining Hodgkin and Reed-Sternberg cells was estimated by counting all Hodgkin and Reed-Sternberg cells in five representative high power fields in routine haematoxylin and eosin stained sections, followed by counting stained Hodgkin and Reed-Sternberg cells in match-

ing high power fields in sequential sections. An average of 500 cells were counted in each case. A staining score of less than 5% meant that Reed-Sternberg cells could be found only after intense searching; a score of 10% was necessary before positive staining cells could be identified with relative ease suitable for routine practice. The intensity of staining in each case was graded on an arbitrary + to +++ scale and a total intensity score calculated by simple addition of the number of pluses in all 20 cases was scored for each method.

Results

These are summarised in the table. Using standard staining methods, Dako-M1 (CD15) stained less than 5% of Reed-Sternberg cells in 14 of 20 cases and in no

Immunohistochemistry of Reed-Sternberg cells in 20 cases of Hodgkin's disease

Antibody	Negative	Percent positive cells					Staining intensity			
		1-4	5-9	10-24	25-50	50-100	+	++	+++	Score
Anti-CD15 (Dako-M1)	6	8	3	3			11	3		17
Anti-CD15* (Dako-M1)	6	4	2	6	2		4	5	5	29
Anti-CD15 (Leu-M1)	2	8	3	6	1		12	8		24
Anti-CD15* (Leu-M1)		3	1	8	6	2	4	8	8	44
Anti-CD15/IgM (Leu-M1)	1	5	3	5	6		6	5	9	39
Anti-CD15/IgM* (Leu-M1)		2	1	5	5	7	1	2	17	56
Anti-CD30 (Ber-H2)	1	3	6	3	5	2	9	6	4	33
Anti-CD30* (Ber-H2)			1	8	3	8	1	10	9	48

*Microwave.

case were more than 25% of the cells stained; staining intensity was weak overall with 11 cases graded + and 3 ++ (total score 11 + 6 = 17). Microwave heating improved the results considerably but they were still unsatisfactory for routine practice.

Leu-M1 (CD15) was marginally more sensitive than Dako-M1 with standard methods, but after microwave heating there was striking improvement; in only three cases was there staining of less than 5% of Hodgkin and Reed-Sternberg cells.

As has been reported by others,¹⁴ the sensitivity of Leu-M1 (CD15) was considerably improved when a specific rabbit anti-mouse IgM bridge was substituted, but the results did not achieve the same level of sensitivity as those obtained after microwave heating. The best results by far were obtained when substitution of the more specific bridge was combined with microwaving. Using this combination, both the number of positively staining Hodgkin and Reed-Sternberg cells and the staining intensity were dramatically enhanced (fig A-D). The staining reaction of granulocytes was equally intensified.

The number of Hodgkin and Reed-Sternberg cells staining with Ber H2 (CD30) and their staining intensity was strikingly enhanced after microwave treatment of sections (fig E-H). Moreover, the often "muddy" cytoplasmic staining was converted to crisp staining of the cell membrane with punctate staining of the Golgi region. Use of microwaves also resulted in a much cleaner background, and an unexpected finding was the loss of plasma cell staining (fig G, H), a well recognised phenomenon when routine methods are used to stain CD30 antigen in paraffin wax sections.^{12,13}

Discussion

Following the discovery of CD15 and CD30 antigen expression by Hodgkin and Reed-Sternberg cells there was initial optimism that these antigens would provide diagnostic markers of Hodgkin's disease. These hopes were soon tempered by the demonstration of CD15 and CD30 antigens in cells of other lymphomas.¹⁷⁻¹⁹ It is only in Reed-Sternberg

cells, however, that both antigens are regularly expressed together and this dual expression of CD15 and CD30 antigens remains the best available immunohistochemical aide in the diagnosis of Hodgkin's disease,^{12,13,19} particularly as both antigens can be shown in paraffin wax sections. Unfortunately, satisfactory demonstration of both CD15 and CD30 antigens is frequently dogged by negative results and, in many cases, weak and uneven staining.^{13,20} Some of these problems are related to variations in the antibody, the nature of the fixative, and the requirement for optimal protease digestion of tissue sections before staining. Thus in a multicentre study of immunohistochemical markers of Hodgkin's disease reported by Ree *et al*,¹³ Hodgkin and Reed-Sternberg cells were negative for CD15 antigen in 43.6% and 23.1% of cases fixed, respectively, in formalin and B5, while corresponding figures for CD30 antigen were 8.8% and 38.5%. LeBrun *et al*¹⁴ were able to reduce the number of CD15 negative cases of Hodgkin's disease and improve the intensity of CD15 antigen staining by using Leu M1, a mouse IgM monoclonal CD15, in association with a goat anti-mouse μ chain bridge instead of the less specific rabbit anti-mouse immunoglobulin. This suggested that difficulty in demonstrating CD15 antigen lay in the detection system rather than in the expression of the antigen itself.

The use of microwave heating to enhance immunohistochemical staining was first reported by Shi *et al* in 1991.¹⁵ These authors used microwaves in association with a variety of metal salts but Catoretti *et al*¹⁶ have described satisfactory results using a simple citrate buffer. In formalin fixed tissues microwave heating may enhance immunoreactivity used alone, together with protease digestion or have no effect; the results depend on the particular antigen under study.

Our study has shown a dramatic effect of microwave heating alone, without the need to pretreat tissues with proteases, on the results of CD15 and CD30 antigen immunostaining. Using Leu-M1, the more sensitive antibody to CD15, microwave heating alone enhanced tissue reactivity more than the substitution

of a specific anti-mouse IgM bridge, but microwave heating together with the more specific bridge produced the best staining in terms both of numbers of positive cells and staining intensity. The effect of microwave heating on CD30 antigen staining is equally dramatic, resulting, in all cases, in easily observable Hodgkin and Reed-Sternberg cells with much improved, crisp membrane and Golgi region staining and reduction in background staining.

The scientific basis whereby either protease digestion or microwave heating retrieves antigens is unknown. After the first descriptions of the use of proteases in immunohistochemistry there was some anxiety that protease treatment might alter antigens in such a way as to cause spurious results but none has been reported during the many years of experience with the technique. Similar reservations might well be justified regarding the effects of microwaves, but the failure of CD30 to stain plasma cells in sections heated with microwaves, as opposed to those treated with a protease, suggests that microwave heating is, if anything, less likely to alter antigens because CD30 (Ber-H2) does not stain plasma cells in cryostat sections.¹¹

The use of microwave heating to retrieve antigens in paraffin wax sections is, clearly, going to have a profound effect on the practice of immunohistochemistry. The microwave technique is the method of choice for the demonstration of the Hodgkin and Reed-Sternberg cell associated antigens CD15 and CD30.

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