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EVALUATION OF IMMUNOPHENOTYPE IN DIFFUSE LARGE B CELL LYMPHOMA

Pathology

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ABSTRACT

INTRODUCTION: Diffuse large B cell lymphoma represents a very heterogeneous group of tumors and different subtypes have been identified by genetic testing. IHC algorithms have been identified as a surrogate to genetic testing, by classifying DLBCL cases into Germinal centre group (GCB) and Activated B cell (ABC) group. Identification of c MYC expression and double expressor lymphoma (DEL) have been shown to correlate with poor prognosis in DLBCL. Present study aim at classifying DLBCL according to HANS algorithm and identifying c MYC expression and DEL by applying different immune markers with the help of IHC.

MATERIAL AND METHODS: A prospective study of 50 cases of biopsy proven nodal DLBCL was conducted. Different immune markers were applied as per the required IHC panel of DLBCL, cases were classified into prognostic subtypes, and c MYC expression and DEL groups were identified.

RESULTS: out of 50 cases of nodal DLBCL, 18% were of GCB and 82% were of ABC group. c MYC expression was observed in 40% cases whereas DEL cases were 16%.

CONCLUSION: DLBCL cases should be classified into prognostic sub types by applying immunohistochemical algorithms and study of expression of adverse prognostic markers should be done by IHC to plan better treatment for these patients.

KEYWORDS

DLBCL, Immunophenotype

INTRODUCTION

Diffuse Large B cell Lymphoma (DLBCL) is a neoplasm of proliferating large cells, which harbor a B cell phenotype. DLBCL is the most common Non-Hodgkin Lymphoma (NHL), accounting for 35%-40% of all cases [1-3]. DLBCL represents a very heterogeneous group of tumors. DLBCL can arise in a nodal (lymphoid) or extranodal site, including unusual sites, such as testis, bone, and lung, and tends to have an aggressive biological nature, but is highly responsive to combination chemotherapy. DLBCL can also occur in the setting of immunodeficiency, primary or acquired in recipients of solid organ transplants or in individuals with Human Immunodeficiency Virus (HIV) infection[4,5].

DLBCL usually arises *de novo* but can represent transformation of an indolent lymphoma, such as follicular lymphoma (FL)[6,7] chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL) in the so-called Richter's transformation[8], and marginal zone lymphoma (MZL) or nodular lymphocytic predominant Hodgkin lymphoma (NLPHL)[9]. Gene expression profiling (GEP) has identified different DLBCL subtypes, bearing prognostic significance [10]. At least three main subtypes of DLBCL were initially identified based on the cell of origin (COO) of the groups, all with underlying clear clinical, biologic and genetic peculiarities[11,12]: germinal centre B cell-like (GCB) DLBCL, expressing genes that are hallmarks of normal germinal center B cells; activated B cell-like (ABC) DLBCL, lacking expression of germinal center B cell-restricted genes and possibly arising from post-germinal center B cells that are arrested during plasmacytic differentiation; primary mediastinal B cell lymphoma (PMLBCL), expressing several genes that characteristically expressed also in Hodgkin Reed-Sternberg cells, and possibly derived from thymic B cells. The latter represents less than 10% of all DLBCL and is now considered a separate entity in the WHO classification[1].Because it is currently impractical to perform microarray analysis on every patients with DLBCL, immunohistochemical profiles analysis have been proposed to divide DLBCL patients into prognostic groups based on the cell of origin of the tumor (Table 1) and as surrogate of GEP[13,14-21]. A few studies have used the immunohistochemical expression of CD10, BCL-6, or MUM1 to classify cases of DLBCL into GCB and non-GCB groups, but without a direct comparison with microarray, leading to conflicting results.[22,23] The algorithms of Choi[24] and Hans[25] appear the best ones in predicting cell of origin as defined by GEP and in predicting the outcome.[17] HANS system employs CD10, BCL6 and MUM1 and 30% cut-offs for reactivity.[25] GCB tumours are CD10+ or BCL6+/ CD10–/MUM1- and non-GCB tumours are CD10–/ MUM1/IRF4+ (BCL6 can be either positive or negative). We have made significant progress in recent years in understanding the pathogenetic role and prognostic impact of MYC in DLBCL. MYC translocations are present in 5–15% of cases of DLBCL. Many (although not all) studies have shown that patients with DLBCL associated with MYC rearrangement have a worse prognosis.[26-31] Double hit lymphoma (DHL) was defined by Aukema and colleagues[32] as a B-cell lymphoma that carries MYC rearrangement and another oncogene rearrangement, usually BCL2 but less often BCL6 and rarely BCL3 or CCND1. Double expressor lymphoma (DEL), also known as double positive lymphoma, is defined as a DLBCL in which MYC and BCL2 are overexpressed as shown by immunohist ochemistry.[33] Cut-offs for MYC of 40–50% and BCL2 of 50–70% have been used in the literature. In most studies, the 40% and 50%, respectively, have been used and these cutoffs are recommended in the revised WHO classification.[34]In this study we will focus on classifying the DLBCL cases into GCB and non GCB groups and further separate the double expressor lymphoma groups as per the latest WHO guidelines 2016.The aims and objectives of this study were to study the Immunophenotype in DLBCL, to subgroup DLBCL into germinal centre B cell group (GCB) and activated B cell (ABC) according to Hans Algorithm by applying Immunohistochemistry and to identify the double expressor DLBCL cases which are positive for both c MYC and BCL 2, hence to identify the potentially complex karyotype and poor prognosis group of DLBCL cases.

MATERIAL AND METHODS:

A prospective study of 50 cases of nodal DLBCL has been conducted. The cases having lymph node biopsy sent to our institute, GCRI, Ahmedabad and diagnosed on histomorphology as DLBCL were included in our study. All the clinically relevant data have been retrieved from the patients case file, including the clinical history, lab investigations, radiological findings and biopsy reports. All the slides and paraffin embedded tissue blocks of these histopathologically diagnosed cases of nodal DLBCL were collected. Sections (3µm) of formalin-fixed, paraffin-embedded tissue are tested for the presence of antigen using the ventana benchmark XT auto immunostainer using ultra view DAB detection kit. Specimens were deparaffinised using the EZ prep buffer and antigen retrieved by CC1 buffer at ph 9. All slides are incubated with various antibodies according to the required IHC panel for DLBCL as per the recommended guidelines. These are Immunohistochemistry These are C-myc (Ep121), CD 20 (L26), BCL 2 (124), BCL 6 (GI191E/A8), MUM 1 (MRQ-41), CD 10 (SP 67), CD 2 (MRQ -11), CD 5 (4C7), MIB 1 (MIB1), PAX 5 (24). The slides coated with the desired antibody was then examined under microscope for the immunoreactivity keeping in mind the benchmark cut off

values for each marker to be considered as positive. 30% was the general cut off values above which their expression was considered to be positive, (as per the HANS algorithm). The expression of MYC and BCL 2 varies considerably, in part depending upon the threshold used to define positivity. In most studies, BCL 2 is considered positive if > 50% of the tumor cells are positive and cMYC considered positive if > 40% of the tumor cells nuclei are positive (as per latest WHO 2016 guidelines). Co-expression of both these proteins was considered as a case of double expressor lymphoma. All the cases were finally classified into either GCB or non GCB (ABC) groups according to the HANS algorithm.

RESULTS

Age wise distribution of cases (GCB vs ABC)

The median age group at presentation was 59 years and ranging from 28-83 yrs. Our study had total 32 male (64%) and 18 female (36%) out of total 50 cases of nodal DLBCL. In our study, we found that 5/9 cases (55%) of GCB and 29/41 cases (71%) of ABC group patients belonged to ≥50 yrs of age.

Table 1 : Age wise distribution of DLBCL cases (GCB vs ABC)

Age (in yrs)	No of cases (GCB)	No of cases (ABC)	Total Cases
20-29	-	1	7
30-39	4	6	10
40-49	-	5	5
50-59	2	12	14
60-69	3	8	11
70-79	-	8	8
≥80	-	1	1
Total	09	41	50

Distribution of cases according to site of lymph node involved

Cervical group of lymph nodes were the most common group involved by DLBCL in our study (42/50), followed by inguinal in 4 cases, axillary in 3 and para aortic lymph node in 1 case out of total 50 cases under study.

Immunophenotype of 50 cases of nodal DLBCL under study

Immunophenotypic analysis of 50 cases of nodal DLBCL showed expression of different markers and then we interpreted the results after microscopic examination of the IHC slides. First, the DLBCL cases were classified into GCB and ABC groups by analysing the expression of CD 10, BCL 6 and MUM 1, by applying the HANS algorithm. By the help of this algorithm, we found total 9 cases (18%) to be in GCB and 41 cases (82%) in ABC group.

Table 2 : classification of cases according to Hans Algorithm

Immunoreactivity	GCB (09 cases)	ABC (41 cases)
CD 10+	7	-
CD 10- BCL 6+ MUM 1-	2	-
CD 10- BCL 6-	-	27
CD 10- BCL 6+ MUM1+	-	14

C MYC expression in DLBCL

Table 3 : showing c MYC expression results

marker	Positive	Negative
cMYC	20/50 (40%)	30/50 (60%)

c MYC antibody was applied in all the 50 cases under our study out of which positive expression (cut off>40%) was found in 20 cases (40%) and negative in rest of the 30 cases (60%).

Table 4 : showing cases according to strength of positivity of c MYC

c MYC expression	40%	50%	60%	70%	80%	≥80
No of cases (20)	11	4	1	3	1	-

Out of the total 50 cases, 11 cases (22%) showed positivity of 40% (just around cut off value), 4 cases (8%) showed positivity of 50%, 1 case (2%) showed 60% positivity, 3 cases (6%) showed 70% and 1 case (2%) showed 80% positivity for c MYC. In a way it can be concluded that 5 cases out of our 50 cases of DLBCL (10%) showed strong positivity of 60% or more.

Table 5: showing c MYC reactivity in GCB vs ABC groups

Immunoreactivity	GCB (09)	ABC (41)
c MYC +	2/9 (22%)	18/41 (44%)
c MYC –	7/9 (78%)	23/41 (56%)

Our analysis revealed that out of 20 c MYC positive cases, 18 cases were in ABC group (18/41= 44%) and only 2 cases were in GCB group (2/9= 22%), thus demonstrating a strong difference of c MYC expression in both the group..

Table 6 : showing strength of c MYC expression in GCB vs ABC groups

c MYC +	40%	50%	60%	70%	80%
GCB (02)	-	-	-	1	1
ABC (18)	11	4	1	2	-

If we compare the strength of positivity of c MYC in both the groups, only 2 cases under the GCB group were positive for c MYC and both showed a strong positivity of 70% and 80% respectively and rest all of the 7 cases of GCB group were negative. In ABC group, only 2 out of 18 c MYC positive cases (11%) showed strong positivity of 70%. Majority of them, 11 out of 18 cases (61%) showed positivity of 40% (just near the cut off value.

BCL 2 Expression in DLBCL

Table 7 : showing BCL 2 expression results

Immunoreactivity	GCB	ABC	total
BCL 2 +	2/9 (22%)	15/35 (43%)	17/44 (38%)
BCL 2 -	7/9 (78%)	20/35 (57%)	27/44 (62%)

BCL 2 antibody was applied in 44 cases out of the 50 cases under our study (In 9 GCB and 35 ABC cases) out of which 17 showed positivity for BCL 2 (cut off value of 50% or more) and rest 27 were negative. On comparing the 17 BCL 2 positive cases in the GCB vs ABC groups, we found that 2 BCL 2 positive cases (out of 44 tested) belonged to GCB group (2/9=22%) whereas remaining 15 of the BCL 2 positive cases were of ABC group. (15/35=43%) This comparison showed that BCL 2 expression was more common in ABC group in our study.

c MYC+ BCL 2 dual expression in DLBCL

Table 8 : showing BCL 2, c MYC expression and cases with dual expression

Immunoreactivity	No of cases
BCL 2 +	17/44 (38%)
c MYC +	18/44 (38%)
Both +	07/44 (16%)

Since we wanted to study the incidence of double expressor lymphoma (DEL) cases under our study, we analysed the cases which showed positive expression for both c MYC and BCL 2. We found 7 such cases of double expressor lymphoma **(16%)** in our study.

Table 09 : showing dual positive and dual negative expression of c MYC and BCL 2 in GCB vs ABC groups

Immunoreactivity	GCB	ABC
c MYC+ BCL 2+	NIL	7/7 (100%)
c MYC– BCL 2-	5/9 (55%)	13/41 (32%)

On comparing the double expressor cases in GCB vs ABC groups, it was found that all 7 of the c MYC + BCL 2 positive cases were in the ABC group and none of the GCB group cases showed double expression of c MYC and BCL 2. Five GCB cases (55%) and 13 ABC cases (32%) were negative for both c MYC and BCL 2.

We studied only the expression of c MYC and BCL 2 antigens by applying the immunohistochemistry. So we were able to identify only the double expressor lymphoma cases showing expression of both c MYC and BCL 2. Genetic confirmation was not done in our patients under study.

CD 5 expression in DLBCL

Table 10 : showing CD 5 reactivity in DLBCL

Immunoreactivity	Positive	Weak positive	Negative
CD 5	7/13 (54%)	1/13 (08%)	5/13 (38%)

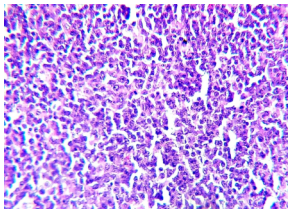
In our study, CD 5 was applied in total 13 cases and its positive expression was found to be in 8 cases (62%)

Table 11 : showing CD 5 expression in GCB vs ABC groups

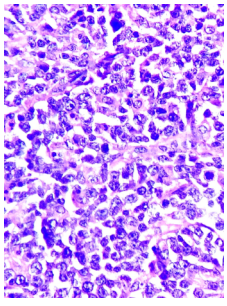
CD 5	GCB	ABC	Total
Positive	NIL	7	07 (54%)
Weak positive	NIL	1	1 (08%)
negative	1	4	5 (38%)
Total	01	12	13 (100%)

Out of 8 cases positive for CD 5, all 8 belonged to ABC group (100%) and one out of five CD 5 Negative cases was of GCB group, rest four belonged to ABC group.

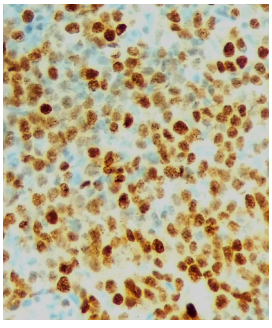
PHOTOGRAPHS



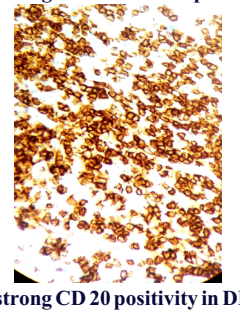
H&E section: Lymph node Bx of same case of DLBCL (20x)



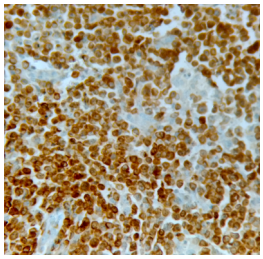
H&E section (40x): Large cells with prominent nucleoli evident in this same case of DLBCL



IHC (40x) showing strong c MYC nuclear positivity in DLBCL



IHC (40x) showing strong CD 20 positivity in DLBCL



IHC (40x) showing strong positivity of BCL 2 in the same case of DLBCL (This case was labelled as Double Expressor Lymphoma)

DISCUSSION

A total of 50 nodal DLBCL diagnosed on biopsy from July 2016 till September 2018 were enrolled in the study and immunophenotype of all these cases were studied. A comparative analysis has been done with similar studies across the world.

AGE AND SEX

The median age at presentation was 55 yrs in our study (age range 28-83 yrs). L Norasetthada et al35 in their study in 2015, showed that the median age in nodal DLBCL cases was 54.9 yrs. Young-Ha Oh et al36, showed in their study in 2005 that the median age of the nodal DLBCL under their study of 51 cases was 59 yrs. Also in our study, we found that 44% of the GCB cases (4/9) were in the age ranging from 30-39 yrs, while in this age range, only 17% cases (7/41) belonged to ABC group. Our study showed that GCB was more common in younger age group than ABC. There were 18 female (36%) and 32 male (64%) in our study. Young-Ha Oh et al had 21 female (41%) and 30 male (59%) while L Norasetthada had 47% female and 53% male in their study of 585 nodal cases of DLBCL. All these studies point towards a slight male preponderance, but lesser than our study, which might be due to low health orientation towards female in our setting.

CD 10, BCL 6 and MUM 1 expression

Our study showed CD 10 expression in only 7/50 (14%) cases and CD 10 was negative in 43/50 (86%) cases. This is slightly lower than similar studies on nodal DLBCL. Young Ha et al showed CD 10 expression to be around 22% in their study. CD 10 expression has been shown to carry a better prognosis in literature and various studies, (chang et al and ohshima et al).[37,38]

21 cases in our study showed positive BCL 6 expression (42%) and 29 were negative for BCL 6 (58%). This was close to the study conducted by young Ha et al where it was positive in 39% of cases. BCL 6 expression has been shown to be associated with a better prognosis in studies (Hans etal, Uccella et al)[25,39]. In our study BCL 6 expression was 77% in GCB and 34% in ABC group.

MUM 1 expression was found to be in 41 out of 50 cases (82%) in our study which points towards bad outcome as pointed out in studies conducted by Muris et al, Hans etal, chang et al.21,25,37 Muris et al showed MUM 1 positivity of 65% in their 71 cases study of nodal DLBCL which is lower than our observation. MUM 1 was positive in 22/33 cases (67%) of nodal DLBCL cases, and only in 10/30 cases (33%) of extranodal cases, in a study conducted by Ting Li et al.[40]

Comparative studies	CD 10 positivity	BCL 6 positivity	MUM 1 positivity
Gonzalo etal [18]	31%	63%	59%
Armando etal[41]	26%	70%	54%
Young Ha etal[36]	22%	39%	31.4%
Muris etal[21]	23%	58%	65%
Ting Li etal[40]	30%	42%	67%
Present study	14%	42%	82%

GCB vs ABC group (nodal DLBCL)

In our study, after applying Hans algorithm, we could classify the cases into GCB and ABC group. Only 9 out of 50 cases (18%) were categorized into GCB group and 41 cases (82%) were in ABC group. This observation is much higher than other studies where the GCB cases varied from 34% in the study of Muris et al[21] upto 47% in the study conducted by Armando et al.[41] However studies on immunophenotype of nodal DLBCL are very few and more studies should be needed to see the trend of GCB vs ABC group.

c MYC and BCL 2 expression

Expression of c MYC protein has been under extensive research in DLBCL patients and many studies have shown that c MYC over expression points towards poorer prognosis, particularly when c MYC and BCL 2 express together in the same patient and are described as double expressor lymphoma.[33]

In our study, c MYC expression was observed in 20/50 cases (40%) with a cut off value of 40%. In most studies, cut off value of 40% and 50% have been used for c myc and this is also recommended by revised WHO classification. Johnson et al[33] Agata M et al[42] used 40% cut off for c MYC to be considered positive. Agata M et al had 27/93 cases (29%) positive for c MYC in their study while Johnson et al also showed 29% of their cases to be positive for c MYC. Kluk et al[43] showed that all

cases of DLBCL with c MYC rearrangement can be identified by immunohistochemistry when the expression of c MYC is > 50%. They showed that Primary DLBCL with > 50% positive MYC positive tumor nuclei have increased MYC activity and inferior overall survival. They showed 10 cases of primary DLBCL out of 56 cases (18%) to have c MYC positivity of > 50%.

Green TM et al[44] showed c MYC positivity of 17% in their study, with a cut off value of 70%. At this cut off, they showed that 65% of them had a MYC Rearrangement. Our present study also showed a strong c MYC expression of > 60% in 5/50 cases (10%) which is at par with the study of kluk et al and close to study of Green TM et al. In our study, c MYC expression was found in 20 cases out of which 18/41 cases (44%) of ABC group showed c MYC expression. This shows really high expression of c myc protein in this category of DLBCL which is close to the study of J Wang et al[45] in which c MYC expression was found in 51% of ABC group.

BCL 2 expression was observed in (38%) cases under our study. Only 22% of the GCB group and 43% of ABC group showed BCL 2 positive expression showing a higher expression in the ABC group. BCL 2 was positive in 21 out of 33 cases of nodal DLBCL (64%) in a study conducted by Ting Li et al[40] and in 9 out of 30 cases (30%) of extra nodal cases. C MYC and BCL 2 dual expression was seen in 7/44 cases (16%) in our study and all 7 cases belonged to ABC group. C MYC and BCL 2 dual expression was found in 127/428 cases (30%) in a study done by kerry J Salvage et al[46] and in 21% of the cases in the study of Johnson et al[33]. Dual expression of c MYC and BCL 2 was seen in 32% of the cases in a study conducted by Yu Ri Kim et al.

Shimin Hu et al[47] showed 157 cases out of 466 to be dual positive for c MYC and BCL 2 (34%) out of which 66% of ABC group showed dual expression of both the proteins. These all studies show that double expressor lymphoma is more common in ABC group and might be a reason for poor prognosis of these patients. However studies have confirmed that both double expression of c MYC and BCL 2 as well as cell of origin classification are independent risk factors for poor survival. MYC translocation in DEL predicts poor prognosis.

In 2 recent German prospective randomized trials in DLBCL, cell-of-origin determination failed to identify prognostic subgroups, whereas dual expression of MYC and BCL2 was highly predictive of poor survival.[48] Cases of DEL include cases of DHL with MYC and BCL 2 rearrangement and also cases without these rearrangements. We have not performed genetic analysis of the cases showing c MYC expression. So we could only classify the cases in DEL group rather than DHL group.

Studies have shown that approx one third of these DEL cases show c MYC rearrangements by genetic analysis. Studies have also shown that DEL patients have an inferior OS than non DEL patients and they show response on newer therapies like R-EPOCH Anita aggarwal et al.[49]

CD 20, PAX 5 and LCA expression

One out of 50 cases (2%) did not show CD 20 Expression in our study. This is consistent with other studies like Sumit gaur et al[50], who showed that 7 out of their 232 DLBCL cases (3%) were CD 20 negative.

MIB 1 EXPRESSION

The impact of MIB 1 expression, which is an index of proliferation on the outcome of patients of DLBCL has been an area of study. Studies show that high MIB 1 expression in DLBCL, particularly in non GCB (ABC) group had the most unfavourable clinical outcome treated with R-CHOP therapy while in GCB group also, the same result was observed, though statistically not significant. Govind babu et al[51]. Using the cut-off value of 70%, Broude A et al[52] found a significant association between Ki-67 PI and overall survival in patients with DLBCL. In our study, 44/50 cases (88%) showed MIB 1 expression of ≥60% and 56% of the cases showed a very strong expression of MIB 1 (≥80%). 8/9 cases of GCB and 20 out of 41 cases of ABC group showed a strong expression of MIB1 (≥80%).

CD 5 Expression

CD 5 expression has been seen in approx 10% cases of DLBCL and Patients of DLBCL with CD 5 expression often have a complex karyotype, usually have ABC type cell of origin and a distinctive gene expression profile yamaguchi M et al[53], Xu Monette et al[54]. Our study showed that 8 out of 13 cases showed positive expression of CD 5 and all of them were of ABC group. This observation indicates that CD 5 marker should be in the panel for immunophenotyping of DLBCL cases and for the above reasons of prognostic significance, the revised WHO classification 2016 recommends that CD 5 expression be assessed in all newly diagnosed cases of DLBCL[34].

CONCLUSION

From the present study on nodal DLBCL, We conclude that the combination of histomorphology and immunohistochemistry is very essential in classifying the DLBCL cases into prognostic types (GCB and ABC group).The majority of patients in our study belonged to ABC group (82%) which confers a poor response to standard chemotherapy. Study of c MYC antigen expression should be performed in cases of DLBCL since its expression has been seen to be associated with c MYC gene amplification and rearrangement in a proportion of c MYC positive cases. c MYC expression in 40% of our cases suggests that many of these c MYC positive patients, having expression of additional adverse prognostic markers are candidates for gene expression profiling and hence can benefit from newer therapies if required. There were 16% cases of double expressor DLBCL in our study which gives a strong message that these patients are candidates for genetic testing to identify cases of double hit lymphomas. High CD 5 expression in 8 out of 13 tested cases show a high proportion of DLBCL cases showing expression of this antigen. CD 5 expression being shown to indicate a complex karyotype, and an ABC type of origin with distinctive genetic expreesion profile, this should be a part of IHC panel for every newly diagnosed cases of DLBCL.

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