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Utility of C4d immunostaining in the first year after pediatric and young adult heart transplantation

Ying Xu, BA,[a] Csaba Galambos, MD, PhD,[b] Miguel Reyes-Múgica, MD,[b] Susan A. Miller, MD,[c] Adriana Zeevi, PhD,[b] Steven A. Webber, MBChB,[c,d] and Brian Feingold, MD, MS[c,d]

From the [a]University of Pittsburgh School of Medicine; and [b]Department of Pathology, University of Pittsburgh School of Medicine; and [c]Division of Pediatric Cardiology, Children's Hospital of Pittsburgh of the University of Pittsburgh Medical Center; and [d]Clinical and Translational Science, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

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BACKGROUND: C4d assessment of endomyocardial biopsies (EMBs) after heart transplantation (HTx) has been widely adopted to aid in the diagnosis of antibody-mediated rejection (AMR), yet it remains unclear whether or not to assess all patients routinely and with what frequency/duration. In this study we sought to evaluate the utility of routine C4d immunostaining in the first year after pediatric and young adult HTx.

METHODS: We reviewed pre-transplant alloantibody and clinical data, including serial EMB reports, on all 51 patients who received HTx at our center since we instituted routine C4d staining of all first-year EMBs. C4d was considered positive if diffuse capillary staining ($\geq 2^+$) was present. Rare/focal capillary staining or absence of staining was considered negative.

RESULTS: Twenty-six of 406 first-year EMBs (6%) were C4d$^+$ in 6 (12%) patients. Sixty-five percent of all C4d$^+$ EMBs occurred by 30 days post-transplant. Five of 6 patients had pre-transplant donor-specific antibody (DSA) $\geq$4,000 MFI. The sixth patient had neither pre-transplant anti-HLA antibodies nor a positive donor-specific cytotoxicity crossmatch (DSXM), but there was clinical concern for AMR. Among the entire cohort, 5 of 10 patients with pre-transplant DSA $\geq$4,000 MFI and/or a positive DSXM were C4d$^+$ compared with only 1 of 41 without (50% vs 2%; $p = 0.001$).

CONCLUSIONS: In the first year after HTx, C4d$^+$ occurred early and only in children and young adults with pre-transplant DSA or with clinical suspicion of AMR. Although our data suggest that assessment limited to the first 90 days post-transplant in patients with pre-transplant DSA $\geq$4,000 MFI may be appropriate in the absence of clinical concern for AMR, further research is needed to determine the optimum strategy for post-transplant surveillance.

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Although much has been learned in the last decade about risk factors for and adverse outcomes associated with antibody-mediated rejection (AMR) after heart transplantation, there is continued uncertainty about the appropriate work-up of endomyocardial biopsies (EMBs) for AMR.[1] The 2005 consensus report of the International Society for Heart and Lung Transplantation (ISHLT) recommended immunostaining for AMR not be performed routinely but should be reserved only for those EMBs showing hallmark pathologic features of AMR.[2] The difficulty with this approach is that classic histologic features of AMR are not readily apparent on all EMBs and interpretation is somewhat subjective.[3] The recent ISHLT working formulation for pathologic diagnosis of AMR recommends surveillance

Reprint requests: Brian Feingold, MD, MS, Division of Pediatric Cardiology, Children's Hospital of Pittsburgh of UPMC, 4401 Penn Avenue (FP 5129), Pittsburgh, PA 15224. Telephone: 412-692-5540. Fax: 412-692-6991.

E-mail address: brian.feingold@chp.edu

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immunostaining be performed at 2 and 4 weeks after transplant and then at the time of serum alloantibody assessments (i.e., 1, 3, 6 and 12 months).[4] It also recommends that C4d assessment of EMBs be included, at a minimum, in the diagnostic panel, regardless of whether immunofluorescence (IF) or immunohistochemistry (IC) is utilized.

Since May 2007 we have prospectively utilized routine IC staining for C4d of all first-year EMBs. The purpose of the present study was to evaluate the utility of routine IC staining for C4d in the first year after pediatric and young adult heart transplantation.

Methods

After obtaining institutional review board approval, we reviewed the medical records of all patients who underwent heart transplantation at the Children's Hospital of Pittsburgh of UPMC, since we began routine assessment for C4d on all first-year EMBs. Patients who had at least one EMB through April 2011 were included in this study. Demographics, pre-transplant alloantibody testing, retrospective crossmatch results and clinical data were reviewed. Pre-transplant alloantibody assessment consisted of complement-dependent cytotoxicity (CDC) panel-reactive antibody (PRA) testing in all patients and an anti-HLA antibody profile using the Luminex platform (LABScreen; One Lambda, Canoga Park, California) in all but 2 patients, both of whom had CDC PRA 0% and negative enzyme-linked immunoassay (ELISA) anti-HLA antibody assessments. Clinical data included EMB reports and serial hemodynamics with temporally linked echocardiogram findings. Based on these data, we defined graft dysfunction as present if any of the following were observed: shortening fraction $<28\%$ or decrease in shortening fraction of $>12\%$ from prior echocardiogram; pulmonary artery wedge pressure >24 mm Hg; mixed venous oxygen saturation $<56\%$; or cardiac index <2.2 liters/min/m^2.

Data from EMB reports were abstracted by one investigator (Y.X.) who was blinded to the clinical information. Because we could not exclude that a pathology diagnosis of AMR was made solely on the basis of C4d$^+$ immunostaining, we did not record any mention of AMR from the EMB report. Instead the EMB reports were reviewed for acute cellular rejection (ACR) grade; the presence or absence of C4d immunostaining; and the presence or absence of histologic features that have been ascribed to cardiac AMR, which include endothelial activation, intracapillary neutrophils, intracapillary macrophages, intracapillary thrombi, hemorrhage and edema.[1,2,4–7] We then quantified an "AMR score" for each EMB by assigning 1 point to each histologic feature of cardiac AMR described and summing these values. We chose to weigh each feature equally because of the current uncertainty regarding the histologic findings of cardiac AMR, including the histologic features of "early" AMR and the significance of intravascular macrophages.[4] Also, because EMBs were interpreted during the clinical care of these patients between 2007 and 2011, the 2011 working formulation guidelines[4] were not available to be applied in real time.

Assessment for C4d was performed by paraffin IC as previously reported.[8] Diffuse brown-colored staining of capillary endothelial cells ($\geq 2^+$) was interpreted as positive C4d staining.[9] Staining of other vessels or serum was not considered positive.

All patients received thymoglobulin induction and corticosteroids during the first 5 to 7 days after transplantation. Tacrolimus was initiated on Days 2 to 4 post-operatively and patients were maintained on tacrolimus plus adjunctive therapy (either sirolimus or mycophenolate mofetil). In addition, patients with a positive donor-specific CDC crossmatch received daily plasmapheresis for 3 to 5 days after transplantation with continuation of plasmapheresis based on clinical status, alloantibody profile and EMB findings. These patients were also maintained on weaning doses of corticosteroids during the first year post-transplant. Surveillance EMBs were typically performed 6 to 8 times in the first year at 7 to 14 days and 1, 2, 4, 6 to 7, 9 to 10 and 12 months, with infants having slightly fewer EMBs.

Statistical analysis

Data are presented as mean $\pm$ standard deviation, median (range) or count (percent). Group comparisons were made using Wilcoxon's rank-sum test and the Fisher's exact test, as appropriate. The Kruskal–Wallis test was used to analyze associations of AMR score with graft dysfunction and C4d$^+$. Data were analyzed using STATA, version 10.1 (StataCorp LP, College Station, TX), and all comparisons were 2-sided with significance level of 0.05.

Results

Study cohort

Fifty-one patients met the inclusion criteria. Their median age at transplantation was 6.6 years (15 days to 20.4 years) and reasons for transplant were cardiomyopathy/myocarditis (59%), congenital heart disease (31%) and re-transplantation (10%). The cohort was 58% male, 68% white and 16% black. Median pre-transplant CDC PRA was 0% (range 0% to 89%) and 10 patients (20%) had a positive donor-specific CDC crossmatch ($n = 2$), pre-transplant donor-specific antibody (DSA) $\geq$4,000 mean fluorescent intensity (MFI) ($n = 3$) or both ($n = 5$). Four other patients (8%) had at least 1 pre-transplant DSA 1,000 to 3,999 MFI with a negative donor-specific CDC crossmatch. Two patients (4%) who did not have pre-transplant Luminex assessment had pre-transplant CDC PRA of 0% with negative ELISA anti-HLA antibody assessments, and negative donor-specific CDC crossmatches.

All but 2 recipients (96%) are alive as of the time of this report, with a median post-transplant follow-up of 2.9 years (range 1.0 to 4.9 years). Two infant recipients died at 27 and 67 days post-transplant having had 1 and 2 EMBs, respectively. One died of complications of adenovirus and the other of severe ACR or recurrent myocarditis. Neither was pre-sensitized, had a positive donor-specific CDC crossmatch, or had C4d$^+$ EMB.

Endomyocardial biopsies

A total of 406 first-year EMBs were assessed for C4d. The median number of first-year EMBs per patient was 8 (range 1 to 15). One first-year EMB among the cohort was inadvertently not stained for C4d. It was obtained on Day 161 post-transplant from a non-allosensitized male in whom all other first-year EMBs ($n = 6$) were C4d$^-$.

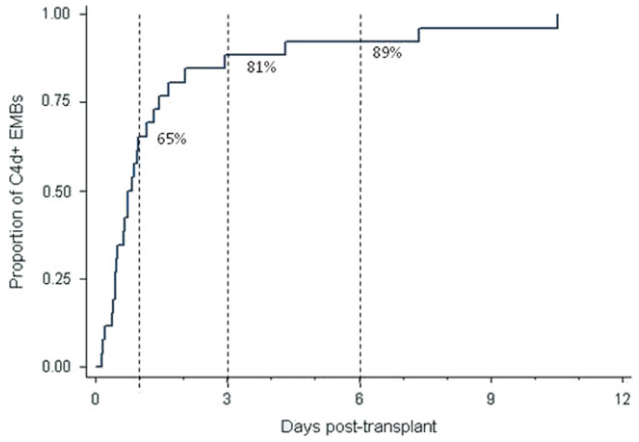

Figure 1 Cumulative incidence of C4d$^+$ EMBs during the first post-transplant year.

A total of 26 EMBs (6%) were C4d$^+$. Seventeen C4d$^+$ EMBs (65%) occurred in the first month post-transplant (Figure 1). The distribution of C4d$^+$ EMBs stratified by ACR grade is shown in Figure 2. Most C4d$^+$ EMBs (73%) occurred in the absence of ACR (Grade 0).

As shown in Table 1, all C4d$^+$ EMBs occurred in 6 patients (12%). Five of these patients (83%) had a pre-transplant DSA $\geq$4,000 MFI with 4 of the 5 also having a positive donor-specific CDC crossmatch. All of these patients had $\geq$2 C4d$^+$ EMBs. One patient with neither pre-transplant anti-HLA antibodies by Luminex nor positive donor-specific CDC crossmatch had a single C4d$^+$ EMB. This patient also had elevated filling pressures, borderline low cardiac index, worsening ventricular ectopy, and an AMR score of 4 on his C4d$^+$ EMB. He was treated with pulse corticosteroids and plasmapheresis for 5 days and, on follow-up assessment 7 days later, had resolution of C4d$^+$, normal filling pressures and improved histology (AMR score = 1).

The timing of all first-year EMBs in the 6 patients with C4d$^+$ is shown in Figure 3. Median time to first C4d$^+$ EMB was 10.5 days (5 to 16 days). All patients with C4d$^+$ immunostaining were positive on their first or second EMB, and once their EMB became C4d$^-$, none had recurrence of C4d$^+$ during the first year post-transplant. There was no significant difference in first-year EMB follow-up duration between C4d$^+$ patients and C4d$^-$ patients (326 [range 279 to 362] days vs 305 [range 9 to 365] days, respectively; $p = 0.23$).

Four patients with pre-transplant DSA 1,000 to 3,999 MFI had a total of 32 EMBs, all of which were C4d$^-$. Among the entire cohort, 5 of 10 patients with pre-transplant DSA $\geq$4,000 MFI and/or a positive CDC crossmatch had C4d$^+$ on EMB compared with only 1 of 41 of patients without pre-transplant DSA $\geq$4,000 MFI and a negative CDC crossmatch (50% vs 2%; $p = 0.001$). The 5 patients with pre-transplant DSA $\geq$4,000 MFI and/or a positive CDC crossmatch who had no C4d$^+$ tended to have a fewer number of DSA (median 1 [0 to 5] vs 3 [1 to 7]; $p = 0.07$) and fewer DSA $\geq$4,000 MFI (median 1 [0 to 1] vs 2 [1 to 6]; $p = 0.03$) than the 5 patients with DSA $\geq$4,000 MFI and/or a positive CDC crossmatch with C4d$^+$.

Figure 4 shows the distributions of: (1) C4d$^+$ EMBs; and (2) graft dysfunction, stratified by AMR score. Although half of all C4d$^+$ EMBs were observed with AMR score $\leq$1, C4d$^+$ was more common as AMR score increased ($p = 0.0001$). Similarly, 70% of all episodes of graft dysfunction occurred with EMBs showing AMR scores $\leq$1, yet graft dysfunction was increasingly prevalent as AMR score increased ($p = 0.0009$). Taken together these data show that both C4d$^+$ and graft dysfunction were more commonly present among first-year EMBs showing multiple histologic features of AMR. Finally, C4d$^+$ was observed more often in EMB showing graft dysfunction than not (19% vs 5%; $p = 0.001$).

Discussion

In this single-center analysis of all first-year EMBs prospectively immunostained for C4d, we found that paraffin IC C4d$^+$ occurred almost exclusively in patients

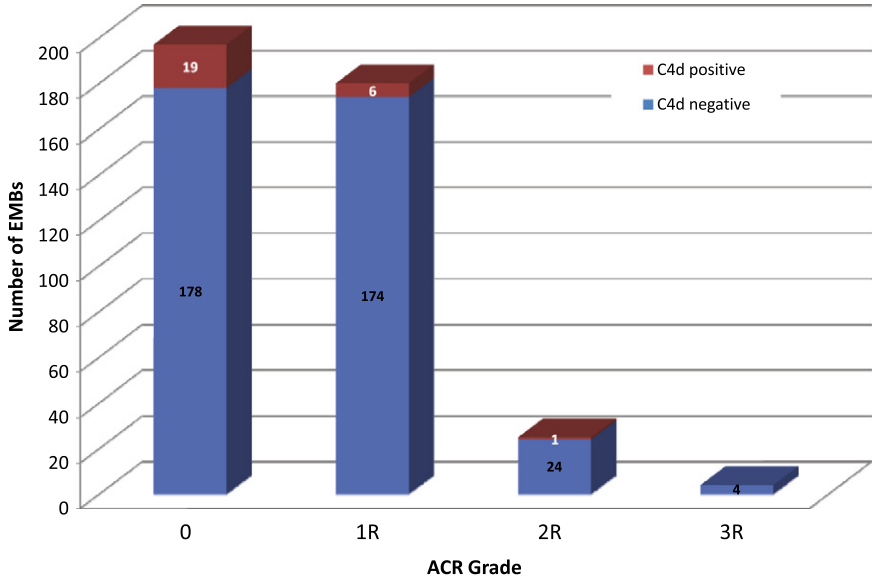

Figure 2 Distribution of C4d$^+$ EMBs by grade of ACR.

Table 1 Characteristics of Patients with ≥1 C4d$^+$ EMB

Patient	Gender	Race	Age at Tx (years)	Diagnosis	Pre-Tx CDC PRA (%)	Pre-Tx DSA ≥4,000 MFI	CDC XM	Number of C4d$^+$ EMBs
1	F	Non-white	14	CHD	4	Yes	Positive	2/10
2	M	White	2	CHD	89	Yes	Positive	5/9
3	M	White	13	CHD	0	No	Negative	1/11
4	F	White	14	ReTx	0	Yes	Positive	9/9
5	M	White	6	CHD	29	Yes	Positive	5/9
6	M	White	16	CHD	75	Yes	Negative	4/12

CDC, complement-dependent cytotoxicity; CHD, congenital heart disease; DSA, donor-specific antibody; MFI, mean fluorescence intensity; Tx, transplant; ReTx, re-transplant; XM, crossmatch.

with a positive donor-specific CDC crossmatch or significant pre-transplant DSA. Furthermore, C4d$^+$ occurred early after transplantation and, after resolution, did not recur during the first year post-transplant. Perhaps the most clinically useful finding is that, among 41 children and young adults without pre-transplant DSA ≥4,000 MFI or a positive donor-specific CDC crossmatch, only 1 had 1 C4d$^+$ EMB during the first post-transplant year and this was in the context of classic histologic features and abnormal hemodynamics.

In our review of the literature, we found no other pediatric-based studies that addressed routine, first-year cardiac allograft C4d immunostaining. Among studies of adult recipients, there were widely divergent incidence rates for C4d$^+$ reported during the first year post-transplant, ranging from 17% to 84% of recipients with ≥1 C4d$^+$ EMB.[10–12] The time of earliest C4d$^+$ EMB in these studies (61 days and 179 days) was also later than in our cohort (10.5 days). It is unclear whether differences in the proportions of recipients with pre-transplant DSA and/or positive donor-specific CDC crossmatch between these studies and ours could explain these variations. However, our findings are consistent with the fact that AMR commonly occurs within the first 8 to 12 weeks after transplantation.[13,14]

We also found that C4d$^+$ was associated with graft dysfunction and was more common on EMBs with multiple histologic features of AMR. Although these findings confirm the approach to the diagnosis of AMR taken by the 2005 revised ISHLT grading scheme,[2] we believe our finding that C4d$^+$ was not specific to AMR (50% of C4d$^+$ EMBs in our sample had AMR scores of ≤1) is of greater importance because of the current uncertainty about "asymptomatic AMR"[1] and the definition of "early AMR."[4,15] A similar lack of specificity was reported by Rodriguez et al in adult cardiac transplant recipients in whom only 5 of 20 patients with C4d and/or C3d by IF had clinical features of AMR.[16] At present, it is unclear whether C4d$^+$ alone, in the absence of other features of AMR (hallmark histologic features, graft dysfunction, circulating donor-specific alloantibodies), is clinically meaningful. Although Kfoury et al reported that "asymptomatic" AMR portends worse outcomes in adult cardiac transplant recipients, their diagnosis of "asymptomatic" AMR relied on a combination of histologic features and immunostaining, and not on isolated C4d.[17] In two studies that tested the association of C4d with outcomes, the findings were contradictory. Although Fedrigo et al reported higher mortality among C4d$^+$ adult heart allograft recipients, Lai et al found C4d$^+$ alone was not associated with outcomes.[12,18] Also, data from the ABO-incompatible renal transplant literature show that diffuse C4d$^+$ staining of peritubular capillaries on surveillance biopsies is common, occurring in 70% to 80% of such grafts as compared with 30% to 40% of renal allografts transplanted across a positive donor-specific crossmatch, and is not associated with

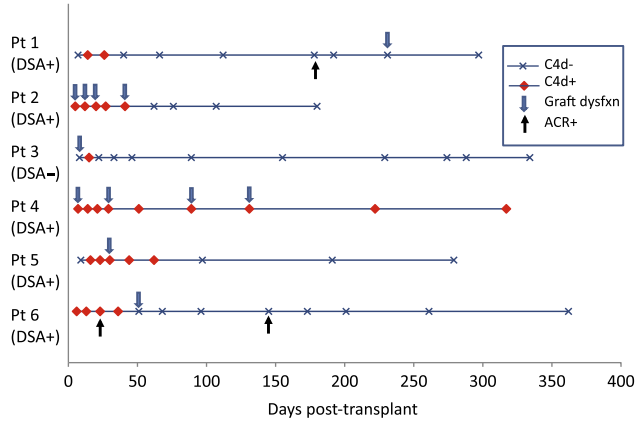

Figure 3 Chronology of first-year EMBs among patients with ≥1 C4d$^+$ EMB. ACR, acute cellular rejection; dysfxn, dysfunction.

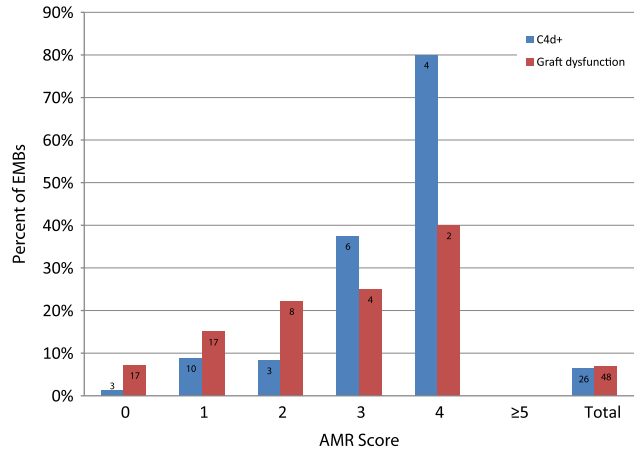

Figure 4 Distribution of C4d$^+$ EMBs and graft dysfunction by AMR score.

adverse outcomes.[19] Our study did not show that $C4d^+$ was associated with post-transplant survival, but our follow-up time was relatively short, and it is possible that a longer follow-up may have shown $C4d^+$ to have prognostic significance.

Our findings suggest that a strategy of routine C4d immunostaining of all first-year EMBs is not warranted. Rather, an alternative strategy that enables targeted C4d surveillance should be pursued, particularly early after transplantation, in those recipients with the greatest likelihood for $C4d^+$. Possible surveillance strategies could include: (1) routine C4d immunostaining in all children until 90 days post-transplant, with continued assessments in those who are $C4d^+$ at the stopping point; or (2) restriction of routine C4d immunostaining to those with significant pre-transplant DSA (i.e., $\geq$4,000 MFI), a positive donor-specific CDC crossmatch, and/or clinical or histologic concern for AMR. Both of these strategies would have detected all $C4d^+$ EMBs in our cohort while resulting in fewer surveillance assessments for C4d than the recommendations of the 2011 working formulation.[4] It is important to note that neither of these strategies considers the recipient who is beyond 1 year post-transplantation.

It should be noted that our study was a single-center analysis; however, we reviewed data on $>$400 EMBs in 51 recipients, which represents the largest series to date in the pediatric and young adult heart transplant literature. Also, our study relied solely on data from the clinical record. This is important limitation because it means that we did not utilize a systematic, blinded re-review of all EMBs by a single pathologist. Rather, pathology findings were documented by 1 of 5 pediatric pathologists on a rotational basis in the course of clinical care from 2007 to 2011. Although all pathologists are experienced in the interpretation of pediatric allograft EMBs, with each reporting at least 75 EMBs annually, no checklist is used. Thus, a lack of uniformity in the interpretation of EMBs may have occurred, which could have influenced our findings. However, because uncertain or concerning findings were reviewed among the group of pathologists as they arose, we believe that the potential influence of such variability on our findings is limited.

With the exception of recipient survival, our analysis has considered clinical and EMB data in the first post-transplant year only. Although this observation period was unavoidable due to our clinical practice of routine C4d immunostaining only during the first post-transplant year, we believe that our analysis is clinically relevant and informative because cardiac AMR is most common early after transplantation. Nonetheless, late post-transplant AMR is also well recognized. The characterization of DSA and EMB findings, including C4d, late after transplantation will thus be important to further our understanding late AMR. Unfortunately, we did not have data on post-transplant DSA, including persistent pre-formed DSA and de novo alloantibodies, and thus we are not able to address this topic. However, ongoing studies at our center now include serial assessments for DSA and C4d after transplantation.

In conclusion, we found paraffin IC $C4d^+$ occurred almost exclusively in patients with a positive donor-specific CDC crossmatch or pre-transplant DSA $\geq$4,000 MFI. When present, $C4d^+$ occurred early post-transplant and, after resolution, did not recur during the first post-transplant year. Although associated with graft dysfunction and hallmark pathologic features of AMR, most $C4d^+$ EMBs occurred in the absence of these circumstances. Taken together, the data suggest that a strategy of routine immunostaining of all first-year EMBs for all children and young adults after heart transplantation is not warranted. Further investigations with larger numbers of patients and EMBs are needed to determine the appropriateness of our strategy, which consists of surveillance C4d immunostaining only in the first 90 days among patients with pre-transplant DSA, supplemented by selective assessment when there is clinical and/or histologic concern for AMR.

Disclosure statement

The authors have no conflicts of interest to disclose.

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