

Evidence for a disease-promoting effect of *Staphylococcus aureus*-derived exotoxins in atopic dermatitis

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Background: The skin of patients with atopic dermatitis (AD) exhibits a striking susceptibility to colonization with *Staphylococcus aureus*. Some strains of *S aureus* secrete exotoxins with T-cell superantigen activity (toxigenic strains), and abnormal T-cell functions are known to play a critical role in AD.

Objective: Our purpose was to examine the impact of superantigen production by skin-colonizing *S aureus* on disease severity.

Methods: In a cross-sectional study of 74 children with AD, the presence and density of toxigenic and nontoxigenic strains of *S aureus* was correlated with disease severity. In a subgroup of patients the T-cell receptor V β repertoire of peripheral blood and lesional T cells was investigated and correlated with individual superantigen activity of skin-colonizing *S aureus*.

Results: Fifty-three percent of children with AD were colonized with toxigenic strains of *S aureus* producing staphylococcal enterotoxin C, staphylococcal enterotoxin A, toxic shock syndrome toxin-1, staphylococcal enterotoxin B, and staphylococcal enterotoxin D in decreasing frequency. Children colonized with toxigenic *S aureus* strains had higher disease severity compared with the nontoxigenic and *S aureus*-negative groups. Patients colonized with toxigenic *S aureus* exhibited shifts in the intradermal T-cell receptor V β repertoire that correspond to the respective superantigen-responsive T-cell subsets.

Conclusion: The data demonstrate that *S aureus*-released exotoxins can modulate disease severity and dermal T-cell infiltration. (J Allergy Clin Immunol 2000;105:814-9.)

Key words: Atopic dermatitis, *Staphylococcus aureus*, staphylococcal enterotoxins (SEA, SEB, SEC, SED, TSST-1)

Abbreviations used

AD:	Atopic dermatitis
cfu:	Colony-forming units
CLA:	Cutaneous lymphocyte-associated antigen
SAg:	Superantigen
SCORAD index:	Scoring atopic dermatitis
SEA:	Staphylococcal enterotoxin A
SEB:	Staphylococcal enterotoxin B
SEC:	Staphylococcal enterotoxin C
SED:	Staphylococcal enterotoxin D
TCR:	T-cell receptor
TCR V:	Variable region on the β -chain of T-cell receptor
TSST-1:	Toxic shock syndrome toxin-1

Atopic dermatitis (AD) is a chronically relapsing inflammatory dermal manifestation of the atopic syndrome that is pathogenetically determined by abnormal T-cell functions.¹ In accordance, the histopathologic features of affected skin are characterized by perivascular infiltrations of predominantly CD4⁺ T cells and monocytes.² In addition to their own proinflammatory potential, these cells are involved in local recruitment and functional modulation of other effector cells including mast cells and eosinophils.³⁻⁶ However, the mechanisms that govern T cell accumulation in affected skin remain to be fully delineated.

In this respect, the prominent role of *Staphylococcus aureus* skin colonization as a factor contributing to the exacerbation of AD has been well established.⁷⁻⁹ An attractive concept is that, because of the disruption of the skin barrier, *S aureus*-released proinflammatory products may gain access to the dermis. Besides several well-described molecules secreted by most strains of *S aureus*-like α -toxin, protein A, and less well-characterized pyrogenic exoproteins,¹⁰ some strains of *S aureus* produce exotoxins with T-cell superantigen (SAg) activity including enterotoxins A to E and G as well as toxic shock syndrome toxin-1 (TSST-1).^{8,11-15} The aim of this study was to determine the impact of exotoxin production by skin-colonizing *S aureus* on disease severity and the presence of T-cell subsets in lesional skin.

METHODS

Study populations

From April 1996 to May 1997, all patients admitted to the department of clinical immunology because of severe atopic dermatitis, according to the criteria of Hanifin and Rajika as modified by Samp-

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son,¹ were enrolled in a cross-sectional study. Seventy-four children with AD (median age 26 months, minimum age 3 months, maximum age 184 months) and 25 nonatopic healthy control subjects (median age 73 months, minimum age 3 months, maximum age 110 months) were investigated. Only patients who had not used either topical or systemic antimicrobial drugs for at least 14 days before investigation were included. Topical steroids (betamethasone 0.03% or 0.05%) were applied twice daily independently of the colonization status. None of the children used systemic steroids. Severity of AD was determined according to the SCORAD index.¹⁶ Informed parental consent was obtained. The study was approved by the university ethics committee.

Isolation of *S aureus* from skin and nose

For the isolation of *S aureus*, contact samples from both unaffected and eczematous skin were obtained. Neck, wrist, elbow, and erosive eczematous lesions were systematically investigated with use of Rodac blood agar plates (diameter 5.6 cm) that were subsequently incubated for 48 hours at 32°C. Growth of *S aureus* was identified by testing typical colonies for coagulase activity.¹⁷ Colonization was quantified by counting the number of colony-forming units (cfu) per agar plate and subsequent calculation of colonies per square centimeter. Nasal carriers were identified by incubating standardized samples obtained from both nares by sterile cotton tips on blood and mannitol salt agar for 48 hours at 32°C.

Detection of *S aureus*-derived exotoxins

To identify the production of exotoxins, a defined inoculum of *S aureus* (10 colonies of a 24-hour subculture on blood agar) was incubated in RPMI 1640 protein-free cell culture medium for 48 hours at 37°C. The filter-sterilized supernatants (0.45 µm) were tested for the presence of exotoxins using the TSST- and SET-RPLAR latex agglutination test (Unipath, Hampshire, UK) according to the manufacturer's instructions.¹⁵ A sample of the same culture filtrate diluted 1:200 in fresh cell culture medium was used to stimulate PBMCs in vitro. The mitogenic activity of *S aureus*-derived supernatants was tested in a whole blood proliferation assay as described earlier.¹⁸

Analysis of T-cell receptor Vβ repertoire by flow cytometry

PBMCs were obtained at the time of skin biopsy by Ficoll (Pharmacia) gradient density centrifugation of fresh heparinized blood. The cells were directly analyzed by flow cytometry to determine the individual native T-cell receptor Vβ (TCR Vβ) repertoire. TCR Vβ analysis as previously described with use of a panel of 24 monoclonal anti-human TCR Vβ antibodies.¹⁹ The following FITC-labeled mAbs were generally used: anti-Vβ 2, anti-Vβ 3, anti-Vβ 3.1, anti-Vβ 6.1, anti-Vβ 6.7, anti-Vβ 9, anti-Vβ 11, anti-Vβ 13.1, anti-Vβ 14, anti-Vβ 16, anti-Vβ 17, anti-Vβ 18, anti-Vβ 20, anti-Vβ 21.3, and anti-Vβ 22 (Immunotech, Hamburg, Germany) and anti-Vβ 5.1-3, anti-Vβ 8, and anti-Vβ 12 (DPC, Bad Nauheim, Germany). The pattern of TCR Vβ expression was expressed as percentage of CD3⁺ T cells.

Skin biopsy

Cutaneous punch biopsy specimens were taken from chronically affected skin of the upper part of one leg and immediately snap frozen in liquid nitrogen and stored at -80°C. Serial 5-µm cryostat sections were immunostained with the following mAbs: anti-CD3 (clone UCHT1 obtained from Dako, Glostrup, Denmark) and anti-human Vβ 3.1, Vβ 12.1, Vβ 13.6, Vβ 16, Vβ 17, Vβ 18 T-cell receptor (TCR) (Immunotech), and Vβ 5.1 (DPC), all FITC labeled.

Because the FITC-labeled bound antibodies had to become visualized in the frozen tissue sections, the following method was applied. After incubation with the primary FITC-labeled antibody, mouse

anti-FITC mAb was used. This incubation was followed by the application of a bridging rabbit antimouse antibody and the alkaline-phosphatase-anti-alkaline phosphatase complex. The bound alkaline phosphatase became visible with use of New Fuchsin as a chromogen.²⁰

Immunohistologic features were evaluated by an experienced histopathologist blinded for the identity of the samples. The total number of T cells within the dermis was assessed by counting the CD3-positive cells. The percentage of T cells expressing the various variable regions of the β-chain of TCR was calculated after counting the labeled cells in the corresponding areas of the immunostained serial sections.

Statistical analysis

Statistical analysis was performed with the chi-square test and Wilcoxon's test for independent samples. Linear regression analyses were performed to examine the impact of (1) *S aureus* colonization and (2) *S aureus*-derived exotoxins on disease severity.

RESULTS

Prevalence of toxigenic and nontoxigenic strains of *S aureus*

Of the group of 74 children with AD, 60 (81%) were colonized by *S aureus* on skin. SAg-producing strains were obtained from 40 (53%) of these AD patients but from only 1 (4%) control subject (chi-square test, $P < .001$). Toxigenic strains were found to secrete staphylococcal enterotoxin C (SEC), staphylococcal enterotoxin A (SEA), TSST-1, staphylococcal enterotoxin B (SEB), or staphylococcal enterotoxin D (SED) in decreasing frequency. Nine patients (23%) harbored strains secreting at least 2 different toxins. All supernatants negative for the above exotoxins were additionally tested for undefined mitogenic activity, which was found in 7 patients with AD and in none of the controls (Fig 1).

Correlation between *S aureus* colonization and severity of AD

Severe dermatitis was observed significantly more often in children colonized by toxigenic than by nontoxigenic strains of *S aureus* (Fig 2, A). In the group of children colonized by toxigenic *S aureus* strains, 58% ($n = 23$) presented with a SCORAD score of > 50 points compared with 35% ($n = 7$) of children colonized with nontoxigenic strains. Only one patient with a SCORAD score of > 50 points was present in the group without *S aureus* colonization (chi-square test, $P < .01$). The correlation between the degree of colonization and disease severity is demonstrated in Fig 2, B.

The influence of exotoxin production on the SCORAD score was determined as $R^2 = 0.3$ (ie, 30% of the SCORAD score variance is explained by exotoxin production), whereas infection with *S aureus* revealed $R^2 = 0.5$. Additionally, it was found that 3 exotoxins, SED, SEB, and SEC, together have an impact on a SCORAD score of $R^2 = 0.23$.

Presence of SAg-responsive T-cell subsets in afflicted skin

In an attempt to explain pathophysiologically the impact of exotoxin production on disease severity, we investigated 4 patients who had SEB-positive *S aureus*

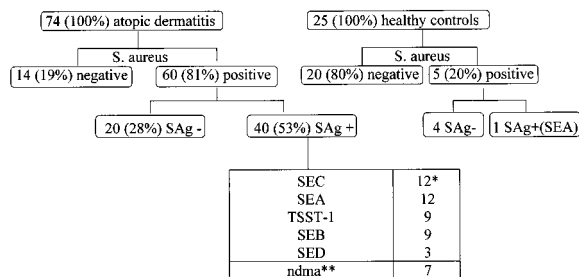


FIG 1. Prevalence of toxigenic and nontoxigenic strains of *S aureus* on the skin of children with AD and of healthy control subjects. Asterisk, Number of children colonized with *S aureus* producing the respective exotoxin; some strains produced more than one toxin (9 patients); two asterisks, number of children colonized with strains of *S aureus* producing undefined mitogenic activity (*ndma*) as determined by lymphocyte transformation assay.

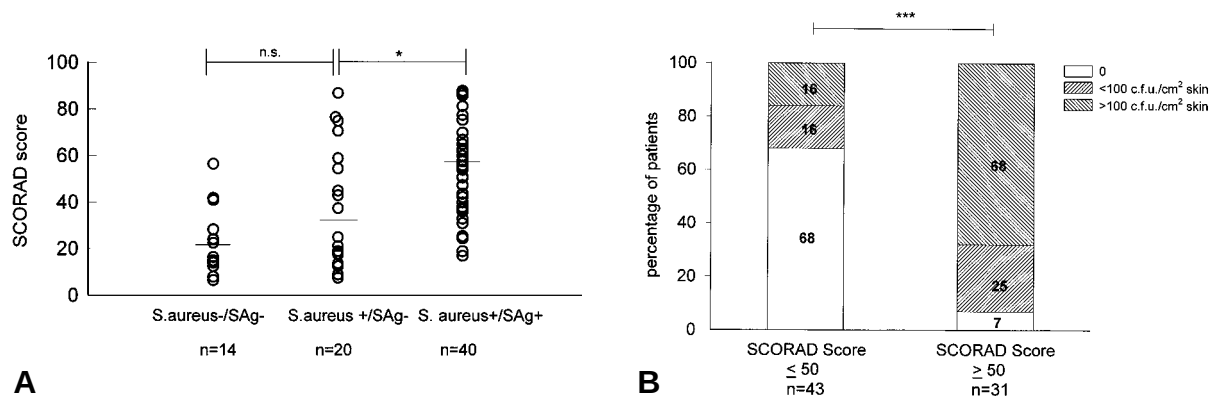


FIG 2. **A**, Severity of AD as determined by SCORAD score in children colonized by nontoxigenic versus toxigenic strains of *S aureus*. Solid lines, Median SCORAD scores. Asterisk, $P < .05$ (Wilcoxon 2-sample test). There was no difference in the use of topical steroids between the groups of *S aureus*-colonized children, irrespective of their SAg production. n.s., Not significant. **B**, Degree of bacterial colonization in patients with mild and moderate dermatitis (SCORAD score ≤ 50 points) in comparison to patients with severe dermatitis (SCORAD score ≥ 50 points). Colonization was quantified by counting the number of cfu per agar plate and subsequent calculation of colonies per square centimeter. Three asterisks, $P < .0001$ (chi-square test). Data are derived from contact samples at neck. Samples for wrist, elbow, and erosive eczematous lesions evaluated same results (data not shown).

and 5 patients colonized with nontoxigenic *S aureus* for both (1) the response of peripheral blood lymphocyte cells to *S aureus*-derived culture filtrate (data not shown) and (2) the presence of SAg-responsive T-cell subsets in affected skin (Fig 3). The results of fluorescence-activated cell sorter analysis obtained in native peripheral blood were compared with the frequencies of the respective T-cell subsets infiltrating *S aureus*-colonized skin. Although SAg nonresponsive T-cell subsets did not preferentially accumulate in skin, the observed preferential in vitro expansion of T cells positive for these TCR V β subsets known to be responsive to SEB suggests that SAg were the major driving force in *S aureus*-derived exoproducts and the preferential accumulation of the respective subsets. Patients colonized with SEB-secreting *S aureus* strains expressed in skin-infiltrating T cells have a higher percentage of cells positive for TCR V β 3.1, TCR V β 12.1 (Wilcoxon 2-sample test, $P < .05$), and TCR V β 17 ($P < .01$) but not in the peripheral blood T cells.

Tissue sections of 2 typical patients are presented in Figs 4 and 5. Differences between patients could shown in the TCR V β 3.1, TCR V β 12.1, and TCR V β 17 expression, which are recognized by the staphylococcal SAg SEB.

DISCUSSION

Increased susceptibility to *S aureus* is well established in AD. Most studies revealed that about 80% of patients with AD are colonized^{7-9,12-15} and may profit from eradication of the bacteria (eg, by antimicrobial therapy⁷). Although the clinical evidence is persuasive, the mechanism of the disease-promoting effect of *S aureus* remains to be elucidated. In this respect, it has been proposed that bacterial invasion or the penetration of *S aureus*-derived soluble factors may play a proinflammatory role.^{3,7-15} However, although the typical host response to infections with *S aureus* is an acute polymorphonuclear inflammation, histopathologic features of affected skin in AD patients are dominated by perivascular T-cell and monocytic infiltrations. We here demonstrate that *S aureus*-derived exotoxins with SAg activity contribute significantly to disease severity as quantified by the SCORAD score. Detailed analysis of the TCR V β repertoire of lesional T cells revealed that a comparatively high percentage of T cells in fact expressed TCRs known to be responsive to exactly those exotoxins produced by the strain of *S aureus* isolated from the respective skin lesions.

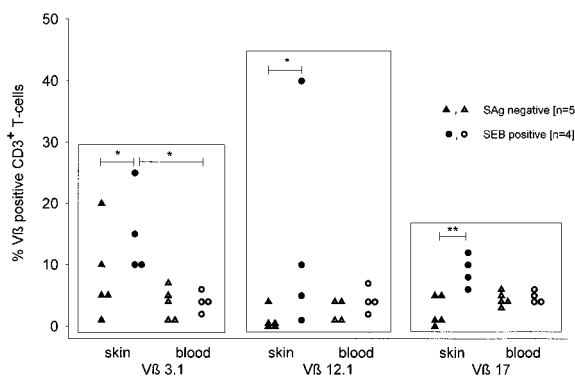


FIG 3. Frequency of TCR V β subsets in native CD3⁺ PBMCs and lesional skin. Cutaneous punch biopsy specimens were taken from randomly selected patients (SCORAD index >50 points for more than 3 years) colonized with SEB-positive (4 patients) as well as nontoxicogenic strains of *S aureus* (5 patients), respectively. The site of biopsy was not treated with steroids for at least 3 weeks.

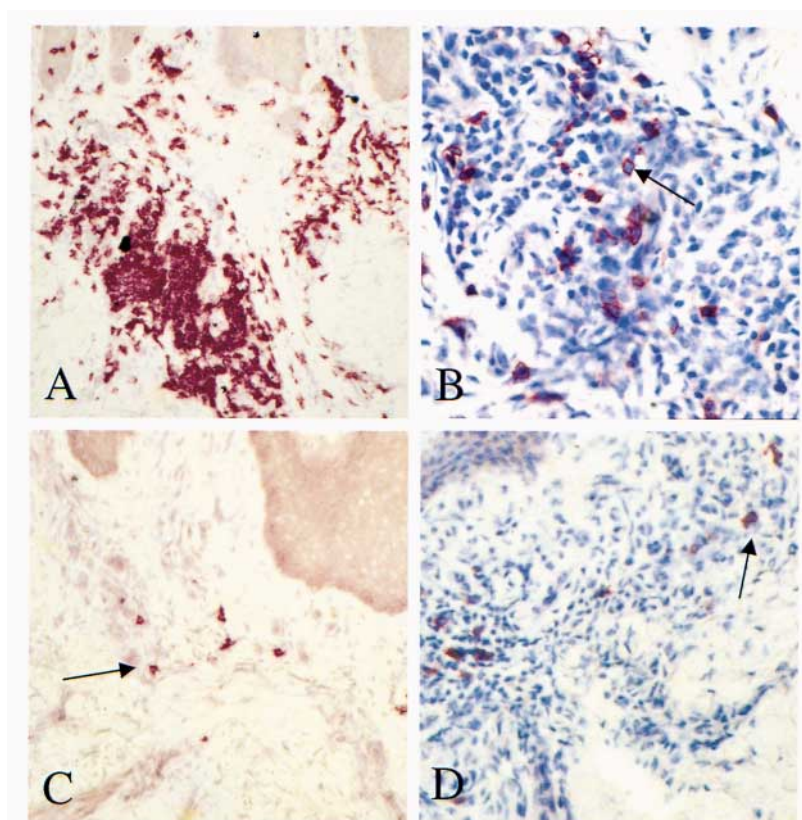


FIG 4. Patient demonstrates severe chronic dermatitis (SCORAD score 87 points) and was colonized with SEB-secreting *S aureus* (>100 cfu/cm² skin). Percentage of total (A), TCR V β 3.1+ (B), TCR V β 12.1+ (C), and TCR V β 17+ CD3⁺ T cells (D) in chronically affected skin.

In analogy to toxic shock syndrome and Kawasaki disease as well as rheumatoid arthritis,²¹ the observed preferential accumulation of T-cell subsets in afflicted skin may explain the disease-promoting effect of *S aureus*-derived exotoxins.

Therefore our data support but extend the observation by Ha et al²² that staphylococcal SAg and the corresponding T-cell subsets may act as pathogenetically relevant factors in a subgroup of patients with AD.

The contribution of T cells in perpetuating AD has been well established.²³ In subgroups of patients, food antigen-specific and aeroallergen-specific T cells have been isolated from the skin. In all cases, however, the frequencies of antigen-specific T cells in the skin was extremely low, so the presence of these T cells may just be the result of an inflammation-induced (nonspecific) recruitment of memory T cells.

Recently, the group of Yadate et al²⁴ indicated poly-

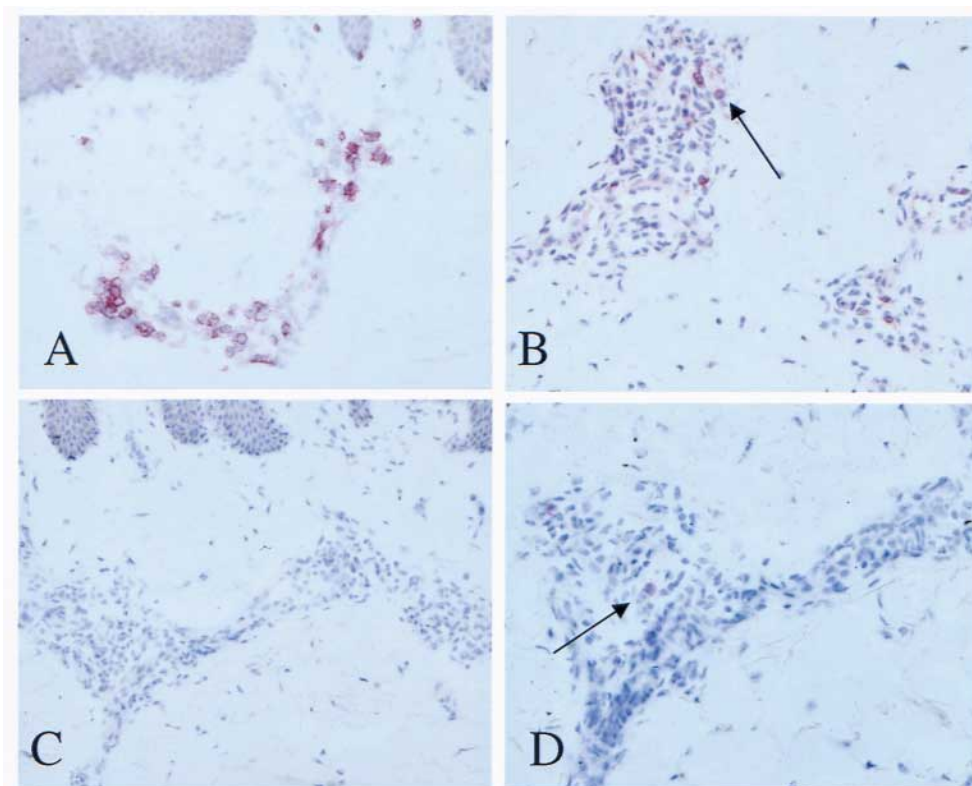


FIG 5. This patient also has severe chronic dermatitis (SCORAD score 76 points) but was colonized with non-toxicogenic *S aureus* (>100 cfu/cm² skin). Percentage of total (A), TCR V β 3.1+ (B), TCR V β 12.1+ (C), and TCR V β 17+ CD3+ T cells (D) in chronically affected skin.

clonal and oligoclonal expansion of certain T-cell subsets after stimulation of PBMCs from AD patients with SAGs such as SEA and SEB. To further elucidate the role of SEB in the pathogenesis of AD, we have recently developed a humanized SCID mouse model in which the epidermal application of SEB to mice reconstituted with PBMCs from AD patients not only caused marked dermal T-cell infiltration but also inflammatory lesions consistent with those observed in AD.²⁵ These observations found their analogy in studies in which the epidermal application of SEB to human skin induced dermatitis-like symptoms.²⁶

Additional evidence for a disease-modulating effect of SAG has been provided by a recent publication from Strickland et al.²⁷ The authors analyzed the TCR-V β repertoire of cutaneous lymphocyte-associated (CLA)-positive peripheral blood T cells from AD patients colonized with toxigenic and nontoxigenic *S aureus* strains. All patients in the group colonized with toxigenic strains manifested significant SAG-appropriate TCR V β skewing within the CLA-positive T-cell subset. Torres et al²⁸ found that circulating skin-homing T cells of patients with active AD contain an increased percentage of cells bearing TCR V β 2- and TCR V β 5.1-expressing cells.

SAGs consist of a group of high-molecular-weight proteins defined by a unique pattern of T-cell stimulation. In contrast to conventional antigens, SAGs do not require

classic presentation by antigen-presenting cells. Instead, they can directly interact with α/β TCRs. The site of engagement, resulting in the expansion and activation of certain T-cell subsets, has been mapped to the TCR V β element for some superantigens.²⁹⁻³¹ That this in fact may take place in skin of patients with AD colonized by toxigenic strains of *S aureus* is suggested by our finding that a majority of T cells found in lesional skin expressed a T-cell receptor V β repertoire that corresponded to the SAG produced by the colonizing *S aureus* strain.

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