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Mouse Strain Susceptibility to Trypanosome Infection: An Arginase-Dependent Effect

Sébastien Duleu,*† Philippe Vincendeau,* Pierrette Courtois,* Silla Semballa,* Isabelle Lagroye,† Sylvie Daulouède,* Jean-Luc Boucher,‡ Keith T. Wilson,§ Bernard Veyret,¶ and Alain P. Gobert*†‡¶

We previously reported that macrophage arginase inhibits NO-dependent trypanosome killing in vitro and in vivo. BALB/c and C57BL/6 mice are known to be susceptible and resistant to trypanosome infection, respectively. Hence, we assessed the expression and the role of inducible NO synthase (iNOS) and arginase in these two mouse strains infected with Trypanosoma brucei brucei. Arginase I and arginase II mRNA expression was higher in macrophages from infected BALB/c compared with those from C57BL/6 mice, whereas iNOS mRNA was up-regulated at the same level in both phenotypes. Similarly, arginase activity was more important in macrophages from infected BALB/c vs infected C57BL/6 mice. Moreover, increase of arginase I and arginase II mRNA levels and of macrophage arginase activity was directly induced by trypanosomes, with a higher level in BALB/c compared with C57BL/6 mice. Neither iNOS expression nor NO production was stimulated by trypanosomes in vitro. The high level of arginase activity in T. brucei brucei-infected BALB/c macrophages strongly inhibited macrophage NO production, which in turn resulted in less trypanosome killing compared with C57BL/6 macrophages. NO generation and parasite killing were restored to the same level in BALB/c and C57BL/6 macrophages when arginase was specifically inhibited with Nω-hydroxy-nor-arginine. In conclusion, host arginase represents a marker of resistance/susceptibility to trypanosome infections. The Journal of Immunology, 2004, 172: 6298–6303.

Trypanosomes of the brucei group develop extracellularly and are responsible for trypanosomiasis in both humans and animals in Africa. Human African trypanosomiasis is considered as a re-emerging disease, and hundreds of thousands of people are currently infected (1). The disease is partially controlled by B cell responses to variant surface glycoprotein. However, other components of both innate and adaptive immune response are involved in resistance. Thus, a Th1 cell response to parasite Ags is associated with relative resistance to African trypanosomes (2).

When stimulated with Th1 cytokines (3) or with microbe-derived products (4, 5), macrophages express the inducible NO synthase (iNOS), which synthesizes large amounts of NO by oxidation of L-arginine. NO is known to be a major effector molecule in macrophage-mediated cytotoxicity, and therefore macrophage-derived NO is a key component of the defense against microbial agents (6), including trypanosomes (7). Furthermore, arginase, another enzyme that metabolizes L-arginine, can be induced in various immunological responses (3, 4, 8, 9). Two isoforms of arginase have been identified: the cytoplasmic arginase I and the mitochondrial arginase II catalyze the same reaction, which is the conversion of L-arginine into urea and L-ornithine (10). Because arginase competes with iNOS for their common substrate, it is established that arginase can inhibit NO production (10). We have previously reported that iNOS and arginase are both induced in macrophages from Trypanosoma brucei brucei-infected mice (11). This coexpression has a critical biologic consequence in infected mice because macrophage arginase inhibits NO production and NO-dependent trypanosome killing (11).

Various studies have reported a resistance of C57BL/6 mice and a susceptibility of BALB/c mice to parasitic infections (12, 13), including trypanosomiasis (14). In addition, it was previously shown that NO production by macrophages from infected C57BL/6 mice is greater than that from BALB/c mice (15, 16), and a correlation between the level of NO production and the susceptibility of mouse strains to parasitic infections has been suggested. Since an increased arginase to iNOS ratio is essential for parasitic survival, we hypothesized that differential susceptibility of mice to trypanosome infection is related to differences in arginase induction.

In this study we report that in the early stage of T. brucei brucei infection, up-regulation of both arginase I and arginase II mRNA expression is greater in macrophages from BALB/c mice than expression in macrophages from C57BL/6 mice, whereas iNOS mRNA up-regulation is identical in cells from both mouse strains. Similarly, arginase activity is higher in macrophages from infected BALB/c than from C57BL/6 mice. The abundant macrophage arginase activity in BALB/c mice favors parasite growth by strongly inhibiting NO production and trypanosome killing, whereas...
C57BL/6 macrophages exhibit more effective NO-dependent parasite killing. NO production and trypanosome killing were increased similarly in both types of mice when arginase was specifically inhibited. Therefore, we postulate that the difference in parasite susceptibility observed between BALB/c and C57BL/6 mice is due to the different levels of arginase expression that inhibit NO-dependent trypanosome killing and favor parasite growth.

Materials and Methods

Reagents

For cell culture and RNA analysis, all the reagents were obtained from Life Technologies (Gaithersburg, MD). 1-[14C]Guanido-1-[14C]arginine (1-[14C]arginine, specific activity: 51.5 mCi/mmol) was purchased from NEN (Cambridge, MA). The specific arginase inhibitor N\(^{\text{f}}\)-hydroxy-nor-\(\text{L}\) arginine (norNOHA), which is not a substrate for iNOS, was synthesized as previously described (17). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Parasites

The Antat 1.1 E clone of \(T.\ brucei\) was obtained from Institute of Tropical Medicine (Antwerp, Belgium). Parasites were maintained in vivo in Swiss mice and purified from blood by chromatography on DEAE cellulose.

Mice, cells, and culture conditions

All animals were housed under conventional conditions, given water and chow ad libitum according to institutional guidelines. Seven- to ten-week-old female BALB/c and C57BL/6 mice (Iffa Credo, Saint-Germain-sur-l’Aresse, France) were i.p. infected with \(2 \times 10^5\) trypanosomes. Peritoneal macrophages from control or infected BALB/c and C57BL/6 mice were harvested, and purity assessed as previously described (7). Peritoneal macrophages (\(5 \times 10^6\)/well in 24-well plates) or J774 cells, a murine macrophage cell line, were cocultured with or without purified \(T.\ brucei\) (\(5 \times 10^7\)/well) in 500 \(\mu\)l of HBSS or using an MEM Select-amine kit supplemented with 1% FBS, 20 mM HEPES, 2 mM sodium pyruvate, 10 \(\mu\)g/ml gentamicin, and 3% glucose, at 37\(^\circ\)C in a humidified 5% CO\(_2\) atmosphere. Variable concentrations of \(\text{L}\)-arginine, 500 \(\mu\)M norNOHA, and/or 10 \(\mu\)M \(\text{N}^{\text{f}}\)-\(\text{L}\) arginase, 500 \(\mu\)M \(\text{N}^{\text{f}}\)-(1-iminoethyl)-\(\text{L}\)-lysine (\(\text{N}^{\text{f}}\)-NIL), a specific iNOS inhibitor, were added in the medium when required.

mRNA analysis

Purified peritoneal macrophage total RNA was isolated with TRIzol reagent. Subsequently, 2 \(\mu\)g of RNA were reverse transcribed using a set of oligo(dT) primers and 25 \(\mu\)l of Superscript II Reverse Transcriptase. The resulting cDNA (1 \(\mu\)l) was then amplified by classical PCR and by real-time PCR using 0.5 \(\mu\)l of TaqDNA polymerase and primers for iNOS, arginase I, arginase II, and \(\beta\)-actin under the following PCR conditions as previously reported (8): 1) PCR products were run on 1.5% agarose gel and quantified by the Imagequant (Molecular Dynamics) software. 2) Amplified cDNA was labeled with the SYBR-Green PCR Assay (Roche, Mannheim, Germany) and with 0.16, 0.16, 0.16, and 0.06 pmol/\(\mu\)l each of iNOS, arginase I, arginase II, and \(\beta\)-actin primers, respectively; PCR products were detected with a LightCycler apparatus (Roche). One real-time PCR cycle consisted of the following: 94\(^\circ\)C for 30 s, 60\(^\circ\)C for 30 s, 60\(^\circ\)C for 45 s. Relative mRNA expression was calculated as suggested by the manufacturer.

Measurements of arginase activity

Arginase activity was measured on purified macrophage by a colorimetric method as previously described (11). Briefly, 10 \(\text{mM MnCl}_2\) and 0.5 \(\text{M L-arginine were successively added to macrophage lysates for 1 h at 37}\(^\circ\)C. The reaction was stopped by the addition of an acid solution, and the urea formed by arginase was analyzed by addition of \(\alpha\)-nitrosopropioniphenone at 100\(^\circ\)C for 45 min. The colored product was quantified by absorption at 540 nm.

In addition, macrophage arginase activity was assayed by the measurement of the conversion of \(\text{L-}[1^4\text{C}]\text{arginine to }[1^4\text{C}]\text{urea. Macrophages (4 } \times 10^6\text{/well in 24-well plates) from control or } T.\ brucei\text{-infected mice were cultured in HBSS containing 2 mM L-arginine and 0.1 } \mu\text{M } \text{L-}[1^4\text{C}]\text{arginine. The same medium was incubated without cells as a negative control. After 18 h, 150 } \mu\text{l of supernatant were removed and added to scintillation fluid (3 ml) in counting vials. The percentage of } \text{L-}[1^4\text{C}]\text{arginine converted to }[1^4\text{C}]\text{urea was calculated as previously described (18).}

Nitrite assay

In culture supernatant, NO\(_2\) concentration was determined spectrophotometrically at 540 nm after reaction with the Griess reagents as previously described (7).

Statistical analysis

Analyses were performed with the Statview 5.0 software. The Student-Newman-Keuls test was used for comparisons between multiple groups. For single comparisons between two groups, the Student t test was used.

Results

Infection rates of BALB/c and C57BL/6 mice infected with \(T.\ brucei\) brucei

The course of infection was compared between BALB/c and C57BL/6 mice infected with \(T.\ brucei\) brucei. In BALB/c and C57BL/6 mice, \(T.\ brucei\) brucei grew dramatically in the host’s blood 2 days after the infection (Fig. 1A). However, C57BL/6 mice had a 2 log order lower level of parasitemia in comparison with BALB/c mice, after 4 days of infection (Fig. 1A). Similarly, parasite load in the peritoneum was significantly more important in BALB/c mice vs C57BL/6 mice (Fig. 1B). These data indicate that, in our experimental conditions, BALB/c mice are relatively more susceptible to \(T.\ brucei\) brucei infection than are C57BL/6 mice.

Induction of arginase and iNOS mRNA expression in macrophages from \(T.\ brucei\) brucei-infected mice

After 6 days of infection, mRNA levels of arginase I, arginase II, and iNOS were determined in peritoneal macrophages by RT-PCR. In both BALB/c and C57BL/6 mice, the three genes were up-regulated in infected mice in comparison with uninfected animals (Fig. 2A). However, the expression of arginase I and arginase II mRNAs were greater in BALB/c macrophages compared with C57BL/6 macrophages, whereas iNOS transcripts were induced at 800 \(\mu\text{l}\) of a 250 mM acetic acid solution, pH 4.5, containing 100 mM urea and 10 mM \(\text{L-arginine. After the addition of Dowex resin (HCR-W2; Sigma-Aldrich), the mixture was centrifuged at } 120 \times g\text{ for 5 min. Supernatants (500 } \mu\text{l}) were removed and added to scintillation fluid (3 ml) in counting vials. The percentage of \(\text{L-}[1^4\text{C}]\text{arginine converted to }[1^4\text{C}]\text{urea was calculated as previously described (18).}

FIGURE 1. Time course of \(T.\ brucei\) brucei infection. A, BALB/c (□) and C57BL/6 (●) mice were infected with \(2 \times 10^7\) trypanosomes. The blood was harvested at indicated time, and the parasite number was determined with a hemocytometer. B, In mice infected for 5 days, parasites were enumerated with a hemocytometer in the peritoneal content collected after an i.p. injection of 4 ml PBS. A and B, Each point is the mean ± SEM of one experiment performed on three mice. * \(p < 0.05\) vs BALB/c mice.
infected BALB/c, 45.3% cultured in the presence of 0.4 mM L-arginine was observed during induction by macrophages from BALB/c and C57BL/6 mice cultured at day 6 postinfection. The data shown are representative of three mice. The data represent the mean ± SEM of three mice. *p < 0.05, **p < 0.01 vs BALB/c macrophages.

the same level in both type of macrophages (Fig. 2A). This observation was confirmed by the analysis of mRNA levels by real-time PCR (Fig. 2B): the up-regulation of arginase I and arginase II mRNAs was ~3.5- and ~2.5-fold more important in macrophages from infected BALB/c compared with infected C57BL/6 mice, for arginase I and arginase II, respectively. The iNOS mRNA was dramatically fold increased by 3169 ± 290 and 2590 ± 450 in BALB/c and C57BL/6 mice, respectively (no significant difference between BALB/c and C57BL/6; n = 3 mice).

Time course of arginase and iNOS activities in macrophages from infected mice

An increase in the macrophage arginase activity was observed during the disease in both BALB/c and C57BL/6 mice (Fig. 3A). However, arginase activity was constantly greater in macrophages from infected BALB/c compared with those from C57BL/6 mice. The arginase activity level was 2.4 ± 0.9-fold greater in BALB/c than in C57BL/6 macropahes at day 6 postinfection. These results were confirmed by the measurement of arginase activity by a radioassay. The percentages of conversion of L-[14C]arginine to [14C]urea were as follows: uninfected BALB/c, 23.3 ± 8.1% (n = 3 mice); uninfected C57BL/6, 12.9 ± 3.8% (n = 3 mice); infected BALB/c, 45.3 ± 2.9% (p < 0.05 vs uninfected BALB/c, n = 5 mice); infected C57BL/6, 17.4 ± 5.1% (p < 0.01 vs infected BALB/c, n = 5 mice).

As depicted in Fig. 3B, a time-dependent increase of NO2− production by macrophages from BALB/c and C57BL/6 mice cultured in the presence of 0.4 mM L-arginine was observed during the disease. However, the levels of NO were inversely related to the arginase activity presented in Fig. 3A, with significantly less NO produced by infected macrophages from BALB/c mice compared with C57BL/6 mice. At day 6 postinfection, NO2− concentration was decreased by 2.2 ± 0.3-fold in the macrophage supernatants of BALB/c compared with those of C57BL/6 mice.

Direct activation of macrophage arginase by trypanosomes

Arginase induction was assessed by semiquantitative analysis of mRNA from peritoneal macrophages of C57BL/6 and BALB/c mice cocultured with T. brucei brucei for 18 h. As shown in Fig. 4A, arginase expression was increased in macrophages activated by trypanosome when compared with unstimulated cells. Up-regulation of macrophage arginase I and arginase II mRNAs was ~3.2- and ~5-fold greater in BALB/c than in C57BL/6, respectively (Fig. 4A).

Macrophage arginase activity was also directly induced by trypanosomes in both strains of mice (Fig. 4B). Nonetheless, although basal arginase activity was similar in BALB/c and C57BL/6 mice, the increase in parasite-induced arginase activity was higher in macrophages from BALB/c mice compared with those from C57BL/6 mice. Arginase activity was 2.4 ± 0.2-fold and 3.4 ± 0.5-fold greater in BALB/c macrophages vs C57BL/6 macrophages after 36 h and 72 h coculture, respectively. Moreover, increase of trypanosome-induced macrophage arginase activity, after 72 h of coculture, was not altered by the use of 10 µg/ml polymyxin B in the supernatant (n = 3 mice); for BALB/c macrophages, arginase activity was increased by 4.29 ± 0.04- and 3.84 ± 0.28-fold above control macrophages when cells were stimulated by T. brucei brucei and by T. brucei brucei plus polymyxin B, respectively; for C57BL/6 macrophages, arginase activity was increased by 2.12 ± 0.08- and 2.11 ± 0.04-fold above control when cells were cocultured with T. brucei brucei and with T. brucei brucei plus polymyxin B, respectively. However, the increase of arginase activity in control macrophages stimulated with 10 ng/ml Escherichia coli LPS was significantly inhibited by ~90% and ~65% when polymyxin B was added to the cultures of BALB/c and C57BL/6 macrophages, respectively (data not shown; p < 0.01 for both, n = 3 mice). Arginase activity was also induced directly by trypanosomes in J774 cells by ~7-fold when compared with unstimulated macrophages (data not shown). No significant increase of arginase activity was observed in macrophages from either mouse type cultured alone for 72 h. Arginase activity was
not detected by the colorimetric assay in a trypanosome lysate obtained with 10^6 parasites.

When assessed by real-time PCR after 18 h stimulation, iNOS mRNA expression was found to be increased by ~0.4- and ~0.1-fold above control for BALB/c and C57BL/6 macrophages, respectively (data not shown, n = 3 mice). Similarly, NO\textsubscript{2} was not detected in the supernatants of BALB/c and C57BL/6 macrophages or of J774 cells stimulated by trypanosomes (data not shown). These results demonstrate that macrophage iNOS was not directly induced by *T. brucei brucei*.

**Inhibition of macrophage NO production and NO-dependent trypanosome killing by arginase**

Because we have previously reported that macrophage NO production and NO-dependent trypanosome killing is inhibited by the endogenous arginase activity (11), we reasoned that the difference in arginase activity levels observed between BALB/c and C57BL/6 mice could be associated with different effects on parasites, and that the availability of L-arginine substrate could also modulate NO production and hence, parasite killing.

In cocultures of purified *T. brucei brucei* and macrophage from infected mice, NO\textsubscript{2} concentration was measured and the results are presented in Fig. 5A. At 0.2 and 0.4 mM L-arginine, NO production by macrophages was significantly less produced by BALB/c-infected mice in comparison with macrophages from C57BL/6 infected mice. When additional substrate (0.6 mM L-arginine) was used, this effect was attenuated. Additionally, NO was produced at the same level by macrophages from BALB/c- and C57BL/6-infected mice when norNOHA, an arginase inhibitor, was added to the cocultures.

An L-arginine-dependent trypanosome killing by macrophages was observed (Fig. 5B). However, trypanosome survival was significantly less affected by macrophages from infected BALB/c mice vs C57BL/6 mice (Fig. 5B). In the presence of norNOHA, parasites were identically killed by macrophages from C57BL/6- or BALB/c-infected mice (Fig. 5B).

The growth of trypanosomes cocultured 24 h with macrophages from uninfected mice was not significantly modified by the use of 0.6 mM L-arginine (BALB/c, 7.8 ± 0.7 × 10^5 *T. brucei brucei*; C57BL/6, 6.8 ± 1.9 × 10^5 *T. brucei brucei*, n = 2 mice) or norNOHA (BALB/c, 3.3 ± 1.4 × 10^5 *T. brucei brucei*; C57BL/6, 4.9 ± 2.1 × 10^5 *T. brucei brucei*, n = 2 mice). When L-NIL was used in the 24-h cocultures of macrophages from infected mice and trypanosomes containing 0.6 mM L-arginine, NO\textsubscript{2} levels were 3.9 ± 1.4 μM and 2.8 ± 1.6 μM in supernatants of BALB/c and C57BL/6 macrophages, respectively (data not shown; p < 0.001 for both vs the same supernatants without L-NIL, n = 4 mice). Consequently, in these cocultures, the toxic effect of BALB/c and C57BL/6 macrophages from infected mice on trypanosomes was significantly inhibited by the use of L-NIL. Parasite numbers were as follows: BALB/c plus L-NIL, 7.6 ± 2.3 × 10^5 *T. brucei brucei*; C57BL/6 plus L-NIL, 6.0 ± 0.8 × 10^5 *T. brucei brucei* (data not shown; p < 0.01 for both vs the same supernatants without L-NIL, n = 4 mice).

**Discussion**

This study highlights the difference in the increase in macrophage arginase activity between susceptible and resistant trypanosome-infected mice. In the present study, we show that arginase activity in macrophages from *T. brucei brucei*-infected mice, or in naive macrophages activated ex vivo by trypanosomes, is higher in susceptible BALB/c mice than in resistant C57BL/6 mice. In the early stage of *T. congolense* infection, macrophage arginase activity was also shown to be higher in BALB/c than in C57BL/6 mice (14).
Moreover, we demonstrate that the high level of arginase activity in BALB/c macrophages was directly linked to inhibition of NO-dependent parasite killing, whereas C57BL/6 macrophages produced more NO because they express less arginase, and are thus able to have a cytotoxic effect on trypanosomes locally in the peritoneal cavity.

We report in this study that during experimental trypanosomiasis both arginase I and II mRNAs are up-regulated in macrophages from infected mice. Similar to our observations, both arginase isoforms are induced in murine peritoneal macrophages stimulated by serum (19) or by LPS (20), as well as in the RAW 264.7 macrophage cell line activated by 8-bromo-cAMP (21). The question raised by our data is how arginase is induced in macrophages during experimental trypanosomiasis? We demonstrate that arginase I, and to a lower extent arginase II, are directly induced in macrophage by trypanosomes, suggesting that arginase activation could represent an ability of the pathogen to escape the host innate immune system. Arginase activation could occur directly by the stimulation of macrophages by parasites or indirectly by the effect of macrophage-derived factors induce by trypanosomes. Thus, the identification of trypanosome factor(s) that stimulate arginase expression and of the associated cellular mechanisms is underway in our laboratory. Two findings support the contention that endotoxin contamination was not responsible for the increase of trypanosome-induced macrophage arginase activity: 1) polymyxin B treatment of macrophages/T. brucei brucei cocultures did not result in an alteration of the enhancement of arginase activity; and 2) macrophage iNOS was not induced by trypanosomes, whereas endotoxins are known to stimulate iNOS expression in macrophages. Nonetheless, in trypanosome-infected mice, the production of IL-4, IL-10, and TGF-β is increased during trypanosomiasis infection, principally in BALB/c mice (16, 22). Because those three cytokines are potent activators of arginase in murine macrophages (3, 19, 23), they could also play a major role in arginase induction in vivo. In support of this hypothesis, it was established that BALB/c macrophages activated by IL-4, IL-10, or TGF-β expressed more arginase activity than macrophages from C57BL/6 mice (24). In addition, the treatment with an anti-IL-4 of mice infected with *Leishmania* enhances NO production and the regression of lesions (25). Beside the classical Th1/Th2 response, a striking difference in macrophages from resistant and susceptible mice has been pointed out: M-2 macrophages from BALB/c mice have a propensity to express arginase in comparison to M-1 macrophages from C57BL/6 mice (26). Therefore, numerous activation pathways, including the trypanosome factors or the host cytokine response, might be responsible for arginase I and arginase II induction in vivo during experimental trypanosomiasis. The identification of the missing link between trypanosome infection and arginase induction could provide new insights to understand the pathophysiology of the disease and to develop new strategies to fight parasitic infections.

In bacterial or parasitic acute infections, NO is described as a resistance factor elaborated by the host to fight pathogens (7, 27, 28). Therefore, macrophage arginase can be considered as an escape mechanism induced by pathogens interacting with macrophages. Similarly, the arginase possessed by microbial pathogens can be a survival factor by inhibiting NO production (26) or by increasing polyamine synthesis (29). However, arginase can also be considered as a protective factor for the host by controlling NO production, which can limit tissue damage or immunosuppression normally caused by high levels of NO (30, 31). This paradoxical effect of the iNOS/arginase balance was evidenced in the murine model of *Chlamydia* infections (32). In a long-term infection of mice with *Chlamydia psittaci*, C57BL/6 mice expressed less arginase II, synthesized more NO, and exhibited more severe lung inflammation compared with BALB/c; conversely, in an acute model of the disease, C57BL/6 mice were healthier than BALB/c mice. Similarly, in other chronic model of African trypanosomiasis, namely mice infection with the line GUTat 7.2 of *T. brucei* (31) or with *T. brucei rhodesiense* (33), it has been shown that NO is not essential for the control of the infection in vivo, whereas from our experiments, it appears that NO production is beneficial for the host in the early stage of infection, at the local level. Thus, we hypothesized that NO possesses opposing effect according to the chronology of the infection, as previously reported for other pathologies (34–36). In addition, it can also be envisaged that the protective role of NO against pathogens is tissue specific.

Besides its role as an inhibitor of NO production, arginase also favors polyamine synthesis by producing L-ornithine, which is metabolized by ornithine decarboxylase (10). Polyamines are essential for the development of trypanosomes because they facilitate synthesis of DNA and trypanothione (37). We can speculate that susceptible BALB/c mice produce more L-ornithine and more polyamines than resistant C57BL/6 mice, according to their respective levels of arginase activity. Therefore, by providing more polyamines, BALB/c mice might also favor trypanosome multiplication.

In conclusion, arginase pathway might represent a factor of resistance and susceptibility in parasitic infection. Arginase inhibitors might have important therapeutic applications by locally restoring NO production and decreasing polyamine synthesis, and further investigations are warranted to determine the effect of arginase at the systemic level during experimental trypanosomiasis and in other infections.

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