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Immunoprophylaxis in Guinea Pigs with *Mycobacterium indicus pranii* (Mw) in Combination with Standard Chemotherapy of *M. tuberculosis* Infection Improves Lung Pathology

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Abstract

Tuberculosis continues to be a major global health problem. BCG (Bacillus Calmette–Guerin) has been used as a vaccine to control tuberculosis, however, the protective value of the vaccine has been reported be highly variable across different populations. During the last three decades, several workers have investigated the potential of protective efficacy of *Mycobacterium indicus pranii* (MIP or Mw) against mycobacterial infections (leprosy and tuberculosis) which has been used as an immunoprophylactic tool and also as an adjunct to chemotherapy. The present study, reports the beneficial effect of prior immunization with MIP in terms of improvement in histopathological findings and reduction of bacterial burden, as augmenter to the effects of chemotherapy in experimental tuberculosis.

Keywords: *Mycobacterium tuberculosis*; Immunoprophylaxis; *Mycobacterium indicus pranii* (MIP or Mw); Histopathology

Introduction

BCG vaccine is being used in several countries since 1921 to control TB infection. BCG has been found to have protective role in childhood extra-pulmonary and progressive TB, but in adults it shows limited effect, thus alternatives are needed [1,2]. Several alternatives have been studied with significant reduction of *Mycobacterium tuberculosis* bacterial burden [3-6]. For the last 25 years several workers have investigated the potential protective/therapeutic efficacy of *Mycobacterium indicus pranii* (known as Mw) against mycobacterial infections as adjunct to chemotherapy in animal models [7-10]. Mw was also found to show protective as well as immunotherapeutic effect in human population against tuberculosis [11] and leprosy [12-18].

It has been reported that Mw not only reduces bacterial growth but also contributes to clearance of granulomatous lesions in the lung tissue, when used as additional immunotherapy during the course of chemotherapy following infection [19,10,9] Mw was also found to be effective when used as a immunoprophylactic tool prior to infection and subsequent standard chemotherapy by bringing a reduction in bacterial counts in guinea pig animal model [20]. These studies have attributed the significant reduction in bacterial burden to boosted immunity via cytokines and chemokines expression following Mw vaccination/immunization in animal models such as guinea pig. In other studies, it has been reported, that the ID93/GLA-SE vaccine has effective adjuvant effect and the DcysH vaccine provides protection against tuberculosis infection and improves histopathology [21,22]. In recent times, it has been also reported that Mw immunization used by different routes (by the aerosol or parenteral route) reduces bacterial burden with low dose approximately 50 bacilli/lung in experimental model [10,19,9]. Further, we have reported that Mw reduces bacterial burden from lung tissue with standard chemotherapy when used as a immune prophylactic tool in guinea pig animal model [20].

As the earlier studies [9,10] provided insufficient information on immunoprophylactic effect of Mw with respect to histopathological evolution of disease in guinea pig lung tissue following *M. tuberculosis* (H37 Rv) aerosol infection, this study was designed and mainly focused on lung tissue to explore the histopathological outcomes subsequent to prior immunization with heat killed Mw (MIP) followed by infection with high dose of *M. tuberculosis* (H37Rv) aerosol and standard chemotherapy at different stages of disease progression.

Materials and Methods

M. tuberculosis H37Rv Strain and Heat killed (MIP) Mw

For this study the *M. tuberculosis* H37Rv (TMC-102) was obtained from Mycobacterial Repository of our Institute. Middlebrook 7H9 medium was used to culture H37Rv and stocks of this growth were stored at -80°C as previously reported and used for aerosol infection [23]. Heat killed Mw was provided by Cadila Pharmaceuticals Ltd (India).

Experimental animals

Healthy out-bred female guinea pigs (Hartley strain) were obtained from Chaudhary Charan Singh Haryana Agriculture University, Hisar, Haryana, India. All these animals were housed and maintained under standard environment. All the animals were housed in micro-isolator caging, number of guinea pigs per cage was 3 in a biosafety level 3 facility with a 12 h–12 h light-dark cycle, and temperature range 21-24°C; were fed with pellets of feed supplemented with Bengal gram and green; vitamin C supplementation in water at animal experimentation facility (NJIL&OMD, Agra, India.) and used for experimentation as per institutional guidelines. A modified Karnofsky animal health status scoring method was used including weight, food intake habits, activity etc., [24]. Health monitoring for activity and food intake was done at the time of administering chemotherapy and all parameters including weight were recorded at 4,8,12 weeks by the study team. This study was permitted by the Animal Ethical Committee (AEC) of NJIL& OMD, Agra, India.

Grouping of animals

In this study, five groups of animals were studied in two batches of experiments and each group of both batches included three animals at each and every time point. All the experiments in this study were executed in duplicate. The groups of animals were as follows: (1) Normal Healthy (NH) guinea pigs included as a control group, no infection was given in this group. (2) Infected (Rv) Group: The animals from this set were infected with *M. tuberculosis* (H37Rv) and no treatment was given. (3) Immunoprophylaxis (RvMw) group: In this group animals were immunized twice (dosage: 5×10^8 bacilli in 0.1 mL volume) subcutaneously with heat killed Mw with one month of interval and after one month of second dose of Mw, all the animals were infected by aerosol route (described below) as reported earlier [20]. (4) Chemotherapy (RvCh) group: Standard chemotherapy was started orally after twenty eight days of infection. (5) Immunoprophylaxis+infection+chemotherapy (RvChMw) group: In this group animals were immunized twice with heat killed Mw prior to infection as described above and standard chemotherapy was started as in group 4.

Aerosol infection

Animals in all the study groups were infected (except controls) by aerosol route on same day with high dose (4.37×10^7 bacilli/mL) of *M. tuberculosis* (H37Rv) via advanced inhalation exposure system (Glas-Col, USA) standardized to deliver 250–300 bacteria into the lungs. High dose of bacilli was given to evaluate the protecting efficacy of used vaccine candidate against infection as previously advocated and described [25,20].

Chemotherapy to animals

Animals were treated after 28 days of infection with standard chemotherapy of first line drugs (RIF 10 mg/kg, ETH 15 mg/kg, INH 5 mg/kg, PZA 25 mg/kg per body weight) in solution form equipped as per average~400 g body weight of animals. All doses were prepared in 50% sucrose to increase palatability. Animals were orally administered each dose (0.5 mL) in the back of the mouth prior to feeding, for five days/week till completion of the study period.

CFU determination

A group of experimental animals (n=3) were sacrificed after 24 hrs post infection to confirm the delivered amount of bacteria. To evaluate the effect of different therapeutic interventions on the bacterial burden and the lung pathology, total six animals were sacrificed from each group at each different time points (4, 8 and 12th week) post challenge. Lungs removed from these animals were homogenized in normal saline and plating was performed with 100 µL from different dilutions of homogenate (Neat, 1:10, 1:100, 1:1000) on to Middlebrook 7H11 agar plate for obtaining bacterial growth. Growth of Mycobacterial colonies (CFUs) were enumerated after incubation for 3-4 weeks at 37°C.

Histopathology

The dissected lungs of guinea pig were fixed in 10% BF (buffered formalin) for twenty four hours. Fixed tissue was placed in cassettes which were then transferred to a tissue processor. Within the tissue processor the specimens underwent dehydration in an ascending series of alcohols, cleared in xylene and subsequently embedded in paraffin wax. The tissue processor was programmed such that the specimens spent the desired length of time in each chamber. The above processed tissue specimens were cooled and transferred to a plastic mould with the tissue surface resting on the base of the mould for making blocks. The blocks thus obtained were fixed on to a wooden base (block) and 5 µm thick sections were cut with rotatory microtome (Lieca, Germany) which were then transferred on to coated glass slides dry fixed using hot plate at temperature <500°C for 30 min. The lung sections were then stained with H&E, and Fite's acid fast stain. Three lung sections from each animal of each group were evaluated histopathologically.

Pathology of infected lung tissue lesions was evaluated with a histological grading system and the slides scorings were done in blinded fashion [26]. The granulomatous lesions was graded on the basis of following parameters: percentage infiltration fraction in the section assayed; number of lesions; lesion defined as diffuse/granulomatous; diameter of granuloma; presence/absence of necrosis; cell populations; AFB positivity/negativity. The microscopic observation of stained slides was carried out using Olympus B×51 (Olympus, Japan) microscope.

Statistical analysis

Using graphpad prism 5 software, two ways Analysis of Variance (ANOVA) was done to compare the data of CFU counts obtained from different experimental sets of animals with "Bonferroni posttests", multiple comparisons between groups was performed. Values of $P<0.05$ were considered as statistically significant.

Results

Effect of different therapeutic interventions on bacterial burden

The bacterial load was compared among different therapeutic groups (Infected but untreated -Rv, Immunoprophylaxis plus infection -RvMw and combination of Immunoprophylaxis+infection and Chemotherapy -RvChMw) following infection of virulent strain *M. tuberculosis* (H37Rv). Bacterial load 4.37×10^7 bacilli/ml was delivered by aerosol chamber. One day post exposure each animal from each group sacrificed and approximately >240 bacilli (per g lung tissue) was

recorded at day one. Animals were treated with standard chemotherapy orally after 4th week of infection. In infected (Rv) group the bacterial load was observed highest at 4th week of infection in comparison of other time points (8th and 12th week). In Immunoprophylaxis (RvMw) group where all the animals received two doses of heat killed vaccine, no significant ($p>0.05$) bacterial reduction was observed at any time point as compared to the Rv group. In chemotherapy (RvCh) group, the standard chemotherapy reduced bacterial burden by about 0.7 logs and 2.4 logs at 8th and 12th week time point of infection, respectively, as compared to infected (Rv) and immunoprophylaxis (RvMw) group, while no significant ($p>0.05$) reduction were observed at the 4th week of infection. Combination of Immunoprophylaxis+chemotherapy (RvChMw) reduced bacterial load by about 1.64 logs, while 1.0 log of bacterial load reduced in chemotherapy (RvCh) group as compared to the infected (Rv) and immunoprophylaxis (RvMw) group, at 8th week time point of infection which is significant ($p<0.05$). Moreover, combination of Immunoprophylaxis+chemotherapy (RvChMw) group reduced CFUs by about 4.0, 4.1 and 1.6 logs, significantly ($p<0.05$) as compared to infected group (Rv), immunoprophylaxis (RvMw) and chemotherapy (RvCh) group, respectively, at 12th week time point of infection (Figure 1).

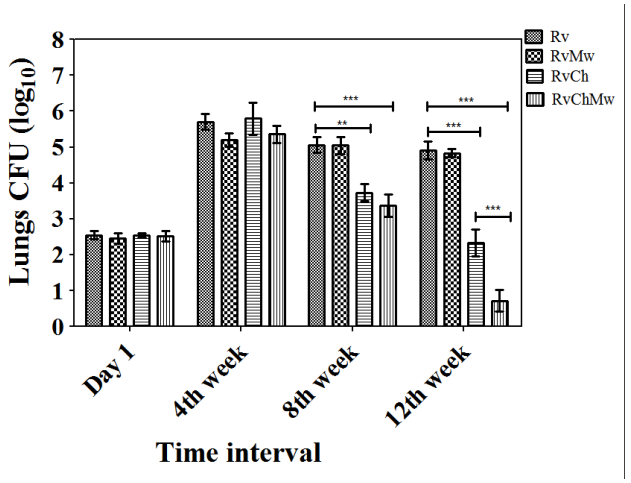

Figure 1: Graph showing the CFU counts at different time points in lung tissue of guinea pig.
Different treatment groups compared with infected (Rv) group - a significant decrease in CFU count in treated (standard chemotherapy) groups (RvCh and RvChMw) at 8th and 12th week. immunoprophylaxis+chemotherapy (RvChMw) groups showed significant ($P<0.0001$) CFUs reduction as compared to other groups. (Marked ***). No significant difference was observed in immunoprophylaxis (RvMw) versus infected (Rv) group at any time point post infection ($P>0.05$). Day one CFUs indicate the initial bacterial load in the lungs and the log value at 4th week indicates bacterial load when chemotherapy was started. (n=6, log10 CFU mean ± SD of *M. tuberculosis* H37Rv).

Localisation (clearance) of *M. tuberculosis* in lung tissues (derived from experimentally infected guinea pigs)

Fite-Faraco staining showed the localisation of *M. tuberculosis* (AFB positivity) in infected tissue of all the sections of lungs of animals from different therapeutic groups (Rv, RvMw, RvCh, RvChMw) except control (NH) group. M. tuberculosis could be seen in infected (Rv), group including immunoprophylaxis (RvMw), chemotherapy (RvCh), and immunoprophylaxis plus chemotherapy (RvChMw) group of animals at 4th and 8th week time point of post infection, while in treated group (RvChMw), *M. tuberculosis* AFB could not be seen in the tissue sections at 12th week time point of post infection.

Histopathological analysis of guinea pig lung tissue treated with different therapeutic interventions

At 4th week: Multiple and the confluent granulomatous lesions (mean diameter =700±50 µm) with central necrosis, mean of infiltration fraction up to 65 ± 5%, and lesions (mean number=7.3 ±2) were observed, at 4th week post infection in infected (Rv) group. In immunoprophylaxis (RvMw) group (prior immunized with Mw then infected with H37Rv), granulomatous lesions (up to approximately 750 ±100 µm in diameter) composed of tight accumulation of lymphocytes and macrophages cells with necrosis, mean of infiltration fraction of up to 60 ± 10 %, and lesions (mean number =6.5 ± 1.5) could be seen. In chemotherapy (RvCh) group, the lesions were seen in the form of peri-bronchial granuloma close to blood vessel, up to approximately 600 ± 50 µm in diameter, mean infiltration fraction 65.66 ± 12.5% and number of lesions (mean=6.3 ± 2) were observed. In immunoprophylaxis+chemotherapy (RvChMw) group, peri-bronchiolar granuloma and mixed granulomas were seen up to approximately 640 ± 40 µm in diameter, mean of infiltration fraction 60 ±10% and number of lesions (mean=7 ± 1.5) with necrosis were observed, while no granulomatous lesions were observed in controls (NH) .

At 8th week: The confluent granulomas (mean=500 ± 100 and 650 ± 150 µm in diameter) with central necrosis, mean infiltration fraction up to 40 ± 10% and 45 ± 5% and number of lesions (mean=6 ± 2, and 4.5 ± 1.5) were observed in infected (Rv) and immunoprophylaxis (RvMw) group respectively, at 8th week post infection. The animal set of chemotherapy (RvCh) and combination of chemotherapy + immunoprophylaxis (RvChMw), had granulomas (mean=475 ± 50 and 250 ± 50 µm, respectively) in diameter, and mean of infiltration fraction 25 ± 5 % and 10 ± 2.5 % respectively, whereas when number of lesions (mean number=9 ± 1.5 and 4 ± 2, respectively) were recorded at 8th week of infection, while no granulomatous lesions were observed in controls (Table 1).

At 12th week: The granulomatous lesions were considered by accumulation of macrophages along with bounded epithelioid and lymphoid cells, which is present in not only infected group but all the treated groups also. Confluent granulomas (mean=1000 ± 100, and 812.5 ± 50 µm in diameter) with central necrosis, mean infiltration fraction up to 70 ± 10 % and 57.5 ± 5 % and number of lesions (mean=8.5 ± 2 and 8 ± 2) were observed in infected (Rv) and immunoprophylaxis (RvMw) group, respectively, at 12th week post infection. In the chemotherapeutic groups, animals were treated with standard chemotherapy (described in section 4.7.4) with and without Mw showed small scattered granulomas, mean diameters 200 ± 75 µm and 100 ± 50 µm, mean infiltration fractions 22.5 ± 7.5% and 6 ± 2.5% and number of lesions (mean=9 ± 3 and 2.5 ± 1.5) were observed in chemotherapy set (RvCh) and combination of chemotherapy with immunoprophylaxis (RvChMw) group respectively. RvChMw animal set showing less infiltration (6 ± 2.5%) and lesion number (2.5 ± 1.5) as compared to other groups at 12th week time point post infection, while no granulomatous lesions were observed in controls (Table 2). At 12

week time point, a significant weight gain was observed in RvChMw group as compared to infected (Rv) and immunoprophylaxis (RvMw) groups (Table 3).

Parameters	Different therapeutic groups of animals			
	Rv	RvMw	RvCh	RvChMw
IF(% mean ± SD)	40 ± 10	45 ± 5 (p>0.05)	25 ± 5 (p<0.05)	10 ± 2.5 (p<0.01)
Lesions (mean ± SD)	6 ± 2	4.5 ± 1.5 (p>0.05)	9 ± 1.5 (p>0.05)	4 ± 2 (p<0.05)
Granuloma dimension (μ) ± SD	500 ± 100	650 ± 150 (p>0.05)	475 ± 50 (p>0.05)	250 ± 50 (p<0.05)
Necrosis	++	+	+/-	-
Cell population	MØ,L	MØ,L	MØ,L	MØ,L
AFB	+	-	-	-
IF: Infiltration; MØ: Macrophages; EC: Epithelioid cells; L: Lymphocytes; GC: Giant cells; + : present; –: absent				

Table 1: Differences among different groups on the basis of scoring parameters for histopathology at the 8th week post infection.

Parameters	Different therapeutic groups of animals			
	Rv	RvMw	RvCh	RvChMw
IF (% mean ± SD)	70 ± 10	57.5 ± 5.0 (p<0.05)	22.5 ± 7.5 (p<0.05)	6.0 ± 2.5 (p<0.01)
Lesions (mean ± SD)	8.5 ± 2.0	8.0 ± 2.0 (p>0.05)	9.0 ± 3.0 (p>0.05)	2.5 ± 1.5 (p<0.01)
Granuloma dimension (μ) ± SD	1000 ± 100	812.5 ± 50 (p<0.05)	200 ± 75 (p<0.01)	100 ± 50 (p<0.01)
Necrosis	+	++	-	-
Cell population	MØ,L	MØ,L	MØ,L	MØ,L
AFB	+	-	-	-
MØ: Macrophages; EC : Epithelioid cells; L : Lymphocytes; GC : Giant cells; + : present; -: Absent, IF: Infiltration				

Table 2: Differences among different groups on the basis of scoring parameters for histopathology at 12th week post infection.

Discussion

The guinea pig is considered as a standard animal model for tuberculosis, having specified similarities in the immuno-pathological response to pulmonary TB infection; therefore, it has been extensively used as an important tool in the research and assessment of new vaccines. *Mycobacterium indicus pranii* (Mw), is a well-established immuno-modulator which provides protection against slow growing mycobacterial infections in humans as well as in animals [14,9,8,16]. We performed a comprehensive assessment of host response to drug treatment in the guinea pig model with or without Mw (*Mycobacterium indicus prranii*) used as a immunoprophylactic tool, where the guinea pig develops primary lesions with necrosis alike to those that develop in naturally occurring infection in humans. Distinct from conventional mouse model of tuberculosis, immunologically naïve guinea pig develops a variety of lesions, including primary lesions with necrosis that are affected by drug treatment in combination with immunoprophylaxis of Mw.

Time point	Average body weight in gm (± SD) of different group of Animals (n=6)				
	NH	Rv###	RvMw##	RvCh	RvChMw#
4 week	480 ± 40	416 ± 36 (p>0.05)	486 ± 36 (p<0.05)	490 ± 50 (p<0.05)	423 ± 23 (p<0.05)
8 week	530 ± 30	470 ± 20 (p>0.05)	516 ± 16 (p>0.05)	532 ± 16 (p>0.05)	500 ± 33 (p>0.05)
12 week	600 ± 50	460 ± 20 (p<0.05)	440 ± 40 (p>0.05)	550 ± 50 (p<0.05)	596 ± 36 (p<0.05)
NH-Normal Healthy, Rv- Infection only, RvMw- immunoprophylaxis, RvCh-Chemotherapy, RvChMw- combination of immunoprophylaxis + chemotherapy.					
# One animal dead, ## Two animal dead, ### Three animals dead during the study.					
The average body weight of guinea pig showing the significant improvement in combination of immunoprophylaxis +chemotherapy (RvChMw) group as compared to infected group Rv and Immunoprophylaxis (RvMw) at 12 week.					

Table 3: The average body weight in gram (± SD) of guinea pig during the course of standard chemotherapy after four week of infection in different groups of animals.

The present study quantified the response to drug therapy with or without prior immunization with Mw using gross necropsy and histological evaluation at the time of necropsy. In the present study we also examined bacterial burden in granulomatous lung tissues of guinea pig which were treated with drugs in *M. tuberculosis* infected animals which has prior immunoprophylaxis with Mw.

It has been reported that Mw is effective in accelerating bacterial clearance, and increases immune response in leprosy infection [17,12,15,7] as well as improved histological changes in leprosy patients in combination with chemotherapy. The employment of Mw as immunotherapy in combination with MDT improves reversal reaction in multibacillary patients [12,15]. Another study in different phases reported the beneficial role of Mw as immunoprophylactic in leprosy contacts as well as booster vaccination to sustain protection [27]. In a 10-13 years survey, Mw used as immunoprophylactic tool against active pulmonary tuberculosis infection, reduced the incidence of tuberculosis [14]. Mw as immunotherapeutic agent has been observed to reduce the period of sputum conversion with standard chemotherapy [11].

Recently the role of Mw as a immunotherapeutic and immunoprophylactic agents in animal model against tuberculosis infection has been addressed [10,20]. The usage of Mw as therapeutic vaccine against pulmonary tuberculosis was observed to provide protection from infection in experimental animals [9,19]. Another reports by Guleria et al., and Singh et al., showed that Mw induced protection via CD4 and CD8 T lymphocytes against tuberculosis infection in mice model [8,23].

It was previously reported that, prior immunoprophylaxis (with two doses) and subsequent chemotherapy after infection was effective in inhibition of growth of MTB, (no CFU was observed from 6th week till 10th week in RvChMw group), in comparison with treatment alone, when the chemotherapy was started second day of infection [20]. In the current study, chemotherapy was given after 28 day of infection to

evaluate the outcome of two doses of Mw prior immunization on the course of disease with standard chemotherapy following high dose (Approximately 50 bacilli/ lung used by Gupta et al [10], 10 CFU by McMurray et al [28], 100-200 CFU by Lim et al [29], 200 bacilli/lung used by Faujdar et al., [9], 100 CFU used by Williams et al [25] of M. tuberculosis (H37Rv) aerosol infection.

In the present study the combination of prior immunoprophylaxis with Mw and subsequent chemotherapy (RvChMw) has been found to reduce bacterial load significantly ($p<0.05$) (Figure 1) from the lung and improve lung pathology (Table 1 and 2) compared with other groups (RvMw and RvCh); this group (RvChMw) also showed better weight gain than the infected (Rv) and immunoprophylaxis (RvMw) groups (Table 3). The AFB positivity in all the groups (Rv, RvMw, RvCh and RvChMw) at 4th week of infection except control (NH), shows equal distribution of bacterial burden in the lung. AFB could not be seen in the lungs at 8th and 12th week after infection in treated groups with chemotherapy+immunoprophylaxis (RvChMw) while AFB detected at 8th week post infection in chemotherapy (RvCh) group. There was reduction in the size of granulomas and number of lesions in animals who had combination of prior immunoprophylaxis and subsequent chemotherapy (RvChMw) compared to other groups studied (Rv, RvMw and RvCh) (Tables 1 and 2). The granulomatous lesions and accumulated macrophages and lymphocytes resolved in RvChMw group more rapidly. In this study, the improvement of histopathological findings and better reduction in bacterial load (at 8th and 12th weeks), indicates that the prior immunization with Mw was found to be effective with standard chemotherapy and shows better recovery in the lung. Thus the study suggests prior vaccination to improve lung tissue pathology by reducing granulomas and bacterial burden.

Beneficial immunoprophylactic role of Mw in animals [8,19,20] and also humans [14] has been reported previously. This protection is, however, partial. The data reported in this study show that prior immunization with Mw also helps in improving the lung pathology of animals when these are treated with chemotherapy. This also adds to the findings of earlier report on the effect of this intervention on bacterial load [20]. This is an important finding and if extrapolated to humans, persons vaccinated with Mw when getting the disease may have better response to treatment. This will have to be confirmed by well-designed studies in humans.

Conclusion

To conclude, MIP has not been found to provide partial protection alone against the high *M. tuberculosis* infection, but even when infection progressed, the animals with prior immunization with MIP (Mw) treated with chemotherapy had better bacteriological and histopathological outcome. The combination of prior immunization with MIP and chemotherapy achieved greater success in terms of reduction in bacterial burden, the size of granulomas and lesion numbers and thus improved lung tissue pathology induced by the disease.

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Conflict of interest statement

None.

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