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Patterns of cytokine response in bcg vaccinated and BCG non-vaccinated children in the age group of 5-8 years in Chennai

S.Kalpana, S.Prabha*,V.Kumarasamy* and Manjula Datta

Department of Epidemiology, The Tamil Nadu Dr.M.G.R.Medical University, Guindy, Chennai, Tamil Nadu India
**Tuberculosis Research Centre, Chennai.*

ABSTRACT

This study was conducted to understand the patterns of cytokine response in response to stimulation of lymphocytes with different mycobacterial antigens in BCG vaccinated and non-vaccinated children in the age group of 5-8yrs in Chennai. BCG vaccinated and non vaccinated children Peripheral Blood Mononuclear Cells were isolated and cultured with different mycobacterial antigens in RPMI medium and then after 48 hrs the supernatant was collected and the in vitro levels of Il-2, IFN-γ, TNF-alpha, IL-4, IL-10 and TGF-beta were estimated in BCG vaccinated and non-vaccinated Mantoux positive and Mantoux negative children using sandwich Enzyme Linked Immunosorbent Assay (ELISA). Different antigens elicit different cytokine response irrespective of the group; however the response in a Mantoux positive group which has received BCG vaccination appears to be much lower than the BCG non-vaccinated Mantoux positive group. While no definite distinguishing pattern could be made out, the findings strongly suggest that the four groups are different from each other in terms of their ability to secrete cytokines in unstimulated cells as well as in response to external stimulation.

Keywords: BCG vaccination, Cytokines, immune response, Mantoux, PBMC.

INTRODUCTION

The paradox of BCG is that it is simultaneously the most widely used and the most controversial of all vaccines today[1]. According to Expanded Programme of Immunization statistics, 50% of all children born in the world in 1985 received BCG vaccination . The present policy of the WHO is to vaccinate newborns with BCG as an integral part of the EPI since all available information indicates high protection by BCG against childhood tuberculosis particularly severe forms like meningitis and military tuberculosis[2] even in areas where there is not much evidence for protection in the adult population [3]. Only the USA and the Netherlands have not used BCG on a national scale [4]. Although BCG Vaccination has been given for over a century, the mechanism by which it induces protective immunity is not known. We do not even know what the markers of immunity to Tuberculosis are. The immunological changes produced by BCG in the human body, strangely, has not been a topic of study; consequently, very little is known about the immune parameters such as the cytokines in vaccinated individuals. BCG being a mycobacterium should normally induce a reaction to tuberculin which can be expected to be lifelong.

But in a large proportion of individuals it wanes. We do not know what this means. We also do not know what this implies for protection from Tuberculosis. Thus a large grey area regarding the protective efficacy needs to be studied: Therefore, a lot more work needs to be done in order to understand the alterations in the immune state following BCG vaccination. However no attempt has been made to determine the profile of cytokines in vaccinated and non-vaccinated healthy children. It is necessary to study the antigen recognition pattern and the cytokine profile of vaccinated and non-vaccinated, tuberculin test positive and tuberculin test negative individuals in order to understand the immune responses to BCG vaccination. Such a study will also provide the basis for correlating results of *in vitro* assay with the tuberculin responses following BCG vaccination.

MATERIALS AND METHODS

This study was carried out among school children in the rural area of Chingleput district in Tamil Nadu. The Cytokine Assays were carried out in the Tuberculosis Research Centre. This study was approved by the ethical committee of Tamil Nadu Dr. M.G. R medical University.

STUDY SUBJECTS

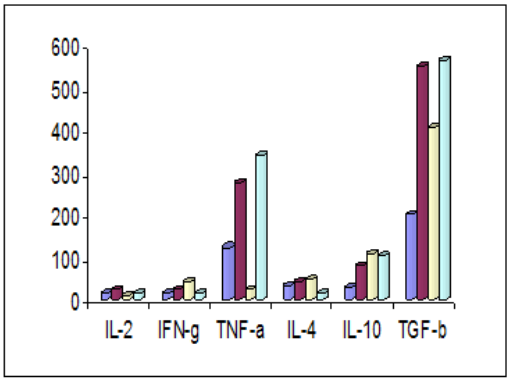
The total subjects recruited for the study were one hundred and five, of which 61 were males and 44 were females. Children who belonged to an age group of 5-10 years were selected from the Government middle school, Nandivaram, Guduvanchery, Chengalpet, as it was felt that the immune responses would have been established in this age group as compared to younger children, but the NTM infection would be insignificant. Study subjects were divided into four groups based on their BCG status and the positivity for Mantoux test. After enrollment physical examination was done by pediatrician and chest x ray was taken to findout any lesions and then Mantoux was given to all the children. After 72hrs Mantoux reading was taken and grouped in BCG vaccinated and non-vaccinated, Mantoux positive and negative. Then 20ml of blood collected and PBMC were separated and stimulated with different mycobacterial antigen(BCG,CF,PPD, and PHA). After 48hrs the supernatant from the cell culture was separated and stored for cytokine estimation. IFN-gamma, TNF-alpha,IL-2,IL-4,TGF-beta and IL-10 were estimated using ELISA method.(BD kits).

The Data was entered into SPSS data base. The statistical analysis was performed using SPSS software, version 10.0. As the data were not normally distributed, non-parametric tests were carried out to find the significance of the observed differences. Comparisons of data were analyzed by Mann-Whitney, group analysis was done using Wilcoxon rank sum, Kruskal-Wallis tests.

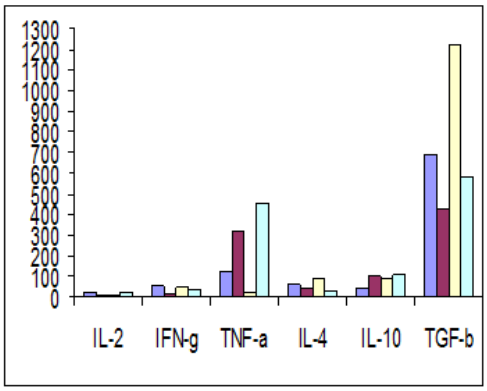
RESULTS

One hundred and five healthy school children were recruited in this study of which 26 were BCG vaccinated Mantoux positive (17 male, 9 female) 34 were BCG vaccinated Mantoux negative (19 male, 15 female) 22 were BCG non-vaccinated Mantoux positive (11 Male, 11 Female) and 23 were BCG negative and Mantoux negative children (14 Male, 9 Female). Positive reaction size ranged between 5 – 11 mm. A total of 250 children were screened to get this sample size. The age of the study subjects was between 5 – 10 years and the median age was 8 years. The socio economic status of their parents was generally poor: 77 % of them was daily wage workers, 10% of them were not working and rest of them were doing business or were employed. Of the total study subjects forty four children were born at home and rest of them were born in hospital. *Vaccination Status was not related to the place of delivery*. Of the total subjects, six were homeless and their family was migrating from a place to place. Sixty seven percent were from rural area and 33 % of them were from semi urban. Only one child had a contact history of tuberculosis. None of them had primary complex. Fifteen percent of them had Recurrent Respiratory Infection (RRI) and only one child had Acute Respiratory Infection (ARI). None of them had any chronic disease.

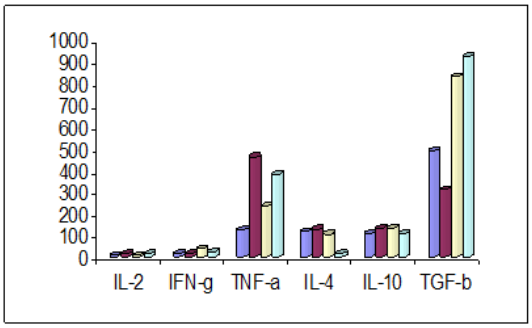
Graph 1: PATTERNS OF CYTOKINE IN PHA STIMULATION (µg/ml)



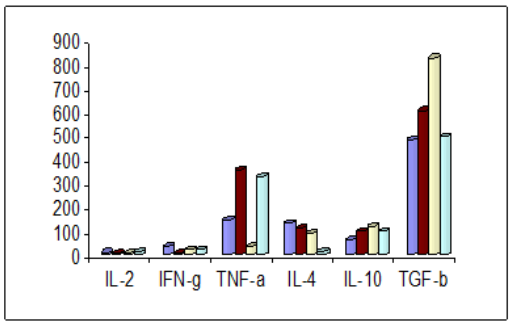
Graph 2: PATTERNS OF CYTOKINE FOR PPD STIMULATION (µg/ml)



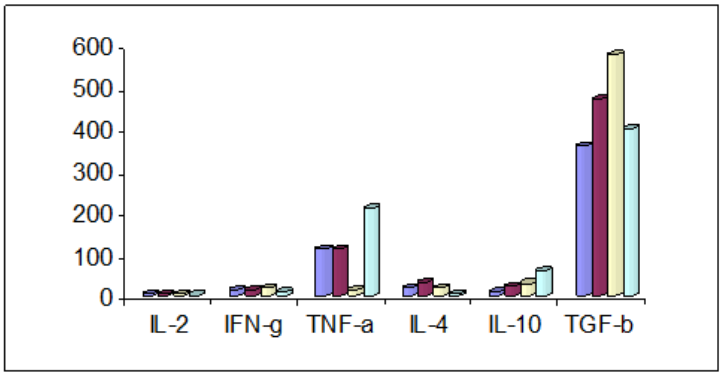
Graph -3 PATTERNS OF CYTOKINE IN CF STIMULATION (µg/ml)



Graph- 4 PATTERNS OF CYTOKINE FOR BCG STIMULATION (µg/ml)



Graph 5 : PATTERNS OF CYTOKINE IN CONTROL (µg/ml)



The difference in the cytokine response upon antigen stimulation with respect to control levels within each group and the comparison of differences in cytokine levels between the groups are explained in the following sections. The former is to find the pattern of cytokine response to antigens in toto and the latter is to find the impact of vaccination and Mantoux status on the in vitro cytokine response to mycobacterial antigens. In this study, the secretion of pro-

inflammatory cytokine IFN-γ was significantly higher for PPD stimulation in all the four groups than for other mycobacterial stimulation as expected (p-value 0.002, 0.023,0.004, 0.001,K.W- 33.499). The response to BCG stimulation was significantly raised in Mantoux positive group irrespective of vaccination. (P-value0.001 and 0.008) and for the CF stimulation significant raise was observed only in non-vaccinated Mantoux Positive group (p.value-0.001). IL10 is less in the vaccinated groups compared to the unvaccinated group irrespective of Mantoux status. (p-value, 0.001) (Table-2) IL-10 response to PPD appears to depend both on the group and on the antigen used for stimulation. (p-value 0.002,0.006,) It is lower in the Mantoux positive children as compared to Mantoux negative children. PPD elicited response was more among the Mantoux positive individual irrespective of vaccination. (p-value,0.001) Inversely BCG elicited response was more among vaccinated children irrespective of Mantoux status.(p-value,0.017) CF elicited response was not dependent on either vaccination or Mantoux status.(p-value, 0.400) Thus there was a pattern of response with respect to the antigen used for stimulation.

There was significant difference observed in the cytokine levels in the un-stimulated specimens (controls) in four groups irrespective of vaccination and Mantoux status. This was seen particularly in the TNFα, IL4, and the TGFβ; but not so much in the other cytokines.

The cytokine response to PHA, which was used as a positive control. The levels are higher, but the pattern is almost identical. Thus, the four groups appear to be dissimilar even with respect to background level of cytokines, and to stimulation with nonspecific antigens. The reason for this is unclear, and it may represent background stimulation.

How do the four groups behave in response to stimulation with CF, BCG and PPD? The following graphs shows the levels of cytokine secreted to different antigen stimulation among four groups. The secretion of pro-inflammatory cytokine IL-2, IFN-γ and TNF–α was not significantly higher for PPD stimulation than for other mycobacterial stimulation as expected. Thus the PPD may not be the specific protein which helps to induce the pro-inflammatory cytokines.

The response of the cytokines to PPD in the four groups is dissimilar. Except with respect to TGF-□, this is elevated in all the four groups. The maximum level is in the nonvaccinated Mantoux positive group followed by the nonvaccinated Mantoux negative group. Thus vaccination appears to suppress the secretion of TGF□IL10 is less in the vaccinated groups compared to the unvaccinated group irrespective of Mantoux status. TNF-□ is higher in the Mantoux negative groups compared to the Mantoux positive irrespective of vaccination.

The cytokine secretion response to stimulation with culture filtrate is different. The TGFβ levels are higher in the nonvaccinated group compared to the vaccinated group irrespective of Mantoux status. The TNF□ is raised in the Mantoux negative groups irrespective of vaccination.IL10 and IL4 is higher in the vaccinated as compared to the nonvaccinated group. IL2, and IFN□ appear to be similar in all the four groups.

The response to BCG is closer to that with PPD rather than with CF. TGF□ is raised in all the four groups, but the levels are lesser in the vaccinated groups. TNF□ is higher in the Mantoux negative group compared to the Mantoux positive. This difference is seen in both the vaccinated and unvaccinated groups. IL10 is less in the vaccinated groups compared to the unvaccinated groups, irrespective of Mantoux status. IL4 is more in the vaccinated groups compared to the nonvaccinated groups. IFN□ is higher in the vaccinated Mantoux positive group compared to the other groups. IL2 is similar in all the four groups.

The maximum levels of IL2 stimulation appeared to be in response to PPD in the Mantoux positive children and to BCG in the Mantoux negative children. Taking the Non Vaccinated Mantoux negative children as naïve, the secretion of IL-2 in the BCG vaccinated Mantoux negative is significantly less ($p<0.05$). Similarly, the levels in the Mantoux positive vaccinated children is lower than that in the Mantoux positive unvaccinated children, although the levels in the Mantoux positive children are much higher than the Mantoux negative group.

In naïve children, i.e. the nonvaccinated, Mantoux negative group, the TNF□ levels are lower than the other groups, irrespective of stimulation. Among the other three groups the response is maximum in the vaccinated Mantoux positive and least in the vaccinated Mantoux negative in response to PPD. However the levels of TNF-α in the vaccinated Mantoux negatives are higher than in the unvaccinated Mantoux negative unlike the other two. BCG

elicits the highest response in the unvaccinated Mantoux positives, and almost no response in the unvaccinated Mantoux Negatives. The response to CF is least in the Unvaccinated Mantoux negatives and almost similar in the other three groups.

The IL-4 stimulation response appears to depend on both the group and the antigen used for Stimulation. Response to PPD is higher in the non vaccinated children irrespective of Mantoux status. Response to BCG and culture filtrate is highest in the Mantoux negative group irrespective of vaccination status. Levels of IL-4 in the vaccinated Mantoux positives are higher than those in the vaccinated Mantoux negative individuals.

The response of TGF-□ seems to be different from all the other cytokines($p<0.05$). Response to stimulation with PPD is higher among the Mantoux positives in the vaccinated children and higher among Mantoux negatives in the unvaccinated children. Response to BCG is higher among Mantoux negatives, and does not appear to be influenced by vaccination status. Response to CF is higher among Mantoux Positives compared to Mantoux negatives among the vaccinated children, but higher in the Mantoux negatives among the unvaccinated children.

Patterns of Cytokine response among the four groups in response to different antigen stimulation is shown in the graphs 1-5. The pattern seems to be similar in all the groups irrespective of vaccination and Mantoux status. The similarities or differences between the groups appear to depend largely on the antigen used for stimulation.

The IL-2: IL-10: AND TGF-b: pattern of IL-2,IFN-□, IL-4 and IL-10 is similar in all the groups, showing low levels, and TNF-□ and TGF-□□showing similar pattern of raised levels in all the groups. The same pattern was observed even in control.

When we compare individual cytokines between the four groups categorized by the stimulating antigen, we find that the antigen strongly influence s the resulting cytokine levels. Taking the controls as the baseline levels, IL2 shows better response to PPD as compared to other antigens in the Mantoux positive groups. The levels of IL2 in the four groups in response to PPD are different from that in response to BCG or CF. There is a similar pattern in the IFN□ but not in the TNF□. However, for the Th2 cytokines IL-4:IL10: and TGF-□□ the patterns are the same between the four groups irrespective of the antigen used for stimulation.

Different antigens elicit different cytokine response irrespective of the group. PPD elicited response was more among the Mantoux positive individual irrespective of vaccination. Inversely BCG elicited response was more among vaccinated children irrespective of Mantoux status. CF elicited response was not dependent on either vaccination or Mantoux status. Thus there was a pattern of response with respect to the antigen used for stimulation.

However the response in a Mantoux positive group which has received BCG vaccination appears to be much lower than the BCG non-vaccinated Mantoux positive group. Thus BCG appears to suppress the innate immune response. Mainly this suppression was more in TH1 cytokine response except in TNF-□ which appear to be higher. Thus, TH2 appear to be maximum in BCG negative Mantoux negative group and it was suppressed in BCG negative Mantoux positive group. This pattern was not so evident with TGF□Considering IFN-□ across all types of stimulation the secretion was minimal in non vaccinated Mantoux negative group.

While no definite distinguishing pattern could be made out, the findings strongly suggest that the four groups are different from each other in terms of their ability to secrete cytokines in unstimulated cells as well as in response to external stimulation. Thus, the response of population to BCG appears to be different in those who are Mantoux negative compared to those who are mx positive; it is also different from the unvaccinated Mantoux negatives and the unvaccinated Mantoux positives. Hence, the Mantoux status after BCG may reflect the immune capacity of the individual and may not be the effect of simple waning. It may not even reflect the capacity of the individual to respond to different antigenic stimulation. What this means for the susceptibility of the individual to develop tuberculosis can only be answered by a large scale follow-up study. While the number of subjects in this study is sufficient to show significance between absolute differences, larger numbers are needed to elicit definite patterns in each of the four groups

DISCUSSION

This study has, for the first time looked at the four naturally occurring groups - viz, BCG vaccinated and non-vaccinated skin test positive and negative children. Also for the first time the pattern of six cytokines together been studied in the children. This profile in response to three antigens – CF, BCG and PPD has been studied. Such a comprehensive assay of cytokine generation, has, to our knowledge, has not been taken up so far. The present study addresses this issue and tests whether the *in vitro* cytokine response to mycobacterial antigens can serve as an indicator to delineate the vaccinated and non-vaccinated children, who are Mantoux positive or negative after the vaccination had been given at birth as per national policy under the routine programme conditions..

This study attempts to understand the profile of the six cytokines studied, and to see if demonstrable differences exist between groups. Most of the studies use PPD as antigen to determine the profile of immune responsiveness that distinguishes patients with TB from healthy tuberculin-positive controls and we have used both CF and BCG antigens also because we did not know which antigen seems to be more effective in eliciting a specific pattern of immune responsiveness to tuberculin-positive healthy individuals. The simultaneous use of the three antigens has shown up some interesting findings. The high levels of IL-2 secretion in BCG vaccinated Mantoux positive group for PPD and BCG stimulation showed that IL-2 is playing some role in altering the Mantoux reaction. Those who are Mantoux negative after BCG vaccination are significantly different from those who are Mantoux positive. This response is seen with BCG stimulation as well as with PPD stimulation. Hence these two groups of individuals would appear to be immunologically different with respect to their IL2 status. On Stimulation with PPD there is a significant difference only between the Mantoux negative and positive. There is no difference in the response between vaccinated Mantoux positive and unvaccinated Mantoux positive to PPD stimulation, but there is a difference to BCG stimulation. Therefore it is possible that a positive Mantoux some years after BCG vaccination is given would indicate fresh infection rather than immunity.

This pattern of difference is seen in all the cytokines studies. Hence, it may be reasonable to state that the Mantoux reaction should not be taken as a measure of immunity conferred by BCG. IFN-□ response is one of the well-taken correlates of protective immune response. There are previous reports on the increased IFN-□ response exhibited by the Mantoux positive individuals and its correlation with the DTH response [5]. Our observation does not support this as even the Mantoux negative children pictured a significant IFN-□ response to PPD stimulation *in vitro* pointing out the crucial importance of the demographic and environmental setting for the efficacy of BCG vaccination. Nonvaccinated Mantoux positive group alone showed CF-specific IFN-□ response for which the reason is unknown. The BCG-specific IFN-□ response by BCG- MX+ subjects indicates the prior exposure to environmental mycobacteria that shares cross-reactive antigens with *M. bovis*. The response of vaccinated Mantoux positive subjects can be attributed to the presence of memory T-cells generated due to the prior vaccination. But a study reported that the secretion of IL-2 and IFN-□ by the PBMC was significantly ($P<0.05$) high in the vaccinated Mantoux positive's than the unvaccinated children. And they observed that in majority of the BCG vaccinated children, the stimulation of specific TH1 cells seem to be considerably high, in short-term in vitro cultures.[6] In rural Malawian population they have shown that the IFN-□ response to PPD of *M. tuberculosis* correlates strongly with skin test responses to *M. tuberculosis* PPD RT23 [7]**.** This is similar to our findings.

The different response seen to the culture filtrate in our study may be because of the presence of these specific antigens in the culture filtrate. Hence it would be interesting to study the profile of response to a battery of antigens and then follow up the children for development of TB. Such a study is likely to be very costly, and is beyond the scope of this thesis.

The lack of IFN-□ response by BCG+ MX- may be due to absence of memory cells and indicates that the BCG vaccination is ineffective in these children. Because of the non-exposure to environmental mycobacteria and non-vaccination status, the BCG- MX-group did not show any BCG specific response. Higher levels of IFN-γ were detected in PPD stimulation in vaccinated and Mantoux positive groups showed that BCG vaccination converts the Mantoux reaction in children. Why this occurs only in some children is a point that should be studied further.

In our study, the TNF-□ levels increased significantly in all the stimulated conditions irrespective of vaccination and Mantoux Status. The reason for the insignificant production of TNF-□□to BCG stimulation by BCG-M+ group is

unknown. Also the *in vitro* pattern of TNF-□□response to mycobacterial antigens did not match with the IFN-γ secretion pattern. This supports the previous observation by Lihao Chen,**,** which suggest that the TNF-α does not control type 1 immune activation by directly regulating IFN-γ production[8]. In contrast, EP Moura, study showed that the tuberculosis patients had significantly raised TNF-alpha production and they suggest that this cytokine may be involved in the destruction of pulmonary tissues.[9]

The IL-10 secretion was significantly higher in all the stimulated conditions implying that the response of this cytokine is mycobacterial antigen-specific and is independent of the vaccination status and exposure to environmental mycobacteria. Notably, the IL-10 levels were high for the CF stimulated conditions in all the groups. The absence of difference in IL-10 levels in vaccinated and non-vaccinated children indicates that prior BCG vaccination does not alter IL-10 production. This is in accordance with the previous report by Das *et al* in pre and post vaccinated adults. This is supported by the present observation of increased TNF-□ and IL-10 levels in the culture supernatants. [10]

The IL-4 response was significantly higher in all the stimulated conditions in all the children. This implies that the production of this cytokine is antigen-specific and is indifferent to vaccination and Mantoux status. There is no apparent variation pattern in *in vitro* IL-4 secretion among different groups for mycobacterial antigens. If this IL-4 component to the response evoked by BCG is detrimental, then one would expect the efficacy of BCG to be variable, particularly in areas close to the equator where the Th2 response to M. tuberculosis is likely to be particularly high. Similarly, repeat BCG vaccination might be hazardous in the same environments because it might boost the IL-4, rather than the IFN- □□component. In this study, we could not find any significant response in the IL-4 production irrespective of vaccination and Mantoux status.

In the present study, there was no regular pattern of TGF-β response to the mycobacterial antigens *in vitro* within the groups and the variation pattern between the groups, thus obscuring the conclusions to be drawn. The increased TGF-β was reported in patients with pulmonary tuberculosis and tuberculous pleuritis [11]. In this study, they found increased TGF-□ levels even in healthy children and this supports the present report that the TGF-β enhances IL-10 production.

CONCLUSION

Prior vaccination with BCG did not influence the *in vitro* cytokine response in pediatric population. Thus this study has shown up significant differences in the cytokine levels in the groups, and differences in the capacity of the lymphocytes from children in the four groups to respond to stimulation. The response seems to depend on the antigen used for stimulation, rather than on the group. Thus No definite pattern could be demonstrated for each group. The implications of these findings are significant. Vaccination does not alter the cytokine response. But the pattern seems to depend on the antigen used for stimulation. It may be possible to identify a pattern; but further studies and larger studies are needed to define the implications for prediction of clinical course of the infection or to define immunity in trials of new vaccines. Definitely, a pattern seems to be of more value than a single cytokine.

Limitations of the Study

The cytokine pattern has been studied only in PBMC. Children have not been actively followed up. But they were asked to report to the health facility in case of any illness over a period of 6 months to one year. None had reported with any illness. This could be because we had actively screened out only healthy children, or because they preferred other sources of health care. Only Crude culture filtrate antigen had been used due to the non-availability of funds.

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