Does the contamination of dendritic cells by lymphocytes skew the differentiation of monocytes into macrophages? A pilot study

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Abstract

Immunotherapy based on dendritic cells is currently a very promising therapy for cancer. Methods for the generation of dendritic cells (DCs) in vitro *are now being widely investigated. The cellular composition of the culture and functional ability of generated DCs is strongly dependent on precursor cells, culture micro-environment and the type of maturation stimuli. The aim of our study was to investigate the influence of different factors on the differentiation of peripheral blood monocytes into DCs or macrophages. Dendritic cells were generated from adherent monocytes in RPMI medium supplemented with rhGM-CSF, rhIL-4, and autologous serum of lung cancer patients (n=14) and from healthy donors (n=14). The phenotype and morphology of the cultured cells were established in a flow cytometer, as well as by light and confocal microscopy. The level of lymphocyte contamination in the cultures was about 20% in non-small cell lung cancer (NSCLC) patients and 30% in healthy donors. Despite high levels of IL-6 and IL-10 in autologous serum, the monocytes in the NSCLC patients differentiated efficiently in immature DCs CDla^{+}/CD14^{low} or CD1a^{+}/CD14^{-}. The expression of CD14 antigen and percentage of CD14^{bright} cells (macrophages) were significantly higher in cultures from the healthy donors compared to cultures from NSCLC patients. The high number of macrophages was visualized by light and confocal microscopy. The functional status of monocytes and/or cytokines released by lymphocytes during the generation of DCs could modify the differentiation of monocytes into macrophages or DCs.*

Key words: *dendritic cells, monocytes, macrophages, cell culture, lung cancer.*

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Introduction

Vaccination with dendritic cells (DCs) is one of the promising methods in adoptive immunotherapy for cancer. Monocytes from peripheral blood, as well as stem cells from peripheral blood, bone marrow, or umbilical cord blood are the main sources of DCs generated *in vitro* [1]. The separation of monocytes in a magnetic field (MACS) or during apheresis and elutriation may be useful in large-scale generation of DCs [2, 3]. In immunology protocols, the purification of monocytes is also carried out in adherent cultures which provide a favourable environment for the isolation of monocytes. However, impurities in lymphocytes are constantly observed during the generation of DCs [4].

DCs are usually generated in a medium supplemented with autologous or allogenic serum. The medium includes not only growth factors, but also agents which inhibit the development and differentiation of cells. The concentration of IL-10, TGF-ß or IL-6 in cancer serum is higher than in healthy serum. Therefore, AB serum from healthy donors could serve as a beneficial medium supplementation. However, the "clearest" condition for the generation of DCs is supplied by commercially available serum-free medium, such as CellGrow DC or AIM-V. The transfor-

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mation of monocytes into immature DCs is generally stimulated by GM-CSF, IL-4 or IL-15. This process could be modified by the cytokines from serum supplementation or by the factors released by residual lymphocytes in DC' cultures. It should be noted that the monocytes of cancer patients may be defective. Monocytes from healthy donors could differ from the monocytes of cancer patients in the expression of cytokine and chemokine receptors, as well as in their ability to produce specific cytokines [1, 5].

Given the significant role of the environmental and cellular composition of the culture in the generation of DCs, we studied the process of the transformation of monocytes into immature DCs or macrophages in non-small cell lung cancer (NSCLC) patients and healthy donors.

Material and Methods

The study population consisted of 14 NSCLC patients (mean age 61.75±9.9 years). The control group consisted of 14 healthy donors (mean age 51.7±13 years).

Serum was obtained by the centrifugation (3.000 rpm/10min) of peripheral blood samples taken from each subject and then were frozen in aliquots at –80°C, and subsequently thawed for examination. The serums were not mixed. Serum was used for supplementation of the culture medium and for measurement of the cytokines using the ELISA method.

Peripheral blood was drawn from the NSCLC patients into heparinized tubes, in accordance with protocols accepted by the Local Ethics Committee. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Lymphoprep, Nycomed, Norway). After isolation, the PBMCs were resuspended in RPMI 1640 medium (BioWhittaker, Belgium), supplemented with 1% v/v Penicillin-Streptomycin (Sigma, Germany), seeded into 6-well tissue culture plates in an appropriate culture micro-environment, and left to adhere for 1.5 hours at 37°C in a humidified 5% CO_2 atmosphere.

After the adherence time, non-adherent cells were removed and the culture plates were washed in cold PBS without Ca^{2+} and Mg^{2+} (Biochrom AG, Germany) in order to obtain a fraction of adherent $CD14^+$ monocytes. The adherent cells were then cultured in medium supplemented with 10% autologous serum, together with 500 IU/ml rhIL-4 (Strathmann, Germany) and 1.000 IU/ml rhGM-CSF (Gentaur, Belgium). Thereafter, the cytokines were added every 3 days.

After 48 and 96 hours, and 5 days of culturing, the adherent cells were harvested by incubation with 0.25%/0.02% trypsin/EDTA solution (Biochrom AG, Germany). The cells obtained were washed in PBS without Ca^{2+} and Mg^{2+}, and resuspended prior to immunophenotyping and microscopy analysis.

To characterize the phenotype of the cells generated, FITC-, PE- or TC-conjugated monoclonal antibodies (Caltag, USA) were used: mouse anti-human CD14, mouse anti-human CD1a, mouse anti-human CD45/CD14. The cells were incubated for 20 minutes in the dark and afterwards washed in PBS without Ca^{2+} and Mg^{2+}. The cells were analyzed by means of an FACS Calibur flow cytometer (Becton Dickinson, USA) equipped with a 488-nm argon laser. An acquisition gate was established based on FSC and SSC, that included only the monocytes/macrophages population and excluded lymphocytes. After acquisition, the cells were analyzed using CellQuest software.

The level of the cytokines examined was estimated in serum, using commercial kits: Quantikine HS Human IL-10 ELISA Kit (R & D Systems, USA), Human IL-6 High Sensitivity Kit (Bender, Austria), and a Human TGF-β1 Kit (Bender, Austria), as well as ELx800 equipment (Bio-Tek Instruments, USA).

Confocal microscopy (Carl Zeiss, Germany) analysis was used to evaluate the morphology of the immature DCs and macrophages generated. Digital analysis of the images was performed with LSM 5 Pascal software, version 3.0.

Data are presented as the mean value ± standard deviation (SD). For evaluation of the statistical significance, the U Mann-Whitney test was used for comparison of the unpaired group data. The Wilcoxon matched pairs test was applied for comparing two variables. The Spearman correlation test was used for correlation observations. A level of $p<0.05$ was accepted as significant.

Results

The level of serum IL-10 and IL-6 was significantly higher ($p<0.01$) in the NSCLC patients compared to the control donors (Table 1).

After 48 and 96 hours, culture contamination by lymphocytes was slightly higher in the healthy donors than in the NSCLC patients (Table 2).

The percentages of $CD1a^+/CD14^+$ cells and $CD1a^+/CD14^-$ cells in 2-day and 4-day cultures were similar in both groups examined. However, the percentage of $CD14^{bright}$ cells was significantly higher, while the percentage of $CD14^{low}$ was lower in the cultures from healthy donors compared to cells cultured from NSCLC patients (Table 2, Fig. 1). After 5 days of culture, the macrophages were shown in both light and confocal microscopy scans in both examined groups (Fig. 2).

Table 1. Concentration of cytokines in serum of NSCLC patients and healthy donors

	IL-6 (pg/ml)	IL-10 (pg/ml)	TGF-β1 (ng/ml)
Control group	0.15±0.16*	2.96±2.18**	5.32±6.11
NSCLC patients	2.57±5.73*	9.57±10.43**	9.08±14.56

** p<0.01; ** p<0.001.*

Table 2. Lymphocyte contamination (mononuclear cells gate) and phenotype of monocytoid cells (monocyte/macrophage gate) after 48 hours and 96 hours culturing

		48 hours	**96 hours**
Lymphocyte contamination (%)	Control group	30.97±17.81	18.00±13.48
	NSCLC	21.79±13.26	14.22±11.97
$CD1a^+/CD14^+$ cells (%)	Control group	86.53±6.85	89.47±4.56
	NSCLC	89.64±7.02	87.30±10.64
$CD1a^+/CD14^-$ cells (%)	Control group	11.01±6.24	9.24±4.81
	NSCLC	6.67±6.85	7.44±9.53
$CD14^{bright}$ cells (%)	Control group	26.88±24.42*	51.44±20.35**
	NSCLC	7.57±4.72*	23.49±14.87**
$CD14^{low}$ cells (%)	Control group	61.27±23.06**	39.89±17.76**
	NSCLC	84.64±8.28**	66.15±20.06**
CD14 expression (MFI)	Control group	974.90±886.90	1606.00±751.60***
	NSCLC	282.20±166.30	434.10±354.40***
CD1a expression (MFI)	Control group	151.90±64.20	319.60±92.50*
	NSCLC	113.30±47.60	230.10±97.60*

** $p<0.05$; ** $p<0.005$; *** $p<0.0005$.*

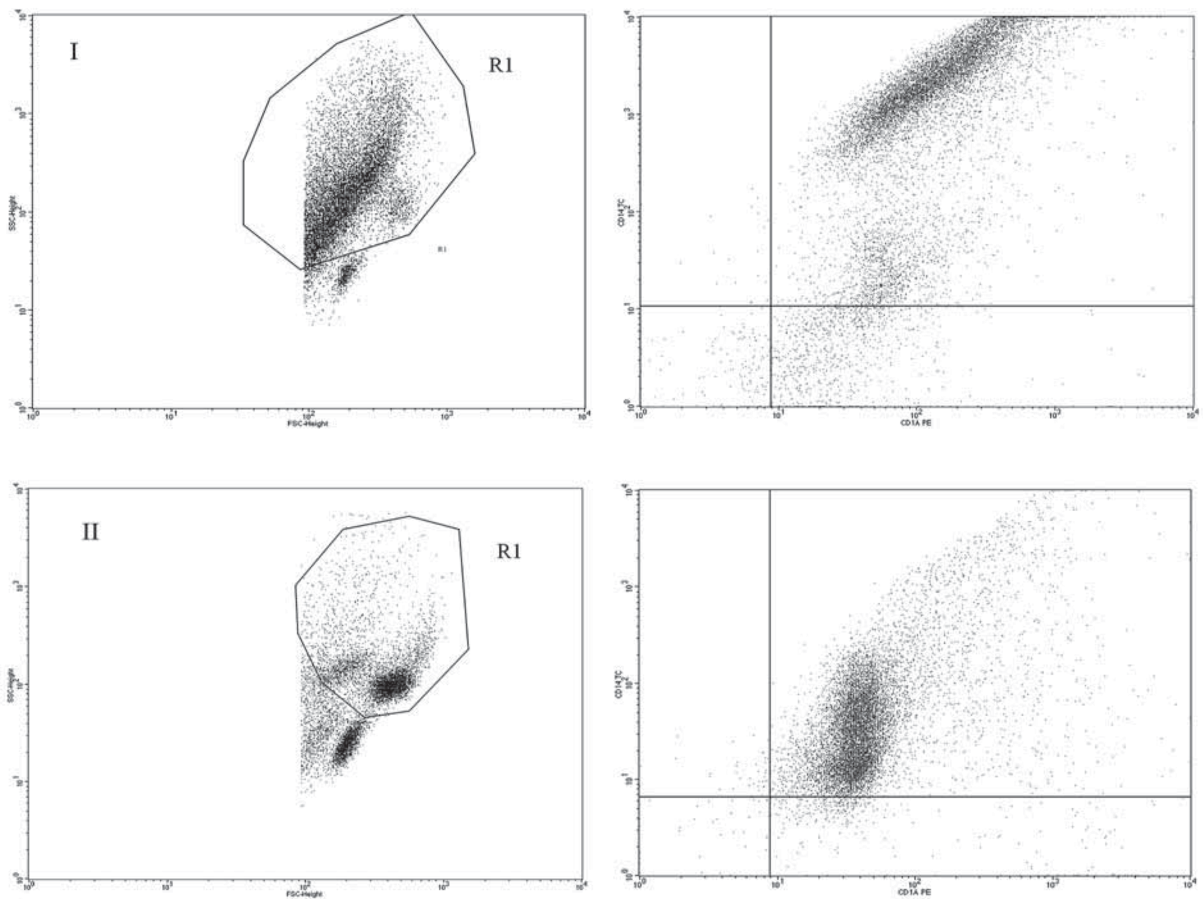

Fig. 1. Flow cytometric analysis of monocytoid cells (Region 1) generated for 96 hours in medium supplemented with autologous serum in healthy donors (I) and in NSCLC patients (II). Plots on the left demonstrate FSC (ordinate) and SSC (vertical axis) parameters. Plots on the right demonstrate CD1a (ordinate) and CD14 (vertical axis) expression of DCs and macrophages

The expression of CD14 antigen was significantly higher in cells from the healthy donors than in cells from the NSCLC patients. After 96 hours, the expression of CD1a antigen in the cultured cells was significantly lower in the NSCLC group of patients compared to the control donors. Moreover, in both examined groups, the expression of CD1a antigen was significantly higher ($p<0.05$) in cells cultured for 96 hours than in those cultured for 48 hours (Table 2).

In both examined groups, in the 48-hour cultures the percentage of lymphocytes significantly positively correlated with the percentage of $CD1a^{+}/CD14^{-}$ cells ($R=0.513$, $p<0.05$) and significantly negatively with $CD1a^{+}/CD14^{+}$

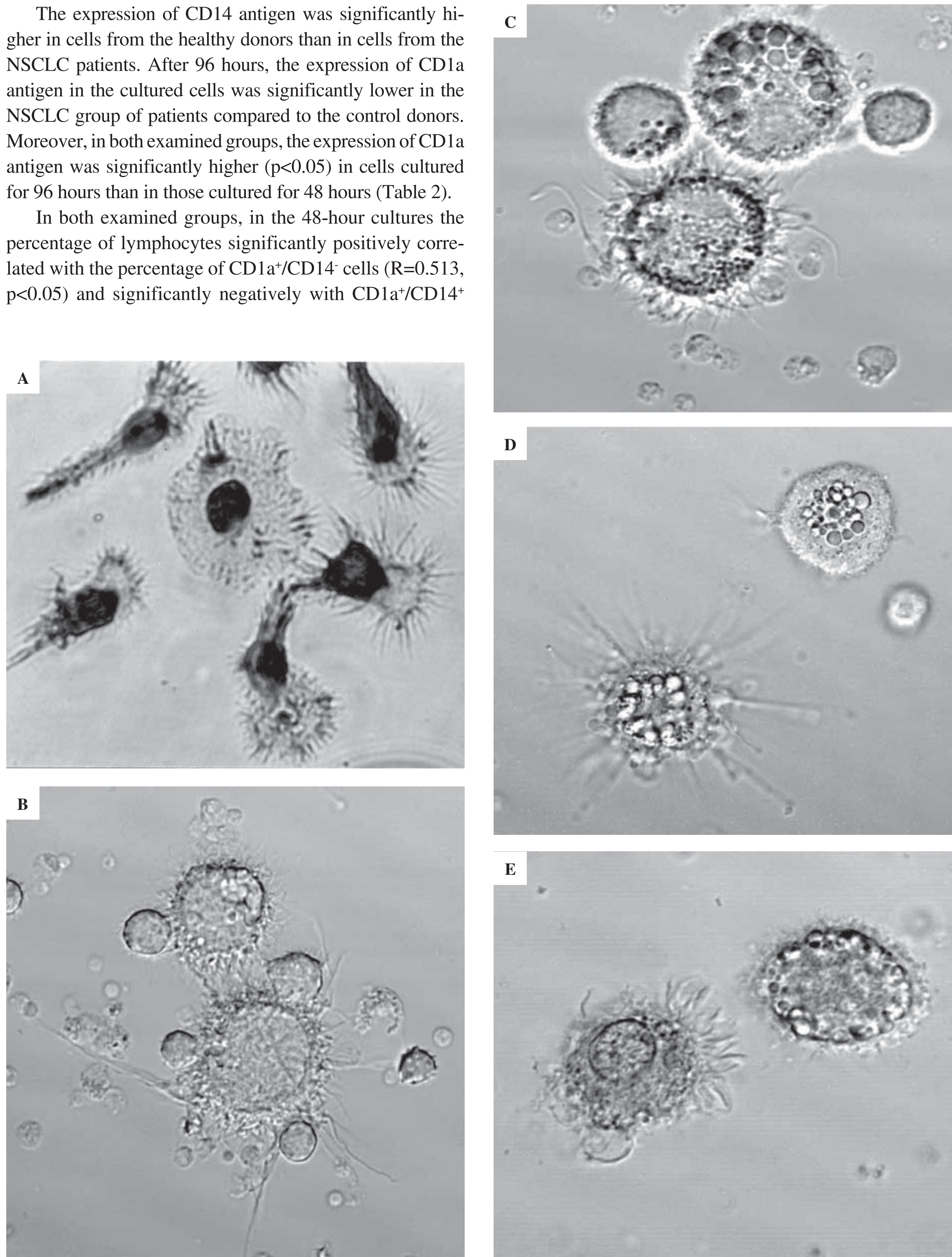

Fig. 2. DCs and macrophages generated in 5 days from peripheral blood of healthy donors (A-C) and NSCLC patients (D-E)

cells (R=-0.494, p<0.05). In the control donors, the percentage of 48-hour cultures contaminated with lymphocytes insignificantly positively correlated with the percentage of $CD14^{low}$ cells detected in the cultures after 96 hours (R=0.539, p=0.11). Moreover, in the NSCLC patients the percentage of $CD1a^+/CD14^-$ cells significantly positively correlated with the autologous serum IL-10 level (R=0.833, p<0.01). A significant negative correlation was found between the concentration of IL-6 serum and the expression of CD1a antigen on cells from the NSCLC patients (R=-0.721, p<0.05).

Discussion

It is widely known that during their differentiation into DCs, monocytes lose their CD14 antigen expression and transform into a $CD1a^+/CD14^-$ immature population of DCs with a high ability to initiate specific immune responses. Monocytes differentiated into macrophages in the presence of M-CSF are characterized by a high CD14 expression, and by potent phagocytic and non-specific bactericidal and anti-tumoural activities [6]. The delicate balance between macrophages and DCs observed during the generation of DCs may play a key role in the effectiveness of anti-cancer vaccines.

Peripheral blood monocytes can differentiate into DCs or macrophages, depending on various environmental factors. The differentiation of monocytes into immature DCs could be stimulated by IL-4 and GM-CSF, or IL-13 and IFN-α. Addition of the transforming growth factor ß (TGF -ß) or exposure of the monocytes to GM-CSF plus IL-15 led to the generation of DCs with features of Langerhans cells. DCs derived from monocytes can be matured in a monocyte-conditioned medium in the presence of IL-1ß, TNF-α, IL-6, prostaglandin E_2, toll-like receptor ligands, or CD40L [2, 7, 8]. In contrast, the macrophage colony-stimulating factor (M-CSF) is also a potent macrophage differentiation factor. IL-6 and IL-10 also shift monocyte differentiation from DCs to macrophages [9, 10].

IL-6 and IL-10 are produced by tumour cells and activated Th2 lymphocytes [11]. Avila-Moreno et al. found that the soluble factors from lung cancer cell lines caused an increase in the expression of myelomonocytic markers and altered the differentiation of monocytes *in vitro* from DCs to macrophages [12]. Fibroblasts are another source of IL-6. Chomarat et al. has shown that co-cultures of monocytes and fibroblasts in the presence of GM-CSF and IL-4 yield $CD14^{bright}$ cells displaying macrophage morphology [10, 13]. IL-6 up-regulates expression of the functional M-CSF (CD115) and the consumption of autocrine M-CSF by the monocytes, thereby switching their differentiation from DCs to macrophages [14].

We proved that the concentration of IL-6 and IL-10 was significantly higher in the serum of cancer patients than in the serum of healthy donors. Nevertheless, the transformation of monocytes into macrophages seems to be independent of the presence of these cytokines in the autologous serum used for medium supplementation. In the cultures of monocytes from healthy donors we found a large population of macrophages, despite the low concentrations of IL-6 and IL-10 in the serum.

It is postulated that IFN-γ also switches monocyte differentiation from DCs to macrophages, at least partly, *via* an autocrine production of M-CSF and IL-6 by the monocytes. IFN-γ is released during the early and late stages of the immune response by the natural killer (NK) cells and activated Th1 cells. Delneste et al. cultured human monocytes in medium supplemented with GM-CSF and IL-4, with or without IFN-γ. The presence of IFN-γ, together with GM-CSF and IL-4, resulted in a sustained and dose-dependent up-regulation of M-CSF and IL-6 production by monocytes. Monocytes, in the presence of GM-CSF, produced a higher level of M-CSF when stimulated by IFN-γ plus IL-4 than with IFN-γ alone [15].

According to these results, we hypothesize that contaminated lymphocytes could be responsible for the macrophages instead of the generation of DCs during the culture of monocytes. This effect is independent of the percentage and number of lymphocytes, and is probably connected with the function of the lymphocyte and cytokine network. The phenotype of monocytes, especially the expression of cytokine receptors, could also play a role in the differentiation of cells. Surprisingly, the generation of immature DCs in the NSCLC patients was more efficient than in the healthy donors. We surmise that the quality of the generated DCs may be improved by anti-M-CSF factors.

Therefore, is it possible to generate DCs after monocyte enrichment in adherent cultures from healthy donors? TNF overcomes IL-6-driven macrophages differentiation by down-regulating the expression and internalization of the M-CSFR on activated monocytes. Furthermore, macrophages generated by the culturing of monocytes with M-CSF can convert into DCs upon exposure to GM-CSF and IL-4, even at a late stage of differentiation [13]. For instance, Suzuki et al. have shown that both mature macrophages and blood monocytes from NSCLC patients could differentiate into DCs, and might be a useful source of DCs for immunotherapy [16]. These results suggest that the initial proportion of DCs, macrophages and lymphocytes in *in vitro* cultures is favorable for terminal DCs generation as a result of the dynamic alteration in the components of the culture micro-environment.

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