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Analysis of IL-1α, bFGF, TGF-β1, IFNγ, MMP-1, and CatD Expression in Multinuclea Macrophages *In Vitro*

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The incidence of mono- and multinuclear cells and their expression of pro- and antifibrotic factors were studied in cultured peritoneal macrophages from intact and BCG-infected mice. Generally, the expression of factors increased with an increase in the number of nuclei per cell. However, the expression was higher in macrophages from BCG infected mice, except the cells with 3 and more nuclei, extremely rarely expressing IL-1α in cultures from intact and BCG-infected animals. The number of macrophages with 3 and more nuclei, expressing CatD, was comparable with the number of mono- and binuclear macrophages. Presumably, this was determined by various mechanisms of formation of multinuclear (3-5 and more nuclei) macrophages, for example, by amitosis.

Key Words: *multinuclear macrophages; intact and BCG-infected mice; expression of pro- and antifibrotic factors*

Important role of macrophages in inflammation process during all of its stages is well known. One of its stages, the so-called reparative, as a rule manifests by fibrosis in foci of destruction of differentiated cells. However, in pathologies associated with activation of the mononuclear phagocyte system (MPS), *e.g.* in influenza during the acute period of disease, fibrosis manifestations are found in the lungs of mice in parallel with destruction of organs outside these zones [3]. Fibroplastic activity of fibroblasts in the tuberculous granulomas of the liver increased by 14 times and the content of fuchsinophilic fibers by 16 times 90 days after infection with BCG vaccine mycobacteria; no necrobiotic processes were detected in these granulomas [5]. On the other hand, 2.5-3% of the organ parenchyma in these animals was occupied by necrotic foci and about 4% by fuchsinophilic tissue outside these foci during various periods of observation [5].

Hence, fibrotic process is not always associated by destructive processes and can be "reparative" (replacing). Tuberculosis is a classical granulomatosis, which manifests by formation of extremely numerous so-called granulomas, consisting of macrophages and their derivatives, in the organ interstitium. The "core" of a mature granuloma by day 90 after infection of animals by BCG mycobacteria (more than 70% in the liver [5] and up to 90% in the lymph nodes [4]) can consist of epithelioid mononuclear and binuclear and also multinuclear cells — macrophageal derivatives, part of which have a great hydrolytic potential [4]. However, the mechanisms of their formation, probable role in organ destruction and fibrosis are not amply studied [1,4]. Multinuclear and polyploid MPS cells, spontaneous destructive and fibrotuc processes are characteristic of tuberculous and other granulomatoses (about 70 diseases), and fibrosis is their serious complication resistant to therapy [4].

We studied *in vitro* expression of cytokines and hydrolytic enzymes, essential for destruction, inflammation, and fibrosis of mammalian organs, by macrophages with different numbers of nuclei under conditions of their coculturing with BCG vaccine *M. tuberculosis*.

MATERIALS AND METHODS

The study was carried out on peritoneal transudate macrophages (PMP) from male BALB/c mice (aged 2 months; 21-22 g), intact and infected intraperito-

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neally by BCG vaccine mycobacteria for creation of conditions for guaranteed mycobacteria-PMP contact. Peritoneal transudation was collected 90 days after injection of BCG vaccine (0.5 mg in 0.25 ml isotonic NaCl), as the highest concentrations of epithelioid cells with one or more nuclei were found in tuberculous granulomas of mouse viscera *in vivo* 90 days after infection [4,5]. PMP cultures were incubated for 48 h on slides in plastic plates in 2 ml medium 199 with 10% fetal calf serum [2]. The cultures were fixed in 4% formaldehyde in PBS (pH 7.3).

The incidence of PMP (mononuclear and with 2, 3, and more nuclei) was evaluated under an Axiostar Plus microscope (Carl Zeiss) at ×400. Six specimens of PMP cultures were examined, 100 macrophages per sample selected at random. The expression of cytokines (IL-1α, bFGF, TGF-β1, and IFNγ) and enzymes (MMP-1 and CatD) in these cells was studied by the indirect immunocytochemical method with visualization by means of Anti-Rat Ig HRP Detection Kit (biotin—streptavidin—peroxidase complex; BD Pharmingen) and Novocastra diagnostic kits of monoclonal antibodies. The expression of cytokines and enzymes was detected by specific staining (light-brown) of the cytoplasm, after which the percentage of PMP with different numbers of nuclei was evaluated. Total count of PMP (irrespective of number of their nuclei) was taken for 100%.

The results were processed by Statistica 8.0 software. The significance of differences between the means was evaluated by nonparametric Mann—Whitney test. The differences between the means were significant at $p<0.05$.

RESULTS

Cells with 1, 2, 3, and more nuclei were detected in PMP cultures from intact and BCG-infected animals. Control cultures contained 4.2±0.4% binuclear macrophages and 0.8±0.2% PMP with 3 and more nuclei, cultures from BCG-infected animals — 6.4±0.5 and 2.4±0.2%, respectively. The counts of cells with 1, 2, 3, and more nuclei, expressing profibrotic cytokines (IL-1α, bFGF, and TGF-β1) [7,9,11,12] and antifibrotic factors (IFNγ, MMP-1) and CatD protease [6,8,10] in PMF cultures from intact and BCG-infected animals generally increased with an increase in the number of cell nuclei; however, their levels were higher in PMP cultures from BCG-infected mice (Table 1). Peritoneal macrophages with 3 and more nuclei from intact and BCG-infected mice were an exclusion: they expressed IL-1α so rarely, that the values of this expression were taken for 0 (Table 1). The percentage of multinuclear PMP expressing CatD differed little from the level of mononuclear PMP (Table 1). The level of cells expressing all factors except IL-1α was higher in cells of all types (by the number of nuclei) in PMP cultures from BCG-infected animals than in control cultures (Table 1).

Multinuclear PMP in cultures from intact and infected animals presumably formed by amitotic division of cell nuclei or by fusion of mononuclear, rarely binuclear, macrophages (in infected mice) [1,2]. Presumably, if these cells formed by acytokinetic amitosis [1,2], the formation of PMP with 5-6 and more nuclei could lead to further uneven distribution of the genetic material. This fact and low number of cells of this kind in PMP cultures could be responsible for extremely low level of cells expressing IL-1α (Table 1). No multinuclear Langhans cells (a sort of marker cell for tuberculous granuloma) containing several tens of nuclei much more rare *in vivo* than epithelioid cells [4] were found in granulomas initiated *in vivo* by BCG vaccine *M. tuberculosis* and unfortunately, these cells could not be reproduced *in vitro*.

TABLE 1. Percentage of Mononuclear and Multinuclear PMP, Expressing Profibrotic and Antifibrotic Factors, in PMP Cultures from BALB/c Mice (n=6; $M±m$)

Factors	Number of nuclei in PMP					
	Intact mice			BCG-infected mice		
	1	2	≥3	1	2	≥3
IL-1α	4.2±0.3	5.0±0.3	0	7.0±0.4*	9.3±1.3*	0
bFGF	16.3±1.5	48.2±3.1^{o}	58.9±6.9^{o}	95.3±1.0*	95.0±8.7*	83.3±8.0*
TGF-β1	18.5±1.5	32.6±3.0^{o}	42.9±4.3^{o}	24.8±2.2*	48.9±4.4*^{o}	89.7±9.0*$^{o+}$
IFNγ	11.3±1.2	21.9±2.1^{o}	63.5±4.5^{o+}	84.0±3.2*	83.1±6.6*	78.3±3.5*
MMP-1	31.5±2.9	47.6±5.0^{o}	69.2±7.0^{o+}	87.5±1.8*	84.4±4.9*	92.3±7.0*
CatD	77.5±2.6	65.9±1.3^{o}	66.7±7.0	91.0±2.2*	72.2±7.0^{o}	86.4±9.0

Note. n, number of culture specimens. $p<0.05$ in comparison with *control for comparison of groups, ^{o}mononuclear PMP, and $^{+}$binuclear PMP within the group.

We have demonstrated previously in influenza A/H1N1 that lung alveolocytes and particularly macrophages expressed TGF-β and SMAD-2 [3] mediating epithelial—mesenchymal transition with subsequent acquisition of the fibroblast-like phenotype by epitheliocytes. These data and the present findings indicate a high regulatory potential of macrophages for initiation of fibrotic process in disease and determining hyperactivation of SMP. On the other hand, they are also characterized by manifest antifibrotic activity, which is proven by a high level of PMP expressing CatD and particularly MMP-1 (its activity manifesting at neutral pH) outside the zones of destruction, but in zones presumably formed by just forming fibrous connective tissue.

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