Dynamics of Structural Transformations of BCG Granulomas and Expression of TNF-α and Granulocyte-Macrophage CSF by Macrophages *In Vitro*

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The spleens were isolated from mice at different times after BCG infection and BCG granulomas were explanted and cultured *in vitro*. Cell migration, chemoattractant potential, and expression of TNF-α and granulocyte-macrophage CSF (GM-CSF) by macrophages migrated from granulomas were evaluated in granulomas. The number of macrophages able to migrate; migrating out of granulomas, expressing TNF-α and GM-CSF decreased with increasing the time after infection. The number of cells in "dissociating" granulomas correlates with the number of macrophages containing live BCG mycobacteria in the cytoplasm.

Key Words: *BCG-induced granulomas; macrophages; TNF-α; granulocyte-macrophage colony-stimulating factor; in vitro*

It is generally accepted that cells are confined to granulomas via a gradient of chemoattractants released by macrophages (MP) and other granulomatous cells [3,7,9]. These include granulocyte-macrophage CSF (GM-CSF) and TNF-α [3,5,10]. Live mycobacteria present in granuloma MP are believed to be the major granuloma-forming factor determining granuloma formation and stabilization during tuberculosis [2,3]. MP produce cytokines such as GM-CSF and TNF-α contributing to the formation of granulomas. However, the patterns of expression of these cytokines in granulomas are insufficiently studied in relation to the "dissociation" resulting in their involution [3].

Here we studied the features of GM-CSF and TNF-α expression by MP of BCG-induced granulomas in the spleen explanted later in tissue cultures and the dynamics of structural changes in granulomas.

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MATERIALS AND METHODS

The experiments were performed on male BALB/c mice weighing 22-23 g kept under standard vivarium conditions with free access to water and food. The experiments were carried out in accordance with Federal Law on the Protection of Cruelty to Animals from 01.01.1997 and Directive 86.609.EEC. Granulomatosis was induced by intravenous injection of mycobacterium BCG vaccine (0.5 mg in 0.25 ml isotonic aqueous solution of NaCl). In 1, 2, and 3 months, the mice were killed by cervical dislocation under ether anesthesia. BCG-granulomas were isolated from the spleen by mechanical disintegration [11,12] followed by fractionation and centrifugation in culture medium at 15*g* [6,8,11]. Granuloma cell suspension was transferred into culture wells with sterile coverslips. Granulomas were explanted to tissue cultures 1, 2, and 3 months postinfection, when typical BCG granulomas were formed in the spleen [3]. The concentration of MP isolated from the spleen was determined in a Goryaev chamber. "Pure" granulomas from granuloma cell suspensions without free splenocytes were cul-

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tured for 1 or 3 days in α-MEM containing 5% fetal bovine serum and used to study MP migration under an inverted microscope. The cultures were then fixed in 4% aqueous solution of neutral formalin, stained for 5 minutes with 0.5% aqueous safranin and embedded in Canada balm (cells down) to visualize cells of the outer layer of granulomas and cells migrating from them. MP and epithelioid cells (EC) were identified by morphological features and ingestion of zymosan granules [1]. Cytomorphology of cell cultures was analyzed by bright field microscopy after safranin staining and by differential interference contrast microscopy of unstained preparations. Migrating activity of granuloma MP was estimated by the percentage of granulomas with out-migrating cells (MP and EC), and mean number of MP and EC, migrating from granulomas (N_1), which was calculated by the formula: $N_1=N_2/N_3$, where N_2 is the total number of cells (MP or EC) located completely or partially outside the granuloma (provided that this was sufficient for their cytomorphological identification), N_3 is the number of granulomas around which EC or MP were counted. The size of granulomas (diameter, μ) was estimated after they were photographed using an AxioVision Z1 microscope (Zeiss) followed by computer morphometry of calibrated digital images using morphometric module of AxioImager software.

To indirectly estimate chemoattractant potential of granulomas, macrophage migration to BCG granulomas was studied. For this purpose, "mixed" cultures of granulomas and spleen MP derived from the same animals infected with mycobacterial BCG were used. MP (1.5×10^5) were placed into well plates; the seeding density was chosen so that at the moment of explantation no more than 1-2 free MP were found within the radius of 50 μ around granulomas. Cell activity was assessed by microphotographs of cultures, which were morphometrically analyzed with AxioVision 8 software module. Since the size of isolated granulomas (their maximum diameter) ranged from 40 to 80 μ, a square with a side of 300 μ was specified as the test area for digital images. Increased MP count in the test area around the granulomas at the estimation time indicated increased number of MP migrating to granulomas. To indirectly estimate the possible error due to MP migrating from granulomas, diameters of granulomas in mixed cultures were additionally measured.

To study GM-CSF and TNF-α expression in MP migrating from granulomas, explanted granulomas were cultured for 1 and 2 days. MP migrating out of granulomas were studied by indirect immunocytochemical method with the system based on biotin-streptavidin-peroxidase complex (we used BD Pharmingen™ Anti-Rat Ig HRP Detectioin Kit C.N. 551013). The cells were fixed in 4% aqueous solution of neutral formalin. For unmasking investigated differentiation clusters, antigen retrieval was performed using Triton X-100 (0.3% solution in phosphate buffer). GM-CSF and TNF-α expression in MP was analyzed using monoclonal antibodies to the following cytokines (Becton Dickinson): anti-mouse GM-CSF (Rat Anti-Mouse, Isotype: Rat IgG2a; clone MP1-22E9), anti-mouse TNF- α (Rat Anti-Mouse, Isotype: Rat IgG1; clone MP6-XT22). Nuclei were counterstained with 1% aqueous solution of methyl green. The percentage of MP producing TNF-α and GM-CSF migrating out of granulomas was assessed. To indirectly estimate the levels of chemoattractant potential of granulomas determined by TNF-α and GM-CSF expression in granuloma MP, the comparison group was used comprising cultures of free mouse spleen MP not contributing to granuloma formation, which were isolated 1,2, and 3 months after intravenous BCG injection. BCG mycobacteria in MP were visualized by fluorescent microscopic analysis after staining with acridine orange [4].

The data were processed by methods of variation statistics using Statistica 7.0 software. The results are presented as arithmetic mean and standard error ($M\pm m$). The significance of differences of mean values between the groups was determined by nonparametric Mann–Whitney test. Correlation analysis was performed by Spearman's rank correlation.

RESULTS

Immediately after isolation from the spleen and explanation, granulomas looked like a ball-shaped clusters of cells closely adjacent to each other (Fig. 1). Granulomas isolated after 1, 2, and 3 months after infection were comparable by size namely 65.9±5.9, 69.3±6.1 and 71.2±6.5 μ. In the first 2-3 h of culturing, granulomas were compact dense cell formations without evidence of cell migration. The first signs of MP migration out of granulomas (MP appear from the lower layer of cells) were recorded after 5-7 h of culturing. On days 1-3 of culturing, the number of MP migrating out of granulomas was gradually increasing (Fig. 2, Table 1). After 5 days, some small granulomas were completely "dissociated" into clusters of loosely arranged cells.

The highest migration activity of granuloma cells was noted 1 month after infection of mice with BCG mycobacteria (Table 1). After 1 day, out-migrating cells, mainly MP, were detected in 35% granulomas. By day 3, their percentage was 96.7%. The number of MP migrating out of granulomas increased more than 3 times and the size of BCG granulomas decreased by 30-40% indirectly characterizing the "level of dissociation" of granulomas *in vitro*. On day 3, a small amount of EC was revealed among migrating MP. Reduced migratory activity of granuloma cells was

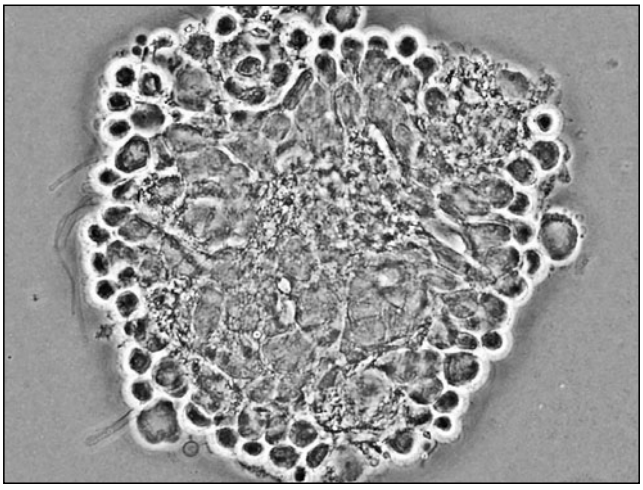

Fig. 1. BCG granuloma from BALB/c mouse spleen cultured *in vitro* 2 months after mycobacterial BCG infection; 1 h after explantation. Bright field microscopy, ×400.

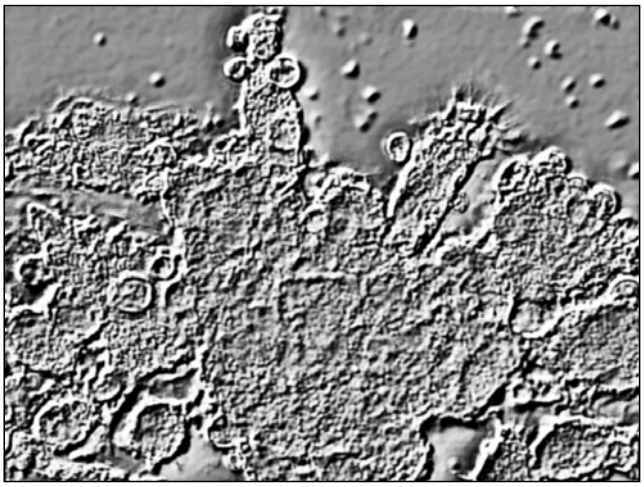

Fig. 2. BCG granuloma from BALB/c mouse spleen explanted and cultured *in vitro* for 2 months after BCG infection and migrating cells; day 3 of culturing. Differential interference contrast microscopy, ×600.

reported 2 months after their induction. It was lowest in cultured granulomas explanted from the spleen 3 months after *in vivo* induction.

"Mixed" cultures of granulomas and MP used for the assessment of chemoattractant capacity of granulomas showed the most intensive MP migration to granulomas 1 month after their induction *in vivo* (Table 1). By day 2 of culturing, the number of MP migrating to granulomas increased by 3 times compared to day 1 and by day 3 by more than 5 times. MP migration to granulomas was significantly decreased 2 months after their induction *in vivo*. Because the size of granulomas in mixed cultures did not change on days 1 and 2, this indirectly indicated that in mixed cultures the number of out-migrating MP was low (less than in the culture of "pure" granulomas) and did not affect significantly the accuracy of evaluation of granulomas chemoattractant potential.

The number of MP migrating from granulomas producing TNF-α decreased with increasing the time elapsed after BCG infection (Table 2). The number of MP migrating from granulomas and expressing TNF-α on day 3 of culturing was relatively high compared to the number of TNF-α-producing MP on day 1 of culturing in granulomas explanted 1 month after BCG infection. In 2 months after BCG infection, even lower number of these cells was detected. In 3 months, the number of granuloma MP expressing TNF-α did not differ significantly on day 1 and 2 of culturing. The percentage of TNF-α producing MP explanted from splenic granulomas in infected mice was lower than the percentage of TNF-α producing MP migrating from granulomas. These differences were most pronounced in 1 month after BCG infection. TNF-α expression in splenic and granuloma MP did not differ in 3 months after infection.

It was found that the percentage of MP expressing GM-CSF was approximately 2 times higher than those expressing TNF-α in populations of both spleen and granuloma macrophages. With "aging" of BCG granulomas, the pool of out-migrating cells capable of expressing GM-CSF decreased. The number of MP expressing GM-CSF was maximum in the "1-month-old" granulomas on day 2 of culturing. A similar result was obtained in 2 months after infection. This may indicate elimination of granuloma-forming factor (mycobacteria) activating the production of GM-CSF and TNF-α in MP population, which are regarded as factors that induce and prolong the genesis of granuloma in the "aging" granulomas.

Thus, the number of cells migrating from the BCG-induced granulomas decreases with increasing the time elapsed after their formation in murine spleen. This is probably due to several factors including reduced chemoattractant potential in the epithelioid cell granulomas due to elimination of mycobacteria and weakened gradient of chemoattractants originating from granulomas; starting fibrosis in granulomas observed in 3 months after induction of BCG-induced granulomas *in vivo* [4]; enhanced adhesion capacity of granuloma cells [7,10]; increased content of granuloma EC [3,4], which migration ability is limited by finger-like processes emerging between them.

On 5 day of culturing of granulomas explanted 1 month after BCG infection, more than 30% relatively small granulomas consisting of 40-50 cells were completely "dissociated" into separate cells forming a sort of single-layer cell "islands", two-dimensional structures of macrophage granulomas. In the center of forming macrophagal granulomas MP that contained live BCG mycobacteria in the cytoplasm were identified by fluorescence microscopy. The number of

TABLE 1. Characteristics of BCG Granulomas and Their Cell Components after Explantation from the Spleen of BALB/c Mice *In Vitro* (*M*±*m*)

Time after infection with BCG	Culture time, days (h)	Diameters of granulomas in "pure" cultures, μ	Percentage of granulomas with MP migrating from them	Number of MP migrating from granulomas	Number of EC migrating from granulomas	Number of MP migrating to granulomas in the cultures "mixed" with MP	Diameters of granulomas in the cultures "mixed" with MP, μ
1 month	0 (3)	65.9±6.1	0	0	0	1.2±0.5	65.2±6.3
	1	63.3±5.9	9.7±2.4	2.6±0.3	0.1±0.1	7.2±0.7**	64.9±5.6
	2	55.7±5.3	89.4±5.2**	8.3±0.5**	0.3±0.1	25.7±1.9**	61.3±5.1
	3	48.8±4.7**	96.7±5.5**	15.7±0.9**	1.1±0.2*	-	-
2 months	0 (3)	69.3±6.2	0	0	0	1.4±0.6	69.1±5.8
	1	68.1±5.4	5.2±1.9	1.7±0.2	0	4.6±0.8*	69.0±5.5
	2	66.2±4.5	70.9±4.8**	4.2±0.4*	0.2±0.1	13.1±1.4**	67.8±4.7
	3	57.3±4.1*	87.1±5.1**	9.7±0.6**	0.5±0.2	-	-
3 months	0 (3)	71.2±6.5	0	0	0	1.1±0.5	70.9±6.2
	1	70.1±6.3	1.2±1.1	1.1±0.1	0	2.3±0.9	70.7±6.1
	2	69.5±5.8	15.4±2.4**	2.4±0.3	0	5.4±1.7*	69.9±5.4
	3	67.4±5.2	21.9±2.9**	3.9±0.4*	0.1±0.1	-	-

Note. *p<0.05, **p<0.01. When estimating diameters of granulomas, differences were revealed between 3-h incubation and 1-, 2- and 3-day incubation. When estimating the percentage of BCG-induced granulomas with cells migrating from it and the number of migrating cells, differences were revealed between 3-h incubation and 2- and 3-day incubation. When estimating the number of MP migrating to BCG granuloma, differences were revealed between 3-h incubation and 1- and 2-day incubation.

TABLE 2. Expression of TNF-α and GM-CSF by MP from BCG Granulomas Explanted from BALB/c Mouse Spleen and Cultured *in vitro*, and "Free" Spleen MP of BCG-Infected Mice (*M*±*m*)

Time after infection with BCG	Culture time, days	Percentage of granuloma MP expressing		Percentage of spleen MP expressing	
		TNF-α	GM-CSF, %	TNF-α	GM-CSF, %
1 month	1	6.2±0.6**	11.9±1.2**	1.5±0.2	3.5±0.4
	2	9.2±0.7**	18.4±1.4**	1.8±0.3	4.2±0.5
2 months	1	4.6±0.5*	9.5±0.9**	2.1±0.3	2.7±0.3
	2	6.4±0.6**	13.3±1.1**	2.6±0.4	3.3±0.4
3 months	1	3.7±0.4	6.4±0.5*	2.4±0.3	2.1±0.2
	2	4.3±0.3*	8.6±0.8*	2.5±0.2	2.6±0.3

Note. *p<0.05, **p<0.01. Differences were estimated between the groups of spleen MP cultures and cultures of MP migrated from granulomas on days 1 and 2 of culturing.

cells that belonged to the "granulomas dissociated into cells" positively correlates with the number of MP within them containing live BCG mycobacteria (r=0.59; p<0.05). The number of granuloma MP expressing GM-CSF positively correlated with the size of granulomas (r=+0.48; p<0.05). Thus, these data are consistent with the hypothesized [2,3] high granuloma-forming activity of live mycobacteria. From this point of view, the number of cells producing GM-CSF or TNF-α in granulomas should be considered as a manifestation of such activity.

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