Arginase Expression and NO Production by Peritoneal Macrophages in Th1 and Th2-Dependent Immune Response

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Production of NO by peritoneal macrophages remained unchanged, while the expression of arginase by these cells considerably increased during the development of Th2-dependent immune response (immunization with ovalbumin). The development of Th1-dependent immune response (immunization with BCG) was accompanied by expected increase in NO secretion and paradoxical increase in arginase activity, which attested to the existence of unknown regulatory mechanisms aimed at suppression of the developed Th1-dependent immune response.

Key Words: *arginase; nitric oxide; macrophage; immune response*

Macrophages are an important element of the system of natural resistance and acquired immunity. On the one hand, they are the source of antimicrobial factors and mediators determining the type, duration, and magnitude of the inflammatory reaction. On the other hand, macrophages together with dendritic cells act as antigen-presenting cells, thus determining the type and magnitude of the subsequent immune response.

It is currently accepted that macrophage activation proceeds via two opposite pathways: classical and alternative. Classical activation of macrophages is induced by Th1 cytokines (primarily, IFN-γ) and leads to the appearance of inflammatory characteristics, whereas Th2 cytokines (IL-4, IL-10) induce alternative activation and the appearance of antiinflammatory properties of macrophages. Macrophages activated via classical and alternative pathways promote the development of Th1- and Th2-dependent immune response, respectively [3,6].

These two types of macrophages are well characterized. Macrophages activated via the classical pathway produce chemoattractants for neutrophils, natural killers, and lymphocytes, secrete proinflammatory cytokines, including IL-1β, IL-6, and TNF-α. Macrophages activated via the alternative pathway produce other chemoattractants, secrete antiinflammatory agents, *e.g.* inhibitor of IL-1 receptor, transforming growth factor-β, IL-10 [3,6].

These two groups of macrophages differ by arginine metabolism: in macrophages activated via the classical pathway, arginine is metabolized to NO and citrulline under the action of inducible NO synthase, while in macrophages activated via the alternative pathway, arginase converts arginine to urea and ornithine [9].

Most studies on classical and alternative activation of macrophages were performed *in vitro*. However, it remains unclear whether these groups of macrophages appear during the immune response in the organism, when various factors determining the formation of functional properties of macrophages act simultaneously. *In vivo* studies in general agree with the data obtained *in vitro* [5,10]. On the other hand, enhanced expression of inducible NO synthase during bronchial asthma, a pathology mediated by Th2 type immune response, is well documented [1,13].

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Thus, it was interesting to study parameters of classical and alternative activation of macrophages during Th1- and Th2-dependent immune response. Here we studied NO production by peritoneal macrophages and expression of arginase in these cells during the development of qualitatively different types of immune response.

MATERIALS AND METHODS

Experiments were carried out on 190 male and female BALB/cY mice (age 8-16 weeks) obtained from the nursery of Institute of Pharmacology, Tomsk Research Center, Siberian Division of Russian Academy of Medical Sciences. The animals were kept under conditions of partial barrier system (22±2°C, 60-70% humidity, 12 h:12 h day/night regimen) and received sterile granulated fodder and boiled water adjusted to pH 4-4.5 with hydrochloric acid.

Immune response was induced by injection of ovalbumin and BCG vaccine (N. F. Gamaleya Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences). Ovalbumin (100 μg) with 5 mg aluminum hydroxide (Sigma) in 0.1 ml 0.9% NaCl was injected subcutaneously into the hip, BCG (100 μg) in 0.1 ml incomplete Freund adjuvant (Difco) was injected intracutaneously near the tail base. The interval between the successive injections was 14 days. Control mice received the same volume of 0.9% NaCl according to the same scheme. The material was sampled on day 7 after single or triple immunization.

Immediate type hypersensitivity (ITH) reaction was induced by intravenous injection of ovalbumin (10 μg in 0.2 ml 0.9% NaCl) to immunized mice. The reaction was classified as follows: weak reaction (depressed mode, rumple hair), moderate (cyanosis of extremities, clonic seizures, pareses, and palsies), and severe (all symptoms are clearly seen, the animals die within 2-4 h).

For modeling of the adoptive variant of delayed type hypersensitivity (DTH) reaction, intact animals were injected under the hind-paw aponeurosis with 0.03 ml mixture of BCG (20 μg per mouse) and syngeneic splenocytes (10^8 cells per mouse) from intact (control paw) and immunized (experimental paw) animals. The inflammatory reaction was evaluated by paw edema (developed after 24 h). To this end, the animals were sacrificed, the paws were cut at the level of the ankle joint and weighed. The index of the reaction was calculated by the formula:

$$IR\ (\%)=100\times(Ex-C)/C,$$

where *Ex* and *C* are the weight of the experimental and control paws, respectively.

It is known, that injection of ovalbumin induces primarily Th2-dependent immune response [2,11]. On the contrary, injection of BCG induces Th1 differentiation of lymphocytes [4,12]. Functional state of macrophages was evaluated by activity of arginase and production of NO by peritoneal macrophages isolated from mice immunized one or three times with ovalbumin or BCG. In parallel, we measured important parameters of the immune system: secretion of IFN-γ and IL-2, which increases during Th1- and decreases during Th2-mediated immune response, and the capacity of lymphocyte to induce ITH and DTH during sensitization with ovalbumin or BCG, respectively.

The animals were sacrificed by cervical dislocation, peritoneal macrophages were isolated, suspensions of myelokaryocytes and splenocytes were filtered through 4-layer capron filter, washed, and resuspended in culture medium containing RPMI-1640 (Sigma), 10% FCS (ISN), 20 mM HEPES (Sigma), 0.05 mM 2-mercaptoethanol (Sigma), 50 μg/ml gentamicin (Sigma), and 2 mM L-glutamine (Sigma). The cells were cultured at absolute humidity and 5% CO_2.

Arginase activity was determined using a modified method [9]. Freshly isolated peritoneal macrophages (10^6) cells were incubated with 0.1 ml 0.1% Triton X-100 for 30 min at room temperature and constant shaking, 0.1 ml 25 mM Tris-HCl and 0.035 ml 10 mM manganese chloride were added for arginase activation and the mixture was incubated at 56°C for 10 min. After that 0.5 M L-arginine (pH 9.7, Sigma) was added to 0.05 ml lysate and incubated at 37°C for 60 min. The reaction was stopped by adding 0.8 ml mixture of concentrated sulfuric and phosphoric acids with water (1:3:7 v/v/v). Urea concentration in the obtained solution was measured using Mochevina-450 kit (Bio-LA-Test) according to the applied protocol on a spectrophotometer (540 nm). The amount of arninase catalyzing the formation of 1 mmol urea per min was taken as enzyme activity unit.

The content of IFN-γ and IL-2 were measured in biotests by stimulation of NO production [14] and proliferation of IL-2-dependent lymphocytes in the presence of T cell supernatants obtained after 24-h culturing of splenocytes (5×10^6 cells/ml) with 4 μg/ml concanavalin (Con A, Sigma). For obtaining IL-2-dependent lymphocytes, splenocytes from intact mice (4×10^6/ml) were cultured with 4 μg/ml Con A for 36 h, the mitogen was then removed and lymphocytes were incubated for 72 h with the test supernatant (1/3 volume); ^{3}H-thymidine was added to the incubation medium 16 h before the end of incubation and incorporation of the isotope was

TABLE 1. Arginase Activity and NO Production in Peritoneal Macrophages during Immunization with Ovalbumin and BCG ($X \pm m$)

Number of immunizations	Group	NO, nitrite concentration, μM	Arginase, U/10^6 cells
Ovalbumin			
1	Control	40.7±5.6	17.5±3.8
	Experiment	29.4±11.4	16.3±2.2
3	Control	27.9±7.0	12.1±1.9
	Experiment	18.4±6.4	25.0±3.8*
BCG			
1	Control	32.5±2.8	21.4±5.5
	Experiment	38.8±12.8	18.4±2.1
3	Control	24.0±5.8	17.4±2.3
	Experiment	53.6±7.3*	30.7±4.0*

Note. Here and in Table 2: *$p<0.005$ compared to the control.

measured on a β-counter. The amount of IFN-γ was determined by the concentration of nitrites in supernatants obtained after 24-h culturing of freshly isolated myelokaryocytes and T cell supernatant using Griess reagent.

The data were processed statistically using Student's *t* test.

RESULTS

Single injection of ovalbumin and BCG had little effect on NO-synthase and arginase activities of macrophages (Table 1). Three injections of ovalbumin increased arginase activity 2.7 fold, while the intensity of NO production remained unchanged. Three injections of BCG increased expression of both NO synthase (2.2 fold) and arginase (1.8 fold).

Injection of the provoking dose of the antigen (ovalbumin) on day 7 after single injection of ovalbumin induced a weak anaphylactic reaction in all animals (each group $n=10$), none animals died (Table 2). Cytokine production in these animals did not differ from the control values. Administration of the provoking dose of ovalbumin to 3-fold immunized animals induced severe anaphylactic reaction leading to death of 92% animals. The production of IFN-γ and IL-2 by lymphocytes sharply decreased.

In case of immunization with BCG, splenocytes of singly immunized mice did not induce DTH reaction, while lymphocytes from 3-fold immunized animals induced pronounced inflammatory reaction in intact mice. Enhanced cytokine production by spleen lymphocytes was observed only in animals 3-fold sensitized with BCG. These findings suggest that single injection of ovalbumin or BCG was insufficient for immunity activation; 3 sensitizing injections induced the development of Th2- or Th1-dependent immune response, respectively.

Functional properties of macrophages were preserved, no signs of immunity activation were observed after single immunization with ovalbumin and BCG. The increase in arginase activity after triple immunization with ovalbumin against the background of developed Th2-type immune response is quite expectable, because Th2 cytokines *in vitro* stimulate arginase expression and NO synthesis.

The results obtained after triple immunization with BCG do not agree with the existing concept on the effect of Th1 and Th2 types on macrophage activation. The increase in NO synthase activity agrees with published data obtained in *in vitro* experiments [9], while the increase in arginase activity is surprising and cannot be explained by direct effect of Th1 cytokines. Hence, the formation of *in*

TABLE 2. Secretion of Cytokines and Induction of ITH or DTH by Lymphocytes from Mice Immunized with Ovalbumin and BCG ($X \pm m$)

Number of immunizations		Group	IFN-γ, % of control	IL-2, % of control	DTH (index of reaction) or ITH (dead animals, %)
Ovalbumin	1	Control	100.0±15.7	100.0±10.4	0
		Experiment	103.2±11.3	92.0±11.2	0
	3	Control	100.0±3.9	100.0±10.9	0
		Experiment	53.7±4.6*	41.2±6.8*	92
BCG	1	Control	100.0±10.6	100.0±10.1	0.4±1.6
		Experiment	95.3±8.9	118.8±9.7	1.5±1.9
	3	Control	100.0±7.3	100.0±16.1	0.4±1.6
		Experiment	150.1±12.8*	175.6±18.6*	36.5±4.3*

vivo Th1-dependent immune response modulates other systems (*e.g.* neuroendocrine system), which, in turn, produce signals increasing arginase activity. The role of these signals can be played by glucocorticoids (production of glucocorticoids is stimulated during inflammatory reactions [7]) or some other unknown regulatory mechanisms suppressing the developed immune response.

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