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Inactivation of M111 Protein Gene Modifies *Streptococcus Pyogenes* Interactions with Mouse Macrophages *In Vitro*

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Immunomodulatory properties of *S. pyogenes* protein M111 were studied on the model of Gurov strain and its isogenic mutant not expressing M protein. Mouse resident peritoneal macrophages were incubated with bacteria and generation of nitroxide and superoxide anions and production of IL-6, IL-10, and IL-17 were evaluated. Protein M111 modified macrophage response: it exhibited antiphagocytic activity, prevented ROS formation, and stimulated the production of anti-inflammatory cytokine IL-10. The results suggested that this protein could serve in the bacteria as a factor suppressing the host defense forces and promoting the realization of the strategy beneficial for pathogens — escape from the host immune defense.

Key Words: *Streptococcus pyogenes; M protein; macrophages; superoxide radicals; cytokines*

Streptococcus pyogenes is a gram-positive bacterium of group A Streptococcus, highly prevalent in humans. One of the main factors of *S. pyogenes* virulence is surface M protein, characterized by antiphagocytic activity and involved in adhesion to the surface epithelium and subsequent invasion [4]. About 200 serotypes of group A streptococcus M proteins are known, differing by structure and functional characteristics [14]. Just a little part of them is studied — the most prevalent M serotypes of *S. pyogenes*.

The behavior of M proteins towards phagocytosis varies. The majority of the studied M proteins are characterized by antiphagocytic activity; however, some of them, for example, M4, are inessential for streptococcus phagocytosis [9]. The main mechanism preventing the capture of bacteria is the capacity of M proteins to fix antibodies and the complement and thus block their opsonizing capacity [5]. In addition, proteins M5 and M6 block macrophage phagocytosis of streptococci not associated with opsonins [3]. The M1 proteins suppress the intracellular digestion process, thus promoting survival of streptococci inside human granulocytes [13]. The effects of *S. pyogenes* M111 protein on phagocytosis and the digestive capacity of phagocytes are not studied.

We study the effects of *S. pyogenes* protein M111 gene inactivation on the process of streptococcus phagocytosis (ROS capture and production) and the cytokine production by mouse peritoneal macrophages.

MATERIALS AND METHODS

S. pyogenes clinical strain Gurov, serotype M111, was a kind gift from Acad. V. A. Chereshnev. A strain incapable of M protein expression (Gurov *emm*⁻) was created by the electroporation method [2] as follows. The gene site coding for the central part of M protein was cloned in *p7ermB* integrative plasmid, incapable of replication in streptococci. Streptococcal clones were tested for the presence of the target construction by PCR for the insert area and subsequent sequencing

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of the amplicon. *S. pyogenes* were cultured in Todd—Hewitt medium with 0.5% yeast extract at 37ºC.

Experiments were carried out on male hybrids F1(CBA×C57Bl/6), 18-20 g obtained from Rappolovo Breeding Center. Resident macrophages from intact mice were isolated by peritoneal lavage and incubated (2 h) in 96-well plates in RPMI-1640 with 10% fetal calf serum (FCS). The monolayer was washed from nonadhesive cells, and viable streptococcal cells were added to peritoneal exudate cells (PEC) in bacteria:PEC proportion 1:3, after which the plates were incubated for 2 h. Ampicillin (25 µg/ml) was then added and incubation was carried out for 22 h more. The viability of PEC monolayer under these conditions, evaluated by Trypan Blue staining, was at least 95%. The production of nitrites and NBT test were evaluated by spectrophotometry as described previously [1]. *E. coli* 055B5 LPS (Sigma) in a concentration of 50 ng/ml and phorbol ester (Sigma) in a concentration of 1 µg/ml served as the standard activators. In addition, cytokines IL-6, IL-10, and IL-17 in the supernatants of PEC cultured with the bacteria as described previously were measured using ELISA kits (Affimetrix). *E. coli* LPS in a concentration of 1 µg/ml served as the standard activator. The phagocytic activity of macrophages was studied in a PEC monolayer after incubation (30 min, 37ºC) with viable *S. pyogenes* in the bacterium:PEC proportion 5:1 with 10% thermoinactivated FCS without antibiotics. The phagocytic index (percentage of phagocytosed cells) and phagocytic number (number of phagocytosed bacteria per phagocyte) were counted in preparations stained by azure—eosin.

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

RESULTS

In order to clear out the effect of M111 protein removal on phagocytosis, PEC were incubated with one of the two bacterial strains: initial *S. pyogenes* Gurov and its isogenic mutant Gurov *emm⁻*. The phagocytic number and phagocytic index were higher in phagocytosis of the mutant *vs.* initial strain (Fig. 1), which indicated the antiphagocytic role of M111 protein at the stage of bacterium capture. As the study was carried out with inactivated FCS, blocking of the complement activation and antibody binding seemed to be inessential. Presumably, M111 protein bound other humoral factors present in FCS [5] or blocked the recognition of streptococci by means of pattern-recognizing macrophage receptors, which was demonstrated for M5 and M6 proteins [3].

In order to evaluate the production of nitrites, macrophages were cultured with bacteria for 24 h. However, mouse macrophage culturing with viable *S. pyogenes* led to macrophage death in just 8 h [7], and hence, the coculturing conditions had to be determined. In order to detect the optimal conditions, macrophages were cultured with *S. pyogenes* in various proportions and the viability of phagocytes was evaluated. As a result, a very low concentration of the bacteria was selected, with subsequent addition of the bacteriostatic antibiotic dose.

Both *S. pyogenes* strains, initial and devoid of M protein, similarly stimulated the production of nitrites (Fig. 2, *a*). We studied the effects of *S. pyogenes* M proteins on macrophage production of nitrites for the first time. The data on *S. pyogenes* M1 protein capacity to stimulate NO production by endothelial cells *in vitro* are scanty [12].
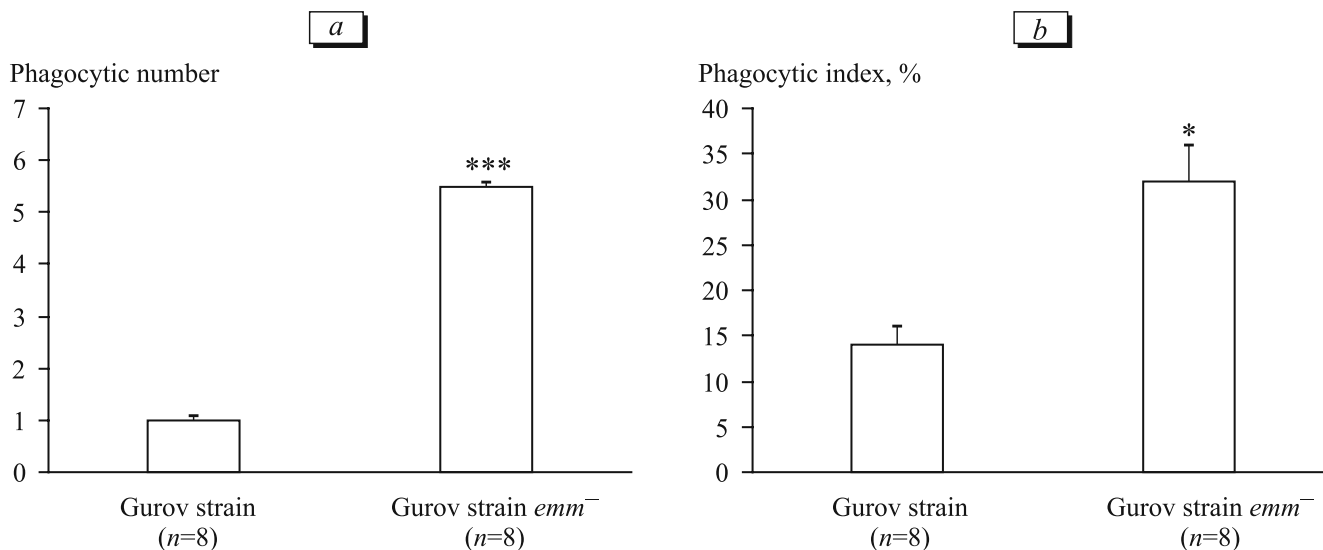

Fig. 1. Phagocytosis of *S. pyogenes* by peritoneal macrophages. *$p < 0.05$, ***$p < 0.001$ in comparison with incubation with Gurov strain.
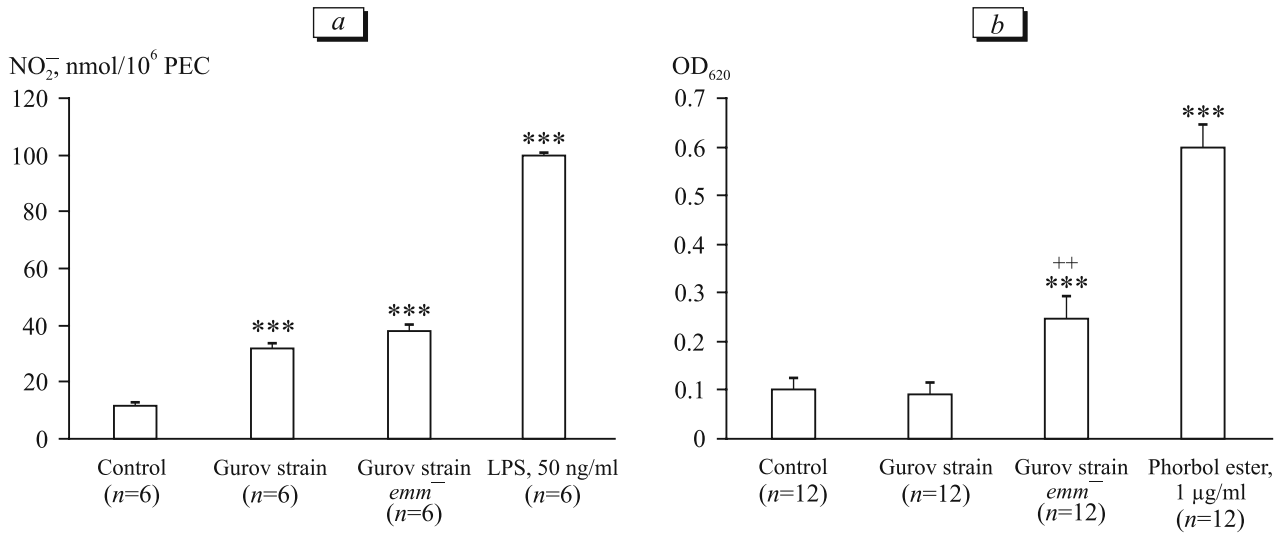
Fig. 2. Effects of *S. pyogenes* on nitrite production (*a*) and NBT reduction (*b*) by PEC. ***$p<0.001$ in comparison with the control, ++$p<0.01$ in comparison with Gurov strain.
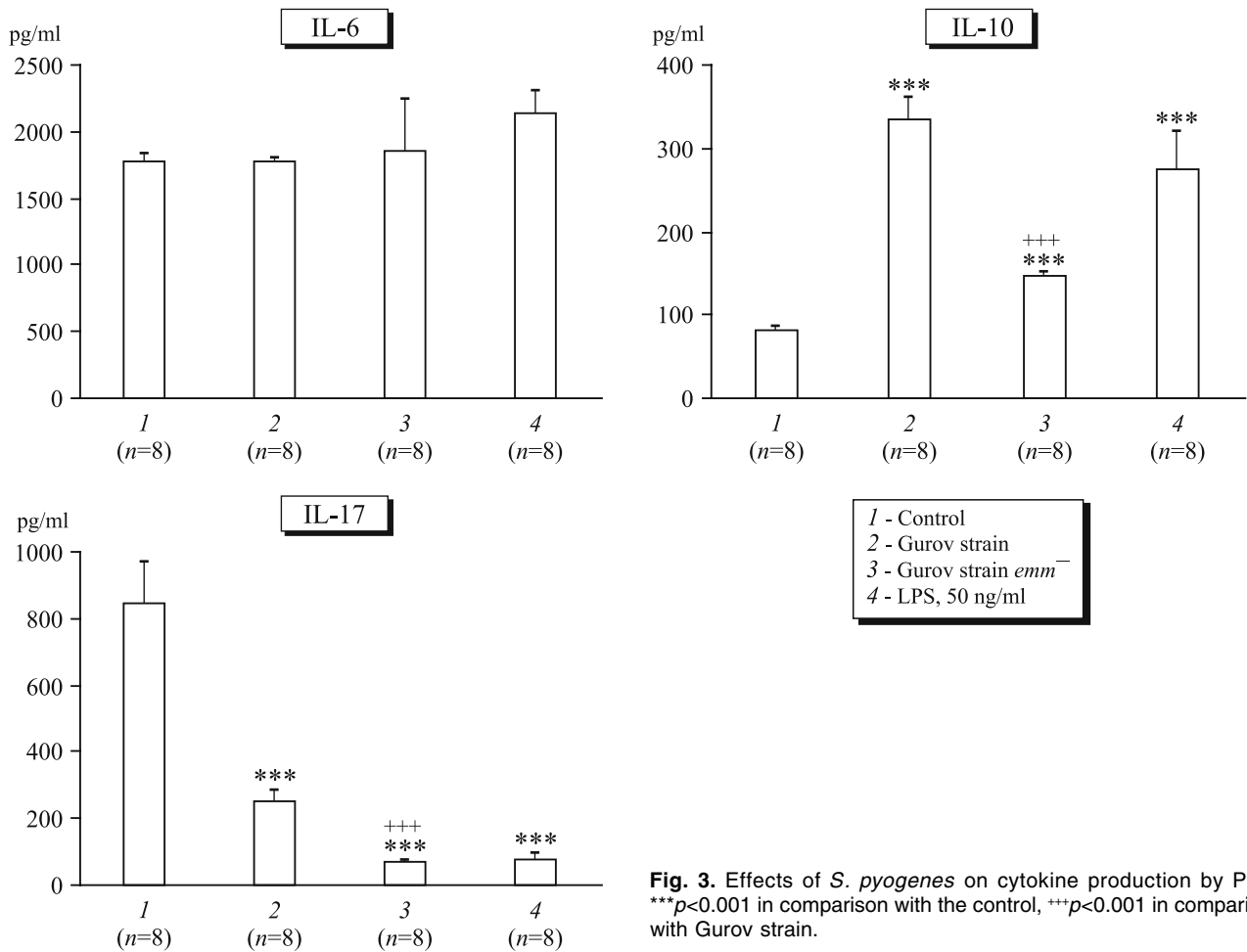

Fig. 3. Effects of *S. pyogenes* on cytokine production by PEC. ***$p<0.001$ in comparison with the control, +++$p<0.001$ in comparison with Gurov strain.

1 - Control
2 - Gurov strain
3 - Gurov strain *emm⁻*
4 - LPS, 50 ng/ml

However, NBT test (Fig. 2, *b*) showed significant differences between the strains: strain Gurov did not stimulate ROS generation, while strain Gurov *emm⁻* increased it. Similar data on stimulation of the oxidative burst in human blood granulocytes were obtained for proteins M2 and M9 in studies of mutant strains

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devoid of M protein in comparison with the parental strains [9]. One of the possible explanations of high production of ROS in response to Gurov *emm⁻* strain was greater capture of the bacteria, detected by phagocytosis reaction, observed also by other authors [9]. However, low concentration of the bacteria in our study and long incubation were expected to eventuate in complete phagocytosis of the bacteria of both strains. Hence, it seems that protein M111 could prevent the formation of oxidative radicals in macrophages. This effect was described for *S. pyogenes* M1 protein, which blocked in human granulocytes the fusion of phagosomes with azurophilic granules, containing myeloperoxidase essential for ROS generation [13].

The impact of M protein removal for macrophage production of cytokines was studied for two proinflammatory cytokines IL-6 and IL-17, exhibiting protective effects in experimental infection with group A streptococci [6], and one anti-inflammatory IL-10, with immunosuppressive activity [11]. *S. pyogenes* proteins M1, M3, M5, and M49, used alone, caused an inflammatory response in human blood monocytes, specifically, induced the production of IL-6 in these cells *in vitro* [8], while protein M5 directly reacted with human blood T cells and caused IL-10 synthesis in them [10].

The effects of the parental Gurov strain and its isogenic mutant on the production of IL-6 did not differ, in contrast to the production of two other cytokines (Fig. 3). The parental Gurov strain activated the production of IL-10 much more intensely than strain Gurov *emm⁻*, which fact suggested that the presence of M111 protein was essential for inducing the production of this anti-inflammatory cytokine by macrophages. Resident macrophages and neutrophils served as the first-line defense, providing the early stage defense from the majority of bacterial pathogens. For this reason, the capacity of protein M111 to induce the production of IL-10 was an unfavorable factor, as this cytokine could suppress the congenital immune response and promote the formation of T-regulatory cells.

As for IL-17 production, both studied strains were capable of suppressing it (Fig. 3), the mutant strain Gurov *emm⁻* exhibiting significantly higher activity in this respect than the parental Gurov strain. IL-17 promoted neutrophil attraction and effectively protected from extracellular pathogens, including *S. pyogenes*. Our results indicated that protein M111, a surface factor of *S. pyogenes* virulence, promoted the persistence of macrophageal IL-17 response. Presumably, this was a part of the pathogen strategy, associated with *S. pyogenes* survival in neutrophils due to M proteins [13].

Our study has shown that *S. pyogenes* protein M111 modifies the early macrophageal response: a strain with the intact M protein gene, in contrast to the isogenic strain, exhibits antiphagocytic activity,

prevents the formation of ROS, and more actively stimulates the production of anti-inflammatory cytokine IL-10. The results suggest that in the bacteria this protein serves as a factor aimed at suppression of the host defense forces and promoting the realization of a strategy beneficial for pathogens — escape from the host immune defense.

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