Membrane-Attack Complexes and Membrane Complement Inhibitors on Leukocyte Surface during Combined Exposure to Meningococcal Lipopolysaccharide and Complement

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Leukocytes from donors and subjects with terminal complement component deficiency were incubated for 30 min at 37°C in autologous serum with or without meningococcal lipopolysaccharide (LPS), followed by treatment with monoclonal antibodies to membrane-attack complexes (MAC) and complement inhibitors and cytofluorometry. After incubation in normal serum, about 97% granulocytes and 65% lymphocytes expressed CD55 and CD59. MAC were detected on only 5% of both types of cells. After incubation with the serum and meningococcal LPS, up to 40% granulocytes expressed MAC, expression of CD55 was decreased, and of CD59 virtually did not change. MAC were not detected in cells incubated in the serum with terminal complement component deficiency. LPS-dependent MAC-mediated hyperactivation of granulocytes may play an unfavorable role in meningococcal infection.

Key Words: *lipopolysaccharide; complement; membrane inhibitors*

The effect of lipopolysaccharide (LPS), an endotoxin of gram-negative bacteria, one of the most interesting and clinically important bioactive compounds, has been extensively studied at the cellular level and at the level of whole organism [2,4]. Endotoxins bind virtually all blood cells, modifying their activity and stimulating a complex of inflammatory reactions; injection of endotoxins to animals leads to lethal shock. In many septic conditions, for example, meningococcal infection, blood level of meningococcal LPS (MLPS) strictly correlates with the disease severity, development of complications (shock, polyorganic failure), and mortality [1,2]. Some membrane molecules in human cells bind LPS, but these "receptor molecules" are not true receptors, because they lack the cytoplasmic signal part [4]. Therefore, the initial component (or components) in the chain of intracellular events triggered by endotoxins remains unknown.

Our clinical observations showed that patients with a hereditary deficit of terminal components of the complement (DTCC) develop meningococcal infection thousands times more often than subjects with normal complement system. However, meningococcal infection never caused death in subjects with DTCC; the disease ran a comparatively benign course without infectious toxic shock [1,8]. Moreover, we demonstrated that *in vitro* incubation of human granulocytes with MLPS induces their lysis only in the presence of serum with normal complement activity, *e. g.* in autologous serum from healthy donors. Incubation of granulocytes in normal serum with sublytic concentrations of MLPS led to cell activation, specifically, increased the generation of active oxygen forms [1,10].

These data brought us to a working hypothesis [1,8,10] that MLPS (and probably other LPS) after

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adsorption on blood and endothelial cells promotes binding of terminal components of the complement to the membrane (directly or through initial components previously bound to MLPS), thus leading to the formation of the so-called membrane-attack complex (MAC). Water and ions penetrating through MAC (circular pore) cause cell hyperactivation and, if the pores are numerous, cell death. In particular Ca^{2+} entry through MAC accompanies platelet activation by thrombin and complement and sublethal effect of the complement on antibody-coated neutrophils [6,7].

Normally, excessive activation of the complement system and attack of the autologous complement (autocomplement) on blood and endothelial cells are prevented by many complement inhibition proteins [1,5]. Some of these inhibitors are present in the plasma, and some are located on the outer plasma membrane [5, 12]. Proteins CD59 (protectin, MIRL, HRF20), CD55 (DAF), CD46 (MCP), and CD35 (CR1) are membrane complement inhibitors. These inhibitors are present on cells of different mammals and are species-specific: human inhibitors protect cells from human complement only [5]. CD59 prevents the assembly of MAC on cell membranes, other inhibitors accelerate the dissociation of C3 and C5 convertases [5]. Inactivation of these inhibitors *in vitro* with monoclonal antibodies sharply increases leukocyte (or erythrocyte) lysis induced by autocomplement [3,5,11]. Similarly, *in vivo* a congenital or acquired deficiency of these proteins reduces cell resistance to autocomplement and leads to autoimmune disease, *e. g.,* paroxysmal nocturnal hemoglobinuria [11]. These inhibitors in different combinations have been revealed on all cells contacting with the complement system [3,5,11,13]. The protective effect of inhibitors is not absolute and can be overcomed: human IgM-coated neutrophils are lyzed in the presence of high concentrations of human serum [1,7,9].

Therefore, direct detection of the complement MAC on the surface of human leukocytes after incubation with endotoxin (MLPS) in the presence of normal serum will be an additional verification of our working hypothesis. *A priori* there are at least two causes of MAC assembly on the leukocyte surface. First, LPS can bind to cell surface [4] and activate the complement: LPS in a solution activates the complement by the classical and alternative pathway [14]. Second, LPS binding to cell membrane may regulate (decrease?) the expression of membrane complement inhibitors [12] and provoke the assembly of MAC. Therefore, the second task of our study was to examine the expression of CD55 and CD59 after incubation with LPS.

Additionally, we evaluated the expression of complement inhibitors on cell surface in subjects with DTCC after incubation in autologous serum. There are no publications on this problem. Normally the expression of inhibitors can be partially regulated by the negative feedback principle, *i. e.* a weak sublytic attack of autocomplement stimulates an increase in the number of inhibitors on the cell surface. If so, the expression of CD55 and CD59 is decreased in subjects with DTCC due to lack of the lytic activity of the complement in them because of the absence of C8 (or C7) [1].

MATERIALS AND METHODS

LPS from *Neisseria meningitidis* group B (strain B-8 isolated in Cuba from a patient with generalized infection) isolated by the Westphal's method and purified according to Adams [8] was kindly provided by Dr. A. M. Gracheva. Murine monoclonal antibodies to MAC (B7), CD55 (BRIC110), and CD59 (A35) were a gift from Dr. B. P. Morgan (University of Wales) [7].

Mononuclear cells (lymphocytes and monocytes) and polymorphonuclear cells (granulocytes) were isolated from donor blood and blood of patients with DTCC by single-stage gradient separation in Mono-Poly Resolving Medium (ICN-Flow). Granulocyte and mononuclear fractions, twice washed with phosphate buffer were suspended (individually) and incubated (30 min, 37°C) in autologous serum with or without a sublytic dose of meningococcal LPS (10 μg/ml), washed in cold buffer with 0.2% NaN_3 and 0.1% human serum albumin (Serva). The cells were then suspended in 200 μl phosphate buffer with 0.2% azide, 2% autologous serum, and 25 μg/ml corresponding monoclonal antibodies (B7, BRIC110, A35), and incubated for 30 min at 4°C. Then the cells were washed twice and incubated in 100 μl buffer with 2% autologous serum, azide, and 4 μl goat FITC-conjugated antibodies to murine immunoglobulins (Becton Dickinson) for 30 min at 4°C. Then the cells were again washed, suspended in 300 μl cold phosphate buffer with azide and albumin, and their fluorescence was measured on a FACScan cytoflorimeter (Becton Dickinson).

Subpopulations of typical solitary granulocytes and lymphocytes were isolated from the totality of registrations by a standard method, using their optic properties (lateral and anterior diffusion). A negligible number of damaged cells (less than 3-5%) was detected by red autofluorescence and excluded from the analysis. Green FITC-dependent fluorescence reflecting binding of the first antibodies was characterized by three methods:

1) the percentage of positive (antigen-carrying) cells with fluorescence surpassing the threshold level was estimated for antibodies of each type in all experiments and the mean number of antigen-positive cells under certain conditions was estimated;

2) mean fluorescence of cells of a certain type under certain conditions, which provided information

on the content of the antigen, was determined and averaged similarly;

3) the whole range of fluorescence (from zero to the 1024th channel of the analyzer) was divided into 8 equal intervals and the percentage of cells with fluorescence within each interval was determined.

RESULTS

Low basal fluorescence of cells incubated without first antibodies (cell autofluorescence and fluorescence induced by nonspecific binding of FITC-labeled second antibodies) in comparison with specific fluorescence (Fig. 1) allowed us to accurately determine the threshold fluorescence intensity between antigen-positive and antigen-negative cells. The mean value (median) of nonspecific fluorescence corresponded to the 210th channel of the analyzer for granulocytes and to the 150th channel for lymphocytes.

After incubation in the serum all granulocytes were CD59- and CD55-positive (98 and 96% antigen-carrying cells, respectively, Fig. 1, Table 1). Distribution of CD59 and CD55 on granulocytes was characterized by a clearly expressed maximum in the range of the 470th channel of the analyzer. Under these conditions, the distribution of CD59 and CD55 on lymphocytes was more leveled (Fig. 1). In some experiments we recorded a bimodal distribution with two peaks for the 300th and 430th channels; this pattern was less pronounced on averaged fluorescence spectra. Some lymphocytes (65%) were identified as antigen-positive (Table 1), while others carried no or little antigens (undetectable amounts). MAC were detected on the surface of only 5-10% cells of both types (Table 1).

After incubation in serum with MLPS, the expression of CD55 on both cells slightly decreased, while the expression of CD59 did not change (Table 1). Up to 40% granulocytes carried detectable number of MAC, while the number of MAC-positive lymphocytes remained practically the same. It is noteworthy that the content of MAC-positive granulocytes under these conditions (incubation with sublythic doses of LPS) was practically equal to the number of granulocytes lyzed after incubation with 50 μg/ml MLPS in media with normal complement activity [1,10].

The next step was the detection of MAC on the surface of leukocytes from subjects with DTCC. Cells and sera from Russian patients with C8 (C8β subunit) or C7 deficiency were used. The results with C8 and C7 deficiency were the same, and were therefore united. No more than 5% leukocytes from patients with DTCC and stained with antiMAC antibodies possessed fluorescence surpassing the threshold value after incubation in autologous serum with or without LPS (Table 1). The presence on the cell surface of a neo-epitope C9, to which antibodies were produced, is hardly possible under these conditions (because of the absence of C7 or C8 in the serum disrupting the chain of MAC assembly), and therefore these results suggest that: 1) anti-MAC B7 antibodies may nonspecifically bind to 3-5% cells; 2) the actual number of MAC-positive cells after incubation in normal serum without LPS is lower than shown in Table 1 (about 4%); 3) large number of MAC-positive granulocytes detected after

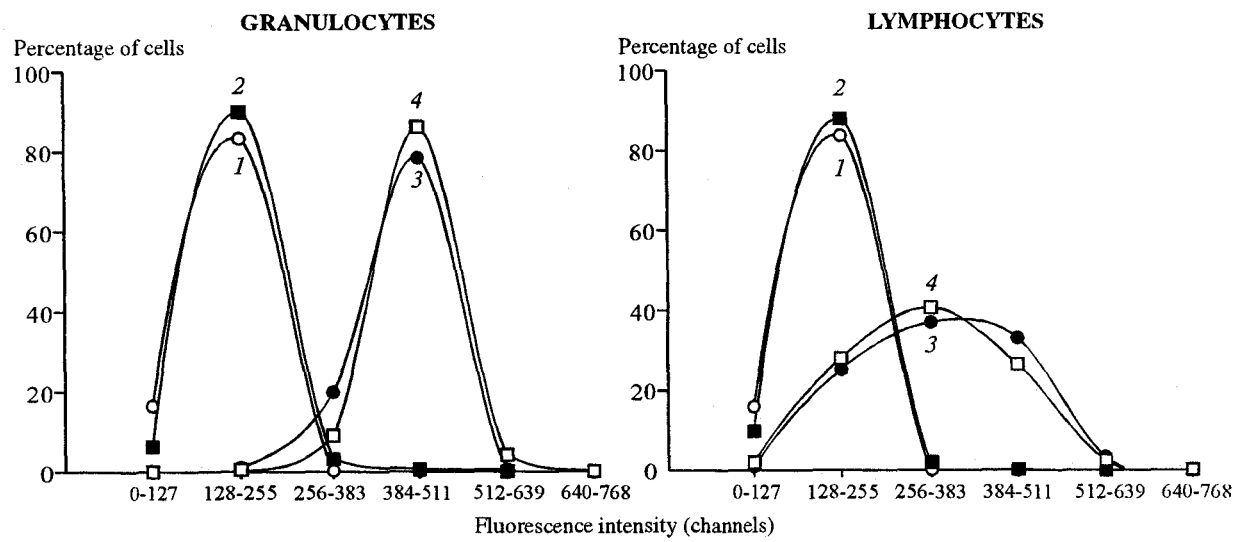

Fig. 1. Expression of CD59 on human leukocytes after incubation in autologous serum with or without meningococcal lipopolysaccharide, detected by treatment with murine anti-CD59 and second goat antimurine FITC-conjugated antibodies; flow cytofluorometry (means of 6 experiments). *1*) without antibodies (autofluorescence); *2*) treatment with second FITC-conjugated antibodies (control); *3,4*) treatment with anti-CD59 and then second FITC-conjugated antibodies after incubation in autologous serum without (*3*), or with 10 μg/ml meningococcal lipopolysaccharide (*4*).

TABLE 1. Expression of Membrane Complement Inhibitors CD55 and CD59 and MAC on the Surface of Leukocytes from Donors and Patients with DTCC after Incubation with Autologous Serum (AS) and MLPS (Mean±Standard Deviation)

First and second antibodies used for detecting surface antigen, primary incubation medium (30 min)	Donors				Patients with DTCC			
	percentage of antigen-positive cells		increment of fluorescence intensity[1]		percentage of antigen-positive cells		increment of fluorescence intensity[1]	
	lymphocytes	granulocytes	lymphocytes	granulocytes	lymphocytes	granulocytes	lymphocytes	granulocytes
Goat antimurine Ig-FITC antibodies								
AS (control 1)	2.6±1.6	2.6±1.6			3.4±2.0	3.0±1.6		
AS+10 μg/ml MLPS (control 2)	1.5±1.4	1.5±1.8	1±4	8±10	3.8±1.2	3.4±2.0	10±20	10±30
Murine A35 anti-CD59, antimurine Ig-FITC antibodies								
AS	65±4	98.5±0.6	220±50	240±40	70.0±5.5	88.5±10.0	220±50	220±60
AS+10 μg/ml MLPS	63±5	97.5±0.6	200±50	260±40	70.5±4.0	72.0±20.5	210±20	190±30
Murine BRIC110 anti-CD55, antimurine Ig-FITC antibodies								
AS	65±4	96.0±3.5	210±30	240±40	64.5±9.0	77.5±15.0	200±30	165±50
AS+10 μg/ml MLPS	56±4	85±16	185±30	190±50	67.0±6.0	69.0±20.0	200±40	150±40
Murine B7 anti-MAC, then antimurine Ig-FITC antibodies								
AS	7±6	10±10	2±15	48±30	5.2±1.8	6.5±3.0	10±15	5±25
AS+10 μg/ml MLPS	13±8	29±14*	14±9	90±40*	5.6±0.9	7±4+	10±25	0±30+

Note. [1]Mean fluorescence of cells (number of fluorimeter channel) treated with antibodies to CD59 or CD55 or MAC minus mean fluorescence of cells treated only with second FITC-conjugated antibodies (control 1). $p<0.05$: *vs.* control 2 or incubation without MLPS; +*vs.* incubation with normal serum (Mann—Whitney's test).

their incubation in normal serum with LPS is not an artifact.

After incubation in autologous serum, lymphocytes of patients with DTCC express the same number of CD55 and CD59 as the lymphocytes from donors. This number does not change after incubation in complement-deficient serum with LPS (Table 1). Under similar conditions, granulocytes of patients with DTCC carry a lesser number of CD59 and CD55 (89 and 78% positive cells, respectively). The number of CD59- and CD55-positive granulocytes further decreases after incubation in complement-deficient serum with LPS (Table 1).

These data indicate that during acute LPS-dependent activation of the complement, MAC can assembly on the surface of granulocytes without essential changes in the expression of membrane complement inhibitors on the same cells. At present we do not know which complement component first reacts with LPS in each situation. Three variants are possible: LPS → antibodies to LPS → C1, LPS → C1, and LPS → alternative pathway components [14]. We cannot rule out the possibility of direct binding of LPS with C5 followed by assembly of MAC on cell surface. Though MAC are detected on the surface of only 30-40% granulocytes, we failed to detect neutrophil populations notably differing by the expression of complement inhibitory proteins. LPS binding to the membrane can impair the function of these proteins: LPS can serve as "safe binding site" for complement components or cause overexpression of complement receptors. Another phenomenon underlying differences in complement resistance may be as follows: cells actively resist the attack of the complement by removing MAC from their surface and controlling the metabolic shifts caused by it. This process is energy-dependent and is regulated by cell calcium and other bioactive substances [6,7].

In additional experiments with Ca^{2+}-sensitive fluorochrome we showed granulocyte activation by Ca^{2+} enter through channels formed by MAC on cell surface [1]. The increase in Ca^{2+} concentration to 150-200 μM is sufficient for (or is associated with) activation of different cells. *In vivo* granulocytes, hyperactivated or damaged as a result of this process, contribute to the development of polyorganic failure and infectious toxic shock often accompanying meningococcal infection as its most grave complication [1,2]. The use of membrane inhibitors CD55, CD59, and CD35 manufactured by biotechnological methods as soluble proteins seems to be promising in therapy of endotoxic shock and other conditions associated with autoaggressive attack of the complement [15].

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