Correlation between endotoxin-neutralizing capacity of human plasma as tested by the limulus-amebocyte-lysate-test and plasma protein levels

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In the present study the endotoxin-neutralizing capacity of human plasma obtained from healthy volunteers was determined by use of the limulus-amebocyte-lysate test. The extent of the endotoxin-neutralizing capacity which showed a very broad variation was correlated to the plasma levels of some proteins which are believed to contribute to endotoxin transport and detoxification. The plasma levels of α_2-macroglobulin and transferrin, the major transport proteins, as well as the levels of IgG, IgA and IgM and apoprotein A were not significantly correlated to the neutralizing capacity. Only the apoprotein B, the major apoprotein of the low density lipoprotein fraction shows a significant correlation. Together with previously published results it should be concluded that low density lipoproteins are involved in the endogenous endotoxin-neutralizing reaction occuring in human plasma, at least when high doses of endotoxin are administered. Nevertheless the endotoxin-neutralizing reaction seems to be a very complex multistep process.

Endotoxin neutralization; Plasma; Plasma protein

1. INTRODUCTION

Endotoxins (lipopolysaccharide; LPS) are believed to represent the main pathogenetic mediators of Gram-negative bacteria [1,2]. The clinical symptoms of Gram-negative sepsis can be reproduced either by bacteria or endotoxin which is a major constituent of the cell wall of these bacteria [3]. Although bacteremia can be treated by antibiotics, very little knowledge exists about antiendotoxic agents. On the other hand, plasma is known to be a very potent endotoxin inhibitor [5]. In order to elucidate the nature of the endotoxin-inhibiting factors of human plasma, one approach is the characterization of the endotoxin-binding plasma constituents. The main endotoxin carrier system in blood is believed to be represented by the high density lipoprotein fraction [6,7]. Although some aspects of the LPS toxicity could be inhibited by the high density lipoproteins in vitro and in vivo, HDL-complexed LPS retained the ability to induce hypotension and death in a rabbit shock model [8,9]. Hypotension and death, however, could be prevented by lipoprotein-free serum fractions. In 1977, Johnson et al. described a plasma protein which is able to neutralize endotoxin in vivo [10]. A further characterization of this protein was not possible at that time. Flegel et al. [11] were able to demonstrate the inhibition of the endotoxin-induced release of tumor necrosis factor from isolated monocytes by human low density lipoproteins. To our knowledge there are no further in vivo studies concerning a possible benefit of the low density lipoproteins in preventing hypotension and death in the endotoxic shock. In 1987, the endotoxin-binding capacity of human transferrin and Gc globulin was demonstrated [12]. Furthermore, α_2-macroglobulin was shown to bind endotoxin in a specific manner [13]. In a prospective clinical study we demonstrated a strong correlation between the plasma levels of these proteins and the outcome of Gram-negative sepsis [14]. As shown by use of the limulus-amebocyte-lysate test, the endotoxin-neutralizing capacity of human plasma could be enhanced by addition of these endotoxin-binding proteins [15]. In order to obtain further information about the nature of the endotoxin-neutralizing capacity of human plasma, we developed a new method for determining the endotoxin-neutralizing capacity by the limulus-amebocyte-lysate test and tried to correlate the neutralizing activity with the levels of the described proteins presumed to be involved in the neutralizing reaction.

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2. MATERIALS AND METHODS

From 39 healthy volunteers blood was taken by puncture of the cubital vein under sterile conditions. The coagulation system of the samples was inhibited by the addition of 15 IE heparin/ml blood. Platelet-poor plasma was prepared by immediate centrifugation at $2000 \times g$ for 15 min. The samples were stored for one week at $-20°C$, before determining the endotoxin-neutralizing capacity and the protein levels.

The plasma levels of transferrin, α_2-macroglobulin, apoprotein A, apoprotein B, IgG, IgM, IgA, haptoglobin and α_1-acid glycoprotein were determined nephelometrically by use of the Bering nephelometer Analyser 100 (Behring Co., Marburg, Germany). Antisera as well as

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further buffers needed for the nephelometric determinations were also purchased from Behring Co. The inter- and intra-assay variation coefficients were in the range of 5% for all proteins tested.

The limulus-amebocyte-lysate test was delivered from Byk-Sangtec (Dietzenbach, Germany), together with the endotoxin standard, which is adjusted to the EC 5 standard of US Food and Drug Administration. The test itself was performed as a chromogenic two-step assay and endpoints were determined in microtiter plates (Greiner Co., Nürtingen, Germany). The chromogenic substrate, Pefachrome, was obtained from Pentapharm Co. (Basel, Switzerland).

The following solutions were used in the limulus-amebocyte-lysate test:

Solution A: lysate, solubilized in pyrogen free water according to the recommendations of the manufacturer
Solution B: chromogenic substrate, 10 μmol in 6.6 ml H_2O
Solution C: buffer, 0.05 mol/l Tris-HCl, pH 9.0, containing 0.2 mol/l NaCl
Solution D: acetic acid 20%

50 μl of the sample were incubated with 50 μl of the lysate for 25 min at 37°C. Afterwards 100 μl of the chromogenic substrate (solution B diluted 1:2 with solution C) was added and further incubated at 37°C for three min. The reaction was stopped by adding 200 μl solution D. The color formed, *p*-nitroaniline, was quantified at 405 nm in a spectrophotometer of SLT Co. (Salzburg, Austria), suitable for microtiter plates. The endotoxin content of unknown samples was determined according to a standard curve which was set up in parallel. Linearity was obtained from 0.06 EU/ml to 0.6 EU/ml.

In order to follow up the endotoxin-neutralizing capacity of plasma, 20 μl of the NP2 standard (lipopolysaccharide of *Salmonella abortus equi*, 100 ng/ml corresponding to 1000 EU/ml) were added to 180 μl plasma and incubated for 60 min at 24°C. After dilution 1:10 with isotonic sodium chloride the endotoxin content was determined according to the standard curve, without further inactivation, usually performed when the endotoxin content of biological samples should be determined. Time sequence studies showed that maximal inactivation was achieved after 20 min and did not change any more for up to 24 h.

The endotoxin content of the 1:10 diluted sample amounted to 10 EU/ml. The endotoxin recovery ranged from 0.15 to 0.6 EU/ml; these values are given in Fig. 1.

3. RESULTS AND DISCUSSION

For a long time plasma has been known to be a very potent endotoxin inhibitor [5,16]. Although there are some hints concerning the nature of the endotoxin-inhibiting plasma constituents, experimental studies yielded quite inconsistent results. Up to now the main endotoxin carrier system seems to be represented by the high density lipoproteins [6,7]. This lipoprotein fraction, however, could not diminish the endotoxin-induced hypotension in animals [9]. On the contrary, this capacity was found in a lipoprotein-free serum fraction. Other authors also demonstrated endotoxicity-influencing action in lipoprotein-free protein fractions [10,15]. Summarizing these data, there is no generally accepted hypothesis concerning the nature of the endotoxin detoxification process in plasma. In order to contribute to the solution of this question which is obviously of major clinical importance we developed a test to determine the endotoxin-neutralizing capacity based on the limulus-amebocyte-lysate test. The principle of our test simply consists in adding endotoxin to plasma and determining the endotoxin recovery without any further inactivation. Warren et al. demonstrated in 1985 that the limulus-amebocyte-lysate test is a simple method in order to determine the endotoxin-neutralizing capacity. In a turbidimetric assay they found the neutralizing capacity as determined in the LAL test correlated to the antipyrogenic activity in the rabbit fever test, so that a direct effect of plasma on the enzymatic cascade of the limulus lysate seems to be improbable [17]. By use of this assay the endotoxin-inactivating capacity of plasma samples of 39 healthy volunteers was determined. There was no correlation to sex or age, although the absolute value of recovery varies from below 0.15 EU/ml to 0.6 EU/ml.

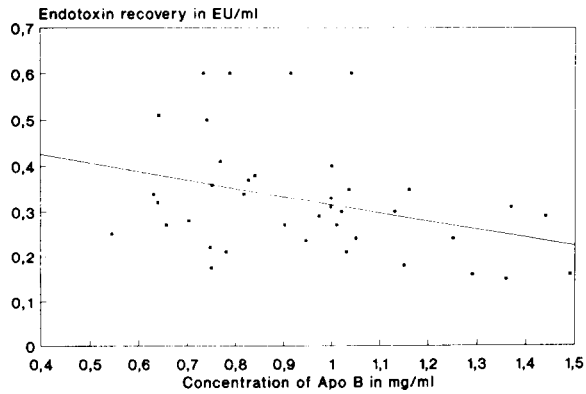

Fig. 1. Linear correlation between the endotoxin recovery and the plasma level of apoprotein B. The ordinate shows the endotoxin recovery in EU/ml, on the abscissa the plasma levels of apoprotein B are depicted. The endotoxin content of the sample amounted to 10 EU/ml.

Recently we described endotoxin-binding capacity of human transferrin and α_2-macroglobulin [12,13]. According to the previously cited studies, also the high density lipoprotein fraction or the low density lipoproteins seem to be involved in the endotoxin transport. In order to elucidate a relationship between the endotoxin neutralization and the plasma constituents we determined the plasma levels of transferrin, α_2-macroglobulin, apoprotein A, and apoprotein B. Furthermore the levels of the acute phase reactants α_1-acid glycoprotein and haptoglobin were determined in order to exclude any stress reaction which would be seen as an increase of the plasma levels. In all samples no increase above the normal range could be observed. Also the volunteers did not report any signs of disease in the last 4 weeks.

The acute phase reactants haptoglobin and α_1-acid glycoprotein did not correlate to the endotoxin-neutralizing capacity (results not demonstrated). The same fact passes for transferrin and α_2-macroglobulin (Table I). As would be expected, also the plasma levels of IgG, IgA and IgM did not show any correlation. Although transferrin and α_2-macroglobulin possess a high affinity to endotoxin [13], a contribution to the neutralizing

Table I

Correlation coefficients and statistical significance

	Apo A	Apo B	Transferrin	α_2-macroglobulin	IgG	IgM	IgA	Apo B (reciprocal correlation)
Correlation coefficient	0.005	−0.33	0.16	−0.05	−0.18	−0.11	−0.17	0.47
$P<$	0.97	0.04	0.3	0.79	0.27	0.52	0.3	0.003

The table demonstrates the coefficients of a linear correlation between the levels of the different proteins and the endotoxin recovery. Furthermore the P value is given. As to the case of the plasma levels of apoprotein B, a reciprocal correlation ($1/y = ax + b$) was tested and a highly significant result obtained was also demonstrated. The high coefficient of the reciprocal correlation implies that the correlation is not a linear one. Obviously the same differences of the concentration of apoprotein B at very low protein levels lead to a greater difference of the endotoxin-neutralizing capacity, as the same differences of the protein concentration in the higher concentration range of apoprotein B.

activity seems to be ruled out, at least in the assay used in this study. These results are also in contrast with a clinical study, in which a strong correlation between the plasma levels of transferrin and α_2-macroglobulin and the outcome of Gram-negative sepsis was demonstrated [14]. Also, an experimental study was able to show an endotoxicity-influencing effect of transferrin. However, it should be taken into account that the endotoxin concentrations found in plasma of septic patients, are in the pg/ml range, as well as the endotoxin concentrations which were used in the previous study showing that the influence of transferrin on endotoxicity was quite low [19]. In the assay used in the present study, ng amounts of endotoxin were added to plasma. So an explanation of these inconsistent results could be that at low endotoxin concentrations an inhibiting effect is possible or even probable. At pharmacological doses both proteins cannot contribute to the neutralizing action.

The Ig classes also did not exhibit any correlation with the endotoxin-neutralizing activity. Perhaps these results obtained for the immunoglobulins could be explained in an analogous way. Furthermore the blood was obtained from healthy volunteers, and not after immunization with endotoxin. Experimental studies that showed an anti-endotoxic effect of the immunoglobulin fractions were usually performed with hyperimmune sera [20,21]. Warren et al. also demonstrated the lack of immunoglobulins and some other serum proteins in a system similar to that one used in our experiments to contribute to the endotoxin-neutralizing capacity [17]. They did not find a correlation with the levels of antilipid A antibodies, since the levels of these specific immunoglobulins found in non-immunized men are very low. This protein class would be expected not to contribute to the inactivation process in our system. As to the case of apoprotein A, there is also no correlation, which is in good agreement with the results of Abdelnoor et al. who showed the lack of endotoxin-neutralizing activity of high density lipoproteins in vivo [9].

The only correlation betwen the neutralizing activity and the serum level of a protein was observed in the case of apoprotein B (Fig. 1). This correlation is quite low, as can be seen in Table I and also in Fig. 1, where the serum levels of the protein are given on the abscissa and the endotoxin recovery is depicted on the ordinate. The correlation is a negative one, meaning that the higher the endotoxin recovery, the lower the protein levels determined. Similar results were reported by Flegel et al. [11] who used isolated monocytes. They found that the inhibition of the endotoxin-induced release of tumor necrosis factor from isolated monocytes depends on the low density lipoproteins because LDL-free serum exhibited no inhibition of the TNF release. These authors described the necessity to incubate serum with endotoxin for at least 3 h, in order to see any effect. In our system we tested different incubation times between plasma and endotoxin from 20 min to 24 h and found maximal inactivation already after 20 min. Shorter incubation times were not studied because this parameter should be considered with caution. The inactivation process was not stopped before the samples were diluted and added to the limulus-amebocyte-lysate test. So during the 35 min of incubation between sample and lysate the inactivation process can go on. Nevertheless, in our system the inactivation seems to run off much faster than in the monocyte system. In spite of this, the correlation in our assay is in principal in accordance with the cell culture study.

The main question arising from our results, however, is: is there any physiological significance of the LDL-endotoxin interaction? As described above, the endotoxin amounts which were added to plasma were nearly 100 times higher than those ones found in human disease. So our results possibly only reflect an unspecific mechanism occurring when the endogenous and pathophysiologically more important specific neutralizing activity is exhausted. Nevertheless, there could be a therapeutic implication if also in in vivo experiments an antiendotoxin effect of LDL can be observed. Our results, together with the former study of Flegel et al. should justify animal experiments to elucidate the anti-endotoxin process in plasma, in order to develop a specific anti-endotoxin therapy in the future, which is one of the most urgent problems of intensive care medicine.

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