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Analysis of Bypass Activation of C3 by Endotoxic LPS and Loss of this Potency

M. P. DIERICH, D. BITTER-SUERMANN, W. KÖNIG, U. HADDING, C. GALANOS AND E. T. RIETSCHEL

Institut für Medizinische Mikrobiologie der Johannes-Gutenberg-Universität, 65 Mainz/ Germany and Max-Planck-Institut für Immunbiologie, 78 Freiburg i. Br./Germany

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Summary. Endotoxic lipopolysaccharides prepared from *Salmonella minnesota* smooth form (LPS-S) and rough form, R 595 (LPS-R) activate C3 in guinea-pig serum as is demonstrated by C3 kinetics and generation of anaphylatoxic activity. The activation depends on the presence of certain serum factors, one of which is the protein (SF) interacting with the cobra venom factor, on the presence of Mg^{++}, the temperature and on dose of LPS applied.

This turnover of C3 is terminated at 37° within 2 minutes (LPS-R) to 12 minutes (LPS-S) and then reaches a plateau. The amount of C3 consumption, i.e. the level of the plateau, is typical for given concentrations of LPS-S and LPS-R (intermediate plateau). Above a certain LPS-concentration additional LPS does not induce further C3 turnover (maximal plateau). While the 'maximal plateau' may be explained by a limitation of the factors needed for the LPS dependent consumption of C3, the 'intermediate plateau' and the termination of the action on C3 are not well understood. It is hypothesized that coating of the LPS molecule by serum protein, e.g. albumin, inactivates LPS-S and LPS-R with regard to their action on C3. C3 and C5 in normal serum concentrations appear not to be involved. It is suggested that an additional mechanism for termination of LPS action is a rapid loss of activity of LPS-induced intermediates.

INTRODUCTION

Endotoxic lipopolysaccharides (LPS) interact with the complement system (C) through preferential consumption of the 6 terminal complement components (Gewurz, Shin and Mergenhagen, 1968). This interaction takes place with the same efficiency in C4 deficient guinea-pig serum and thus does not depend on C1, C4 and C2 but represents an alternative pathway of C-activation starting with C3 (Frank, May, Gaither and Ellman, 1971).

The influence of normal serum on LPS-containing enterobacteriaceae has been thoroughly investigated in kinetic studies (Glynn, 1969; Davis, Gemsa and Wedgwood, 1966). In these reports bacterial killing and bacteriolysis served as parameters for the reaction based on the concept: antibody-complement-lysozyme. In that sequence the role

Definition and nomenclature of the complement system follow the bulletin of the WHO (1968).

of a so-called natural antibody represents an open question. It is not intended to contribute to this problem by the experiments reported in this paper. But as a hypothesis we suggest that the serum factors of the alternative pathway might serve as a link inducing the interaction of LPS with C.

It is the purpose of this paper to investigate in normal guinea-pig serum the LPS induced consumption of C3 as a key-component and primary LPS-C interaction site. The kinetics and quantitative aspects of the C3-turnover will be analysed as well as 'detoxification' phenomena with regard to the action of LPS on C3.

MATERIALS AND METHODS

Lipopolysaccharides (LPS)

Three different LPS-preparations were used. (a) LPS of *Salmonella minnesota* smooth form (LPS-S); (b) LPS of *S. minnesota* rough form, R 595 (LPS-R), (c) Lipid-A coupled to BSA. These preparations were made as described previously (Galanos, Rietschel, Lüderitz and Westphal, 1971).

Cobra venom factor (VF)

VF was used in chemically pure form (Bitter-Suermann, Dierich, König and Hadding, 1972).

Cobra venom co-factor (SF)

SF was used in a functionally pure form (Bitter-Suermann *et al.*, 1972). It seems to be the guinea-pig analogue to the human C3-proactivator (C3PA).

SF-depleted guinea-pig serum

Guinea-pig serum was deprived of SF by several adsorptions at 37° with cobra venom factor (VF) coupled to sepharose with the bromcyanide method (Edelman, Rutishauser and Millette, 1971). The adsorption was thought to be complete if no further C3-turnover in the sample could be induced by new sepharose-VF.

Mg^{++}-and Ca^{++}-free guinea-pig serum

To guinea-pig serum EDTA was added to a final concentration of 0·05 M. Ten millilitres of this serum were passed twice through a Sephadex G-25 column (5×20 cm) equilibrated with a 0·02m Tris-HCl buffer, pH 7·5 plus NaCl to a final concentration of 0·2 M. The twice chromatographed serum samples were dialysed for 48 hours against the same Tris-HCl buffer to remove the remaining minimal amounts of Mg^{++}, Ca^{++} and EDTA salts. This serum did not lyse sensitized erythrocytes, not even in the presence of Mg^{++} or Ca^{++} alone. But addition of both ions led to strong lysis.

Standard buffer

As standard buffer veronal buffered saline (VBS) was used, in some cases gelatine was added to a final concentration of 0·1 per cent.

Albumin, γ-globulin

Bovine serum albumin and γ-globulin (Beriglobin) were purchased from Behring Werke AG., Marburg/Germany.

Test for C3

The test was performed with EAC142 cells and C5-C9 in excess. The amounts of haemolytically active C3 are given as optical density values at 412 nm which are directly proportional to the amount of C3 present in the test sample (appropriate dilution of the incubation mixture) and inversely proportional to the turnover of C3 in the incubation mixture. They are expressed as site-forming units (SFU) (Bitter-Suermann, Hadding, Melchert and Wellensiek, 1970) in case the turnover of C3 exceeds the amount demonstrable within one dilution range by optical density values.

Every test includes three controls: ——— EAC142 cells plus C5-C9; - - - - - - EAC142 cells plus buffer; (1) 100 per cent lysis of EAC142.

Tests for anaphylatoxic activity were performed as described (König, Dierich, Bitter-Suermann and Hadding, 1972a).

RESULTS

In the following experiments we dealt with two different LPS-preparations: LPS-S, LPS-R and in a few experiments with Lipid-A. Our interest was focused on the interaction with C3.

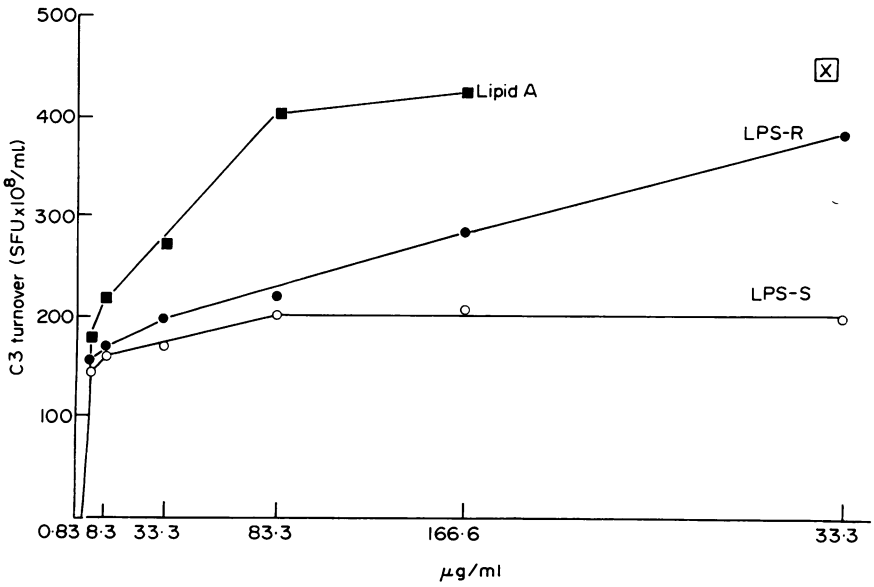

FIG. 1. C3-turnover by LPS-S, LPS-R, Lipid-A. On the abscissa the amount of LPS in μg LPS/ml incubation mixture is given. The incubation mixture consisted of 100 μl guinea-pig serum+200 μl solution of LPS in H_2O. The mixture was kept 20 minutes at 37°. The ordinate gives the amount of C3 turnover expressed as SFU. ×, input of haemolytically active C3.

On incubation of 200 μl LPS solution together with 100 μl guinea-pig serum a turnover of C3 takes place dependent on the amount and preparation of LPS applied (Fig. 1). With increasing amounts of LPS the total C3 turnover increases. But, when calculated per μg LPS, the relative turnover decreases. The efficiency of the three endotoxins increases markedly in the sequence LPS-S, LPS-R, Lipid-A. As shown in Fig. 2, the turn-

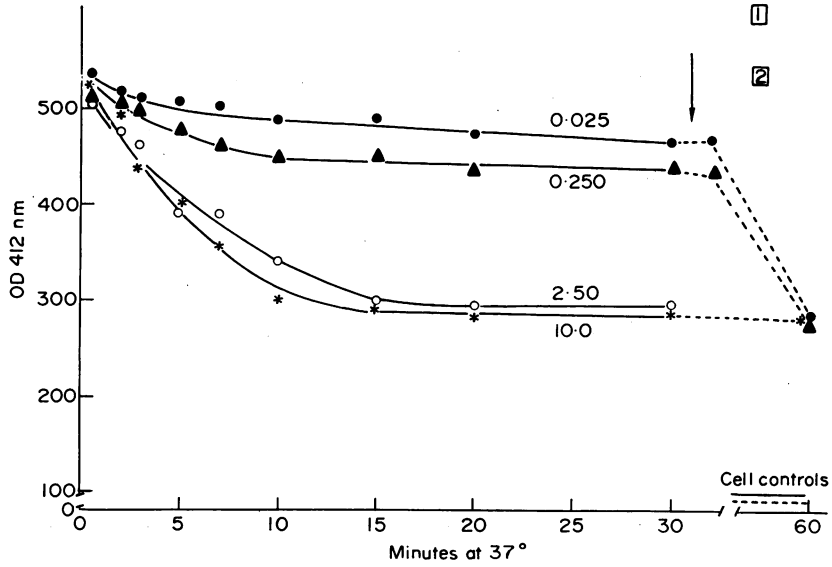

FIG. 2. Time dependence of C3 turnover in guinea-pig serum by increasing amounts of LPS-S. Ordinate: optical density values at 412 nm obtained from the haemolysis test. The values are obtained from an appropriate subdilution of the mixture after the given incubation time. High values correspond to high amounts of C3, i.e. low consumption of C3. Low values correspond to high consumption, i.e. high LPS activity. 1, 100 per cent lysis of the test cells. 2, Maximal amount of C3 in the incubation mixture. Cell controls: see Materials and Methods.

The figures on the curves represent the amount of LPS-S in μg/test mixture. Such a test mixture consisted of 100 μg guinea-pig serum + 100 μl LPS-S solution in distilled water. At different time intervals the amount of haemolytically active C3 was determined. After 32 minutes a further 10 μg LPS-S were added to the mixtures containing 0·025, 0·25 and 10 μg LPS-S. The amount of haemolytically active C3 was determined immediately and 30 minutes later after incubation at 37°.

over induced by LPS-S takes place during the first 12–15 minutes. After that no further loss of C3 occurred, the values forming a plateau, although an excess of C3 was still available. The position of the respective plateaux is a function of the LPS–S input. With a certain LPS-S concentration (in Fig. 2 ca. 10 μg/incubation mixture) a plateau is reached which cannot be lowered by further addition of LPS-S or fresh guinea-pig serum. Provisionally this plateau is the 'maximal plateau'. In some experiments we noticed that by adding excessive amounts of LPS-S the level of the plateau went up again, so that beyond a certain concentration LPS-S seems to be less efficient, as was also noted by Galanos *et al.* (1971) testing not C3 but CH50-units. In contrast to the maximal plateau the plateaux gained with smaller concentrations of LPS-S can be lowered by further LPS-S input. But even then the plateau values do not exceed those of the maximal one. We call the second type the 'intermediate plateau'. Neither type changes for at least 6 hours, the longest time they were followed up.

Similar plateaux were obtained with LPS-R (Fig. 3), but there are several differences in comparison with LPS-S: First, the C3-turnover by LPS-R is faster. Second, the level of the LPS-R maximal plateau is lower representing more C3 turnover as can be seen in Fig. 4. This Fig. shows a time course experiment, where the C3 turnover in guinea-pig serum is continuously measured during the sequential addition of LPS-S, LPS-R and VF. Third, the LPS-S maximal plateau can be lowered by addition of LPS-R, but only to the level of the LPS-R maximal plateau. Not shown in Fig. 4 is the fact that in repeated

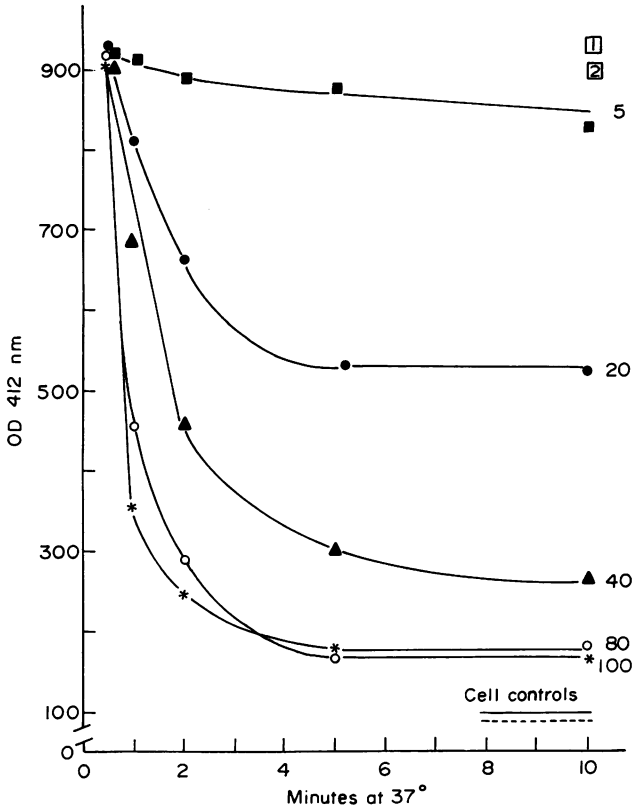

FIG. 3. C3 turnover by increasing amounts of LPS-R. Ordinate (1) and (2), cell controls, see Fig. 2. The figures by the curves give the amount of LPS-R as μg/ml test mixture. Such a test mixture consisted of 100 μl guinea-pig serum+200 μl LPS-R solution (LPS-R in H_2O).

experiments it was never possible to depress the LPS-R maximal plateau by addition of LPS-S. A further depression of the LPS-R and LPS-S maximal plateau can be provoked by addition of cobra venom factor (VF) (Fig. 4). That suggests that the serum factor (SF) needed for C3 turnover by VF is still available in a sufficient amount. Apparently other co-factors are limited for the LPS-system. In an incubation mixture of 100 μl guinea-pig serum and 100 μl LPS-S solution (100 μg/ml H_2O) a 1 : 20 dilution of the serum causes a reduction of C3 turnover. Applying a 1 : 50 dilution almost no C3 consumption was noticed.

To elucidate the involvement of SF in the turnover of C3 by LPS we used two types of sera, an SF depleted one and a serum in which the SF was heat inactivated (50°, 30 minutes). With both sera no C3 turnover could be provoked on incubation with LPS. Addition of purified SF to both sera led to a turnover of C3 by LPS, which was very limited in the SF depleted one. Both experimental conditions point to a role for SF in the co-factor system of LPS dependent C3 turnover. The role of Mg^{++} and Ca^{++} in the LPS-complement interaction was also investigated. It was found that addition of LPS to a serum containing 0·01 M EDTA does not lead to turnover of C3. To demonstrate which ion is needed, we used an ion-deprived serum. By adding Mg^{++} and/or Ca^{++} in increasing amounts we could show that only Mg^{++} is required. In a mixture of 100 μl

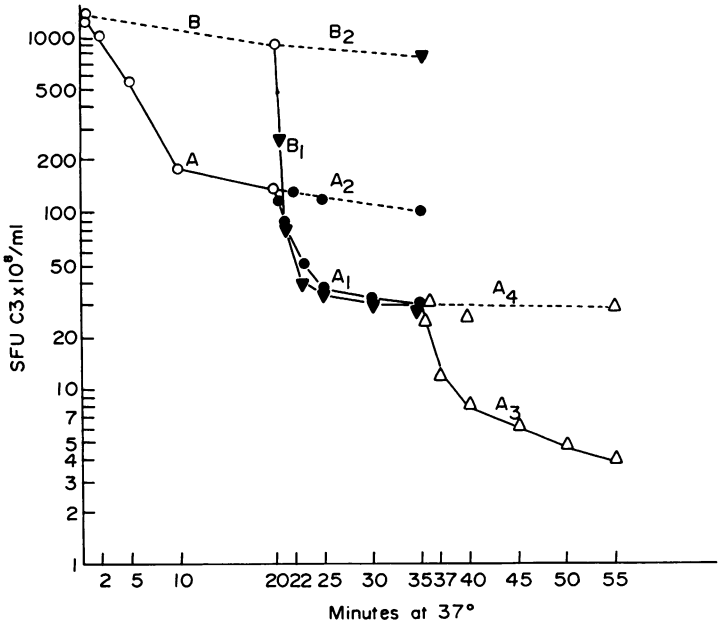

FIG. 4. C3 turnover in guinea-pig serum by sequential addition of LPS-S, LPS-R and VF. Ordinate (in a logarithmic scale): amount of haemolytically active C3 given as SFU available in the test solution A, guinea-pig serum+LPS-S (85 μg LPS-S/ml incubation mixture) corresponding to LPS-S maximal plateau. B, guinea-pig serum control without LPS-S. At 20 minutes A_1 and B_1 and their respective controls A_2 and B_2 started. A_1, incubation mixture A+LPS-R (250 μg LPS-R/ml incubation mixture) corresponding to LPS-R maximal plateau. A_2, incubation mixture A without addition of LPS-R. B_1, incubation mixture B+LPS-R (250 μg LPS-R/ml incubation mixture). B_2, incubation mixture B without addition of LPS-R. At 35 minutes A_3 and its control A_4 started. A_3, incubation mixture A_1+VF (1·5 mg VF/ml incubation mixture). A_4, incubation mixture A_1 without VF. By addition of LPS-R, VF or H_2O to the incubation mixtures at 20 and 35 minutes the guinea-pig serum was diluted only to a minimal extent.

ion-free guinea-pig serum and 100 μl LPS-S solution (100 μg/ml H_2O) a concentration of 5×10^{-4} M Mg^{++} was optimal.

During our experiments with ion-free serum we found that it was necessary to add Mg^{++} at the very beginning to the mixture of ion-free guinea-pig serum and LPS at 37° to obtain the maximal LPS effect on C3. If Mg^{++} was added with a delay of up to 10 minutes to the incubation mixture a reduced or even no C3 turnover was provoked during a further 20 minutes of incubation at 37°. As demonstrated in Fig. 5 the possibility of initiating C3 consumption by Mg^{++} and the degree of the consumption was a function of the time and temperature of preincubation. Short preincubation prior to the addition of Mg^{++} or low temperature facilitated high C3 turnover.

A total reduction of the potency of LPS-S in inducing C3 turnover can also be obtained by preincubating LPS-S or LPS-R for one minute in a 4 per cent albumin solution or to a lesser degree in a 1 per cent solution (0·25 per cent gave no inhibition). In contrast, incubation of LPS-S with 1 per cent γG, 0·05 per cent gelatin or purified C3 (1200 μg/ml) or purified C5 (350 μg/ml) had no effect on its capacity to consume C3. It may be possible that these substances at a concentration far exceeding that in normal serum have an effect. On the other hand, the presence of phosphate in a concentration of 0·002 M also reduced the LPS induced C3 turnover. 0·1 M was completely inhibiting. Phosphate exerts

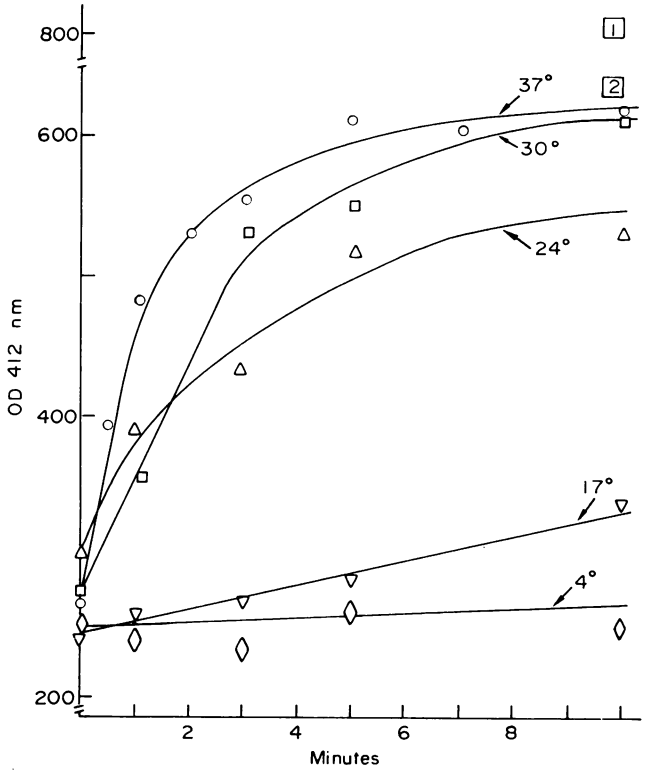

FIG. 5. Inactivation of LPS-S by ion-deprived serum. Ordinate, 1 and 2, see Fig. 2. The incubation mixture of 100 μl ion-deprived serum and 100 μl LPS-S solution (200 μg/ml H_2O) was kept at 4°, 17°, 24°, 30° and 37° for different time intervals up to 10 minutes. After that 10 μl $MgCl_2$-solution (5×10^{-3} m) were added. This mixture was then immediately brought to 37° for a further 40 minutes. After this the amount of haemolytically active C3 was determined. High amounts of haemolytically active C3 are equivalent to a low turnover, i.e. a high inactivation rate of LPS; low C3 amounts correspond to a low inactivation rate.

its effect by interaction with Mg^{++} for the inhibition can be prevented by addition of sufficient Mg^{++}.

These data are to be kept in mind when one discusses the hypothesis that the end of the C3 turnover by LPS and the beginning of the plateau respectively are determined by a complete decay of the enzymatic activity of an LPS–co-factor complex directed towards C3. In the ion deprived serum or where LPS was preincubated in high concentrations of albumin inactivation occurred without the existence of a complex active against C3. The reason for the inactivation might be an unspecific coating of the active centre of the LPS molecule.

So the question remained unanswered whether decay of a LPS–co-factor complex contributes to the inactivation of LPS with respect to the C3 turnover parallel to the coating. We, therefore, added EDTA (0·01 M) to the mixture of guinea-pig serum and LPS-R during the phase of the maximal C3 turnover (10–120 seconds). This stopped the Mg^{++} dependent formation of LPS–co-factor complexes. So any C3 turnover after addition of EDTA would have been induced by active complexes present at the time of addition of the ligand. We expected a turnover of C3 especially since among other C3

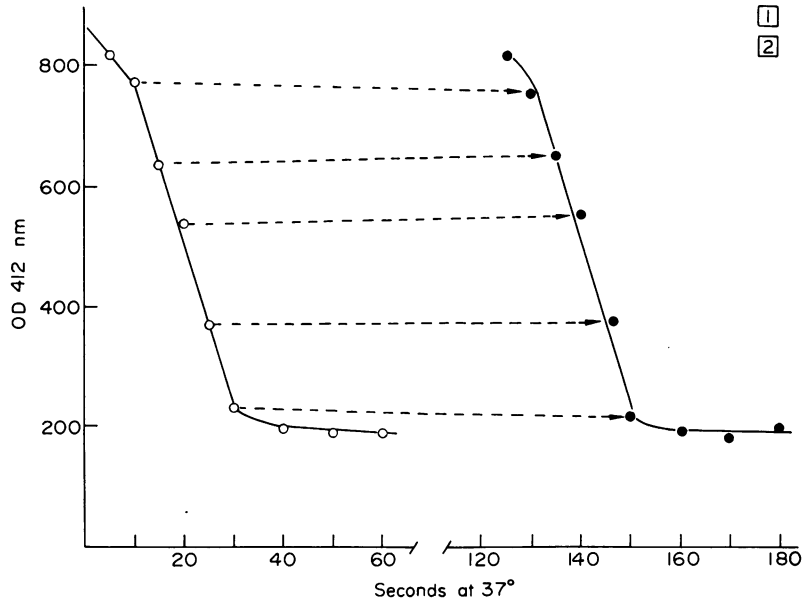

FIG. 6. Interruption of the LPS-induced action on C3 in guinea-pig serum by addition of EDTA. Ordinate, 1 and 2, see Fig. 2. 1400 μl guinea-pig serum + 800 μl LPS-R solution (500 μg/ml H_2O) were incubated at 37°. After certain time periods (0–60 seconds) two samples of 50 μl were taken, one to determine the amount of haemolytically active C3 present in the solution (○). To the other 5 μl EDTA-solution were added to obtain a final concentration of 0·01 m. This mixture was kept for a further 2 minutes at 37°. After this period the amount of haemolytically active C3 was determined again (●). The dotted lines connect the values of the respective two test samples.

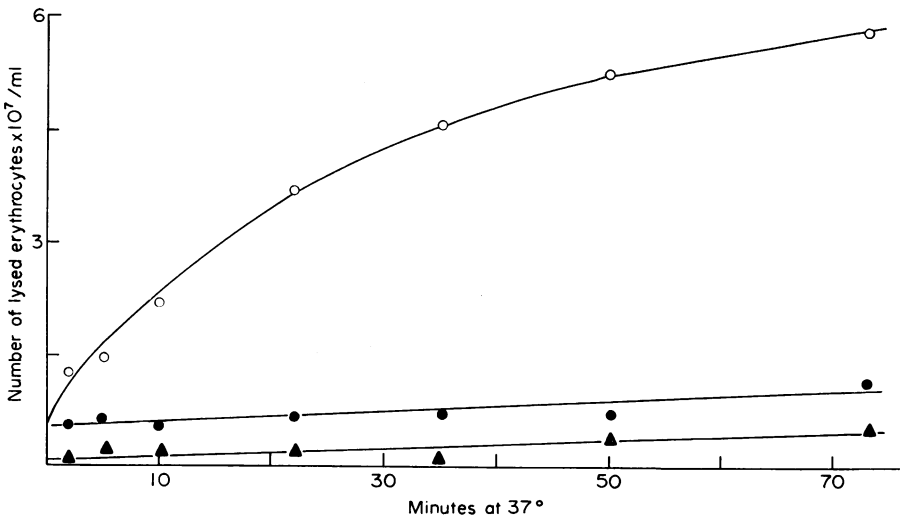

FIG. 7. Lysis of erythrocytes by LPS-R in an autologous system of E^{gp} and $serum^{gp}$. 500 μl E^{gp} ($\times 10^9$/ml VBS) + 500 μl LPS-R solution (500 μg/ml 0·15 M NaCl) incubated 60 minutes at 37°, washed three times in VBS, cells corrected to 1×10^9 cell/ml VBS. These cells were named E^{LPS}. In a control experiment 500 μl LPS-R solution were replaced by 0·15 m NaCl. These cells were named E-control. These two cell pools were used during a further incubation of up to 70 minutes at 37°. (▲) 300 μl E-control (1×10^9/ml VBS) + 300 μl $serum^{gp}$ + 300 μl NaCl. (●) 300 μl E (1×10^9/ml VBS) + 300 μl $serum^{gp}$ + 300 μl LPS-R solution (500 μg/ml NaCl). (○) 300 μl E^{LPS} (1×10^9/ml VBS) + 300 μl $serum^{gp}$ + 300 μl NaCl.

converting enzymes the C3 convertase and the VF·SF-enzyme (Bitter-Suermann *et al.*, 1972), once generated, act in the presence of EDTA. But as shown in Fig. 6 no C3 turnover at all could be observed. Any action on C3 was stopped immediately. The same was true for the consumption of C5 to C9 (unpublished data).

To demonstrate that the consumption of native C3 in guinea-pig serum by LPS correspond to an activation of C3 we tried to induce lysis of unsensitized guinea-pig red blood cells by autologous guinea-pig serum. If the erythrocytes were incubated first in a LPS-solution, then washed three times in VBS and then exposed to guinea-pig serum there was a much better lysis of E in comparison to the lysis obtained by simultaneous incubation of E, serum and LPS (Fig. 7). So one can assume that the high degree of lysis is based on the fixation of LPS-R, prior to its inactivation in the fluid phase, to the endotoxin receptor of E (Arend and Springer, 1971) and the activation of complement components in close contact to the membrane. The same experiments were performed in ion-free serum reconstituted with Mg^{++}. Lysis occurred proportional to the Mg^{++} concentration.

Another proof of activation of C3 and C5 by LPS was obtained from the fact that Lipid-A as well as LPS-R and LPS-S generated anaphylatoxic activity on incubation with guinea-pig serum. As can be seen (Fig. 8) the fast contraction is followed by a long-lasting one. The latter could not be prevented by antihistaminic substances like Casantin (10(2-diethylamino-ethyl)phenothiazine) (König, Dierich, Bitter-Suermann and Hadding, 1972a).

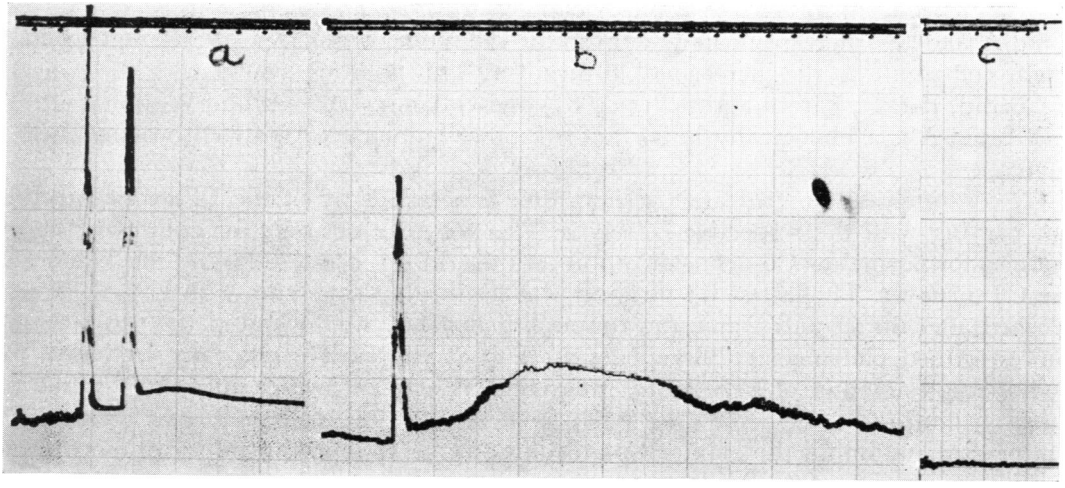

FIG. 8. Generation of anaphylatoxin activity by incubation of guinea-pig serum with Lipid-A. (a) Contraction of guinea-pig ileum provoked by histamine (1×10^{-7} M). (b) Fast contraction of guinea-pig ileum induced by the mixture of guinea-pig serum and Lipid-A (50 μg/ml guinea-pig serum). This mixture had been kept at 37° for 30 minutes. After the fast contraction a slow long-lasting one appeared. (c) Control: Lipid-A without guinea-pig serum.

DISCUSSION

It is reasonable to assume that the activation of the terminal complement components in normal serum by LPS is induced via the alternative pathway of complement activation.

According to Galanos and Parant (manuscript in preparation) a natural antibody against LPS is responsible for a part of the LPS induced action on complement. This so-called natural antibody may be part of the complex co-factor system that is utilized by different LPS for the activation of C3 and thereby the bypass-inducing centre is located on the LPS molecule. Alternatively such a natural antibody may react with LPS as an antigen and then induce the bypass reaction analogous to the γ1-antibody of the guinea-pig (Sandberg and Osler, 1971; König, Bitter-Suermann, Dierich and Hadding, 1972b). That does not exclude an activation via the classical pathway, i.e. C1, C4 and C2 provided there are specific antibodies available after immunization with LPS.

The following data are in favour of the alternative pathway: 1. LPS induced a turnover of the terminal complement components in normal as well as in C4-deficient guinea-pigs (Frank *et al.*, 1971). 2. Anti-C2-antibodies did not reduce the C3-turnover by LPS in normal guinea-pig serum (Marcus, Shin and Mayer, 1971). 3. The results presented here show that one of the co-factors of the alternative pathway—which is needed in the cobra-venom (Bitter-Suermann *et al.*, 1972) and γ1-model (König *et al.*, 1972b) is also involved in the LPS-induced turnover of C3. 4. C3-turnover by LPS is dependent only on Mg^{++} without any need for Ca^{++}, which is in agreement with previous findings of Pillemer, Schoenberg, Blum and Wurz (1955).

The total C3-turnover differed for the three LPS-preparations and increased per μg LPS in the sequence LPS-S, LPS-R and Lipid-A. Higher LPS-concentrations were less effective when the relative turnover of C3 was calculated per μg. The Lipid-A moiety is common to the three types of LPS used and becomes more and more accessible from the S- over the R-form to the free Lipid-A coupled to BSA. The increase in accessibility of Lipid-A and the increase in its w/w ratio for the sequence LPS-S, LPS-R and Lipid-A might contribute to the increasing C3 turnover. This is in agreement with the concept of Chedid, Parant, Parant and Boyer (1967) and Galanos, Lüderitz and Westphal (1971) that Lipid-A is a biologically important substance common to the LPS-bearing enterobacteria.

C3 consumption stopped after a short time independently of the LPS-concentration but varying with the respective LPS-type. This was true not only for guinea-pig serum but also for normal or C6-deficient rabbit serum, which did not differ in their kinetics of the C3 turnover. The fastest C3 turnover was seen in human serum with LPS-R. It was finished after 30 seconds–2 minutes (unpublished data). At the end of the turnover the curves formed plateaux at different levels (Figs 2 and 3). The plateaux are stable for hours. Small amounts of LPS led to 'intermediate' plateaus, which could be lowered by further addition of the same LPS-type down to the respective 'maximal' plateau. A simple explanation for the maximal plateaux would be a limitation of one or more of the cofactors needed for C3 consumption.

The SF, which seems to be the guinea-pig analogue of human C3PA, is in absolute terms not limited in sera after formation of maximal plateaux, since it is still available for interaction with VF followed by marked C3 turnover. The participation of SF in the LPS induced C3 consumption was demonstrated using SF depleted serum. In that reagent LPS was unable to consume C3.

It is difficult to explain the fact that LPS-R is able to lower a LPS-S induced 'maximal' plateau. Even though both endotoxins are prepared from *S. minnesota* strains, it is assumed that LPS-R uses in normal serum the limited amount of co-factors in a different or more effective way than LPS-S and thus can still act when the limits of LPS-S action have been reached.

The events leading to rapid termination of the LPS-induced C3 consumption remained obscure. The following data have to be evaluated in the discussion of inactivating mechanisms.

1. Preincubation of LPS in albumin solution equivalent to the albumin concentration in normal sera inactivated LPS in less than 1 minute with regard to the induction of C3 turnover. This can be viewed as coating or masking of the active site of LPS. Purified C3 or C5 did not lead to such a coating.

2. The turnover of C3 via LPS is Mg^{++} dependent and can thus be prevented by EDTA. Phosphate ions are also able to inhibit the reaction. Fifty per cent inhibition is reached in serum with 0·01 M.

3. As revealed by addition of Mg^{++} at different time intervals to LPS in ion-free serum (Fig. 5) the loss of C3 consuming potency took place with the same velocity as in normal serum, even though no C3-cleaving enzyme had been generated. The curves in Fig. 2 and Fig. 5 (37°) thus reflect corresponding mechanisms of LPS inactivation. Since it is unknown at which step of the reaction sequence Mg^{++} is needed, various intermediates therefore might be formed. A possible masking mechanism of either the LPS molecules or the mentioned intermediates might be the underlying event for inactivation. In addition, a a very rapid decay of intermediates has to be considered. But it is unlikely that the decay of a distinct intermediate common for all tested LPS-preparations is responsible for the velocity of the inactivation. Applying the different LPS the velocity for the inactivation of the C3-consuming principle varied markedly (2–15 minutes) in the same serum batch.

4. As mentioned under (2) EDTA inhibits the formation of a LPS-induced C3-consuming principle. In analogy to known C3-converting enzymes $C\overline{42}$ and VF-SF (Dierich, Bitter-Suermann, König and Hadding, 1971), we expected a C3 turnover in the presence of EDTA once the C3-converting principle had been formed (Gewurz, Pickering, Snyderman, Lichtenstein, Good and Mergenhagen, 1970). But addition of EDTA to a mixture of LPS and serum at the time of maximal C3 turnover stopped immediately the C3-consumption (Fig. 6). This fact might be interpreted in the following ways: (a) The last step of the LPS-induced reaction, i.e. the C3 turnover proper, is Mg^{++} dependent. (b) EDTA destroys instantaneously the active principle. (c) As mentioned, EDTA only prevents the further formation of C3-converting principle, but this would imply that the C3 consuming principle already present at the time of EDTA-addition is active only for seconds. The curves for C3 turnover would then reflect the summation of the effect of very short-lived activities.

5. In contrast to Marcus, Shin and Mayer (1971), we were unable to isolate LPS-complexes active against isolated C3 (unpublished data). This would be in accordance with the discussed very short-lived activity. From these data we tend to assume that the potency of LPS to induce C3 turnover is terminated by coating or masking of the active centre of LPS and possibly of active intermediates on the LPS molecule. The complexes which are generated by LPS before coating become inactive nearly instantaneously either by rapid decay or by rapid inactivation mediated by serum proteins.

In the whole spectrum of the biological activities of endotoxin interaction with the complement system plays an important role. This interaction leads to activation of terminal complement components as demonstrated by lysis of LPS-coated erythrocytes and the generation of anaphylatoxic peptides. The widely used term 'detoxification' has therefore to include the loss of LPS-activity directed against complement. In contrast to

the range of several hours given in the literature for overall detoxification (Skarnes, 1970) we found an inactivation range of minutes with regard to a primary point of LPS-induced attack on the complement system, i.e. C3. Taking into account the generation of biologically active fragments derived from complement components with chemotactic and anaphylatoxic activity, i.e. C3a and C5a, and the influence on other systems, e.g. the clotting system (Zimmermann and Müller-Eberhard, 1971) it is reasonable that the detoxification of these substances or secondary effects might require longer. The role of C3 as mediator for endotoxin shock was recently underlined (Fong and Good, 1971).

The question whether the data presented here are pertinent to the lysis of LPS carrying bacteria in normal serum, which starts with a log-phase of 5–10 minutes (Davis *et al.*, 1968), has to be clarified. It is reasonable to assume, that our *in vitro* experiments have been focused on those events, which take place during this log-phase.

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NOTE ADDED IN PROOF

After submission of this paper we received C4-deficient guinea-pigs from Dr M. M. Frank (N. I. H. Bethesda, Washington D.C., U.S.A.). With sera from these animals we have confirmed the results reported here.