INTERACTIONS OF THE COMPLEMENT SYSTEM WITH ENDOTOXIC LIPOPOLYSACCHARIDES IN IMMUNOGLOBULIN-DEFICIENT SERA*

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Endotoxic lipopolysaccharides (LPS)1 have a potent ability to interact with complement (C) during incubation in normal mammalian serum (2, 3). Lesions indicative of terminal C component activation appear on LPS (2, 4) while, concomitantly, C components are consumed and C-dependent biologically active by-products (neutrophil chemotactic factor and anaphylatoxin) are generated (5–7). As a result, it has been postulated that certain toxicities induced by endotoxins may be mediated via the C system (8, 9).

One striking feature of the LPS-C interaction in fresh guinea pig serum is that marked consumption of the six terminal C components (C3-C9) occurs, even though minimal consumption of the earlier-acting C components (C1, C4, and C2) is detected (5). When hyperimmune serum is added to the reaction mixtures, pronounced consumption of C1, C4, and C2, as well as C3–C9, is readily seen (3). This has raised the question of whether LPS activates the C3–C9 components via the usual pathway of antibody–C1–C4–C2 (10–12) or via an alternative pathway.

Hence, the present investigation was concerned with the role of the factors usually responsible for activation of C, classical humoral antibodies, in initiating the LPS-C interaction. The requirement of antibody for the consumption of C by LPS was investigated. Initial attempts to deplete antibodies to LPS by selective absorptions proved noncritical, in part because of solubilization of LPS (2). Hence, the LPS-C interaction was studied in bovines, porcines, and humans with developmental agammaglobulinemia (13–15), chickens with experimental agammaglobulinemia (16), and humans with agammaglobulinemia syndromes (17).

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1 The following abbreviations are used in this paper: AGG, agammaglobulinemia; BSA, bovine serum albumin; CVF, cobra venum factor; and LPS, endotoxic lipopolysaccharide.
Materials and Methods

Endotoxin lipopolysaccharides (LPS).—Biologically active LPS were prepared from Viellenova alcalens (2), wild type Salmonella minnesota (4) and the R595 mutant of Salmonella minnesota (4) by the phenol-water extraction procedure of Westphal and Lüderitz (18). Serratia marcescens LPS (3) was prepared by the trichloroacetic acid extraction procedure of Boivin et al. (19).

Cobra venom factor (CVF).—The complement “inactivating” factor was extracted from Naja haje Egyptian cobra venom by a modification (20) of the procedure by Nelson (21).

Immune complexes.—Crystalline bovine serum albumin (BSA) was obtained from Pentex Inc., Kankakee, Ill. Rabbit anti-BSA was obtained from Hyland Laboratories, Los Angeles, Calif., and contained 240 μg antibody nitrogen per ml. BSA and rabbit anti-BSA were reacted at equivalence for 24 hr at 4°C in the presence 0.01 M EDTA. The precipitate was washed twice in EDTA-saline and resuspended in saline. Final protein concentrations were determined by the assay of Lowry et al. (22).

Immunoglobulin-Deficient Sera.—Sera from several different types of immunoglobulin-deficiency states were available for study. Serum from newborn humans was separated from cord blood samples. Serums from newborn bovine and pigs were taken immediately postpartum prior to any ingestion of colostrum. Precolostral serum specimens from representative pathogen-free, disease-free agammaglobulinemic Minnesota miniature piglets were generously provided by Drs. Y. B. Kim and D. W. Watson (14). Sera from chickens with agammaglobulinemia experimentally induced by bursectomy and irradiation in the newborn period (16) were kindly provided by Drs. G. Alm and R. D. A. Peterson. Sera from humans with agammaglobulinemic syndromes were collected from patients previously described (23, 24) at the University of Minnesota Hospitals.

Methods.—Assays of hemolytic C in human (25), bovine (24), porcine (26), and chicken (27) serum, and assays for neutrophil chemotactic factor (6) and anaphylatoxin (7) in piglet serum were performed as previously described. Preincubations of LPS and the test sera, unless otherwise described, were performed with 0.1 ml of test serum in 1.0 ml of Veronal-buffered saline, supplemented with calcium and magnesium ions (10).

Formation of an LPS-X intermediate capable of fixing the terminal C components has been described by Shin et al. (28). In the present adaptation, 300 μg V. alcalens LPS in 0.5 ml of buffer was incubated with 0.5 ml of human serum at 37°C for intervals of 15–120 min. The mixtures were centrifuged at 13,000 rpm for 45 min at 0°C, the supernates discarded, and the LPS-serum complexes resuspended in 0.5 ml of buffer. These were added to 1.5 ml of 1:10 guinea pig C-EDTA, and the mixtures incubated at 37°C for 60 min; the amounts of terminal C components (C3–C9) fixed during this incubation were determined in the usual way (25).

Quantitation of the immunoglobulins in the human sera was performed by radial diffusion assay (29). Assays for immunoglobulin (14), phage neutralizing capacity (14), and sheep hemolysins (30) also were performed as previously described.

RESULTS

I. Developmental Agammaglobulinemia.—Newborn and gestational members of several species are known to be retarded in their development of gamma globulins generally and specific antibodies in particular. Therefore, we first tested the ability of LPS to fix C in several of these sera.

2 Generously provided by Dr. O. Lüderitz.

3 Generously provided by Dr. A. Nowotny.
**Human:** Human newborns have deficient serum concentrations of IGM, IGA, and the C-fixing sheep cell hemolysin, while they have IGG titers generally as great or greater than their mothers at the time of birth. (Table I). Nonetheless, incubation of *V. alcalescens* LPS with these sera led to consumption of large amounts of hemolytic C (Table I, Fig. 1).

**Bovine:** The bovine has a six layered placenta which acts as a barrier to transfer of maternal proteins to the fetus; hence, the bovine embryo is more deprived of gamma globulins than is the human (13). This can be seen in the gamma globulin and sheep cell hemolysin titers shown in Table II. When these sera were preincubated at 37°C with LPS derived from any of four organisms (*S. marcescens, V. alcalescens, S. minnesota* wild type, and *S. minnesota R595* mutant), pronounced consumption of C ensued (Table II). Hence, LPS had the capacity to deplete C even in these immunoglobulin-deficient sera. Even an LPS devoid of the O antigen (the R595 mutant of *S. minnesota*), which lacked the antigen against which natural antibodies frequently are directed, could effectively consume C in these sera. C consumption was seen both in embryo sera (the 65 cm stage, corresponding to the 3rd trimester) and in precolostral specimens of serum.

**Piglet:** The pig has a placenta which is even less permeable to passive transfer of maternal proteins than is that of the bovine (31). Specimens of pig embryo serum was taken on the 77th and 90th day of the 115-120 day gestational period, as well as in the immediate postnatal period prior to colostral feedings. These sera were found to be devoid of gamma globulins by immunoelectrophoresis, and to have either absent or trivial amounts of “natural” antibodies against the msp-2 phage and the sheep red cell (Table III). Nonetheless, when reacted with LPS from four separate bacterial species, pronounced consumption of C was observed.

**Watson-Kim piglet model:** The isolation of the porcine embryo from certain

### TABLE I

| Source of sample | IGG mg/100 ml serum | IGA mg/100 ml serum | IGM mg/100 ml serum | IGD mg/100 ml serum | Sheep hemolysin Ctt6O/ml | Total C Ctt6O/ml | Available C consumed by LPS % |
|-----------------|---------------------|---------------------|---------------------|---------------------|------------------------|-----------------|-----------------------------|
| Cord 1          | 1400                | 7                   | 7                   | 2                   | 2                      | 15              | >80                         |
| Cord 2          | 940                 | 6.5                 | 9                   | 0.1                 | 2                      | 25              | 66                          |
| Cord 3          | 970                 | 5                   | 8                   | 1                   | 2                      | 26              | >80                         |
| Maternal 1      | 1300                | 217                 | 168                 | 5.3                 | 240                    | 30              | 47                          |
| Maternal 2      | 940                 | 135                 | 87                  | 2.7                 | 130                    | 43              | 39                          |
| Maternal 3      | 940                 | 190                 | 82                  | 16.5                | 910                    | 42              | 50                          |
Fig. 1. (A) Hemolytic antibodies to sheep erythrocytes in sera from agammaglobulinemic, normal, and new-born individuals. (B) C consumption by *S. marcescens* LPS (20 μg) in 0.1 ml of sera from agammaglobulinemic, normal, and new-born individuals.
of the maternal proteins led Watson and Kim to establish a methodology for deriving agammaglobulinemic newborn piglets, which they have extensively characterized (14). These investigators worked with the disease-free, pathogen-free Minnesota miniature pig colony and were able to obtain precolostral blood samples from embryos which had less than $2.5 \times 10^{-6}$ mg gamma globulin per 100 ml of serum, while the corresponding maternal sera had approximately $5 \times 10^5$ mg gamma globulin per 100 ml serum. When the effects of various LPS preparations upon the C activity of these sera were tested, it was again found that each was effectively C-consuming (Table IV): substantial depletion of C occurred during the preincubation periods.

Previous findings indicated that the consumption of C by LPS leads to the generation of neutrophil chemotactic factors and anaphylatoxins from the C system in fresh guinea pig serum (6, 7). We tested whether these factors could also be derived in these agammaglobulinemic sera. As is shown in Figs. 2 and 5, there was clear development of both these factors. However, the total amount of factor generated in the newborn sera always was less than that generated in comparable amounts of adult sera.

We conclude that in sera from the available models of developmental agammaglobulinemia, LPS can effectively induce consumption of C and, further, bring about the generation of anaphylatoxin and neutrophil chemotactic activities.

It should be noted that newborns in each of the three species tested had C titers lower than those of their mothers. This disparity in C available made it difficult to compare the newborn and adult sera quantitatively with respect to their relative abilities to respond to given amounts of LPS with C depletion. On the basis of the per cent of available C consumed, the degree of C depletion

| Age of animal | γ-globulin | Sheep hemolysin | Total C | Available C consumed |
|---------------|------------|----------------|--------|---------------------|
|               | mg/100 ml blood | units/ml |        | S. marcescens | S. alcaligenes | S. minnesota (R395 mutant) | S. minnesota (wild type) |
| 65 cm embryo | < 50 | < 2 | 25 | > 80 | 80 | 80 | 80 |
| Newborn       | < 50 | < 2 | 42 | > 80 | 80 | 80 | 80 |
| Adult         | 620 | 230 | > 80 | > 70 | > 70 | > 70 | > 70 |
| Adult         | 620 | 242 | > 80 | > 70 | > 70 | > 70 | > 70 |
| Adult         | 620 | 228 | > 80 | > 70 | > 70 | > 70 | > 70 |
in the immunoglobulin-deficient newborns was similar to that observed in the nearly normal globulinemic adults. However, on an absolute basis, since there was much less C available, less total units of C were fixed.

This deficiency of the newborn sera was dramatized by their decreased ability

### TABLE III

*C Consumption by Endotoxic LPS in Immunoglobulin-Deficient Sera of Fetal and Newborn Pigs*

| Age of animal | γ-globulin (immunoglobulin) | Phage (mono-2) neutralizing activity (K values) | Sheep hemolysin | Total C | S. marcescens | V. alcaligenes | S. minnesota (R595 mutant) | S. minnesota (wild type) |
|---------------|----------------------------|---------------------------------------------|----------------|---------|---------------|---------------|--------------------------|--------------------------|
| 77 day embryo | 0*                         | <0.001                                     | 2              | 14      | >60           | 14            | --                       | --                       |
| 90 day embryo | 0                          | 0.006                                      | 2              | 28      | >80           | --            | 27                       |                          |
| 3 day newborn | 0                          | ---                                        | ---            | 55      | 64            | 53            | --                       | --                       |
| Adult         | +                           | 0.081                                      | 3100           | 133     | 72            | 81            | 87                       | 56                       |
| Adult         | +                           | 0.079                                      | 540            | 160     | 69            | 79            | 67                       | 43                       |
| Adult         | +                           | 0.052                                      | 175            | 134     | 55            | 74            | 83                       | 51                       |

* 0 indicates absence, + indicates presence of γ-globulin.

### TABLE IV

*C Consumption by Endotoxic LPS in Immunoglobulin-Deficient Precolostral Miniature Piglet Serum*

| Age of animal | γ-Globulin | Sheep hemolysin | Total G | S. marcescens | V. alcaligenes | S. minnesota (R595 mutant) | S. minnesota (wild type) |
|---------------|------------|----------------|---------|---------------|---------------|--------------------------|--------------------------|
| Newborn       | <2.5 × 10^4 | 17             | >75     | >75           | --            | --                       | --                       |
| Newborn       | <2.5 × 10^4 | <10            | >78     | >78           | >78           | >78                      | >78                      |
| Sow           | 5 × 10^9   | 44             | 66      | 75            | --            | --                       | --                       |
| Sow           | 5 × 10^9   | 875            | 57      | 40            | 74            | 54                       | 67                       |

to generate neutrophil chemotactic factor(s) and anaphylatoxin(s), and (see below) by their decreased ability to form, upon incubation with LPS, an intermediate capable of inducing consumption of the terminal C components in a mixture of serum-EDTA.

It is not yet clear whether the C depletion observed in the newborn sera results from a very efficient use of subdetectable amounts of immunoglobulin, or whether alternative nonimmunoglobulin factors are involved in initiating these reactivities.
II. Experimental Agammaglobulinemia in the Chick.—In the past 4 years, the experiments of Cooper et al. (16) have resulted in a means for experimentally producing an agammaglobulinemic animal. This was achieved by removal of the bursa of Fabricius in the chicken, followed by X-irradiation. Animals so pretreated were available for study. Such chickens have been shown to be unable to respond with antibody formation, even after repeated stimulation, to a variety of antigens including the Brucella abortus organism. Representative birds had less than 2 mg gamma globulin per 100 ml blood detectable by immunodiffusion against anti-chicken gamma globulin antiserum (compared to an age-matched normal with 1000 mg gamma globulin per 100 ml blood), yet had clearly detectable C activity. When reacted with LPS, pronounced consumption of C ensued (Table V). Therefore, LPS effectively consumed C in the sera of animals with the presently available model of experimental agamma-

**TABLE V**

| Source of sample                  | γ-globulin mg/100 ml blood | Total C units/ml | Available C consumed by LPS % |
|-----------------------------------|---------------------------|-----------------|------------------------------|
| Normal                            | 1000                      | 100             | 95                           |
| Bursectomized-irradiated          | 2                         | 180             | 94                           |

**Fig. 2.** Demonstration of factor(s) which contract the guinea pig ileum after the addition of 0.2 ml of an incubation mixture containing 200 μg of *V. alcalescens* LPS in 1.0 ml of serum from (A) precolostral pathogen-free, disease-free piglet and (B) postpartum maternal pig (generously provided by Drs. Y. B. Kim and D. W. Watson).
globulinemia, as well as in the available models of developmental agammaglobulinemia (AGG).

III. Complement Consumption in the Sera of Humans with Agammaglobulinemia Syndromes.—Since the introduction of penicillin to clinical medicine, it has become apparent that certain AGG states exist in man which are associated with a propensity to infection. Sera from certain of these individuals were available for study.

The marked deficiency of gamma globulins in a group of 11 such patients is represented in Fig. 1A by the greatly decreased titer of natural antibody against sheep erythrocytes, compared to the readily demonstrable titer of such C-fixing antibodies in normal children. When 20 μg S. marcescens LPS was interacted with these sera, consumption of C clearly ensued, despite the immunoglobulin deficiency (Fig. 1B); however, the per cent and the total amount of C consumed was less than that consumed by normal individuals. It is not yet clear whether multiple pathways to C depletion exist, so whether this represents a quantitative deficiency in the only pathway, or a qualitative deficiency of one of multiple pathways remains to be ascertained. It is striking that even newborns, who also had decreased amounts of the C-utilizing hemolysin for sheep cells, were able to consume a greater per cent of the available C than were the AGG patients.
It has been found that AGG individuals are deficient in C1 and C1q (24, 32) as well as in gamma globulins generally. Hence, the ability of LPS to deplete C in these sera was compared with the C-depleting ability of immune complexes

**TABLE VI**

*Formation of C3-C9-Consuming Intermediate by Incubation of Endotoxic LPS and Serum for 15 Min at 37°C*

| Serum source          | Units C3-C9 consumed | C3-C9 consumed % |
|-----------------------|----------------------|------------------|
| Swiss-type AGG        | <50                  | <10              |
| Bruton-type AGG       | 150                  | 17               |
| Late-occurring AGG    | 180                  | 21               |
| Normal newborn        | 220                  | 26               |
| Normal adult          | 800                  | >90              |

* 860 units available.

![Graph](image)

**Fig. 4.** *V. alcalescens* LPS (300 µg in 0.5 ml) was incubated for varying intervals at 37°C in 0.5 ml of serum of normal, newborn, and agammaglobulinemic individuals; the formation and decay of a classical C3 (C3-C9)-fixing intermediate on the LPS is shown. The agammaglobulinic sera performed this function more poorly even than newborn sera.

and CVF, neither of which require additional immunoglobulin to deplete C in normal sera. It can be seen in Fig. 3 that patients with AGG reacted normally to both these agents. This is compatible with the interpretation that their
Fig. 5. Chemotactic response of rabbit neutrophils to factor(s) generated by 5 µg of *V. alcalescens* LPS incubated in various pig sera (× 450). (A) Precolostral pathogen-free, disease-free piglet serum or maternal serum alone; (B) precolostral piglet serum (0.1 ml) plus LPS; and (C) maternal serum (0.1 ml) plus LPS.

decreased ability to react with LPS results from their immunoglobulin deficiency.

The C-consuming efficiency of LPS in these sera was studied in still another way. Shin et al. (28) found that the interaction of LPS and fresh mammalian serum leads to formation of an unstable intermediate provisionally termed
“LPS-X”, somewhat reminiscent of properdin-zymosan complex of Leon (33). LPS-X has the capacity to fix the classical third component of C (C3–C9) in the presence of EDTA. Assay of LPS-X formation allows comparing the ability of test sera to form a classical C3-fixing intermediate with the amount of terminal C components in the indiactor step held constant, an important point since in the previous experiments there were larger amounts of C3–C9 available in the serum of older individuals. The ability of various human sera to form this intermediate with identical amounts of LPS was tested. It was found that AGG patients, as well as new-borns, were deficient in their ability to do so (Fig. 4, Table VI). They formed much less LPS-X than did normal adults. Hence, the role of immunoglobulin in formation of this intermediate, and in consumption of C generally, seems likely.

In parallel experiments, precolostral bovine and porcine sera were also found to be greatly inferior to normal adult sera of the respective species in forming the LPS-X complex.

DISCUSSION

It was found that bacterial endotoxins (LPS) could induce C consumption in a wide variety of immunoglobulin-deficient sera, including those from representative subjects with developmental AGG, experimentally-induced AGG and clinical AGG syndromes. The LPS-C interaction proceeded even in sera which contained less than \(2.5 \times 10^{-6}\) mg/ml gamma globulin. Hence, large amounts of immunoglobulins clearly are not necessary in order for this interaction to proceed. However, whether small amounts of antibody either qualitatively not recognizable as immunoglobulin or quantitatively below the detection range of most assay systems were required to initiate these interactions remained uncertain.

Various investigators have postulated that antibody is necessary for LPS to initiate certain of its various biologic effects including C consumption (34–36). Others have entertained the possibility that LPS contains groups either with direct toxicities or which activate host enzyme systems without mediation by antibody (37–40). The present investigation shows that if classical antibody is necessary for the initiation of the C-dependent portion of these host reactivities, then extremely small amounts suffice to satisfy this requirement.

Certainly there is a question of whether classical antibodies are necessary at all to induce consumption of the terminal C components in normal and immunoglobulin-deficient sera. The existence of alternative pathways which lead to C activation without a requirement for antibody is possible. Pillemer and his colleagues earlier had postulated that there is such an alternative pathway (41), but ultimately showed that it, too, was dependent upon antibody. However, various bacterial and mammalian enzymes have been shown to activate certain of the C components (42–44). More recently, work with a nontoxic factor de-
rived from several species of cobra (21, 45) has shown that the entire terminal C component sequence (C3–C9) can be activated by factors which seem not to include antibody or the earlier-acting C components (20, 46). Hence, it is conceivable that LPS can activate the terminal C components without the presence of antibody.

At present, we favor the hypothesis that antibody is involved in the initiation of LPS-C interactions. The decreased total amounts of C consumed in the immunoglobulin-deficient sera compared with immunoglobulin-normal sera, the decreased per cent, as well as total amount of C consumed in sera of patients with clinical AGG syndromes, the decreased amounts of neutrophil chemotactic factor and anaphylatoxin generated in immunoglobulin-deficient sera compared to immunoglobulin-normal sera, and the markedly impaired ability of the immunoglobulin-deficient sera to form a terminal C component-consuming intermediate (LPS-X), all support this view. However, this does not rule out that other nonimmunoglobulin-requiring pathways lead to consumption of the terminal C components.

Hence, we believe that LPS efficiently interacts with trace amounts of immunoglobulin in serum in a way which leads to consumption of the terminal C components. It does so in a way which consumes very small, if any, amounts of the known early-acting C components (C1, C4, and C2). Whether each of these also is involved in the C consumption induced by LPS, whether the basis of C utilization by LPS resides in a particular immunoglobulin, in other serum proteins, or in special substrates in the LPS, and whether certain or all of the early-acting C components are bypassed in the LPS-C interaction, remains to be seen.

In any case, the ability of LPS to consume C even in serum markedly deficient in immunoglobulin speaks for the potential role of the LPS-C interaction throughout development as well as in later life.

SUMMARY

Bacterial lipopolysaccharides (LPS) derived from a variety of organisms effectively induced C consumption in humans, bovines, and porcines with developmental agammaglobulinemia; birds with experimental agammaglobulinemia; and humans with agammaglobulinemia syndromes. This interaction proceeded even in precolostral piglet sera which contained less than $2.5 \times 10^{-6}$ mg/ml gamma globulin, and led to generation of neutrophil chemotactic factor and anaphylatoxin in these sera. Hence, the LPS-C interaction can proceed in sera markedly deficient in immunoglobulin.

The question of whether immunoglobulins can be bypassed in the LPS-C interaction, or whether they are regularly utilized in a way so efficient that their participation is masked, was considered.
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