BACTEROIDES FRAGILIS ENDOTOXIN

AND FETAL GROWTH RETARDATION

- EXPERIMENTAL STUDIES IN THE PREGNANT GUINEA PIG -

BACTEROIDES FRAGILIS ENDOTOXINE
EN FOETALE GROEIVERTRAGING

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Abbreviations

bw	= body weight
c.f.u./ml	= colony forming units/milliliter
CIE	= counterimmunoelectrophoresis
D	= dalton
DNA	= deoxyribonucleic acid
EDTA	= ethylenediaminetetraacetic acid
GAL	= galactose
GLU	= glucose
GLcN	= glucosamine
GLcNAc	= N-acetylglucosamine
H	= heptose
HF	= hydrofluoric acid
IFN-γ	= interferon-γ
Il-1	= interleukin-1
Il-6	= interleukin-6
KDO	= 2-keto-3-deoxyoctonate
LPL	= lipoproteinlipase
M	= mol
m-RNA	= messenger ribonucleic acid
MTT	= (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
nm	= nanometer
P	= phosphate
PBS	= phosphate-buffered saline
PG $F_{1\alpha}$	= prostaglandin $F_{1\alpha}$
PG I_2	= prostacyclin
PROM	= premature rupture of membranes
S_1-S_4	= sugar residues
TBA	= thiobarbituric acid assay
TLC	= thinlayer chromatography
TNF-α	= tumor necrosis factor α

Chapter 1

GENERAL INTRODUCTION

The influence of systemic and intrauterine infections in pregnant women on the course and outcome of pregnancy has been extensively investigated. There is good evidence that infection during pregnancy can be associated with abortion[25,134,143], stillbirth[55,57], preterm rupture of membranes (PROM)[132,138], preterm labor and delivery[129] and low birthweight[17,140,146]. Many studies focus on the effects of gram negative bacteremia in clinically manifest or subclinical decidual-amniochorionic infections and the role of endotoxins or endotoxin-induced mediators in the etiology of PROM and the induction and maintenance of preterm labor and delivery[59,110,130].

Endotoxins, cell wall antigens of gram negative bacteria, have been widely studied in connection with the diversity of biological responses elicited by these macromolecules in vivo and in vitro; they proved to be major factors contributing to the pathophysiologic mechanisms involved in gram-negative bacteremia and septic shock[115,126]. They have been characterized as lipopolysaccharides (LPS) and identified and immunochemically analyzed with respect to gram-negative bacteria, especially the aerobic *Enterobacteriaceae*. Recent research has revealed that endotoxic lipopolysaccharides are capable to elicit in the host the release of a variety of host effector molecules, including inflammatory mediators and immunoregulatory cytokines[27,50]. This capacity of lipopolysaccharides appears to be responsible for the pathophysiologic mechanisms involved in gram-negative sepsis[5,45,68].

The bacterial flora involved in intrauterine infections is complex, with a prevalence of *Escherichia coli* and *Bacteroides* species of the gram negative group[41,60]. Most experimental studies of the influence of bacteria or endotoxins on the development of complications of pregnancy were performed with the aerobic *E.coli*. The anaerobic *Bacteroides* species, the most commonly isolated anaerobic bacteria in obstetric and gynecologic infections[22], have been investigated less frequently. It is claimed that lipopolysaccharides isolated from *Bacteroides* species exhibit only weak biological activity[101,142], but it was demonstrated that *B.fragilis* is involved in abortion, stillbirth[111,143] and PROM[41,141], and that *B.fragilis* and *B.bivius* are associated with the

development of preterm labor and birth in subclinical and clinically manifest infections[93,94,111,112]. Culture supernatants of *B.fragilis* have been shown to stimulate the release of prostaglandin E_2, known to stimulate uterine contractions, from amnion cells[12,97].

The significance of gram negative bacteria and endotoxins as a risk factor of abnormal fetal growth has been investigated only occasionally. Subclinical decidual-amniochorionic infections were shown to be associated with fetal loss and impaired or retarded fetal development[25,117]; a few clinical studies have revealed a correlation between the presence of gram negative aerobes (*Escherichia coli, Chlamydia*) or anaerobes (*Bacteroides* species) in the amniotic cavity and low birthweight[83,112,146]. In a large multicenter study among a cohort of 13.914 pregnant women Germain et al.[56] showed that the presence of anaerobic gram-negative rods of the genera Bacteroides, Prevotella and Porphyromonas in the vaginal and cervical flora of pregnant women during the second trimester of pregnancy was significantly associated with an increased relative risk of fetal growth retardation. For studies dealing with the influence of infections on birthweight corrected for gestational age an exact dating of the pregnancy is mandatory, but many of the clinical studies do not differentiate between low birthweight because of prematurity and because of fetal growth retardation ("small-for-gestational age" neonates). Animal models permit exact dating of the gestation, and the effects of infections with *Campylobacter*[117], *Escherichia coli* and *Bacteroides bivius*[112] and of endotoxins isolated from *E.coli, Salmonella typhimurium, Shigella dysenteriae* and *Vibrio cholerae* on fetal growth were investigated in such models[53,121,125,128,167]. Sublethal doses of bacteria or endotoxins were administered intravenously, intraperitoneally or subcutaneously in mice and rabbits, in an attempt to mimick bacterial infection; and abortion and impaired fetal growth were observed repeatedly, but fetal growth retardation at the end of gestation was reported in only few studies[29,30].

This thesis presents the results of experimental studies in the pregnant guinea pig designed to evaluate the effects of *B.fragilis* endotoxin on fetal growth and to explore the physiologic and pathologic reactions in the host.

The pregnant guinea pig was chosen as an experimental model because of the similarity of the guinea pig and human placenta, which are both hemochorial. Moreover, the

length of gestation in the pregnant guinea pig, ca. 65 days, allows the development of a sufficiently high anti-endotoxin antibody response before delivery.

The following questions were specifically addressed:

- Does *B.fragilis* endotoxin elicit a specific immune response in the pregnant guinea pig and the fetus?
- What is the impact of *B.fragilis* endotoxin on fetal growth?
- Is there an effect of *B.fragilis* endotoxin on maternal and fetal metabolism?
- Does *B.fragilis* endotoxin stimulate the release of inflammatory mediators in the pregnant animal?
- Is it possible to describe basic structural features in *B.fragilis* endotoxin that might explain the biological activity of this lipopolysaccharide in vivo and in vitro during pregnancy?

The answers to these specific questions will be presented and discussed in chapters 2-7.

Chapter 2

IMMUNE RESPONSE TO BACTEROIDES FRAGILIS ENDOTOXIN IN THE PREGNANT GUINEA PIG*

2.1. Introduction

The gram-negative *Bacteroides* species are frequently involved in intrauterine infections, often in association with other microorganisms[40,60,78,129]. Though *Bacteroides* species have been isolated from amniotic fluid in cases of clinically manifest infections, little is known about the specific immune response to these bacteria in apparently healthy and clinically infected pregnant women and their fetuses. Gibbs et al.[57] measured Immunglobulin G (IgG) against *B.bivius* in serum and amniotic fluid from healthy pregnant women and from pregnant patients with clinical intraamniotic infections, with and without *B.bivius* contamination in the amniotic fluid. They found significantly higher antibody titers in patients with intraamniotic infection in which *B.bivius* was cultivated from amniotic fluid than in women with clinical symptoms of infection but negative amniotic fluid cultures, and in healthy controls. Evaldson et al.[42] found specific IgG against *B.fragilis* in 14 sera and 12 amniotic fluid samples obtained from 30 pregnant women without signs of infection. No correlation was observed between the levels of antibody titers in serum and those in amniotic fluid. There are several studies indicating impaired cell-mediated immunity during pregnancy[69,160]; the influence of pregnancy on the humoral immune response is not yet fully explored and seems to depend on the nature of the antigen and the species of the host.

This chapter presents the results of an experimental study in the pregnant guinea pig, designed to evaluate the immune response to *B.fragilis* endotoxin in mother and fetus, and to compare it with the response in the nonpregnant animal.

* *The main substance of this chapter was published in: Beckmann I, Meisel-Mikołajczyk F, Leszczynski P, Wallenburg HCS. Int Arch Allergy Appl Immunol 1990;93:222-226.*[9]

2.2. Material and Methods

2.2.1. Extraction and isolation of endotoxin

B.fragilis IPL E 323 culture collection reference strain was cultivated in yeast-broth medium[162] at 37°C for 48 hs. The culture was centrifuged at 1500 g and the deposit collected. The bacterial bulk was extracted with aqueous phenol according to Westphal et al.[163]. The water phase was dialyzed against tap water and distilled water, filtered through a Schott G 5 filter, concentrated and lyophylized. The resulting substance, the crude endotoxin, was dissolved in phosphate-buffered saline (PBS) pH 7.4. The solution was boiled for three min and used for immunization procedures and serological tests.

2.2.2. Animals

All animals were of the same batch of virgin albino Dunkin-Hartley guinea pigs and bred in our laboratory facilities. The guinea pigs were kept in individual cages in a controlled environment (19°, 50 % humidity, light-dark circle 13/11 h), and were fed commercial guinea pig pellets, hay, and water ad libitum. The total food intake was carefully recorded. Vitamin C was added to the drinking water twice weekly. In pregnant animals, the first day of gestation was defined as the second day of the opening of the vaginal membrane[35]. Five healthy pregnant animals were used for the sampling of control blood and amniotic fluid.

2.2.3. Immunization of guinea pigs

a. *Intramuscular with formalinized bacteria.* Five nonpregnant virgin guinea pigs (median weight 543 (527-593) g) were immunized with a vaccine of formalin-killed *B.fragilis* IPL E 323 bacteria suspended in saline in a concentration of $2x10^{10}$ c.f.u./ml. Two injections of 0.25 ml vaccine diluted 1:1 with complete Freund's adjuvant each were given into the right and left hind limb on day 0. A booster injection of 0.25 ml vaccine diluted 1:1 with incomplete Freund's adjuvant was given on day 30. The final titer was determined on day 49.

2. *Intramuscular with B.fragilis IPL E 323 endotoxin.* Five nonpregnant virgin guinea pigs (median weight 543 (527 - 593) g) and 12 pregnant guinea pigs (median

weight on gestational day 30 803 (701-928) g) were immunized with *B.fragilis* IPL E 323 crude endotoxin dissolved in PBS pH 7.4. For the first two injection schedules the crude endotoxin was dissolved in 0.1 ml PBS and for the subsequent injections in 0.2 ml PBS. The endotoxin solutions were emulsified in equal volumes of either complete or incomplete Freund's adjuvant (Sigma, St.Louis, U.S.A.), and injected intramuscularly into one or both hind limbs. The following scheme of immunization was applied: day 0 (in pregnant animals day 30 of pregnancy): 100 μg endotoxin/kg body weight (bw); day 6: 200 μg endotoxin/kg bw; day 12: 400 μg endotoxin/kg bw; day 18: 800 μg endotoxin/kg bw; day 24: 1000 μg endotoxin/kg bw. Preliminary investigations revealed that complete Freund's adjuvant should be used in order to reach sufficiently high antibody titers during immunization with *B.fragilis* endotoxin. Accordingly, complete Freund's adjuvant was used for the first immunization, whereas all booster injections were given with incomplete Freund's adjuvant. The final titer was determined on day 31/32 (day 61/62 of pregnancy).

2.2.4. Sampling of maternal and fetal blood and amniotic fluid

After completion of the immunization scheme (for pregnant guinea pigs on day 61 of pregnancy), the animals were anesthetised with an intramuscular injection of ketamin-hydrochloride (15 mg/kg bw) and xylazine-hydrochloride (2 mg/kg bw). The nonpregnant and pregnant animals were weighed and bled by heart puncture. In the pregnant animals, the abdomen was opened, and amniotic fluid samples were withdrawn from the amniotic cavities. The fetuses were then removed and weighed. Blood was taken from the fetuses by heart puncture.

The blood samples were kept at 37°C for 30 min until clotting was complete. The amniotic fluid and blood samples were then centrifuged at 4°C and 1500 g for 10 min, and the amniotic fluid supernatants and the sera were stored at -20°C until analysis.

2.2.5. Reference sera

Reference sera against *B.fragilis* IPL E 323 bacteria were prepared in rabbits as described by Meisel-Mikołajczyk and Puczyńska[107]. The hemagglutination titers against *B.fragilis* endotoxin were 1:1280 - 1:2560.

2.2.6. Inactivation of IgM antibodies with dithiothreitol

According to Olson et al[118], equal volumes of a 0.01 M solution of dithiothreitol (Sigma, St.Louis, Mo.,USA) in PBS pH 7.4 and antiserum diluted 1:5 in PBS were incubated at 37°C for 30 min. Controls containing equal parts of PBS and 1:5 diluted antiserum were incubated simultaneously. After incubation, inactivated sera and controls were titrated in hemagglutination tests.

2.2.7. Serological tests

a. *Hemagglutination tests.* Hemagglutination tests were performed on microtiter plates as described previously[11], with endotoxin-coated formalinized sheep erythrocytes as antigens.

b. *Passive hemolysis test.* These tests were performed according to the method of Kontrohr and Peterffy[93]. Fresh sheep erythrocytes coated with *B.fragilis* IPL E 323 endotoxin were used as antigens; normal guinea pig serum, absorbed with uncoated sheep erythrocytes, served as the source of complement. After inactivation of complement by incubation at 56°C for 30 min, the hemolytic activity of the guinea pig sera was determined spectrophotometrically at 413 nm; the final serum titer was defined as 25 % hemolysis.

c. *Coombs test.* The indirect Coombs test was performed according to the modifications of Wagner and Kuhns[156] with commercial rabbit immunoglobulin against guinea pig immunoglobulins (Dakopatts, Glostrup, Denmark) as second antibody.

A serum concentration of two dilutions (1:2) below the final hemagglutination titer was chosen as the baseline antiserum dilution. This solution was than further diluted (1:2) in PBS (pH 7.4) and 100 μl of each serum dilution were incubated with 100 μl of a 1% formalinized sheep erythrocyte suspension, coated with endotoxin *B.fragilis* IPL E 323. After incubation at 37°C for 3 hs, the erythrocytes were centrifuged at 1500 g for 10 min, washed three times with PBS and resuspended in 100 μl PBS. Rabbit anti-guinea pig Ig immuno-globulin was diluted 1:100 in PBS (pH 7.4) and 25 μl of this solution were added to 25μl erythrocyte suspensions in microtiter plates. The results were read after 3 hs at 37°C and overnight incubation at 4°C.

d. *Crossed immunoelectrophoresis.* First-dimension (Grabar) immuno-electrophoresis of the endotoxin (2 mg/ml PBS, pH 7.4) was performed as described

previously[11] in agarose gel (1% agarose in Laurell buffer pH 8.4[159]) on microscope slides at 3 V/cm for 150 min. Second-dimension electrophoresis of the separated antigenic fractions into reference serum was carried out on 5x5 cm glass plates at a field strength of 5 V/cm for 6 hs at 10°C. The reference serum was a rabbit antiserum against formalin-killed bacteria *B.fragilis* IPL E 323, with either PBS or guinea pig immune serum in the intermediate gel. After washing with PBS and drying, the precipitates were stained with Coomassie Brilliant Blue G 250 according to Weeke[159].

2.2.8. Statistical evaluation

The Wilcoxon rank-sum test was used to analyze differences in fetal weight between the group of pregnant guinea pigs immunized with *B.fragilis* crude endotoxin, and nonimmunized pregnant control animals. A value of $p < 0.05$ was taken to represent statistical significance.

2.3. Results

At no time throughout the period of endotoxin - or placebo - administration any serious maternal illness was noted. A ruffled coat was observed in most endotoxin-treated animals and disappeared two days after endotoxin-injections.

2.3.1. Antibodies in nonpregnant guinea pigs after immunization with formaline-killed B.fragilis bacteria and with endotoxin from B.fragilis.

The antibody titer against *B.fragilis* endotoxin before immunization was < 1:10 in all animals. Data on the response to intramuscular immunization with killed bacteria and extracted endotoxin are presented in table 2.1.

No differences were apparent between the antibody response of sera obtained after immunization with killed bacteria and the extracted crude endotoxin. Immunization with killed bactera as well as crude endotoxin elicited IgM, IgG and "Coombs" incomplete antibodies with a slightly higher rate of IgM and lower content of complement-fixing and incomplete antibodies in sera obtained by immunization with the endotoxin.

Table 2.1. Serological response to intramuscular immunization with *B.fragilis* IPL E 323 endotoxin and formalin-killed bacteria in individual nonpregnant guinea pigs

Immunization with	Titers hemagglutination*	hemolysis	Coombs
	1:160/160	1:160	1: 5120
formalin-	1:320/320	1:160	1: 5120
killed	1:640/160	1:320	1:10240
bacteria	1:160/ 80	1: 80	1: 5120
	1:320/ 40	1: 10	1: 5120
	1: 640/ 80	1: 80	1: 5120
	1:1280/160	1: 40	1: 5120
endotoxin	1: 320/ 80	1: 80	1: 5120
	1: 160/ 40	1: 80	1: 2560
	1: 320/ 40	1: 40	1: 2560

* : total antibody titer/dithiothreitol-resistent titer

sampling of guinea pig blood after immunization with bacteria on day 49, after immunization with crude endotoxin on day 31 after the first endotoxin injection.

The crossed immuno-electrophoresis (CIE) - pictures (figure 2.1. b,c) show that endotoxin as the immunizing agent produces the same antibody spectrum as full bacteria. Figure 2.1. also shows that antibody fractions reacting with *B.fragilis* endotoxin are the same in rabbit reference serum against bacteria as in guinea pig sera against bacteria or endotoxin (Fig.2.1. a,b,c).

2.3.2. Antibodies in nonpregnant and pregnant guinea pigs after immunization with B.fragilis endotoxin

Intramuscular immunization with *B.fragilis* endotoxin was performed in 5 nonpregnant and 12 pregnant guinea pigs. The antibody titer against the endotoxin before immunization was < 1:10 in all animals. For pregnant guinea pigs, the immunization started on day 30 of gestation. One animal aborted and died after the fifth immunization on day 55; in 11 animals, the immunization scheme was completed.

The immune response to *B.fragilis* endotoxin in nonpregnant and pregnant guinea pigs was compared by means of passive hemagglutination (IgG and IgM), passive hemagglutination after inactivation of IgM by dithiothreitol (IgG), passive hemolysis (complement-fixing antibodies IgG_2), and Coombs test (incomplete antibodies). The results are presented in table 2.2.

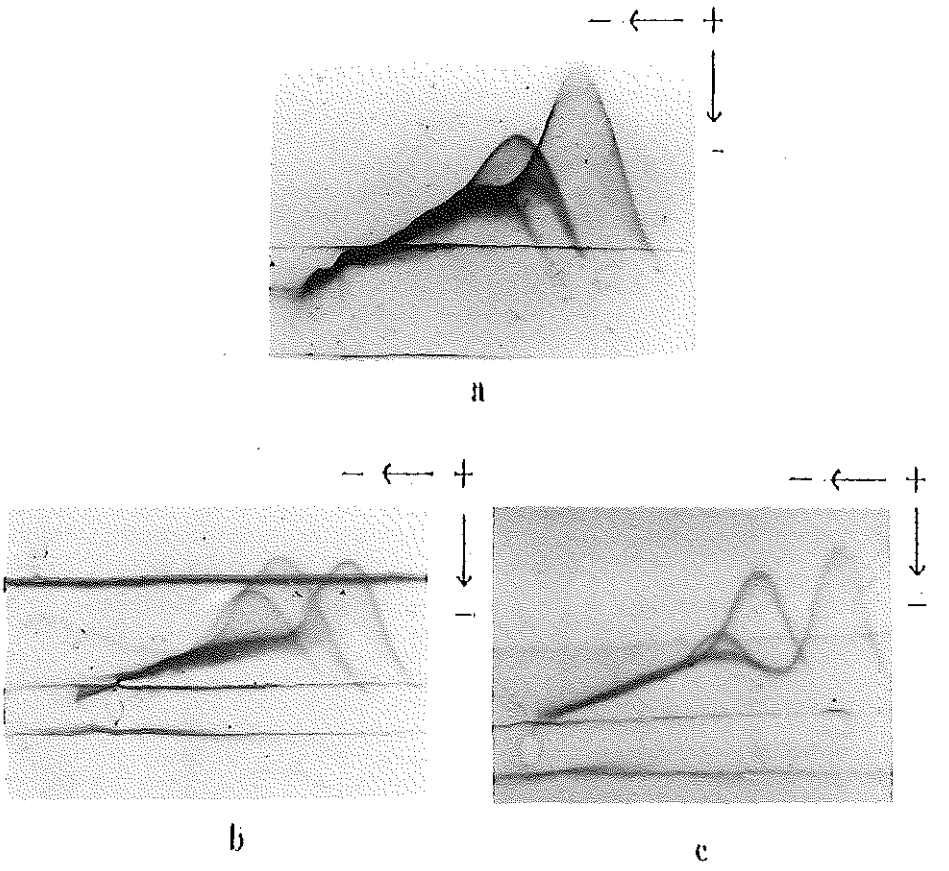


Figure 2.1. a,b,c: Crossed immunoelectrophoresis (CIE) with endotoxin isolated from B.fragilis IPL E 323 and rabbit antiserum against B.fragilis

a) CIE of endotoxin E 323 with blank intermediate gel

b) CIE of endotoxin E 323 with guinea pig (nonpregnant) antiserum against bacteria E 323 in the intermediate gel

c) CIE of endotoxin E 323 with guinea pig (nonpregnant) antiserum against E 323 endotoxin in the intermediate gel

15 µg endotoxin, separated in the first dimension electrophoresis, was run in the second dimension against 100 µl rabbit antiserum (bacteria) in 1700 µl agarose. Intermediate gels (600 µl agarose) contained in a) 60 µl PBS, in b) 60 µl guinea pig (nonpregnant) antiserum (bacteria), in c) 60 µl guinea pig (nonpregnant) antiserum (endotoxin). The cathodic gel (300 µl) contained in a) 35 µl rabbit antiserum, in b) 35 µl guinea pig antiserum (bacteria) and c) 35 µl guinea pig antiserum (endotoxin).

Table 2.2. Antibody response to intramuscular immunization with B.fragilis IPL E 323 endotoxin in nonpregnant and pregnant guinea pigs

Nonpregnant guinea pigs.				Pregnant guinea pigs.			
no.	Titers			No.	Titers		
	hemagglutination*	hemolysis	Coombs		hemagglutination*	hemolysis	Coombs
1	1: 640/ 80	1:80	1:5120	6	1:1280/320	1:160	1:5120
2	1:1280/160	1:40	1:5120	7	1: 640/160	1: 80	1:5120
3	1: 320/ 80	1:80	1:5120	8	1: 160/ 40	1: 40	1: 640
4	1: 160/ 40	1:80	1:2560	9	1: 640/ 80	1: 80	1:5120
5	1: 320/ 40	1:40	1:2560	10	1:1280/ 80	1: 40	1:5120

* Total antibody titer / dithiothreitol-resistant titer

Sampling of guinea pig blood on day 31 after the first endotoxin-injection

Precipitating antibodies in guinea pig immune sera obtained from nonpregnant and pregnant guinea pigs were investigated in crossed immunoelectrophoresis (CIE) as shown in Figure 2.2.(a,b).

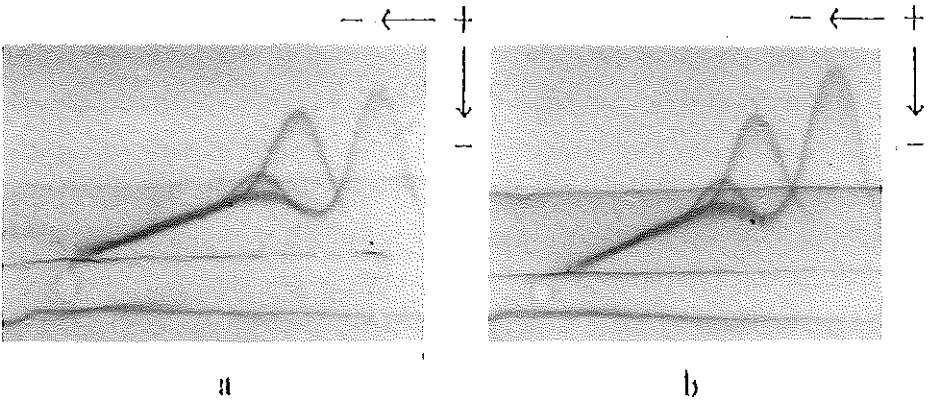


Figure 2.2. a,b.: Crossed immunoelectrophoresis (CIE) with crude endotoxin isolated from B.fragilis IPL E 323 and rabbit antiserum against B.fragilis

a) CIE of crude endotoxin E 323 with guinea pig (nonpregnant) antiserum against E 323 crude endotoxin in the intermediate gel

b) CIE of crude endotoxin E 323 with guinea pig (pregnant) antiserum against E 323 crude endotoxin in the intermediate gel

15 µg endotoxin, separated in the first dimension electrophoresis, was run in the second dimension against 100 µl rabbit antiserum (bacteria) in 1700 µl agarose. Intermediate gels (600 µl agarose) contained

in a) 60 µl guinea pig (nonpregnant) antiserum (endotoxin),

in b) 60 µl guinea pig (pregnant) antiserum (endotoxin).

The cathodic gel (300 µl) contained in a and b 35 µl guinea pig antiserum (crude endotoxin)

No differences in hemagglutinating -, complement fixing - and incomplete antibodies or the IgG \ IgM ratio were observed between nonpregnant and pregnant guinea pigs. The CIE picture obtained with serum from endotoxin-immunized pregnant guinea pigs revealed the same antibodies at similar concentrations as present in serum from nonpregnant animals after immunization with crude endotoxin.

2.3.3. Antibodies against B.fragilis endotoxin in maternal and fetal sera and amniotic fluids of pregnant guinea pigs.

The antibody response to *B.fragilis* endotoxin in 11 pregnant animals as determined in maternal and fetal sera and amniotic fluids is shown in table 2.3.

Maternal and fetal sera and corresponding amniotic fluids of 5 nonimmunized pregnant guinea pigs were run with the tests as controls. No antibodies against endotoxin were detected in these sera and amniotic fluids (titers < 1:2 for fetal sera and amniotic fluids). The examination of fetal sera for anti-endotoxin antibodies, before and after treatment with dithiothreitol, revealed IgG and IgM in lower titers than in the corresponding maternal sera as well as a higher percentage of incomplete antibodies. Antibodies against *B.fragilis* endotoxin were detected in low titers in 10 out of 11 amniotic fluids. These antibodies were partially inactivated by dithiothreitol. The data are summarized in table 2.3.

2.3.4. The effect of immunization with B.fragilis endotoxin on fetal weight.

The median fetal weight per litter in 8 immunized animals (with 3 or 4 fetuses per litter) was 10 g less than that in a control group of 8 nonimmunized guinea pigs on day 61/62 of gestation ($p<0.05$), randomly chosen from a large group of healthy pregnant guinea pigs with uncomplicated pregnancies bred in our laboratory.

2.4. Discussion

The main aim of our study was to determine to what extent pregnancy affects the immune response potential of guinea pigs following immunization with *B.fragilis* endotoxin, and whether such immunization induces the formation of antibodies in the fetus and in amniotic fluid. The pregnant guinea pig was chosen as the experimental animal model to assess the impacts of *B.fragilis* endotoxin on mother and fetus during pregnancy. The choice of the guinea pig was based on the structural similarity of the guinea pig and human placenta, which are both hemochorial. Moreover, the length of gestation (ca. 65 days) allows the development of sufficiently high anti-endotoxin antibody titers before delivery.

Table 2.3. Hemagglutination titers in maternal and fetal sera and in amniotic fluid isolated from pregnant guinea pigs after immunization with B.fragilis IPL E 323 endotoxin

Guinea pig no.	Titers in maternal serum		Titers in fetal serum		Titers in amniotic fluid
	hemagglutination*	Coombs	hemagglutination*	Coombs	hemagglutination*
6	1:1280/320	1: 5120	1:256/ 32	1:2560	1: 8/2
7	1: 640/160	1: 5120	1:128/ 64	1:1280	1: 8/2
8	1: 160/ 40	1: 640	1: 16/ 8	1: 320	1: <2
9	1: 640/ 80	1: 5120	1:128/128	1:2560	1: 8/8
10	1:1280/ 80	1: 5120	1: 16/ 4	1: 640	1: 4
11	1: 640/ 40	1: 2560	1: 32/ 16	1: 320	1:16/2
12	1: 320/320	1: 2560	1: 16/ 8	1: 320	1:8/<2
13	1: 640/ 40	1: 5120	1: 64/ 64	1:1280	1: 2
14	1: 640/640	1:10240	1:128/128	1:2560	1: 2
15	1: 320/160	1: 5120	1:128/ 32	1:2560	1:8/<2
16	1: 640/160	1: 5120	1:128/ 16	1:1280	1:8/<2

* : Total antibody titer / dithiothreitol-resistant titer
sampling of guinea pig blood and amniotic fluid on day 31 after the first endotoxin-injection

Crude *B.fragilis* endotoxin was used as the immunogen because this cell wall constituent carries biological activity and presents an important component of the immunogenic complex of these bacteria. *B.fragilis* LPS is immunogenic in mice[157]. CIE with the crude endotoxin as antigen showed that all components of this preparation were immunogenic in guinea pigs. Whereas polysaccharides are nonimmunogenic in guinea pigs, immunogenicity of LPS in guinea pigs has already been described[48,49]. Guinea pig antisera against endotoxin isolated from *B.fragilis* reacted with similar titers as sera against bacteria, but the proportion of incomplete antibodies was lower and the percentage of IgM was slightly higher. The occurrence of IgM in the immune response could be expected since *B.fragilis* is known to induce IgM-synthesis in LPS-responsive mice[149,157].

The endotoxin was administered intramuscularly, which might lead to a slower uptake of the LPS into the maternal circulation than achieved by the usual intravenous or intraperitoneal route. The intramuscular route was chosen to mimick chronic infection.

Various studies have indicated that pregnancy may be associated with a certain degree of immune suppression[69,160]. However, no significant reduction in the immune responsiveness to *B.fragilis* endotoxin was detected in pregnant guinea pigs as compared with that in nonpregnant animals. It should be noted that both the pregnant and nonpregnant animals were immunized using the same schedule, with the incorporation of endotoxin in Freund's adjuvant. The antibody response as measured by IgG and IgM serum antibodies and as demonstrated in CIE and Coomb's test in pregnant animals is similar to that observed in nonpregnant animals, which is in agreement with previously reported data on the antibody response to lipopolysaccharide in pregnant mice[3].

Of interest is the finding that IgM-type antibodies to *B.fragilis* endotoxin were present in fetal sera. The source of the IgM type antibodies in fetal serum is not clear. In rabbits, rats[20,21,96] and guinea pigs[4,21] antibodies are transferred from mother to fetus via the yolk sac splanchnopleur and vitelline vessels, and not across the placenta. The yolk sac splanchnopleur in rabbits does not select for the transmission of different types of γ-globulins, including IgG and IgM[21,67,164]. Transfer of IgM across the yolk sac in guinea pigs would explain our finding of IgM antibodies of the anti-endotoxin type in the amniotic fluid. Another possibility is that the transfer of endotoxin antigen from mother to fetus could induce the formation of IgM type antibodies in the fetal IgM

immunoglobulin fraction. The development of the fetal immune system in guinea pigs reaches immunocompetence about gestational day 35[124,145]; transferred endotoxic LPS could thus induce fetal anti-LPS antibodies. A few experiments, described in chapter 7, were performed to assess the transfer of *B.fragilis* endotoxin into the fetal compartment. The endotoxin was detected in amniotic fluid of two endotoxin-immunized guinea pigs. This finding indicates that the detected fetal anti-endotoxin IgM antibodies could indeed be of fetal origin.

The main visible impact of *B.fragilis* endotoxin on the pregnant guinea pig after repeated administration during the second half of gestation was a significant reduction in fetal weight. Chapter 3 will deal with the question whether or not the reduction in fetal weight is due to the endotoxin itself or to the stress of the immunization procedure, including the use of Freund's adjuvant.

Chapter 3

THE IMPACT OF BACTEROIDES FRAGILIS ENDOTOXIN ON MATERNAL AND FETAL GROWTH*

3.1. Introduction

The effects of endotoxins from gram negative bacteria on course and outcome of pregnancies have been recognized since many years. Injection of sublethal doses of endotoxin into animals has been reported to cause fetal resorption, abortion and still-birth[29,30,55,125,167]. It is also known from clinical studies in women and experiments in animals, that subclinical or mild bacterial infections during pregnancy are frequently associated with impaired fetal developement[32,58,117,143], prematurity[59,112,129] and retarded fetal growth[30,56,83,112,146]. Reported studies in animals were almost exclusively performed in mice or rabbits; injected gram negative bacteria or endotoxins were the aerobic *E.coli, Salmonella* species, *Shigella, Vibrio cholerae* or *Campylobacter*, and anaerobic *Bacteroides* species.

In the study of the immune response in pregnant guinea pigs to *B.fragilis* endotoxin as reported in chapter 2, a markedly reduced fetal weight was observed in endotoxin-treated animals. On the basis of the previous experiments it could not be excluded that the stress of the immunization procedure, including the use of Freund's complete adjuvant, could have had a negative influence on fetal growth.

For that reason, the present study was designed to test the hypothesis that the endotoxin is responsible for the reduction of fetal weight in endotoxin-treated pregnant guinea pigs, and to assess effects of endotoxin on the development of fetal and maternal weight during pregnancy.

* *The main substance of this chapter was published in : Beckmann I, Meisel-Mikołajczyk F, Leszczynski P, Brooijmans M, Wallenburg HCS. Am J Obstet Gynecol 1993; 168: 714-8.*[8]

3.2. Material and Methods

3.2.1. Extraction, isolation and purification of endotoxin

The extraction and isolation of *B.fragilis* IPL E 323 crude endotoxin are described in chapter 2.2.1., the purification of *B.fragilis* endotoxin is described in chapter 6.

3.2.2. Animals

Experiments were performed between days 30 and 61 of gestation in 18 albino Dunkin-Hartley guinea pigs bred in our laboratory facility. The first day of gestation was defined as the second day of the opening of the vaginal membrane. The conditions of animal care are described in chapter 2.2.2. The total intake of food was carefully recorded.

3.2.3. Endotoxin administration

Nine randomly selected animals were injected with a solution of purified *B.fragilis* IPL E 323 endotoxin, dissolved in phosphate-buffered saline (PBS) at pH 7.4, diluted 1:1 with Freund's adjuvant (endotoxin group I). The other nine animals received injections with the solvent only (sham-group). The same scheme of intramuscular injection used in the study described in chapter 2.2.3. was applied. Complete Freund's adjuvant was used for the first injection, all following injections were with incomplete Freund's adjuvant. The nine sham-treated guinea pigs received PBS with complete Freund's adjuvant in the first and incomplete adjuvant in the following injections, according to the scheme followed for the endotoxin-treated animals.

For the evaluation of the impact of *B.fragilis* endotoxin on the course of maternal and fetal weight unpublished data on maternal and fetal weight obtained during the immunization studies with crude endotoxin as reported in chapter 2 were used to compose a third study group of nine endotoxin-treated pregnant guinea pigs (endotoxin group II).

3.2.4. Experimental protocol

Starting on day 28 of pregnancy, the animals were weighed every other day.

Blood was collected on day 30 (maternal) and day 61 (maternal and fetal) by heart puncture under anesthesia obtained with an intramuscular injection of ketamine hydrochloride (15 mg/kg body weight) and xylazine hydrochloride (2 mg/kg bw). The blood samples were kept at 37°C for 30 min until complete clotting. The amniotic fluid and blood samples were then centrifuged at 4°C and 1500 g for 10 min, and the amniotic fluid supernatants and sera were stored at -20°C until analysis.
On day 61 the abdomen was opened under anesthesia, amniotic fluid was sampled, the fetuses and placentas were removed and weighed. In five animals of the endotoxin and sham groups the maternal and fetal livers were removed and weighed.

Antibody titers against *B.fragilis* endotoxin were determined in all blood samples by means of hemagglutination tests on microtiter plates as described in chapter 2.2.7.

3.2.5. Statistical analysis

Data are presented as median (range) throughout. Wilcoxon's rank-sum and rank-sign tests were used to evaluate differences between measured variables. A value of $p < 0.05$ was chosen to represent significance.

3.3. Results

Before the start of the experiment on day 30 of gestation, hemagglutination titers against *B.fragilis* endotoxin were $< 1:10$ in all animals. The nine endotoxin-treated guinea pigs in the study groups I and II responded with a median hemagglutination titer of 1:320 (range 1:40 to 1:640).

At no time throughout the period of endotoxin - or placebo - administration any serious maternal illness was noted. A ruffled coat was observed in most endotoxin-treated animals and it disappeared two days after endotoxin-injections.

3.3.1. Maternal weight

Maternal weight at the beginning of the experiments on day 30 of gestation was not different between the three groups (Table 3.1.). Total maternal weight gain between gestational days 30 and 61 was slightly different between the two endotoxin-groups and the sham-group: maternal weight gain in endotoxin-group I (31 %) was similar to that

in sham-treated controls (36 %), but that in endotoxin-group II (24 %) was significantly lower. When standardized for litter size, the median increase in maternal weight in the two endotoxin-treated groups was 14 %, and 25 % respectively, less than in the sham-treated group ($p < 0.05$). No difference in food intake between groups was observed.
A characteristic example of the course of maternal weight in both groups is shown in figure 3.1.

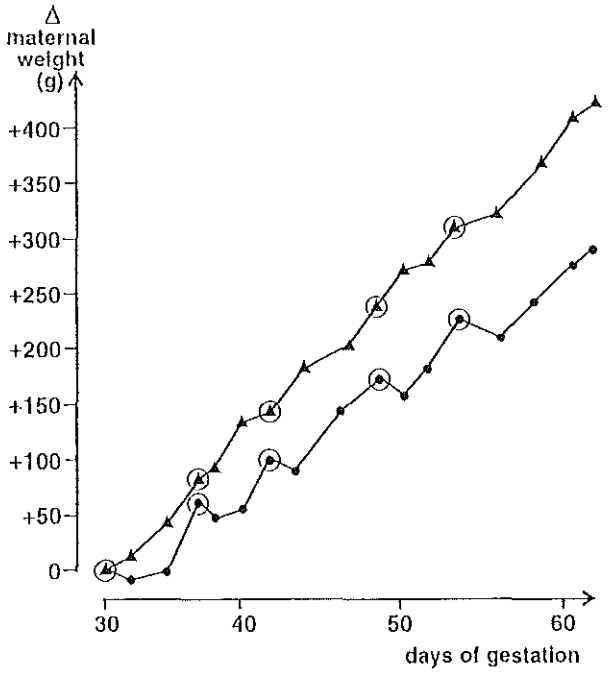


Fig.3.1.: The development of maternal weight in an endotoxin-treated and a sham-treated pregnant guinea pig with four fetuses per litter.

● = *endotoxin-treatment* ▲ = *sham-treatment*

○ = *i.m.injection*

3.3.2. Fetal weight

Two of the 70 fetuses in the endotoxin-groups were stillborn, a nonsignificant difference with one of 32 in the sham-treated animals. One guinea pig which aborted three fetuses after the fifth endotoxin-administration was not included in the two endotoxin-groups. Obvious impaired fetal growth with one to three partly resorbed

fetuses in the uterine horn was observed during dissection in three endotoxin-injected guinea pigs; this was not observed in sham-treated animals. None of the fetuses displayed gross abnormalities.

The total number of fetuses, the number of fetuses per litter, and fetal weights in the three groups are also presented in table 3.1. The number of fetuses per litter was not different between the endotoxin-treated animals and sham-treated controls, but median fetal weight in the endotoxin-treated groups was lower by 12 - 19 % than that in controls ($p < 0.001$).

3.3.3. Placental weight and weight of the fetal liver

No influence of endotoxin - treatment was observed on placental weight and placental weight per litter (Table 3.2.). However, table 3.2. shows that the median weight of the fetal liver in the endotoxin-treated animals was 37 % lower than that in sham-treated controls ($p < 0.0001$).

3.4. Discussion

Intramuscular administration of *B.fragilis* endotoxin in the second half of gestation appears to cause a significant reduction in fetal weight. The experimental design of the study, with controls receiving identical treatment except for the use of endotoxin, excludes a significant effect of stress from the immunization procedure and from the use of Freund's adjuvant.

It is of interest that placental weight is not affected by endotoxin-treatment. That makes it unlikely that the reduction in fetal weight is caused by a reduced uteroplacental supply of nutrients and gases, which would also have affected placental weight. The marked reduction in fetal liver mass in endotoxin-injected guinea pigs indicates that not only fat deposits are reduced but that the endotoxin challenge disturbs fetal metabolism. It is known that endotoxins may interfere with carbohydrate and fat metabolism[14,106,139]. Endotoxins from gram negative aerobes have been shown to influence fetal developement in mice by causing fetal resorption and abortion[29,55,125,128,167].

Table 3.1. The development of maternal and fetal weight in endotoxin-treated and sham-treated guinea pigs.

	P*	Endotoxin-group I (n=9)	Sham-group (n=9)	Endotoxin-group II (n=9)	P*
Maternal weight (g) at 30 days' gestation*$_1$	NS	828 (788-880)	827 (677-930)	824 (738-928)	NS
Maternal weight gain days 30-61 (g)*$_1$	NS	286 (106-347)	301 (235-480)	199 (102-317)	$p < 0.05$
Maternal weight gain per fetus (g)*$_1$	$p < 0.05$	72 (39- 87)	84 (72-123)	63 (34- 79)	$p < 0.01$
Fetuses (total number)	NS	35*$_2$	32*$_3$	35	NS
Fetuses per litter*$_1$ (number)	NS	4 (2- 5)*$_2$	4 (2- 6)*$_3$	3 (2- 5)	NS
Fetal weight (g)*$_1$ per litter	$p < 0.05$	76.6 (45.5-95.4)*$_4$	84.9 (75.3-97.3)*$_4$	65.3 (52.5-75.6)	$p < 0.01$
Fetal weight (g)*$_1$	$p < 0.001$	73.0 (39.1-95.4)*$_4$	83.3 (63.6-103.5)*$_4$	67.0 (44.7-84.7)	$p < 0.001$

*$_1$: results expressed as median (range)

*$_2$: 2 dead fetuses

*$_3$: one dead fetus

*$_4$: dead fetuses excluded

* : Endotoxin-group I (purified endotoxin) vs Sham-group

* : Endotoxin-group II (crude endotoxin) vs Sham-group

NS: Nonsignificant ($p \geq 0.05$)

Table 3.2. The influence of B.fragilis endotoxin on the weight of placenta and fetal liver*

	Endotoxin-group I (n=9)	Sham-group (n=9)	P
Placental weight (g) (n=33)	5.3 (3.3-8.7)	5.2 (4.0-8.4)	NS
Placental weight/litter (g) (n=9)	5.3 (4.9-8.7)	5.5 (4.6-8.1)	NS
Fetal liver weight (g) (n=15)	2.6 (2.4-4.0)	4.1 (3.1-5.0)	$p < 0.0001$
Fetal liver weight/litter (g) (n=5)	2.8 (2.5-3.1)	4.2 (3.7-4.6)	$p < 0.0001$

* All data median (range) NS : Nonsignificant ($p \geq 0.05$)
Endotoxin-group I : purified endotoxin

These conditions were only occasionally observed in studies with the endotoxin of the anaerobe *B.fragilis* in the pregnant guinea pig, where the significant impact was a reduction in fetal weight. These observations may indicate that not only the species of the host but also the biological activity of the infecting bacteria or injected endotoxins may influence the pathologic impact on the course of pregnancy. This hypothesis will be dealt with in chapter 6.

The results of this study are in agreement with our earlier findings as reported in chapter 2 and support epidemiologic observations in pregnant women indicating that infections with gram negative anaerobes during pregnancy are associated with an increased incidence of fetal growth retardation[56,112,146].

Chapter 4 will deal with the impact of *B.fragilis* endotoxin on some of the metabolic processes involved in fetal growth.

Chapter 4

THE IMPACT OF B.FRAGILIS ENDOTOXIN ON MATERNAL AND FETAL METABOLISM*

4.1. Introduction

Fetal growth depends on complex adaptational processes in the mother. A variety of hormones and enzymes regulate the processing of nutrients such as glucose, lipids and aminoacids by the maternal and fetal organism; disturbances in the regulatory mechanisms may lead to impaired fetal development. The results described in the preceding chapter show that a maternal challenge with endotoxin isolated from *B.fragilis* reduced fetal growth in the pregnant guinea pig. From studies in animal models it is known that endotoxins affect the carbohydrate and fat metabolism[14,116,139]. Endotoxins also induce prostaglandin synthesis, presumably throught stimulation of cyclooxygenase activity[84]. This may explain the observed initiation of preterm labor, leading to late abortion and preterm delivery[129,130], in cases of clinically manifest or subclinical maternal infections with *E.coli* or *Bacteroides* species.

This chapter deals with experiments performed in pregnant guinea pigs in order to assess the impact of *B.fragilis* endotoxin on maternal and fetal metabolism of glucose, triglycerides, and prostacyclin, which could be involved in the observed reduction in fetal weight.

4.2. Material and Methods

Experiments were performed in nine endotoxin - and nine sham - treated pregnant guinea pigs between days 30 and 61 of gestation.

* *The main substance of this chapter was published in : Becknann I, Meisel-Mikołajczyk F, Leszczynski P, Brooijmans M, Wallenburg HCS. Am J Obstet Gynecol 1993; 168: 714-8.*[8]

4.2.1. Extraction, isolation and purification of endotoxin

The extraction and isolation of *B.fragilis* IPL E 323 endotoxin are described in detail in chapter 2.2.1., the purification of the endotoxin is described in chapter 6.

4.2.2. Animals

The animals used in this study are the same as those described in chapter 3.2.2. as endotoxin-group I and sham-group.

4.2.3. Endotoxin administration

Nine randomly selected animals were injected with a solution of purified *B.fragilis* IPL E 323 endotoxin dissolved in phosphate buffered saline (PBS) at pH 7.4 (endotoxin-group I). The other nine animals received injections with the solvent only (sham-group). The scheme of intramuscular injections used in this study is described in detail in chapter 2.2.3. Complete Freund's adjuvant was used for the first injection in endotoxin - and sham - treated guinea pigs, all following injections were with incomplete Freund's adjuvant in both groups.

4.2.4. Experimental protocol

Blood was collected on day 30 (maternal) and day 61 (maternal and fetal) by heart puncture under anesthesia as described in chapter 3.2.4. Blood samples were kept for 30 min at 37°C until clotting was complete and then centrifuged at 4°C and 1500 g for 10 min. The sera were stored at -20°C. Blood samples (2.5 ml) for the determination of maternal 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$), a stable metabolite of prostacyclin (PgI_2), were collected in cooled plastic tubes containing 10 μl of heparin and 25 μl of indomethacin (0.1 % in phosphate buffer pH 7.4). The samples were centrifuged for 10 min at 0°C and 1500 g and the plasma was stored at -20°C until analysis.

4.2.5. Analytical procedures

After deproteinization of the sera with 3.5 % perchloric acid at 0°C and neutralization with ice-cold 0.12 mol/L potassium carbonate, glucose was determined spectrophotometrically at 420 nm after reaction with glucose oxidase, peroxidase and

2,2'-azino-di-(3-ethyl-benzthiazoline) - sulfonate[13,79], (Boehringer, Mannheim).

Serum triglycerides were measured as described by Mendez et al.[108] after extraction with heptane and saponification with potassium hydroxide in isopropanol. Glycerol was determined spectrophotometrically at 415 nm after oxidation with periodate and reaction with acetylacetone.

Plasma concentrations of 6-keto-$PGF_{1\alpha}$ were determined with a radioimmunoassay (E.I. Du Pont de Nemours-NEN Research Products, Boston). A Sep-Pak C^{18} cartridge (Waters, Milford, Mass.) was prewashed with 10 ml of absolute ethanol, 10 ml of distilled water, and 2 ml of air. Two ml of plasma were applied to the column, followed by 2 ml of distilled water and 2 ml of air. The prostaglandin metabolite was eluated with 2 ml of absolute ethanol, followed by 2 ml of air, and the eluate was dried at 40°C under a gentle stream of nitrogen. The residue was dissolved in radioimmunoassay buffer, and the assay was performed according to the instruction manual[82].

4.2.6. Statistical analysis

Data are presented as median (range) throughout. Wilcoxon's rank-sum and rank-sign tests were used to evaluate differences between measured variables. A value of $p < 0.05$ was chosen to represent significance.

4.3. Results

No signs of serious maternal illness in the pregnant animals were noticed during the period of endotoxin - and placebo - administration.

4.3.1. Glucose in maternal and fetal plasma

There were no significant differences in maternal serum glucose concentrations determined on days 30 and 61 within groups or between the endotoxin and sham - groups. Maternal and fetal values are presented in table 4.1. Fetal glucose levels on day 61, expressed as percentages of maternal glucose concentrations, were significantly lower in fetuses of endotoxin - treated animals than in those of sham - treated controls.

4.3.2. Maternal and fetal triglyceride levels

Maternal serum triglyceride levels on gestational day 30 were not different between groups. In endotoxin - treated animals maternal triglyceride concentrations on day 61 were significantly higher than those on day 30 ($p<0.02$). Such a rise was not observed in sham - treated guinea pigs. The median triglyceride concentration on day 61 was 59 % higher in the endotoxin - treated compared with the sham - treated group ($p<0.05$). Also in fetal serum the median triglyceride level on day 61 of gestation was 45 % higher in endotoxin - treated animals than in sham - treated controls ($p<0.05$). The results obtained on day 61 of gestation are presented in table 4.1.

4.3.3. 6-keto-$PGF_{1\alpha}$ in maternal plasma

Table 4.1. shows the 6-keto-$PGF_{1\alpha}$ levels in plasma of pregnant guinea pigs after endotoxin and sham - treatment. The plasma level of 6-keto-PGF_{α} of 29(17-93) pg/ml observed on day 61 in the sham - treated group was not different from that determined on day 30 {37(19-70) pg/ml} and before gestation {45{18-61) pg/ml} in the same animals.

The maternal plasma concentration of 6-keto-$PGF_{1\alpha}$ as determined on day 61 in endotoxin - treated animals was found to be 17 times higher than that in sham-treated controls on day 61 ($p<0.001$).

4.4. Discussion

The results of this study show that in the pregnant guinea pig a maternal endotoxin challenge markedly affects glucose, fat and prostaglandin metabolism in the mother and glucose and fat metabolism in the fetus.

It is known since many years that low blood glucose levels occur in several species following infection with endotoxin-producing gram negative bacteria[63,109,168]. Animals injected with endotoxin initially exhibit hyperglycemia which turns into hypoglycemia with a decrease in hepatic and muscular glycogen[71]. Shands et al.[139] and McCallum and Berry[106] found that endotoxin impairs the action of enzymes involved in gluconeogenesis from noncarbohydrate sources and in glycogen synthesis. In the present study significant hypoglycemia was demonstrated only in fetal plasma.

Table 4.1. Maternal and fetal values of serum glucose and triglycerides and maternal plasma 6-keto-$PGF_{1\alpha}$ concentrations on day 61 of gestation in endotoxin-treated and sham-treated guinea pigs

	Maternal values			Fetal values/litter		
	Endotoxin (n=9)	Sham (n=9)	P	Endotoxin (n=9)	Sham (n=9)	P
Glucose (µg/ml)						
Median	2076	1984	NS*	739	912	NS*
Range	1473-2705	1143-2334		328- 975	680-1288	
Glucose, fetal / maternal ratio (%)						
Median	-	-		36	47	$p < 0.05$
Range	-	-		15- 42	33- 80	
Triglycerides (µg/ml)						
Median	511	322	$p < 0.05$	2352	1622	$p < 0.05$
Range	322-1126	248- 472		973-4087	1178-2141	
6-keto-$PGF_{1\alpha}$ (pg/ml)						
Median	499	29	$p < 0.001$	-	-	
Range	412- 798	17- 93		-	-	

* NS : nonsignificant ($p \geq 0.05$)

The lack of hypoglycemia in maternal plasma may be explained by the fact that maternal blood samples were obtained seven days after the last of five endotoxin injections. It should be noted that at this stage elevated hemagglutination titers, indicating marked formation of anti-endotoxin antibodies, were already detected.

In the present study significantly elevated maternal and fetal plasma triglyceride levels were observed in endotoxin-treated animals. The induction of hyperlipidemia with elevated serum triglyceride levels during infection[43,52,135] or endotoxin administration that has been observed in experiments with nonpregnant animals[14,72,136] could be due to the effect of endotoxin on lipoprotein lipase (LPL), the key enzyme of triglyceride metabolism[2,91,135]. Recent research has revealed that these metabolic effects of infection or endotoxin may be caused by cytokines, such as tumor necrosis factor α (TNF-α), produced by endotoxin - stimulated macrophages. Indeed, TNF-α has been shown to be capable of inducing hypoglycemia[14,24,152] and inhibiting lipoprotein lipase activity, thus reducing triglyceride removal. Injection of TNF-α in guinea pigs caused a marked decrease of mRNA levels for LPL in adipose tissue[36]. After the endotoxin challenge we found a marked rise in the concentration of 6-keto-$PGF_{1\alpha}$ in maternal plasma, which indicates an increase in prostacyclin synthesis, assuming the metabolic clearance has remained unchanged. Because maternal 6-keto-$PGF_{1\alpha}$ concentrations in sham - treated animals were not different from control values obtained in untreated pregnant and nonpregnant animals, *B.fragilis* endotoxin appears to stimulate the synthesis of prostacyclin in the pregnant guinea pig. This conclusion is supported by the observations that endotoxins[46,133,1441], and culture medium conditioned with or bacterial products isolated from *B.fragilis*[12,97,98] cause an increase in prostaglandin production by human amnion and decidual cells. Endotoxin - induced enhancement of prostacyclin synthesis has been reported previously[105,114,155] and seems also to be mediated by the action of cytokines.

In conclusion, this study has shown that fetal growth retardation induced in pregnant guinea pigs by *B.fragilis* endotoxin, may be related to the observed alterations in maternal and fetal carbohydrate and fat metabolism, and in prostaglandin synthesis. Further investigation is needed to assess the complicated pathophysiologic interactions in this experimental model, in particular with regard to cytokine action.

The next chapter deals with the putative role of tumor necrosis factor α in the response of pregnant guinea pigs to *B.fragilis* endotoxin.

Chapter 5

ENDOTOXIN CHALLENGE AND RELEASE OF TUMOR NECROSIS FACTOR α IN THE PREGNANT GUINEA PIG*

5.1. Introduction

In the previous chapters the effect of bacterial endotoxin on fetal growth in the pregnant guinea pig was described. After repeated administration of *B.fragilis* endotoxin fetal growth retardation associated with fetal hypoglycemia, fetal and maternal hypertriglyceridemia and a marked increase in maternal plasma prostacyclin concentrations was observed. Similar biochemical changes have been reported to be induced by the cytokine tumor necrosis factor α (TNF-α) in animal experiments and in man. This cytokine, mainly but not exclusively produced by activated macrophages[44,153], interferes with carbohydrate- and fat metabolism[14,152], DNA-biosynthesis[44,81] induces anemia[85,113] and enhances prostaglandin synthesis[114,116,155]. The results as reported in chapter 3 and 4 may indicate that cytokines, especially TNF-α, are involved in the pathophysiologic mechanisms triggered by bacterial endotoxin and leading to fetal growth retardation.

The present study was designed to test the hypothesis that *B.fragilis* endotoxin, administered intramuscularly into pregnant guinea pigs, induces formation of TNF-α, associated with changes in the metabolism of glucose and prostacyclin as previously observed in endotoxin-induced fetal growth retardation.

5.2. Material and Methods

5.2.1. Extraction, isolation and purification of endotoxin

The extraction and isolation of *B.fragilis* IPL E 323 endotoxin are described in detail in chapter 2.2.1., the purification of the endotoxin by treatment with nucleases and ultracentrifugation is described in chapter 6.2.1.

* *The main substance of this chapter is under consideration in : Beckmann I, Lotgering F, Meisel-Mikołajczyk F, Rotmans P, Wallenburg HCS. Am J Obstet Gynecol 1994.*[6]

5.2.2. Animals

Experiments were performed in 12 randomly selected albino Dunkin-Hartley guinea pigs, at 30 days gestation. The first day of gestation was defined as the second day of the opening of the vaginal membrane[35]. The animals were kept in individual cages in a controlled environment (19°C, 50 % humidity, light/dark circle 13/11 h), and were fed commercial guinea pig pellets, hay and water ad libitum. Vitamin C was added to the drinking water twice weekly.

5.2.3. Experimental protocol

On day 30 of gestation a polyethylene catheter was introduced into a carotid artery under general anesthesia, obtained with an intramuscular injection of ketamine-hydrochloride (15 mg/kg body weight) and xylazine-hydrochloride (2 mg/kg bw). After recovery from the operation, as judged by restored weight gain, and 3 - 4 days after surgery, an arterial sample was taken from all animals for the determination of anti-*B.fragilis* antibodies. The animals were then randomly divided into two groups of six, a sham-group with a median weight of 801 (range 695-875) g and an endotoxin-group (median weight 814 (range 636-880) g). At 0 h the experiment was started with the intramuscular administration of 100 μg endotoxin /kg body weight, dissolved in 100 μl sterile phosphate buffered saline (PBS) at pH 7.4 and mixed with 100 μl complete Freund's adjuvant, to the animals of the endotoxin-group. Guinea pigs in the sham-group received an intramuscular injection of 100 μl PBS mixed with 100 μl complete Freund's adjuvant. Blood samples (1 ml) for the determination of TNF-α, glucose and hematocrit were taken from the arterial catheter into heparinized tubes immediately before (sample 0) and 4, 7, 9, 11, 24 and 48 hs after the start of the experiment. For the determination of prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$), the stable metabolite of prostacyclin, blood samples (1.5 ml) were collected at 0, 11, 24 and 48 hs into cooled plastic tubes containing 10 μl of heparin and 25 μl of indomethacin (0.1 % in phosphate buffer pH 7.4). Indomethacin blocks enzymes participating in the biosynthesis of prostaglandins and was used in order to prevent in vitro biosynthesis of prostacyclin by blood monocytes. The samples were immediately centrifuged for 10 min at 1500 g and 4°C and the supernatants stored at -80°C until analysis. After each sampling of heparinized blood without indomethacin, the remaining erythrocytes were resuspended in saline and

returned to the animals through the arterial catheter.

5.2.4. Stimulation of TNF-α release in whole blood.

This experiment was done to compare the time course of release of TNF-α by human and guinea pig monocytes in vitro, after stimulation in vitro with *B.fragilis* and *E.coli* LPS.

A blood sample from a healthy nonpregnant female human donor and from a healthy pregnant guinea pig were collected into commercial EDTA vacuum tubes (Becton-Dickinson, Rutherford, NJ, U.S.A.). 0.5 ml blood was transferred into 6-wells tissue culture plates (Costar, Cambridge Ma, U.S.A.) and diluted 1:10 with RPMI 1640 culture medium with 25 mM Hepes (Seralab Ltd., Drawley Down, U.K.), supplemented with 100 U/ml penicillin, 100 ug/ml streptomycin, 4 mM l-glutamine and 10 % fetal calf serum (Sebak GmbH, Aidenbach, Germany). The blood samples were stimulated with 25 μl PBS (controls), 10 μl lipopolysaccharide (LPS) *E.coli* 0111 B4 (Sigma St.Louis U.S.A.) (1 mg/ml PBS) or 25 μl LPS *B.fragilis* (1 mg/ml PBS) and incubated at 37°C for 48 hs in a humified, 5 % CO_2 atmosphere. Samples (1 ml) were taken after 0, 6, 24 and 48 hs of incubation, centrifuged for 10 min at 1500 g and 4°C and the supernatants were stored at -80°C until analysis.

5.2.5. Analytical procedures

a. Antibody titers against *B.fragilis* endotoxin were determined in all 0-samples by means of a hemagglutination test on microtiter plates, with endotoxin-coated sheep erythrocytes as antigens, as described in chapter 2.

b. TNF-α in plasma and blood culture supernatants was assessed by bioassay[37,39], using the TNF-α-sensitive murine fibrosarcoma WEHI 164 cell line. Cell death was measured after 20 h of incubation at 37°C and 5 % CO_2 by the colorimetric MTT (3-{4,5-dimethylthiaziol-2-yl}-2,5-diphenyltetrazoliumbromide, Sigma) assay[66]. A standard titration curve, prepared with human recombinant TNF-α, was used to calculate TNF-α values from measured cytotoxicity. Neutralization of cytotoxicity in supernatants of human blood cultures was performed with a polyclonal rabbit anti-human recombinant TNF-α antibody (Genzyme Cambridge, U.S.A.). The detection limit of the assay was 12 pg/ml.

c. Hematocrit values were determined by the microcapillary technique.

d. The determination of glucose in deproteinized plasma was performed by spectrometry at 420 nm after reaction with glucose oxidase, peroxidase and 2,2'-azino-di-(3-ethyl-benzthiazoline-sulfonate as described in chapter 4.2.5.

e. Plasma concentrations of 6-keto-$PGF_{1\alpha}$ were determined with a radioimmuno-assay (E.I. Du Pont de Nemours-NEN Research Products) as described in chapter 4.2.5.

5.2.6. Statistical analysis

Friedman's two-way analysis of variance and Wilcoxon's rank-sum and rank-sign tests were used to evaluate differences between variables within groups. A value of $p<0.05$ was chosen to represent significance.

5.3. Results

Before the start of the experiments on gestational day 33 or 34, hemagglutination titers against *B.fragilis* endotoxin were $<1:10$ in all animals.

There were no signs of serious maternal illness during the course of the experiment. A ruffled coat was observed in most endotoxin-treated animals and disappeared 24 to 48 hs after endotoxin injections.

5.3.1. Bioactive TNF-α values in plasma

Bioactive TNF-α was detected in plasma of five of the six animals in the endotoxin group, 9 hs after endotoxin administration, with a significant rise to a mean level of 400 pg/ml at 11 h, followed by a significant fall to levels below the detection limit at 48 hs; in one guinea pig plasma weak TNF-α like cytotoxicity was found only at 24 hs. In the sham-group five out of six animals did not develop any bioactive TNF-α within 48 hs; in one guinea pig plasma weak TNF-α like cytotoxicity was observed at 11 and 24 hs. The results for all animals are presented in table 5.1. and figure 5.1.A.

Table 5.1. Bioactive TNF-α in plasma of pregnant guinea pigs after endotoxin and sham-treatment

hours after injection	Plasma concentrations of TNF-α (pg/ml) endotoxin - group (n=6) median	range	sham - group (n=6) median	range
0	0		0	
4	0		0	
7	0		0	
9	65	0- 210	0	
11	215	0-1640	0	0-54
24	145	30- 600	0	0-36
48	0		0	

5.3.2. Hematocrit values

The course of hematocrit values during the experiment is summarized in table 5.2. and figure 5.1.B.

Table 5.2. Hematocrit in pregnant guinea pigs after endotoxin and sham-treatment

hours after injection	Hematocrit (%) endotoxin - group (n=6) median	range	sham - group (n=6) median	range	P
0	36	34-41	37	33-42	N.S.
4	36.5	33-38	35	32-38	N.S.
7	35	29-40	35	33-39	N.S.
9	34.5	25-40	33.5	31-40	N.S.
11	35	21-38	35	32-42	N.S.
24	24.5*	12-36	35	33-40	* p < 0.03
48	22.5*	18-36	34	31-39	* p < 0.03

In the endotoxin-group hematocrit values were significantly reduced by 33 and 30 % at 24 and 48 hs, respectively, after endotoxin injection. There was no significant change in hematocrit values in the sham-group during the course of the experiment.

5.3.3. Glucose levels in plasma

A slight increase in plasma glucose levels nine to eleven hours after endotoxin injection was observed in five of six guinea pigs, but this increase was statistically not significant. There was a significant decrease of plasma glucose levels four hours after placebo injection in sham-treated animals. The results are summarized in table 5.3 and figure 5.1.C.

Table 5.3. Glucose in plasma of pregnant guinea pigs after endotoxin and sham-treatment*

hours after injection	Plasma concentrations of glucose: endotoxin - group (µg/ml) median	range	sham - group (µg/ml) median	range	P
0	995	823-1538	967	858-1084	N.S.
4	1003	772-1283	817	631-1034*	*p < 0.03
7	969	829-1056	922	736-1268	N.S.
9	1081	898-1173	1014	722-1060	N.S.
11	1131	840-1270	1025	782-1060	N.S.
24	928	878-1090	1036	796-1156	N.S.
48	1040	846-1245	1142	942-2500	N.S.

* endotoxin vs sham group N.S. = nonsignificant ($p \geq 0.05$)

5.3.4. Prostacyclin levels in plasma

6-keto-$PGF_{1\alpha}$ levels in the endotoxin-treated guinea pigs showed a significant rise after 11 hs ($p < 0.03$), whereas no significant changes were observed in the sham-group. The results are presented in table 5.4. and figure 5.1.D.

Table 5.4. 6-keto-prostaglandin-$F_{1\alpha}$ in plasma of pregnant guinea pigs after endotoxin and sham-treatment

hours after injection	Plasma concentrations of 6-Keto-$PGF_{1\alpha}$: endotoxin - group (pg/ml) median	range	sham - group (pg/ml) median	range	P
0	17.1	10.2- 35.0	31.3	19.7-65.4	N.S.
11	65.9	18.8-138.0	36.7	3.8-61.8	* p < 0.03
24	46.8	14.4-195.7	18.2	0.5-50.4	* p < 0.05
48	56.3	20.3-285.0	25.3	20.2-39.3	* p < 0.03

* = endotoxin vs sham-group N.S. = nonsignificant ($p \geq 0.05$)

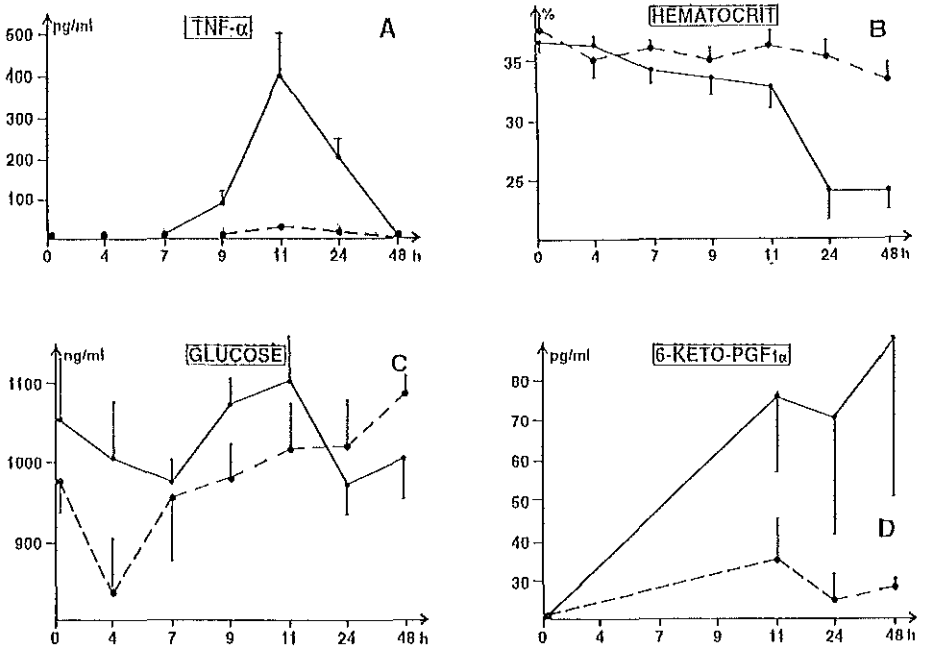


Figure 5.1. TNF-α, hematocrit, glucose and 6-keto-PGF$_{1α}$ in plasma of guinea pigs after i.m. administration of B.fragilis endotoxin or placebo. Endotoxin group = ——, placebo group = - - -, 1A : TNF-α, 1B : hematocrit, 1C : glucose, 1D : 6-keto-PGF$_{1α}$, values as mean [SEM] throughout.

5.3.5. TNF-α released by guinea pig and human monocytes after stimulation with LPS isolated from B.fragilis and *E.coli*

The results of the experiments with *B.fragilis* and *E.coli* endotoxin to stimulate release of TNF-α in guinea pig and human monocytes are shown in figure 5.2.

Cytotoxicity in guinea pig samples followed a course similar to that in human blood. In human samples cytotoxicity was completely neutralized by anti-TNF-α antibody. Maximum secretion of TNF-α following *B.fragilis* endotoxin stimulation was reached in both cultures after 24 hs, whereas *E.coli* LPS stimulated TNF-α release in both cultures with a maximal response after 6 hs. TNF-α peak levels in supernatants of human and guinea pig blood cultures stimulated with *E.coli* LPS were higher than those reached by stimulation with *B.fragilis* endotoxin.

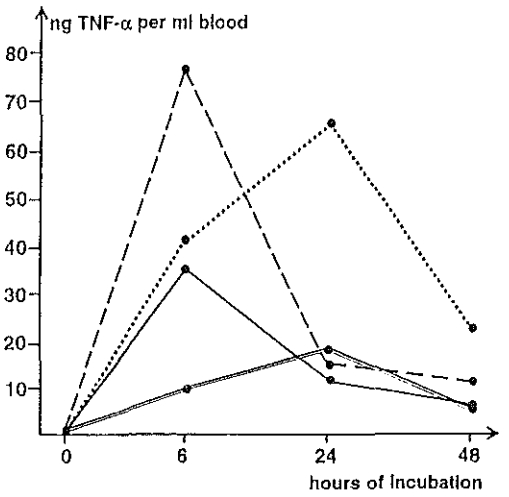


Fig. 5.2. Release of TNF-α from monocytes in whole blood cultures after stimulation with LPS E.coli 0111 B4 and LPS B.fragilis for 0,6,24 and 48 hours. Human blood stimulated with LPS E.coli ——, and LPS B.fragilis ═══; guinea pig blood stimulated with LPS E. coli - - - -, and with LPS B.fragilis ····.

5.4. Discussion

In pregnant guinea pigs TNF-α was detected 9 hs after administration of *B.fragilis* endotoxin with peak values at 11 hs and a return to baseline levels after more than 24 hs. No reports of comparable experiments could be found in the literature. Zuckerman and Bendele[169] found that intraperitoneal administration of *E.coli* LPS (2 mg/kg body weight) into nonpregnant guinea pigs caused a rapid rise of serum TNF-α levels with peak values between less than 0.2 and 180 ng/ml 2 hs after injection and a return to base level after 6 hs. The difference in the time course and peak values of TNF-α between that study and the experiments in pregnant guinea pigs may be explained by the low concentration of LPS injected into the pregnant animals (100 μg/kg body weight) and, in particular, by the intramuscular administration of the endotoxin, resulting in a much slower uptake into the maternal circulation than following intraperitoneal or intravenous injection. The intramuscular route was chosen to mimick chronic infection.

The bacterial origin of the endotoxin may also influence the host's immunologic and

pathophysiologic response. Most studies involving the release of TNF-α after injection of endotoxins in mice used intravenously or intraperitoneally administered LPS from gram negative aerobes, mainly *Enterobacteriaceae* such as *E.coli* or *Salmonella* species. There is evidence that the endotoxin of the anaerobe *B.fragilis* as used in this study is biologically less active than endotoxins isolated from *Enterobacteriaceae*[101,142]. This may add to a difference in peak levels of TNF-α stimulated by *E.coli* or *B.fragilis* endotoxin as observed in the stimulation experiments shown in figure 5.2.

The neutralization of cytotoxicity in human blood supernatants by anti-human-recombinant TNF-α antibody identified the cytotoxic agent as TNF-α. The lack of anti-guinea pig TNF-α antibody and the low and irreproducible cross-reactivity of anti-human TNF-α antiserum with guinea pig TNF-α precluded the exact identification of the cytotoxicity in guinea pig plasma. However, the similar time course of TNF-α release in human and guinea pig blood cultures after stimulation with *B.fragilis* or *E.coli* LPS indicates that the released cytotoxic factor in guinea pig blood is indeed TNF-α. This is supported by the study of Tamatani et al.[150] who isolated and analyzed guinea pig TNF-α after stimulation of guinea pig macrophages by *E.coli* LPS. Additional evidence for this assumption is derived from the fact that the WEHI cell line used in this study to detect cytotoxicity in guinea pig plasma is specifically sensitive to TNF-α and insensitive to the cytokines as interleukin 1α (Il-1α), interleukin 1ß (Il-1ß), interleukin 6 (Il-6) and interferon γ (IFN-γ)[38].

The observation of significantly decreased hematocrit values in endotoxin-treated guinea pigs, 24 to 48 h after endotoxin administration, may be explained by microscopic blood loss in urine or stool, which was not controlled during the experiments, and the capacity of endotoxin or TNF-α to induce anemia in vivo by reducing the lifespan of circulating red blood cells and by interfering with erythropoiesis[85,113,151].

Lethal doses of TNF-α in experimental animals have been shown to result in transient early hyperglycemia followed by hypoglycemia, both characteristic of septic shock[28]. On the other hand, intravenous infusion of sublethal doses of TNF-α in endotoxin-tolerant rats left blood glucose levels unchanged, except for a small and transient increase 90 minutes after the start of the infusion[1]. Significant hyperglycemia was not apparent in the present study, possibly due to the low concentration and low

endotoxicity of *B.fragilis* endotoxin and its slow uptake into the circulation. The observation of a transient slight increase in plasma glucose levels 11 hours after endotoxin administration in five of the six guinea pigs may be an indication of the involvement of TNF-α in an endotoxin-induced effect.

The effect of *B.fragilis* endotoxin on fat metabolism was not investigated in this study. Hypertriglyceridemia is known to occur 16 to 24 hours after intraperitoneal administration of lethal doses of *E.coli* or *Salmonella* LPS in mice[91,136]. In view of the low dose of *B.fragilis* endotoxin used in this study and the late appearance of TNF-α, a significant impact on plasma triglyceride levels during the short time course of these experiments could not be expected.

The observation of a significant increase in circulating prostaglandin $F_{1\alpha}$, 11 and 24 hs after endotoxin injection, is in agreement with results of studies in humans in whom TNF-α was triggered by endotoxin, and was shown to cause increased levels of circulating prostacyclin[155]. An increased production of prostacyclin has also been observed after stimulation of guinea pig macrophages with endotoxin in vitro[147,148].

In conclusion, this study shows that an intramuscular challenge with *B.fragilis* endotoxin in pregnant guinea pigs stimulates the release of TNF-α. The effects of TNFα on carbohydrate metabolism and fat biosynthesis in the pregnant guinea pig, together with the impact of TNF-α on DNA-biosynthesis[44,81], may be responsible for the endotoxin - induced retardation of fetal growth observed in previous experiments.

Chapter 6

STRUCTURAL FEATURES OF BACTEROIDES FRAGILIS ENDOTOXIN*

"The missing link" :2-Keto-3-deoxyoctonate

6.1. Introduction

Endotoxins, constituents of the outer membrane of gram negative bacteria, are classically defined as high molecular weight complexes of lipopolysacharides (LPS) and proteins. There is convincing evidence that the lipopolysaccharide (LPS) moiety is responsible for most of the biological activities exhibited by bacterial endotoxin: pyrogenicity, local Shwartzman reaction, leucopenia and leucocytosis, hypotension, complement activation, mitogenic and blastogenic stimulation of B-lymphocytes and others (reviewed by Galanos and Freudenberg[50]). The capacity of LPS to induce in mononuclear phagocytes synthesis and secretion of a cascade of endogenous mediators involved in the pathogenesis of sepsis or endotoxic shock has received particular scientific attention in the past ten years. Among these mediators are cytokines such as TNF-α, interleukins, arachidonic acid derivatives such as prostaglandin E_2 and prostacyclin, reactive oxygen intermediates, procoagulant factors, platelet activating factor and others (reviewed by Morrison[115]).

Investigations into the structural components of LPS responsible for the initiation of mediator release from mononuclear cells are based on a detailed knowledge of the chemical structure of enterobacterial LPS. LPS from a great number of gram negative bacteria have common features. They consist of three structurally different regions: the lipid A moiety, the core oligosaccharide, and the complex O-specific polysaccharide of

* *The main substance of this chapter was published in: Beckmann I, van Eijk HG, Meisel-Mikołajczyk F, Wallenburg HCS. Int J Biochem 1989;21:661-666*[10].

wild type strains, differing within and between species and carrying the serologic specificity of each strain[126]. The core oligosaccharide with a more limited stuctural diversity within the same bacterial genus consists of an "outer core" with common sugars such as glucose, galactose and N-acetyl-glucosamine, and an "inner core" made up in almost all enterobacterial and many nonenterobacterial LPS of l-glycero-D-manno-heptose and 3-deoxy-D-manno-2-octulosonic acid (2-keto-3-deoxyoctonate) (KDO). The 'inner core' sugars heptose and KDO are often substituted by phosphate, pyrophosphate or phosphoryl-ethanolamine.

KDO is of vital necessity for the bacteria; blocking of the enzyme system leading to biosynthesis of KDO is lethal for gram negative organisms[23,126,127]. The attachment of KDO residues as a link between core oligosaccharide and lipid A is one of the early steps in LPS biosynthesis[123].

Lipid A, consisting of a mono- or di-phosphorylated ß-1,6-linked D-glucosamnine disaccharide substituted with ester and amid-bound long-chain fatty acids, has the same basic structure in practically all gram negative bacteria investigated so far. A schematic representation of the general structure of enterobacterial LPS is shown in figure 6.1.

Studies on the role of structural components of LPS during activation of mononuclear cells have focussed on the lipid A region, which is responsible for many of the toxic activities of LPS[104]. It has been shown to be involved in receptor-mediated binding of LPS to macrophages and monocytes thus inducing a biological response in the mononuclear cell[27,87,166]. The lipid A of *B.fragilis*, though structurally similar to *E.coli* lipid A, differs by its lack of the phosphate group on C_4 in the nonreducing glucosamine and by the number and acylation pattern of long-chain fatty acids. In enterobacterial lipid A the glucosamine disaccharide is substituted by six to seven C_{12}, C_{14} and C_{16} fatty acids, predominantly 3-hydroxytetradecanoic acids. In contrast, lipid A of *B.fragilis* contains per two glucosamine residues five C_{15} and C_{17} branched hydroxylated fatty acids, which are lacking in enterobacterial lipid A, and only traces of the 3-hydroxytetradecanoic acid.

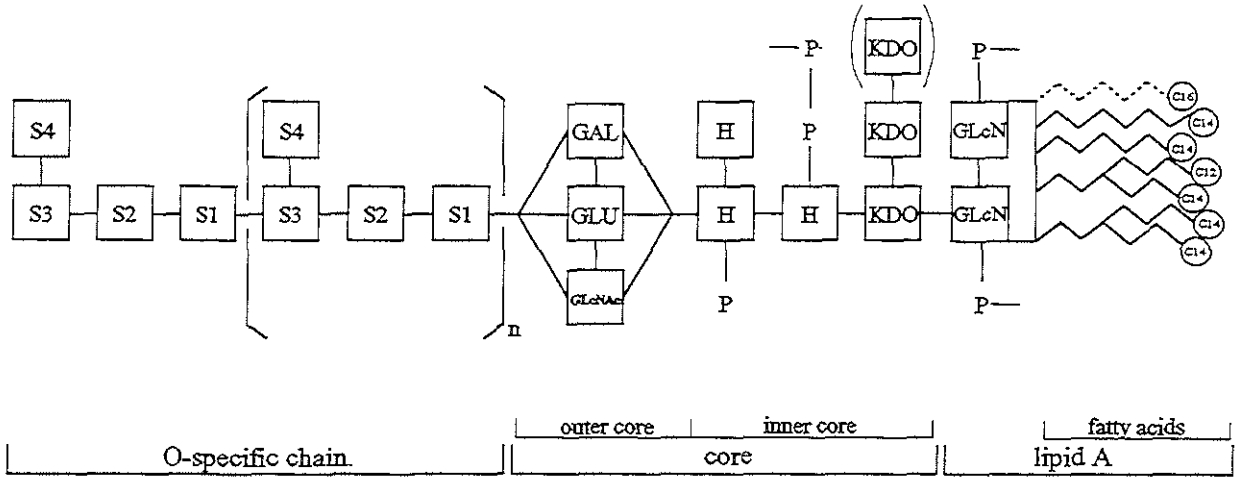


Fig.6.1. Basic structure of an enterobacterial LPS (modified after Lindberg et al[101]
GAL=galactose, GLU=glucose, GlcN=glucosamine, GlcNAc=N-acetylglucosamine, KDO=2-keto-3-deoxyoctonate, P=phosphate, S1-S4=sugar residues.

Experimental evidence for the presence of carbohydrate-binding LPS receptors on macrophages and involvement of the core oligosaccharide structures in the stimulation of macrophages by LPS has been provided by the work of Haeffner-Cavaillon et al. Their results implicate the KDO residues as an important structural feature required for the release of interleukin-1 from macrophages[65,100]. It has been claimed that certain bacterial genera, including the anaerobic *Bacteroides* species, form LPS that are deficient of KDO[75,90]. Both features, KDO deficiency and a different lipid A structure, are held responsible for the low endotoxic activity of these bacteria compared with the activity of *Enterobacteriaceae* such as *E.coli* or *Salmonella* species[101,142].

The presence of KDO is normally detected by means of the widely used thiobarbituric acid assay (TBA)[161], after mild acid hydrolysis of the acid-labile ketosidic linkage of KDO with the following glucosamine disaccharide of lipid A[154]. The TBA detects the 2-keto-3-deoxyaldonic acid configuration (HOOC-CO-CH_2-CH(OH)-) that is present in 2-keto-3-deoxyoctonate and related compounds as 2-keto-3-deoxyheptulosonic acid or N-acetyl-neuraminic acid, a component of various strains of gram negative bacteria[64,103].

Investigations by Le Dur et al.[99] of the TBA-negative LPS of *Bordetella pertussis* revealed that a negative TBA does not necessarily exclude the presence of KDO. In the LPS of *Bordetalla pertussis* KDO was substituted in position C_4 with phosphate and in position C_5 with the sugar chain. Substitution in positions 4 and 5, or 5 and 7, prevents the formation of the thiobarbituric acid reactive fragment HOOC-CO-CH_2-CHO, thus causing a negative TBA[23]. Dephosphorylation of the LPS with 50% aqueous hydrofluoric acid prior to the TBA resulted in a positive response in the latter, thus indicating the presence of phosphorylated KDO in the native LPS. Brade et al.[18] demonstrated for *V.cholerae*, another alledgedly KDO-deficient gram negative bacterium, the presence of phosphorylated KDO after strong acidic hydrolysis. Beckmann et al.[11] detected 2-keto-3-deoxyaldonic acid in endotoxin isolated from *Bacteroides ovatus* after hydrolysis with 2 M HCl. The 2-keto-3-aldonic acid was also detected in LPS isolated from *Aeromonas salmonicidas, V.cholerae* and *Prevotella intermedia (Bacteroides intermedius)* after dephosphorylation of the endotoxins with aqueous 50% hydrofluoric acid[23]. These results prompted us to investigate the presence or absence of KDO in six reference strains of the *Bacteroides fragilis* group, after

dephosphorylation of the endotoxins with hydrofluoric acid.

6.2. Material and Methods

6.2.1. Chemicals

N-acetyl-neuraminic acid, 2-deoxyribose, 2-keto-3-deoxyoctonate (as ammoniumsalt), crystalline bovine pancreatic ribonuclease and bovine pancreatic deoxyribonuclease were from Sigma, St.Louis, U.S.A. All other chemicals were obtained from Merck, Darmstadt, B R D.

6.2.2. Bacterial strains

Six reference strains of the *B.fragilis* group were used: *B.thetaiotaomicron* NCTC 10582, *B.ovatus* ATCC 8483, *B.vulgatus* ATCC 8482, *B.distasonis* ATCC 8503, *B.fragilis* NCTC 9343 and *B.fragilis* IPL E 323.

6.2.3. Extraction and isolation of endotoxins and purification of a lipopolysaccharide

Bacteria were cultivated in yeast-broth medium[162] at 37°C for 48 hs. The cultures were centrifuged at 1500 g and the deposit collected. The bacterial bulk was extracted with hot aqueous phenol[163]. The water phase was dialyzed against tap water and distilled water, filtered through a Schott G5 filter, concentrated and lyophilized. The resulting substances were used as crude endotoxins.

The crude endotoxin of strain *B.fragilis* IPL E 323 was purified by ultracentrifugation and nuclease treatment[77]. A 700 mg sample of the crude endotoxin was dissolved in 24 ml distilled water and ultracentrifuged for 1 h at 1 x 10^5 g in a Beckman ultracentrifuge L5-65. Pellet and supernatant were separated, the pellet dissolved in distilled water and lyophilized. Of the resulting substance 70 mg were suspended in 4 ml 0.15 M phosphate buffer pH 7.1, and incubated with ribonuclease and deoxyribonuclease in an enzyme:substrate ratio 1:50 for 60 min at 37°C. After inactivation of the enzymes (12 min at 65°C) the solution was recentrifuged for 90 min at 1 x 10^5 g in the ultracentrifuge. The supernatant was discarded, the pellet dissolved in distilled water, undissolved particles removed by centrifugation at 2000 g for 10 min, and the lipopolysaccharide was isolated by lyophilization of the clear supernatant.

All experiments with crude endotoxin of *B.fragilis* and related strains and with the purified LPS of *B.fragilis* were performed in triplicate.

6.2.4. Dephosphorylation of crude endotoxins and purified LPS

Dephosphorylation was performed according to Caroff et al.[23]. A 5 mg sample of endotoxin or LPS was suspended in 300 μl aqueous 50% hydrofluoric acid (HF) and kept in sealed polypropylene tubes at 4°C for 48 hs. Volatile material was removed at room temperature by a stream of nitrogen, the residue dissolved in 300 μl distilled water and again dried by a stream of nitrogen. The dry residue was kept at -20°C for further analysis.

6.2.5. Determination of KDO in crude endotoxins and LPS by the thiobarbituric acid assay (TBA)

A 2 mg sample of genuine LPS or 3 mg crude endotoxin or the corresponding residues after dephosphorylation with HF, were suspended in 50 resp. 100 μl 0.25 M HCl and kept in sealed tubes for 8 min at 100°C. The hydrolysates, after cooling in ice-water, were centrifuged at 4°C, (2000 g and 10 min), the residue washed with 50 resp. 100 μl distilled water, centrifuged at 2000 g and supernatant and washing water combined. Aliquots of these solutions were used for the TBA and/or thinlayer chromatography. The TBA was performed according to the method of Karkhanis et al.[88]. Aliquots of the hydrolyzed substances were transferred to fresh tubes and the volume adjusted to 100 μl with 0.125 M HCl. A 50 μl sample of 0.05 M HJO_4 in 0.125 M H_2SO_4 was added and the solutions were kept at room temperature for 30 min. Excessive periodate was destroyed with 200 μl of 2% $NaAsO_2$ in 0.5 M HCL. A 400 μl sample of 0.6% thiobarbituric acid in water was added, the solutions were kept at 100°C for 15 min and, when still hot, diluted with 400 μl dimethylsulfoxide. Absorption values at 549 nm were measured in a Zeiss photometer PM 2 DL, spectra registered in a Beckman spectrophotometer DK 2.

For determinations with synthetic KDO, aliquots corresponding to 4-20 nM KDO in water, were adjusted with HCl to 100 μl 0.125 hydrochloric solutions.

6.2.6. Thinlayer chromatography of KDO, related substances and hydrolysates

Commercially prepared TLC-plates with linear spotting zones (Whatman Inc., Clifton, U.S.A.) type LK 5 D, 0.25 mm thick, were used. The chromatograms were developed in n-butanol:acetic acid:water 2:1:1[80], with the tank being equilibrated with the solvent before development of a chromatogram. Samples of synthetic KDO, 2-deoxyribose and N-acetylneuraminic acid were applied as solutions in water,

hydrolysates as solutions in 0.125 M HCl. Each chromatogram was run twice. After drying, the plates were stained with the thiobarbituric acid reagent[19].

6.2.7. Determination of nucleic acids

A sample of 1 mg substance was dissolved in 10 ml 0.01 M NaOH and the nucleic acid content determined by absorption spectrometry at 260 nm with pure DNA as standard.

6.3. Results

6.3.1. Purification of the endotoxin

The yield of purified LPS after nuclease treatment and repeated ultracentrifugation of the crude endotoxin was 7.6 %. The nucleic acid content of the crude endotoxin (70.5 %) was reduced to 12.4 % in the LPS after the second ultracentrifugation.

6.3.2. Reactivity of endotoxins in TBA before and after dephosphorylization

All preparations, crude endotoxins obtained from the six reference strains, and purified LPS of strain IPL E 323, before and after dephosphorylation, were investigated in the TBA after short hydrolysis in 0.25 M HCl. Before hydrofluoric acid treatment, the endotoxins did not form the characteristic absorption maximum at 549 nm in the TBA, whereas all six endotoxins after dephosphorylation reacted with spectra as shown in figure 6.2. for the crude endotoxin of *B.fragilis* IPL E 323.

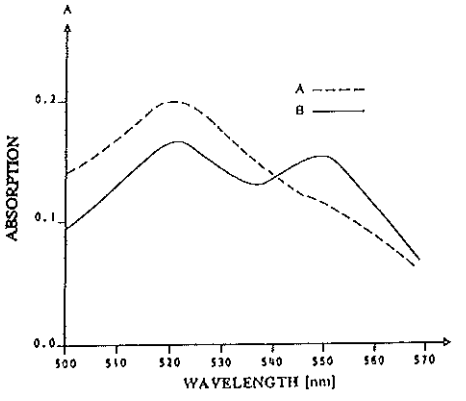
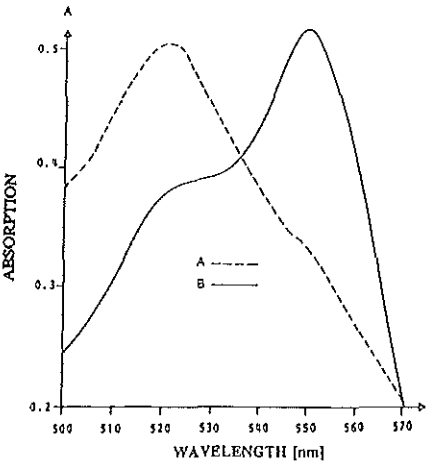


Fig.6.2. TBA response of 1 mg crude endotoxin, isolated from B.fragilis IPL E 323, before (A) and after (B) dephosphorylation with 50% hydrofluoric acid.

The reactivity of the purified LPS in TBA before and after dephosphorylation is presented in figure 6.3.



6.3. TBA-response of 1 mg purified LPS, isolated from B.fragilis IPL E 323, before (A) and after (B) dephosphorylation with 50% hydrofluoric acid

6.3.3. The identity of 2-keto-3-deoxyaldonic acid

The identity of the 2-keto-3-deoxyaldonic acid detected by TBA in the LPS of IPL E 323 was further investigated by means of thinlayer chromatography. N-acetylneuraminic acid, 2-deoxyribose and 2-keto-3-deoxyoctulosonic acid were applied together with the acidic hydrolysate of the dephosphorylated LPS. The R_f-value of the TBA-reactive substance in the hydrolysate was identical with that of synthetic KDO as demonstrated in figure 6.4.

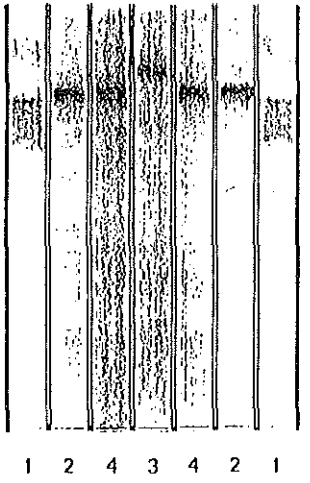


Fig.6.4. Thinlayer chromatography of reference substances and the acidic hydrolysate of B.fragilis IPL E 323 LPS in n-butanol:acetic acid:water 2:1:1.
Stain: thiobarbituric acid reagent.
lane 1: N-acetylneuraminic acid,
lane 2: KDO,
lane 3: 2-deoxyribose,
lane 4: hydrolysate of LPS B.fragilis IPL E 323.

6.3.4. Quantification of KDO in B.fragilis IPL E 323 LPS

KDO is acid-labile and, when released from a lipopolysaccharide by acidic hydrolysis, it is partially destroyed during the procedure. In order to evaluate the amount of KDO lost during dephosphorylation and hydrolysis, 100 μg KDO were dissolved in 300 μl aqueous hydrofluoric acid and treated as described for endotoxins and LPS. After hydrolysis at 100°C in 0.25 M HCl for 8 min the KDO-content of the solution was determined quantitatively by means of the TBA. Of the employed KDO, 20 % were lost after treatment with HF and hydrolysis, as being calculated from reference values obtained for untreated, unhydrolyzed KDO. The KDO-content of *B.fragilis* IPL E 323 LPS was determined from the absorbtion at 549 nm in the TBA, a destruction of 20% of the liberated KDO during dephosphorylation and hydrolysis, and a nucleic acid content of 12.4% and was calculated to be approximately 15 nM/mg LPS.

6.4. Discussion

It was shown in this chapter that KDO is a normal constituent of endotoxins isolated from *B.fragilis* and the five related strains. The reactivity in the native endotoxin and the positive reaction after treatment of the latter with hydrofluoric acid, a highly specific dephosphorylating agent[102], indicates that KDO in these endotoxins is substituted by at least one phosphate group. KDO can also be liberated from *Bacteroides* endotoxins by 2 M HCl, but is easily destroyed during the strong acidic hydrolysis[11].

Dephosphorylation of the LPS - bound KDO prior to mild acidic hydrolysis, as applied for the TBA, has the advantage of a less rigid destruction of the liberated KDO. The reduction of reactivity during the TBA was mainly a result of the mild acidic hydrolysis, as shown by the quantitative determinations conducted with synthetic KDO.

The identification of the 2-keto-3-deoxyaldonic acid as ketodeoxyoctonate was achieved by means of thinlayer chromatography in which the acid comigrated with synthetic KDO and could be clearly differentiated from N-acetylneuraminic acid.

Compared with KDO-values reported for enterobacterial LPS with 3 KDO

molecules in the inner core, the KDO content in the LPS of *B.fragilis* IPL E 323 is low. This is in accordance with reports of other nonenterobacterial LPS with only one molecule phosphorylated KDO in the inner core[23,95,127].

Results of investigations on involvement of KDO-containing inner core structures of LPS in the stimulation of mononuclear cells to release TNF-α are still controversial. Studies with monoclonal antibodies directed against epitopes in the inner core structure of LPS seem to indicate that these regions are also involved in the development of endotoxicity in general, and in the induction of TNF-α release from macrophages[126]. In this context the findings reported by Haeffner - Cavaillon[65] are of interest; they show that the weak induction of interleukin-1 by LPS of *V.cholerae* and *B.pertussis* with one phosphorylated KDO in the inner core, was increased after removal of the phosphate group of KDO by treatment with hydrofluoric acid, thus involving the KDO residue in the events leading to Il-1 release from mononuclear cells.

In conclusion, the results presented in this chapter show that KDO, probably one of the structural requirements for endotoxic activity, is present in *B.fragilis* endotoxin. Phosphorylation of the ketodeoxyoctonate and a low KDO content of the LPS may be structural features of the *B.fragilis* LPS contributing to a lower endotoxicity as compared to that of enterobacterial endotoxins.

Chapter 7

BACTEROIDES FRAGILIS ENDOTOXIN IN THE FETAL COMPARTMENT AND SOME OF ITS (PHYSICO)CHEMICAL CHARACTERISTICS*

7.1. Introduction

Following parenteral administration lipopolysaccharides (LPS) have been detected in various tissues of several animal species[47,92] and in blood cells such as mononuclear cells, granulocytes and platelets[33,54,87]. *E.coli* LPS could be demonstrated in liver, lung and spleen for at least four weeks after intravenous injection in rats[47]. The few reports on the capacity of endotoxin to cross the maternal/fetal barrier show controversial results. Evidence for transplacental transfer of *E.coli* and *S.typhi* antigens in pregnant women was provided by Cramer et al.[31]. Dzvonyár et al.[34] detected *E. coli* endotoxin in the fetal placenta, skin, kidney, lung and liver of endotoxin-treated pregnant rabbits, but Parant and Chedid[26,122] failed to demonstrate *E.coli* or *S.enteritidis* endotoxin in the fetal compartment after injection into pregnant mice. Romero et al.[130] showed that *E.coli* endotoxin did not cross chorioamniotic membranes in vitro.

In the study of the immune response to crude *B.fragilis* endotoxin in pregnant guinea pigs reported in chapter 2, IgM-type anti-endotoxin antibodies were observed in fetal plasma and amniotic fluid. As discussed in chapter 2, this could be due to transfer of IgM across the yolk sac, but it may also be a consequence of transfer of *B.fragilis* endotoxin from mother to fetus followed by induction of production of specific fetal IgM. The possibility that the endotoxin could have penetrated the placental barrier in the pregnant guinea pig was assessed in experiments in amniotic fluid from two endotoxin-treated and two sham-treated guinea pigs and some (physico)chemical characteristics of the endotoxin were determined.

* *The methodological approach to detect B.fragilis endotoxin in amniotic fluid is accepted for publication as : Beckmann I, de Graaff K, Meisel-Mikołajczyk F, Wallenburg H.C.S. Detection of Bacteroides fragilis endotoxin in amniotic fluid by counterimmunoelectrophoresis. Antonie van Leeuwenhoek 1994[7].*

7.2. Material and Methods

7.2.1. Chemicals and materials

Trypsin (bovine pancreas) and pronase (*Str.griseus*) were from Sigma (U.S.A.), all other chemicals from Merck Germany).

Ultrafilters UFC3 LCCOO (molecular weight (MW) limit MW < 5000 D) and UFC3 LGCOO (MW limit MW < 10000 D) were from Millipore B.V., Etten-Leur, the Netherlands.

7.2.2. Antisera

Rabbit antiserum against *B.fragilis* IPL E 323 was prepared as described in chapter 2.

Goat anti-rabbit antiserum was a gift from Dr.B.Kickhöfen, Max Planck Institut für Immunbiologie, Freiburg, Germany.

7.2.3. Amniotic fluid samples

Amniotic fluid samples from two endotoxin-treated and two sham-treated guinea pigs that were used as experimental animals in the study described in chapter 3, and amniotic fluid from a nonimmunized healthy guinea pig were assessed for the presence of *B.fragilis* endotoxin. The amniotic fluids were obtained on gestational day 61, centrifuged for 10 min at 4°C and 1500 g and stored at -20°C until analysis.

7.2.4. Extraction, isolation and purification of B.fragilis IPL E 323 LPS

The extraction of *B.fragilis* IPL E 323 bacteria with phenol/water, the isolation of crude endotoxin and purification of the LPS by nuclease treatment and ultra-centrifugation are described in chapter 6.

7.2.5. Single radial immunodiffusion (Mancini) test for the detection of B.fragilis LPS in amniotic fluid

Microscope slides were precoated with a solution of 0.1% agarose in Laurell buffer pH 8.4. Agarose (1% in Laurell buffer pH 8.4) kept at 56°C, was supplemented with *B.fragilis* IPL E 323 antiserum (1:5 diluted with PBS) in a concentration of 20 μl

serum / 2 ml agarose. Of this mixture 2 ml were put on precoated slides. After solidification of the agarose layer holes with a diameter of 3.5 mm were cut and filled with an antigen solution in amniotic fluid, amniotic fluid of experimental animals or controls (amniotic fluid of healthy nonimmunized guinea pigs). The slides were kept in a humid chamber at 4°C for 48 hs. Antiserum was washed out of the agarose layer by PBS at 4°C in a humid chamber for 72 hs with repeated changing of the washing fluid. To each hole 3 µl of goat anti-rabbit antiserum were added, the slides were again incubated for 24 hs and washed for two days with PBS. After drying, the precipitates were stained with Coomassie Brilliant Blue G 225 as described by Weeke[159].

7.2.6. Ultrafiltration and counterimmunoelectrophoresis for the detection of B.fragilis LPS in amniotic fluid

Amniotic fluid was heated for 5 min at 100°C in order to dissociate antigen/antibody complexes and centrifuged at 1500 g and 4°C for 10 min. 400 µl of the supernatant were transferred into an ultrafilter (5000 D) and centrifuged for 60 min at 2000 g and 20°C. This procedure resulted in an concentration factor of approximately 10.

Counterimmunoelectrophoresis (CIE) was performed on microscope slides precoated with 0.1 % agarose in Laurell buffer pH 8.4 and covered with 2 ml of agarose gel (1 % in Laurell buffer). Two rows of holes (diameter 3.5 mm) were cut with a distance of 3 mm between rows. Six µl of the testsolution (amniotic fluid and controls) were put into the holes on the cathodic side of the slide, 8 µl of antiserum were put into the holes on the anodic side. After electrophoresis for 45 min at a field strength of 3.2 V/cm and 20°C, the slides were incubated over night at 4°C in a humid chamber, washed for 48 hs at 4°C with PBS and stained with Brilliant Blue G 225.

7.2.7. Grabar immunoelectrophoresis of B.fragilis IPL E 323 endotoxin

The electrophoresis was performed as described in chapter 2.2.7.d. After electrophoresis of the endotoxin and application of the antiserum to the central trough the slides were kept in a humid chamber at 4°C for 24 to 48 hs until immunoprecipitates were formed. After extensive washing in PBS at pH 7.4 for 48 hs the agarose layers were dried and stained with Brilliant Blue G 225.

7.2.8. Periodate oxidation of B.fragilis IPL E 323 endotoxin

B.fragilis endotoxin was oxidized with periodate[11] and the oxidized product compared in Grabar immunoelectrophoresis with an endotoxin sample, sham-treated with the same procedure but with phosphate buffer instead of sodium periodate.

One ml of a 0.05 M sodium periodate solution in phosphate buffer (pH 7.0) was added to a solution of 2 mg crude endotoxin in 1 ml phosphate buffer, and the mixture was incubated at 4°C in the dark for five days. Periodate was destroyed by addition of 150 µl diethyleneglycol, the mixture dialyzed over night against distilled water and the solution concentrated under vacuum to a final volume of 1 ml, to which 900 µg NaCl were added.

7.2.9. Enzymatic digestion of B.fragilis IPL E 323 endotoxin

Crude *B.fragilis* endotoxin was treated with the proteolytic enzymes trypsin and pronase to assess the presence of a protein component.

A volume of 100 µl of a solution of 1 mg trypsin / ml phosphate buffered saline pH 7.4 (PBS) and 100 µl pronase (1 mg/ml PBS) were added to 0.5 ml of a solution of endotoxin in PBS (1 mg/ml). The mixture was kept at 37°C for 20 hs, and the enzymes were inactivated by addition of formaldehyde to a final concentration of 0.2%.

7.3. Results

7.3.1. Endotoxin B.fragilis IPL E 323 in amniotic fluid samples

Figure 7.1. shows the results of a Mancini test with amniotic fluids obtained from two endotoxin-injected, one sham-treated and one nonimmunized guinea pig. The detection limit for *B.fragilis* IPL E 323 endotoxin in this test was 125 ng/ml.

A distinct precipitate was visible around the hole which contained amniotic fluid from an endotoxin-treated guinea pig (hole 5). The concentration of *B.fragilis* endotoxin in amniotic fluid of the second guinea pig (hole 2) is lower than in the first one and near the detection limit for this assay. There were no precipitates around holes which contained amniotic fluid from a nonimmunized (hole 1) or sham-treated guinea pig (hole 6). Amniotic fluid with added *B.fragilis* endotoxin or purified LPS reacted with a solid precipitation ring (holes 3 and 4).

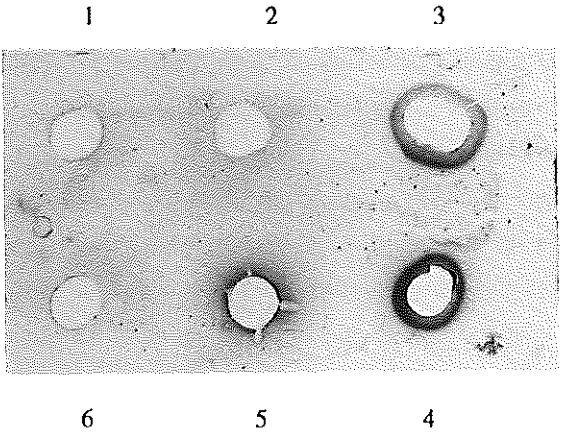


Fig. 7.1. Mancinitest with B.fragilis IPL E 323 rabbit antiserum (bacteria) and goat anti-rabbit Ig-antiserum in the agarose layer and 1: Amniotic fluid (AF) of a nonimmunized guinea pig, 2: AF of an endotoxin-treated guinea pig, 3: AF with crude endotoxin B.fragilis 3 µg/ml, 4: AF with LPS B.fragilis 3 µg/ml, 5: AF of an endotoxin-treated guinea pig, 6: AF of a sham treated guinea pig

Samples of amniotic fluid of endotoxin and sham-treated guinea pigs were concentrated and investigated in CIE as shown in figure 7.2.a. and b.

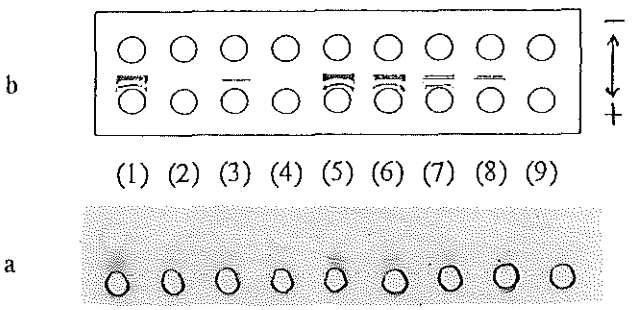


Fig. 7.2.a. and b. a:photographic picture; b:schematic drawing. Amniotic fluid of two endotoxin-treated and two sham-treated guinea pigs in the CIE test. Row to the anode: rabbit anti-B.fragilis IPL E 323 antiserum (bacteria), row to the cathode: (1) AF of guinea pig I after endotoxin-treatment, (2) AF of a sham-treated guinea pig, (3) AF of guinea pig II after endotoxin treatment, (4) AF of a sham-treated guinea pig, (5) - (8) LPS B.fragilis (1 µg/ml) diluted 1:1 with amniotic fluid of a immunized healthy guinea pig, (9) Amniotic fluid of a healthy nonimmunized guinea pig.

The CIE - picture shows that antigenic components of *B.fragilis* endotoxin, reacting specifically with the homologous antiserum, were detected in amniotic fluid of two endotoxin-treated guinea pigs. No immune precipitates were visible in the amniotic fluid of the two sham-treated animals.

The immune precipitate in amniotic fluid of the second endotoxin-treated guinea pig was faintly perceptible on the original slide; it is obscure on the photographic picture but indicated on the schematic drawing. The detection limit for the assay was 500 ng/ml, which is equivalent to 50 ng/ml after tenfold concentration of the amniotic fluid.

7.3.2. The antigenic components of B.fragilis IPL E 323 endotoxin

The antigenic pattern of *B.fragilis* IPL E 323 crude endotoxin and purified LPS which were used in the studies reported in this thesis, was assessed by Grabar immunoelectrophoresis. The results are shown in Fig.7.3.

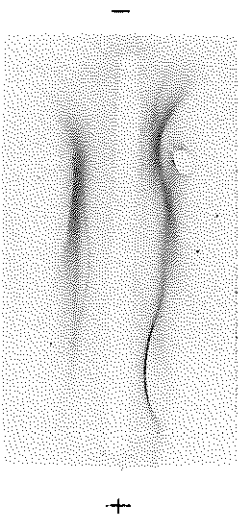


Fig. 7.3.: Grabar immunoelectrophoresis with crude endotoxin and purified LPS from B.fragilis IPL E 323 as antigens and rabbit anti - B.fragilis IPL E 323 antiserum. Right: B.fragilis IPL E 323 crude endotoxin, left: B.fragilis IPL E 323 purified LPS. Middle: B.fragilis IPL E 323 antiserum (rabbits).

The Grabar immunoelectrophoresis shows that the crude endotoxin is antigenically heterogenous. The purified LPS contains less, but still several, distinct components.

After periodate treatment of the endotoxin all antigenic components present in the original and the sham-treated endotoxin, had disappeared. Endotoxin treated with the proteolytic enzymes trypsin and pronase gave the same picture as the original endotoxin.

The capacity of antigenic components in *B.fragilis* LPS to pass the ultrafiltration membrane, used for concentration of amniotic fluid before CIE, was investigated. *B.fragilis* LPS was added to amniotic fluid of a healthy, nonimmunized guinea pig and concentrated by filtration through ultrafiltration membranes with molecular weight limits of 5000 and 10000 D. Residues and filtrates were investigated in Grabar immunoelectrophoresis and CIE. In the filtrate of the 5000 D filter no antigenic components were detectable by CIE; the residue (> 5000 D) contained all antigenic fractions of the original LPS.

After concentration of the LPS solution in amniotic fluid with a 10000 D ultrafilter all antigenic components of the original solution were still present in the residue, but CIE of the filtrate showed that some antigenic components had passed the filtration membrane. The CIE results are presented in figure 7.4.

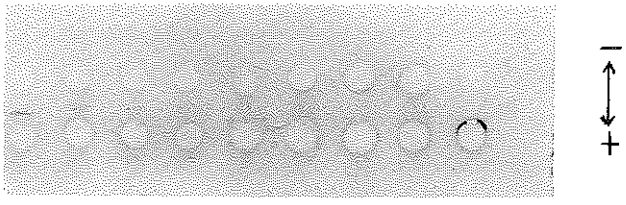


Fig.7.4. CIE with the filtrate of a solution of B.fragilis LPS in amniotic fluid after concentration with a 10000 D ultrafilter (row to the cathode), and rabbit anti-B.fragilis antiserum (bacteria) (row to the anode).

7.4. Discussion

The results of a small number of experiments with Mancini tests and counterimmunoelectrophoresis to assess the transfer of *B.fragilis* endotoxin through the placental barrier show that antigen-reacting components of the endotoxin were present in amniotic fluid. In both tests amniotic fluid from sham-treated guinea pigs, before and after concentration, did not produce any immune precipitate; the observed precipitate is specific for *B.fragilis* endotoxin. The concentration in the two samples from endotoxin-treated animals was low, in one sample approximately between 250 and 125 ng/ml, in the second sample less than 50 ng/ml. This sample produced only a faint immune precipitate at the detection threshold of both assays. The results are in accordance with

observations of Dzvonyár et al.[34], who investigated the distribution of P^{32}-labeled *E.coli* endotoxin in pregnant rabbits and their fetuses and found the majority of endotoxin in maternal and fetal placentas and fetal skin and kidney, and only low quantities in blood and amniotic fluid.

The transfer of maternally injected *B.fragilis* endotoxin to the fetus as already observed for *E.coli* endotoxin in pregnant rabbits[34] would explain the presence of specific IgM in fetal sera and amniotic fluid and may be a challenge for fetal immunocompetent cells to produce immunologic mediators.

Additional experiments performed to characterize the crude and purified endotoxin used in all studies of this thesis show that it is antigenically heterogenous. Purification by nuclease treatment and ultracentrifugation removed the majority of nucleic acids and reduced the number of antigenic components in the LPS which, in immunoelectrophoresis, still displays at least three antigenically different components. This is in accordance with a study of Hofstad[73], who reported similar results with the same strain of *B.fragilis*.

Kasper et al.[90] determined the molecular weight of the LPS monomer isolated from a related *B.fragilis* strain as approximately 12000 D. Filtration of the LPS isolated from *B.fragilis* IPL E 323 in amniotic fluid through ultrafilters with a molecular weight limit > 10000 or 5000 D showed that the smallest antigenically reactive component in the LPS preparation used in the studies reported in this thesis had a molecular weight between 5000 and 10000 D, lower than the value determined by Kasper for the LPS of *B.fragilis* NCTC 9343 but in accordance with estimated values for LPS with a similar basic structure[48].

LPS from *B.fragilis* strains, extracted and purified by different methods, can be contaminated with capsular polysaccharide antigens of high molecular weight (approximately 1.5×10^5 D^{120}), which are also important virulence factors of the species *B.fragilis*[119]. A complete separation of both cell wall antigens with nonaggressive methods, which leave the primary structure and conformation of these macromolecules intact, is not easily achieved and was not the aim of this study. But the molecular weight range, the presence of KDO which is absent in the capsular polysaccharide, and the biological activities of the crude endotoxin and purified LPS used in these studies indicate that these preparations indeed contain the endotoxic cell wall lipopolysaccharide.

Chapter 8

GENERAL DISCUSSION

Endotoxins (lipopolysaccharides) are important factors in the development of the clinical sequelae of infection of the pregnant woman with gram negative bacteria. Some of the complications could be associated with the capacity of endotoxin to elicit the release of effector molecules which regulate the pathophysiologic response of the host[27,50]. Most studies on this subject deal with endotoxins isolated from gram negative aerobes such as *Enterobacteriaceae*, but the anaerobic *Bacteroides* species are the most often isolated anaerobic bacteria in obstetric and gynecologic infections[22], and they are known to be involved in abortion[111,143] and preterm labor[93,94,111,112]. These organisms have also been shown to be associated with low birthweight; but the pathophysiologic mechanisms in mother and fetus have not been explored.

This thesis presents an attempt to assess effects of endotoxin isolated from *B.fragilis* on the maternal and fetal organism and especially on fetal growth, with the pregnant guinea pig as the experimental animal. The endotoxin was injected intramuscularly, a route of administration that results in a slower uptake into the maternal circulation than intravenous or intraperitoneal injection. This route was chosen to mimick chronic bacterial infections. The choice of a guinea pig model was based on the similarity of the human and guinea pig placenta and the length of gestation, which allows for immunization schedules with sufficient antibody production.

In the first study presented in chapter 2, the antibody response to *B.fragilis* endotoxin was investigated. Pregnancy may be expected to be associated with a nonspecific immune suppression in the context of tolerance to the fetal allograft, as observed in pregnant guinea pigs after immunization with haptenated protein antigens[69,160]. However, the humoral immune responsiveness to *B.fragilis* endotoxin in the pregnant animal was unsuppressed. There was no decrease in maternal IgM levels in the early primary response, and no impaired IgG response in the later stages. The response to proteinic antigens is T-cell dependent, whereas *B.fragilis* LPS has been reported to mediate in mice T-independent B-cell stimulation[76,86,165]. At the end of gestation fetuses responded with IgM and IgG antibodies to maternal immunization with endotoxin. The

source of IgM antibodies could be maternal if pregnant guinea pigs, like pregnant rabbits, transfer IgM through the yolk sac splanchnopleur to the fetus[21,67]. But the IgM in fetal sera could also be produced by antigen-stimulated fetal B-cells. The results of two experiments, described in chapter 7, suggest that the immune system of the fetus may be stimulated by antigenic components of *B.fragilis* endotoxin transferred into the fetal compartment.

The first study showed that crude *B.fragilis* endotoxin administered intramuscularly during the second half of pregnancy was associated with a reduction in fetal weight. Further experiments to assess the influence of *B.fragilis* endotoxin on the development of maternal and fetal weight are described in chapter 3. These experiments confirmed the observations that *B.fragilis* endotoxin, administered over the second half of gestation, indeed reduces fetal weight significantly. The observation of an unaffected placental weight but a significantly reduced fetal liver mass points towards an effect of *B.fragilis* endotoxin on maternal and fetal metabolism with an unimpaired uteroplacental blood flow. This metabolic effect was investigated in more detail in the experiments described in chapter 4.

An endotoxin challenge in the pregnant guinea pig resulted in fetal hypoglycemia, maternal and fetal hypertriglyceridemia, and in increased prostaglandin $F_{1\alpha}$ levels in maternal plasma. These experimental findings show that the known impact of enterobacterial endotoxins on glucose- and fat metabolism and prostaglandin synthesis[46,71,91] are also produced by endotoxin from the anaerobic *B.fragilis*. These metabolic consequences of an endotoxin challenge could be involved in the observed reduction of fetal growth.

Because it is known that the observed metabolic changes can be produced by tumor necrosis factor-α[28,36,84,155], released mainly but not exclusively from macrophages after stimulation with endotoxin, the possible contribution of this potent cytokine was investigated in more detail in the experiments reported in chapter 5. It was shown that intramuscular administration of *B.fragilis* endotoxin stimulated in first instance the release of bioactive TNF-α, followed subsequently by decreased hematocrit values, increased prostacyclin levels and a slight and transient increase in plasma glucose concentrations. By comparison with intravenous or intraperitoneal administration of enterobacterial LPS, the induction of TNF-α release after intramuscular injection of

B.fragilis endotoxin was slow and resulted in lower levels of TNF-α. In vitro experiments, to compare the induction of TNF-α release in guinea pig and human blood monocytes by *E.coli* and *B.fragilis* endotoxin, confirmed the results obtained in vivo in pregnant guinea pigs. The slow release of TNF-α with low top levels is most likely caused by the intramuscular route of administration, with a slow uptake of the endotoxin into the circulation, and could also be attributed to a "low endotoxicity" of the anaerobic *B.fragilis* by comparison with the highly endotoxic *E.coli*[101,142].

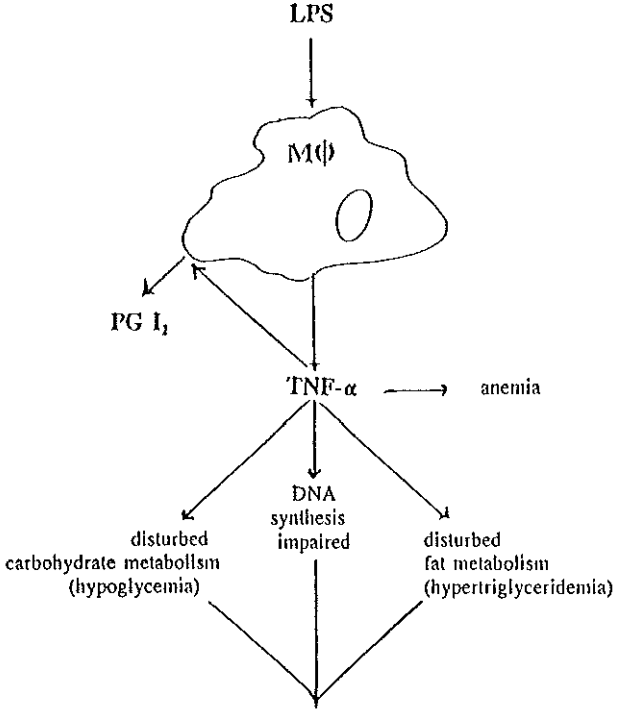
It has been suggested in the literature[75,90] that *Bacteroides* species lack certain structural features involved in the expression of endotoxic activities such as the induction of interleukin-1 release from macrophages. However, the results described in chapter 5 indicate that injection of *B.fragilis* endotoxin induced in pregnant guinea pigs the release of another cytokine, TNF-α. In the study presented in chapter 6, it was demonstrated that one of the missing structural features, the "inner core" constituent 2-keto-3-deoxyoctonic acid (KDO), is present in the endotoxin of *B.fragilis*, but that substitution with a phosphate group not only prevented its detection in previous studies but could also be a cause of a reduced capacity to induce cytokine release. This assumption is supported by the observation that dephosphorylation of KDO in *V.cholerae* LPS increased the capacity of this LPS to induce the release of interleukin-1 from macrophages[65].

KDO as an inner core constituent seems to be involved in the binding of LPS to LPS receptors on the macrophage which are responsible for the transmission of the signal for cytokine release[27]. Whereas the strong induction of release of TNF-α by the highly endotoxic LPS of *E.coli* and other *Enterobacteriaceae* leads to early abortion or fetal death, the lower endotoxicity of the *B.fragilis* LPS in a low-dose influx during chronic infection may lead to a less marked but still effective impact on fetal metabolism and growth.

In conclusion, the studies presented in this thesis show that *Bacteroides fragilis* endotoxin, when injected into the pregnant guinea pig, stimulates a humoral immune response, reduces fetal weight, induces the release of TNF-α and prostacyclin into the maternal circulation, and induces changes in maternal and fetal carbohydrate and fat metabolism that are related to a decrease in the fetal liver mass. These results support

the hypothesis that *Bacteroides fragilis*, a common constituent of the cervical and vaginal flora and an important contributor to intrauterine infections, may induce through its endotoxin the release of TNF-α by maternal and/or fetal mononuclear cells. The maternal and fetal metabolic changes observed after injection of *B.fragilis* endotoxin in pregnant guinea pigs may also occur in case of subclinical intrauterine infection with *Bacteroides fragilis* in pregnant women and, together with the known impact of TNF-α on DNA biosynthesis, could lead to fetal growth retardation. A tentative scheme of mechanisms leading to fetal growth retardation by *B.fragilis* endotoxin is presented in the figure.

POSSIBLE MECHANISMS
LEADING TO FETAL GROWTH RETARDATION
AFTER INFECTION OF THE PREGNANT MOTHER
WITH BACTEROIDES FRAGILIS



Results of clinical and epidemiologic research performed during the last two decades suggest a link between subclinical infections of the pregnant woman and an unfavorable pregnancy outcome manifested in preterm labor and low birth weight. Low birth weight due to preterm delivery or fetal growth retardation constitutes a major perinatal problem that contributes significantly to neonatal mortality and morbidity.

Progress in the development of new rational approaches to prevention and treatment can only be achieved by further research to unravel the complicated pathophysiologic relationship between subclinical decidual-amniochorionic bacterial infection, the maternal and fetal immune system, the release of bioactive mediators, and maternal-fetal responses. The studies presented in this thesis were designed to make a small contribution to this goal.

SUMMARY

The studies presented in this thesis deal with the effects of *Bacteroides fragilis* endotoxin on the maternal and fetal organism, in particular on fetal growth, with the pregnant guinea pig as the experimental animal.

CHAPTER ONE is a general introduction to the thesis. The available literature on the influence of clinically manifest or subclinical gram-negative aerobic and anaerobic decidual-amniochorionic infections on course and outcome of pregnancy is briefly reviewed. Release of inflammatory mediators and immunoregulatory cytokines induced by endotoxic lipopolysaccharides could be involved in many of the pathophysiologic mechanisms leading to fetal growth retardation, but the evidence is limited and fragmental. For that reason the questions addressed in the thesis concern the immune response to *B.fragilis* endotoxin in the pregnant guinea pig and the fetus, and the effects on maternal and fetal metabolism and fetal growth in relation to the biological activity of endotoxic lipopolysaccharide and cytokine release.

CHAPTER TWO deals with the antibody response in mother and fetus after intramuscular administration of *B.fragilis* endotoxin. Comparison with nonpregnant guinea pigs showed that the humoral immune responsiveness to *B.fragilis* endotoxin, measured by IgM - and IgG levels, was unsuppressed by pregnancy. At the end of gestation IgM and IgG antibodies against *B.fragilis* endotoxin were present in maternal and fetal sera and amniotic fluid. The detection of *B.fragilis* endotoxin in amniotic fluid, described in chapter 7, suggests that the fetal immune system might be stimulated by antigenic components of *B.fragilis* endotoxin transferred to the fetal compartment. Intramuscular administration of the endotoxin during the second half of gestation was associated with a reduction in fetal weight.

CHAPTER THREE presents an experimental study to explore the influence of *B.fragilis* endotoxin on the development of maternal and fetal weight. Repeated intramuscular administration of the endotoxin during the second half of pregnancy significantly reduced maternal weight gain and fetal weight. The observation of an unaffected

placental weight but a significantly reduced fetal liver mass points towards an effect of *B.fragilis* endotoxin on maternal and fetal metabolism.

CHAPTER FOUR describes experiments designed to investigate the influence of *B.fragilis* endotoxin on maternal prostacyclin synthesis and maternal and fetal carbohydrate and fat metabolism. The endotoxin challenge resulted in increased maternal serum levels of prostaglandin $F_{1\alpha}$, fetal hypoglycemia and maternal and fetal hypertriglyceridemia.

CHAPTER FIVE presents a study on the release of tumor necrosis factor α (TNF-α) after intramuscular injection of *B.fragilis* endotoxin into the pregnant guinea pig. It was shown that *B.fragilis* endotoxin induces the release of TNF-α, followed by a slight transient hyperglycemia, a significantly reduced hematocrit and a significantly increased concentration of prostaglandin $F_{1\alpha}$ in maternal serum. In vitro experiments confirmed the capacity of *B.fragilis* endotoxin to induce TNF-α release in human and guinea pig macrophages.

CHAPTER SIX describes results of a study designed to explore structural features of the endotoxin which are involved in the expression of biological activity. In earlier investigations 2-keto-3-deoxyoctonate (KDO), an 'inner core' constituent of entero and non-enterobacterial lipopolysaccharides participating in the stimulation of cytokine release by activated macrophages, was claimed to be absent in *Bacteroides* lipopolysaccharides. In contrast to these earlier observations, the presence of KDO in endotoxins isolated from six different strains of the *B.fragilis* group was detected after dephosphorylation of the endotoxins with 50 % hydrofluoric acid. Phosphorylation of this essential structural component of endotoxins may contribute to the, in comparison with the *E.coli* or *Salmonella* species less severe, but still effective, impact of *B.fragilis* endotoxin on fetal growth and metabolism.

CHAPTER SEVEN deals with experiments designed to assess the possible transfer of maternal endotoxin into the fetal compartment. *B.fragilis* endotoxin was detected by single radial immunodiffusion and counterimmunoelectrophoresis in concentrated

amniotic fluid of guinea pigs after repeated intramuscular injection of the endotoxic lipopolysaccharide during the second half of gestation. A few (physico)chemical characteristics of the endotoxin used for all studies included in this thesis are described. The purified lipopolysaccharide was antigenically still heterogenous, with a molecular weight of the smallest fraction between 10000 and 5000 D. All serologically reactive components were carbohydrates, protein components were not observed.

CHAPTER EIGHT presents a general discussion of the results of the studies described in this thesis. The results support the hypothesis that *B.fragilis*, when present in the uterine tissues of the pregnant woman, may induce through its endotoxin the release of TNF-α by macrophages, which could be responsible for pathophysiologic changes in the maternal and fetal carbohydrate and fat metabolism. Together with the known impact of TNF-α on DNA biosynthesis these effects could lead to fetal growth retardation.

SAMENVATTING

In dit proefschrift wordt dierexperimenteel onderzoek beschreven bij de drachtige cavia naar de effecten van het endotoxine van *Bacteroides fragilis* op het moederlijke en foetale organisme, in het bijzonder op de groei van de foetus.

HOOFDSTUK EEN geeft een algemene inleiding. In het kort wordt de beschikbare literatuur besproken met betrekking tot de invloed van klinisch manifeste of subklinische gram-negatieve aerobe en anaerobe infecties van de decidua en het amniochorion op het verloop en de uitkomst van de zwangerschap. Endotoxine lipopolysacchariden induceren het vrijkomen van ontstekingsmediatoren en cytokines, die betrokken zouden kunnen zijn bij veel van de pathofysiologische mechanismen die leiden tot foetale groeivertraging. De wetenschappelijke basis voor deze hypothese is echter beperkt en fragmentarisch. Daarom wordt in dit proefschrift getracht een aantal vragen te beantwoorden met betrekking tot de immuunrespons van de drachtige cavia en haar foetus op endotoxine van *B.fragilis* en de effecten op het moederlijke en foetale metabolisme en de foetale groei in samenhang met de biologische activiteit van endotoxine lipopolysacchariden en daardoor geïnduceerde cytokines.

HOOFDSTUK TWEE geeft een beschrijving van experimenten met betrekking tot de antilichaam-reactie in moeder en foetus na intramusculaire toediening van het endotoxine van *B.fragilis*. In vergelijking met de niet-drachtige cavia wordt de humorale immuunreactie op *B.fragilis* endotoxine, gemeten aan de hand van IgM en IgG concentraties, niet onderdrukt door de zwangerschap. Aan het einde van de zwangerschap waren IgM en IgG antilichamen tegen *B.fragilis* endotoxine aanwezig in het moederlijke en foetale serum en in vruchtwater. De bevinding dat *B.fragilis* endotoxine kan worden aangetoond in vruchtwater, zoals beschreven in hoofdstuk 7, wijst erop dat het foetale immuunsysteem zou kunnen worden gestimuleerd door antigene componenten van het endotoxine, die zijn overgegaan naar het foetale compartiment. De intramusculaire toediening van endotoxine in de tweede helft van de dracht ging samen met een duidelijk verlaagd foetaal gewicht.

HOOFDSTUK DRIE beschrijft een experimenteel onderzoek naar de invloed van het endotoxine van *B.fragilis* op de ontwikkeling van het moederlijke en foetale gewicht. Herhaalde intramusculaire toediening van endotoxine tijdens de tweede helft van de zwangerschap leidde tot een significante vermindering van de moederlijke gewichtstoeneming en van het foetale gewicht. Het gewicht van de placenta werd door toediening van endotoxine niet beïnvloed, maar het gewicht van de foetale lever was significant lager dan in de controlegroep. Dit wijst op een effect van het endotoxine van *B.fragilis* op de moederlijke en de foetale stofwisseling.

HOOFDSTUK VIER geeft een verslag van experimenten gericht op het onderzoeken van de invloed van endotoxine van *B.fragilis* op de moederlijke synthese van prostacycline en op het moederlijke en foetale metabolisme van koolhydraten en vetten. Toediening van endotoxine aan de drachtige cavia leidde tot een toeneming van de concentratie van prostaglandine $F_{1\alpha}$ in moederlijk serum, foetale hypoglykemie, en moederlijke en foetale hypertriglyceridemie.

HOOFDSTUK VIJF beschrijft een onderzoek naar de vorming van tumor necrosis factor-α (TNF-α) na intramusculaire toediening van het endotoxine van *B.fragilis* aan de drachtige cavia. Toediening van *B.fragilis* endotoxine induceert de afgifte van TNF-α, gevolgd door een voorbijgaande moederlijke hyperglykemie, een significant verlaagde hematocrietwaarde en een significant toegenomen concentratie van prostaglandine $F_{1\alpha}$ in moederlijk serum. Door middel van experimenten in vitro werd bevestigd dat het endotoxine van *B.fragilis* in staat is om de vorming van TNF-α door macrofagen van de mens en de cavia te stimuleren.

HOOFDSTUK ZES geeft de resultaten van een onderzoek naar structurele eigenschappen van endotoxine die betrokken zijn bij de expressie van de biologische activiteit. Resultaten van eerdere onderzoeken zouden erop wijzen, dat 2-keto-3-deoxyoctonate (KDO), een onderdeel van de "inner core" van entero- en niet-enterobacteriële lipopolysacchariden dat van belang is voor het stimuleren van de afgifte van cytokines door geactiveerde macrofagen, afwezig was in het lipopolysaccharide van *Bacteroides*. In tegenstelling tot de resultaten van die onderzoeken werd de

aanwezigheid van KDO aangetoond in endotoxinen geïsoleerd uit zes verschillende stammen van de *B.fragilis* groep, na defosforilering met 50% hydrofluorzuur. Fosforilering van deze essentiële structurele component van endotoxinen draagt waarschijnlijk bij aan het, in vergelijking met *E.coli* of *Salmonella*, geringere effect van het endotoxine van *B.fragilis* op de groei en het metabolisme van de foetus.

HOOFDSTUK ZEVEN beschrijft experimenteel onderzoek naar de transplacentaire overgang van aan de moeder toegediend endotoxine naar de foetus. Het endotoxine van *B.fragilis* werd aangetoond door middel van enkelvoudige radiale immunodiffusie en counterimmunoelectroforese in geconcentreerd vruchtwater van drachtige cavia's na herhaalde intramusculaire toediening van het endotoxine lipopolysaccharide tijdens de tweede helft van de dracht. Tevens wordt een aantal (fysisch-)chemische eigenschappen beschreven van het endotoxine dat werd gebruikt voor alle experimenten beschreven in dit proefschrift. Het gezuiverde lipopolysaccharide was in antigeen opzicht heterogeen, met een moleculair gewicht van de kleinste fractie tussen 10.000 en 5.000 D. Alle serologisch reactieve componenten waren koolhydraten, eiwitbestanddelen werden niet aangetoond.

HOOFDSTUK ACHT geeft een algemene bespreking van de resultaten van de onderzoeken, die worden beschreven in dit proefschrift. De resultaten steunen de hypothese dat het endotoxine van *B.fragilis*, indien aanwezig in de uterus van de zwangere vrouw, macrofagen kan induceren tot afgifte van TNF-α, dat verantwoordelijk kan zijn voor pathofysiologische veranderingen van het moederlijke en foetale metabolisme van koolhydraten en vetten. Samen met het bekende effect van TNF-α op de biosynthese van DNA kan dit leiden tot foetale groeivertraging.

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