ABORTIFACIENT EFFECTS OF *VIBRIO CHOLERAE* EXO-ENTEROTOXIN AND ENDOTOXIN IN MICE

GABRIEL J. GASIC, TATIANA B. GASIC AND JEROME F. STRAUSS, III

*Departments of Pathology and Obstetrics and Gynecology,
University of Pennsylvania School of Medicine, Philadelphia,
Pennsylvania 19174, U.S.A.*

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Summary. To study antifertility properties of microbial toxins, exo-enterotoxin and endotoxin from *Vibrio cholerae* were injected intravenously into mice at different times during pregnancy. The two substances induced termination of pregnancy, but the patterns of abortifacient activity were different. Exotoxin terminated pregnancy in mice when administered between Days 4 and 10 of gestation, but abortifacient activity was reduced in animals more than 10 days pregnant; exogenous progesterone did not protect the pregnancies. Endotoxin was most effective in terminating pregnancy when injected after mid-gestation and the active principle was heat-stable; exogenous progesterone was not able to prevent the effects of endotoxin. Animals treated with endotoxin on Day 17 often gave birth to live young prematurely; indomethacin reduced the incidence of premature littering. The results demonstrate that exo- and endotoxins have antifertility properties and both appear to act on intrauterine targets rather than inducing progesterin deficiency.

INTRODUCTION

Maternal bacterial and viral infections during pregnancy are associated with an increased incidence of fetal death, congenital malformations, placental abruption, premature rupture of membranes and pre-maturity (for review see Charles & Finland, 1973). The factors responsible for these sequelae have not been elucidated. Since it is possible that substances released from the invading organisms cause these phenomena, we have undertaken a study of the effects of purified microbial toxins on pregnancy. In the present study, we examined the effects of administration of exo-enterotoxin and endotoxin from *Vibrio cholerae* to mice at different stages of gestation. Although *V. cholerae* are not common pathogens in obstetrical practice, their products were studied because of the availability of both exo- and endotoxin.

MATERIALS AND METHODS

Mice of the CF-1 strain (Carworth Farms, New York) were maintained in a controlled environment with 14 hr light/10 hr darkness and were mated to

males of proven fertility. The presence of a vaginal plug following evening mating was designated Day 1 of pregnancy.

Purified cholera exo-enterotoxin (exotoxin) and cholera toxin B subunit (lot 3/11/71), a protein which is immunologically identical to exotoxin but lacking biological activity, were isolated by the method of Finkelstein & LoSpalluto (1970; see also LoSpalluto & Finkelstein, 1972) and supplied by the National Institute of Allergy and Infectious Diseases; 40 ng exotoxin are equivalent to 1 Craig Lb Unit (Craig, 1971).

Exotoxin and cholera toxin B subunit were dissolved in tris-EDTA buffer (0.5 M-tris, 0.001 M-EDTA, 0.003 M-Na₂CO₃, 0.2 M-NaCl, pH 7.5) and were diluted with phosphate-buffered saline (PBS) before injection. In control experiments, exotoxin was inactivated by heating to 80°C for 30 min.

A fraction containing endotoxin was generously supplied by Dr R. A. Finkelstein, University of Texas Southwestern Medical School. This endotoxin-rich material eluted as a leading peak of a Sephadex G-75 chromatography during purification of the exotoxin (Finkelstein & LoSpalluto, 1970). The endotoxin fraction was dissolved in PBS and heated to 80°C for 30 min to inactivate contaminating exotoxin. All injections of exotoxin, cholera toxin B subunit and endotoxin were given into the tail vein in a volume of 0.25 ml.

Progesterone or progesterone and oestrone (Sigma Chemical Co.) were administered subcutaneously in sesame oil. Indomethacin (Merck, Sharp and Dohme) was suspended in a solution of 1% Emulphor EL-620 (G.A.F. Co.) in 0.9% saline and injected subcutaneously.

In some experiments, pregnant mice were killed at different intervals following treatment and examined for the condition of implantations; in others, they were killed after parturition and the young were counted and assessed for malformations and viability. In one study, mice were bilaterally ovariectomized through lumbar incisions under ether anaesthesia on Day 6 of pregnancy and then treated with 500 ng of either exotoxin or denatured exotoxin on the day of surgery and also on Day 8. Hormonal replacement therapy with either 2 mg progesterone/day and a single dose of 0.5 µg oestrone on the first treatment day, or a combination of 2 mg progesterone and 0.5 µg oestrone was begun 2 hr before ovariectomies were performed and continued daily until mice were killed on Day 15.

RESULTS

Cholera exotoxin

Single injections of 1000 ng exotoxin in the peri-implantation period prevented gestation in about 60% of mice, while pregnancy continued normally in the majority of animals receiving 1000 ng heat-inactivated toxin (Table 1). The mean number of embryos in or young born to mice receiving exotoxin and remaining pregnant was similar to that observed in mice treated with inactivated toxin and there were no gross malformations observed in the conceptuses.

Administration of two 500 ng injections 48 hr apart resulted in almost total suppression of pregnancy when treatments were given between Days 4 and 10 of pregnancy (Table 1). When mice treated on Days 4 and 6 of gestation were

killed within 5 days following the second exotoxin injection, the uteri contained the debris of resorbing implantations. When killed at later intervals, the only evidence of previous pregnancy was the presence of small mesometrial scars. Administration of exotoxin on Days 6 and 8 or Days 8 and 10 and autopsy 5 to 8 days after the last injection, resulted in haemorrhagic lesions in the placentae and fetal membranes, and subsequent total resorption of the conceptuses within a few days.

Table 1. Effect of cholera exo-enterotoxin and cholera genoid on pregnancy in mice

Group	Days of treatment	Dose/ injection (ng)	Day of autopsy	Denatured exotoxin		Exotoxin	
				No. of mice pregnant/no. treated	No. of embryos/ pregnant mouse	No. of mice pregnant/no. treated	No. of embryos/ pregnant mouse
1	4	1000	20	10/10 (100)	*	4/11 (36.4)	*
2	5	1000	19	11/18 (61.1)	12.5 ± 0.7	6/17 (35.3)	11.8 ± 1.3
3	4 and 6	500	18	15/17 (88.2)	11.4 ± 0.8	1/18 (5.6)	11
4	6 and 8	500	13	14/17 (82.4)	12.2 ± 0.6	1/18 (5.6)	8
5	8 and 10	500	18	6/8 (75.0)	10.5 ± 1.2	0/9 (0)	
6	12	1000	20	4/4 (100)	*	8/8 (100)	*
7	13	833	20	11/11 (100)	*	12/12 (100)	*
8	13	1000	20	6/6 (100)	*	9/9 (100)	*
9	14	1000	20	2/2 (100)	*	4/4 (100)	*
10	14 and 16	660	20	11/11 (100)	*	12/12 (100)	*
11	12 and 14	1000	19	6/7 (85.7)	12.0 ± 0.6	9/17 (52.9)†	8.9 ± 1.3
12‡	6 and 8	500	14			9/10 (90)	13.1 ± 0.5
13‡	6 and 8	5000	14			4/4 (100)	12.3 ± 3.0

Values are Means ± S.E.M.; figures in parentheses are percentages.

* Six to fourteen live young/mouse.

† Expulsion of conceptuses observed in some animals.

‡ Cholera genoid.

Treatment of mice on or after Day 12 with doses of exotoxin which terminated early pregnancy did not result in disruption of gestation and the treated mice gave birth to healthy young (Table 1). Increasing the dose of exotoxin to two injections of 1000 ng did cause termination of pregnancy in about half of the treated mice and a reduction in the number of viable conceptuses in other animals (Table 1). Some of the mice whose pregnancies were terminated apparently expelled the products of conception since no debris was present *in utero* at the time of autopsy. Cholera genoid displayed no abortifacient activity when administered in doses up to 5 µg/injection (Table 1, Groups 12 and 13).

To determine whether exotoxin terminates pregnancy by inhibiting the secretion of ovarian hormones, we attempted to reverse the actions of exotoxin with exogenous progesterone or progesterone in combination with oestrone. In preliminary studies, daily administration of 2 to 4 mg progesterone alone or in combination with 1 µg oestrone did not prevent resorption of implantations in mice treated with 500 ng exotoxin on Days 6 and 8, but did delay the onset of resorption by several days. A more definitive study was then carried out with ovariectomized mice. All thirteen mice receiving progesterone and the exotoxin contained resorbing implantations when killed, but 8/11 mice receiving progesterone and the heat-inactivated toxin had apparently normal conceptuses

(9.0 ± 1.4 , S.E.). The induction of embryo resorption by exotoxin in the progesterone-treated mice again progressed at a slower rate compared to that usually observed in mice given no hormonal supplement. Addition of oestrone to the hormonal therapy did not prevent the action of exotoxin in sixteen mice and the incidence of pregnancy (9/16) and survival of embryos (5.6 ± 1.4) was also reduced in control mice.

Cholera endotoxin

In contrast to the ng quantities of exotoxin required to terminate gestation, μg doses of the lipopolysaccharide fraction were needed to interfere with pregnancy and the active principle was heat stable. Mice tolerated the intravenous injection of $15 \mu\text{g}$ endotoxin and this was used as the standard dose, the only significant side effect being diarrhoea.

Table 2. Effect of cholera endotoxin on pregnancy in mice

Group	Days of treatment*	Day of autopsy	PBS		Endotoxin	
			No. of mice pregnant/no. treated	No. of embryos/pregnant mouse	No. of mice pregnant/no. treated	No. of embryos/pregnant mouse
1	4	18	20/23 (87.0)	12.8 ± 0.4	23/30 (76.7)	6.0 ± 1.3
2	5	18	13/15 (86.7)	13.8 ± 0.6	6/15 (40.0)	11.8 ± 1.0
3	4 and 6	10	12/14 (85.7)	11.4 ± 0.8	3/19 (15.8)	10.0 ± 1.0
4	6	10	6/6 (100)	13.8 ± 1.0	1/12 (8.3)	15
5	8	14	11/13 (84.6)	12.3 ± 0.7	6/16 (37.5)	11.7 ± 1.4
6	12	20	8/8 (100)	†	0/12 (0)	
7	14	19	5/5 (100)	11.4 ± 1.3	0/6 (0)	

Values are Means $\pm$ S.E.M.; figures in parentheses are percentages.

* $15 \mu\text{g}$ per injection.

† Six to fourteen live young/mouse.

Administration of endotoxin on Day 4 of gestation did not result in significant inhibition of pregnancy, but the number of viable implantations at autopsy was approximately half the number found in mice receiving PBS (Table 2). In many of the mice treated with endotoxin on Day 4, the implantations were crowded in the uterus, unlike the uniform distribution in controls. There was some suppression of pregnancy in mice given endotoxin on Day 5, but the average number of implantations in animals remaining pregnant was nearly normal (Table 2). During the early post-implantation period (Days 6 to 8), administration of endotoxin terminated pregnancy in a significant number of mice, but endotoxin was most effective in ending pregnancy when injected on or after Day 12 when fetal death was observed in all mice receiving a single $15 \mu\text{g}$ injection (Table 2).

In mice treated on Days 12 and 14, vaginal bleeding was observed within 24 hr and resorption of the conceptuses was advanced when mice were killed 48 to 96 hr later. In many of these animals, intraperitoneal adhesions were observed at necropsy. Premature parturition was induced in one-half to two-thirds of the mice receiving endotoxin on Day 17 of pregnancy. The apparently

normal young were born approximately 36 hr after endotoxin treatment (Table 3), but the offspring were neglected by their mothers and subsequently died. Animals that did not experience premature parturition were found to have resorbed their embryos.

To determine whether cholera endotoxin interferes with gestation by causing failure of the CL, progesterone was administered. Fourteen mice were treated with 4 mg progesterone/day and fourteen with sesame oil on Days 12 to 14 beginning 2 hr before 15 μ g endotoxin was administered on Day 12. None of the mice had viable implantations when killed on Day 15.

Table 3. Premature parturition induced by cholera endotoxin and its prevention by indomethacin

Group	Treatment*	No. of mice	No. of mice resorbing implantations (%)	No. of mice giving birth prematurely† (%)	No. of mice pregnant (%)
1	PBS	8	0	0	8 (100)
2	Endotoxin	18	5 (27.8)	12 (66.7)	1 (5.6)
3	Endotoxin + vehicle	11	5 (45.5)	6 (54.6)	
4	Endotoxin + indomethacin	10	9 (90.0)	1 (10.0)	

* Endotoxin (15 μ g) in PBS (phosphate-buffered saline) or PBS alone was administered on Day 17 of pregnancy and animals were killed on Day 19. Other mice received, in addition to endotoxin, 0.1 ml of 1% Emulphor EL-620 in saline or 50 μ g indomethacin in the same vehicle twice daily on Days 17 and 18 with the first injection being given 2 hr before the endotoxin was administered.

† In our laboratory, CF-1 mice begin to litter during the night of Day 19 and all animals have given birth by mid-morning of Day 20.

The ability of indomethacin, an inhibitor of prostaglandin biosynthesis, to prevent the premature delivery and fetal death resulting from endotoxin treatment on Day 17 was tested. Mice received 50 μ g indomethacin or the vehicle twice daily on Days 17 and 18 were killed on Day 19 of pregnancy. The incidence of premature parturition was reduced to 10% in mice treated with indomethacin, but the conceptuses died and were resorbed (Table 3).

DISCUSSION

Cholera exotoxin is a protein which causes the acute diarrhoea of clinical cholera by altering the flow of salts and water in the intestinal lumen. The initial step in exotoxin action appears to be the stimulation of mucosal adenylate cyclase (Sharp, 1973), but the exotoxin is also capable of stimulating adenylate cyclase in a number of other tissues (see Cuatrecasas, 1973). If stimulation of adenylate cyclase is a requisite step in exotoxin action, the task of determining the specific site(s) at which the exotoxin acts to interrupt pregnancy promises to be difficult since 3',5'-cyclic adenosine monophosphate is a universal intracellular regulator of metabolism and cell proliferation.

The antifertility activity of the exotoxin is associated with the active protein

since the ability to terminate pregnancy is lost upon heat denaturation, and cholera toxin, a biologically inactive protein which appears to be derived from the exotoxin molecule, does not display abortifacient activity. These doses are well below the LD₅₀ which is 6500 ng for CF-1 mice weighing 21 to 25 g (R. S. Northrop, personal communication), but exotoxin administration did produce a marked increase in food and water consumption and increased urination and defaecation (Gasic & Gasic, 1972). The exotoxin does not appear to cause ovarian failure because exogenous steroids (present study), LH, HCG and prolactin (unpublished observations) cannot protect pregnant mice against its action.

We currently believe that exotoxin acts by damaging the decidua, placenta, and/or embryos since the earliest signs of implantation degeneration are haemorrhagic lesions of the placenta and fetal membranes. The reduced effectiveness of the exotoxin when administered on or after Day 12 of pregnancy is of considerable interest since it is around Day 12 that the mouse placenta reaches morphological and functional maturity (Gardner & Allen, 1942). It is possible that the mature placenta prevents the exotoxin from reaching the embryo. We do not have, at present, an explanation for the ability of exogenous progesterone to retard the action of exotoxin, although the supplemental progestin might hasten placental maturation and thus protect the conceptus.

The cholera endotoxin possesses antigestational activity which is most prominent when administered to mice during the last half of pregnancy. Endotoxin preparations from other organisms will also disrupt pregnancy and this action appears to be characteristic of microbial lipopolysaccharides (Zahl & Bjerknes, 1943, 1944).

The mechanism by which bacterial endotoxins interfere with pregnancy has not been established, but from the present results and those of others (Chedid *et al.*, 1962; Harper & Skarnes, 1972) they do not appear to cause ovarian failure. Others have suggested that endotoxin damages the capillaries of the decidua and placenta. Injections of endotoxin directly into the embryos have no adverse effects upon pregnancy but haemorrhages in decidual and placental tissue result from endotoxin administration to the mother (Zahl & Bjerknes, 1943; Rieder & Thomas, 1960; McKay & Wong, 1963; Parant & Chedid, 1964). Haemorrhages may result from the release of vasoactive substances such as serotonin since treatment of pregnant mice with serotonin will cause placental haemorrhages and high doses of serotonin antagonists reputedly protect mice against the abortifacient actions of endotoxin (Parant & Chedid, 1964).

It has recently been suggested that prostaglandins are involved in the mechanism of action of endotoxins because administration of abortifacient doses of *Salmonella enteritidis* endotoxin to pregnant mice causes a marked increase in plasma, uterine and urinary prostaglandin F content (Skarnes & Harper, 1972; Harper & Skarnes, 1972). Prostaglandin F_{2α} will terminate pregnancy when administered to laboratory animals, domestic species and human and non-human primates and will also induce premature parturition when administered in late pregnancy (Labhsetwar, 1974). Our observations suggest that prostaglandins are probably not involved in the fetal death resulting from endotoxin treatment, since indomethacin did not prevent this action of endotoxin.

However, the incidence of premature birth was reduced by indomethacin treatment, indicating that prostaglandins may mediate this effect. Essentially identical results were reported by Skarnes & Harper (1972), although these authors later concluded that prostaglandins may also induce the fetal death resulting from toxin treatment (Harper & Skarnes, 1972).

Although abortion has been observed in clinical cholera (Hirschhorn *et al.*, 1969), it has not been attributed to the direct action of toxins on the reproductive system since the vibrios and their products are generally thought to be restricted to the intestinal lumen. However, our studies do illustrate that microbial products may play a significant role in pregnancy wastage. The abortifacient properties of bacterial lipopolysaccharides now seems well established but our observations clearly demonstrate that exotoxins have potent antigestational activity which is distinct from that of endotoxin. These findings should direct further attention to the role of soluble products of microorganisms in the pathogenesis of abnormal gestation.

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