Glycogen-induced intrauterine leucocytosis and its effect on mouse blastocyst implantation *in vivo* and *in vitro*

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Summary. Intrauterine instillation of heat-deaggregated glycogen in virgin mice induced a marked but transient luminal infiltration of polymorphonuclear leucocytes (PMNL). Similar injections of glycogen 2 days *post coitum* resulted in termination of pregnancy in the majority of treated females. Embryos recovered on Day 4 of pregnancy from the glycogen-treated females, before the expected time of implantation, had developed to the cavitated blastocyst stage, and were capable of undergoing characteristic outgrowth when cultured *in vitro*. A small proportion, however, appeared abnormal and did not develop *in vitro*. Cavitated blastocysts obtained from control mated females did not undergo outgrowth *in vitro* in the presence of intact PMNL, homogenates of these cells or a low molecular weight (<1500) fraction of the homogenates and after culture appeared similar to the abnormal blastocysts recovered from glycogen-treated uteri. It is proposed that the anti-fertility effect of PMNL infiltrates is due to a low molecular weight component of these cells which acts *in utero* and is cytotoxic to preimplantation blastocysts before their hatching from the zona pellucida.

Introduction

Despite the effectiveness and popularity of intrauterine devices (IUDs) as a means of contraception in women, their precise mode of action is unknown, although it has been suggested that interference with the processes of implantation is involved (Eckstein, 1971; Huber, Piotrow, Orlans & Komner, 1976). It is apparent that a fuller understanding of their mode of action may lead to the development of more effective devices and a reduction of undesirable side effects. Many studies suggest that the anti-fertility effects are mediated by the consistently observed influx of polymorphonuclear leucocytes (PMNL) into the uterus resulting from the inflammatory stimulus of the IUD. It has been claimed that fertility is inversely related to the surface area of the IUD in contact with the uterine endometrium (Davis & Lesinski, 1970) and to the number of PMNL cells present (El Sahwi & Moyer, 1970).

Intrauterine injections of PMNLs, macrophages and their extracts have produced marked reduction in fertility in rats (Bo, Krueger & Sain, 1976; El Sahwi & Moyer, 1977a). Macrophages, PMNL cells and their homogenates also arrest the *in-vitro* development of 2-cell embryos beyond the 4–8-cell stage (Smith, El Sahwi, Wilson & Moyer, 1971; El Sahwi & Moyer, 1977b) and prevent the morula–blastocyst transformation (Parr, 1969). Whether these embryotoxic effects occur *in vivo* is unknown.

Intrauterine injection of heat-deaggregated glycogen, a potent chemotactic agent, induces intrauterine leucocytosis and affects fertility in the rat (Anderson & Alexander, 1979). In the present studies we have used this system to examine the effect of intrauterine leucocytosis upon fertility in the mouse and to determine the stage at which embryonic development is affected *in vivo*.

Materials and Methods

Mice and operative techniques

Mature female mice of the inbred strains A, C57BL/6 and C57BL/10 and the randomly breeding BM colony were used. Timed pregnancies were obtained by caging 3–4 females overnight with a male of proven fertility and observing the presence of vaginal plugs the following morning. The day of detection of the plug was designated Day 1 *post coitum* (*p.c.*) of pregnancy.

Intrauterine injections were performed following mid-ventral laparotomy on animals anaesthetized by a single injection of 2.5% tribromoethanol with 2.2% tetra-amyl alcohol in distilled water at a dose of 0.1 ml/10 g body weight. Solutions were introduced by injection at the utero-cervical junction and a ligature was applied to prevent leakage. In virgin mice, 15 μ l of a 10% heat-deaggregated oyster glycogen (Type II, Sigma Chemical Co., St Louis, Missouri, U.S.A.) in Dulbecco A and B phosphate-buffered saline (DPBS), prepared before the operation by heating at 100°C for 10 min, were injected into one uterine horn and 15 μ l DPBS were injected into the other horn. In pregnant mice, one horn received 15 μ l glycogen or DPBS whilst the other horn was left untreated.

In fertility studies, mice were killed on Day 9 of pregnancy by cervical dislocation and the number of implantation sites, including resorptions, in each uterine horn was recorded. The Student *t* test was employed for all statistical analyses.

Collection and quantification of intrauterine cellular infiltrate

At timed intervals after injection animals were killed by cervical dislocation and the uterine horns were excised, cleared of fat, and flushed with 1 ml DPBS. This suspension was centrifuged at 800 *g* for 5 min and the pellet was resuspended in 0.1–1 ml cell diluting fluid (0.2% acetic acid) and cells were examined and counted in a haemocytometer. For histological studies aliquots of the suspensions (in DPBS) were smeared on microscope slides and air dried.

For in-vitro embryo cultures, intact intrauterine cell infiltrates were obtained under sterile conditions from uterine flushings of virgin animals 24 h after intrauterine injection with a sterile 10% glycogen solution. These cells were resuspended in RPMI-1640 culture medium (Gibco-Biocult, Glasgow, U.K.) with added 10% fetal calf serum, 0.2% sodium bicarbonate, penicillin and streptomycin, at 20×10^6 /ml. Sterile homogenates of such cells (20×10^6 cells/ml) were prepared with a glass homogenizer followed by centrifugation for 5 min at 2000 *g* to remove cellular debris, under sterile conditions. High molecular weight (>1500) fractions of the homogenates were obtained under sterile conditions by applying aliquots (200 μ l) to a Sephadex G15 column (total volume of 1 ml in syringe barrels) equilibrated in, and eluted with, RPMI-1640 culture medium, and collecting 200 μ l fractions. High and low molecular weight fractions were gauged by the addition of blue dextran (high) and potassium ferricyanide (low) to the column.

Embryo collection and culture

Embryos were obtained by aseptic flushings of uterine horns with RPMI-1640 culture medium between 11:00 and 14:00 h on Day 4 of pregnancy. After microscopic examination groups of embryos (2–5) were transferred to wells of microtitre plates (Sterilin, Teddington, Middlesex, U.K.) containing 0.1 ml RPMI-1640 culture medium. Cultures were incubated at 37°C in a humid atmosphere of 5% CO₂ in air for 72 h, after which they were examined under a binocular microscope and scored for the number of blastocyst outgrowths. To examine the effects of intrauterine infiltrate cells and their homogenates, aliquots of 50 μ l were added at the start of the culture period, to give a final concentration of 1×10^6 cells, or their equivalent, per culture well. This concentration of PMNL cells has previously been demonstrated to inhibit the

development of 4–8-cell embryos (El Sahwi & Moyer, 1977b). The high and low molecular weight fractions were added to culture wells in aliquots of 50 μ l.

Histological procedures

Smears of the intrauterine cellular infiltrates were fixed in methanol for 5 min and subsequently stained with diluted Giemsa solution (1:10) for 10 min. Blastocyst outgrowths were fixed in absolute alcohol for 45 min and, after drying, stained with diluted Giemsa solution for 45 min.

In virgin females given intrauterine glycogen injections, segments of uterine horns were isolated by ligation, excised, fixed in neutral buffered formalin, sectioned and then stained with haematoxylin and eosin or trichrome blue for microscopic examination.

Results

Effect of intrauterine administration of glycogen in virgin females

As shown in Text-fig. 1, administration of glycogen to virgin A-strain female mice attracted a large number of cells to the uterine lumen, the maximal response being observed 24 h after injection; the response was apparent by 12 h, but fell dramatically by 72 h. The number of cells recovered from glycogen treated horns was significantly greater than that from untreated or DPBS-treated horns ($P < 0.01$). DPBS, however, induced a significant amount of infiltration when compared to values for untreated horns ($P < 0.05$) but the maximal infiltration occurred at 48 h after injection (Text-fig. 1). Identical responses were observed in virgin females of all the inbred strains and the BM outbred colony.

Text-fig. 1. Time course of leucocyte accumulation in uterine horns of virgin mice after a single injection of 10% glycogen in DPBS or DPBS. Values are mean $\pm$ s.e.m. for the number of horns indicated. Hatched area indicates numbers of leucocytes recovered from untreated horns (2 s.e.m.).

Examination of stained smears of uterine flushings revealed that the principal cell type present in all infiltrates was the polymorphonuclear leucocyte (PMNL) (95–100%). This finding was supported by microscopic examination of cells found within the lumen in uterine sections.

Effect of intrauterine administration of glycogen in pregnant females

Effect on fertility. Glycogen injections administered on Day 2 of pregnancy resulted in a dramatic reduction in the number of implantation sites present on Day 9 (compared with untreated horns, $P < 0.001$), with only 1/12 uterine horns containing conceptuses (Table 1). Intrauterine administration of DPBS also significantly reduced fertility compared to untreated controls ($P < 0.001$), but was not as effective as glycogen ($0.01 < P < 0.02$) (Table 1). It therefore appeared that the relative anti-fertility effects of glycogen and DPBS reflected their relative efficacy in inducing intrauterine PMNL infiltration.

Table 1. Effect on fertility (total no. of implantation sites/uterine horn) of intrauterine instillation of glycogen or DPBS in pregnant female mice*

Glycogen-treated horn	Untreated horn	DPBS-treated horn	Untreated horn
0	1	5	5
0	4	4	6
4	1	0	4
0	4(1)	0	4
0	3(3)	0	3
0	2(1)	1(1)	3
0	5	1(1)	7
0	3	3(1)	3
0	6(4)	0(3)	7
0	7	0	3
0	3	0	5
0	4		
Mean $\pm$ s.d.			
0.33 $\pm$ 1.11	3.58 $\pm$ 1.75	1.44 $\pm$ 1.77	4.5 $\pm$ 1.49

* Injections given at Day 2 *p.c.* and animals examined at Day 9 *p.c.* Values in parentheses indicate resorbing fetuses.

Effect on embryonic development in vivo. Since normal implantation had not occurred in the majority of females treated with glycogen on Day 2 of pregnancy, the embryos were flushed from these females just before the expected time of implantation to determine whether the glycogen had already exerted an effect on embryonic development. As shown in Table 2, the proportion of embryos recovered at the morula stage was similar to that in untreated controls. Although the percentage of embryos at the cavitated blastocyst stage recovered from glycogen-treated horns was less than from untreated horns (57% compared to 84%), several embryos recovered from the glycogen-treated horns were considered to be 'degenerate' blastocysts. These embryos were still encased in a zona pellucida, but exhibited an overall shrinkage of the blastocoele and inner cell mass and abnormal blastomere morphology. It therefore appeared that intrauterine glycogen did not affect development of embryos before the cavitated blastocyst stage.

Effects of intrauterine cellular infiltrate on blastocyst outgrowth in vitro

Since intrauterine glycogen administration appeared to inhibit implantation but not development of the embryo to the cavitated blastocyst stage *in vivo*, the effects of the infiltrating PMNL cells and their homogenates were tested in an in-vitro blastocyst outgrowth system.

Table 2. Effect on embryonic development of intrauterine instillation of glycogen in pregnant females*

	Glycogen-treated uteri		Untreated uteri	
	No. of embryos recovered (%)	% outgrowth <i>in vitro</i>	No. of embryos recovered (%)	% outgrowth <i>in vitro</i>
No. of morulae/total	6/37 (16%)	83	7/45 (16%)	57
No. of cavitated blastocysts/total	21/37 (57%)	52	38/45 (84%)	76
No. of 'degenerate' blastocysts/total	10/37 (27%)	0	0	—

* Glycogen was administered to females on Day 2 *p.c.* and embryos recovered from the uterine horns between 11:00 and 14:00 h on Day 4 *p.c.*

Under appropriate conditions, blastocysts will hatch from the zona pellucida, attach to the surface of the culture vessel and form a monolayer of trophoblast cells surrounding a knot of inner cell mass (Sherman & Wudl, 1976; Jenkinson, 1977).

Cavitated blastocysts obtained from untreated pregnant females on Day 4 of pregnancy were cultured in the presence of intrauterine infiltrate flushings at a concentration approximating the maximum cell number observed per uterine horn after glycogen administration. No blastocyst outgrowths were detected in any wells containing intrauterine cells (0/12) as compared to a 65% outgrowth rate in control wells (11/17). After culture with intrauterine cells, all blastocysts appeared identical to the 'degenerate' blastocysts observed in the flushings from glycogen-treated pregnant females. This effect was irreversible; there was no development when blastocysts were re-cultured in fresh cell-free medium.

Addition of an homogenate of the PMNL cells, at an equivalent concentration, reduced the rate of blastocyst outgrowth to 12% (1/8). Those blastocysts not attaching at the end of the 72-h culture period exhibited features identical to those of the 'degenerate' blastocysts. Addition of a low molecular weight (<1500) fraction of the homogenate totally inhibited blastocyst outgrowth whereas addition of a high molecular weight fraction was without effect.

Groups of embryos recovered on Day 4 of pregnancy from glycogen-treated uterine horns were divided into morulae, cavitated blastocysts and 'degenerate' blastocysts and similarly cultured for 72 h. As shown in Table 2, the morulae and cavitated blastocysts exhibited an outgrowth rate comparable to that observed with control embryos, whilst none of the 'degenerate' blastocysts showed outgrowth.

Discussion

A single intrauterine injection of a chemotactic agent, heat-deaggregated glycogen, induced a marked infiltration of cells, primarily polymorphonuclear leucocytes, into the uterus of the virgin mouse. This influx was transient, with the number of cells approaching control values 72 h after injection. Although this was comparable to the kinetics of the response in non-pregnant rats (Anderson & Alexander, 1979) the similar response observed in injected pregnant mice (G. T. Waites, unpublished observations) was not evident in pregnant rats, in which the infiltrate remained for at least 5 days (Anderson & Alexander, 1979). As in the rat, this treatment in the mouse produced potent anti-fertility effects when given on Day 2 of pregnancy. Although the carrier fluid employed (DPBS) also affected fertility, to a lesser but nonetheless significant degree, this was considered to reflect the significant chemotactic properties of this reagent. These

observations support the concept that the cellular infiltrate induced by an intrauterine contraceptive device could mediate the observed anti-fertility effects of the device.

Although no evidence of implantation was seen in the infertile uterine horns on Day 9 of pregnancy, as assessed by absence of decidualization, in those females untreated with glycogen, embryos were recovered from the uteri at 12:00 h on Day 4 of pregnancy. The proportion of embryos at the morula stage of development was similar to that observed in untreated controls, and although the proportion of cavitated blastocysts was lower, this discrepancy was accounted for by a number of abnormal blastocysts observed only in glycogen-treated uterine horns. Both the morulae and the cavitated blastocysts from treated horns underwent blastocyst outgrowth *in vitro* at rates similar to those of embryos from control uteri.

On Day 2 of pregnancy, when the glycogen was administered, embryos are normally present in the oviduct at the 4-cell stage, and develop to 16–32-cell morulae before entering the uterus (New, 1966). The glycogen-induced infiltrate had no effect on this oviducal period of embryonic development. This observation contrasts with those of studies in which PMNL cells, macrophages and their products inhibit the development of embryos beyond the 4–8-cell stage *in vitro* (Smith *et al.*, 1971; El Sahwi & Moyer, 1977b), but is in accordance with results for women that indicate that IUDs are less effective in protecting against ovarian or tubal pregnancies (Lehfeldt, Tietze & Gorstein, 1970). The present observations suggest that intrauterine PMNL cells cannot gain access to the oviduct in the mouse. However, why morulae should undergo their conversion, and the majority of cavitated blastocysts thus formed remain functional in the undoubted presence of the intrauterine infiltrate, is uncertain.

The fact that some abnormal non-viable blastocysts, i.e. unable to undergo outgrowth *in vitro*, are recovered on Day 4 of pregnancy suggests that cavitated blastocysts are eventually susceptible to some embryotoxic effect of the PMNL cells. That these abnormal blastocysts are produced by the action of the PMNL cells upon cavitated blastocysts is suggested by the observation that the cells or their homogenates inhibit blastocyst outgrowth *in vitro*, and result in 'abnormal' blastocysts in culture. It is possible that blastocysts require a certain period in contact with PMNL cells before they succumb to the embryotoxic effect, or that some alteration in the zona pellucida must occur before the toxic factors can gain access to the embryo. The fact that abnormal blastocysts recovered from glycogen-treated uteri are still enclosed in the zona and that normal cavitated blastocysts killed *in vitro* with PMNL cells or their homogenates also retain their zona strongly suggests that the embryotoxic factor(s) operates by killing the blastocyst at the initiation of the hatching process. This would support the conclusions of Hurst *et al.* (1978) that embryonic development in IUD-bearing mice is blocked at the blastocyst-hatching stage.

In females treated with glycogen there was complete absence on Day 9 of pregnancy of the decidualization expected when blastocysts are present during the period of endometrial sensitivity (Finn & Porter, 1977). It is possible that the intrauterine leucocytosis also affected the preparation of the endometrium for implantation and/or uterine epithelial blastocyst interaction. Martin & Finn (1970) have observed that IUDs prevent progestational differentiation of the luminal epithelium and luminal closure and have suggested that these effects could mediate the antifertility action of IUDs. Since untreated contralateral horns also exhibit alteration in epithelial morphology, cellular infiltrates are not thought to be involved (Martin & Finn, 1978). Although these effects may be associated with the presence of an IUD, it is unlikely that they are involved in the anti-fertility action of glycogen-induced cellular infiltrates since there is no apparent inhibition of implantation in the untreated contralateral uterine horns.

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