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Induction of Uterine Leukocytosis and Its Effect on Pregnancy in Rats

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ABSTRACT

Glycogen, a leukocyte chemotactic agent, was used to induce transient leukocytosis in rat uteri during pregnancy. Large numbers of polymorphonuclear leukocytes (PMNLs) were attracted to the uterine endometrium and lumen after a single intrauterine injection of soluble glycogen (ligated horns) or glycogen-releasing gelatin beads (nonligated horns). Glycogen treatment on Days 3 through 5 of pregnancy (Day 5 = implantation) caused complete termination of pregnancy and significantly reduced fertility on Days 2 and 6 of pregnancy. However, fertility was unaffected by glycogen treatment after Day 6 of pregnancy even though a substantial leukocytic response was also elicited by glycogen in rats during later stages of gestation. This study indicates that glycogen-induced uterine leukocytosis effectively terminates pregnancy before and during the implantation stage. We propose that leukocyte chemotactic agents such as glycogen may prove clinically useful as abortifacient agents or may be used to improve the contraceptive efficiency of intrauterine devices.

INTRODUCTION

Although intrauterine devices (IUDs) are widely used for human contraception, their mechanisms of action have not been clearly defined. It is often stated that a better understanding of the sites and modes of action of IUDs could lead to the development of more effective and better accepted methods of fertility regulation.

Most recent studies indicate that the antifertility effect of the IUD is exerted in the uterus and there is increasing evidence that inflammatory cells attracted to the uterus by IUDs mediate certain of the contraceptive effects. Davis and Lesinski (1970) reported that the contraceptive efficiency of IUDs in women is directly correlated to the surface area of the IUD in contact with the uterine endometrium and the degree of inflammation elicited by local irritation to the uterine tissues. Other investigators have demonstrated an inverse correlation between fertility and the number of inflammatory cells in IUD-bearing uteri (Parr et al., 1967; El Sahwi and Moyer, 1970). Such observations have led to studies in experimental animals on the antifertility effects of inflammatory cells and their products. In rats and rabbits, transfer of viable polymorphonuclear leukocytes (PMNLs) or PMNL extracts to the uterine lumen during early pregnancy causes a marked reduction in fertility (Bo et al., 1976; El Sahwi and Moyer, 1977). In addition, inflammatory cells and their products have been shown to be toxic to preimplantation embryos in vitro (Parr, 1969; Smith et al., 1971; Parr and Shirley, 1976; El Sahwi and Moyer, 1977).

In the present study, we used a new approach to investigate the antifertility effects of intrauterine leukocytes. Transient uterine leukocytosis was induced in rat uteri on various days of pregnancy by introduction of a physiologic leukocyte chemotactic agent, glycogen, into the uterine lumen. Our purpose was to determine whether this technique would terminate pregnancy and if so which stages of pregnancy would be affected.

MATERIALS AND METHODS

Preparation of Chemotactic Factors

Intermediate molecular weight glycogen from rabbit liver (Type II, Sigma Chemical Co.) was used in most of the experiments because it proved to be the most potent leukocyte chemotactic factor tested by us in the rat uterus. Glycogen solutions of various concentrations were prepared by the heating of glycogen-saline mixtures to 100°C for 10 min for deaggregation of the glycogen molecules. Glycogen solutions containing 20% gelatin (Difco Laboratories) were heated to 100°C for 10 min, then poured into shallow petri

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dishes and solidified at 4°C. Gelatin beads (∿1 X 3 mm) were made by insertion of 25 μl capillary tubes into the solidified gelatin layer.

In preliminary experiments various other leukocyte chemotactic agents were tested in rat uteri: zymosan-activated serum prepared as described by Ward (1968), *Escherichia coli* extract (Ward et al., 1968), spermatozoa homogenates (Clark and Klebanoff, 1976) and the peptides, N-formyl-L-methionyl-L-phenylalanine and N-formyl-L-methionyl-L-leucyl-L-phenylalanine (Sigma Chemical Co., St. Louis, MO).

Animal Studies

Virgin sexually mature female Sprague-Dawley rats were used for initial studies to measure the relative potency of various leukocyte chemotactic factors and the dynamics of the elicited leukocytic response. Pregnant rats were used in subsequent experiments. Mature nulliparous female Sprague-Dawley rats were mated with males of proven fertility and the day that sperm were found in vaginal smears was considered Day 1 of pregnancy. Later observations of embryos, resorption sites, or corpora lutea at laparotomy provided confirmation of pregnancy.

Sterile intrauterine injections were made in virgin and pregnant rats via laparotomy. In some animals, uterine horns were ligated above the cervix with 4-0 silk suture. In groups receiving soluble chemotactic factors, Hamilton microsyringes were used to inject 0.02 ml amounts into the ovarian end of the uterine lumen. The left uterine horn received an injection of chemotactic factor solution; the right horn received saline only and served as a control. In other groups, capillary tubes fitted with stylets were used for placement of glycogen-releasing gelatin beads in the uterine lumen. The tapered tip of a capillary tube was inserted into the lumen through a small opening made at the ovarian end of the uterine horn with a 20 gauge needle. In these animals, the right (control) horns received gelatin only.

Animals used for leukocyte quantification and histological studies were anesthetized with ether and killed by exsanguination at various times post-treatment. Cells were flushed from the uterine horns with saline, centrifuged at a high speed (8,000 X g) for 5 min and then resuspended in 0.02 ml diluting solution (0.5% glacial acetic acid and 0.1% toluidine blue in distilled water). The cells were counted on a hemocytometer and the numbers of PMNLs, macrophages and lymphocytes in each sample were recorded. In some animals, a segment of the ovarian end of the uterus was isolated from the rest of the uterus by ligation prior to the flushing procedure and was subsequently removed, fixed in buffered formalin, sectioned (6 μm) and stained with hematoxylin and eosin for histological examination.

Animals used for fertility studies were killed on Day 15 of pregnancy and the number of embryos, resorption sites and corpora lutea on each side was recorded. Results from the experimental (chemotactic-factor treated) and control (saline or gelatin treated) horns were compared directly and were also compared to results from untreated animals.

We performed an additional experiment to determine the long term effects of glycogen treatment on fertility. We allowed one group of rats that had received plain gelatin in the right uterine horn and glycogen-releasing gelatin beads in the left horn on Day 5 of pregnancy to recover for 30 days. Any pups delivered were removed immediately. Vaginal smears were examined daily to determine when normal estrous cycles resumed. Thirty days after glycogen treatment, the females were again caged with fertile males. When advanced pregnancy (Day 18 ± 2) was detected by abdominal palpation, the rats were killed and the numbers of embryos, resorption sites and corpora lutea on each side were recorded. All data were subjected to analysis of variance or to the Wilcoxon rank sum test (nonparametric data) to determine significant differences between treatment groups (Sokal and Rohlf, 1969).

RESULTS

Leukocyte Quantification Studies

Few PMNLs were recovered from nonligated rat uterine horns that received soluble chemotactic factors. However, substantial leukocytic responses were elicited in uterine horns that were ligated at the cervix at the time of injection. Of all the leukocyte chemotactic agents tested in this system, 10% glycogen exerted the most powerful leukocytic effect (Table 1). The

TABLE 1. Number of leukocytes recovered from ligated rat uterine horns 12 h after intrauterine injection of various leukocyte chemotactic factors.

Saline only (control)	$<10^3$
Sonicated sperm extract	2.0 ± 10^5
E. coli extract	2.5 ± 10^4
Zymosan activated serum	8.0 ± 10^3
1% F-met-leu-phe	2.5 ± 10^5
1% F-met-phe	6.0 ± 10^4
0.01% Glycogen	5.3 ± 10^4
0.1% Glycogen	8.0 ± 10^4
1% Glycogen	3.25 ± 10^5
10% Glycogen	5.75 ± 10^5

[a]Minimum of 3 horns used for each test material.

dynamics of the leukocytic response induced by injection of 10% glycogen into ligated horns are shown in Fig. 1. Large numbers of PMNLs first appeared in the uterine lumen 6 h after glycogen injection. In nonpregnant animals, the leukocytic effect was transient: the maximum number of PMNLs (2.2×10^5) was recovered at 24 h and the cells disappeared from the lumen within 72 h. However, when intrauterine glycogen injections were given on Day 5 of pregnancy, the number of PMNLs and other inflammatory cells recovered from the uterus steadily increased and on Day 10 of pregnancy $2.1 \pm 0.8 \times 10^6$ PMNLs were recovered/uterine horn. Fewer than 10^3 PMNLs were recovered from saline injected control horns at any time in nonpregnant and pregnant animals.

We found that gradual release of glycogen into the uterine lumen from biodegradable 20% gelatin matrices caused a transient influx of PMNLs into nonligated uteri (Fig. 2). The peak leukocytic response occurred in these animals at 12 h and subsided within 48 h. Gelatin alone (used as a control) also exerted a moderate leukocytic effect, but the response was significantly less than that observed in horns treated with the gelatin-glycogen combination (3.6×10^4 PMNLs/horn vs 3.9×10^5 PMNLs/horn, $P < 0.025$).

Fertility Studies

The fertility data from animals given intrauterine injections of saline or 10% glycogen during early pregnancy are presented in Fig. 3.

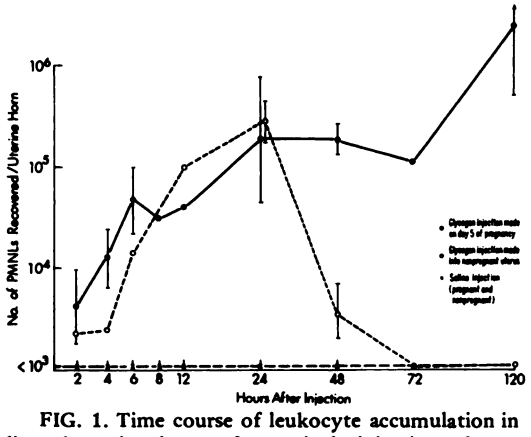

FIG. 1. Time course of leukocyte accumulation in ligated uterine horns after a single injection of 10% glycogen in saline. Key points represent the mean no. of PMNLs ± SEM from 3 uterine horns. Points without standard errors represent single observations.

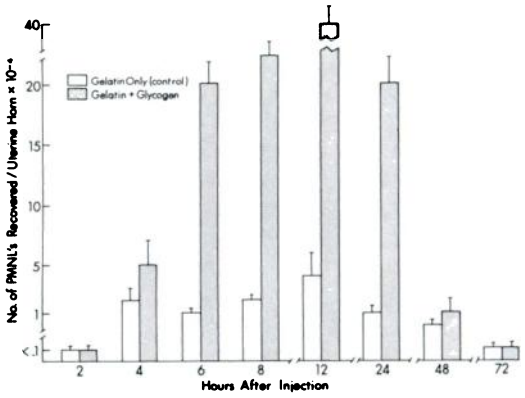

FIG. 2. Number of leukocytes recovered from nonligated uterine horns treated with glycogen-releasing gelatin beads or gelatin (n = 3 for each value; mean ± SEM plotted).

When the cervix was not ligated at the time of injection, neither saline nor soluble glycogen injections significantly affected fertility. On the other hand, when injections were made into ligated uterine horns, fertility was affected on certain days of early pregnancy. Ligated horns treated with 10% glycogen on Days 2 through 5 of pregnancy were completely infertile and fertility was also markedly reduced in ligated horns treated with glycogen on Day 6 of pregnancy. The significance of the glycogen effect was masked, however, because the ligated control horns treated with saline on Days 2 through 6 of pregnancy were also subfertile (2.1 ± 0.7 embryos/saline treated horn vs 6.2 ± 1.1 embryos/untreated horn, $P < 0.01$). However, saline treated horns were rarely infertile and there was a significant difference in fertility between control and experimental horns treated on Day 4 of pregnancy (3.0 ± 0.3 embryos/saline treated horn vs 0 embryos/glycogen treated horn, $P < 0.05$). Neither glycogen nor saline injections caused a significant reduction in fertility in ligated horns after Day 6 of pregnancy.

Fertility was also affected when glycogen-releasing gelatin beads were inserted into nonligated uterine horns during early pregnancy (Fig. 4). No viable embryos were recovered on Day 15 of pregnancy from horns that received glycogen-releasing gelatin on Days 4 and 5 of pregnancy and fertility was also significantly reduced in animals treated on Days 2, 3 and 6 of pregnancy ($P < 0.01$, when fertility of glycogen treated horns was compared with that of untreated horns). However, control horns that

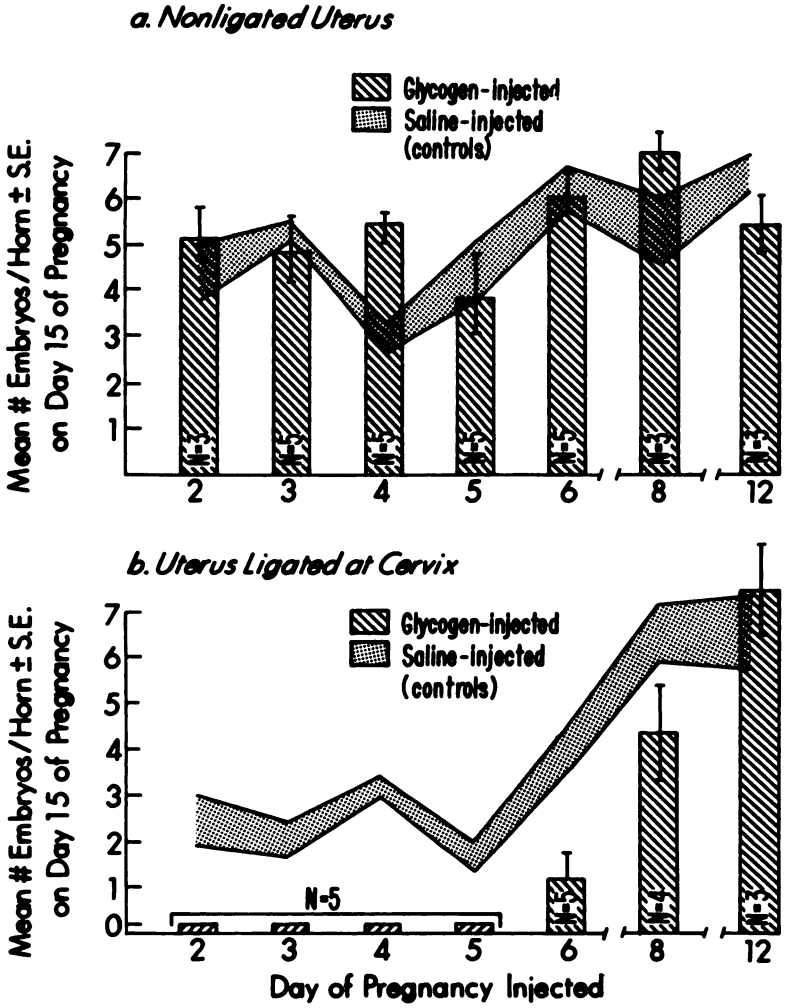

FIG. 3. Fertility after injection of 10% glycogen into ligated and nonligated uterine horns on a given day of pregnancy.

received plain gelatin early in pregnancy were also subfertile (3.4 ± 0.9 embryos/gelatin treated horn vs 6.2 ± 1.1 embryos/untreated horn, $P < 0.01$) and there was a significant difference in fertility between gelatin and glycogen-gelatin treated horns only on Days 4 and 5 of pregnancy. As was the case with soluble glycogen, glycogen-releasing gelatin did not affect fertility when inserted into the uterine lumen after Day 6 of pregnancy.

Results from an experiment to determine the long term effect of glycogen treatment on fertility indicate that uterine horns treated with glycogen-releasing gelatin beads 30 days earlier were as fertile as untreated or gelatin treated horns (Table 2). Glycogen treated rats resumed normal estrous cycles within 20 days of termination of pregnancy and all but one became pregnant on the first mating (30–35 days after termination of pregnancy).

Histologic Results

Margination of PMNLs in uterine blood vessels was first observed 4 h after treatment with soluble glycogen (ligated uteri) or glycogen-releasing gelatin beads (Fig. 5). Large numbers of PMNLs were found in the uterine endometrium and lumen 6 h after treatment (Fig. 6). Fetuses were not found in uterine lumina in sections taken 24, 48 or 72 h after glycogen treatment on Days 4 through 6 of pregnancy. However, resorption sites containing

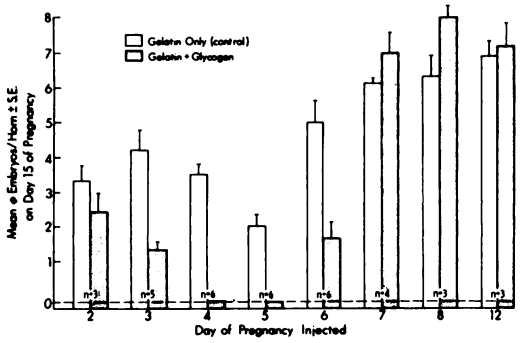

FIG. 4. Fertility after insertion of glycogen-releasing gelatin beads into nonligated uterine horns on a given day of pregnancy.

placental tissue were found in most horns treated with glycogen during early pregnancy (Fig. 7). These sites attained maximum size (3 × 2 mm) on Day 15 of pregnancy, then regressed and disappeared by Day 25 of pregnancy. Invasion of implanted fetal tissue by PMNLs was not observed in the sections examined.

Rats treated with glycogen after Day 6 of pregnancy also manifested massive leukocytic responses, but the leukocytes did not penetrate the outer fetal membranes (Fig. 8) and did not apparently affect fetal development.

DISCUSSION

We tested various leukocyte chemotactic factors in the rat uterus and found that glycogen is a particularly potent leukocytic agent in the uterine environment and induces a variety of leukocytic responses whose magnitude and dynamics depend on the mode and time of administration of glycogen. However, none of the chemotactic factors including glycogen elicited a measurable leukocytic response unless the cervix was ligated at the time of injection, or unless the factor was released gradually into the uterine lumen from a biodegradable matrix. We have observed in previous studies that materials pass quickly from the rat uterus into the vagina. When leukocytes or erythrocytes are transferred to the rat uterine lumen, 90% disappear from the lumen within 5 min and can be recovered in the vagina (Anderson, unpublished observation). We postulate that soluble chemotactic factors drain from the uterus before they exert a leukotactic effect unless their passage is impeded by ligation of the cervix or by entrapment of the chemotactic factor within a biodegradable matrix. We observed that glycogen induced transient uterine leukocytosis with a peak response at ~24 h in nonpregnant uteri. Injection of glycogen into ligated uterine horns during early pregnancy produced a more pronounced and

TABLE 2. Fertility in animals mated 30 days after treatment with glycogen-releasing gelatin beads.

Day of pregnancy treated[a]	Treatment	Mean no. embryos/ uterine horn ± SEM[b]
2	Gelatin	9.0 ± 2.05
2	Gelatin + glycogen	3.0 ± .80
3	Gelatin	4.33 ± 2.28
3	Gelatin + glycogen	3.67 ± 1.75
4	Gelatin	3.33 ± 1.59
4	Gelatin + glycogen	8.0 ± 1.24
5	Gelatin	6.0 ± 1.61
5	Gelatin + glycogen	8.33 ± .92
6	Gelatin	5.67 ± .97
6	Gelatin + glycogen	4.33 ± 2.29
Total: gelatin treated (n = 15)		5.86 ± 1.7
Total: gelatin + glycogen treated (n = 15)		5.46 ± 1.4[c]

[a]Gelatin or gelatin + glycogen injected into uterus during first pregnancy. Three rats were treated in each group.

[b]Fertility in second pregnancy.

[c]None of the individual or total mean values from either gelatin or glycogen + gelatin treated groups was significantly different from a control value of 4.87 ± 1.24 embryos/untreated horn (n = 8).

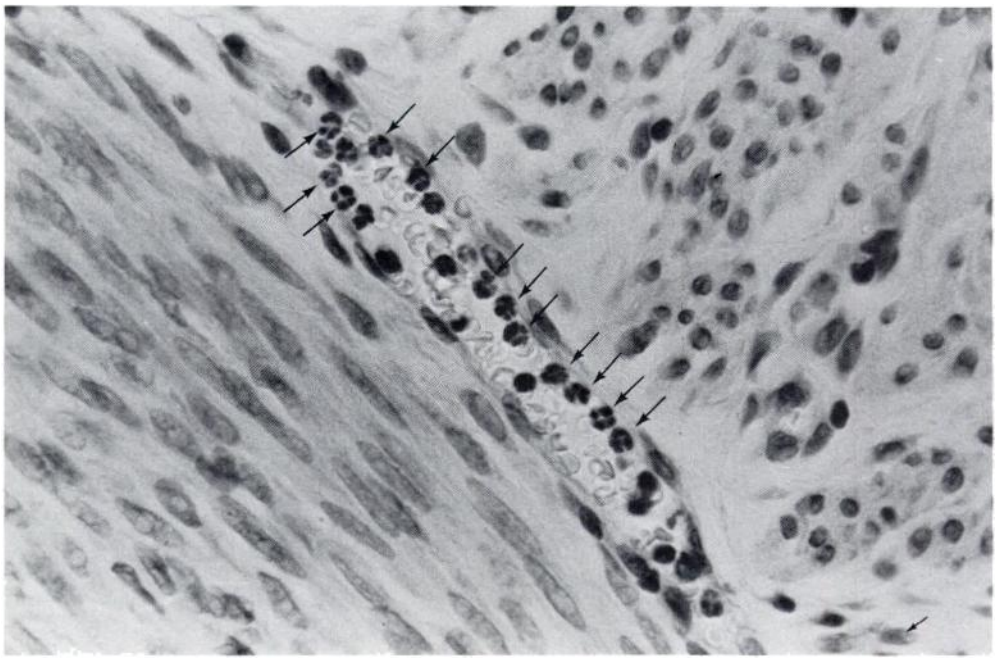

FIG. 5. Margination of leukocytes in uterine blood vessels 4 h after glycogen injection. X 640.

sustained leukocytic effect, probably because of increased blood flow to the uterus during pregnancy, increased permeability of the uterine blood vessels and because considerable cellular debris is present in these horns which no doubt potentiates the inflammatory response. The enhanced leukocytic effect during pregnancy in ligated horns is at least initiated by glycogen because control horns (ligated, saline injected) do not support leukocytic responses of the same magnitude.

Uterine leukocytes have been implicated as mediators of some of the antifertility effects of IUDs and this study provides further evidence to support this theory. We found that a single intrauterine injection of glycogen, which induces a marked leukocytic response, causes pregnancy termination when administered to rats during early pregnancy. It is unlikely that the glycogen injections per se caused the observed antifertility effect because glycogen and its breakdown product, glucose, are not cytotoxic and because very small volumes (0.02 ml) and low concentrations (0.1%–10%) were used. Since other investigators have shown that leukocytes are embryotoxic (Parr, 1969; Smith et al., 1971; Parr and Shirley, 1976; El Sahwi and Moyer, 1977), it is probable that the leukocytes attracted to the uterus by glycogen mediated the antifertility effect observed in our studies. We suggest that our model provides more convincing proof than previous cell transfer studies that leukocytes actually do mediate contraceptive effects since we used autologous effector leukocytes and did not use in vitro procedures that could affect the viability and activity of the cells.

There is still considerable debate as to whether fertilization and implantation occur in the presence of an IUD. The results of this study provide information concerning which stages of pregnancy are affected by intrauterine leukocytes and may help to further define the contraceptive role of leukocytes in IUD bearing uteri. Pregnancy was terminated by our leukocytosis induction technique on Days 2 through 5 but was not affected after Day 6 of pregnancy. Implantation reportedly is initiated in rats late on Day 5 of pregnancy (Dickmann and De Feo, 1967). We found implantation sites containing placental tissue but no viable embryos in animals treated during both preimplantation and implantation stages of pregnancy, indicating that implantation occurred in animals treated even 2 days prior to the implantation stage. Therefore, it appears that leukocytes, their products, or both, are embryocytotoxic or arrest fetal development during or

immediately after implantation. Since it has been observed that large numbers of PMNLs migrate to the uterine lumen immediately after normal mating in several species including rodents (Yanagimachi and Chang, 1963) and primates (Hafez and Jaszczak, 1973), it is not likely that leukocytes mediate a contraceptive effect on Day 1 of pregnancy. In fact, Marston and Chang (1975) showed that removal of IUDs before Day 2 of pregnancy will allow gestation to proceed normally in most rats. There is some evidence that contact between spermatozoa and leukocytes is required for capacitation to occur (Soupart, 1970). A recent report by Hurst et al. (1978) also indicates that brief association of fertilized mouse eggs with uterine leukocytes does not necessarily affect their subsequent development. Postimplantation embryos apparently are protected from possible harmful effects of uterine leukocytes by the fetal membranes since leukocytic infiltration of fetal tissues was not observed in postimplantation embryos.

Intrauterine devices apparently exert multiple contraceptive effects and the primary contraceptive mechanisms may vary from species to species. In sheep, IUDs reportedly prevent sperm from migrating through the fallopian tubes by affecting uterine and oviductal contractions (Hawk, 1967). In the rabbit there is evidence that IUDs prevent implantation by stimulating a higher concentration of prostaglandins in the uterus (Saksena and Harper, 1974). In rodents it has been shown that luminal fluid from IUD bearing uteri is toxic to preimplantation embryos (Joshi and Kraemer, 1970; de Boer and Anderson, 1971). The toxic factor(s) have not been identified and may be produced by inflammatory cells since it has been demonstrated that leukocyte homogenates are toxic to embryos in vitro (Parr, 1969; Smith et al., 1971; Parr and Shirley, 1976; El Sahwi and Moyer, 1977). In humans, researchers have recovered viable sperm and both fertilized and unfertilized ova from fallopian tubes of women with IUDs (Morgenstern et al., 1966; Noyes et al., 1966), indicating that IUDs do not suppress ovulation or completely inhibit sperm transport or the fertilization process. The most widely accepted theory at present is that IUDs in women interfere in some manner with the implantation process (Eckstein, 1971; Huber et al., 1976). This late contraceptive effect in women may be mediated by intrauterine leukocytes since we demonstrated in our rat study that leukocytes

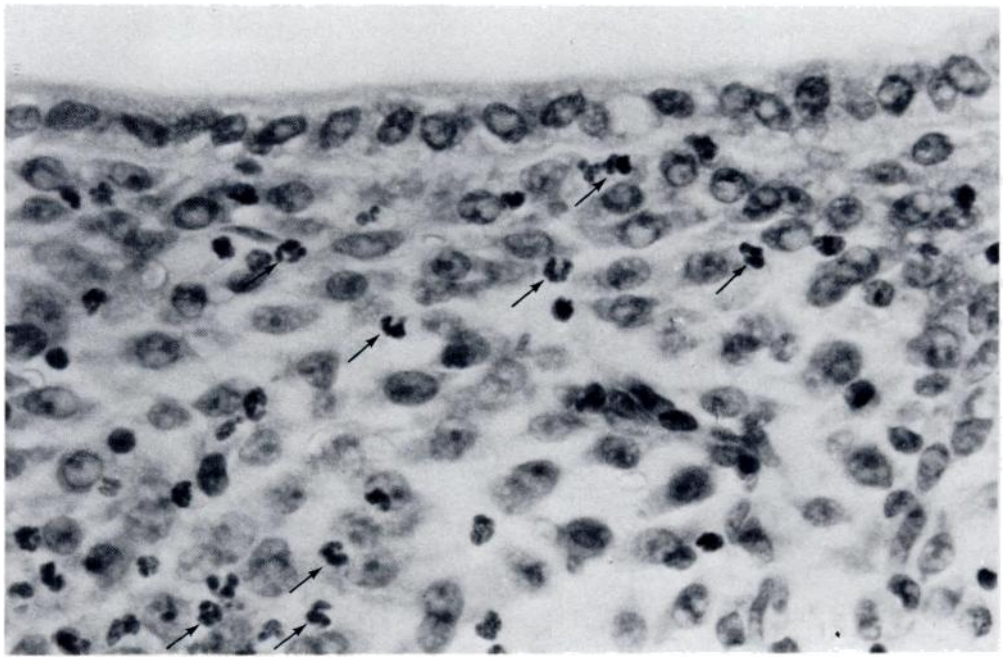

FIG. 6. Leukocytes (arrows) in uterine endometrium 6 h after glycogen injection. ×640.

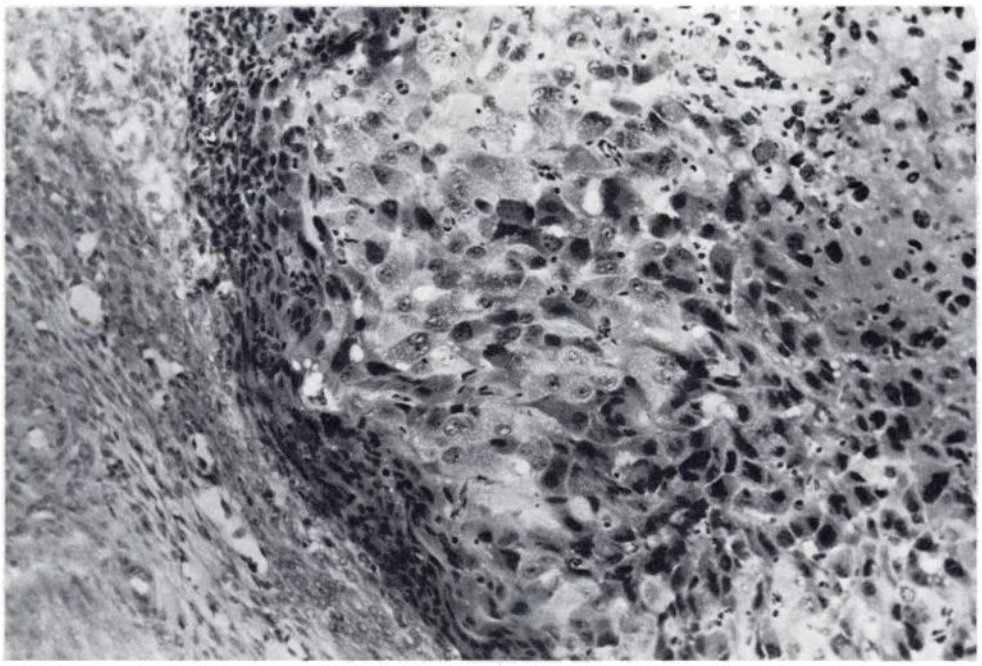

FIG. 7. Placental tissue found on Day 15 of pregnancy in a uterine horn treated with glycogen on Day 4 of pregnancy. X160.

affect pregnancy during the implantation stage. We usually found placental remnants in rat uteri when leukocytosis was induced before or during implantation, but to date there is no histological evidence to indicate that implantation occurs in women with IUDs. However, in 3 recent studies, a human chorionic gonadotropin-like substance was detected in luteal phase blood and urine samples from a large percentage (10–20%) of women with IUDs (Beling et al., 1976; Landesman et al., 1976; Hodgen et al., 1978), which suggests that transient implantation of at least trophoblast tissue may commonly occur in women bearing IUDs. Implanted fetal tissue probably does not reside long in these cases, because menstrual cycles are reportedly no more irregular in women with IUDs than in the normal population (Eckstein, 1971).

Our demonstration that a leukocyte chemotactic factor can be used to terminate early pregnancy with no effect on subsequent fertility suggests at least 2 potential clinical applications for such factors. It may be possible to induce abortion during early pregnancy by instilling a small amount of a leukocyte chemotactic factor into the uterine lumen via the cervix. Since a low concentration of a physiological substance would be used, this technique could prove to be less traumatic than many abortion techniques currently in use. Possible disadvantages of this technique which require further study are the possibilities that 1) infection would occur; 2) such a technique may be effective for only a short period near the time of implantation; and 3) placental tissue may implant and remain embedded in the uterine wall. A second, more promising contraceptive application of leukocyte chemotactic factors is their use to enhance the antifertility effectiveness of IUDs. Gradual release of small concentrations of leukocyte chemotactic factors would enable small IUDs cast in a shape readily tolerated by the uterus to elicit infiltration of inflammatory cells comparable to that induced by larger nonmedicated IUDs. If inflammatory cells or their products mediate antifertility effects (and there is good evidence that they do) these chemotactic-factor-releasing IUDs may be as effective and more acceptable than IUDs currently in use.

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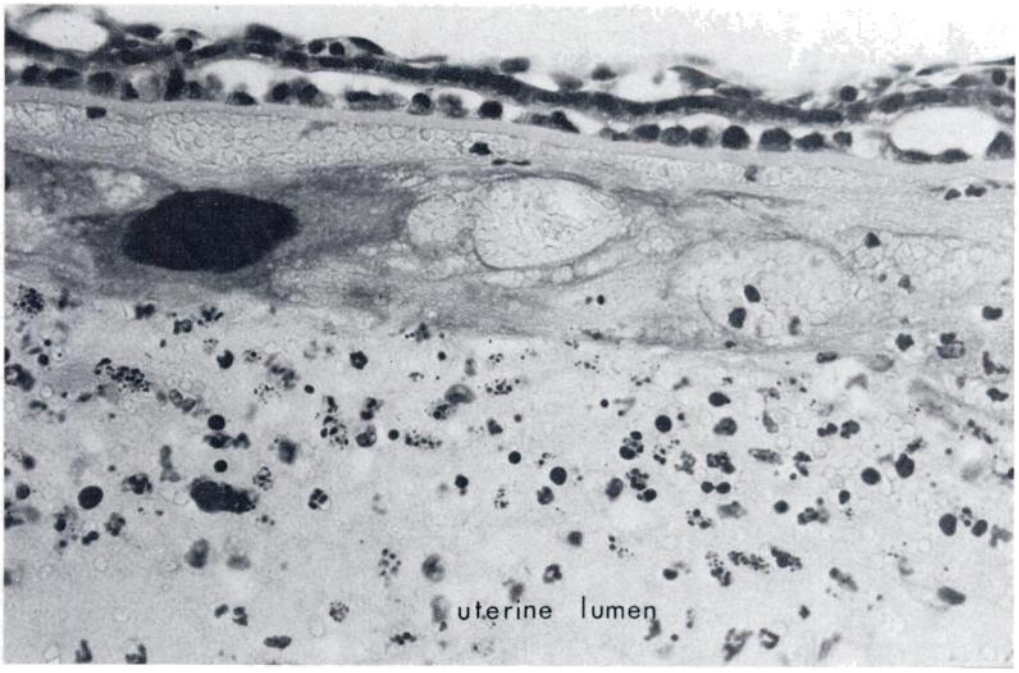

FIG. 8. Outer membranes of an 8-day-old embryo in a glycogen treated uterine horn. Postimplantation embryos are apparently protected from adverse effects of leukocytes by fetal membranes. X 400.

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