Perforin-expressing Granulated Metrial Gland Cells in Murine Deciduoma

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Summary

It has previously been shown that granulated metrial gland (GMG) cells of the pregnant uterus express abundant quantities of the lymphocyte pore-forming protein, perforin. No perforin was present before implantation of the embryo, but large numbers of perforin-producing GMG cells were observed after implantation, which coincides with decidualization of the uterus. The possible source of the activation factors responsible for perforin gene induction in GMG cells was studied here with the pseudopregnancy model, in which cervical stimulation of mice during estrus leads to a series of hormonal changes resembling those seen in pregnancy, but in the absence of an embryo. Subsequent stimulation of the uterus of pseudopregnant mice with oil causes the stimulated portion of the endometrium to differentiate into decidual tissue. Perforin-containing GMG cells were in fact present in the deciduomata, but not in adjacent nondecidualized tissues of the same mice. These results suggest that maternal factors associated with decidual tissue are responsible for the local expression of perforin in GMG cells.

Several cytotoxins have now been isolated from the granules and supernatants of cytolytic lymphocytes. The best characterized is a pore-forming protein known as perforin or cytolysin, which is present in the granules of all the cytolytic lymphocytes studied to date, CTL, NK cells, lymphokine-activated killer cells, and γ/δ cells (1–4). In vitro, perforin is induced in CTL/NK cells by several stimuli, including IL-2, lectins in the presence of phorbol esters, and allogeneic cells. In vivo, perforin-producing cells have been observed during pathological conditions where large amounts of IL-2 might be generated, such as in autoimmune diseases and acute viral infections (2–4).

The mouse uterus during pregnancy contains a large population of granulated metrial gland (GMG)[1] cells, which resemble LGL in several respects (5), including the presence of perforin-rich granules. GMG cells are derived from precursors in the bone marrow, and exhibit the markers of NK cells, eg., asialo GM_1 and LGL-1 (6–8). Interestingly, perforin is found in GMG cells at levels higher than those observed with any pathological condition studied until now (6, 9). In view of the remarkable expression of perforin in the uterine tissue, we sought to determine whether perforin synthesis in these cells is stimulated by the hormones derived from fetal or from maternal tissues. This question was addressed by measuring perforin synthesis in pseudopregnant mice. In this model system, the hormonal changes associated with pregnancy can be induced by cervical stimulation of female mice in estrus. Injection of oil into the uteri of these mice several days later results in the formation of deciduoma, which allows for the study of GMG cell differentiation in the absence of an embryo. Thus, it is possible to unequivocally assign any differentiation effects to activation signals from the maternal side.

Materials and Methods

Pseudopregnant Mice. Sexually mature virgin female mice of the CD1 (Charles River Breeding Laboratories, Wilmington, MA) and ICR strains (Harlan-Sprague Dawley, Indianapolis, IN) were used. CD1 animals were individually housed in a partitioned cage with a mature male mouse to induce regular estrous cycles. Vaginal smears were done daily until cornification was seen. Pseudopregnancy was induced in CD1 mice by cervical stimulation with a small glass rod (day 0 of pseudopregnancy). ICR female mice were mated to a vasectomized male, and the day a copulation plug was found in the vagina was considered day 1 of pseudopregnancy. Mice with diestrus smears for 5 d or more after stimulation were considered to be pseudopregnant. Deciduoma formation was induced on day 4 of pseudopregnancy by injection of 20–40 μl peanut oil into the uterine lumen, through the uterine wall at the uterotubal junction during laparotomy. Alternatively, 20–40 μl peanut oil was injected into the uterine lumen via the transcervical route. Mice were anesthetized with ether and killed by rapid decapitation 0, 120, 168, and 216 h after injection of oil. Control mice were injected with oil,

[1] *Abbreviation used in this paper:* GMG, granulated metrial gland.

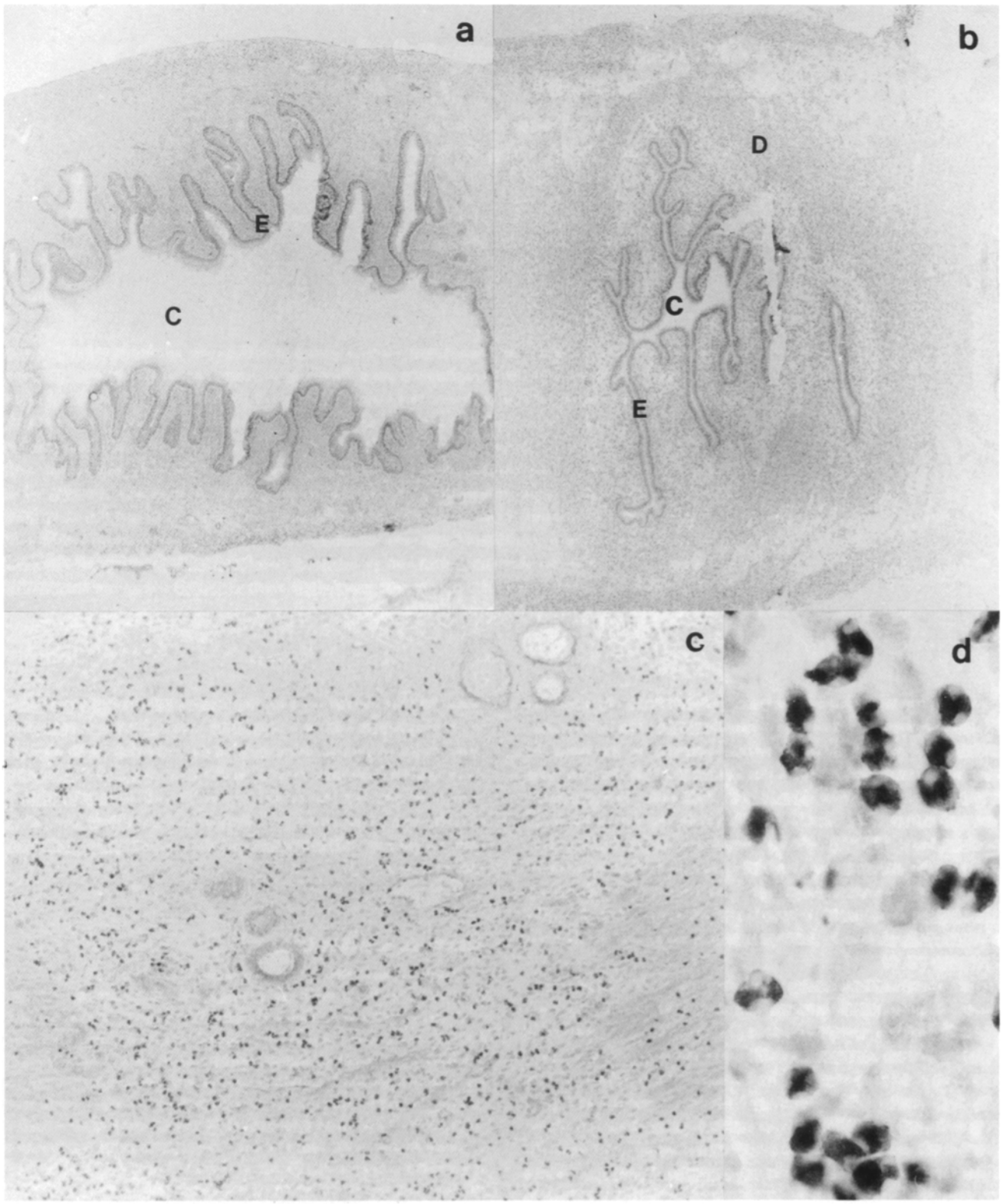

Figure 1. (*a*) Sagittal section of the uterus on day 4 of pseudopregnancy stained with toluidine blue (*C*, uterine cavity; *E*, epithelium); ×25. (*b*, *c*, and *d*) Uterine sections 120 h after injection of oil. (*b*) Toluidine blue staining; note the distention and decidualization of the uterus (*D*, decidual tissue); ×25. (*c* and *d*) Immunoperoxidase staining with anti-mouse perforin polyclonal antiserum; ×50 (*c*) and ×250 (*d*).

or pseudopregnant mice injected with PBS. These were all killed at the same times.

Histological Examination. The uteri from pseudopregnant animals were rapidly cut into small pieces and fixed with 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, overnight. The tissues were post-fixed with 2% osmium tetroxide in 0.1 M phosphate buffer for 30 min, dehydrated in a graded ethanol series, and embedded in Epon 812. Semi-thick sections (0.5 μm) were stained with toluidine blue and examined with a light microscope. Ultrafine sections (<70 nm) were contrasted with uranyl acetate and lead citrate, and examined with a JEOL-100 EX electron microscope.

Immunohistochemistry. Uteri from pseudopregnant or control mice were rapidly frozen in isopentane cooled to −40°C with liquid nitrogen. Cryostat sections (6 μm) for immunoperoxidase staining were cut and dried onto gelatin-coated slides. The sections were fixed with picric acid–saturated ethanol containing 10% formalin for 10 min, rinsed in 0.1 M phosphate buffer, and treated with 0.5% H_2O_2 to quench endogenous peroxidase. After preincubation with 5% normal goat serum, the slides were incubated for 24 h with rabbit polyclonal anti–mouse perforin antisera (10). The slides were washed with 0.01 M PBS, incubated successively with biotin-conjugated goat anti–rabbit IgG and avidin-biotinylated peroxidase complex (Vector Laboratories, Burlingame, CA), and reacted with diaminobenzidine tetrahydrochloride (Polyscience, Chicago, IL).

Sections for immunofluorescence staining were dried onto poly-L-lysine-coated slides, fixed in acetone for 10 min and methanol for 1 min, and incubated sequentially in the following reagents with intermediate washes in PBS: donkey gammaglobulin, rabbit polyclonal anti–mouse perforin serum, and rhodamine-conjugated donkey anti–rabbit IgG. Alternatively, sections were incubated in donkey gammaglobulin, FITC-monoclonal rat anti–mouse Thy-1.2 (Becton Dickinson & Co., Mountain View, CA) plus mouse monoclonal IgM anti-perforin (11), rabbit anti–mouse IgM (μ chain–specific; Zymed Laboratories, San Francisco, CA) plus mouse IgG at 50 μg/ml (Jackson Immunoresearch Labs, West Grove, PA), and rhodamine-conjugated donkey anti–rabbit IgG. Control sections were incubated with PBS, nonimmune rabbit serum, or purified mouse IgM (Zymed Laboratories), followed by the secondary reagents listed above. Staining was not observed in control sections.

In Situ Hybridization. In situ hybridization was performed as described (9). Briefly, cryostat sections (6 μm) were mounted onto polylysine-coated slides, fixed in 4% paraformaldehyde, rinsed, and dehydrated in ethanol. Slides were then acetylated and incubated with prehybridization buffer for 3 h. Hybridization was done at 50°C for 2 d, using ^{35}S-labeled sense or antisense mouse perforin RNA probes. After washing, slides were dipped in Kodak NTB2 emulsion and stored dry in light-tight boxes at 4°C for 4 d before developing.

Results and Discussion

A complete estrous cycle in mice occurs every 4 or 5 d. A condition known as pseudopregnancy can be induced by cervical stimulation of mice during estrus. Pseudopregnancy is characterized by hormonal changes typical of pseudopregnancy, and it can cause the cessation of estrous behavior for up to 2 wk; for comparison, during normal pregnancy, parturition takes place 21 d after fertilization.

Fig. 1 *a* shows the sagittal section of a uterus 4 d after initiation of pseudopregnancy. Injection of oil into pseudopregnant mice induces endometrial proliferation and differentiation to form a deciduoma, causing the uterus to be substantially thicker than in nonpregnant or pseudopregnant mice (Fig. 1 *b*). No perforin-producing cells were found in the uterus of control mice (data not shown). As perforin has been previously detected in GMG cells of the pregnant uterus (6, 9, 12), it became of interest to determine whether the deciduoma by itself might secrete the factors required for perforin synthesis in the uterus. As seen by immunoperoxidase staining with polyclonal antiperforin antisera, perforin was in fact present in a large number of cells in the deciduomata

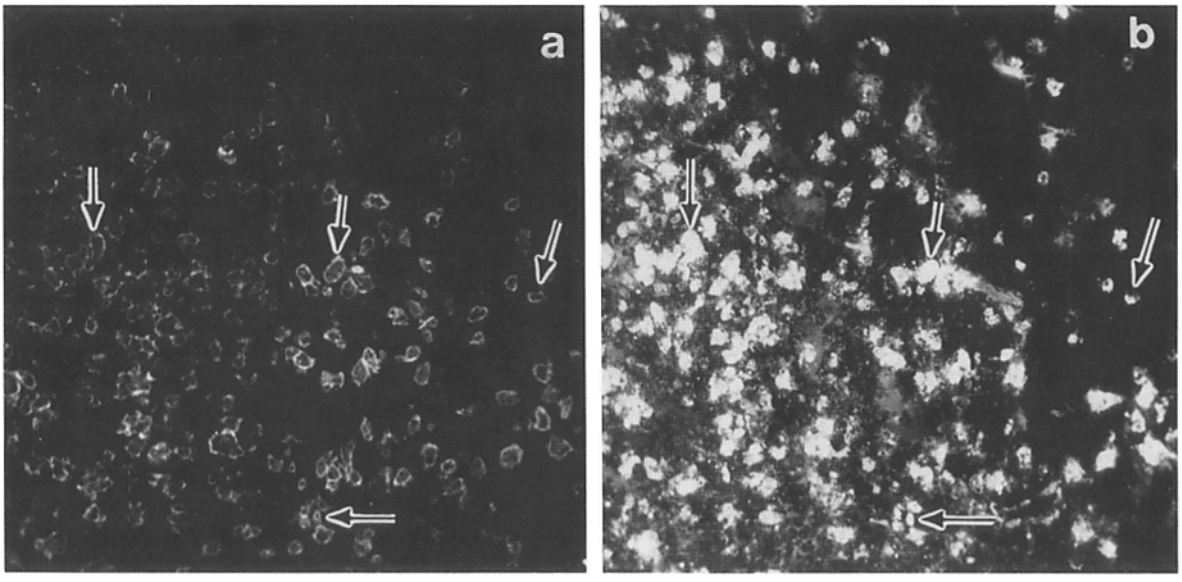

Figure 2. Double immunofluorescence staining with mAbs to mouse Thy-1.2 (*a*) and perforin (*b*), in uterine sections 120 h after injection of oil. Arrows indicate the same cells in the two micrographs. Perforin-containing GMG cells were observed only in the mesometrial decidua at this time; ×90.

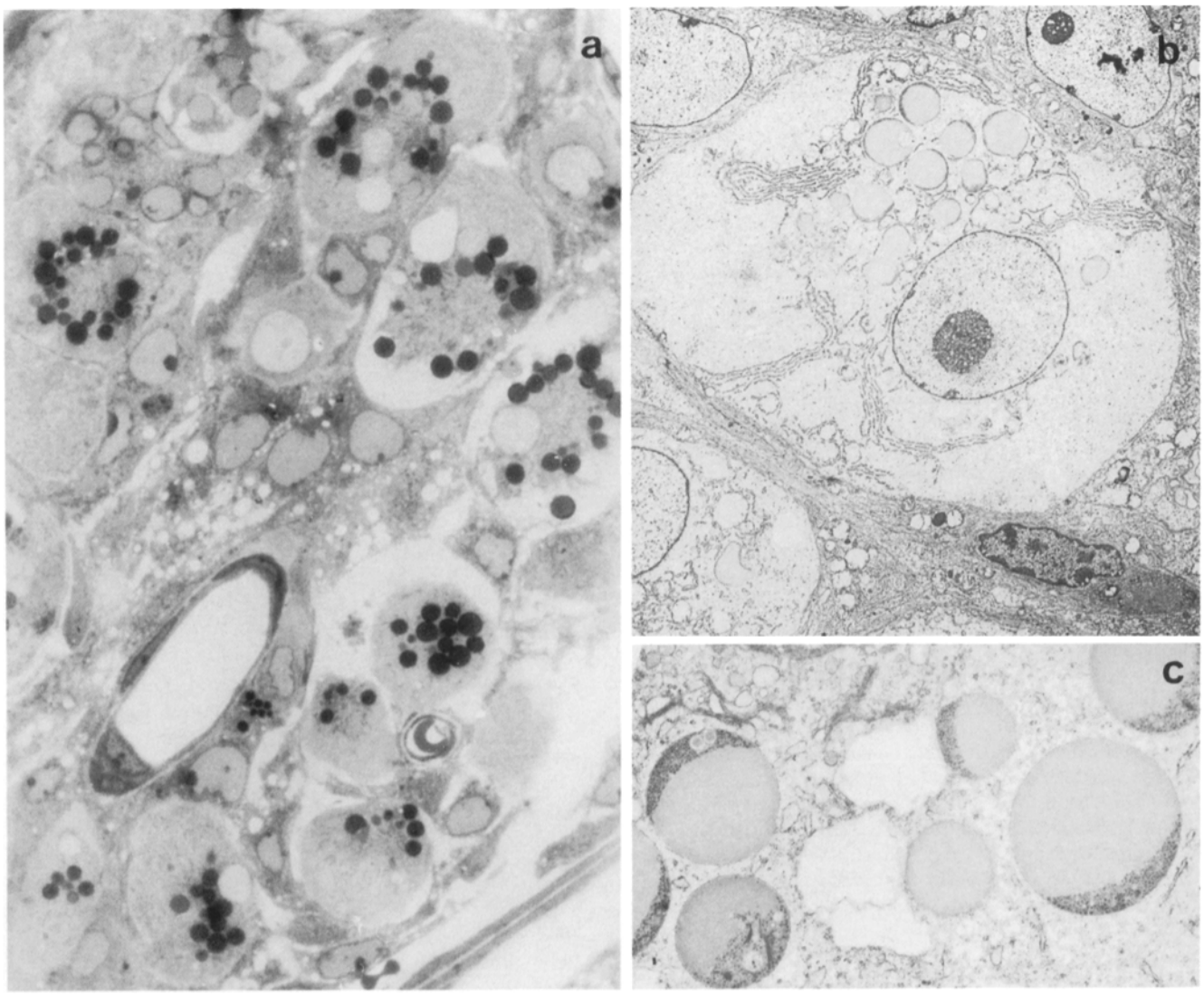

Figure 3. GMG cells in the mesometrial triangle on day 10 of pseudopregnancy (6 d after oil injection). (*a*) Thick section stained with toluidine blue showing GMG cells with numerous granules and a pale cytoplasm. The GMG cells lie between the smaller decidual cells; ×500. (*b*) Electron micrograph of a GMG cell; ×2,000. (*c*) Higher magnification of granules shows the characteristic core and cap structure; ×12,500.

(Fig. 1, *c* and *d*). Double immunofluorescence staining with mAbs to perforin and Thy-1.2 (Fig. 2) showed that the perforin-containing cells are GMG cells, identified by the surface expression of Thy-1.2 (5–7). Perforin-producing cells are restricted to deciduomata. They were not seen in any other region of deciduoma-containing uteri, analogously to the case for normal pregnancy, as perforin-positive cells were found in decidualized tissue but never between implantation sites. In pseudopregnancy, the number of perforin-containing cells increased between 120 and 216 h after uterine stimulation with oil, and there was a concomitant increase in the size and cytoplasm/nucleus ratio of the cells.

On day 10 of pseudopregnancy (6 d after oil injection), GMG cells with prominent cytoplasmic granules were also identified on histological examination. The GMG cells are up to 50 μm in diameter, with large granules of 2–3 μm (Fig. 3 *a*). In addition to the granules, the cells were identified by their round nuclei and pale cytoplasm. On electron microscopy, the nucleoli were prominent, and the cytoplasm contained abundant rough endoplasmic reticulum (Fig. 3 *b*). The granules (Fig. 3 *c*) were characterized by a core and a crescentic cap composed of small vesicles. These granules were recently shown to contain perforin (12).

Perforin mRNA was demonstrated in decidual tissue by in situ hybridization at 120 h after oil stimulation (Fig. 4). Perforin mRNA was abundantly expressed at this time, and the cells strongly hybridizing with the antisense riboprobe were seen only in sections adjacent to those containing perforin-positive cells by immunostaining. The distribution of labeled cells was similar with both the immunoperoxidase and in situ hybridization techniques. The sense riboprobe did not hybridize with any of the sections.

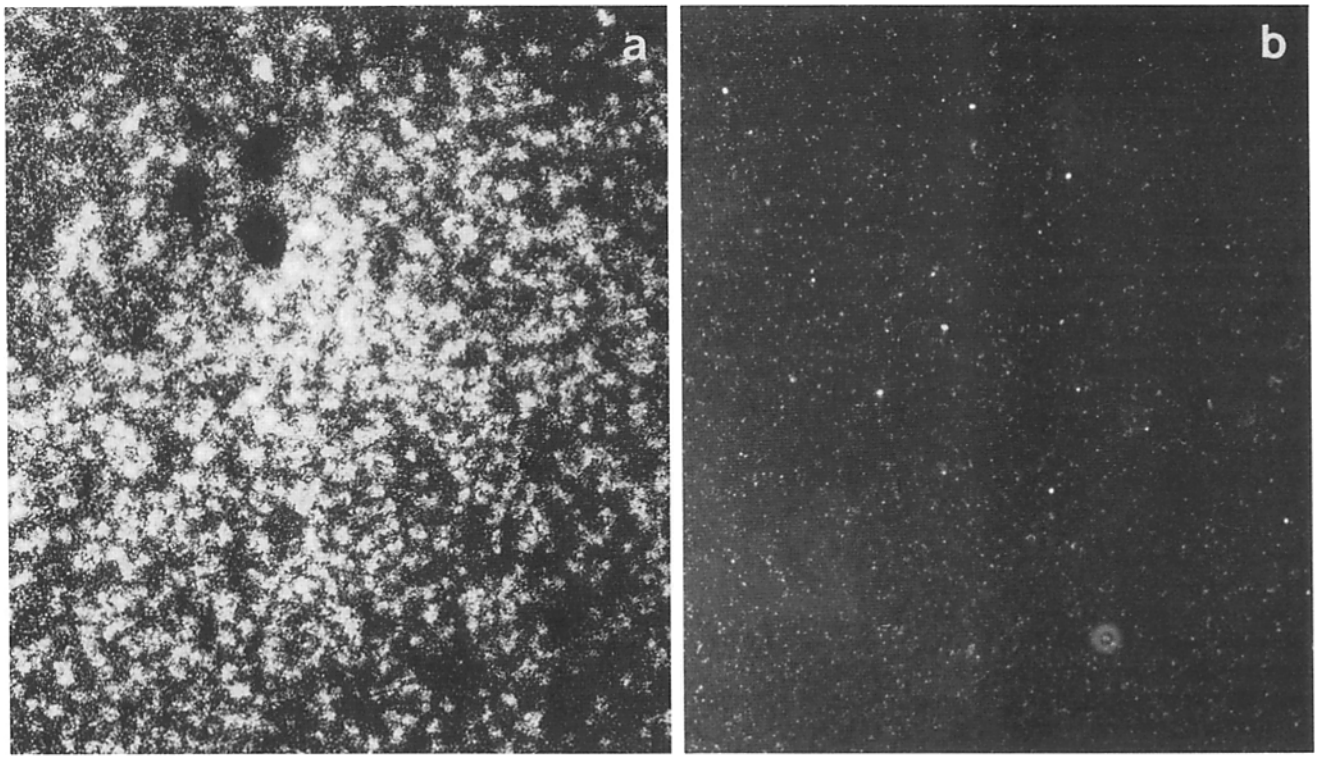

Figure 4. Dark-field photomicrographs of adjacent sections of uterus, 120 h after injection of oil, hybridized with perforin antisense (*a*) and sense (*b*) riboprobes; ×80.

GMG cells with prominent cytoplasmic granules were readily identified on histological examination, as previously described for normal pregnancy (9, 12). In addition to their granules, the cells were identified by their round nuclei and pale cytoplasm. By electron microscopy, the nucleoli are prominent, and the cytoplasm contains abundant rough endoplasmic reticulum. The granules of GMG cells from pseudopregnant mice are characterized by a core region and a crescentic cap composed of small vesicles, as found in GMG cells during normal pregnancy (12).

The presence of perforin in GMG cells in deciduomata indicates that maternal rather than fetal factors are responsible for perforin induction. Moreover, their absence in nondecidualized pseudopregnant uteri indicates that local decidual factors are responsible for perforin induction, rather than the systemic hormonal factors responsible for pregnancy or pseudopregnancy. Our results are consistent with those of Stewart (13), who identified GMG cells in deciduomata but not in contralateral, nondecidualized horns of pseudopregnant mice. We demonstrate here that perforin is present in these GMG cells, similarly to what was found for GMG cells during normal pregnancy (6). In addition, these results are in line with our previous observation that perforin mRNA is expressed during normal pregnancy only after implantation has taken place, suggesting that the same signals that induce decidualization also induce perforin expression (9).

GMG cells, in both pregnancy and in deciduomata, express perforin at higher levels than seen in viral infections or other diseases where activated cytotoxic cells are seen (9). The presence of fetal tissues in pregnant uterus complicates the biochemical identification and characterization of the uterine factors that induce perforin in cytolytic lymphocytes. The present results suggest that deciduomata of pseudopregnancy offers a more suitable model tissue for studying the uterine induction of perforin in cytolytic cells.

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