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Immunoregulatory activity of supernatants from short-term cultures of mouse decidual tissue*

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Summary. Supernatants from short-term in-vitro cultures of decidual tissue, obtained from the uteri of pregnant mice from Days 4 to 13 *post coitum* (Day 1 = day of mating), were assessed for immunoregulatory activity by their addition to a mixed lymphocyte reaction (MLR), an in-vitro analogue of the afferent arm of the immune response. All culture supernatants tested possessed inhibitory activity in the MLR, although the extent of inhibition was affected by seeding density, length of culture, and the day of pregnancy from which decidual tissue was obtained. Inhibitory activity produced by decidual cultures increased from Day 4 to reach a maximum on Day 8, and then declined to Day 11. Two morphologically distinct cell types were present in all decidual cultures; flat dendritic cells, considered to represent decidual cells, and small round cells, but whether immunoregulatory factors are associated with both is uncertain. The results suggest that decidual tissue could fulfil a role in the local partial blockade of the afferent arm of the maternal immune response during pregnancy.

Introduction

Implantation of the blastocyst in the maternal uterus induces the differentiation of endometrial cells to form the decidual tissue of pregnancy. Many functions have been attributed to this tissue, including nutrition of the embryo, an endocrinological role, and as a barrier to trophoblast invasion (see Finn, 1971). More recently, various immunological effects have been reported to be associated with the decidua and many cells bearing markers associated with immunologically active cells have now been identified in this tissue (see Bell, 1983). The extent of the development of decidualization in the mouse is dependent upon antigenic differences between the maternal and paternal strains and also on the reproductive history and immunological status of the mother (Hetherington, 1972; Hetherington & Humber, 1975).

During implantation in the mouse the embryo is surrounded by immunoglobulins, which are localized later on immunoglobulin-binding cells within the decidua (Bernard, Ripoche & Bennett, 1977). Many authors have described Fc receptor-bearing cells (Bernard, Scheid, Ripoche & Bennett, 1978; Rachman, Bernard, Scheid & Bennett, 1981; Kirkwood, 1981), which appear to be present in the decidua in different proportions, dependent upon the stage of pregnancy. Later in pregnancy, Ia antigen- and complement receptor-bearing cells have also been identified in the

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decidua basalis (Jenkinson & Searle, 1979; Kirkwood, 1981; Searle, Bell & Billington, 1983). Allogeneic grafts transplanted to uteri of early pregnant or pseudopregnant rats, when decidual tissue could develop beneath them, and to deciduoma-containing rabbit uteri, survived significantly longer than grafts placed in the uteri of cyclic rats or elsewhere in the body (Beer & Billingham, 1974; Dodd, Andrew & Coles, 1980). Prostaglandins, which have been detected in homogenates of rat decidua and cultured decidual cells, have been implicated in the demonstrated in-vitro suppression of antibody synthesis induced by decidual tissue homogenates (Antebi, Bauminger, Zor & Lindner, 1975; Globerson, Bauminger, Abel & Peleg, 1976). These observations support the contention that the decidua may fulfil an important role in maternal–fetal immunological interactions (Beer & Billingham, 1974; Hetherington & Humber, 1975; Bell, 1979a, 1983).

Supernatants from cultures in which decidual cells arise *in vitro* by differentiation from uterine stem cells (Bell & Searle, 1981) partly inhibit lymphocyte responses, i.e. recognition and proliferation, in a mixed lymphocyte reaction (MLR) (Kirkwood & Bell, 1981), a reaction considered to represent an analogue of the afferent arm of the immune response. In the present study, the reactivity of supernatants from short-term cultures of decidual cells isolated from uteri of females at various stages of pregnancy has been tested to investigate whether normal gestational decidual tissue produces immunoregulatory factors able to inhibit a similar mixed lymphocyte reaction.

Materials and Methods

Animals. Male C57BL and Balb/c mice were used for the mixed lymphocyte reactions. Pregnant females were obtained by caging 3–4 female A strain mice overnight with an A strain male, and the morning on which vaginal plugs were detected was designated Day 1 of pregnancy.

Tissue, cultures and supernatants. Primigravid females were killed by cervical dislocation. On Day 4, pregnancy was verified by the detection of blastocysts in the uterine flushings. On Day 5, implantation sites were visualized by i.v. injection of 50 μl of 1% pontamine sky blue solution 10 min before being killed. To prepare Day 4–5 'decidual' tissue for culture, it was necessary to use the whole uterus and, as a control for possible contributions from non-decidual elements, uteri of virgin mice were treated in the same manner. At later stages of gestation (Days 6–13), the uterus was cut open along the antimesometrial axis and the conceptuses with their surrounding decidual tissue were removed. The decidual tissue, the extent of which varied with the stage of gestation, was peeled away from the underlying embryonic tissues with watchmaker's forceps (except at Day 6 when the whole implantation site with the embryo was used). All the decidual tissue from one Day 6–13-pregnant female or the whole uteri from virgin and Day 4–5-pregnant females was minced, washed once in phosphate-buffered saline (Dulbecco A and B; Oxoid, Basingstoke, U.K.) (DPBS) and incubated in 0·1% trypsin in DPBS at room temperature for 10–15 min, either with continuous agitation, provided by a magnetic stirrer (for uteri from virgin and Day 4–9-pregnant females) or pipetting (for decidual tissue from Day 10–13-pregnant females). The cell suspension was then prepared as described previously (Bell & Searle, 1981; Kirkwood, 1981). Aliquants (0·4 ml) of suspension containing $1{\cdot}0 \times 10^5$ cells were added to the wells of leucocyte migration plates (Sterilin, Teddington, Middlesex, U.K.) and incubated at 37°C in a humid atmosphere of 5% CO_2 in air. After 6 h, when cells had adhered to the bottom of the wells, supernatants of these cultures were discarded and replaced by fresh RPMI-1640 medium (Gibco (Europe), Paisley, U.K.). It was considered that this procedure would remove toxic cell debris and products from the cell cultures. The supernatants were removed 24 or 48 h later and stored at −20°C in sterile tubes.

All these procedures were carried out under sterile conditions.

Microscopic observation. After 24 or 48 h of culture, the plates were rinsed and fixed in absolute alcohol for 45 min and, after drying, stained with diluted Giemsa solution (1 : 10) for 45 min. The approximate nuclear area of the cells was estimated as described previously (Bell & Searle, 1981).

Mixed lymphocyte reaction (MLR). Single cell suspensions were prepared from the spleens of male Balb/c and C57BL mice, treated with 0·83% NH_4Cl solution to lyse the red blood cells, washed twice with large volumes of phosphate-buffered saline and adjusted to 4 or 5 × 10^6 cells/ml in Hepes-buffered RPMI culture medium. The C57BL responder cells were stored at 4°C whilst the C57BL and Balb/c stimulator cells were treated with 40 μg mitomycin C/ml at 37°C for 30 min and then washed three times in large volumes of PBS. Both stimulators and responders were then resuspended in bicarbonate-buffered RPMI-1640 culture medium which had been supplemented with 5 × 10^{-5} M-2-mercaptoethanol. Immediately before the cells were ready, selected supernatants were thawed and six 25-μl aliquants of each supernatant were placed in the wells of flat-bottomed microtitre plates (Sterilin Ltd). Stimulator and responder cells (0·1 ml of each) were placed in each well and the contents thoroughly mixed. For each supernatant, 3 wells were set up with a mixture of C57BL responder and Balb/c stimulator cells (allogeneic mixture) and 3 wells were set up with C57BL responder and C57BL 'stimulator' cells. A further control was included on each plate by setting up a similar pair of triplicate wells with 25 μl PBS rather than with an experimental supernatant. The plates were incubated in moist, air-tight boxes gassed with 5% CO_2 and air at 37°C for 72 h. Then 50 μl PBS containing 2 μCi [^{3}H]thymidine (Radiochemical Centre, Amersham, U.K.) were added to each well and incubated in moist air-tight boxes gassed with 5% CO_2 and air at 37°C for 4–6 h. The contents of each well were then harvested on individual glass-fibre papers under vacuum filtration conditions and the filters were washed 3 times each with cold PBS, 5% trichloracetic acid and absolute alcohol. The filters were dried and counted using a xylene-based scintillation fluid.

The results were expressed as the average c.p.m. of 3 replicate wells with the standard deviation of the mean, or as the stimulation index (SI). The percentage of inhibition of MLR and syngeneic background was calculated as described previously (Kirkwood & Bell, 1981).

Student's *t* test was used for statistical analysis, *P* values of $< 0{\cdot}05$ were considered to represent significant deviation from control values.

Results

Effect on the MLR of supernatants from short-term cultures of decidual tissue obtained from Days 4–13 of pregnancy

Supernatants from short-term (48 h) in-vitro cultures of uterine decidual tissues from syngeneically mated A strain mice (Days 4–13 of pregnancy) were tested in a one-way mixed lymphocyte reaction (MLR) between C57BL responder and Balb/c stimulator lymphocytes. Table 1 shows the results from a representative experiment; all culture supernatants significantly inhibited the MLR. Results were compared to a control in which saline was added, because it was considered possible that culture supernatants could be depleted of nutrients. The inhibition of the MLR was not due to any inherent cytotoxicity of the supernatants since the % viability of lymphocytes was similar in test and controls at the end of the MLR (91–95% viable as assessed by eosin dye exclusion). Significant inhibition of incorporation of radioactivity into the syngeneic background cultures was also observed but this was normally less than the inhibition observed in the allogeneic mixtures (Table 1).

The degree of inhibition of the MLR by supernatants was affected by a number of factors.

Effect of source of tissue for cultures. As shown in Table 1 and Text-fig. 1, the extent of suppression of the MLR by supernatants varied according to the day of pregnancy on which the uterine/decidual tissue was obtained for culture. The MLR was suppressed by supernatants from cultures of tissue obtained on the day of implantation, Day 4, compared to cultured control virgin uterine tissue $0{\cdot}01 < P < 0{\cdot}02$. The level of inhibition exhibited by supernatants of cultured tissue rose from Day 4 to 7, to reach a maximum on Day 8 ($P < 0{\cdot}001$). This level fell significantly from Days 8 to 11 ($P < 0{\cdot}01$), to rise, although not significantly, on Days 12 and 13. When the level of

Table 1. A representative experiment to investigate the effect of decidual cell supernatants from short-term cultures on the MLR

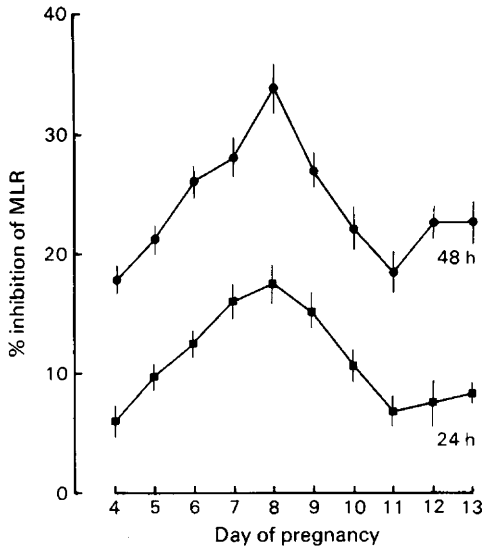
Supernatant additive	Lymphocyte cultures: Syngeneic (C57BL vs C57BL†)	Lymphocyte cultures: Allogeneic (C57BL vs Balb/c†)	SI‡	% inhibition: MLR‡	% inhibition: SB‡
Saline (control)	21337 ± 1082	66748 ± 1520	3·13	—	—
Supernatant§ (day of pregnancy)					
4	17218 ± 339**	48805 ± 1233***	2·83	30	19
5	17093 ± 2349**	42247 ± 1732***	2·47	45	20
6	15454 ± 455***	40852 ± 243***	2·64	44	27
7	14321 ± 1040***	38236 ± 1304***	2·67	47	33
8	15377 ± 1714***	38068 ± 617***	2·47	50	28
9	12973 ± 245***	42298 ± 2978***	3·26	35	39
10	18935 ± 1833*	49554 ± 3092***	2·61	33	11
11	20038 ± 428	53556 ± 5453***	2·67	26	6
12	19248 ± 1201*	46835 ± 5835***	2·43	39	10
13	14924 ± 1016***	46277 ± 4051***	3·1	31	30
Virgin	20428 ± 482	64115 ± 2329	3·13	4	4

Significance levels compared to control value: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

† Cells treated with mitomycin C.

‡ SI = stimulation index, % inhibition of mixed lymphocyte reaction (MLR) and syngeneic background (SB) (see 'Materials and Methods').

§ From decidual cells or uterine tissue cultured for 48 h at a seeding density of 1×10^5.



Text-fig. 1. The effect of supernatants from short-term cultures of decidual tissue obtained during pregnancy on the MLR. Cells were prepared from tissues obtained at an appropriate day of pregnancy and supernatants were obtained 24 or 48 h after the start of decidual tissue culture. Each supernatant was tested in triplicate wells of the MLR assay. The results are expressed as % inhibition of MLR and bars represent mean ± s.e. of 3–5 cultures.

inhibitory activity of supernatants obtained 24 h after culture was examined, a similar profile, but at a lower level of activity, was obtained, suggesting that the release of the factor(s) into the supernatants continued for at least 48 h of culture (Text-fig. 1). Supernatants from cultures of uterine tissue obtained from randomly cyclic virgin females did not produce any significant effect on the MLR compared to the saline control (Table 1).

Effect on the MLR of time of addition of supernatants. The MLR comprises an initial recognition phase ($\leqslant$ 12 h) followed by a phase of lymphocyte proliferation. To determine when these factors exert their effect on the MLR, experiments were designed in which supernatants from cultures of decidual tissue (obtained from Day 8 of pregnancy) were added at 0, 1, 2, 4, 6 and 24 h after the initiation of the MLR (Table 2a). The results demonstrated that supernatants were still inhibitory even when added after the recognition phase of MLR, suggesting that the inhibitory factors were active primarily during the proliferative phase.

Effect of cell number and length of tissue culture. In the previous experiments, $1{\cdot}0 \times 10^5$ decidual cells were added to each well, before removing the supernatants 24 or 48 h after culture. However, modification of the number of cells or time of incubation affected the degree of inhibition of the MLR. The major portion of the inhibitory material was produced between 24 and 48 h of culture, a further 24 h of culture having no additional effect (Table 2b). Increasing the seeding density reduced the inhibitory activity of supernatants, but reduction of the cell number slightly enhanced the degree of inhibition (Table 2c).

Table 2. Factors affecting the activity of supernatants from decidual cell cultures on the MLR

Supernatant additive	Lymphocyte cultures: Syngeneic (C57BL vs C57 BL†)	Lymphocyte cultures: Allogeneic (C57 BL vs Balb/c†)	MLR inhibition (%)‡
Saline (control)	29250 ± 3854	61701 ± 2438	—
(a) Time of supernatant addition after start of MLR			
*0 h	14893 ± 777	35271 ± 5909	37
24 h	16928 ± 1827	41236 ± 3062	25
(b) Duration of decidual cell culture			
24 h	24467 ± 3194	52328 ± 3821	14
*48 h	14893 ± 777	35271 ± 5909	37
72 h	17929 ± 2519	42149 ± 5922	25
(c) Decidual cell number			
$1{\cdot}5 \times 10^5$ cells/0·4 ml	18019 ± 1608	40424 ± 6404	31
*$1{\cdot}0 \times 10^5$ cells/0·4 ml	14893 ± 777	35271 ± 5909	37
$0{\cdot}5 \times 10^5$ cells/0·4 ml	19879 ± 3289	39125 ± 5073	40

* Standard conditions, i.e. decidual tissue obtained on Day 8 of pregnancy; $1{\cdot}0 \times 10^5$ cells cultured; and cultured for 48 h.

† Cells treated with mitomycin C.

‡ % inhibition of mixed lymphocyte reaction (MLR).

Microscopic examination of cultures

Examination of all cultures revealed the presence of two major cell populations. Irrespective of the stage of pregnancy from which decidual tissue was obtained, the major population consisted of flat, mono- or bi-nucleate, spindle-shaped or stellate cells (Pl. 1, Fig. 1), the cytoplasmic and nuclear area of which increased after 48 h in culture when prepared from decidual tissue on Days 4–6 (nuclear areas: Day 4, 140 ± 40 μm^2; Day 5, 390 ± 210 μm^2; Day 6, 530 ± 200 μm^2). Small numbers of flat dendritic cells achieved nuclear areas of 1700 μm^2, similar to the decidual cells arising by in-vitro differentiation (Bell & Searle, 1981). The second population was represented by small round cells, diameter 7–10 μm, which did not alter during culture, and comprised 15–20% of the cell

numbers (Pl. 1, Figs 1–3). A third minor population was observed in cultures obtained from Day 8 to 11 decidual tissue, which were flat and ovoid, and whose nuclear and cytoplasmic area greatly enlarged over this period (Pl. 1, Figs 2 & 3).

Discussion

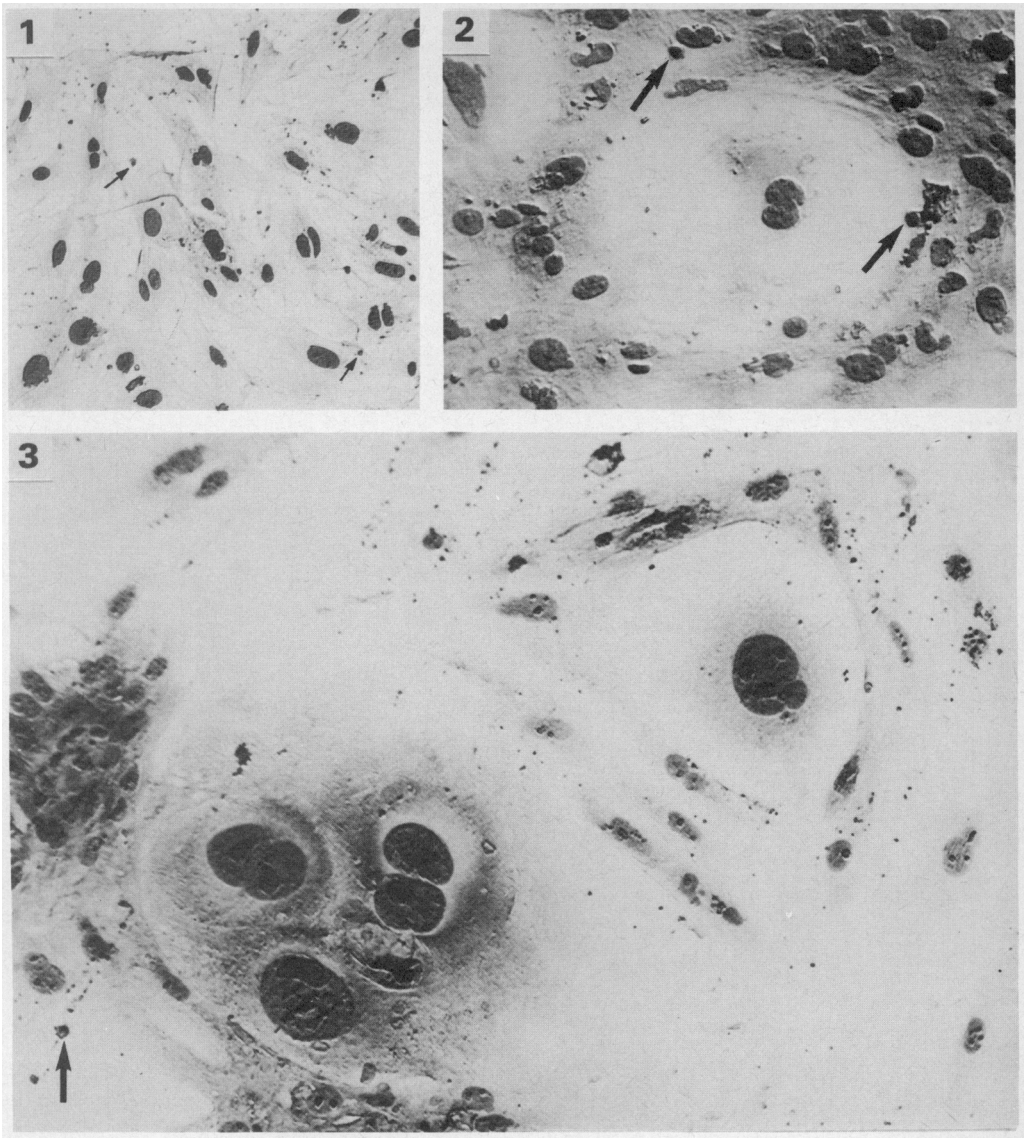
The results of the present study demonstrate that supernatants obtained from short-term cultures (up to 48 h) of decidual tissue isolated from uteri of syngeneically mated mice can suppress the recognition and proliferation responses of lymphocytes in the MLR. The level of suppression varied with the gestational age of the tissue, being maximal with decidual tissue obtained on Day 8 of pregnancy, the period of maximal development of the decidua *in vivo* (Finn, 1971). The factors in the supernatants do not exhibit immunological specificity with respect to the histocompatibility antigen system since they are also inhibitory in a third party MLR assay, and their production by decidual tissue obtained from syngeneically mated females suggests that it is an inherent property of decidual tissue rather than induced by exposure to foreign histocompatibility antigens of the fetus. It would therefore appear that these non-specific factors would be produced by decidual tissue irrespective of the genotype of the paternal strain.

The inhibition of the MLR was not due to toxicity of the decidual tissue culture supernatants, as shown by the lack of effect on lymphocyte viability during culture. The MLR is comprised of an initial antigen recognition by the responder lymphocyte population followed by its subsequent proliferation. The observation that substantial inhibition of the MLR is still achieved by addition of the supernatants after the recognition phase (i.e. after 12 h) suggests that a major action of the factor(s) is the inhibition of subsequent lymphocyte proliferation. Supernatants also produce inhibition of mitogen-induced lymphocyte proliferation and thymocyte proliferation (Badet, Bell & Billington, 1983) and, as shown in the present study, inhibition of lymphocyte proliferation as detected by inhibition of syngeneic cultures (Table 1). However, supernatants are also able to inhibit the recognition phase of the MLR because (1) the level of inhibition of the MLR (i.e. due to recognition and proliferation) in almost all cases exceeds the level of inhibition of syngeneic cultures (i.e. due to proliferation alone) and, (2) factors responsible for inhibition of lymphocyte and thymocyte proliferation are separable from MLR inhibitory factors (Badet *et al.*, 1983).

In the present study the maximum inhibition observed in the MLR was 50% and it remains to be determined whether the generation of cytotoxic T cells in the MLR is similarly affected. In normal murine pregnancy, anti-paternal specific cytotoxic T cells are reported to be rare or absent (Smith, Burton, Barg & Mitchell, 1978; Wegmann, Waters, Drell & Carlson, 1979; Gottesman & Stutman, 1980) and anti-paternal humoral immune responses are restricted to a few inbred strains (Kaliss, 1973; Bell & Billington, 1980, 1981). Non-specific inhibitory factors produced locally from decidual tissue could provide an explanation for such observations (see Billington & Bell, 1982).

It is apparent that at least two morphologically distinct cell populations are present in the short-term decidual cultures, analogous to those observed in an in-vitro decidual cell differentiation system (Bell & Searle, 1981), which possess characteristic cell surface markers. The flat dendritic cells, which undergo dramatic alterations in cytoplasmic and nuclear area, not only during culture but with increasing gestational age of the tissue, express maternal histocompatibility antigens and lack surface Fc receptors and immunoglobulin (Searle *et al.*, 1983). A sub-population of these cells appears to express Ia antigens from Day 14 of pregnancy (Jenkinson & Searle, 1979; Searle *et al.*, 1983). Of the second population of small round cells, at least a proportion appear to express Fc receptors (Kirkwood, 1981) and possess macrophage-like characteristics (Searle *et al.*, 1983), and are probably identical to Fc receptor-bearing cells identified in decidual tissue suspensions (Bernard *et al.*, 1978). It has been suggested that these small round cells represent a cellular infiltrate, recruited into the decidua during decidualization (Bell, 1983), whereas the former population is of true decidual cells arising by differentiation of stromal fibroblasts *in situ* (Bell &

PLATE 1



Decidual cells explanted from mice on Days 6, 8 or 9 of pregnancy, seeded at 1×10^5 cells/well and examined after 48 h in culture (see 'Materials and Methods').

Fig. 1. Explanted at Day 6 of pregnancy. Major population of flat dendritic cells are mono- or bi-nucleate with extensive cytoplasm. Arrows indicate the small round cells. × 137.

Fig. 2. Explanted at Day 8 of pregnancy. Appearance of population of flat ovoid cells, mono- or bi-nucleate with large cytoplasmic area (central cell). Arrows indicate the small round cells. × 244.

Fig. 3. Explanted at Day 9 of pregnancy. Note dramatic increase in nuclear area of ovoid cells (see Fig. 2). Arrows indicate the small round cells. × 244.

(*Facing p.* 356)

Searle, 1981). Which of these cell populations is responsible for the production of the active component(s) of culture supernatants is unknown, although there is evidence that it is a cellular infiltrate in the decidua. Cells originally identified in the draining lymph nodes, which produce a soluble non-specific immunosuppressive substance (Clark & McDermott, 1978; Clark, McDermott & Szewczuk, 1980), have also been detected in decidual tissue (Clark & McDermott, 1981) and may constitute a proportion of the small round cells observed in the cultures. The nature and relationships of these soluble factors to other decidual tissue products (Umapathysivam & Jones, 1978; Bell, 1979b; Rosenberg, Maslar & Riddick, 1980; Sutcliffe, Bolton, Sharp, Nicholson & MacKinnon, 1980) is unknown, but appear to be associated with two protein fractions (Badet *et al.*, 1983).

It is clear that decidual tissue during short-term in-vitro culture produces soluble factors, the production of which reflects the development of the decidua *in vivo*, and which exhibit the capacity to effect a partial afferent blockade of an in-vitro analogue of the cell-mediated immune response. These results not only suggest that the production of the factors observed in cultures of uterine cells in which decidual cell differentiation occurs (Kirkwood & Bell, 1981) are also produced *in vivo*, but support the in-vivo transplantation studies (Beer & Billingham, 1974; Dodd *et al.*, 1980) showing that decidual tissue can partly block the rejection of intrauterine skin allografts. The fact that MLR inhibitory factors have not been detected in the peripheral serum of the pregnant mouse (Smith, 1978) suggests that these factors may operate locally at the decidua–placental interface and/or at the local draining lymph nodes to prevent proliferation, and possibly differentiation, of allosensitized lymphocytes, and be produced by migratory cells moving between the draining lymph nodes and the developing decidua. All viviparous animals bearing histoincompatible embryos face the problem of circumventing the immune responses which ensure the elimination of non-self, but the mechanisms that have evolved to achieve this may be multi-factorial and vary in individual species (Billington & Bell, 1982). The production in many mammals of decidual tissue beneath the implanting embryo, which may fulfil many functions in pregnancy, not only provides an ideal site for the production of such non-specific immunosuppressive factors involved in the protection of the fetal allograft, but links their production to pregnancy irrespective of the particular genetic status of the fetus.

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