Preparation of a population of macrophages from human third trimester decidua

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Introduction

Preterm labour occurs in ~6–8% of all pregnancies, but it accounts for at least 50% of the handicapped infants. The biochemistry of preterm labour remains incompletely understood, although during the last few years it has become clear that activation of the inflammatory response within the decidua is important (Romero et al., 1989a; Kelly, 1994). This is particularly apparent in the special case of infection-associated preterm labour, in which the organisms induce the production of cytokines and prostaglandins which are present in amniotic fluid at increased concentrations (Romero et al., 1988, 1989b,c, 1990, 1991). It is clear that bacterial products can induce the production of cytokines and prostaglandins by the decidua in vitro (Casey et al., 1989; Romero et al., 1989d, 1990; Mitchell et al., 1990). In addition, a number of cytokines, particularly interleukin-1β (IL-1β), induce decidual prosta- glandin output (Mitchell et al., 1990; Ishihara et al., 1992; Kent et al., 1993), which suggests that these cytokines have a role to play in labour. Increased concentrations of IL-1β are present in amniotic fluid during normal term delivery (Romero et al., 1992), implicating an inflammatory response in labour at all gestational ages, although the factors controlling cytokine production in term labour have not been identified. These findings indicate that cytokines are likely to be key regulators of human labour, and therefore the control of decidual cytokine production needs to be investigated.

The third trimester decidua of human pregnancy is a complex tissue which contains a number of cell types, including stromal cells (53%), macrophages (19%), granulocytes (16%), T-lymphocytes (8%) and large granular lymphocytes (4%) (Vince et al., 1990). Small numbers of other cell types may be present (e.g. vascular endothelial cells and B-lymphocytes), but these have not been quantified. The cytokines implicated in preterm labour are those of the inflammatory pathway, specifically IL-1β, IL-6, IL-8 and tumour necrosis factor-α (Romero et al., 1989b,c, 1990, 1991). In other tissues these cytokines are produced by macrophages, so it is a reasonable supposition that this also applies to the decidua. Recent papers describe the isolation of a pure population of decidual macrophages (Vince et al., 1990, 1992) using a fluorescence-activated cell sorting (FACS) procedure. In our hands, attempts to use this method to provide cells for functional studies were limited by cell viability (<80%), which decreased the survival of the cells in culture. The small numbers of cells available (normally 1–2 × 10⁶ macrophages) impose severe limitations on the experiments which could be carried out, so we investigated alternative methods based on the separation of cell populations of up to 10 × 10⁶ macrophages at a time, rather than individually as in FACS. Here we describe a method to isolate an enriched population of decidual macrophages by an immunomagnetic method (Miltenyi et al., 1990) and maintain them in culture for at least 3 days. These cells can be used to investigate how cytokine production by decidual macrophages is controlled, but such studies lie beyond the scope of this paper.

Materials and methods

Fetal membranes were obtained from women delivered by elective Caesarean section at term (>37 weeks of gestation). All the women had normal pregnancies, and none were treated with anti-platelet or anti-inflammatory drugs. Immediately after delivery, the fetal membranes were cut from the placenta and placed in phosphate-buffered saline (PBS) with antibiotics (1% gentamycin–penicillin–streptomycin) for transport to the laboratory, where all other steps were performed under sterile conditions. The method used to generate cell populations enriched in macrophages is shown in Figure 1 and is described in more detail below. The generation of decidual cells has been described in detail previously (Vince et al., 1990; Khan et al., 1991). In brief, the decidual tissue was scraped from the maternal surface of the membranes to provide 2–5 g of tissue, and subjected to a two-stage enzyme digestion with dispase followed by a mixture of collagenase–hyaluronidase–DNase (Vince et al., 1990).
These cells were washed by centrifugation and cultured in macrophage serum-free medium (MSFM) (MSFM; Life Technologies) at 37°C in 5% CO₂, 95% air for 24 h.

These mixed decidual cells were subsequently harvested with a sterile cell scraper, passed through a 50 μm nylon mesh to remove cell debris, washed and counted prior to magnetic separation. The magnetic cell sorter (MACS; Miltenyi Biotech, available from Eurogenetics, UK) was developed to separate peripheral lymphocytes by labelling cells with appropriate antibodies coupled to super-paramagnetic beads. The labelled cells were retained within a metal matrix by a high-intensity magnetic field, while unlabelled cells were washed away. The labelled cells (magnetic fraction) could be eluted from the magnetic field and recovered after removal from the field. Decidual cells were smeared on microscope slides, fixed and stained with antibody EMB 11 as described above. Data from each of the fractions obtained from the separation process (negative, wash and positive preparations) compared with the total unseparated population is shown in Figure 4. The enrichment factor (percentage of LP9+ cells in the positive fraction/percentage of LP9+ cells in the unseparated population) was 3.9 in this experiment. Using this procedure in a series of experiments (Table II), we obtained a mean enrichment factor of 3.82 ± 0.34-fold for macrophages. The characteristics of these antibodies are summarized in Table I.

The data obtained from 23 separate cell preparations are shown in Figure 2. From these data it may be seen that the greatest labelling in each preparation was observed with one of three antibodies (Ham 56, EMB11 and LP9), whereas antibodies to CD14 and HLA-DR were bound to a lesser extent. Direct immunocytochemistry of these antibodies was performed on cell preparations showing the proportion of LP9+ cells in each of the fractions obtained from the separation process (negative, wash and positive preparations) compared with the total unseparated population is shown in Figure 4. The enrichment factor (percentage of LP9+ cells in the positive fraction/percentage of LP9+ cells in the unseparated population) was 3.9 in this experiment. Using this procedure in a series of experiments (Table II), we obtained a mean enrichment factor of 3.82 ± 0.34-fold for macrophages. The characteristics of these antibodies are summarized in Table I.

Results

The first stage of the study was to identify the best antibody to use for the magnetic bead separation of macrophages from the other decidual cells. Preliminary studies on five separate cell preparations showed that five antibodies were appropriate for further study, namely anti-CD14, anti-HLA-DR, Ham 56, EMB11 (CD68) (all from Dako) and LP9 (Serotec). The characteristics of these antibodies are summarized in Table I. The data obtained from 23 separate cell preparations are shown in Figure 2. From these data it may be seen that the greatest labelling in each preparation was observed with one of three antibodies (Ham 56, EMB11 and LP9), whereas antibodies to CD14 and HLA-DR were bound to a lesser extent. Direct comparison of the antibody binding (Figure 3) showed that LP9 and Ham 56 (which are IgM antibodies) bound to the largest numbers of cells within the preparations and gave similar data. LP9 was chosen for the remainder of the studies, as it is more specific for macrophages (see Table I). EMB11, an IgG antibody, was used to double-label the cells and assess the numbers of macrophages in the preparation by immunocytochemistry.

A typical FACS profile showing the proportion of LP9+ cells in each of the fractions obtained from the separation process (negative, wash and positive preparations) compared with the total unseparated population is shown in Figure 4. The enrichment factor (percentage of LP9+ cells in the positive fraction/percentage of LP9+ cells in the unseparated population) was 3.9 in this experiment. Using this procedure in a series of experiments (Table II), we obtained a mean enrichment factor of 3.82 ± 0.34-fold for macrophages. The original cell preparations ranged from 8.0 to 18.2% LP9+ cells, and the enriched samples from 31.2 to 80.4% LP9+ cells (Table II). These cells were cultured in MSFM for 3 days and showed no loss of viability, as assessed by Trypan Blue exclusion. At the end of the culture period, the cells were stained with antibody EMB11 as described above. Data from experiment 3 are shown in Table II: this sample was chosen as representative because the number of macrophages in the
Table I. Characteristics of antibodies used to stain decidual cell preparations

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Source</th>
<th>Specificity</th>
<th>Applications</th>
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<tbody>
<tr>
<td>CD14</td>
<td>Dako, Serotec</td>
<td>Myeloid cells</td>
<td>Smears, frozen sections, FACS</td>
</tr>
<tr>
<td>Ham 56</td>
<td>Dako</td>
<td>Macrophages</td>
<td>Smears, frozen sections, FACS</td>
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<tr>
<td>HLA-DR</td>
<td>Dako</td>
<td>Endothelium</td>
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<td>EMB11</td>
<td>Dako</td>
<td>B-lymphocytes</td>
<td>Smears, frozen sections, FACS</td>
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<tr>
<td>LP9</td>
<td>Serotec</td>
<td>Macrophages</td>
<td>Frozen sections</td>
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FACS = fluorescence-activated cell sorting.

Discussion

Enriched cell populations must be derived from a mixed cell population so as to study the functions of the component cells of human decidua. The MACS separation method allows us to generate populations of decidual macrophages which are ~90% pure by immunocytochemistry and contain ~10^6 cells. The enriched cells retain the ability to produce prostaglandin E2, and are therefore a useful model to study the functions of decidual macrophages in vitro. MACS has been used to separate a variety of cells from peripheral blood (Pflueger et al., 1990; Farace et al., 1992; Manyonda et al., 1992; Kato and Radbruch, 1993; Semple et al., 1993), and more recently from other tissues (Schmitz et al., 1993; Clarke et al., 1994). The purity of most preparations was >90% (Manyonda et al., 1992; Kato and Radbruch, 1993; Semple et al., 1993; Clarke et al., 1994), although follicular dendritic cells and megakaryocytes proved more difficult to isolate and enrichments to 78.4 and 65.0% respectively were obtained (Schmitz et al., 1993, 1994). These data indicate that the enrichment of decidual macrophages is comparable with other cell types. The basic technique of magnetic cell sorting has been described in Materials and methods, but we found that it was necessary to alter the protocol to accommodate the characteristics of third trimester human decidual cells. In particular, the cells are large and sticky, leading to blockage of the separation column. In our laboratory we have used the MACS system on peripheral blood lymphocytes without needing any intervening stages such as overnight culture, indicating that the method developed reflects decidual cell characteristics rather than any technical problems.

The purity of macrophages in one of the enriched populations was assessed to be 49% by FACS (Figure 4) and to be ~90% by immunocytochemistry (Figure 5), and the yield was 7.6 × 10^6...
addition, the cells in the enriched fraction should be LP9
+ macrophage cells do not stain even lightly with EMB11. In found in this population, which would indicate that non-conflict with the data from the macrophage-depleted population (Figure 5D); no positive cells (lightly or heavily stained) were argued that these cells are not macrophages, but this would in the MACS-enriched population (Figure 5C). It might be lightly stained cells which make up the majority of the cells cells. In addition, this is consistent with me large numbers of interest while MACS also selects the less strongly stained marker expression (Figure 5A and C), and that FACS may select only those cells with relatively high levels of an antigen macrophages are heterogeneous with respect to cell-surface must lie in the enrichment methods used. We suggest that in both studies were the same, so the differences in the data preparations relatively rich in macrophages as the starting material.

The variation in antigen expression was not limited to EMB11. The common macrophage markers used in previous studies (CD14, HLA-DR) were expressed at lower levels than expected, as determined by FACS (19% previously, ~10% in our study; Vince et al., 1990). These findings also conflict with the findings of Bulmer and Johnson (1984), from which we would anticipate CD14 > HLA-DR, whereas we found the opposite (Figure 3). Both CD14 antibodies tested gave similar levels of expression, indicating that this was a genuine finding and not dependent on the antibody used. It is not clear why this is the case, but the number of macrophages detected with LP9 and Ham 56 (~25%) agrees with the previous data (Vince et al., 1990). An analysis of 23 different decidual preparations (Figure 2) showed that five preparations (numbers 2, 3, 8, 19 and 20) had <10% macrophages present by all five markers tested. The most likely explanation is a loss of cell-surface markers during tissue digestion, leading to an apparent lack of macrophages. An assessment of macrophage numbers in each preparation can be performed during the cell culture step prior to MACS enrichment of the macrophage numbers.
Enrichment of human decidual macrophages

Figure 5. Expression of LP9 by cells from (A) the original decidual cell preparation, (C) a macrophage-enriched population and (D) a macrophage-depleted population. (B) Negative staining with an irrelevant antibody. Data shown are typical of three different cell preparations. Original magnification ×100. In (C) there are strongly stained (*), lightly-stained (†) and non-stained (→) cells.

and thus allows the selection of appropriate cell preparations for enrichment and minimizes the use of damaged or macrophage-poor preparations. From the data in Figure 2, we anticipate that 75–80% of the preparations would be appropriate for the MACS method.

In conclusion, we have accomplished the original aims of
this study and generated large numbers (10×10⁶ cells) of macrophages of high purity (~90%). These populations can be used for studies on the functions of decidual macrophages, and they need to include an assessment of whether variations of antigen expression are related to any variations in decidual macrophage biochemistry. In particular, it is not clear whether the differences in antigen expression reflect enzymatic damage or biological variation in decidual macrophages.

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References

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