

# Endometrial leucocytes: expression of steroid hormone receptors

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## Abstract

**Background**—Stromal leucocyte populations in human endometrium comprise T cells, macrophages, and phenotypically unusual endometrial granulated lymphocytes. Their proportions vary during the menstrual cycle and, in particular, endometrial granulated lymphocytes increase in number in the late secretory phase. The stimulus responsible for these cyclical changes is unknown but it is likely that the steroid hormones oestrogen and progesterone play a role.

**Aims**—To define further the expression of steroid hormone receptors by leucocytes in non-pregnant and pregnant human endometrium.

**Methods**—Frozen and paraffin wax embedded sections of endometrium from non-pregnant women and early pregnancy decidua were labelled using single and double immunohistochemical techniques with monoclonal antibodies directed against oestrogen and progesterone receptors and various leucocyte subpopulations.

**Results**—Despite the prominence of CD56 positive endometrial granulated lymphocytes in late secretory phase endometrium and early pregnancy decidua, double immunohistochemical labelling showed no evidence of expression of either progesterone or oestrogen receptors by these cells or other endometrial leucocyte populations.

**Conclusions**—Rather than acting directly, steroid hormones are likely to influence endometrial leucocyte populations indirectly via products of endometrial stromal or epithelial cells that express steroid hormone receptors.

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Keywords: endometrium; steroid hormone receptors; granulated lymphocyte

The endometrium is a steroid sensitive organ that responds to oestrogen and progesterone in a cyclical fashion. The morphological cyclical response has been well documented.<sup>1,2</sup> The stromal leucocyte populations in human endometrium also change during the menstrual cycle.<sup>3-5</sup> Endometrial stromal leucocytes consist of T cells that remain, numerically, relatively stable during the cycle, macrophages that increase in number as the cycle progresses towards menstruation, and phenotypically unusual endometrial granulated lymphocytes. Endometrial granulated lymphocytes increase

in number dramatically in the late secretory phase of the menstrual cycle; if conception and implantation occur, endometrial granulated lymphocytes continue to increase in number and comprise around 70% of the stromal leucocytes in first trimester decidua.<sup>3,6</sup>

Immunohistochemical studies have demonstrated active proliferation of endometrial granulated lymphocytes in the late secretory phase of the menstrual cycle<sup>7</sup> but the mechanisms that control the changes in endometrial leucocyte numbers are not known. In view of their cyclical nature and the influence on decidualisation, it appears likely that they respond to the sex steroid hormones that influence the menstrual cycle—oestrogen and progesterone. This could be a direct effect on the leucocytes, which must in consequence express the appropriate receptors, or an indirect effect mediated by products from other endometrial cells, which themselves express steroid receptors.

That leucocytes might be stimulated directly by sex steroids has been suggested by reports that peripheral blood CD8 positive T lymphocytes express progesterone receptors during pregnancy,<sup>8</sup> although earlier binding studies excluded androgen, oestrogen, and progesterone receptors on peripheral blood lymphocytes, including those activated by phytohaemagglutinin.<sup>9</sup> Progesterone is reported to inhibit lymphocyte proliferation but this action is not blocked by RU486, a progesterone (and glucocorticoid) antagonist, suggesting a mechanism not related to direct stimulation of progesterone receptors.<sup>10,11</sup> Immunohistochemical studies of leucocytes in non-pregnant human endometrium have detected oestrogen receptor expression by T cells in aggregates in the basal endometrium<sup>12</sup>; however, King *et al* reported conflicting results, having shown no evidence of sex steroid hormone receptor expression by endometrial leucocytes.<sup>13</sup> The latter study was confined to the general leucocyte population (CD45 positive), without examination of specific leucocyte subgroups.

This study was designed to examine more closely the possible expression of sex steroid receptors by endometrial leucocytes in pregnant and non-pregnant endometrium to clarify the mechanism of regulation of endometrial leucocyte numbers.

## Materials and methods

### FROZEN TISSUE

Fresh endometrial tissue was obtained from 15 hysterectomy specimens (five proliferative, five early secretory, and five late secretory) where

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the operation was performed for non-endometrial pathology such as fibroids, benign ovarian cysts, or persistent cervical intraepithelial neoplasia. All specimens had normal endometrial histology in keeping with the menstrual dates. Blocks (5–10 mm cubes) were snap frozen in liquid nitrogen cooled isopentane. Decidual tissue, identified macroscopically by its opaque grey-white appearance, was obtained from five early gestation suction terminations of pregnancy with fully informed consent. Decidual tissue was irrigated in normal saline to remove excess blood and snap frozen in a similar way in 5–10 mm cubes. All tissues were stored at  $-70^{\circ}\text{C}$ .

Frozen sections of endometrium and decidua (6  $\mu\text{m}$ ) were cut on to poly-L-lysine (Sigma Chemical Co, Poole, Dorset, UK) coated slides, air dried overnight at room temperature, fixed in acetone for 10 minutes at room temperature, and stored sealed at  $-20^{\circ}\text{C}$  until use. Haematoxylin and eosin staining was performed on all sections to check orientation and to ensure that an adequate amount of tissue with normal well preserved morphology was included.

#### PARAFFIN WAX EMBEDDED TISSUE

Sections (3  $\mu\text{m}$ ) of late secretory phase endometrium ( $n = 5$ ) and normal first trimester decidua ( $n = 5$ ) from similar patient groups to those described above, were cut from archival paraffin wax blocks on to 3-aminopropyltriethoxysilane (APES) (Sigma) coated slides. Late secretory phase endometrium and decidua were used because maximal changes in endometrial leucocyte numbers occur in these samples. After the sections were deparaffinised and rehydrated, antigen unmasking was achieved by microwaving for  $2 \times 5$  minutes in either 0.01 M sodium citrate buffer, pH 6.0 (BDH Chemical Company Ltd, Poole, Dorset, UK)<sup>14 15</sup> or, for ER-6F11 (see table 1), in 1 mM EDTA buffer, pH 8.0 (BDH). After cooling, immunohistochemical staining was performed as outlined below.

#### MONOCLONAL ANTIBODIES

Antibody specificities, dilutions, and sources are given in table 1. ER-LH2 and PGR were used to identify the distribution of oestrogen and progesterone receptors by immunohistochemical labelling in frozen and paraffin wax sections. ER-LH2 was less satisfactory when used on paraffin wax embedded tissues and,

therefore, it was substituted by ER-6F11, which gave good quality staining in paraffin wax embedded sections after high temperature antigen retrieval.

Leucocyte populations were identified by single and double immunohistochemical staining procedures using antibodies to CD45 (leucocyte common antigen), CD3 (T cells), and CD14 (macrophages). Endometrial granulated lymphocytes were identified using antibodies to CD56 and in paraffin wax embedded sections this was confirmed using the MT-1 antibody.<sup>16</sup>

#### IMMUNOHISTOCHEMICAL LABELLING PROCEDURES

##### Single labelling

Single immunohistochemical labelling was performed using the Vectastain Elite ABC kit (Vector Laboratories, Peterborough, UK) and secondary and tertiary reagents were prepared according to the kit instructions.<sup>17</sup> After initial rehydration for 10 minutes in 0.05 M Tris buffered 0.15 M saline, pH 7.6 (TBS; Sigma), sections were overlain for 30 minutes with serum from the same species in which the secondary antibody had been raised to block non-specific binding. The slides were then incubated with primary monoclonal antibody at the appropriate dilution in TBS for 60 minutes. After washing in TBS, biotinylated secondary antibody was applied for 30 minutes, followed by further washing and incubation with ABC reagent for 30 minutes. A third wash was followed by incubation with the peroxidase substrate, amino-ethyl carbazole (AEC; Vector), to give a red reaction product. This reaction was monitored microscopically and stopped after  $\sim 15$  minutes with excess water. The sections were counterstained lightly with Mayer's haematoxylin.

##### Double labelling

Double immunohistochemical labelling was performed to detect any coexpression of steroid receptors by endometrial leucocyte populations. Sections were labelled initially for leucocyte antigens with the ABC method described above. After development of the red reaction product, sections were washed in TBS for 10 minutes, overlain with blocking serum for a further 30 minutes, and incubated with the second primary antibody directed against either the oestrogen receptor or the progesterone receptor for 60 minutes. After further washing in TBS, sections were incubated for 30 minutes with biotinylated secondary antibody washed in TBS and incubated with ABC alkaline phosphatase (Vectastain alkaline phosphatase kit) for 30 minutes. The reaction was developed using the alkaline phosphatase substrate kit III (Vecta Blue; Vector); sections were incubated at room temperature until the appropriate blue colour developed, usually  $\sim 10$  minutes. The reaction was stopped by excess water. Double labelled slides were not counterstained. Table 2 shows the double labelling combinations prepared.

All slides were mounted initially in Supermount (BioGenex, San Ramon, USA) and,

Table 1 Monoclonal antibodies

Antibody	Specificity	Source	Dilution	
			Frozen	Paraffin
ER-LH2	Oestrogen receptor	Novocastra	1/30	1/10
ER-6F11	Oestrogen receptor	Novocastra		1/10
PR	Progesterone receptor	Novocastra	1/100	1/100
CD3	T lymphocytes	Dako	1/200	
CD3	T lymphocytes	Novocastra		1/100
CD14	Macrophages	Dako	1/20	
CD45	Leucocyte common antigen	Dako	1/200	1/200
CD56	Natural killer cells	Novocastra	1/200	
CD56	Natural killer cells	Vector		1/100
MT-1	(CD43) Natural killer cells, T cells	Novocastra		1/40

Novocastra, Newcastle upon Tyne, UK; Dako, High Wycombe, Bucks, UK; Vector, Peterborough, UK.

Table 2 Double labelling combinations

Antibody	Frozen sections		Paraffin sections		
	ER-LH2	PR	ER-LH2	ER-6F11	PR
CD45	Yes	Yes	Yes	Yes	Yes
CD3	Yes	Yes	No	Yes	Yes
CD56	Yes	Yes	No	Yes	Yes
CD14	Yes	Yes	No	No	No
MT-1	No	No	Yes	Yes	Yes

ER, oestrogen receptor; PR, progesterone receptor.

after overnight drying, with DPX mountant (BDH) and coverslips.

#### CONTROLS

Frozen or fixed sections of tonsil were included in all runs as positive controls for leucocyte markers. The distribution of oestrogen and progesterone receptors in human endometrium and myometrium is well documented and stromal, glandular, and myometrial cells therefore acted as inbuilt positive controls. Negative controls were performed for each tissue by replacement of the primary antibody with serum. For each double labelled section the primary antibody was omitted from each stage of the technique in turn. The different antibody/enzyme/substrate combinations were also reversed in the double labelled runs to confirm consistency of the reaction patterns with the different enzyme labels. The single labelled and double labelled slides were compared to confirm consistent distribution of labelling in each tissue and to check that no spurious double labelling had occurred.

#### ANALYSIS

All sections were analysed qualitatively for double and single labelling.

#### Results

Large numbers of CD45 positive cells were identified throughout the endometrium (fig 1A) and decidua, with aggregates adjacent to glands and vessels (fig 1B). CD3 positive cells were present in all the frozen tissues, with aggregates in the stratum basalis, particularly adjacent to glands (fig 1C). There was a pronounced increase in CD56 positive cells in secretory phase and decidual tissues compared with proliferative endometrium, findings complemented by the increased number of MT-1 positive cells in formalin fixed, paraffin wax embedded tissues (fig 1D). MT-1 also reacted with T cells in the endometrium. Only small numbers of CD14 positive cells were identified in all frozen tissues studied.

In frozen and fixed tissues, the oestrogen receptor was expressed in both the epithelium and stroma in proliferative and secretory phase endometrium (fig 1A–D), although glandular staining was reduced in the mid and late luteal phases. Oestrogen receptor staining was present throughout the decidual stroma (fig 1E and 1F). Only oestrogen receptors were expressed by epithelial cells of decidualised endometrium.

The progesterone receptor was expressed to a variable degree in some glands in proliferative endometrium but, in general, glands were

negative in the luteal phase (fig 2A and 2B). Progesterone receptors were expressed throughout the stroma in both phases of the cycle (fig 2A–D) in non-pregnant endometrium and throughout the decidual stroma (fig 2E). The results of single labelling of both frozen and paraffin wax embedded sections were in keeping with previous observations.<sup>3 18 19</sup>

There was no evidence of double labelling of either the oestrogen receptor or the progesterone receptor in any of the leucocyte populations in any of the sections examined (fig 1A–E). In particular, the T cell aggregates in the stratum basalis (CD3 positive and MT-1 positive) did not show steroid receptor binding (fig 1C). CD56-positive cells (and MT-1 positive cells in paraffin wax sections) did not express oestrogen or progesterone receptors at any stage of the cycle (figs 1C, 2B, 2C, 2F, and 2G).

#### Discussion

The cyclical effects of the sex steroids oestrogen and progesterone on the endometrium are integral to the menstrual cycle and the histological changes have been well documented.<sup>20 21</sup> Immunohistochemical studies have characterised the glandular and stromal distribution of sex steroid receptors during the menstrual cycle.<sup>18 22 23</sup> Although the cyclical changes in endometrial leucocyte populations are dramatic and have been well documented, the mechanisms controlling these changes have not been defined.

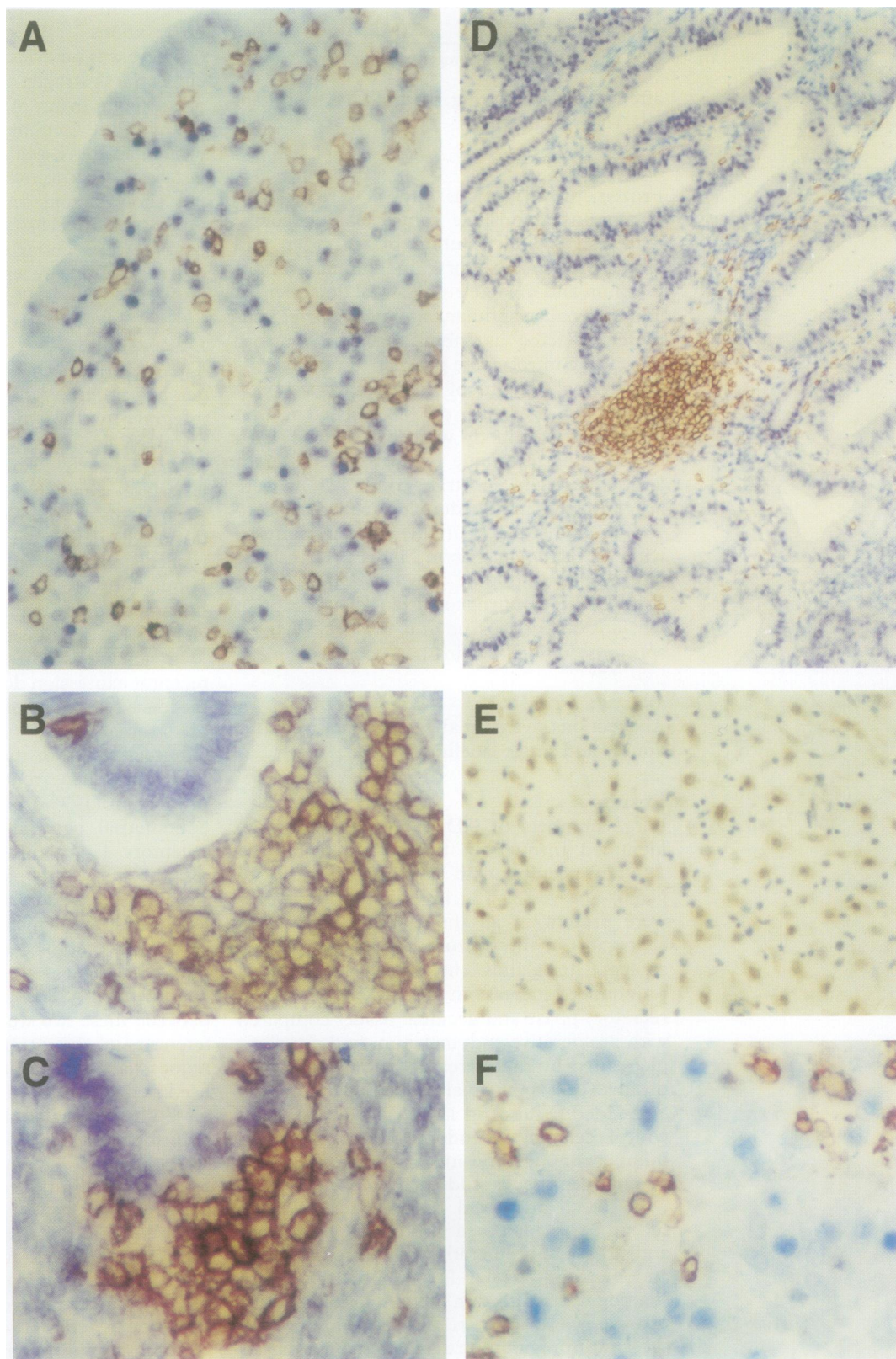
In general, studies of endometrial steroid receptor expression do not mention specifically the leucocytes that comprise a large proportion of stromal cells, particularly in the late secretory phase of the cycle. Tabibzadeh and Satyaswaroop<sup>12</sup> reported that a small proportion (1–7%) of T lymphocytes in basal endometrial aggregates expressed oestrogen receptors. This contrasts with the results of the present study, in which sex steroid receptors were not detected on any group of endometrial leucocytes, and which also showed absence of oestrogen receptors on T lymphocytes in lymphoid aggregates in the basal layer of the endometrium. The discrepancies in results between these two studies could be due to differences in the immunohistochemical techniques used. Tabibzadeh and Satyaswaroop<sup>12</sup> used peroxidase labelled secondary antibodies for both primary antibodies in double immunohistochemical labelling. In the present study two different enzyme systems were used. Furthermore, in the earlier study, endogenous peroxidase activity was revealed by 4-chloro-1-naphthol, resulting in a blue reaction product,<sup>12</sup> and it is not clear how this was differentiated from specific staining with CN-H<sub>2</sub>O<sub>2</sub>, which also produced a blue reaction product. The red and blue reaction products used for double immunostaining techniques in the present study were easier to distinguish between than the blue and brown/black reaction products of the previous study. Differences may also result from the use of different monoclonal antibodies with different specificities. The present study used two anti-oestrogen receptor



antibodies (ER-LH2 and ER-6F11) and two antibodies to detect endometrial granulated lymphocytes (CD56 and MT-1). Consistent and compatible results were obtained throughout the study. The use of both paraffin wax embedded and frozen sections indicates that

the tissue preparation alone is not responsible for the negative findings.

Recently, King *et al* reported similar results.<sup>13</sup> Fixed tissue was examined and double immunostaining techniques similar to those used here were used, although the study was



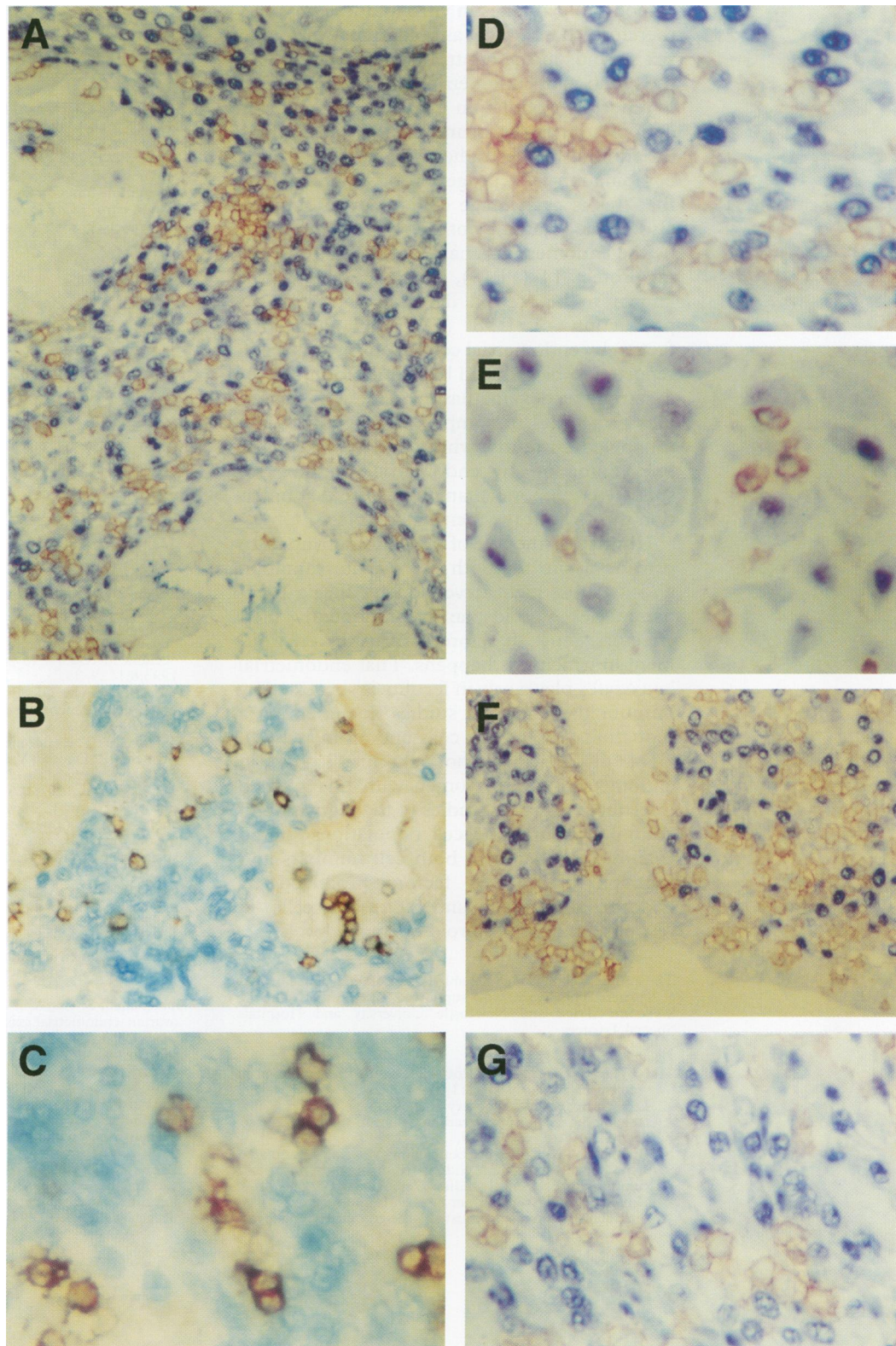
**Figure 1** Double immunohistochemical labelling: paraffin wax section of secretory endometrium, antibodies ER6F11/CD45 (A); paraffin wax section of secretory endometrium, antibodies ER6F11/CD45 (B); paraffin wax section of secretory endometrium, antibodies ER6F11/MT-1 (C); paraffin wax section of secretory endometrium, antibodies ER6F11/MT-1 (D); paraffin wax section of decidua, antibodies ER6F11/CD45 (F). In all cases leucocyte cell membranes are labelled red and the oestrogen receptor is demonstrated by blue nuclear reactivity. Single immunohistochemical labelling: paraffin wax section of decidua showing brown oestrogen receptor nuclear positivity in non-leucocyte stromal cells, antibody ER6F11 (E).



confined to labelling for oestrogen and progesterone receptors and CD45 (leucocyte common antigen), because of the limitations of anti-CD56 in fixed sections. Furthermore, the authors failed to demonstrate specific oestrogen receptor labelling in decidual tissue. This may be owing to the relative unsuitability of ER-LH2 for paraffin wax embedded sections.

In the current study, it was found that a newer antibody, ER-6F11, gave greatly improved labelling in paraffin wax embedded endometrial sections and also confirmed oestrogen receptor expression in decidual tissue, as has been reported elsewhere.<sup>19</sup>

Although the present study excludes the possibility of a direct effect on endometrial



**Figure 2** Double immunohistochemical labelling: paraffin wax section of secretory endometrium, antibodies PR/CD45 (A); frozen section of secretory endometrium, antibodies PR/CD56 (B); frozen section of secretory endometrium, antibodies PR/CD56 (C); paraffin wax section of secretory endometrium, antibodies PR/CD45 (D); paraffin wax section of decidua, antibodies PR/CD45 (E); paraffin wax section of secretory endometrium, antibodies PR/MT-1 (F); paraffin wax section of secretory endometrium, antibodies PR/MT-1 (G). In all cases leucocyte cell membranes are labelled red and the progesterone receptor is demonstrated by blue nuclear reactivity.

leucocytes by progesterone and oestrogen, it does not exclude an indirect effect via stromal or epithelial mediators such as cytokines. Endometrium derived cytokines include interferon  $\gamma$  (IFN $\gamma$ ), interleukin 1 (IL-1), tumour growth factor  $\alpha$  (TGF $\alpha$ ), TGF $\beta$ , granulocyte-macrophage colony stimulating factor (GM-CSF), CSF-1, IL-6, and tumour necrosis factor  $\alpha$  (TNF $\alpha$ ).<sup>24-25</sup> Leucocytes are known to respond to cytokines in vitro and it is possible, therefore, that they act as second messengers to sex steroids in the control of proliferation and the function of endometrial leucocytes. Laird *et al* reported modulation of IL-6 production by endometrial epithelial and stromal cell cultures on addition of oestradiol,<sup>26</sup> either alone or in combination with progesterone, later showing that this is cycle stage dependent.<sup>24</sup> IL-6 can also be modulated by other peptides produced by the endometrium, namely IL-1 and placental protein 14 (PP14), which are themselves known to be steroid dependent.<sup>27</sup> Similarly, production of IL-8 (neutrophil activating factor) by endometrial cells in culture has been reported to be reduced by the addition of progesterone or dexamethasone. These are examples of a series of complex interactions being untangled currently, which may represent the fine tuning of any endometrial immune response to temporal changes in steroid stimulation throughout the menstrual cycle.

Ultimately, the role of the endometrium is to provide a site for both embryo implantation and its subsequent development during pregnancy. Sex steroids, and consequently their effectors, must therefore control the environment in which this happens. That endometrial leucocytes play a role in fertility is suggested by immunohistochemical studies documenting a reduction in CD8+ T cells and an increase in CD4+ T cells throughout the luteal phase in endometrium from women with unexplained infertility.<sup>28</sup> Further studies of the regulation of endometrial immune cell populations should examine regulation of both cell numbers and function by soluble factors produced by endothelial stromal and epithelial cells in response to altered steroid hormone levels.

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