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Immunological aspects of endometriosis

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The immune system probably plays a role in the onset and development of endometriosis. A general picture can be proposed. In some women refluxing endometrial cells are not destroyed, either because the patient is genetically programmed not to respond to endometrial antigens, or because the reflux is so abundant that the scavenging capacity of the peritoneal immune cells is overloaded. Refluxing cells could be protected due to an abnormal adherence to the mesothelium which exceptionally expresses certain adhesive molecules. Undestroyed, these endometrial cells would cause an inflammation with activation of macrophages. Not only does the peritoneum protect these endometrial cells, but it also produces abnormal quantities of chemotactic and angiogenic cytokines (interleukin-8). Macrophages facilitate development via growth factors such as transforming growth factor β. Immunosuppressive factors block the cytotoxic activity of natural killer (NK) cells. Activated macrophages present antigens of endometrial cells to T cells which will co-operate with B cells to synthesize autoantibodies. Synthesized antibodies protect the ectopic endometrium and could worsen the dysfunction of local NK cells. A vicious circle is set up involving all the partners of the immune system. It is as yet impossible to pinpoint the triggering mechanism. The primary defect could be localized on the endometrium, macrophages already activated by an extrinsic factor (infection, spermatozoa, chemical substances), the uterus or the tubo-uterine junction. The two pathophysiological theories put forward to explain endometriosis are linked by a defective immune system. Indeed, once the vicious circle is set up, growth and angiogenic factors could induce metaplasia of the already irritated mesothelium.

Key words: autoimmunity/cellular immunity/cytokines/endometriosis/endometrium/integrins/peritoneum

Immunological aspects of endometriosis

Endometriosis remains a frequent and puzzling disease. Even its reality is still disputed (Koninckx, 1994; Moen, 1995). Numerous hypotheses have been put forward to explain the presence of ectopic endometrial tissue and stroma. The first theory, proposed by Meyer in 1919, implies that chronic irritation of the peritoneum by infections or chemical substances induces metaplasia of peritoneal mesothelial cells by provoking their transformation into endothelial tissue. Clinical and experimental data to support this theory are scarce. If the mesothelium is capable of metaplasia, then endometriosis should be encountered in men, and also in all coelomic epithelium of the organism. In recent studies, endometriosis was not induced in rats subjected to chronic irritation of the peritoneum with peritoneal fluid obtained from women with endometriosis (Ramey and Archer, 1993). Sampson (1927), having observed the low frequency of endometriosis in amenorrhoeic or hysterectomized women, and having objectively observed retrograde menstrual reflux, proposed another theory whereby, in some patients, retrograde tubal endometrial reflux during menstruation could allow the implantation and development of endometrium in the peritoneal cavity. There are numerous features that lend support to the implantation theory. Peritoneal fluid recovered from women during menstruation contains endometrial

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cells that are capable of proliferation and adhesion in 98% of these women (Kruitwagen *et al.*, 1991). Progressively, these cells, which possess two important features essential for implantation (proliferation and adhesion capacity), disappear, only to be found again at the end stage of the follicular phase. Some women are capable of eliminating this reflux menstruation since it occurs in >90% of women, a frequency far greater than the incidence of endometriosis (Halme *et al.*, 1983a). It has been reported that 1–7% of women suffer from endometriosis (Barbieri, 1990). As early as 1941, Levander had attempted to link these two previous theories. Menstrual reflux in some patients would overload their scavenging capacity either due to the abundance of the reflux or due to a defective scavenging system. The presence of this abnormal menstrual reflux would irritate the peritoneum. By defending itself, the peritoneum would secrete activating and growth factors, which facilitate implantation and growth and could thus induce metaplasia (Levander, 1941). This unifying theory is supported by modern immunological concepts. The immune system participates in the homeostasis of the peritoneal cavity. Modifications in its functioning have been advanced to explain endometriosis and its consequences. Our purpose is to identify the contribution of the immune system in the pathogenesis of endometriosis, by underlining for each theory advanced the difficulties encountered to distinguish causative mechanisms from secondary abnormalities subsequent to the presence of ectopic tissue.

Endometriosis: disease of cell-mediated immune response

T lymphocytes have a regulatory and effector role. They are capable of destroying a specific target by a cytotoxic mechanism (cytotoxic T cell) and of communicating with accessory cells and lymphocytes in order to induce (helper T cell) or contrarily to suppress (suppressor T cell) the immune system.

Several observations in women and in the female monkey are in favour of a defective T cell response in endometriosis: cutaneous anergy to autologous endometrial antigens (Dmowski *et al.*, 1981), reduction of the proliferative response of T cells stimulated by autologous endometrium (Dmowski *et al.*, 1981; Steele *et al.*, 1984), reduction of the cytotoxicity of circulating T lymphocytes (Gilmore *et al.*, 1992). For some authors, this reduction of the cytotoxicity of T lymphocytes is specific against stromal cells (Vigano *et al.*, 1991).

Non-specific immune responses are identical for normal women and those who have endometriosis. T lymphocyte counts as well as those of T lymphocyte derivatives are unmodified (Gleicher *et al.*, 1984). Some authors have reported that the ratios of helper T cells to suppressive T cells are abnormally raised in the peripheral blood and peritoneal fluid of women with endometriosis (Hill *et al.*, 1988; Cunningham *et al.*, 1992). A reduction of activated T cells (CD25+ CD3+ lymphocyte subpopulation) has been observed in the peritoneal cavity of women with endometriosis (Ho *et al.*, 1995).

Ectopic implantation of endometrium in the female monkey is facilitated if the immune system is altered by chemotherapy or radiotherapy (Wood *et al.*, 1983; Fanton and Golden, 1991). The incidence of endometriosis in Belgium (one of the highest in the world; Koninckx *et al.*, 1991) has been suggested to be associated with the high level of dioxin pollution in this country. In the USA, the annual increase of hysterectomies due to endometriosis parallels that of dioxin pollution. The high incidence of endometriosis could be linked to the severity of dioxin pollution, which is known to suppress cell-mediated immunity (Smith, 1995). Endometrial lesions are more severe in rhesus monkeys chronically exposed to dioxin for several years (Rier *et al.*, 1993).

In healthy women without endometriosis, endometrial cells that flow back are recognized by the immune system before being destroyed. The question under debate is why these autologous cells are recognized by the immune system. However, there are data indicating that the immune system can recognize and respond to normal self-antigens without initiating an autoimmune disease (Weksler *et al.*, 1981). In addition, the peritoneal environment of healthy women may modify the immunogenicity of these retrograde endometrial cells, thereby facilitating their elimination by the immune system.

In women with endometriosis it is possible that alterations in T cell-mediated immunity facilitate implantation of the endometrial fragments or cells in ectopic locations (Dmowski *et al.*, 1994), either directly interfering with T cell cytotoxicity or indirectly altering their cooperation with other cell types of the immune network [macrophages, natural killer (NK) cells, B cells].

Endometriosis: disease of humoral-mediated immune response

The possibility of an antigen–antibody reaction was proposed initially by Weed and Arquembourg in 1980. Using immunofluorescence techniques, these authors demonstrated the presence of immunoglobulin (Ig) A antibodies and C3 complement fraction on the uterine endometrium. These observations, together with the decrease of total complement proteins, could be an indication of an autoimmune

response with local activation and consumption of complement factors by the antigen–antibody complexes.

Studies designed to investigate the implication of antibody response in endometriosis have looked at the following: (i) the local precipitation of immunoglobulins and/or complement factors, (ii) the difference between serum and peritoneal concentrations of non-specific factors (IgA, IgM, IgG, C3,C4, total complement fraction), (iii) the demonstration of an autoimmune response, either specific for the endometrium or a more generalized response.

Local precipitation of immunoglobulins and of complement

Since the initial report by Weed and Arquembourg (1980), the precipitation of IgA, IgM, IgG and complement fraction C3,C4 in the endometrium has been confirmed by many authors (Saifuddin *et al.*, 1983; Badawy *et al.*, 1984; Wild and Shivers, 1985; Bartosik *et al.*, 1987; Kreiner *et al.*, 1986).

The specificity of these precipitations is poor, as IgG precipitates can be encountered in pelvic inflammatory disease (Kreiner *et al.*, 1986; Bartosik *et al.*, 1987). Recently, factors regulating the activation of complement have been identified in endometrial cells (D'Cruz and Wild, 1992). The presence of local precipitation of antibodies and of activated complement factors cannot be considered to be final evidence of a specific immune response, since it has been proven that endometrial cells themselves are capable of synthesizing complement factors (Isaacson *et al.*, 1990; Bischof *et al.*, 1994).

Differences between the concentrations of non-specific circulating factors (IgA, IgM, IgG, C3,C4, total complement)

Studies comparing results of the concentrations of non-specific factors are contradictory. With regard to serum and peritoneal concentrations of immunoglobulins, some authors have reported a decrease, particularly of IgA (Meek *et al.*, 1988), others have reported no difference (Badawy *et al.*, 1984; Gilmore *et al.*, 1992), and still others have reported an increase in concentration (Gleicher *et al.*, 1987). A positive correlation between immunoglobulin concentrations and the clinical stage of endometriosis has even been reported (Taylor *et al.*, 1991).

Measurements of complement factors also differ. For women with endometriosis, C3 and C4 concentrations are increased in some reports (Badawy *et al.*, 1990; Taylor *et al.*, 1991), identical to those of normal women in other studies (Gilmore *et al.*, 1992), or, in recent studies, decreased (Gleicher *et al.*, 1987; Meek *et al.*, 1988). The decrease of complement factors is considered to be secondary to a local consumption of complement factors by immune complexes, evoking the possibility of a local autoimmune response.

Demonstration of an autoimmune response

Studies of the humoral immune response identify two major abnormal autoimmune responses in endometriosis: (i) an antibody response aimed at endometrial antigens and (ii) a polyclonal response as is encountered in autoimmune disorders.

Several techniques, i.e. immunodiffusion (Meek *et al.*, 1988; Badawy *et al.*, 1990), passive haemagglutination (Chihal *et al.*, 1986; Mathur *et al.*, 1988; Badawy *et al.*, 1990), Western blot (Mathur *et al.*, 1988), immunofluorescence (Mathur *et al.*, 1988) and enzyme-linked immunoassay (Kennedy *et al.*, 1990a; Wild *et al.*, 1991; Odukoya *et al.*, 1995b), have confirmed the presence of anti-endometrium antibodies in serum (Mathur *et al.*, 1988) and peritoneal fluid (Halme and Mathur, 1987) as well as on endometrial implants (Saifuddin *et al.*, 1983). Several authors (Badawy *et al.*, 1990) reported that the antibody titre was higher in the serum than in the peritoneal fluid. This could be due to the fact that, since antigen–antibody reactions take place in the peritoneal cavity, the presence of antigen–antibody complexes could influence the results obtained. Furthermore, the local accumulation of prostaglandins could modify the antibody response. No study has been able to demonstrate a correlation between the severity of the anatomical lesions in endometriosis and the antibody titres (Garza *et al.*, 1991). Immunohistochemical studies have demonstrated that anti-endometrial antibodies are directed against the glandular component of the endometrium (Kennedy *et al.*, 1990b). Results obtained by immunofluorescence before or after destruction of the Fc fragment of immunoglobulins in the sera of patients with endometriosis are identical, thereby indicating that the fixation of immunoglobulins is indeed specific, since it is the $F(ab')_2$ fragment of the immunoglobulin which recognizes the antigen (Wild and Shivers, 1985).

Some authors, having been unsuccessful in demonstrating the presence of anti-endometrial antibodies, insist on the need to develop more specific techniques, as these antibodies seem to have a poor affinity and a variable reactivity depending on the tissue involved (Switchenko *et al.*, 1991; Fernandez-Shaw *et al.*, 1993).

A double immunofluorescence technique allows distinction between a specific fixation and background noise due to endogenous IgG, thus improving specificity. With this technique, endogenous IgG can be detected in the endometrial stroma of women with or without endometriotic lesions. It is unlikely that the presence of IgG locally

reflects an autoimmune phenomenon (Fernandez-Shaw *et al.*, 1993). With this technique the presence of anti-endometrial antibodies is more frequently and more abundantly revealed in cases of endometriosis (Fernandez-Shaw *et al.*, 1993). These antibodies react both with autologous endometrium and ectopic endometrium (Fernandez-Shaw *et al.*, 1993; Kim *et al.*, 1995). Double immunofluorescence has demonstrated that, in endometriosis, autoimmune antibodies are aimed against the glandular component, confirming other studies, but also against endometrial vessels, thus allowing for the possibility of a generalized autoimmune disease (Gleicher *et al.*, 1987).

Other studies have investigated the relationship between atypical autoantibodies (lupus anticoagulant, anticardiolipin, antinucleotide, antiphospholipids) and endometriosis. Indeed, the majority of patients suffering from endometriosis present with autoimmune abnormalities (Confino *et al.*, 1990). Results of searches for autoantibodies depend directly on the number of autoantibodies investigated. For example, if 16 antibodies are tested, the result will be positive at least for one antigen in 65% of patients; if only antinuclear antibody is tested, only 10–25% of patients will be positive. If a large number of antibodies are used, including antibodies directed against phospholipids, histones and polynucleotides as well as antithyroid antibodies, positivity will be encountered in at least 85% of patients with endometriosis (Gleicher *et al*., 1987). It has also been demonstrated that 70% of women suffering from endometriosis have an antibody directed against carbonic anhydrase. This antibody is present in several autoimmune diseases (Kiechle *et al.*, 1994).

There are two opposing schools of thought concerning the significance of these autoimmune abnormalities. The first one links endometriosis with certain types of infertility, particularly failure of implantation, in a single entity characterized by the presence of autoantibodies (Gleicher *et al.*, 1987; Dmowski *et al.*, 1994; Nip *et al.*, 1995). Controversies concerning the association of these antibodies and endometriosis are due to the ignorance of microscopic forms of undiagnosed endometriosis. The severity of autoantibody abnormalities is not correlated with the clinical symptoms of endometriosis but with the incidence of infertility and the risk of miscarriage (Branch *et al.*, 1986; Gleicher *et al.*, 1989). The search for autoantibodies in patients with endometriosis should be considered a necessary investigation, thereby allowing a functional classification of the disease that takes into account the lack of precision of both descriptive and prognostic classifications. The argument supporting these investigations rests upon the observation that the reduction of autoantibodies improves the fertility of patients with endometriosis (El-Roeiy *et al.*, 1988). If this hypothesis is true, autoantibodies would thus permit ectopic endometrium to escape the immune surveillance by helper and cytotoxic T cells, probably by masking antigenic sites. Immunomodulators may be the next generation of therapeutics for the management of endometriosis and associated infertility. More evidence of activated B cells in endometriosis has recently been found by detection of the high serum concentration of soluble CD23 produced by activated B cells from their membranes. The serum concentration of soluble CD23 decreased significantly on treatment with danazol (Odukoya *et al.*, 1995a).

For the second school of thought, there is an absence of correlation between the severity of the endometriosis and the autoantibody titre, contrary to what is observed in other autoimmune disorders, where the increase of antibody titre is correlated with increasing severity of the disease; this thus implies that the production of autoantibodies is a secondary event (Oosterlynck, 1993).

Besides an inappropriate immune response, an absence of humoral response against specific endometrial antigens (22 and 18 kDa) has recently been demonstrated in the peritoneal cavity of women with endometriosis (Berkova *et al.*, 1996). Patients with endometriosis exhibit the same peritoneal immunological profile as that described in women with unexplained infertility (Gleicher, 1994). The significance of this defect is unknown; however, it is tempting to speculate that subjects without visible endometriosis who have lower antibody titres and infertility could represent a precursor stage of endometriosis.

Endometriosis: disease of the endometrium

Several features support the view of abnormality of the endometrium in endometriotic patients (Kikuchi *et al.*, 1993). Ectopic endometrium differs from its native intrauterine partner by its response to steroids, by the presence of steroid-specific receptors (Lessey *et al.*, 1989) and by the expression of epidermal growth factor (EGF) and of its receptors (Megela *et al.*, 1991).

Anti-endometrial antibodies are specifically directed against one or several endometrial antigens (Wild *et al.*, 1991). Western blot analysis, which allows a separation of the various endometrial antigens according to their molecular weight before incubating them with the sera of patients containing marked antibodies, has permitted the identification of two specific antigens in endometriosis which are expressed by endometriotic lesions and by the endometrium of endometriotic patients (Garza *et al.*, 1991).

Using Western blot, Rajkumar *et al.* (1992) have demonstrated the presence of circulating autoantibodies binding to 52 and 58 kDa endometrial antigens in all women, while in women suffering from endometriosis, they have demonstrated an antibody binding to a 34 kDa antigen.

There is no correspondence between the concentration of antibodies and the severity of the illness as defined by the American Fertility Society (AFS; Garza *et al.*, 1991). Endometriotic patients could be genetically determined either to significantly express these antigens or to recognize them and to develop an immunological response (Frey, 1957; Ranney, 1971; Malinak *et al.*, 1980; Lamb *et al.*, 1986). However, endometriotic patients do not possess a particular human leukocyte antigen (HLA) profile (Simpson *et al.*, 1984).

Several autoimmune diseases have an exaggerated HLA expression in the tissues involved (Grave's disease, diabetes, Hashimoto's disease). In line with these diseases, ectopic and eutopic endometria of patients with endometriosis express HLA-DR antigens excessively (Ota *et al.*, 1993).

Endometrial cells of patients with endometriosis seem to be abnormally recognized by the immune system. Indeed, normal endometrium possess so-called 'self' antigens which should not be recognized by the autologous immune system. However, in-vivo autologous lymphocytes, when in contact with endometrial cells, proliferate significantly even in the absence of endometriosis. Endometrial cells stimulate the proliferation of lymphocytes; this is probably mediated by cytokines. Endometrial cytokines are produced either after hormonal or immunological stimulation. Interleukin 6 (IL-6), which is produced by the normal endometrium, could be one of the lymphocyte stimulators (Buyalos *et al.*, 1992; Tabibzadeh and Sun, 1992). In contrast, in patients with endometriosis, autologous endometrial cells are incapable of initiating such a lymphocytic proliferation (Steele *et al.*, 1984). The endometrium could be constitutionally incapable of secreting cytokines, or of obtaining the necessary stimulation, or it could produce inhibitors of proliferation (Wang *et al.*, 1987). Immunosuppressive factors released by endometriotic lesions have been identified (Hirata *et al.*, 1994).

Several studies are in favour of a certain independence of the endometrium, once outside the uterine cavity. The endometrium is capable of synthesizing complement factor C3. In the castrated mouse, this secretion is oestrogen dependent for the intrauterine endometrium whilst ectopic endometriotic fragments do not require oestradiol. Endometriotic tissue contributes to the establishment of a favourable environment by locally secreting complement factors which are chemotactic for lymphocytes and macrophages (Isaacson *et al.*, 1991).

Eutopic and ectopic endometria of endometriotic patients secrete chemotactic substances when stimulated by certain cytokines [IL-1 and tumour necrosis factor α (TNFα)]. Very chemotactic substances for macrophages include monocyte chemotactic protein-1. Stimulated under identical conditions, the endometrium of normal patients does not secrete such substances (Akoum *et al.*, 1995). Chemotactic substances for macrophages are produced in a cyclic fashion by the endometrial stroma, with a peak during the luteal phase (Leiva *et al.*, 1991). Steroid hormones contribute to this regulation (Lee *et al.*, 1990). The endometrium of women suffering from endometriosis secretes larger amounts of these chemotactic substances, with a loss of the cyclical pattern (Leiva *et al.*, 1994). This hormone dependency reflects an intrinsic modification of the endometrium in women with endometriosis.

The endometrium secretes numerous cytokines: γ-interferon (IFNγ), IL-1 and IL-6, and their specific receptors are present (Tabibzadeh, 1991; Tabibzadeh *et al.*, 1995a,b). Certain locally produced cytokines, such as IFNγ, inhibit the proliferation of the endometrium (Van Le *et al.*, 1992). However, the proliferation observed in endometriosis is less than that observed in the normal endometrium (Klein *et al.*, 1994). Endometriotic tissues differ from the normal endometrium by the presence of lymphocytes. Scattered lymphocytes are more numerous in endometriosis and cells containing IFNγ are more numerous. The presence of IFNγ receptors in endometriotic lesions may imply their possible role in the regulation of the proliferation of endometriotic lesions (Klein *et al.*, 1994).

Studies on rats have demonstrated the presence of a specific protein, Endo 2, on implanted peritoneal endometrial tissue. The immune system influences the production of this protein. Pentoxifylline, an immunosuppressive agent, induces a significant reduction of the size of peritoneal implants and a significant reduction of Endo 2 protein in rats (Nothnick *et al.*, 1994).

The susceptibility of endometrial cells to lysis by NK cells is closely connected with the expression of HLA class I molecules. The number of major histocompatibility complex (MHC) class I molecules has been reported to be higher in ectopic than eutopic cells. Down-regulation of MHC class I molecules enhances susceptibility to lysis. Both local cytokine-mediated (IFNγ up-regulates the expression of MHC class I molecules) and hormone-controlled mechanisms can be postulated to regulate the expression of these membrane molecules (Semino *et al.*, 1995).

Cultured human endometrial cells are constitutively able to release secreted intercellular adhesion molecule-1 (sICAM-1). This molecule is a member of the immunoglobulin (Ig) supergene family and functions as a ligand for lymphocyte function-associated antigen-1 (LFA-1), which is a member of the leukocyte integrin family. The NK cell-mediated immunological recognition of endometrial targets, which when defective has been claimed to be the crucial stimulus for initiation and progression of endometriosis (see below), is LFA-1/ICAM pathway dependent (Vigano *et al.*, 1994a). Shedding of ICAM-1 has been demonstrated to be one of the mechanisms by which neoplasic cells escape immunosurveillance. sICAM could be one of several endometrial factors endowed with the ability to influence the immune system, since endometrial stromal shedding is greater in patients with advanced stages of endometriosis than in women without the disease (Somigliana *et al.*, 1996).

Endometriosis: disease of the macrophage

Macrophages constitute 85% of the cells in peritoneal fluid; the remaining 15% are shed cells and lymphocytes. The peritoneal fluid is thus equipped to defend itself against aggression. The number of macrophages varies during the menstrual cycle, with a peak occurring in the postmenstrual period. Macrophages are essential to clear the peritoneal cavity of endometrial debris, old spermatozoa and follicular cells. In the event of bilateral tubal obstruction, the postmenstrual increase of macrophages does not occur (Halme *et al.*, 1983b). Macrophages are equipped with several systems to intervene: release of cytokines, phagocytosis and cytotoxicity. The means used depends on the level of activation of the peritoneal macrophages. Peritoneal macrophages have an enzymatic activity greater than that of circulating monocytes. The level of activation varies during the menstrual cycle. Acid phosphatase activity, which is weak at the beginning of the cycle, increases during the luteal phase.

In women with endometriosis, macrophages are more numerous (Haney *et al.*, 1981) and at a higher level of activation (Muscato *et al.*, 1982; Chacho *et al.*, 1986; Dunselman *et al.*, 1988). Several biological markers reflect this hyperactivation (Halme *et al.*, 1984, 1985): (i) larger size of the macrophages (Halme *et al.*, 1984); (ii) release of a substantial amount of complement factors C3 and C4; (iii) an increase of the secretion of lysosomal phospholipase capable of acting on membranous phospholipids, which are the source of arachidonic acid used to synthesize prostaglandins. This could explain the raised concentration of peritoneal prostaglandins in women with endometriosis; (iv) a more significant expression of lysosomal and membranous enzymes (acid phosphatase, acid hydrolase, proteases; Olive *et al.*, 1985) and oxygen metabolites (H_2O_2; Zeller *et al.*, 1987). The production of oxygen derivatives is determined by examining chemoluminescence of macrophages, which is increased in women with endometriosis (Zeller *et al.*, 1987). Circulating monocytes are also at a certain degree of activation, since their stimulation by phorbol myristate acetate gives rise to more chemoluminescence than in controls (Zeller *et al.*, 1987); (v) a membranous expression of a larger number of specific markers of activation (CD14 and HLA DQ; Becker *et al.*, 1995); (vi) a modification of the membrane cytoskeleton. In the presence of antibodies directed against surface antigens (in this case HLA class I and II antigens), immunofluorescence studies have demonstrated the migration of antigen–antibody complexes towards a pole of the cell before their internalization. This phenomenon, termed capping, is present on peritoneal macrophages of endometriotic patients, whilst it is not found on normal macrophages.

Several factors can activate peritoneal macrophages: menstrual reflux, follicular content, sperm antigens, pelvic infections, endometriotic lesions themselves and some chemical substances. The peritoneal fluids of endometriotic women contain cytokines released by T cells. One of them, RANTES, a newly discovered 8 kDa T cell-specific cytokine of the platelet factor 4 gene superfamily, is capable of activating macrophages (Schall, 1991). Its concentration is related to the severity of endometriosis (Khorram *et al.*, 1993).

In endometriosis, peritoneal macrophages are thus more numerous and more aggressive; they contribute in many ways to the initiation, the development and the growth of endometriosis. Peritoneal macrophages are cytotoxic towards endometrial cells in endometriosis, but comparison shows that this cytotoxicity can be reduced in cases of AFS stages III and IV endometriosis. Prostaglandins could impede this cytotoxicity since indomethacin allows stage III and IV macrophages to recover a cytotoxicity comparable to that observed in stages I and II. Treatment with danazol or gonadotrophin-releasing hormone agonist increases this cytotoxicity (Braun *et al.*, 1992).

Macrophages could be involved directly in the adherence of endometrial cells on the peritoneum, by producing more fibronectin (Kauma *et al.*, 1988). Fibronectin is involved in the adhesion of endometrial cells, but more specifically acts as a competent factor, permitting the conversion of endometrial cells from stage G_0 to stage G_1 of the cell cycle, thus rendering them sensitive to growth factors termed progression factors. Oestradiol could be one of

these progression factors. An increased activation of peritoneal macrophages combined with a relative hyperoestrogenaemia could permit a proliferation of retrograde endometrial cells. Local hyperproduction of fibronectin could play a role in the genesis of pelvic adherences (Kauma *et al.*, 1988).

Activated macrophages secrete TNFα (Eiserman *et al.*, 1988; Halme, 1989; Rana *et al*., 1996). TNFα is involved in the genesis of adherence since it leads to fibroblast proliferation and the precipitation of collagen. Generalized symptoms of malaise, body aches and pains reported by patients with endometriosis may be due to systemic effects secondary to an increase in the synthesis of TNFα (Beutler and Cerami, 1986). Vercellini *et al.* (1993), who have not found a plasmatic or peritoneal excess of TNFα, do not reject the hypothesis implicating cytokines, since a membranous form of TNFα has been reported that allows macrophages to concentrate locally (Kriegler *et al.*, 1988).

Some cytokines released by activated macrophages increase the proliferation of endometrial cells (Halme *et al.*, 1988; Koutsilieris *et al.*, 1991; Olive, 1991; Hammond *et al.*, 1993). The concentration of these factors seems to be correlated with the severity of the disease (Zhang *et al.*, 1991). Crossed proliferative reactions, combining the macrophages of healthy patients with those of endometriotic patients in the presence of healthy and endometriotic endometrium, seem to show that this increase in growth requires the presence of a simultaneous combination of activation of macrophages and modification of the endometrium (Braun *et al.*, 1994a). To be sensitive to these growth factors, endometrial cells must express certain cell receptors whose induction depends partially on an external network of cytokines.

IL-1, which is well known for its involvement in the immune response, seems to be implicated in endometriosis (Fahih *et al.*, 1987). The precise influence of IL-1 on the ectopic and eutopic endometrium is as yet unknown, but it seems that IL-1 is capable of modulating stromal endometrial cell growth (Van Le *et al.*, 1992). The effects of IL-1 are modulated by another cytokine which blocks IL-1 cellular receptor: IL-1 receptor antagonist (IL-1ra). Two features emphasize the importance of these two cytokines in endometriosis. IL-1 is found in higher concentrations in the peritoneal fluid of endometriotic patients (Takenani *et al.*, 1992). The secretion of peritoneal macrophages from endometriotic patients seems to be preferentially oriented towards the synthesis of IL-1ra with the worsening of endometriosis (Mori *et al.*, 1992). Immunofluorescence studies demonstrate a different localization of IL-1ra in the eutopic endometrium and in the endometriotic endometrium, thus leading the ectopic endometrium possibly to escape the regulatory influence of IL-1 (Sahakian *et al.*, 1993).

IL-1 and TNFα are capable of modulating the secretion of IL-6 by peritoneal macrophages and stromal endometrial cells (Semer *et al.*, 1991; Rier *et al.*, 1994). The IL-6 concentration is raised in the peritoneal fluid of endometriotic patients (Keenan *et al.*, 1994; Ueki *et al.*, 1994). The significance of the presence of IL-6 is debated as it may stimulate the growth of haematopoietic cells (Kawano *et al.*, 1988), but it appears that it inhibits the growth of stromal and glandular endometrial cells (Tabibzadeh *et al.*, 1989). During in-vitro culture, stromal cells demonstrated the presence of IL-6 receptor. If this inhibiting action is sustained, the increase of IL-6 concentration could be a protective mechanism against the development of endometriosis (Zarmakoupis *et al.*, 1995). Moreover, a progressive rise in expression of IL-6 has recently been demonstrated during the secretory/menstrual phases, suggesting that this cytokine may prepare endometrium for implantation and menstrual shedding (Tabibzadeh *et al.*, 1995a). The experimental induction of endometriosis in rats is accompanied by an increase of peritoneal IL-6 only in the phase of onset of the lesions, and it subsequently disappears (Lim and Schenken, 1993). Recent studies have demonstrated that IL-6 does not have the same effect on the ectopic endometrium as on the intrauterine endometrium. IL-6 could be incapable of inhibiting the proliferation of endometriotic lesions due to a defect in the expression of its specific receptor (IL-6r) (Rier *et al.*, 1995).

Among other cytokines produced by peritoneal macrophages, one must remember transforming growth factor β (TGFβ), whose concentration is raised in peritoneal fluid of women with endometriosis (Oosterlynck *et al.*, 1994a). This cytokine is involved in tissue repair by stimulating the production of extracellular matrix, and by decreasing the secretion of proteases (Williams *et al.*, 1992). When repair is complete, an as yet unknown mechanism stops the production of TGFβ (Border and Ruoslahti, 1992). An excess of TGFβ leads to defective scars and adherences. TGFβ stimulates the proliferation of stromal cells of the endometrium (Hammond *et al.*, 1993).

TGFβ stimulates tumoral angiogenesis. Endometriosis can only develop and proliferate if numerous vessels are formed. Peritoneal fluid of endometriotic patients stimulates angiogenesis on chorioallantoic membranes of chicken, and this could be explained by the peritoneal concentration of TGFα and TGFβ in the peritoneal fluid of these patients (Oosterlynck *et al.*, 1993). Morphological studies of the stromal vascularization of peritoneal lesions have been able to demonstrate that the more active the mitotic activity, the more abundant will be the vessels

(Nisolle *et al.*, 1993). Induction of angiogenesis by these cytokines (TGFβ and TNFα) requires the local expression of certain integrins. Anti-integrin antibodies are capable of blocking angiogenesis. Thus, a potentially therapeutic line of research is opened when one knows the importance of vascularization in endometriotic lesions (Brooks *et al.*, 1994).

Peritoneal macrophages from women with endometriosis show an enhanced ability for IL-8 synthesis. Furthermore, peritoneal fluid concentrations of IL-8 in women with endometriosis correlate with IL-8 production by peritoneal macrophages (Rana *et al.*, 1996). IL-8 is an inducible chemokine synthesized by macrophages in response to TNFα and other pro-inflammatory stimuli (Ryan *et al.*, 1995). The greater concentrations of TNFα and IL-8 observed in women with endometriosis may contribute towards the development of clinical symptoms, especially those related to the gastrointestinal tract. IL-8 has been directly implicated in angiogenic disease. TNF-α- and IL-1-induced angiogenesis may be mediated by induction of endogenous IL-8 (Koch *et al.*, 1992).

Endometriosis: disease of natural killer cells

Natural killer (NK) cells are lymphocytic cells which are one of the first-line defence mechanisms against tumours. NK cells are directly cytotoxic and do not obey the laws of specific immunity. Their aggressiveness is modulated by several cytokines. Circulating NK cells efficiently destroy endometrial cells (Oosterlynck *et al.*, 1991). In patients with endometriosis, the cytotoxic power of circulating NK cells against autologous and heterologous endometrium is diminished (Vigano *et al.*, 1991; Kanzaki *et al.*, 1992; Garzetti *et al.*, 1993; Iwasaki *et al.*, 1993). The sera of these patients contain an inhibiting factor against NK cells (Tanaka *et al.*, 1992). In endometriosis, the endometrium becomes resistant to the cytotoxicity of NK cells, as it is less efficiently destroyed by NK cells obtained from men, compared to endometrium of women without endometriosis (Oosterlynck *et al.*, 1991).

Some authors (Wilson *et al.*, 1994; D'Hooghe *et al.*, 1995), who only found such a reduction in AFS stage III and IV endometrium, reject the notion of this defect as the trigger of the disease, but these same authors did not measure the cytotoxic power present in the peritoneal fluid. In endometriosis the cytotoxic activity of NK cells in the peritoneal fluid is even more inhibited (Oosterlynck *et al.*, 1992; Ho *et al.*, 1995). The reduction of peritoneal NK cytotoxicity is maximal in the follicular phase, during which period retrograde endometrial cells must be destroyed. The reduction is proportional to the severity of the illness according to the AFS classification (Oosterlynck *et al.*, 1992). This reduction seems to be due to a functional alteration of NK cells without a reduction of their number (Opsahl *et al.*, 1994; Ho *et al.*, 1995).

The peritoneal cell environment is altered in endometriosis, with an increase of monocytes. The normally inversed CD4/CD8 ratio is even more pronounced in endometriosis (Oosterlynck *et al.*, 1994b). The peritoneal fluid obtained from endometriotic women can inhibit the cytotoxicity of NK cells obtained from normal women (Hirata *et al.*, 1994). A cytotoxin secreted by macrophages, TGFβ, seems to be the main cause of this inhibition, as pre-treatment of the peritoneal fluid with an anti-TGFβ antibody decreases these inhibiting properties. The peritoneal concentration of TGFβ is raised in women with endometriosis (Mori *et al.*, 1992). Treatment of circulating lymphocytes with IL-2 allows them to recover a cytotoxic activity towards the autologous endometrium similar to that found in controls (Melioli *et al.*, 1993). Investigating circulating T and NK cells according to their degree of differentiation, Kikuchi *et al.* (1993) have shown that there is a reduction of highly differentiated NK cells, which are the most cytotoxic, in endometriosis. For these authors, these abnormalities of NK cells are secondary to endometriosis itself. Indeed, surgical treatment corrects these abnormalities, with a reduction of immature NK cells (CD57+ CD16–) and an increase of moderately (CD57+ CD16+) and of highly differentiated (CD57– CD16+) NK cells. However, a functional evaluation of the cytotoxicity of NK cells after laser resection of the lesions is more in favour of a primitive anomaly of NK cells, since this treatment does not improve their performance (Oosterlynck *et al.*, 1994c).

Steroid hormones could play a role in the alteration of NK function. The reduction of NK activity could be correlated with serum concentration of oestradiol, a hormone whose immunodepressive properties are well known, particularly on NK activity (Garzetti *et al.*, 1993). Since this correlation is only observed in advanced stages of endometriosis (stages III and IV), it would appear that oestradiol is only involved in the evolution of the disease and not in its onset (Di Stephano *et al.*, 1994).

Stress, pain and menstrual reflux lead to an increase of production of β-endorphin by monocytes. β-Endorphin modulates the immune response (Blalock, 1989) by stimulating the action of NK cells (Mathews *et al.*, 1983). The defect of NK activity encountered in endometriosis could be due to a defective secretion under certain circumstances. In women suffering from endometriosis, a decrease of NK activity could be correlated with a decrease of β-endorphin

production by peripheral monocytes (Vercellini *et al.*, 1992).

B and NK cells mutually interact with each other (Yuan *et al.*, 1994). Autoimmune abnormalities have been linked with defective NK cytotoxicity (Takeda *et al.*, 1993). In the mouse it has been demonstrated that NK cells prevent the occurrence of autoimmune diseases and that conversely their removal rapidly induces autoimmune diseases (Jonjic *et al.*, 1992). Autoimmune abnormalities observed in endometriosis and certain forms of sterility could be secondary to a defect of NK cells.

Finally, studies performing xenotransplantation of human endometrial tissue into immunodeficient mice demonstrated the in-vivo importance of NK cells for the growth of endometrial tissue on ectopic sites. Indeed, nude mice, who have a congenital defect of T and B cells, temporarily accept human endometrial grafts. The same nude mice, when treated with NK anticellular serum prior to xenotransplantation, permanently accept endometrial grafts. Scid mice deficient in T, B and NK cells do not reject endometrial grafts (Aoki *et al.*, 1994).

Endometriosis: disease of the peritoneum

Among the numerous theories advanced, the presence of an abnormal peritoneum in patients with endometriosis that is unusually sensitive to refluxed cells cannot be excluded. It could be an abnormal response of adhesive proteins expressed under particular stimulation, such as wound healing (Jonjic *et al.*, 1992). In endometriotic patients, peritoneal cells, even without external stimulation, express several adhesive proteins, such as ICAM-1 and vascular cellular adhesive molecule-1 (VCAM-1). These adhesive proteins could interfere at two levels: (i) by combining with leukocytic integrins, they could participate in the activation of immune cells; (ii) by combining with integrins expressed by refluxing endometrial cells, they would allow the fixation of the cells and the onset of their growth.

Certain cytokines released by activated macrophages, particularly TNFα, can initiate the adherence of stromal endometrial cells by inducing the expression of adhesive proteins on the peritoneum (Zhang *et al.*, 1993). Several studies are under progress to test the hypothesis that, in endometriosis, oestradiol could increase the expression of peritoneal adhesive molecules induced by TNFα, as is observed on the endothelial cells (Cid *et al.*, 1994). Peritoneal macrophages of endometriotic patients contribute to these adhesive phenomena by releasing fibronectin (Kauma *et al.*, 1988). Cell adhesion mediated by the integrin family of cell surface receptors elicits intracellular signals upon fibronectin adhesion. These signals protect cells from apoptosis through overexpression of the Bcl-2 protein (Zhang *et al.*, 1995).

Refluxing endometrial cells during menstruation express several adhesive proteins (E- and P-cadherin), whose role in the onset of endometriosis is retained (Van der Linden *et al.*, 1994). The endometrium of endometriotic women does not express αvβ3-integrin, which normally appears at the beginning of the implantation window around the 20th day of the cycle (Lessey *et al.*, 1994). More than just an element responsible for the onset of endometriosis, abnormalities of integrin expression would more likely be markers of the poor uterine receptivity of women suffering from sterility (Lessey *et al.*, 1995).

The regulating trigger of the expression of β3-integrin is as yet unknown, but two substances have been suggested: TGFβ and IL-1ra. These two factors are produced by implanted endometrium and activated macrophages, which are abundantly present in the peritoneal fluid of endometriotic women (Ignotz *et al.*, 1989).

The peritoneum of endometriotic women could synthesize and release a large amount of IL-8 (Betjes *et al.*, 1993), which attracts and activates macrophages and neutrophils and would have efficient angiogenic properties. IL-8 is synthesized excessively by the peritoneum of patients suffering from endometriosis, probably under the stimulating action of IL-1 and TNFα (Arici *et al.*, 1994).

Rat peritoneum irritated by endometrium produces as yet unidentified factors, resulting in suppression of NK activity which may lead to progression of the endometriotic lesions (Mizumoto *et al.*, 1996).

Conclusion

The immune system probably plays a role in the onset and development of endometriosis. A general picture can be proposed. In some women refluxing endometrial cells are not destroyed, because the patient is genetically programmed not to respond to endometrial antigens, because these endometrial antigens are abnormal or because the reflux is so abundant that the scavenging capacity of the peritoneal immune cells is overloaded. Refluxing cells could be protected due to an abnormal adherence to the mesothelium, which exceptionally expresses certain adhesive molecules. Undestroyed, these endometrial cells would cause a reflex inflammation with activation of macrophages. Not only does the peritoneum protect these endometrial cells, but it also produces abnormal quantities of chemotactic and angiogenic cytokines (IL-8). Macrophages facilitate development via growth factors, angiogenic factors and immunodepressant factors such as TGFβ.

Immunosuppressive factors block the cytotoxic activity of NK cells. Activated macrophages expose antigens of endometrial cells to T cells which will co-operate with B cells to synthesize autoantibodies. Synthesized antibodies protect the ectopic endometrium and could worsen the dysfunction of local NK cells.

A vicious circle is set up involving all the partners of the immune system. It is as yet impossible to pinpoint the triggering mechanism. The primary defect could be localized on the endometrium, on macrophages already activated by an extrinsic factor (infection, spermatozoa, chemical substances), or on the uterus or the tubo-uterine junction. The two pathophysiological theories put forward to explain endometriosis are linked by a defective immune system. Indeed, once the vicious circle is set up, growth and angiogenic factors could induce metaplasia of the already irritated mesothelium.

Investigation of the immune system sheds light on the clinical consequences of endometriosis: subfertility and pain. Adhesions, consequent to the inflammatory response, provide an explanation for pelvic pain worsened by the local hypersecretion of prostaglandins. The disequilibrium of cytokines involved in wound healing (TGFβ, IL-1) could lead to the excessive fibrosis observed in adhesions. Adhesions directly hinder reproduction due to the mechanical lesions they initiate in the pelvis.

More subtle mechanisms are capable of causing damage at the initial stages of reproduction. Cytokines produced by activated macrophages are noxious to the transport (Eiserman *et al.*, 1988) and fertilization of gametes (Chacho *et al.*, 1986), and to the migration and development of the embryo (Fahih *et al.*, 1987; Hill *et al.*, 1987a; Tabibzadeh *et al.*, 1995a). Several cytokines implicated during endometriosis (IL-1, IL-6 and TNFα) are also involved in the implantation process (Chard, 1995). These cytokines form a finely tuned network which is altered during the cycle. Any dysregulation of this network, either overexpression or expression during the wrong time, can interfere with implantation (Tabibzadeh *et al.*, 1995b). In the hypothetical model of implantation proposed by Simon *et al.* (1995, 1996), the IL-1 system is one of the most influential mediators of crosstalk between the embryo and endometrium. The binding of IL-1 to specific receptor IL-1R is a very necessary step in implantation. During endometriosis, a disequilibrium between the cytokines of this system is in favour of IL-1ra, which seems to be produced in excess by peritoneal macrophages. The IL-1ra protein seems to interfere with embryonic attachment (Simon *et al.*, 1994). Several steps of implantation could be affected by anomalies in the IL-1/IL-1R system: (i) IL-1 is a potent inducer of leukaemia inhibitory factor (LIF) expression in endometrial stroma cells (Arici *et al.*, 1995). LIF is a cytokine involved in implantation;. (ii) IL-1/IL-R interaction controls the expression of several integrins whose endometrial expression seems necessary during the implantation window (Lessey *et al.*, 1994, 1995); (iii) proteases involved in trophoblast invasion are closely interrelated with adhesion molecules and are regulated by hormones, growth factors and cytokines, including IL-1 (Simon *et al.*, 1996).

The possibility in animals of manipulations of the immune system [blocking of the activation of macrophages by pentoxifylline (Steinletter *et al.*, 1991a) and of the activation of lymphocytes by calcium blockers (Steinletter *et al.*, 1991b)] is a significant argument in favour of the role of the inflammatory environment encountered in endometriosis. A recent study on the results of in-vitro fertilization in endometriotic patients has shown that the outcome did not depend on the stage and activity of the disease but mainly on the presence of autoantibodies which affect the rate of implantation (Dmowski *et al.*, 1995).

Besides a better understanding of mechanisms involved, these studies could allow the identification of women suffering from subfertility associated with certain abnormalities of the immune system and may prevent the evolution of the disease towards macroscopic forms. Certain therapies could correct these defects of the immune system. In-vitro exposure of lymphocytes to IL-2 normalizes NK function (Melioli *et al.*, 1993). Anti-integrin antibodies, whose role is well known in angiogenesis, halt the progression of induced endometriotic lesions. The efficacy of surgical resection of the lesions will be strengthened by the correction of immunological abnormalities.

Some drugs have revealed themselves to have powerful immunoregulatory functions at therapeutic doses. Danazol inhibits the proliferation of lymphocytes induced by mitogens (Hill *et al.*, 1987b), reduces the production of autoantibodies (Ota *et al.*, 1992), normalizes the plasma concentration of cytokines such as IL-6, IL-2 and the soluble receptor to IL-2 (SIL-2R) (Koumantakis *et al.*, 1994; Odukoya *et al.*, 1995a) and blocks the proliferation of endometrial cells induced by activated macrophages (Braun *et al.*, 1994b). Another molecule which merits further investigation is gestrinone, which blocks the activation of macrophages (Vigano *et al.*, 1994b).

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