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NEW DEBATE

Natural killer cells and reproductive failure—theory, practice and prejudice

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The relationship between peripheral blood natural killer (NK) cells and reproductive failure is one of the most controversial areas in reproductive medicine. Amidst much publicity, peripheral blood NK cell testing is being promoted as a useful diagnostic test to guide the initiation of a variety of immunosuppressive therapies amongst patients with either recurrent miscarriage or infertility. We contend (i) that at present there is no scientific basis for the introduction of NK cell testing into routine clinical practice, and (ii) that the use of immunosuppressant agents based on the results of such testing may potentially be harmful.

Key words: NK cells/peripheral blood/pregnancy/TNF-α

Introduction

The investigation and treatment of couples with reproductive failure is characterized by historical beliefs, anecdotal evidence and the results of small uncontrolled studies. This has lead to the situation where women have been subjected, often at significant financial cost, to treatments such as lymphocyte immunization therapy (LIT), the efficacy of which are at best controversial despite initially promising reports which have not been substantiated in more recent studies (Mowbray *et al.*, 1987; Rai *et al.*, 1996; Scott, 2003).

The arguments supporting an aberrant immune response as being implicated in many cases of reproductive failure have been extensively rehearsed (Coulam *et al.*, 1999; Gleicher, 2002). However, the evidence in favour of these various putative immune or autoimmune causes is scant (Regan *et al.*, 2004). Natural killer (NK) cells are the latest potential immune cause of reproductive failure to enter the clinical arena.

NK cell biology

The name 'natural killer' is evocative. It derives from an in-vitro assay used to detect these cells. Importantly, however, NK cells only kill trophoblast *in vitro* if activated by interleukin (IL)-2, which is not present in the endometrium at the time of implantation (King *et al.*, 1995; Abadia-Molina *et al.*, 1996).

NK cells are lymphocytes that are part of the innate immune system. They express the cell surface antigens CD16 and CD56. CD16 is a low-affinity receptor for IgG complexes and is expressed on the majority of NK cells. It is the receptor responsible for NK-mediated, antibody-dependent cellular cytotoxicity. Based on the intensity of CD56 expression, NK cells may be divided into two populations: $CD56^{dim}$ and $CD56^{bright}$. NK cells that are $CD56^{dim}$ are cytotoxic *in vitro*. In contrast, those that are $CD56^{bright}$ have little cytotoxic ability, but produce immunoregulatory cytokines such as interferon-γ and tumour necrosis factor-α (TNF-α).

NK cell function is tightly regulated by a network of specific activating and inhibitory receptors. In particular, CD69 is one of the earliest cell surface activation markers expressed by NK cells. NK cells also express a variety of killer inhibitory receptors (KIRs) and killing activating receptors (KARs), which recognize HLA-G expressed on extravillous trophoblast (Moffett-King, 2002).

NK cells are found in both peripheral blood and the uterine mucosa. There are, however, important phenotypic and functional differences between NK cells present at the two sites.

The majority (90%) of peripheral blood NK cells are $CD56^{dim}$ and express high levels of CD16; these levels do not fluctuate during the menstrual cycle, and during pregnancy peripheral NK cell numbers and functional activity are suppressed (Sacks *et al.*, 1999; Yovel *et al.*, 2001). In contrast, NK cells are the predominant leucocyte population in the endometrium, particularly in the *decidua basalis* at the implantation site. The number of uterine NK (uNK) cells varies during the menstrual cycle. They are sparse during the proliferative phase, increase significantly throughout the secretory phase, remain in high numbers during early gestation, decrease after 20 weeks gestation and are absent in term decidua (Bulmer and Sunderland, 1984; Bulmer *et al.*, 1991; Trundley and Moffett, 2004). Microarray analysis

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combined with flow-cytometric and RT–PCR studies have demonstrated that the phenotype of uNK cells ($CD56^{bright}$ $CD16^{-}$) is different from that of NK cells in peripheral blood ($CD56^{dim}$ $CD16^{+}$) (Koopman *et al.*, 2003). In addition, Koopman *et al.* were able to demonstrate that uNK cells have an immunoregulatory potential that peripheral blood NK cells do not demonstrate. This suggests that uNK cells either represent a distinct subpopulation of circulating NK cells or that they have undergone some tissue-specific differentiation. Consequently, data derived from studies of peripheral blood NK cells may not reflect what is happening at the feto-maternal interface.

Reproductive role for NK cells

The function of NK cells in governing reproductive outcome has not been demonstrated. Studies of transgenic mice deficient in NK cells demonstrate that uNK cells play a role in uterine spiral artery modification in the placenta (Croy *et al.*, 2003). Several investigators have used murine NK knock-out models to study the effect of NK cells on pregnancy outcome. However, different knock outs have reported differing results. Using Terhost mutants, Croy's group have reported that NK deficient mice have an increased rate of fetal loss (Guimond *et al.*, 1998). In contrast, the fetal resorption (miscarriage) rate is not increased in IL-$15^{-/-}$, NK cell-deficient mice, nor amongst IL-2 receptor knock-outs, which also lack uNK cells (Miyazaki *et al.*, 2002; Barber and Pollard, 2003). These differing results may be explained, at least in part, by the subset of NK cells that is affected by the knock-out.

In human pregnancy, the temporal and spatial distribution of uNK cells suggest they play a role in controlling trophoblast invasion. uNK cell numbers are increased at the time of implantation; they lie in close proximity to the extravillous trophoblast and they express receptors that interact with ligands expressed on trophoblast (Moffett-King, 2002).

NK cells and reproductive outcome

The relationship between peripheral blood NK cells and pregnancy outcome after either IVF or spontaneous conception has been examined in several small observational studies (Aoki *et al.*, 1995; Beer *et al.*, 1996; Fukui *et al.*, 1999; Emmer *et al.*, 2000; Ntrivalas *et al.*, 2001; Yamada *et al.*, 2003; Putowski *et al.*, 2004). Although the underlying aetiology of reproductive failure amongst women in individual studies may well be different, the reports are consistent in associating enhanced NK cell activity with subsequent failure to conceive or miscarriage. These studies have received extensive attention in all media channels. This has led to many patients demanding the performance of these tests and 'treatment' for 'abnormal' results. Is peripheral NK cell testing an important advance or, as we argue, poor science enthusiastically endorsed by its proponents?

There are fundamental flaws in the methodologies used in the published studies. The levels and activation of NK cells is dependent, amongst other variables, on whether whole blood or fractionated mononuclear cells is used, the time of day a sample is taken, whether any physical exercise has been performed, the parity of the patient and whether the samples have been previously frozen (Strong *et al.*, 1982; Porzsolt *et al.*, 1983; Pross and Maroun, 1984; Reichert *et al.*, 1991; Plackett *et al.*, 2004). Furthermore, it is unclear what an abnormal NK cell number is. Whilst traditionally a peripheral NK cell level $>12\%$ of all lymphocytes has been regarded as the cut-off between a raised and a normal level (Beer *et al.*, 1996), this figure is well within the normal range (up to 29%) published by others (Eidukaite *et al.*, 2004). Hence individuals with entirely normal results are being labelled as having raised NK cell numbers.

A recent study published in this Journal illustrates several of the important pitfalls in peripheral blood NK cell testing and the interpretation of the results (Thum *et al.*, 2004). The authors have reported that an increase in the absolute count of activated NK cells ($CD56^{dim}$ $CD16^{+}$ $CD69^{+}$) is associated with a reduced implantation rate amongst women undergoing IVF. By inference it is suggested that (i) this is a useful test for women undergoing IVF, and (ii) therapeutic intervention of some sort to decrease peripheral blood NK cell activation will be of benefit to those women with an increased number of activated NK cells. Are these claims justified?

Peripheral blood NK cells are counted using flow-cytometric analysis. The count will be dependent on (i) the pre-analytical variables we have discussed and (ii) the setting of the lymphocyte gate on the flow cytometer, which is arbitrary and will vary from experiment to experiment. In their manuscript, the authors state that the count for the cell population of interest ($CD56^{dim}$ $CD16^{+}$ $CD69^{+}$) is 1.66×10^6 out of an overall count of 212×10^6 for $CD56^{dim}$ $CD16^{+}$ (0.78%). With numbers so small, variations in the count induced by the settings of the fluorescence-activated cell-sorting parameters can be larger than the claimed differences between groups.

Moving beyond this fundamental issue, do the published data support the hypothesis that peripheral blood NK cell assays are useful tests? The area under a receiver–operating characteristic (ROC) graph is a measure of how well a test (levels of peripheral blood NK cells) divides a cohort of patients into those with a disease (implantation failure following IVF) and those without (implantation achieved). An area of 1.0 represents a perfect test, whilst a figure of 0.5 implies a worthless test. At the cut-off value used by the authors themselves, they have calculated an area of 0.63, demonstrating that the test performs poorly.

Treatment of raised peripheral blood NK cells

Apart from the morbidity generated by NK cell testing, it is of concern that women with 'raised' NK cell levels are being treated with a variety of agents—intravenous immunoglobulin, anti-TNF-α drugs and glucocorticoids—in order to dampen an 'excessive immune response'. Not only is there no evidence base for these interventions, which are potentially associated with significant morbidity, the rationale for their use may be false.

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Hiby *et al.* (2004) recently reported a comparison of the genotypes of (i) maternal uNK cell killer inhibitory receptors and (ii) fetal HLA-C amongst women with and without pre-eclampsia. The genotype combination that results in a maximum inhibitory effect on uNK cell inhibitory receptors was found significantly more often amongst those pregnancies complicated by pre-eclampsia. This suggests that overly inhibited uNK cells cause trophoblast cells to prematurely cease the remodelling of the maternal uterine spiral arteries, which in turn predisposes to the development of pre-eclampsia.

As far as the pharmacological interventions are concerned, intravenous immunoglobulin is a pooled blood product and is associated with anaphylactic response, fever, flushing, muscle pains, nausea and headache (Sherer *et al.*, 2001). Anti-TNF-α agents have been reported to be associated with the development of lymphoma, granulomatous diseases such as tuberculosis, systemic lupus erythematosus-like syndromes, demyelinating disease and congestive cardiac failure (Fleischmann *et al.*, 2004). Of equal concern is that the whilst TNF-α has traditionally been viewed as a cytokine involved in triggering immunologically mediated pregnancy loss, it is also involved in anti-apoptotic signalling pathways and has a regulatory role in cell proliferation. Indeed, studies in TNF-α knock-out mice suggest that this cytokine may play an important role in embryo development and the prevention of structural abnormalities (Toder *et al.*, 2003). These abnormalities may not, of course, be unmasked until later infant life. Glucocorticoids themselves during pregnancy are associated with an increased risk of pre-term delivery secondary to rupture of membranes and the development of pre-eclampsia and gestational diabetes (Laskin *et al.*, 1997). Importantly, glucocorticoid receptors are present in the stromal compartment of the endometrium, thus suggesting they play an important role in decidualization. The effect of exogenous glucocorticoid therapy on the endometrial gene expression profile during decidualization has not been examined.

Conclusions

Patient expectations for an answer and, even better, for a cure for their reproductive failure are ever increasing. This leaves them vulnerable to exploitation by those pedalling new tests and treatments that have little scientific validity. We contend that the present evidence does not support peripheral blood NK cell testing amongst those with reproductive failure. Moreover, despite the competitive environment in which assisted conception services are provided, it is incumbent upon us to lead by example and protect our patients from vocal advocates of tests and treatments that are largely based on pseudo-science.

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