LYMPHOHEMATOPOIETIC CYTOKINES AND THE REPRODUCTIVE TRACT: A BASIS FOR COMMUNICATION BETWEEN THE IMMUNE AND REPRODUCTIVE SYSTEMS

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INTRODUCTION

There can no longer be any doubt about two significant facts concerning the murine reproductive system vis-a-vis maternal immunity. One is that an intact maternal immune response is not necessary in order to produce a viable fetus, especially under gnotobiotic conditions. The second fact is that an immune response from the mother can definitely help to prevent fetal loss whether it might otherwise occur. To establish the first point Croy and her colleagues have shown that mice that are doubly homozygous for the scid and beige mutations (which prevent B and T cell gene rearrangements and knock out most NK activity, respectively) nevertheless reproduce normally under germ free conditions (1). In addition, two different groups have shown that mice that are homozygous for defective beta-2 microglobulin genes and thus virtually lack all class I gene expression at the cell surface could nevertheless reproduce in the homozygous state without obvious difficulty (2, 3). It is currently being argued whether these mice are a bit leaky for class I expression, or whether class I antigens are all together unnecessary for successful reproduction. In any case, many views have had to be modified concerning maternal-fetal immune interactions. On the other hand, there is little doubt that maternal immune stimulation can affect placental and fetal size as well as fetal viability. The evidence for this is reviewed below. The overall conclusion is that while one may not need an immune response to reproduce, it certainly helps in some circumstances. This applies to anti-MHC responses, as reported below.

THE ROLE OF NK-LIKE ACTIVITY IN FETAL RESORPTION AND OF MHC IN PREVENTING IT

By now many groups have confirmed our original observations that immunization can prevent CBA × DBA/2 mice from having abnormal levels of fetal resorption if cells containing paternal MHC haplotype in an immunogenic form are injected into the females 7 days prior to conception (4, 5). There is a
simultaneous increase in fetal and placental size (5) which led me to postulate a role for immune system cytokines in enhancing growth in the fetal-placental unit (6). Baines and his colleagues advanced this area of thinking by finding a correlation between the number of resorbing fetuses and the local prevalence of asialo-GM-1 positive cells, which they inferred to be of the natural killer lineage, although further work needs to be done to clarify this issue. In addition, they found that one could prevent spontaneous fetal resorption by treating the mice with anti-asialo-GM-1 antibody (7, 8). Previous observations had shown that one could also prevent fetal resorption in this strain combination by raising the animals in a germ-free facility, and thus an infectious etiology is likely (9). These observations were extended by Kinsky and his colleagues who reported that poly IC, which is a double-stranded RNA and a potent inducer of splenic NK activity, causes increased spontaneous fetal resorption in a number of strain combinations, particularly inbred ones. One can also induce fetal resorption in pregnant mice through adoptively transferring NK-enriched spleen cells of syngeneic animals that have been priorly treated with poly IC. The effect can be eliminated if the induced spleen cells are treated prior to transfer with anti-asialo-GM-1 antibody plus complement. This suggests that an NK-like cells is involved in the fetal resorption induced by this method. Interestingly, outbred animals are more resistant to this effect than inbred pregnancies (10).

The above observations were extended in collaborative experiments done over the last year between my lab and Dr. B. Singh’s lab of the University of Alberta, along with Drs. Chaouat, Kinsky and Kapovic at Hospital Cochin in Paris, and Dr. P. Kourilsky of the Institut Pasteur. In summary, we have determined that poly IC-induced fetal resorption is reversible by standard alloimmunization in a number of different strain combinations. BM mutant cells also work in C57 × C57 matings. These cells differ from the parental type C57B1/6 animal at a single class I or class II gene locus because of mutational change (11). BM6 is a poorly immunogenic class I mutation and gives no protection whereas BM1 is a strongly immunogenic one and is protective (12). BM12, on the other hand, is a strongly immunogenic class II mutation and also protects. These observations have been confirmed by showing that poly IC-induced abortion can also be prevented by immunizing with L cells that have been transfected with H-2D class I genes that show a high level of expression of that protein. Untransfected L cells, on the other hand, are ineffective. We also have initial experimental indications that synthetic peptides, prepared by Dr. B. Singh at the University of Alberta, which represent the immunogenic domains of the MHC class I H-2K act in a similar manner in terms of preventing poly IC-induced fetal resorption, although further experiments involving other non-MHC peptides are required to properly
control for this effect (Chaouat, G., et al, manuscript in preparation). Thus the initial observations that involved immunotherapy with a particular strain combination that shows spontaneous fetal resorption have been expanded to a more generalized model of induced fetal resorption in which maternal immune responsiveness to defined class I and class II MHC antigens can prevent fetal demise. Since the allo-MHC-based immunotherapy works better in inbred pregnancies, the necessity for paternal-strain MHC antigen specificity has been obviated. Indeed, a strong immune response seems to be the only requirement. In support of this argument, Toder et al. recently reported that treating CBA × DBA/2 mice with complete Freund's adjuvant alone in the footpads can prevent CBA × fetal resorption in this train combination (12).

**CYTOKINE COMMUNICATION BETWEEN THE IMMUNE AND REPRODUCTIVE SYSTEMS**

The above discussion leads to the question of how an immune response can be unnecessary for successful reproduction and yet be contributing in the ways just outlines above. The paradox is only apparent. One answer lies in the possibility that there is a commonality of cytokine signalling within the immune and reproductive systems, with cross-talk between the two. Thus the reproductive system may be visualized as having its own set of regulatory cytokines, some of which are also shared by the immune system. Recent evidence from a number of laboratories, including our own, suggests that this is a plausible working hypothesis. The rudiments of this hypothesis are outlined next.

A recent review (13) covers most of what follows here and therefore a brief summary is given here. After we initially observed that immunization can prevent abortion and that such procedures lead to increased placental and fetal growth, my colleagues and I inquired whether lymphohematopoietic cytokines could serve as growth factors for trophoblast and other placental cells. In vitro studies suggested that trophoblast-like cells in the placenta responded to GM-CSF, CSF-1 and IL-3, (i.e., the CSF family of cytokines) by incorporation of tritiated thymidine and by increased phagocytosis (14). Subsequent studies indicated that long-term mixed culture lines derived from the placenta, that are primarily trophoblastic in nature because of marker studies, show a dependency on CSF cytokines, although this varies substantially from cell line to cell line (15). Confirmation and extension of this work was obtained by Chaouat and Armstrong. They discovered that day 7 1/2 murine ectoplacental cone trophoblast is most responsive to GM-CSF (16). Day 12 trophoblast reacts in a less vigorous manner and day 14 trophoblast is not at all reactive. Pollard and his colleagues (17), as well as Regenstreif and Rossant (18), have shown that CSF-1 is produced in response to pregnancy or pseudopregnancy by the apical
ends of the uterine epithelial cells. Furthermore the \textit{c-fms} protooncogene product, which is a receptor for CSF-1, is present at the messenger RNA level on cells of the spongiotrophoblast layer and on giant trophoblast cells in the mouse, and thus is anatomically juxtaposed to the cell layer secreting CSF-1 from the uterus (17, 18). The \textit{c-fms} receptor product has been reported to be coded for by an unusual messenger RNA with a non-coding exon at the 5' end of the messenger RNA, indicating tissue-specific expression for a cytokine receptor in the human placenta (19). There is as yet no evidence in the case of uterine CSF-1 for immune system involvement in its regulation. On the other hand, its levels increase dramatically under the influence of the steroidal hormones of pregnancy (20). Biologically active CSF-1 can be found in decidually-derived metrial gland cells, which are of bone marrow origin and also contain mRNA coding for leukemia inhibition factor (21). This is a most interesting observation, because LIF is a factor that prevents embryonic stem cell differentiation, whereas when it is applied to leukemia cells it leads to redifferentiation. However, it has not been identified at the protein level in the supernatants of purified metrial gland cells, in contrast to CSF-1 (21).

Curiously, female mice that are doubly homozygous for CSF-1 deficiency (OP/OP mice), produce no detectable CSF-1 and nevertheless are capable of reproducing. However, preliminary observations by Pollard and his colleagues indicate that these animals have fluctuating litter sizes compared to heterozygous controls. Their macrophages are present at only the 5% level of normal (Pollard, J., personal communication, 1990).

**PLACENTAL GM-CSF: ITS NATURE AND FUNCTION**

GM-CSF activity is present in decidual supernatants as measured both by a proliferative cell assay in which one inhibits activity with anti-GM-CSF antibody, as well as ELISA assays (Lin Hui, et al, unpublished observations). To summarize a series of experiments, we have shown that decidual GM-CSF is present in higher levels in allogeneic than in syngeneic pregnancy, and that these levels can be reduced by treatment of the animal with a cocktail of anti-CD4 plus anti-CD8 antibodies. These observations suggest an involvement of the T cell system somewhere along the pathway producing the cytokine. Such treatments, in our hands, can affect placental size and phagocytosis, as well as fetal viability, depending on the strain combination (23, 24, 25). In addition, they are even more effective in reducing placental size and phagocytosis in MRL mice that are autoimmune and have elevated placental weights and phagocytic values. In contrast, such treatment does not affect the relatively small placentas from a variety of nude mice, indicating that T cells are the actual targets for these antibodies (26).

Dr. Hideharu Kanzaki, who was a visiting scientist in my laboratory
last year, set out to localize placental GM-CSF probe, kindly provided by Drs. John Elliott and Vern Paetkau of the University of Alberta. Using frozen sections, he determined that GM-CSF mRNA is strongly clustered in cells of the decidua (27). These cells are of three morphological types, small round cells resembling lymphoid cells, cells which are spindle shaped, resembling fibroblasts, and cells which are of obvious endothelial morphology, surrounding blood vessels. Moving closer to the fetus, one sees clusterings of positive cells in areas of the spongiotrophoblast which are also greater than 90% cytokeratin positive. These cells are large, epithelial-like cells and thus appear to be trophoblasts in nature. However, there is no detectable labelling in the labyrinthine trophoblast and none is seen in any area with the control sense probes. These results indicate that the spongiotrophoblast itself contains messenger RNA which codes for GM-CSF, and therefore suggests that trophoblast both produces and responds to this cytokine, making it obvious to speculate that GM-CSF is autocrine to spongiotrophoblast.

These studies led to a more detailed analysis of the Northern blot patterns exhibited by placental mRNAs for a variety of different cytokines. With the single exception of GM-CSF, other cytokine mRNAs that we examined turned out to be of a normal size pattern when compared to controls. With respect to GM-CSF mRNA, one can only detect it in a Northern blot if poly A+RNA is purified by oligo-DT cellulose chromatography, indicating that it is in low abundance. The pattern that one sees indicates that the standard 1 kilobase band is present as expected. In addition, there are a series of higher molecular weight bands, ranging up to 5.2 kilobases (28). To further analyze this pattern, we have begun a series of mapping experiments to determine genomic sequences contributing to this unique mRNA pattern. Our initial experiments along these lines have used a split probe in which two fragments are generated via restriction enzyme digestion of the whole genomic probe, leading to a 5' and a 3' portion, split roughly in two. Standard 1 kb GM-CSF mRNA is comprised of four exons. The first restriction fragment (GM5') encompasses exons 1 and 2 plus the first 34 nucleotides of exon 3, while GM 3' encompasses the remainder of exon 3 plus exon 4. The GM-CSF full sequence and GM 5' probes produced an identical hybridization pattern. Both detected all five placental GM-CSF transcripts (5.2, 3.9, 2.4, 2.1, 1 kb). In contrast, GM 3' detected only the 2.4 kb placental GM-CSF transcript (barely visible on the actual autoradiograph). All three probes hybridized with equal intensity after equal exposure time to the 1 kb GM-CSF in mRNA extracted from EL4 cells, showing that the GM 3' probe was not defective in a general sense (29). The absence of a hybridization signal with GM 3' for the 2.1 and 1 kb transcripts may be due to the fact that these species appear to be of lowest abundance in placental mRNA. The 5.2 and 3.9 kb transcripts, however, are more abundant
than the 2.4 kb transcript, and since GM 5' hybridized to all five placental GM-CSF transcripts while GM 3' hybridized only to the 2.4 kb transcript, we conclude that the 5.2 and 3.9 kb transcripts are transcribed from some combination of exons 1, 2, and/or 3 but not from exon 4. This raises the possibility that cells in the placenta are capable of generating a series of GM-CSF-related mRNAs that are differentially spliced. It will be of great interest to examine the protein coming from placental cell preparations which may represent an unusual form of GM-CSF relevant to placental function.

A series of experiments done in collaboration with my French colleagues have indicated an important role for GM-CSF and IL3 in preventing fetal resorption. Thus injecting GM-CSF or IL3 intraperitoneally into CBA mice can prevent the spontaneous fetal resorption seen following mating with DBA/2 mice. On the other hand, gamma-interferon, TNF-alpha and IL2 have the opposite effect, leading to increased fetal resorption (30). These latter effects probably relate to the increasing evidence that NK cells are involved in the events leading to spontaneous fetal resorption, as mentioned above. Although the detailed cytokinology of these local NK-like cells needs further study, it is indeed intriguing that GM-CSF seems to antagonize the effect and thus indicates that there is a potentially very interesting interactive network of cytokines which govern the susceptibility and/or resistance of the fetus to spontaneous fetal resorption.

A new view of the reproduction function of GM-CSF is presented by experiments involving humans and rats. We have been collaborating with the laboratory of Dr. Larry Guilbert, who has shown that all three classical human choriocarcinoma cell lines, JEG, JAR and BEWO, secrete GM-CSF. Antibodies specific for GM-CSF partially inhibit the proliferation of the choriocarcinomas, thus suggesting strongly that GM-CSF is autocrine for choriocarcinoma growth. In addition, antibodies to GM-CSF eliminate the ability of human term trophoblast to syncytialize in vitro, a condition which can be reversed and indeed enhanced by adding excess GM-CSF (31, 32). This work possibly relates to that reported by Drs. Sarah Robertson and Bob Seamark who have shown that GM-CSF is present in a T cell-dependent form in the early murine reproductive tract following insemination. In addition, they have shown that GM-CSF, but not CSF-1, promotes mouse embryo implantation in an elegant in vitro culture system (33). In the human system studied by Guilbert and his colleagues, adding GM-CSF to human trophoblast cultures leads to an increased synthesis of human chorionic gonadotropin and human placental lactogen. CSF-1 has a similar effect, although it does not affect syncytialization (32). Corroborative results have been obtained Dr. Kathleen Shiverick and her colleagues at the University of Florida in Gainesville (personal communication, 1990). They have found that rat
trophoblast cultures can be made to secrete clearly defined members of the rat placental lactogen family when they are stimulated with either GM-CSF or CSF-1. Thus it appears that what were formerly considered to be exclusively lymphohematopoietic cytokines may have crucial growth and differentiation functions in the feto-placental unit. They appear to be acting in part by being stimulated and stimulating classical enocrine substances which are known to be essential for reproductive performance. In addition to these observations Deborah Anderson and her colleagues of Harvard University have presented information indicating that GM-CSF inhibits mouse blastocyst attachment to fibronectin-coated wells, again indicating a role for this molecule in modifying the properties of trophoblast membrane (34). In order to better understand these observations, one needs to clarify GM-CSF expression both at the mRNA and at the protein level in the placenta in order to gain insight into how this molecule, having some apparently unique structural features, is designed to carry out these functions at the maternal fetal interface. We also need to better understand how it fits into the network of cytokines that regulate fetal survival as a function of immune-reproductive system interactions. From the in vivo studies reported above, it already appears to be clear that there are positive and negative cytokines with respect to placental growth and function, and the next few years should see clarification of their more detailed roles.

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