Lymphocyte profiles and serum antibodies against neurofilaments in preeclamptic Kuwaiti women

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Objective. It is hypothesized that the pathogenesis of (PE) is accompanied by alterations in immunoregulation that may affect normal turnover of peripheral neurons and release of cytoskeletal components (principally neurofilaments). Since this is expected to alter serum levels of antibodies to neurofilament epitopes, the possibility exists to utilize this parameter as a biomarker for severity of the disorder. **Patients and methods**. Peripheral blood of 23 pregnant women in the third trimester; 13 with PE, 10 healthy pregnant women and 10 non-pregnant controls were evaluated by flow cytometry for major lymphocyte populations and for antibodies to neuronal cytoskeletal elements by Western blot analysis. **Results**. The percentages of CD3+CD16+CD56+, CD4+CD25+, CD8+CD25+, and CD8+HLA-DR populations were significantly increased in normal pregnancy and PE compared to non-pregnant women (p<0.01), dramatic increase of CD4+CD54+ but not CD4+CD45RA populations was observed in PE. Concentrations of autoantibodies for the 200-kDa neurofilament (NFH) was decreased but for the 160-kDa (NFM) was significantly increased in PE. Autoantibodies against the 70-kDa neurofilament (NFL) was significantly decreased in normal pregnancy compared to non- pregnant women (p < 0.05) and further decreased in PE (p < 0.01). **Conclusions**. The present study provides preliminary insight into how peripheral blood anti-neurofilament antibody levels and lymphocyte subpopulations correlate with normal and pre-eclamptic pregnancies. As these studies evolve, such correlations may emerge as valuable tools in medical monitoring, therapy and maintenance of healthy pregnancy.

Key words: Kuwait, Neurofilaments, Pre-eclampsia, Th1 Cells, Western Blot.

Introduction

We have previously shown that serum Butyrylcholinesterase (BuChE) activity in Kuwaiti women afflicted with disorders of pregnancy correlates with immunoregulation in a manner not observed in healthy pregnancies (1). Since BuChE detoxifies pregnancy-threat-

ening neuro - and immunotoxic compounds that are known to have been dispersed into the environment of the Gulf region in 1991 (2, 3, 4) and following the military actions of the 2003 Gulf War. We hypothesize that exposure to these substances may exacerbate pathologic immune processes leading to negative pregnancy outcomes. The present study extends this work in an evaluation of correlations between serum biomarkers of neurodegeneration resulting from organophosphate exposure and lymphocyte subpopulation profiles in women afflicted with pre-eclampsia, a disorder of pregnancy caused by dysregulated maternal immunity (5). It is here hypothesized that pre-eclamptic Kuwaiti women exhibit reduced ability to regulate pregnancy-associated immune activation; and express antibodies to products of pollutant-induced neurodegeneration at levels different from women experiencing normal pregnancies. Autoantibodies may form to cytoskeletal components of pollutant-damaged nerve cells, including: 200-kDa, outer- or high-molecular weight (NFH); 160-kDa, middle or medium-molecular weight (NFM); and 70-kDa, core and low-molecular weight (NFL) neurofilament subunits (6).

Here, serum of pre-eclamptic and healthy pregnant Kuwaiti has been analyzed for content of these antibodies with the objective of establishing parameters for a comprehensive investigation of immunoregulation in a Kuwaiti population.

Patients and methods

Participants in this study included 23 women in the third trimester of pregnancy, 13 afflicted with pre-eclampsia, and 10 experiencing normal pregnancies. 10 healthy, non-pregnant women were enrolled as controls. All were in Kuwait since the 1991 Gulf War. Institutional Ethical Committee Approval was obtained before the commencement of the study and each patient gave her consent before inclusion in the study.

Patient selection criteria

Preeclamptic women had: (a) Blood pressure of 140 mm Hg or higher systolic and 90 mm Hg or higher diastolic on two occasions at least six hours apart on bed rest after 20 weeks of gestation with previously normal blood pressure; (b) proteinuria of 0.3 g or more of protein in a 24-hour urine collection (which usually corresponds with 1+ or greater on a urine dipstick test).

Other features which were signs of severe: (a) Blood pressure of 160 mm Hg or higher systolic and 110 mm Hg or higher diastolic, and proteinuria of 5 g or more in a 24 hour urine collection; (b) oliguria defined as urinary output of less than 500 mL in 24 hours; (c) cerebral or visual disturbances; (d) pulmonary edema or cyanosis; (e) epigastric or right upper quadrant pain; (f) impaired liver function, thrombocytopenia; (g) intrauterine growth restriction. None of the women (patient or control) had evidence of any active infective process such as urinary tract infection or upper respiratory tract infection.

Blood collection and Lymphocyte analysis

Ten ml of venous blood was collected from each patient by venipuncture without use of tourniquet, centrifuged for 10 minutes at 1000 rpm and the serum separated and stored at -70°C until analysis for anti-neurofilament antibodies was carried out. Five ml of peripheral venous blood was collected in EDTA tubes. Fifty µl of blood were incubated for 30 min at room temperature with 5 µl of the flourescein-isothiocyanate (FITC) or phycoerythrin (PE) conjugated monoclonal antibodies (mAb) of interest. FITC- and PE-labeled monoclonal antibodies not reacting with lymphocyte surface antigens were used

as negative controls. After lyses of erythrocytes with Q-prep (Coulter Corporation, Hialeah, FL, USA) followed by fixation with paraformaldehyde, two color fluorescence analysis using an automated flow cytometer (Coulter Altra cell sorter) was performed and analyzed by 3-color flow cytometry for selected lymphocyte subpopulation frequency.

Analysis for Cytoskeletal Proteins

As was reported previously (14), briefly, neurofilament proteins prepared from fresh bovine spinal cord were separated by SDS Gel Electrophoresis, then electrophoretically transferred onto nitrocellulose paper and incubated with serum extracted from each blood sample. Autoradiograms derived from these blots were scanned in a Molecular Dynamics Personal Densitometer SI and quantified, using the gel analysis program IPLab Gel v1.5, to yield serum levels of antibody to each protein.

Statistical analysis

Analysis was performed using multiple comparison analysis of variance (ANOVA) with a post-hoc Tukey test. All statistical analysis were performed using the SPSS for Windows statistical package version 16 (Norusis/SPSS, Inc). A value of $p < 0.05$ was considered statistically significant.

Results

As shown in Table 1, the peripheral blood frequencies of four activated lymphocyte subpopulations (CD4+CD25+, CD8+CD25+, CD8+HLA-DR+, and CD4+CD54+) were observed to be significantly elevated in all pregnant women when compared with their percentages in blood of the non-pregnant group ($p < 0.05$).

The percentages of CD4+HLA-DR+ cells were elevated in PE versus non-pregnant women, but not in healthy pregnant women; and no differences were noted in NK-T (CD3+/CD16+CD56+) cells between all groups. Addi-

Table 1 Lymphocyte subpopulation frequencies in non-pregnant women versus subjects with normal or pre-eclamptic pregnancies

Lymphocyte subpopulation	Non-pregnant (N = 10)	Normal pregnancy (N = 10)	Pre-eclamptic pregnancy (N = 13)
CD3+/CD16+CD56+ [a]	10.4 ± 2.3	22.8 ± 7.4	21.9 ± 6.3
CD4+CD54+ [b]	15.3 ± 2.1	24.9 ± 5.0*	34.9 ± 6.7**
CD4+HLA-DR+ [b]	6.4 ± 1.7	6.8 ± 0.8	9.5 ± 1.7*
CD8+HLA-DR+ [c]	10.6 ± 3.1	26.3 ± 4.5*	27.2 ± 4.1*
CD8+CD25+ [c]	0.7 ± 0.4	6.3 ± 2.1*	7.6 ± 4.8*
CD4+CD25+ [b]	5.3 ± 1.5	14.1 ± 3.8*	19.8 ± 6.9*
CD4+CD45RA+ [b]	38.3 ± 5.2	56.3 ± 4.5*	43.1 ± 4.1¶
CD4+CD45RO+ [b]	47.6 ± 4.1	53.6 ± 5.6	53.0 ± 4.7

Selected activated, memory, and naive lymphocyte species are evaluated by 2-color flow cytometry for percentage representation in CD3+ (a), CD3+CD4+ (b) and CD3+CD8+ (c). Values are given as proportions (%) of lymphocyte subpopulations within each mother population (CD3, CD4 and CD8) ± standard error of the mean (SEM).
* $p < 0.05$ versus non-pregnant subjects
** $p < 0.01$ versus non-pregnant subjects
 ¶ $p < 0.05$ versus women experiencing normal pregnancy

tionally, the percentages of CD4+CD45RA+ T lymphocytes, which may represent either a naive or suppressor phenotype (7), were significantly elevated in subjects experiencing normal pregnancies (p < 0.05), but present at lower frequency in the blood of pre-eclamptic women (p < 0.05).

Figure 1 shows no significant differences in serum levels of anti-NFM and NFH between any of the subject groups. However, compared to non-pregnant women, the concentration of anti-NFL was significantly lower for participants experiencing normal pregnancies (p < 0.05) and for those with (p < 0.01).

Discussion

Maternal immune responses to the feto-placental unit during normal pregnancy include increased percentages of activated T cells in the peripheral blood (7), an effect also observed in the present study (Table 1). If normal immunoregulatory mechanisms are impaired, these cells can cause pre-eclampsia and other disorders of pregnancy (8). This effect may be indicated here by lower CD4+CD45RA+ cells in blood of pre-eclamptic versus healthy pregnant women (Table 1), which confirm
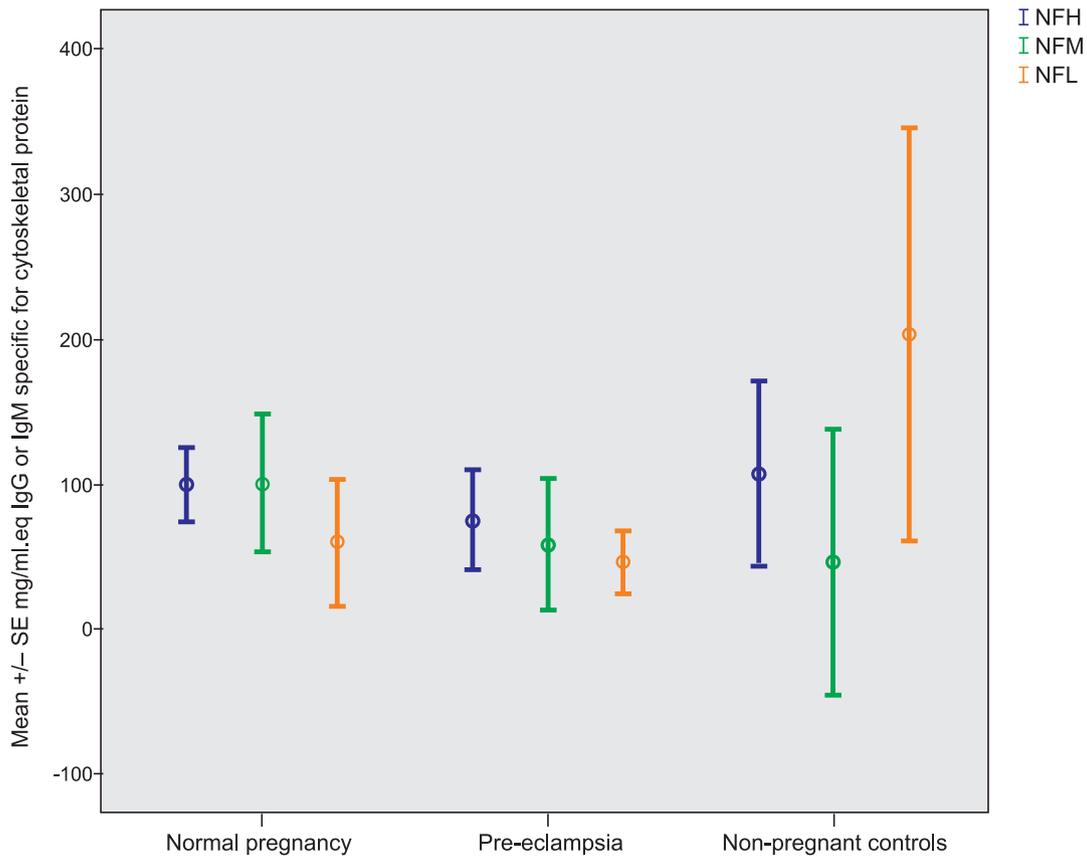

Figure 1 Serum concentration of antibodies to cytoskeletal proteins in non-pregnant women versus subjects with normal or pre-eclamptic pregnancies. Autoantibodies with specificity for 70-kDa (NFL), 160-kDa (NFM), and 200-kDa (NFH) neurofilaments are measured using Western blot analysis. Values are given as ng/ ml equivalence (ng/ ml.eq) IgG or IgM, with specificity for each indicated cytoskeletal protein in serum ± standard error of the mean (SEM). * p< 0.05, ** p< 0.01 versus non-pregnant subjects.

a previously published study in Poland (9). CD45-related signals have been implicated both directly and indirectly, in a variety of lymphocyte functions (10), as a prerequisite for transmitting activation signals through T cell antigen receptor (TCR). Two T receptor populations are expressed on T cells; one linked to the cytoskeleton via its zeta chain. These cytoskeleton linked receptors which make up 30-40 percent of the total number of TCRs are important in TCR mediated signaling (11). Furthemore, oxidative stress is known to be involved in the heat stress-induced down-regulation of TCR zeta chain expression and TCR/CD3-mediated $[Ca^{2+}]$ response in human T lymphocytes (12). Regardless of the cell type, the regulation of actin cytoskeleton is tightly linked to vital biological properties such as polarity, motility, cell to cell contact, exocytosis and proliferation (13). Exposure to certain environmental pollutants causes nerve cell destruction and formation of antibodies to neurofilaments (14). It may also, as suggested by our previous work, contribute to heightened immune activation and exacerbation of pregnancy disorders (1). A separate investigation by our laboratory demonstrated that serum levels of anti-NFL are lower in a group of Kuwaitis afflicted with a Th1-mediated autoimmune disease (psoriasis) than in those with non-psoriatic controls (15). The present study shows that serum levels of anti-NFL are significantly lower in pregnant subjects than in non-pregnant controls, and lower in pre-eclamptic subjects than in the healthy pregnant group. Although data presented in this report does not allow for a precise mechanistic interpretation of these observations, it is possible that lower trunover of NFL may occur as a result of immunoregulatory processes triggered by normal pregnancy and by PE (1, 5). Nevertheless further characterization of this effect will be required before serum anti-NFL titers may be used as diagnostic or therapeutic biomarkers. Preg-

nancy is characterized by a predominantly Th1 profile of immune activity (16), which is exacerbated in pre-eclampsia (17). Furthermore, as shown in Figure 1, serum anti-NFL is significantly lower in healthy pregnant than non pregnant subjects and even lower in preeclamptic women, which suggests that increasing Th1 character may inhibit tissue turnover nerve cells. This normally leads to increased serum levels of antibodies to neurofilaments with increasing age. This interpretation, however, is purely speculative in the absence of additional data. The scope of this study does not allow medically useful conclusions to be drawn regarding correlations between neurofilament antibody expression and the occurrence of PE. However, the data presented here suggests strategies for using these biomarkers in characterizing immunoregulatory disorders in a population such as that of Kuwait subjected to a high toxic burden. Studies currently underway by this laboratory will correlate occurrences of immunoregulatory dysfunction with associated changes in lymphocyte activity and neurofilament antibodies in the context of serum activity of detoxification enzymes such as butyrylcholinesterase. This work is expected to result in more focused therapeutic approaches to treating of a wide range of disorders.

There is abundant evidence of modification of the neurons of the gravid uterus. There is functional denervation of the gravid uterus in the latter part of the pregnancy and renervation in the postnatal period (18), suggested by low α-adrenergic receptor density and absence of neuronal nitric oxide synthase (19). In a recent hypothesis, Quinn proposed a connection between pre-eclampsia and partial uterine innervation, caused by damage to the nerve plexus at the endometrial-myometrial interface which causes impairment of control of a third proliferative, invading trophoblast resulting in the characteristic histological changes (20).

It has been suggested that growth factors like nerve growth factor, vascular endothelial growth factor, and cytoskeleton proteins such as neurofilament produced by nerves and blood vessels may contribute to the process of normal placentation. These processes may be compromised in areas of denervation. Specifically, loss of neural connections between the uterine and renal innervation may cause reduced fetal growth and PE. Neurofilaments are neurone-specific intermediate filament and regulate neuronal cytoskeletons to form the dynamic axonal cytoskeleton. They maintain and regulate neuronal cytoskeletal plasticity through the regulation of neurite outgrowth, axonal caliber and axonal transport (21). It is not yet clear whether there is differential denervation of the gravid uterus in abnormal compared to normal pregnancies, and how they alter the anti-neurofilament levels. In an immunohistochemical study, Khong et al. demonstrated lack of innervation of the spiral arteries, however, nerves were observed in the myometrium in 7 out of 10 normal and 1 in 8 third trimester abnormal placental beds (22).

Cytoskeleton and TCR modification occurs as a result of oxidative stress during pregnancy. Lateral associations between TM helices are also involved in transmission of signals from the activated TCR to downstream effector pathways. Activation of kinases by the phosphatase CD45 appears to be facilitated by CD45-associated protein, which binds kinases with its intracellular domain and CD45 via TM-TM contacts. Recruitment of phosphatidylinositol-3-kinase to the membrane by pp30 (T cell receptor interacting molecule, TRIM) depends on the phosphorylation of intracellular tyrosines on TRIM (23). Stimulation of peripheral blood T cells with phorbol myristate acetate (PMA) or with anti-CD3 monoclonal antibodies resulted in a marked increase in detection of phosphorylated neurofilament

on western blotting, thus indicating that T cell activated through the T-cell receptor associated complex express an intermediate filament usually associated with neurally derived cells (24).

Conclusion

The trigger mechanism that results in the production of antibodies to neurofilaments generally is not known. It has been postulated that such antibodies may represent essential homeostatic mechanism for removing damaged or infected cells (25). This protective mechanism may be deficient in pre-eclampsia. If this is true, then anti-neurofilament could be used as a biomarker of pre-eclampsia in early pregnancy. Any relationship between anti-neurofilament and pre-eclampsia certainly needs more extensive evaluation with a larger sample size in a multi-center collaborative effort.

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