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Abstract

Gene association with *HLA* suggests involvement of immune mediated mechanisms in the pathogenesis of Parkinson's disease (PD). Only a small number of studies have found differences between circulating leukocyte populations in PD patients compared to controls, with conflicting results. To clarify whether there is a circulating leukocyte PD phenotype, we assessed the numbers of T, B and natural killer cells, and monocytes and found a small reduction (15-25%) in CD4+ T and CD19+ B cells in PD. These findings suggest some compromise in immune cells in PD and have potential implications for immune function and the progression of PD.

Keywords

Parkinson's disease; T lymphocytes; B lymphocytes

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder and recent genome-wide association studies (GWAS) have shown an increased risk for PD with variation in the *HLA* (human leukocyte antigen) region of chromosome 6 (Hamza et al., 2010; Do et al., 2011; Nalls et al., 2011; Simon-Sanchez et al., 2011). This region contains a number of genes involved in inflammation and immunosurveillance, strongly supporting an involvement of the immune system in the pathogenesis of PD. In addition, large studies show that regular long-term use of the anti-inflammatory drug ibuprofen significantly reduces the risk of developing PD (Samii et al., 2009; Gagne and Power, 2010; Gao et al., 2011). Of the variants identified, a strong association has been found within a region of *HLA* that encodes the major histocompatibility complex Class II receptor, HLA-DR (Hamza et al., 2010). This cell surface receptor is expressed on antigen presenting cells such as microglia, and increased numbers of HLA-DR positive microglia have been found in close proximity to dead or dying neurons in affected brain regions in PD (McGeer et al., 1988; Imamura et al., 2003). HLA-DR also interacts with receptors on T lymphocytes and both CD4+ and CD8+ T lymphocytes have been identified post-mortem in the brains of PD patients (McGeer et al., 1988; Brochard et al., 2009). Studies in animal models clearly demonstrate a role for T lymphocytes in the development and progression of PD (Benner et al., 2008; Cao et al., 2011). The involvement of the immune system in PD pathogenesis suggests that populations of circulating blood leukocytes may also differ between PD patients and controls.

There have been a small number of studies assessing changes in circulating leukocyte populations in PD patients with conflicting results. In PD, CD4+ T helper cells are

reported as both unchanged and decreased (Chiba et al., 1995; Bas et al., 2001; Hisanaga et al., 2001; Baba et al., 2005; Niwa et al., 2012), CD8+ cytotoxic T cells are reported as both unchanged and increased (Chiba et al., 1995; Bas et al., 2001; Hisanaga et al., 2001; Baba et al., 2005) and B cells are reported as decreased in PD compared to controls using both CD19+ (Bas et al., 2001) and CD20+ activation markers (Niwa et al., 2012). To our knowledge, circulating CD3-CD56+ natural killer and TCRγδ cells have only been examined in single papers with the authors reporting an increase in both cell types (Fiszer et al., 1994b; Mihara et al., 2008), and CD14+ monocytes have not been examined in this disease. In order to verify these findings and resolve any discrepancies, this study has assessed the major populations of circulating blood leukocytes in a cohort of PD patients and controls to determine whether there is a typical PD phenotype.

2. Material and Methods

2.1 Subjects

Eighty-eight (56 male, 32 female) PD patients were recruited for this study with informed consent. All PD patients had a tremor-dominant phenotype and were levodopa responsive. No patient or control subject had a haematological, immune or inflammatory disorder, or was taking immunosuppressive medications. Any patient with a neurologic or psychiatric condition other than PD, strong family history of PD or relative with young onset PD was excluded. The mean (±standard deviation) age of the PD group was 69±9 years and the mean disease duration was 6±5 years. Clinical severity was assessed by the Hoehn and Yahr (H&Y) scale (Hoehn and Yahr, 1967) and Unified Parkinson's Disease Rating Scale part three (UPRDS) (Fahn et al., 1987). The mean (±standard deviation) H&Y score was 2±0.7and the mean UPDRS score was 21±10. Seventy-seven (39 male, 38 female) age and gender matched healthy controls were recruited for

comparison to the PD group. The mean (±standard deviation) age of the control group was 67±10 years. Independent samples T tests and chi-squared tests were performed to confirm that the PD and control groups did not differ by age (t=1.06, p=0.3) or gender (χ^2=2.84, p=0.09).

As a number of studies have suggested that dopamine replacement therapy affects leukocyte populations in peripheral blood (Fiszer et al., 1994a; Blandini et al., 2004; Rajda et al., 2005), medication use was noted and levodopa equivalent dose calculated (Tomlinson et al., 2010). Eight PD patients were assessed pre and post the onset of levodopa medication. All patients were on 300 mg of levodopa for three months before retesting. This study was approved by the Northern Sydney Central Coast Area Health Service Human Research Ethics Committee (Harbour) (EC00333).

2.2 Analysis of human leukocyte populations

Venous blood was collected in sodium EDTA tubes (Becton Dickinson, San Jose, CA) from subjects between 9-11am to minimise the influence of diurnal fluctuation between leukocyte populations. Blood was taken for both a full blood count and flow cytometric quantitation of white cell subsets and specific activation markers. A full blood count was performed using a Coulter counter (Beckman Coulter, Gladesville, NSW, Australia) to allow calculation of absolute numbers from population percentages determined by flow cytometry. The cells were stained with fluorochrome-conjugated (fluorescein isothiocyanate – FITC; peridinin chlorophyll protein complex - Per-CP; allophycocyanin – APC; phycoerythrin - PE) antibodies within 10 minutes of blood collection. All the antibodies were from Becton Dickinson Biosciences (BD Biosciences, San Jose, CA) and used undiluted as recommended. A broad panel of antibodies was used to identify

cellular subsets and activation markers as outlined in Table 1. The cellular subsets included T lymphocytes, B lymphocytes, helper, memory and cytotoxic T lymphocytes, monocytes, natural killer (NK) cells and gamma-delta T lymphocytes, with activation markers as appropriate for the cell population. The samples were analysed within two hours of preparation by a four colour FACScan flow cytometer (BD Biosciences). Ten thousand mononuclear events (lymphocytes and monocytes) were acquired per sample. Flow cytometric data were analysed using the Cell-Quest software. Cell subsets were isolated by selective gating using side and forward scatter parameters and specific antibodies (experimental design shown in Supplementary Table 1). For the activation markers expressed only on activated cells, the results were expressed as percentage of positive cells for that particular marker. The increase in surface antigen expression following activation was measured using the mean of fluorescence for the considered cell subset.

***** Table 1 about here *****

2.3 Statistical analysis

All analyses were performed in SPSS (18.0, IBM, Chicago, USA) with a p value < 0.05. Means and standard errors are provided as appropriate. To determine differences in absolute counts and proportions of lymphocyte/monocyte populations between control and PD groups, multivariate analyses covarying for age and gender were performed. Nonparametric correlations (Spearman's rho) were performed to determine relationships between cell populations and disease duration, H&Y and UPDRS stage. For cell populations that differed significantly between groups, the effect of pre and post levodopa treatment was assessed using paired t tests. Stepwise regression modeling was used to determine which factor (disease duration, H&Y stage, UPDRS stage or levodopa equivalent dose) was the strongest predictor of these changes in PD.

3. Results

For each subject, 10 000 cells were sampled and the absolute numbers of major cell populations determined across the groups. The average numbers of the lymphocyte populations examined were; T cell receptor αβ (TCRαβ)(1145±425), T cell receptor γδ (TCRγδ) (47.9±54), CD4+ T cells (780±296), CD8+ T cells (351±215), CD4+CD8+ T cells (18±32), CD19 B cells (185±110) and CD56+CD3- natural killer cells (240±130). The average number of CD14+ monocytes was 2488±157.

Multivariate analysis of these cell populations co-factoring in age and gender, showed no changes in the numbers of sampled cells labeled for CD14+ (monocytes), CD8+ (cytotoxic T cells), CD56+ (natural killer cells), TCRγδ+ or CD4+CD8+ cells in patients with PD. However, in PD there were significant decreases in TCRαβ+, CD4+ (T helpers) and CD19+ (B) cells compared to controls. For these cell types a decrease of approximately 15-25% in the numbers of sampled cells was observed in the patients with PD (Table 2 and Figure 1).

***** Table 2 and Figure 1 about here *****

Although there were no significant differences between PD and controls in age or gender (see methods), the cell populations varied with both age and gender in the same way in both groups (no group by gender effects, $p>0.95$). Age had a larger effect but was not universal, with the number of CD4+ T helper cells ($p\leq0.03$) and CD19+ B cells ($p<0.0001$) decreasing with age. In contrast, the number of CD14+ monocytes slightly increased with age ($p<0.0001$). The smaller effect of gender was only observed when cell types were assessed by proportion. There were less CD4+ T helper cells in males (9±3% difference, $p=0.04$) but less CD14+ monocytes in females (11±3% difference,

p=0.016). This confirms literature showing age and gender differences in these peripheral blood cell populations, although published results for CD14+ monocytes are inconsistent (Bouman et al., 2004; Kaszubowska, 2008; Agarwal and Busse, 2010; Nyugen et al., 2010).

Differences in subsets of the CD4+ T helper cell population (contributing 9% of the total 10,000 cells analysed) were assessed further. In controls 89% of cells expressed the integrin β1 adhesion marker CD29+, 64% had expression of the memory marker CD45RO+, 28% expressed the IL-2 receptor CD25+ and 19% had expression of the death ligand CD95 (Fas)(Figure 1). When assessing these activation markers in PD, there were significant decreases in the numbers of CD4+ cells expressing CD29+ (19% decrease) and CD45RO+ (14% decrease) between PD patients and controls (Figure 1). However, the proportion of these cells (expressed as a percentage of total CD4+ cells) was increased in PD (CD4+CD29+ increased 2%, CD4+CD45RO+ increased 6%, Figure 1). There was no difference in the numbers or proportion of CD4+ T cells expressing CD25+ or CD95+ (Table 3 and Figure 1).

***** Table 3 about here *****

Differences in subsets of the CD19+ B cell population (contributing 2% of the 10,000 cells sampled) were also analysed. In controls 98% of CD19+ B cells expressed the adhesion marker CD29+, the lipid antigen-presenting molecule CD1d+ and the intracellular adhesion molecule CD54+ suggesting that these markers are expressed on the majority of CD19+ B cells (Figure 1) and should not be independently assessed further. Approximately 1% of CD19+ cells in controls expressed the death ligand CD95+ (Fas) with no difference in their numbers or proportion in PD (Table 3 and Figure 1).

Effect of clinical severity, disease duration and levodopa on peripheral blood cell populations that are altered in Parkinson's disease

Clinical severity was assessed using the H&Y scale as well as the more sensitive UPDRS scale. Both UPDRS and H&Y negatively correlated with the numbers of CD4+ ($p \leq 0.004$) cells, but only H&Y stage negatively correlated with CD19+ cells ($p=0.036$). When assessing the activation markers there was a negative correlation between both clinical severity scores and the numbers of CD4+ cells expressing CD45RO+ ($p \leq 0.003$) and CD29+ ($p \leq 0.005$), and between H&Y stage and the numbers of CD19+ cells ($p \leq 0.008$). Overall, this suggests that as the disease progresses there is an ongoing loss of CD4+ T and CD19+ B cells. The small increases observed in the proportion of CD4+ cells expressing either CD29+ or CD45RO+ activation markers did not vary with clinical severity.

A reduction in the numbers of CD4+CD45RO+ ($p=0.009$) and total CD19+ cells ($p=0.036$) was observed with increasing disease duration. The average decrease in these populations per year was 2% for CD4+CD45RO+ cells and 4% for CD19+ cells. Again, the small increases observed in the proportion of CD4+ cells expressing either CD29+ or CD45RO+ activation markers did not vary with disease duration.

The assessment of eight PD patients before and after the onset of levodopa treatment showed a significant reduction in the numbers of CD19+ cells (15±14% decrease, $p=0.007$) post commencement of medication. This was not observed in cells expressing CD95+. No effect of medication was observed for the other populations.

Stepwise regression modeling revealed that H&Y stage (but not disease duration, UPDRS score or levodopa equivalent dose) was the strongest predictor of the PD changes in all but one of the cell populations (CD4+CD45RO+ memory T cells). There was an average decrease over each H&Y stage of 5.5% and 5% for the number of CD4+ and CD4+CD29+ respectively ($p=0.004$), and 6% for the CD19+ B cell populations ($p=0.007$). The change in CD4+CD45RO+ memory T cells was predicted by both H&Y stage and disease duration ($p<0.0001$). The average decrease in these cells for each H&Y stage was 3.5% and for each progressing year was 2%.

4. Discussion

This study has assessed changes in circulating leukocyte populations in patients with PD versus controls and addresses conflicts in the literature by also assessing the potential impact of clinical variables on such data. We confirm a reduction in CD4+ T helper and B cells in PD patients (Bas et al., 2001; Baba et al., 2005; Niwa et al., 2012) with no change to the numbers of the other major peripheral blood cell types assessed. We found that the decline in CD4+ T helper and CD19+ B cells varied with the clinical stage of disease. Such variability is likely to explain the differences in the effect sizes observed in previous studies. In our study of patients with an average of 6 years duration and a severity stage of H&Y 2, the effect size for the decline in the peripheral blood cell populations was on average 20-25% compared to controls. Assessment of activation markers revealed a more targeted cell-type effect of PD, with some cell types also influenced by disease duration and/or the introduction of levodopa therapy.

Of the CD4+ T helper cells, there was a more selective decrease in the numbers of CD4+CD29+ cells and CD4+CD45RO+ memory T cells. The decrease in numbers of

CD4+CD29+ cells has been shown in a single study before, but only in untreated patients with the suggestion that the reduction in this subtype is reversed with treatment (Bas et al., 2001). Our data show that this effect is observed in treated patients and correlates with the clinical stage of the disease, with an average 5% reduction in CD4+CD29+ cells for each increasing H&Y stage of disease (assuming a linear disease model). Overall, this data suggests that larger populations of patients (34 in previous study, 88 in current study) may need to be studied to see any early effect on this cell type in treated patients with PD. We did observe a slight increase (2%) in the proportion of remaining CD4+ cells expressing the CD29+ activation marker, however given the larger overall decrease in this population compared with controls, it is uncertain what clinical relevance this small proportionate increase may have.

Only one study has examined CD4+CD45RO+ memory T cells in PD with the authors reporting an increase in the proportion of this cell type (Fiszer et al., 1994a). It should be noted that the comparison group in the Fiszer *et al.* study were people suffering from tension headache (Fiszer et al., 1994a) with some evidence that such patients may have peripheral blood cell abnormalities similar to multiple sclerosis (Mix et al., 1990). In agreement with Fiszer *et al.*, our study found a 6% increase in the proportion of remaining CD4+CD45RO+ cells, however we did find a greater 19% decrease in the numbers of this cell population compared to a normal control group. This suggests either a more selective loss of another CD4+ population (possibly CD4+CD45RA+ (Bas et al., 2001)) or that a small proportion of remaining CD4+ cells shift to a memory cell phenotype. Our data also show that the CD4+CD45RO+ memory T cells vary with the clinical severity and disease duration of PD, with an average 2% decline in numbers per

year. Clinical variability in the case types examined and compared will need to be considered for future studies analysing the effect of PD on this cell population.

Studies have reported changes to other CD4+ T helper cell populations, which we were unable to replicate. One study examined the proportion of CD95+ (Fas) expression on CD4+ T cells reporting an increase in PD (Calopa et al., 2010), while several studies report changes in CD4+CD25+ T cells (Bas et al., 2001; Baba et al., 2005; Niwa et al., 2012). The numbers of CD4+CD25+ T cells have been reported to be increased (Bas et al., 2001), and their proportion reported to be increased (Bas et al., 2001), decreased (Baba et al., 2005) or unchanged (Niwa et al., 2012), which may suggest some small transient changes to different functional populations. Although CD4+CD25+ is often used to identify regulatory T cells, this combination also detects effector T cells. Future studies should consider whether additional markers, such as Foxp3+, could improve the accuracy of these results, in addition to looking at the effects of clinical variables.

We did not observe an increase in CD95+ expression on CD19+ B cells, suggesting that the reduction in this population is not due to apoptosis. However, we did find an effect of initial levodopa treatment on CD19+ B cells in patients with PD. There was a 15% decrease in total CD19+ B cells within three months of the initiation of levodopa treatment, but not a strong cumulative effect of treatment on any of the examined cell populations. This supports observations by Bas *et al.* who found similar decreases between treated and untreated PD patients (Bas et al., 2001). It is well known that B cells express dopamine receptors and that even low doses of levodopa (or apomorphine) reduces their proliferation (Meredith et al., 2006), consistent with our findings. In the present study, we found a further reduction in the numbers of CD19+ B

cells over the disease course that correlated with clinical severity (6% reduction per H&Y stage, again assuming a linear disease model). As expected, there was no correlation between increasing H&Y stage and levodopa equivalent dose in our cohort (Spearman Rho=0.12, p=0.3) suggesting that the ongoing reduction in peripheral B cell numbers is a PD effect. A larger cohort of patients tested before and after the onset of levodopa medication is required to confirm the separate effect of PD to that observed following dopamine replacement, and more consideration given to the impact of these factors in any future work on B cells in such patient cohorts.

Interactions between CD4+T and B cells are critical for antibody mediated immunity, and any alterations in these cell populations may compromise immune function in PD. Recent work in experimental autoimmune encephalomyelitis (EAE) suggests that B cells are capable of controlling numbers of circulating CD4+Foxp3+ regulatory T cells (Ray et al., 2012). Depletion of B cells in EAE resulted in reduced numbers of regulatory T cells and increased susceptibility to disease, which was reversed by replacement of B cells using adoptive transfer methods (Ray et al., 2012). In particular, B cells expressing glucocorticoid-induced TNF ligand were crucial to recovery (Ray et al., 2012) and in PD patients, altered glucorticoid – glucocorticoid receptor signaling has been shown in peripheral blood and brain tissue (Ros-Bernal et al., 2011). Reduced CD4+ T cells in PD is one of the more well-studied and consistent findings in the literature (see above) and although one study has reported no difference in CD4+Foxp3+ regulatory T cells between PD and age-matched controls (Rosenkranz et al., 2007), given the low frequency of this population (0.1-0.7% of leukocytes), larger cohorts may be required in order to have significant power to detect small changes. Furthermore, studies in animal models of PD show that increasing the numbers of regulatory T cells provides protection

to the nigrostriatal system (Reynolds et al., 2007). These studies suggest that changes in these cell populations may play an important role in the immune system depression we have observed in PD.

The present study has detailed changes in circulating leukocyte populations in PD and importantly, how they are influenced by clinical severity, disease duration, and the initiation and cumulative impact of dopamine replacement therapies. We found a reduction in both CD4+ T helper and CD19+ B cells that worsened with increasing clinical severity. Our data suggest that clinical variability is likely to account for differences between studies in the literature to date, and it will be necessary to consider these factors further in future studies. Fortunately, the long-term use of therapeutically-beneficial medication did not strongly influence the deficits in these peripheral lymphocyte populations in PD, although a reduction in B cells was observed following the initiation of treatment, as previously reported. Overall, more work is now required to determine the timing and consequences of changes in these cell populations over the course of PD.

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