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Kynurenine, Neopterin and Lipid Peroxidation Levels in Ulcerative Colitis

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Kynurenines are tryptophan metabolites which include an agonist (quinolinic acid) and an antagonist (kynurenic acid) at glutamate receptors on intrinsic myenteric neurons. We have reported raised kynurenine and kynurenic acid in patients with inflammatory bowel disease, raising the possibility of using drugs to modulate the pathway. We have now measure etc. Despite a significant difference in the colitis activity index between active and remission phases, there were no significant differences in the levels of neopterin, kynurenines or lipid peroxidation products. Thus, a change in immune status may not be involved in the transition from active disease to remission, nutritional or endocrine factors may be important. The kynurenine pathway does not appear to be an appropriate target for drug development in this condition.

Key words: Kynurenine, neopterin, lipid peroxidation, ulcerative colitis

INTRODUCTION

The causes of transition from a state of remission into an active state of inflammatory bowel disease are unknown. Neopterin is widely recognised as an indicator of immune status, its levels increasing substantially with infection or inflammation^[1-4]. The kynurenine pathway from tryptophan is also activated in macrophages and neutrophils in response to interferon- γ or interleukin-1 β stimulation^[5,6]. One product of the kynurenine pathway is quinolinic acid, an agonist at glutamate receptors responding to N-methyl-D-aspartate (NMDA). Glutamate is an excitatory neurotransmitter in the central nervous system and probably performs a similar role in the enteric nervous system^[7,8], since receptors for glutamate, including those for NMDA, are known to exist in the myenteric plexus^[9,10]. The activation of glutamate receptors in the intestine increases gut motility^[11,12]. The kynurenines also include kynurenic acid which is an antagonist at several subtypes of glutamate receptors including the NMDA subtype^[13-15].

The levels of kynurenines in patients with mild inflammatory bowel disease compared with the levels in normal control subjects were previously measured, the results revealing increased levels of kynurenic acid^[16]. Since this may indicate a disorder of glutamatergic transmission which could modify intestinal activity and could suggest that the kynurenine pathway would represent a target for new drug development, a single group of patients to assess their levels of kynurenines as they experience successive periods of active disease and of remission was examined.

MATERIALS AND METHODS

Approval for the study was obtained from the Local Research Ethical Committee. Patients were recruited to the study from a gastroenterology clinic and written informed consent was obtained. Eleven patients with ulcerative colitis were recruited of whom 5 experienced a period of active disease resolving into remission during the study. All patients were being treated with sulphasalazine or mesalazine. The patients who went into remission had been treated with oral or intravenous prednisolone. Disease activity was assessed using the Simple Clinical Colitis Index (SCCI)^[17] and remission was defined by the physician's global assessment coupled with an SCCI score of 0-1. The maximum possible score on this scale is 15 plus 1 for each extracolonic feature. The control group was composed of healthy age-matched volunteer subjects.

Standard clinical assays: Blood was collected by venepuncture from patients into plain bottles without anticoagulant and was then frozen and stored at -70°C for subsequent assay. Erythrocyte Sedimentation Rate (ESR) was measured by a Starrsed Automated ESR machine (R and R Mechatronics) which utilises the method recommended by the International Council for Standardization in Haematology^[18], based on the method of Westergren^[19]. C-reactive protein was measured using a Behring Turbitimer.

Neopterin assays: Neopterin levels were measured in 10 μ L aliquots of serum using an immunoassay kit (Immunobiological Laboratories, Germany). All samples were analysed in duplicate. The assay is a competitive ELISA where a peroxidase-conjugated and a non-conjugated antigen compete for a fixed number of antibody binding sites. Following incubation with substrate solution, colour develops and the optical density is read at 450 nm, unknowns being quantified by reference to known standards.

Lipid peroxidation products: An aliquot of 100 μ L of serum was used for quantifying the concentrations of the lipid peroxidation products malondialdehyde and 4-hydroxynonenal measured using a Bioxytech LPO-586 colorimetric assay (Biostat). The analysis involves the reaction of N-methyl-2-phenylindole with these peroxidation products to form a stable chromogenic indolic dimer which is estimated spectrophotometrically at 586 nm. All samples were tested in duplicate.

Kynurenine assays by HPLC: To 480 μ L serum 20 μ L of internal standard were added (1200 μ M 3-nitro-L-tyrosine), followed by 50 μ L 4 M perchloric acid. Samples were vortexed for 30 sec immediately after acid addition, centrifuged at 5000 g for 10 min at 4°C and the supernatant collected. The precipitated proteins were resuspended in 150 μ L water and 50 μ L 4 M perchloric acid, the mixture was vortexed for 30 sec and centrifuged at 5000 g for 10 min at 4°C. Again the supernatant was collected. This washing and centrifugation step was repeated and the three supernatants combined. Combined supernatants were filtered in Whatman Vectaspin Micro Anopore tubes during a further centrifugation at 3500 g for 5 min at 4°C, prior to injection onto the HPLC. A volume of 100 μ L was normally injected.

A Waters HPLC system was used with a C18 reversed phase column (Phenomenex Kingsorb 250x4.6 mm, 5 μ m)^[20]. Samples were analysed using both a dual wavelength UV detector (250 nm for tryptophan

and 365 nm for kynurenine) and a fluorescence detector (excitation wavelength 344 nm and emission wavelength 390 nm for kynurenic acid) connected in series. The mobile phase for UV and fluorescence detection was 50 mM acetic acid, 100 mM zinc acetate containing 3% acetonitrile, based on the composition described by Hervé *et al.*^[21]. Zinc acetate 100 mM was used as it significantly enhances the fluorescence of kynurenic acid^[22]. The internal standard, 3-nitrotyrosine, was quantified at 365 nm. Calibration curves were determined using various concentrations of standard compounds in solution.

Analysis: Data are expressed throughout as mean±S.E. mean. Comparisons between patients in active disease and in remission were made using a paired t test, taking $p < 0.05$ as the limit of significance. Correlations between pairs of variables were assessed using the NSS statistical package and INSTAT, with the Pearson correlation coefficient used to indicate the degree of correlation and $p < 0.05$ being taken to indicate significance. A comparison was also performed between the values for all patients in a state of active disease and those in a state of remission using an unpaired t test. Comparisons between the total group of active disease patients, remission patients and controls were made using a one-way ANOVA followed by the Student-Newman-Keuls post hoc test.

RESULTS

A major problem with clinical research in studies of inflammatory bowel disease is the variation between symptoms and duration of disease activity at time of presentation. Some patients will exhibit an almost regular sequence of relapses and remissions at intervals of a few months, while others will return for examination either days or months after the onset of a relapse phase. It is rare to find two patients who exhibit a similar pattern of disease activity and threshold for seeking medical help. In this study, patients with active disease had begun experiencing problematic symptoms between 1 week and 12 weeks before blood sampling.

The SCCI scores indicated that there was a real difference in disease activity between the active and remission phases. There was a significant difference between the values for this disease index ($p = 0.02$), despite the relatively small number of patients who could be studied across the two periods (Table 1).

Neopterin and lipid peroxidation: Table 1 and 2 summarise the values of neopterin and lipid peroxidation products (malondialdehyde and 4-hydroxynonenal) in those

Table 1: Measured parameters in patients moving from an active disease state into remission during the course of this study (n=5)

| | Active disease state | Remission state |
|--|----------------------|--------------------|
| SCCI score | 5.00±1.34 | 1.00±0.55 (p=0.02) |
| Neopterin (ng mL ⁻¹) | 4.42±1.28 | 3.30±0.65 |
| Peroxidation products (µM) | 0.49±0.16 | 0.38±0.11 |
| Tryptophan (µM) | 46.80±10.48 | 39.19±9.4 |
| Kynurenine (µM) | 2.12±0.35 | 2.36±0.36 |
| Kynurenic acid (nM) | 44.48±9.75 | 38.10±10.75 |
| ESR (mm h ⁻¹) | 24.20±8.11 | 33.60±14.64 |
| CRP (mg L ⁻¹) | 22.94±16.84 | 10.84±6.45 |
| Haemoglobin (g dL ⁻¹) | 13.94±0.31 | 14.04±0.48 |
| White cell count (x10 ⁹ L ⁻¹) | 9.24±1.42 | 8.46±1.92 |
| Platelet count (x10 ⁹ L ⁻¹) | 406.20±88.25 | 365.80±80.76 |

Table 2: Measured parameters in all patients with active disease (n=9) and those in remission (n=7) compared with controls (n=12)

| | All active patients | All remissions | Controls |
|----------------------------------|---------------------|---------------------------|---------------|
| SCCI score | 5.22±0.89 | 0.86±0.40 (p = 0.0012) | N/A |
| Neopterin (ng mL ⁻¹) | 4.55±0.88 | 3.61±0.58 | 4.01±0.38 |
| peroxidation products (µM) | 0.55±0.10 | 0.43±0.08 | 1.13±0.23ab |
| Tryptophan (µM) | 46.00±5.90 | 40.20±6.50 | 58.70±3.10b |
| Kynurenine (µM) | 1.81±0.25 | 2.13±0.32 | 1.78±0.11 |
| Kynurenic acid (nM) | 36.98±7.13 | 34.35±7.86 | 160.00±27.0cd |

a: $p < 0.05$ compared with active patients

b: $p < 0.05$ compared with remissions

c: $p < 0.01$ compared with active patients

d: $p < 0.001$ compared with remissions

patients moving from active disease into remission. No differences were noted in the levels of neopterin or lipid peroxidation products in these states etc. However, peroxidation products were significantly lower in both active and remission conditions compared with the control group.

Routine parameters: Values for ESR, haemoglobin, white cell count and platelet count showed no significant differences between the active and remission states. (Table 1), or between the total pool of active patients compared with those in remission. Although there was a large variation in the range of CRP values, the mean levels during active disease and remission (22.52 ± 10.80 v 10.07 ± 4.96 mg L⁻¹) were not significantly different.

Kynurenines: While the mean level of plasma tryptophan was higher during active disease and kynurenine concentrations were lower, suggestive of a decreased activity of the kynurenine pathway, the differences did not reach statistical significance when compared with the values of patients in remission (Table 1). Tryptophan levels in the patients during remission were significantly lower than in the control group.

Similarly there was no difference in the levels of kynurenic acid between the two states, nor between any of the kynurenines in the total population of patients in

active disease or remission (Table 2). The levels of kynurenic acid, however, were significantly lower in both patient groups compared with the controls.

Correlations: There were significant correlations between the levels of tryptophan and CRP ($p=0.01$) and tryptophan and haemoglobin ($p=0.026$) in the total patient group. There were also very highly significant correlations between platelet counts and CRP ($p=0.005$) and platelets and white cells ($p=0.0009$).

DISCUSSION

Neopterin: Increased levels of immunological markers are usually assumed to relate to the intensity of an inflammatory reaction, caused by the activation of monocytes and macrophages. Neopterin has been widely studied as a possible marker for diseases involving macrophage activation, such as viral infection, malignant disease and autoimmune disorders^[1-4,23]. From studies of inflammatory bowel disease, the levels of neopterin have been correlated with disease severity in Crohn's disease^[12,24,25] while others have reported no correlation^[26], no overall differences in neopterin levels in patients compared with controls^[27] or correlation with disease duration but not severity^[28]. The absence of a significant change in the present study suggests that in ulcerative colitis, as distinct from Crohn's disease, the transition from active disease to remission is not associated with a major activation of macrophages. These results support the detailed analysis by Nielsen *et al.*^[29] who after considering a wide range of potential disease markers including neopterin, concluded that none of the currently available markers were specific or sensitive enough to correlate reliably with inflammatory bowel disease activity.

Similarly, despite the clearly significant difference in disease activity (the SCCAI score) between the active and remission phases of ulcerative colitis in these patients, routine measures of inflammatory status such as ESR do not differ between the two states, making it unlikely that a simple change of generalised inflammatory activity is responsible.

Lipid peroxidation: Activated macrophages also produce reactive oxygen species such as superoxide which induce lipid peroxidation in cell membranes and are believed to contribute to disease progression in inflammatory and auto-immune disorders. While the previous study indicated increased levels of these compounds in patients compared with controls, indicating that the patient group was functioning at a higher level of oxidative stress, the

present study suggests that there is no temporal difference as patients move from active disease into remission. Indeed, the levels measured here lie between the control and patient groups examined in the previous study, probably accounting for the absence of a significant difference. Present results therefore support the report by Koch *et al.*^[30] who could not detect any significant changes in either total antioxidant capacity or the amount of lipid peroxidation products in samples of colon tissue from patients with active or inactive ulcerative colitis.

Kynurenines: The pathway from tryptophan to quinolinic acid is now considered to play a significant role in the regulation of neuronal excitability in the brain^[15]. Quinolinic acid itself has powerful excitatory actions on neurons, acting directly on the N-methyl-D-aspartate (NMDA) receptor to induce depolarisation and neuronal damage^[31,32]. In contrast, the side-arm of the pathway leads to kynurenic acid, which is an antagonist at the NMDA-sensitive and other subtypes of glutamate receptor on neurons^[13-15].

Glutamate can act either as a neurotransmitter in the peripheral nervous system, or at least as a modulator of classical transmitter systems^[7-12]. There is evidence in non-human species for glutamate release from neurons^[33] and for the presence of glutamate receptors in the intestine^[34] with evidence specifically for receptors of the NMDA subtype in the myenteric plexus^[9,10]. These receptors are believed to play a role in gut motility and secretion, activation by glutamate increasing contractile activity^[11]. A lowered level of the glutamate antagonist kynurenic acid could lead to increased activation of the glutamate receptors and thus contribute to the symptomatology of ulcerative colitis.

The enzymes of the kynurenine pathway are activated by immune stimulation, leading to large increases in the generation of the NMDA agonist quinolinic acid and its antagonist, kynurenic acid^[5,6]. The fact that no differences in kynurenine levels have been demonstrable between active and remission patients in this study is consistent with the data on neopterin suggesting that there is no change in macrophage-related inflammatory activity during the transition from active inflammatory bowel disease and remission. On the other hand there are clear differences in the levels of tryptophan and the glutamate antagonist kynurenic acid between patients and controls, the latter being almost 5 fold greater. This low value could lead to increased activation of neuro-excitatory glutamate receptors by endogenous or dietary glutamate, contributing to the susceptibility of patients to inflammatory bowel disease.

However, the data strongly suggested that the switch from remission to active disease is not accompanied by changes in the normally accepted macrophage-derived markers of inflammation. It may be that other factors such as alterations of nutritional or endocrine status may account for the transition, or a greater role for monocyte-related inflammation compared with macrophages, together with the potentially raised neuronal excitability cause by low levels of kynurenic acid.

No differences in the levels of neopterin, lipid peroxidation products or kynurenine metabolites were found between patients with active ulcerative colitis and patients during a period of remission, even when a group of patients was examined who experienced both states during the course of this study. It is concluded that there is no change in immune status during the transition from active inflammatory bowel disease to remission.

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