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Effects of vagus nerve stimulation on pro- and anti-inflammatory cytokine induction in patients with refractory epilepsy

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ABSTRACT

The role of the vagus nerve in controlling and modulating inflammatory responses under physiological conditions has been investigated. The purpose of this study is to assess changes in the immunological state evoked by vagus nerve stimulation in humans, by measuring cytokines produced by peripheral blood mononuclear cells (PBMC). We compared induction of IL-1β, IL-6, IL-8, IL-10 and TNF-α by lipopolysaccharide (LPS)-stimulated PBMC which were isolated from patients treated with vagus nerve stimulation for refractory epilepsy. We observed a significant decrease in IL-8 induction by LPS-stimulated PBMC after 6 months of vagus nerve stimulation in comparison to the pre-stimulation state. No significant changes were seen in the induction of IL-1β, TNF-α, IL-6 or IL-10. The present study shows that cytokine induction by PBMC isolated from patients with refractory epilepsy is altered by long-term vagus nerve stimulation.

1. Introduction

Inflammation is a normal response to a disturbed homeostasis caused by infection, injury or trauma. The onset of inflammation is characterized by the release of pro-inflammatory mediators from activated macrophages (Baumann and Gauldie, 1994). These mediators are responsible for activating an inflammatory cascade and the immune-to-brain communication plays a crucial role in modulating this inflammatory response (Elenkov et al., 2000; Webster et al., 2002). Possible mechanisms by which cytokines signal the brain include blood-borne and neural routes (Banks et al., 1991; Van Dam et al., 1993; Saper and Breder, 1994; Tilders et al., 1994; John and Buckingham, 2003) or indirect pathophysiological changes such as hypotension or hypovolemia (Tkacs and Strack, 1995). Recently, an anti-inflammatory pathway mediated by the vagus nerve was identified as one of these neuro-immunomodulatory pathways (Tracey, 2002). The vagus nerve or tenth cranial nerve accounts for the parasympathic innervation of most visceral organs. Vagal afferent fibers signal the brain when peripheral inflammation occurs (Goehler et al., 2000). These afferent fibers primarily project to the nucleus of the solitary tract located in the brain stem (Berthoud and Neuhuber, 2000). Evidence exists that central immunomodulation is achieved by activation of the hypothalamic-pituitary adrenal (HPA)-axis which regulates cortisol release, the sympathetic nervous system, and a reflex response activating vagal efferent fibers called the cholinergic anti-inflammatory pathway (Pavlov et al., 2003). Following activation of this pathway acetylcholine (Ach) is released in the vicinity of tissue macrophages, leading to an inhibition of cytokine release through interaction with the macrophage nicotinic Ach receptors (Borovikova et al., 2000). Several animal studies, mainly in sepsis models, demonstrated the possibility of influencing the immune response by electrical or chemical stimulation of the vagus nerve (Wu et al., 2007; Borovikova et al., 2000; Bernik et al., 2002). These findings open up new therapeutic opportunities for many inflammatory diseases. Currently, electrical stimulation of the vagus nerve is a treatment for patients with refractory epilepsy and depression. However, the effect of vagus nerve stimulation on the immune system in patients has only been studied in one preliminary report where a significant increase in circulating cytokines after vagus nerve stimulation treatment was observed (Corcoran et al., 2005).

The purpose of this study is to assess the immunological state before and after vagus nerve stimulation in humans by measuring cytokines produced by LPS-stimulated peripheral blood mononuclear cells (PBMC). We compared induction of IL-1β, IL-6, IL-8, IL-10 and TNF-α by LPS-stimulated PMBC isolated from patients treated with vagus nerve stimulation for refractory epilepsy before initiation of the stimulation and after 3 weeks and 6 months of stimulation.

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2. Patients and methods

2.1. Study population

Ten patients with refractory epilepsy (6 females, 4 males) with a mean age of 37 years (range 16–61) were included in this prospective study. All patients were found eligible for implantation with a vagus nerve stimulator (NCP, Cyberonics Inc. USA) as a treatment for refractory seizures. Brain MRI and long-term video-EEG monitoring were carried out in all patients as a part of their presurgical evaluation for epilepsy. All patients took a combination of several antiepileptic drugs (AEDs) (range 2–5) in different combinations. The AED regimen of all patients remained unchanged throughout the study. None of the patients had any known chronic disease besides epilepsy.

Vagus nerve stimulation parameters were adjusted following normal clinical practice i.e. stimulation intensity was increased every 3 to 4 weeks during the first 6 months until side-effects occurred or sufficient seizure reduction was seen. At 6 months of follow-up, the mean stimulation intensity was 1.5 mA (range: 1–1.75).

Clinical patient characteristics such as age at time of implantation, epilepsy type (generalized versus localized) and the presence of a focal lesion visible on brain MRI were correlated with changes in cytokine levels.

Response to vagus nerve stimulation at 6 months was defined as the percentage reduction in mean monthly seizure frequency at 6 months of treatment in comparison to the mean monthly seizure frequency before the start of vagus nerve stimulation. Response to vagus nerve stimulation at 6 months was also correlated with changes in cytokine levels.

Ethics committee approval was obtained (EC 2005/238) and informed consent was signed by the patient or their legal representative, before the start of the study.

2.2. Blood sampling

All blood samples were taken between 8 and 9 a.m. Patients were asked to remain sober and not to take their AEDs until after the blood sample collection.

The first blood sample was taken before the start of vagus nerve stimulation. Patients underwent device implantation 2 to 3 weeks earlier. Device implantation is considered to be minor surgery with subcutaneous placement of a pulse generator and a cuff electrode wounded around the left vagus nerve. Patients were dismissed from hospital 2 days after surgery. None of the patients showed signs of post-operative wound or device infection.

The second blood sample was taken 3 to 4 weeks after the start of vagus nerve stimulation and the third blood sample was taken 6 months after the start of vagus nerve stimulation.

2.3. Isolation and stimulation of peripheral blood monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated by magnetic cell sorting (MACS) using the Human Monocyte Isolation Kit II (Miltenyi Biotec, Amsterdam, The Netherlands). Venous blood (36 ml) was collected into EDTA tubes and immediately processed. PBMCs were enriched using density gradient centrifugation with Ficoll–Pacque (Amersham Biosciences, Roosendaal, The Netherlands). PBMCs were incubated with a cocktail of biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123 and glycophorin A for 10 min at 4 °C, followed by an indirect magnetic labelling using antibiotin microbeads for 15 min at 4 °C. This labelled cell suspension was loaded onto a MACS LS separation column to result in the negative selection of a highly purified untouched monocyte population, confirmed by flow cytometry (>90% purity).

Isolated monocytes were suspended at 10^6 cells/mL in RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 0./5% L-glutamine and 10% foetal calf serum and incubated for 24 h in culture medium (negative control) or medium supplied with 1 μg/ml LPS (Ultra Pure *E. coli* K12 LPS, Invivogen, San Diego, CA, USA). Supernatants were collected and stored at −20 °C prior to analysis.

2.4. Cytometric Bead Array immunoassay

Concentrations of IL-1β, IL-6, IL-8, IL-10 and TNF-α in the culture supernatants were measured simultaneously using a Cytometric Bead Array (CBA) [Human Inflammation Kit, Becton Dickinson (BD), Erembodegem, Belgium] according to the manufacturer's instructions. This technique uses amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. Flow cytometric analysis was performed using a BD FACSCalibur™ scan. Data acquisition and analysis were performed using BD CBA analysis software. Cytokine induction was calculated as the concentration of the cytokine in the culture medium after stimulation minus the concentration in the culture medium of unstimulated monocytes.

2.5. IL-6 and IL-8 ELISA

IL-6 and IL-8 concentrations were assessed in duplo with a sandwich ELISA-technique using the R & D DuoSet human IL-6 and IL-8 ELISA kit (R & D Systems, Abingdon, UK). The assay was run according to the manufacturer's instructions.

2.6. Routine laboratory tests

Routine laboratory tests were performed using standard procedures. In peripheral blood, hemogram, ionogram, renal and liver function, C-reactive protein (CRP), and erythrocyte sedimentation rate were measured, to reveal any acute infection at the time of measurements.

2.7. Statistical analysis

All calculations were performed using SPSS 15.0.

Due to the low number of patients non-parametric statistics were used for the comparison of group medians. Cytokine induction at 3 weeks and 6 months of treatment were both compared with cytokine induction at baseline using the Wilcoxon test. Correlations were analysed using the Spearman correlation test.

3. Results

3.1. Clinical patient characteristics

Ten patients (6F/4M) were included in the study. Eight patients had localized epilepsy, two patients were diagnosed as having generalized epilepsy. Age at time of implantation ranged from 16 to 61 years old. Table 1 gives an overview of patient characteristics.

3.2. Routine laboratory testing

CRP values were below 0.2 mg/dl in all patients at all time points, except for 3 patients who had CRP values of 3.2; 3.5 and 9.5 mg/dl in the blood sample taken after 3 weeks of vagus nerve stimulation. The patient with a CRP value of 9.5 mg/dl showed the highest induction of IL-6, IL-8 and TNF-α and a very high induction of IL-1β at that time point compared to the other patients. Therefore we did not include the results of that patient after 3 weeks of treatment with vagus nerve stimulation for further analysis.

Two patients (patient 3 and patient 8) had low white blood cell count, possibly related to their treatment with AEDs. All other laboratory tests were within the normal range.

Table 1
Patient characteristics.

Patients	M/F	Age at time of implantation (years)	Epilepsy type	Brain MRI	AEDs	% Seizure reduction after 6 months of VNS
1	F	33	L	Normal	LEV, TPM, Clon	53
2	M	32	L	Status after right parietal resection	EXC, LTG, TPM, Clon	46
3	M	16	IGE	Normal	LTG, VPA, ETX, PGB, Clon	93
4	F	49	L	status after left temporal resection	CBZ, PGB, PB	0
5	F	33	L	Sequellae left parietal bleeding	LEV, CBZ, Clon	0
6	F	44	L	Multiple subcortical bifrontal hemosiderine	LEV, TPM, VPA	0
7	M	25	L	Left hippocampal structure loss, general atrophia	VPA, CBZ, ETX	31
8	F	38	L	Extensive damage right hemisphere	LEV, VPA, OXC, Clon	0
9	M	37	L	Normal	VPA, LTG	3
10	F	61	IGE	Normal	LTG, PGB, DPH, Clon	0

Table legend:
L: localized, IGE: idiopathic generalized epilepsy, LEV: levetiracetam, TPM: topiramate, clon: clonazepam, EXC: exegran, LTG: lamotrigine, VPA: valproate, ETX: ethosuximide, PGB: pregabaline, PB: phenobarbital, CBZ: carbamazepine, OXC: oxcarbazepine, DPH: fenytoïne, VNS: vagus nerve stimulation, MRI: magnetic resonance imaging.

3.3. Monocyte responses to LPS stimulation

Very low to absent cytokine release was seen in the supernatant of unstimulated monocytes after 24 h of culture (data not shown). LPS at a concentration of 1 μg/ml induced a marked production of the five cytokines by PBMCs of all patients before, after 3 weeks and after 6 months of vagus nerve stimulation. The highest levels were found for IL-8 and IL-6, followed by IL-1β, TNF-α and IL-10. Levels of IL-6 and IL-8 were reassessed in a higher dilution using ELISA because IL-8 and IL-6 concentrations surmounted the upper detection limit of the CBA kit in the chosen dilution.

An overview of all cytokine measurements is given in Table 2.

After 3 weeks of stimulation, no significant changes were seen in cytokine induction when compared to baseline values. The induction of IL-8 by LPS-stimulated PBMCs was significantly reduced after 6 months of vagus nerve stimulation when compared to baseline ($p<0.05$) (Fig. 1). A decrease of IL-8 of >30% was seen in 6/10 patients. The values of IL-8 induction by LPS-stimulated PBMC for all patients at baseline and after 6 months of VNS are given in Table 3.

No significant changes were seen in the induction of IL-1β, TNF-α, IL-6 or IL-10 after 6 months of stimulation.

3.4. Cytokine induction according to patient characteristics

In the two patients with generalized epilepsy, there was a marked increase in IL-10 induction after 6 months of vagus nerve stimulation, compared to the patients diagnosed with localized epilepsy (Fig. 2). There were no significant differences in cytokine response over time according to age at time of implantation, sex or the presence of a focal lesion visible on brain MRI.

3.5. Cytokine induction according to patient's response to vagus nerve stimulation

No correlation was found between the patients' responses to vagus nerve stimulation therapy at 6 months of follow-up and changes in cytokine induction over time. This was also true when comparing the patient's response with the absolute value of cytokine induction at baseline.

4. Discussion

The present study shows that in vitro cytokine induction by LPS-stimulated PBMCs isolated from patients with refractory epilepsy is altered by long-term vagus nerve stimulation. We observed a significant decrease in IL-8 induction by LPS-stimulated monocytes after 6 months of vagus nerve stimulation of all patients in comparison to the pre-stimulation state. This is the first study in epilepsy patients showing an immunomodulatory effect of electrical stimulation of the vagus nerve.

In the last few years, the role of the vagus nerve in controlling and modulating inflammatory responses under physiological conditions has been described extensively. The afferent vagus nerve detects the presence of inflammation in the periphery, notifies the brain and triggers an anti-inflammatory response (Watkins et al., 1995; Tracey, 2002; Borovikova et al., 2000). This anti-inflammatory response can be generated by activation of the HPA-axis, the sympathetic nervous system, and a reflex response activating vagal efferent fibers (Pavlov et al., 2003; Fleshner et al., 1998). Activation of the HPA-axis and the sympathetic nervous system results in the release of glucocorticoids and catecholamines respectively, both strong regulators of the immune response (Berczi et al., 2009). (Berczi et al., 2009)The efferent arm of the inflammatory reflex is called the cholinergic anti-inflammatory pathway and was first described by Borovikova et al. (Borovikova et al., 2000). Upon activation of this pathway, Ach is released by vagal nerve efferents and inhibits cytokine release through binding on alfa-7 nicotinic Ach receptors located on tissue macrophages (Wang et al., 2003). Several experiments have investigated this immunomodulatory capacity of the vagus nerve. Most of these experiments were performed in animal models for sepsis and resulted in an attenuation of the systemic inflammatory response upon electrical or pharmacological stimulation of the vagus nerve (Borovikova et al., 2000; Wu et al., 2007; Bernik et al., 2002). Only one other study in patients with pharmacoresistant depression investigated the immunological actions of vagus nerve stimulation in humans (Corcoran et al.,

Table 2
Cytokine induction by PBMC.

Cytokine	Baseline ($n=10$)	After 3 weeks VNS ($n=9$)	After 6 months VNS ($n=10$)
IL-10	968 (range 85–1901)	505 (range 261–3263)	675 (range 128–2323)
TNF- α	1007 (range 71–7658)	691 (range 57–3764)	927 (range 22–3571)
IL-1β	7116 (range 3103–34,465)	8274 (range 3299–31,281)	5811 (range 2600–27,995)
IL-6	69,450 (range 38,251–184,922)	67,996 (range 23,712–116,064)	49,869 (range 32,098–147,871)
IL-8	381,116 (range 268,220–836,697)	361,995 (range 210,453–513,996)	301,180 (range 232,525–559,339)*

Values of IL-10, TNF-α, IL-1β, IL-6 and IL-8 induction by PBMC at baseline ($n=10$), after 3 weeks ($n=9$) and after 6 months ($n=10$) of VNS are given. Values are presented as median (range) in pg/ml. *denotes statistical significance ($p<0.05$). VNS: vagus nerve stimulation.

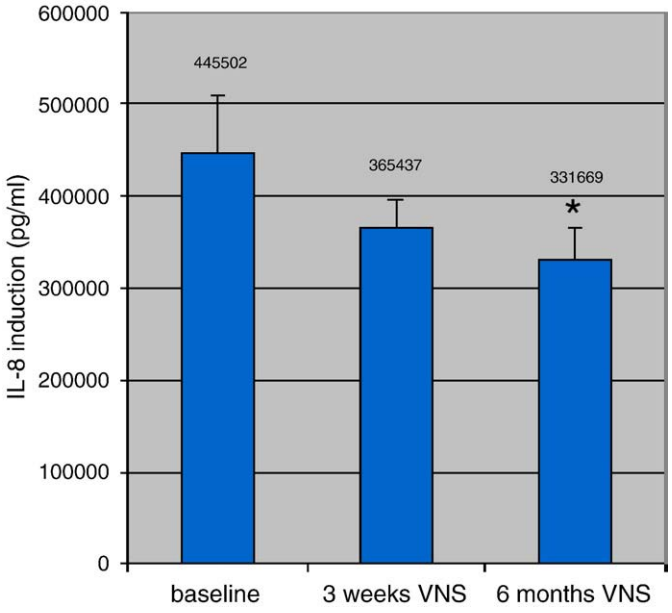

Fig. 1. IL-8 induction after VNS. Mean IL-8 induction in pg/ml by PMBC before, after 3 weeks and after 6 months of vagus nerve stimulation. *denotes a statistically significant ($p<0.05$) reduction of IL-8 after 6 months of vagus nerve stimulation when compared to baseline (Wilcoxon test).

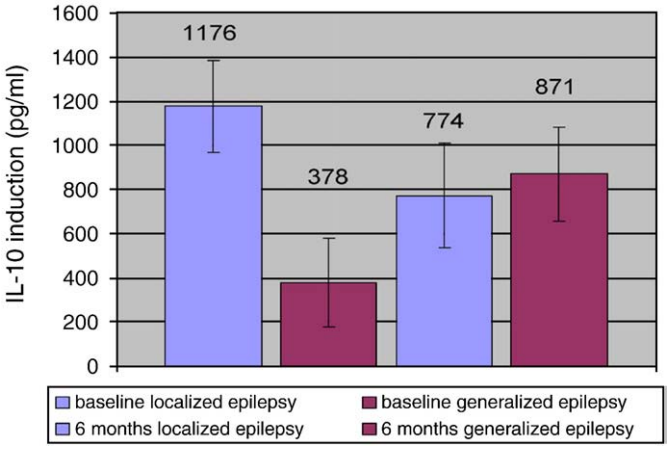

Fig. 2. IL-10 induction according to epilepsy type. Mean IL-10 induction by PBMC before and after 6 months of vagus nerve stimulation in patients with localized epilepsy ($n=8$) and patients with generalized epilepsy ($n=2$). Mean Il-10 decreased from 1176 (SEM: 206) to 775 (SEM: 237) pg/ml in patients with localized epilepsy and increased from 378 (SEM: 293) to 871 (213) pg/ml in patients with generalized epilepsy after 6 months of VNS.

2005). Corcoran and co-workers found a significant increase in plasma levels of IL-6, TNF-α and TGF-β after 3 months of vagus nerve stimulation therapy.

The patients included in this study were all suffering from refractory epilepsy. Electrical stimulation of the vagus nerve is a worldwide accepted treatment for those patients. We hypothesised that vagus nerve stimulation would influence the immunological state through activation of the previously described anti-inflammatory pathways. For this purpose, in vitro cytokine induction by LPS-stimulated PMBC was measured before, after 3 weeks and after 6 months of stimulation. We tried to minimize the influence of external factors on measurements by leaving AEDs unchanged during the whole study period, by taking blood samples in sober patients at the same time of the day, and by measuring CRP values to exclude acute inflammatory episodes.

The data obtained show a significant decrease of IL-8 induction by LPS-stimulated PBMC after long-term vagus nerve stimulation, reflecting a possible modulatory effect of vagus nerve stimulation on immune cell reactivity. IL-8 is a chemokine produced by macrophages and other immune cells and one of the major mediators of the inflammatory response. The primary function of IL-8 is the induction of chemotaxis in its target cells, mainly neutrophil granulocytes (Baggiolini and Clark-Lewis, 1992). The role of IL-8 in epilepsy has not been studied extensively but intrathecal IL-8 synthesis may be associated with seizure activity (Billiau et al., 2007). In a large variety of other inflammatory diseases such as cardiovascular, urogenital and gastric diseases, IL-8 not only plays a central role in the pathogenesis, but also appears a promising biomarker for detecting disease in an early phase or evaluating disease activity (Ono, 2008; Aukrust et al., 2008; di Mario and Cavallaro, 2008; Liu et al., 2009).

Table 3
IL-8 induction by PBMC.

Patients	Baseline (pg/ml)	After 6 months VNS (pg/ml)	% Reduction
1	836,697	559,339	33
2	528,022	315,712	40
3	277,479	309,855	−12
4	707,570	488,894	31
5	526,109	345,609	34
6	401,135	232,525	42
7	361,096	253,720	30
8	268,220	292,505	−9
9	279,570	244,337	13
10	269,121	274,199	−2

Values of IL-8 induction by PBMC at baseline and after 6 months of VNS are given for all patients. Values are presented in pg/ml. VNS: vagus nerve stimulation.

No significant changes were seen in the induction of IL-1β, TNF-α, IL-6 or IL-10 in the group as a whole. However, in the two patients diagnosed with generalized epilepsy, there was a marked increase in IL-10 induction after 6 months of vagus nerve stimulation, compared to the patients with localized epilepsy. To our knowledge, the differential role of IL-10 in localized or generalized epilepsy has not been studied so far. Because only two patients with generalized epilepsy were included in this study, these preliminary results should be confirmed in future experiments containing larger patient groups.

In our study, a large variability of cytokine induction at all time points between patients was observed. This could be due to the different combinations of AEDs patients were taking, as it is known that AEDs influence the production of cytokines (Mlodzikowska-Albrecht et al., 2007). However, AED dosages and combinations were not changed during the study, making patient to patient comparison over time valuable and reliable.

Another explanation for the high inter-patient variability is the fact that the study population was chosen based on their need for treatment with vagus nerve stimulation, and not based on a mutual epileptic disorder. 'Refractory epilepsy' is not a single disease entity, but consists of various epilepsy syndromes with different causes and progress (French, 2007). This is confirmed in our study by the fact that the patients with generalized epilepsy reacted differently compared to those with localized epilepsy regarding IL-10 induction after vagus nerve stimulation.

To conclude, our study showed an anti-inflammatory effect of electrical stimulation of the vagus nerve in humans. This finding opens new perspectives regarding the treatment of many inflammatory diseases.

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