Tropical Biomedicine 40(4): 406-415 (2023)
https://doi.org/10.47665/tb.40.4.005

RESEARCH ARTICLE

Cerebrospinal fluid inflammatory cytokine profiles of patients with neurotropic parasitic infections

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ARTICLE HISTORY

Received: 1 June 2023
Revised: 17 July 2023
Accepted: 17 July 2023
Published: 31 December 2023

ABSTRACT

The pathogenesis of chronic parasitic central nervous system (CNS) infections, including granulomatous amoebic meningoencephalitis (GAE), cerebral toxoplasmosis (CT), and neurocysticercosis (NCC), is primarily due to an inflammatory host reaction to the parasite. Inflammatory cytokines produced by invading T cells, monocytes, and CNS resident cells lead to neuroinflammation which underlie the immunopathology of these infections. Immune molecules, especially cytokines, can therefore emerge as potential biomarker(s) of CNS parasitic infections. In this study, cerebral spinal fluid (CSF) samples from suspected patients with parasitic infections were screened for pathogenic free-living amoebae by culture (n=2506) and PCR (n=275). Six proinflammatory cytokines in smear and culture-negative CSF samples from patients with GAE (n = 2), NCC (n = 7), and CT (n = 23) as well as control (n = 7) patients were measured using the Multiplex Suspension assay. None of the CSF samples tested was positive for neurotropic free-living amoebae by culture and only two samples showed Acanthamoeba 18S rRNA by PCR. Of the six cytokines measured, only IL-6 and IL-8 were significantly increased in all three infection groups compared to the control group. In addition, TNFα levels were higher in the GAE and NCC groups and IL-17 in the GAE group compared to controls. The levels of IL-1β and IFNγ were very low in all the infection groups and the control group. There was a correlation between CSF cellularity and increased levels of IL-6, IL-8, and TNFα in 11 patients. Thus, quantifying inflammatory cytokine levels in CSF might help with understanding the level of neuroinflammation in patients with neurotropic parasitic diseases. Further studies with clinico-microbiological correlation in the form of reduction of cytokine levels with treatment and the correlation with neurological deficits are needed.

Keywords: Cytokines; multiplex suspension assay; Granulomatous amoebic encephalitis; Neurocysticercosis; Cerebral toxoplasmosis.

INTRODUCTION

Chronic parasitic central nervous system (CNS) infections such as granulomatous amoebic meningoencephalitis (GAE), cerebral toxoplasmosis (CT), and neurocysticercosis (NCC), continue to be a health problem, particularly in developing countries (Prandota, 2010; Del Brutto & Garcia, 2021; Raju *et al*., 2022). The persistence of inflammatory processes in GAE and CT, or loss of active immune suppression in NCC, results in parenchymal tissue damage, with severe neurological consequences (Mishra *et al*., 2009; Kot *et al*., 2021). The pathogenesis of these diseases is primarily due to an inflammatory host reaction to the parasite, resulting in symptoms such as headache, migraine, nausea, vomiting, fever, intracranial hypertension, hydrocephalus, ischemia, epileptic seizures, schizophrenia, stroke, focal neurologic deficits, and altered sensorium, in addition to physical obstruction of the flow of cerebral spinal fluid (CSF) (Prandota, 2010; Del Brutto *et al*., 2016; Kot *et al*., 2021).

Prior exposure to Acanthamoeba antigens and the inability of macrophages to phagocytize larger trophozoites result in a hypersensitivity reaction that develops into a granulomatous inflammatory lesion with epithelioid cells and pathogenic T cells that may cause substantial tissue destruction in GAE (Baig *et al*., 2015; Kot *et al*., 2021). In NCC, the *Taenia solium* larva in its vesicular stage lives for several years by blocking the complement system, increasing regulatory T cells, and degrading immunoglobulins, resulting in an anti-inflammatory phase that is asymptomatic (Del Brutto *et al*., 2016). The destruction of larvae by therapeutic treatment or by natural degeneration causes acute or subacute inflammation to colloidal and granular stages or a chronic inflammatory response to the calcified parasite, which is responsible for the severe neuropathology (Garcia *et al*., 2020). *Toxoplasma gondii* establishes

Published by Malaysian Society of Parasitology and Tropical Medicine.

intracellular cysts in the brain in almost one third of the world's population and is asymptomatic in healthy adults (Carruthers & Suzuki, 2007). An imbalance between proinflammatory and anti-inflammatory cytokines, the administration of drugs for some diseases, or decreased T cell influx into the CNS due to AIDS or chemotherapy may result in the reactivation of latent CT and the development of toxoplasma encephalitis (TE), which is characterized by cyst rupture, tachyzoite conversion, and parasite replication within the CNS (Prandota, 2010).

Neuroinflammation during infection is driven by cytokines produced by invading T cells and monocytes, resident astrocytes, and microglia (Becher *et al*., 2017). Pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNFα are primarily produced by monocytes/macrophages but also by other cells. IFNγ is produced by activated T cells, and IL-17A is produced by a subset of CD4 cells called T helper 17 (Th17) cells (Borish & Steinke, 2003; Becher *et al*., 2017). In addition to their unique functions in cellular influx and leucocyte activation for pathogen clearance, these inflammatory cytokines secreted locally in the inflamed CNS act on T cells and macrophages to maintain their pathogenic properties in the presence of parasitic antigens and counteract the natural tendency for resolution of the immune response (Becher *et al*., 2017). Increased levels of some of the cytokines were shown to be an indicator of neuroinflammation and long-term neurologic and cognitive impairment; hence, quantifying them in the CSF of patients with GAE, NCC, and CT can provide valuable information about patients' immune status (Shabani *et al*., 2017; Cuff *et al*., 2020). Currently, there are no published reports on CNS proinflammatory cytokine profiles in patients with GAE and only few reports exist on CSF cytokine profiles in patients with NCC and CT (Kashyap *et al*., 2012; Verma *et al*., 2011). In this study, the levels of six proinflammatory cytokines (IL-1β, IL-6, IL-8, IL17A, IFNγ and TNFα) that are considered important in neurotropic parasitic diseases were measured in smear and culture-negative CSF samples from patients with GAE, NCC, and CT using the Multiplex Suspension assay which has the capacity to detect and quantify multiple cytokines simultaneously in the same sample.

MATERIALS AND METHODS

Clinical samples

From January 2020 to December 2022, a total of 2506 CSF samples from patients with headache, epilepsy and suspected encephalitis, bacterial/viral/tuberculous meningitis, tuberculoma were collected after routine microbiological testing from the Department of Neuromicrobiology, National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, India, which is a tertiary care hospital for neurological disorders. Samples were stored at –20°C until they were tested for PCR and cytokine measurements. The patients' e-records were reviewed to collect demographic characteristics such as age, sex, symptoms, risk factors, clinical history/diagnosis, CSF cell count, and serological status (IgG) for CT and NCC. CSF samples from seven patients with normal-pressure hydrocephalus with no evidence of infection or inflammation were used as controls. The study was approved by the Institutional Ethical Committee (IEC), NIMHANS (No. NIMHANS/IEC (BS & NS DIV.) 12th meeting/2018).

Microbiological investigation for free-living amoeba

CSF samples were initially subjected to the following microscopic investigations: cell count with trypan blue, Gram staining, and Ziehl-Neelsen staining. All samples were cultured on blood agar and McConkey agar (HiMedia) for aerobic bacteria, incubated at 37°C, and observed after 24 h. CSF samples were also cultured on non-nutrient agar (NNA) plates coated with *Escherichia coli.* The plates were sealed with parafilm, incubated at 37°C for five to seven days, and observed under a microscope for amoebic trophozoites and cysts (Khurana *et al*., 2012).

DNA extraction, species-specific 18S rRNA PCR, and sequencing to detect neurotropic free-living amoebae

Genomic DNA was extracted from smear and culture-negative CSF samples (n = 275) that were negative for bacterial, viral, fungal etiologies using a column-based Nucleospin Tissue DNA extraction kit (Macherey Nagel, Germany) according to the manufacturer's instructions. Briefly, the centrifuged deposits of CSF samples were mixed with lysis buffer and proteinase K and incubated at 56°C for 1-3 h, followed by incubation at 70°C for 10 min after adding a second lysis buffer. DNA was extracted with ethanol (99-100%), transferred to the Nucleospin column, centrifuged, washed twice, eluted in kit buffers, and stored at –20°C. The DNA concentration (260 nm) and quality (ratio 260/280 nm) in each sample was measured using NanoDrop (Thermo Scientific). PCR for Acanthamoeba species and *Naegleria fowleri* was done using species-specific primers. The primer sequences and the thermal cycling conditions used are shown in Table 1.

Table 1. Primer sets and thermal cycling conditions used for PCR

Gene	Primers	Size in bp	Thermal cycling conditions		Reference
Acanthamoeba 18SrRNA	JDP1 5'-GGCCCAGATCGTTTACCGTGAA-3' JDP2 5'-TCTCACAAGCTGCTAGGGAGTCA-3'	500	94°C for 5 min 94°C for 1 min 55°C for 1 min 72°C for 1 min 72°C for 10 min	35 cycles	da Rocha-Azevedo *et al*., 2009
Acanthamoeba 18SrRNA	F 900 5´-CCCAGATCGTTTACCGTGAA-3´ R 1100 5´-TAAATATTAATGCCCCCAACTATCC-3´	180	95°C for 2 min 95°C for 15 sec 51°C for 30 sec 72°C for 30 sec 72°C for 10 min	35 cycles	Qvarnstrom *et al*., 2005
N. fowleri ITS-1	Fwl 5'-GTGAAAACCTTTTTTCCATTTACA-3' RV1 5'-AAATAAAAGATTGACCATTTGAAA-3'	310	94°C for 3 min 94°C for 30 sec 47°C for 30 sec 72°C for 30 sec 72°C for 5 min	35 cycles	Panda *et al*., 2015

PCR was performed with 25 µM forward and reverse primers, 5-40 ng of DNA template, and 2× PCR Master Mix (DSS Takara Bio India Pvt. Ltd.) in a 25-µl reaction mixture using a Veriti thermal cycler (AB Applied Biosystems). A nested PCR was done to amplify 18S rRNA for *Acanthamoeba* (500 bp and 180 bp). The *Acanthamoeba* (4B) T4 strain isolated from water was used as a positive control. For detecting *N. fowleri* DNA, ITS-1 PCR was done to amplify a 320-bp fragment. A plasmid harboring the *N. fowleri* ITS-1 region was used as a positive control. Gel electrophoresis was performed on 1.5–2 % agarose gel with ethidium bromide, and bands were visualized using the Gbox gel documentation system (Syngene, India). PCR products from agarose gel were purified using the Nucleospin Gel and PCR Clean-Up kit (Macherey Nagel, Germany) and sent to Madauxin, Bangalore, Karnataka, India, for Sanger-based sequencing in both directions. Identification was performed with BLAST against eukaryotic nucleotide sequences archived in the GenBank database (NCBI).

Cytokine measurement using the Luminex assay

The levels of inflammatory cytokines (IL-1β, IL-6, IL-8, IL-17A, IFNγ, and TNFα) in CSF samples from GAE (n = 2), CT (n = 23), NCC (n = 7), and normal-pressure hydrocephalus (n = 7) patients were measured using the Multiplex Suspension assay (BIO-RAD, USA) according to the manufacturer's instructions (Manglani *et al*., 2019). Briefly, 50 µl of 1× magnetic coupled beads were added to a 96-well assay plate and washed twice with wash buffer. Fifty microliters of standards (eight, four-fold dilutions) and samples (diluted 1:2) were added to the respective wells in duplicate and incubated on a shaker at 850 rpm for 30 min. After washing three times, 25 µl of a 1× biotinylated detection antibody mixture was added for 30 min, and 50 µl of a 1× streptavidin-phycoerythrin was added for 10 min in sequential steps and incubated on a shaker at 850 rpm for 30 min and 10 min, respectively. After washing three times, the beads were suspended in 125 µl of assay buffer and mixed on a shaker at 850 rpm for 30 sec. After calibrating and validating the Bio-Plex 200 system, the standard values were entered in the Bio-Plex manager software. Fifty events were captured for each sample using a gate setting of 5000 (low) and 25000 (high). A range of 0.3 to 60000 pg/ml recombinant cytokines was used to establish standard curves, and the detection limits of the assay for the cytokines were as follows:0.3 pg/ml for IL-1β, 0.36 pg/ml for IL-6, 0.92 pg/ml for IL-8, 2.85 pg/ml for IL-17A, 1.11 pg/ml for IFNγ, and 3.81 pg/ml for TNFα.

Statistical analysis

The nonparametric Mann–Whitney test in the SPSS program (IBM SPSS Statistics 23.0) was used to perform statistical comparisons of the level of cytokines between each of the infection groups and the control group. A p value of <0.05 was considered significant.

RESULTS

Demographic characteristics

The median age of patients in the infection groups was 45 years (range 26-70 years), and 72% (23/32) of them were males. The median age of patients in the control group was 68 years (range 57-76 years), and all were males. The major symptoms of patients with GAE, NCC and CT were as follows: headache (n=14; 44%), seizures (n=12; 38%), fever (n=11; 34%), vomiting (n=6; 19%), upper and lower limb weakness, (n=5; 16%), altered sensorium (n=5; 16%), and hemiparesis (n=5; 16%). Few others had disturbances in gait, memory, speech, vision, and behavior. The major risk factors in these patients were HIV (n=19; 59%), alcoholism (n=9; 28%), hypertension and diabetes mellites (n=3 each; 9%) (Table 2).

Culture and molecular characteristics

The 2506 smear and culture-negative CSF samples were negative for motile amoebae under the light microscope and for free-living amoeba on NNA plates. Of the 275 CSF samples screened for free-living amoeba by PCR, only two were positive for Acanthamoeba 180-bp 18SrRNA, and one of these samples was PCR positive in the brain biopsy sample as well (Figure 1). None of the CSF samples tested was positive for *N. fowleri* DNA.

Elevated IL-6 and IL-8 levels in the infection groups

The control group showed fewer cells (0-2 cells/mm^3) in CSF and very low levels of all the cytokines tested compared to the infection groups, with the exception of marginally higher levels of TNFα, IL-6 and IL-8 in one subject. Significantly higher levels of IL-6 ($p < 0.05$) and IL-8 ($p < 0.05$) were observed in all three infection groups compared to the control samples. In addition, TNFα levels were significantly elevated ($p < 0.05$) in the GAE and NCC groups and IL-17A ($p < 0.05$) in the GAE group compared to the control group (Figure 2). The levels of INFγ and IL-1β were very low in patients in all the infection groups and did not differ significantly compared to controls although there were individual patients in the CT and NCC groups who had elevated levels of these cytokines. Two patients in the GAE group (52-610 cells/mm^3), three patients in the NCC group (15-415 cells/mm^3), and six patients in the CT group (19-280 cells/mm^3) had high CSF cell counts, which correlated with increased levels of IL-6, IL-8, and TNFα. However, four patients in NCC group and 14 patients in the CT group had low CSF cell counts in spite of having higher levels of at least one of these cytokines. In the CT group, three patients with low cell count (0-5cells) showed very low levels of IL-6 (4-11 pg/ml), TNFα (0-6 pg/ml), and IL-8 (27-153 pg/ml), like the control group (Table 2).

DISCUSSION

Persistent production of cytokines or their dysregulation leads to the progression of CNS parasitic diseases from an acute to a chronic phase with neuroinflammatory disorders (Mishra *et al*., 2009). The elevation of cytokine levels is also an important marker for neuroinflammation and cognitive and neurological sequalae as has been shown in cerebral malaria cases (John *et al*., 2008; Cuff *et al*., 2020). In this study, we examined six proinflammatory cytokines in CSF samples of patients with GAE, NCC, and CT and found increased levels of IL-8 in 28 (88%) patients, IL-6 in 25 (78%) patients and TNFα in 17 (53%) patients compared to control subjects suggesting that these three cytokines could be used as markers of neuroinflammation in these neurotropic parasitic diseases.

Although the age of the control patients in our study was higher than in the infection groups, similar levels of cytokines were shown in normal individuals who were under 45 years old and those who were over 65 years old, indicating that age does not influence cytokine production (Kim *et al*., 2011). Similar to the present study, in which 77% of the study subjects were males, others have reported higher numbers of male subjects in their studies, despite females being more prone to inflammatory diseases (Kashyap *et al*., 2012; Cavellani *et al*., 2012; Arce-Sillas *et al*., 2018). In this study, increased levels of cytokines in more than half of the patients did not correlate with CSF cellularity. CSF cell count is generally shown to be an unreliable predictor of the degree of cytokine elevations in CSF (Harrison *et al*., 2021).

Only two of the 275 samples screened for neurotropic free-living amoebae by PCR showed Acanthamoeba 18S rRNA in this study, but they were negative on NNA plates. This could be due to the low number of protozoans in the sample, or they may be nonviable. The PCR finding of Acanthamoeba 18S rRNA correlated with neuroimaging and pathology reports. Currently, PCR is used to identify *Acanthamoeba* DNA in CSF and has been considered an alternative to conventional methods (Qvarnstrom *et al*., 2005). Absence of *N. fowleri*, which causes fulminant primary amoebic meningoencephalitis in culture and PCR could be due to tertiary nature of the hospital. The increased serum levels of anti-toxoplasma

Table 2. Demographic characteristics of patients with neurotropic parasitic infections

Lab. No.	Age/Sex	Symptoms & clinical history	Neuroimaging CT/MRI	Clinical diagnosis	CSF cellularity Cells/mm^3	Risk factors
Granulomatous amoebic meningoencephalitis, n=2, acanthamoeba 18S rRNA PCR positive						
2780/20	70/M	Headache and blurring of vision since 3 months	Amoebic meningoencephalitis	? Viral meningitis	610 Polymorphs: 30% Lymphocytes: 70%	Alcoholic
1594/22	49/M	Left upper and lower limb weakness since 1 week Headache	Meningoencephalitis	? TB brain Biopsy proven amoebic meningoencephalitis	56: Polymorphs: 26 Lymphocytes: 20 Degraded cells:10	Hypertension
Neurocysticercosis, n=7, anticysticercal IgG ELISA positive						
2608/21	47/M	R UL & LL weakness, aphasia	Neurocysticercosis	Vasculitic Encephalopathy	Lymphocytes: 2	DM Alcoholic
3552/21	46/M	Headache Multiple episodes of seizures Decompressive craniectomy R FTP and biopsy of the lesion	Neurocysticercosis with tuberculoma	Disorder of CNS	52 Polymorphs: 2 Lymphocytes: 50	Nil
481/22	50/M	Headache, Multiple episodes of seizure since 4 years	HIV encephalitis	Epilepsy	nil	HIV
747/22	33/M	Seizures No neurological deficits	Neurocysticercosis with pulmonary TB	Cysticercosis	nil	Nil
1602/22	55/M	Fever, headache, vomiting VP shunt system	TBM with hydrocephalus	TBM with Hydrocephalus	15: Polymorphs: 4 Lymphocytes: 7 Degraded cells: 4	Hypertension Alcoholic
2420/22	56/M	Fever, L-FMS, L-Homonymous hemianopia, R- spastic Hemiparesis	Neurocysticercosis	Neurocysticercosis	nil	Chronic liver disease with hepatic encephalopathy
5305/22	56/F	Headache and vomiting Grade 3 papilloedema	Neurocysticercosis	Neurocysticercosis meningitis	415 Polymorphs: 41% Lymphocytes: 56% Degraded cells: 3%	DM
Cerebral toxoplasmosis, n=23, Toxoplasma IgG ELISA positive						
1972/20	44/F	R-blurring of vision Headache	Toxoplasmosis / Cryptococcal meningitis	Cryptococcal meningitis / TBM	Nil	HIV
2064/20	44/M	Seizures since 3 years	Toxoplamosis	Epilepsy	Nil	Nil
2178/20	37/F	Headache, vomiting, weakness No focal neurological deficits	TBM with Hydrocephalus	TBM	5: Polymorphs: 2; Lymphocytes: 3	HIV

2264/20	27/M	Seizures, fever No focal neurological deficits	Toxoplasmosis	TB of nervous system	nil	HIV Pulmonary TB
3058/20	47/F	Fever, vomiting and irrelevant talking, Disoriented Quadriparesis	Intraspinal hypotension with ventral cord herniation	MODS Sepsis	Lymphocytes: 2	HIV
3229/20	26/F	Fever, vomiting & irrelevant talking, No focal neurological deficits	Toxoplasmosis	Bacterial meningitis	43 Polymorphs: 18 Lymphocytes: 25	HIV Alcoholic
3286/20	34/M	Fever, seizures Right Hemiparesis	Toxoplasmosis with tuberculoma	-	Lymphocytes: 2	HIV, Jaundice, Tobacco consumption
3531/20	42/M	Headache, R-hemiparesis, altered sensorium, R-dense hemiplegia	Tuberculoma with TBM	TB of nervous system TBM +IRIS	280 Polymorphs: 2% Lymphocytes: 98%	HIV
4155/20	52/F	Headache, R-eye visual disturbances, cognitive decline Confused, Visual acuity R-HMCF	Toxoplasmosis	Toxoplasmosis	Lymphocytes: 6	HIV
4452/20	50/M	Gait imbalance, memory disturbances. No neurological deficits	Toxoplasmosis/ TB	Toxoplasmosis	Lymphocytes: 25	Anterior wall MI, DM
308/21	44/M	Seizures, fever No focal neurological deficits	TBM with hydrocephalus	Epilepsy	Lymphocytes: 3	Hypertension
1268/21	36/M	Headache, seizure, L-UL weakness Left hemiparesis	Deep CVT	CVT	3: Polymorphs: 1 Lymphocytes: 2	HIV
1468/21	61/M	Seizures Altered sensorium	Toxoplasmosis	Toxoplasmosis	Nil	HIV Alcoholic
2162/21	45/M	Altered sensorium, vomiting and fever, Ataxic gait	Toxoplasmosis	Toxoplasmosis	10: Polymorphs: 1 Lymphocytes: 9	HIV, oral Candidiasis
2520/21	31/F	Headache No neurological deficits	Toxoplasmosis	Toxoplasmosis	Polymorphs: 1	HIV
3563/21	37/M	Loss of appetite, behavioral disturbances, R-UL & LL weakness, seizure, Altered sensorium and R-facial nerve palsy and R-hemiparesis.	Toxoplasmosis	-	nil	HIV Alcoholic
996/22	43/M	Fever, Dysarthria, Non fluent speech, R-UMN facial nerve palsy, R-UL spastic monoparesis	Toxoplasmosis	Toxoplasma meningoencephalitis	105 Polymorphs: 3% Lymphocytes: 90% Degraded cells 7%	HIV
1078/22	35/F	Fever, altered sensorium Glasgow Coma Score: E3V1M5, Neck stiffness	Toxoplasmosis	Toxoplasmosis with TBM	Lymphocytes: 6	HIV
1151/22	50/M	Fever, Loss of appetite, cough, Submandibular gland swelling Global Aphasia, R-UL and LL limb spastic hemiplegia	CNS Tuberculoma + IRIS	Tuberculosis of nervous system	7 Polymorphs: 4 Lymphocytes: 3	HIV
2476/22	43/M	Headache, Seizures Neck rigidity	Tuberculoma with TBM	TBM	19: Polymorphs: 3 Lymphocytes: 6 Degraded cells: 10	Smoker, Alcoholic

2974/22	42/F	Headache Vomiting	TBM with Hydrocephalus	Toxoplasmosis with organ involvement	Lymphocytes: 3	Smoker, Alcoholic
3129/22	34/M	Seizures No neurological deficits	Toxoplasmosis	Toxoplasma meningoencephalitis	5: Polymorphs: 2 Lymphocytes: 3	HIV Alcoholic
4580/22	65/M	L-UL & LL weakness, aphasia Glasgow Coma Score: E1VtM3	Toxoplasmosis	Tuberculoma with TBM	33: Polymorphs: 8 Lymphocytes: 25	HIV HBsAg
Controls, n=7						
172/22	76/M	Tremulousness of L-upper & lower limb, difficulty in walking, urinary disturbances - MPVP shunt	NPH	NPH	nil	-
1400/22	72/M	Difficulty in walking, memory disturbances, urinary urgency -VP Shunt	Idiopathic NPH	NPH	nil	HTN and Type 2 DM, post polio residual paralysis
3272/22	61/M	Urinary disturbances, difficulty in walking, 5 episodes of seizures -VP shunt	NPH	NPH	L1	-
3336/22	64/M	Increased forgetfulness, slowness of activity of daily living -Upward gaze restriction and rigidity.	NPH with vascular dementia	NPH	L2	HTN
4515/22	72/M	Urinary incontinence, gait disturbances-Rigidity in all four limbs	NPH	NPH	nil	DM
4526/22	57/M	Difficulty in walking, memory disturbances, urinary urgency -VP shunt	Idiopathic NPH	NPH	nil	-
4899/22	73/M	Difficulty in walking, memory disturbances, urinary urgency - VP shunt	Idiopathic NPH	NPH	nil	DM, HTN

Note: CNS: central nervous system; CSF: cerebrospinal fluid; CVT: cerebral vein thrombosis; DM: Diabetes mellitus; F: Female; FMS fibromyalgia syndrome; FTP:frontotemporoparietal; HBsAg: Hepatitis B surface antigen; HIV: human immunodeficiency virus; HMCF: hand movement close to face; HTN: hypertension; IRIS: Immune Reconstitution Inflammatory Syndrome; L: Left; M: Male; MI: myocardial infarction; MODS - Multiple Organ Dysfunction Syndrome; NPH: normal pressure hydrocephalus; R: Right; TB: tuberculosis; TBM: tuberculous meningitis; UL & LL: Upper limb and lower limb; UMN: upper motor neuron; VP: ventriculoperitoneal.

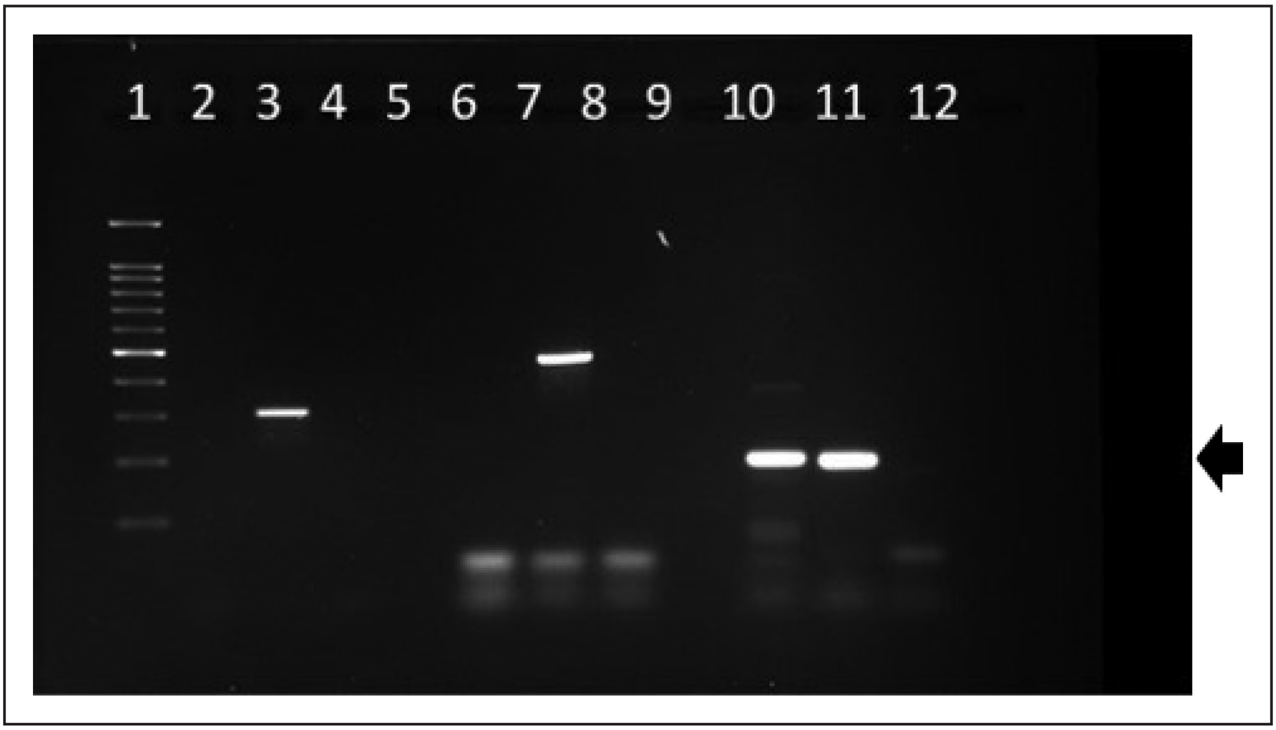

Figure 1. CSF sample showing Acanthamoeba 18S rRNA (180 bp) fragment. Lane1: DNA 100bp ladder; Lane 2: CSF sample 1594; Lane 3: *N. fowleri* ITS plasmid (320bp); Lane 4: Negative control; Lane 6: CSF sample 1594; Lane 7: Acanthamoeba T4 strain (500 bp); Lane 8: Negative control; Lane 10: CSF sample 1594 (180 bp); Lane 11: Acanthamoeba T4 strain (180 bp); Lane 12: Negative control.

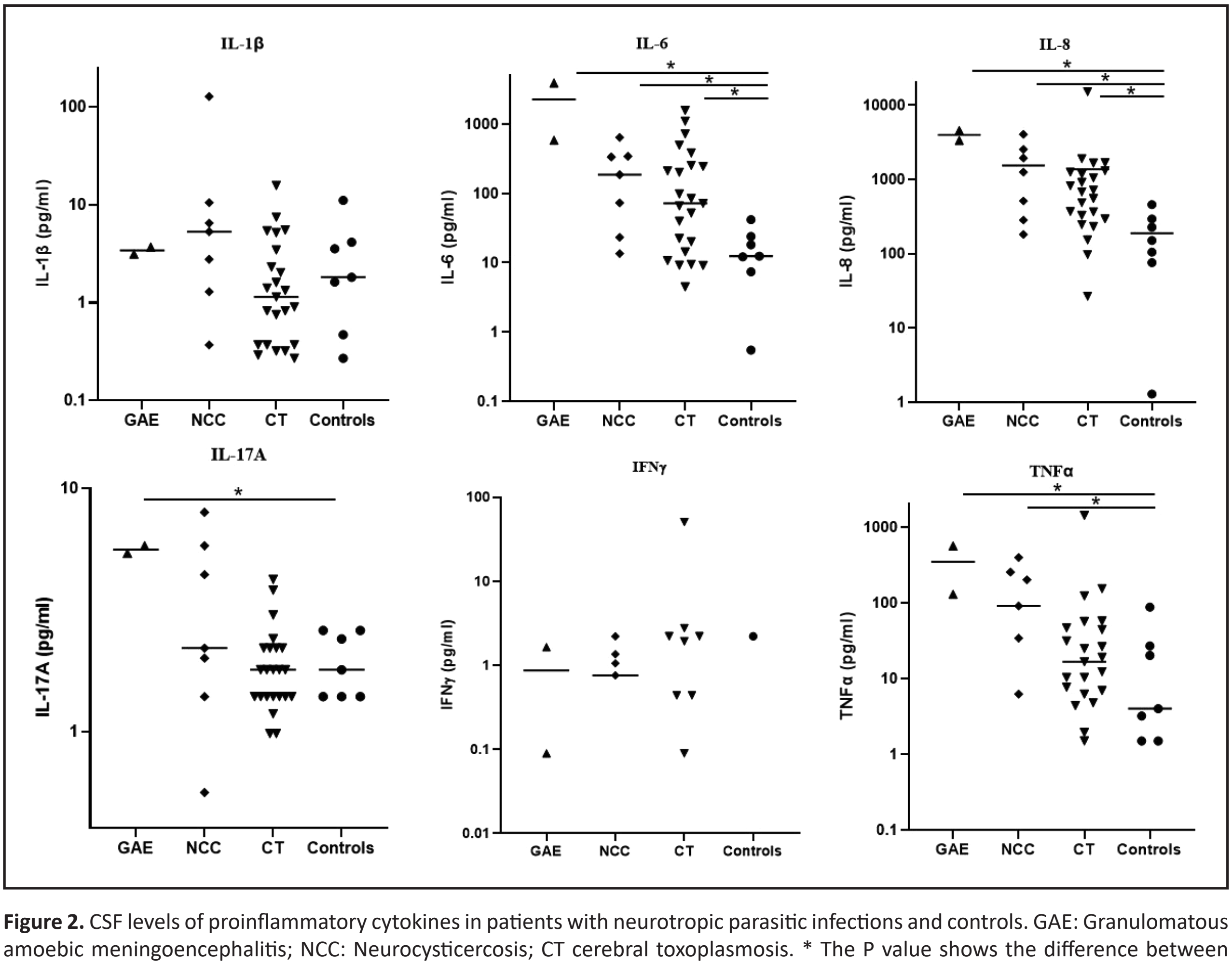

Figure 2. CSF levels of proinflammatory cytokines in patients with neurotropic parasitic infections and controls. GAE: Granulomatous amoebic meningoencephalitis; NCC: Neurocysticercosis; CT cerebral toxoplasmosis. * The P value shows the difference between patients and controls, as calculated by the Mann-Whitney U test.

IgG are characteristic of the active or reactivation phase of CT (Torrey *et al*., 2007). The presence of *T. gondii* IgG antibody correlated with imaging reports in 70% (16/23) of patients in this study. HIV was found to the single most risk factor in Toxoplasma IgG positive patient (78%) that might have predisposed them to CT (Table 2). In this study, five of the seven patients' neuroimaging findings correlated with cysticercal IgG antibody. Although antibody detection does not distinguish between exposure, inactive infection, and active infection in NCC, individuals with multiple viable cysts are shown to be consistently seropositive, and the antibody level increases significantly in patients treated with anti-cysticidal drugs (Garcia *et al*., 2020). Therefore, in addition to imaging techniques, cytokine profiling might help to learn about the stage of the parasitic diseases.

The two patients in the GAE group had increased CSF cell counts, and the levels of IL-6, IL-8, TNFα, and IL-17A were significantly elevated compared to controls. There are no reports on the CNS cytokine profiles of patients with GAE during the chronic stage of the disease. However, it has been shown *in vitro* that cocultures of human monocytes and macrophages with *A. castellanii* trophozoites released proinflammatory cytokines (IL-6, IL-8, IL-12, and TNFα) that could play a role in the development of the inflammatory response in GAE (Mattana *et al*., 2016). The brains of SJL mice infected with *A. castellanii* showed inflammatory cell infiltrate with the predominance of IFNγ producing CD4 T cells (Massilamany *et al*., 2014). Rat microglial cells and murine bone marrow-derived macrophages cocultured with *A. culbertsoni* trophozoites showed increased levels of TNFα and IL-6 (Shin *et al*., 2001; Cano *et al*., 2017). These studies show that proinflammatory cytokines are produced immediately after *Acathamoeba* infection *in vivo* and *in vitro*, and their presence during the chronic phase could lead to immunopathology.

Six out of seven patients in the NCC group in this study showed elevated levels of IL-8, IL-6, or TNFα compared to control subjects. Children with active NCC showed higher IL-6 and TNFα levels in CSF compared to children with inactive (calcified lesions) forms (Aguilar-Rebolledo *et al*., 2001; Kashyap *et al*., 2012). Additionally, in adult patients with NCC, higher levels of IL-6 were detected in CSF from patients with high cerebral blood flow velocity, which is associated with disease severity (Góngora-Rivera *et al*., 2008; Sáenz *et al*., 2012). The increased levels of proinflammatory cytokines in NCC have been shown to decrease after cure or in treatment-resistant patients (Arce-Sillas *et al*., 2018; Harrison *et al*., 2021). *In vitro* studies have also shown upregulation of IL-8 in monocytes in response to *T. solium* antigens (Uddin *et al*., 2010). Rats inoculated with *T. solium* showed increased expression of genes associated with proinflammatory cytokines such as IL-1α, IL-1β, IL-6, IFNγ, TNFα and fibrosis-related proteins including collagen, fibronectin, TGF-β, and arginase in the tissue surrounding the cyst compared to the noninfected tissue, which together may mediate the chronic state of infection (Carmen-Orozco *et al*., 2021). Similar to this study, others have shown low levels of IL-17A, IFNγ, and TNFα in NCC patients (Adalid-Peralta *et al*., 2012; Harrison *et al*., 2021).

In this study, significantly increased levels of IL-6 and IL-8 were shown in CT patients compared to controls. The levels of TNFα were elevated in ten CT patients and IFNγ in one patient. Increased levels of IL-6, IL-8, TNFα, and lymphocyte proliferation were shown in congenitally infected children and their transmitting mothers, suggesting that dysregulated, increased inflammatory responses are related to vertical transmission of *T. gondii* in humans (Gómez-Chávez *et al*., 2020). IFNγ levels have been shown to be higher in asymptomatic individuals than in patients with CT, indicating that this cytokine tended to be higher in individuals whose infections were resolved (Hernández-de-los-Ríos *et al*., 2019). It has been shown in several animal studies that both IFNγ and TNFα and their mRNA expression are significantly elevated in response to *T. gondii* infection during the acute phase, and the levels declined to background levels during chronic stages of TE, similar to NCC (Aviles *et al*., 2008; Moura *et al*., 2016; Tuladhar *et al*., 2019). The levels of IL1β and IL17A were low in CT patients in this study. It has been shown that IL-27 produced by astrocytes regulates inflammation in the CNS during TE by limiting Th-17 cell activity (Stumhofer *et al*., 2006). Therefore, only a few CD4+ IL-17-expressing lymphocytes are seen during the chronic stage of *T. gondii* infection in C57BL/6 mice (Drögemüller *et al*., 2008).

Unlike *Acanthamoeba*, both *T. gondii* and *T. solium* initially coexist with the human host. However, during later stages, reactivation results in heightened immune response and associated symptoms that require medical management. Treatment for neuroinflammation caused by parasitic infections involves the use of drugs to kill the parasites and reduce inflammation. Currently, steroids are used to the suppress immune system. However, their use is associated with significant side effects and sustained parasite viability (Garcia *et al*., 2020). Regulation of cytokines by targeted immunomodulatory therapies may be a better option to prevent complications associated with GAE, CT, and NCC. Several molecules, namely, monoclonal antibodies (anti-TNFα inhibitor, etanercept), somatostatin analogues, nonspecific MMP inhibitor (doxycycline), aptamers, and *Inonotus obliquus* polysaccharide showed promise in experimental systems in the control of parasitic inflammatory responses (Khumbatta *et al*., 2014; Boshtam *et al*., 2017; Mahanty *et al*., 2017; Yan *et al*., 2021). Interestingly, patients who respond to anti-helminthic drugs show upregulation of several genes involved in pro- and anti-inflammatory and immunomodulatory functions, indicating that a pro-inflammatory environment is related to treatment responsiveness and some of them may have a role in neuroprotection (John *et al*., 2008; Cárdenas *et al*., 2014; Arce-Sillas *et al*., 2018). Prevention of neurotropic parasitic diseases can be achieved by immunization/vaccination when available and eradication of parasitic infections by proper sanitation, use of cooked meat, and safe food handling (Hill & Dubey, 2002).

Although the number of patients in each group was small in this study, the increased levels of IL-8, IL-6 and TNFα in majority of the patients show that these three cytokines could be used as markers of neuroinflammation in GAE, NCC, and CT. Testing a larger cohort of patients with CNS parasitic infection will help to confirm this observation. Because IL-1β is secreted in its inactive form, measuring pro-IL-1β levels or intracellular staining by flow cytometric analysis might give a more accurate result (Palomo *et al*., 2015; Hernández-de-los-Ríos *et al*., 2019). The absence of the measured cytokines in a few patients could also be due to polymorphisms in cytokine-coding genes (Hernández-de-los-Ríos *et al*., 2019).

CONCLUSION

Of the 275 samples screened for neurotropic free-living amoebae by PCR, only two samples showed Acanthamoeba 18S rRNA. None of the CSF samples tested was positive for *N. fowleri* DNA. The increased levels of IL-8 in 28 (88%) patients, IL-6 in 25 (78%) patients, and TNFα in 17 (53%) patients, with high CSF cellularity in 11 patients, show that these three cytokines could be used as markers of neuroinflammation in GAE, NCC, and CT. Quantifying these cytokine levels in CSF might help with understanding the level of neuroinflammation in patients with neurotropic parasitic diseases. Further studies with clinico-microbiological correlation in the form of reduction of cytokine levels with treatment and the correlation with neurological deficits are needed.

ACKNOWLEDGEMENTS

This research was funded by the Department of Science and Technology, Ministry of Science and Technology, Government of India, and Daisy Vanitha John is supported by their Women Scientist Scheme (SR/WOS-A/LS-472/2018). The authors thank Dr. Sumeeta Khurana, Post Graduate Institute of Medical Education and

Research, Chandigarh, for providing the Acanthamoeba T4 strain, and Dr. Bijay Ranjan Mirdha, All India Institute of Medical Sciences, New Delhi, for providing the *N. fowleri* ITS plasmid. Authors thank staff at Department of Neuromicrobiology for microbiological and serological assays.

Conflict of Interest

The author declares that they have no conflict of interest.

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