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Global Theme Issue: Poverty and Human Development

Spatially and Genetically Distinct African Trypanosome Virulence Variants Defined by Host Interferon-γ Response

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We describe 2 spatially distinct foci of human African trypansomiasis in eastern Uganda. The Tororo and Soroti foci of *Trypanosoma brucei rhodesiense* infection were genetically distinct as characterized by 6 microsatellite and 1 minisatellite polymorphic markers and were characterized by differences in disease progression and host-immune response. In particular, infections with the Tororo genotype exhibited an increased frequency of progression to and severity of the meningoencephalitic stage and higher plasma interferon (IFN)–γ concentration, compared with those with the Soroti genotype. We propose that the magnitude of the systemic IFN-γ response determines the time at which infected individuals develop central nervous system infection and that this is consistent with the recently described role of IFN-γ in facilitating blood-brain barrier transmigration of trypanosomes in an experimental model of infection. The identification of trypanosome isolates with differing disease progression phenotypes provides the first field-based genetic evidence for virulence variants in *T. brucei rhodesiense*.

Human African trypanosomiasis (HAT) is caused by infection with the tsetse fly–transmitted hemoflagellates *Trypanosoma brucei rhodesiense* (in East and southern Africa) and *T. brucei gambiense* (in West and Central Africa). This disease is reemergent, with a current estimate of >300,000 cases and a further 60 million people at risk of infection [1]. The early (or hemolymphatic) stage of HAT commences 1–3 weeks after an infective fly bite with parasites proliferating within the blood and lymphatic system. Symptoms include general malaise, anemia, headache, pyrexia, weight loss, and weakness. The late (meningoencephalitic) stage of infection coincides with the invasion of the central nervous system (CNS) by parasites and is associated with psychiatric, motor, sensory, and sleep disorders, eventually progressing to a final stage involving seizures, somnolence, coma, and death [2]. Typically *T. brucei rhodesiense* infections are acute, whereas *T. brucei gambiense* infections present as a chronic disease [3]. However, within each subspecies, there are differences in the rate of disease progression. In *T. brucei rhodesiense* infection, large-scale spatial differences in disease presentation have been described, in which "severe" and "mild" disease are associated with "northern" and "southern" disease foci, respectively [4] and in which the mild disease is associated with lower levels of systemic inflammatory response [5]. These differences in disease severity may result from variation in host resistance, genetic variation in parasite virulence, socioeconomic and environmental factors, or a combination of all.

Progression to the late stage requires penetration of the blood-brain barrier by trypanosomes. Recent animal

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model studies have demonstrated that interferon (IFN)–γ plays a critical role in this process by modulating endothelial basement membrane laminin expression and lymphocyte transmigration [6]. Both clinical and experimental animal studies have observed systemically high levels of IFN-γ during trypanosome infection [7–9], and after trypanosome invasion of the CNS there is a direct relationship between the severity of neuropathology and expression of IFN-γ in the brain [10].

Here, we describe 2 spatially distinct HAT foci in eastern Uganda with genetically distinct parasites, allowing us to test the hypothesis that disease progression is determined by parasite genotype and host IFN-γ response.

SUBJECTS, MATERIALS, AND METHODS

Study sites and subjects. Patients with HAT and noninfected control individuals presenting to local hospitals or identified during community surveillance were recruited in eastern Uganda in 2002 and 2003. The Tororo, Iganga, Jinja, and Busia Districts define a common ecotope for the transmission of *T. brucei rhodesiense* by *Glossina fuscipes fuscipes,* which will be referred to henceforth as the Tororo focus, whereas the Soroti District contains a separate *G. fuscipes fuscipes* ecotope, where HAT emerged as a new epidemic in 1998-1999 [11]. Diagnosis was by microscopic detection of trypanosomes in wet blood films, in Giemsa-stained thick blood films, or in the buffy coat fraction after microhematocrit centrifugation [12]. After admission, a detailed physical examination was performed, and neurological involvement was assessed using the Glasgow Coma Score (GCS) [13]. The GCS gives a measure of the degree of impairment of consciousness, with a score of 15 as normal. The ranges 14–12, 11–8, <8 indicate mild, moderate, and severe impairment of consciousness, respectively. A clinical history was taken either from the patient or the attendant relative, and patients were classified on the basis of language to either Bantu, Western Nilo Saharan, or Eastern Nilo Saharan ethnic groups [14]. Stage was determined by examination of cerebrospinal fluid (CSF), using the World Health Organization criteria in which patients with trypanosomes in the CSF and/or a cell count >5 cells/mm^3 were classified as being in the late stage [15]. Early stage infection was treated with suramin and late-stage infection with melarsoprol [9]. Subjects or their guardians signed consent forms after receiving standard information in their local language. Protocols were approved by the Grampian Research Ethics Committee (Aberdeen) and the Ministry of Health (Uganda). Individuals with malarial parasitemia and microfilaremia were excluded from the study.

Blood samples obtained before treatment commenced were collected into EDTA Vacutainers (Greiner) and centrifuged for 10 min at 3000 *g*. Platelet-depleted plasma was aliquoted and frozen immediately in liquid nitrogen. CSF samples obtained as part of normal-stage diagnosis were also frozen and stored in liquid nitrogen. Trypanosome DNA from a subset of case patients was sampled by applying a 200-μL suspension taken from the buffy coat layer to FTA cards (Whatman Bioscience), which were dried and stored at room temperature. Case patients were selected to include representatives from each village and no more than one case per compound/family.

Cytokine assays. IFN-γ, interleukin (IL)–1β, IL-6, transforming growth factor (TGF)–β, and IL-10 concentrations were measured using a solid-phase sandwich ELISA (OptiEIA; BD Pharmingen), as described elsewhere [16]. Biological limits of detection for these assays were 1.8, 10.2, 8.3, 19.2, and 1.6 pg/mL, respectively.

FTA filter preparations and whole-genome amplification. Discs of 1.2 mm in diameter were cut out of each blood spot by use of a Harris Micro Punch (Whatman). The discs were washed 3 times with 200 μL of FTA purification reagent (Whatman) and twice with 200 μL of 1 mmol/L Tris-EDTA buffer (pH 8.0), with incubation for 5 min at each wash. The washed discs were air-dried for 1 h and then used as substrate for whole-genome amplification reactions.

Whole-genome amplification was done using multiple displacement amplification (MDA) technology directly on washed FTA punched discs [17]. Three independent reactions were done for each sample, and products were stored at −20°C.

Polymerase chain reaction (PCR)–based genotyping. One microliter of each MDA product was used as template for PCR, in a volume of 20 μL. The microsatellite loci (ch1/18, ch2/5, ch2/PLC, ch3/5L5, ch4/M12C12, and ch5/JS2) and minisatellite locus (ch3/292) used in this study have been described elsewhere [18, 19]. Both outside and nested PCR primers for each marker are detailed in table 1.

PCR conditions were as follows: PCR buffer (45 mmol/L Tris-HCl [pH 8.8], 11 mmol/L $[NH_4]_2SO_4$, 4.5 mmol/L $MgCl_2$, 6.7 mmol/L 2-mercaptoethanol, 4.4 μmol/L EDTA, 113 $\mu g \cdot mL^{-1}$ bovine serum albumin, and 1 mmol/L each of 4 deoxyribonucleotide triphosphates), 1 μmol/L of each oligonucleotide primer, and 1 U of *Taq* polymerase (Abgene) per 20-μL reaction (2.5 U per 50 μL reaction). For the nested reactions, 1 μL of a 1:100 dilution of first-round product was used as template in the second-round PCR. PCR products were resolved by electrophoresis on a 3% Nusieve GTG agarose gel (Cambrex), stained with ethidium bromide, and visualized under UV light.

Allele size determination. The number and size of alleles amplified in each sample was determined using 1 fluorescently labeled primer for each locus (FAM), followed by separation of products using a capillary-based sequencer (ABI 3100; Applied Biosystems). DNA fragment size was determined relative to a set of ROX-labeled size standards (GS500 markers; Applied Biosystems) using GeneScan software, which allowed resolution to the level of 1 bp.

For the minisatellite locus (ch3/292), allele band sizes were determined on the basis of mobilities relative to a reference stan-

Table 1. Microsatellite outside and nested polymerase chain reaction primer sequences.

	Outside		Nested	
Locus	Primer	Sequence (5'→3')	Primer	Sequence (5'→3')
ch1/18	1/18-C	tataatgcgtttgtgagaat	1/18-A-FAM	tgtgagaatggtactcacgcgctg
	1/18-D	gaagggagggaacagaagcaggg	1/18-B	caacgttagcacacaattcctgtg
ch2/5	2/5-C	tatcgcggttatgtggatttgtgg	2/5-AFAM	atggcgtgtatcacattcgtgatg
	2/5-D	cacaacaaaactgccatgaggtac	2/5-B	ccgttggcattaggcacaagta
ch2/PLC	PLC-G2	ttaagtggacgacgaaataacaaca	2/PLC-GFAM	caacgacgttggaagagtgtgaac
	PLC-H4	ttcaaacaccgtccccctcaataat	2/PLC-H3	ccactgacctttcatttgatcgctttc
ch3/5L5/2	5L5/2-AA	gagcgtacattgcaggtagtgcgtagcg	3/5L5/2-AFAM	gtacgtggttaaccacaacctact
	5L5/2-C	acgaagaaacgaagcaaagaag	2/5L5/2-B	ggaaactgcttaaacttgcgtgag
ch3/292	3/292-G1	cggaaaacgagggtgtgttacgcgac	3/292-G	gctgaacctgtgggcccctcaattg
	3/292-H	gctgaacctgtgggcccctcaattg	3/292-H2	acacccctctccacttcagatac
ch4/M12C12	M12C12-C	aaaacctcatccagtcgcactgg	M12C12-AFAM	tggacacacagaagcctaccg
	M12C12-B	taccctcatcaagtggtcg	M12C12-D	agtgtggtggtgcgtgcaaacttgg
ch5/JS2	JS2-C	agtaatgggaatgagcgtcaccag	JS2-AFAM	gattggcgcaacaactttcacatacg
	JS2-D	gatcttcgcttacacaagcggtac	JS2-B	ctttcttccttggccattgttttactat

dard lane (restriction fragments of λ *Hin*dIII and $\phi\times174$ *Hae*III), as described elsewhere [20].

Genetic analysis. The programs Clustering Calculator [21] and Treeview (version 1.6.6) [22] were used to generate a dendrogram in which multilocus genotypes were compared using unweighted arithmetic average as the clustering method and a pairwise distance matrix of the multilocus genotypes (Jaccard's similarity coefficient) for the input data. This allowed the analysis of the similarity between each multilocus genotype to be determined. The genetic data analysis (GDA) program [23] was used to determine Nei's genetic distance (D) [24] and Wright's fixation index (F_{ST}) [25] between each group of isolates from different foci.

Statistical analyses. The statistical package JMP5 (SAS Institute) was used. The distributions of cytokine concentrations were all right skewed. After log transformation, all approached normality. Log-transformed cytokine values were compared using appropriate parametric tests as indicated in Results, using the linear modeling platform in JMP. Distributional assumptions were tested using Shapiro-Wilks test and Levene's test for homogeneity of variance. Age was stratified as follows: 0–5, 6–14, 15–29, and ⩾30 years. Categorical variables were compared using Pearson's χ^2 or Fisher's exact test.

RESULTS

Disease in Tororo and Soroti Districts caused by distinct genetic variants. One hundred forty-four patients with HAT were recruited during the period January 2002 to August 2003, of whom 115 came from the Soroti focus and 29 from the Tororo focus. Parasite DNA from a subset of 22 subjects in the Tororo focus and 27 subjects in the Soroti focus was subject to whole-genome amplification followed by genotyping with 6 microsatellites and 1 minisatellite marker, by nested PCR. The multilocus genotype of each sample was determined based on the combination of alleles at the 7 loci, revealing 30 distinct multilocus genotypes. To determine the relationship between the isolates, a dendrogram based on Jaccard's similarity index was constructed (figure 1). A *T. brucei rhodesiense* sample, isolated from a patient with sleeping sickness in Malawi in 2002 (NKK/T/02-02) [5], was also included in this analysis for comparison. Two distinct clusters of genotypes (groups 1 and 2 in figure 1) were identified using this approach, and the bootstrap value for the node that separates the 2 groups is high (97 of 100 replications). Group 1 genotypes contain only isolates from Soroti, whereas group 2 genotypes contain only isolates from Tororo, clearly demonstrating that there are distinct parasite genotypes that are responsible for sleeping sickness in the 2 individual foci. This was confirmed by measurement of Nei's genetic distance between the groups (0.143) and by Wright's fixation index (0.071).

Disease characteristics of subjects with HAT in Tororo and Soroti. The disease characteristics at each focus are summarized in table 2. Although there were no significant differences in parasitemia and anemia between the 2 foci, a significantly higher proportion of patients had progressed to the late stage in the Tororo focus at the time of diagnosis. There was a higher frequency of hepatomegaly and splenomegaly and also a small but significant increase in body temperature observed in patients from Tororo, who also exhibited a significantly higher frequency of moderate (GCS of 9–11) or severe (GCS of 8 or less) impairment of consciousness. As this result could be influenced by the higher proportion of late-stage patients in Tororo, the frequency of moderate or severe impairment of consciousness was also compared for late-stage cases only. Again, a significantly higher frequency of patients with a GCS of <12 was found in Tororo

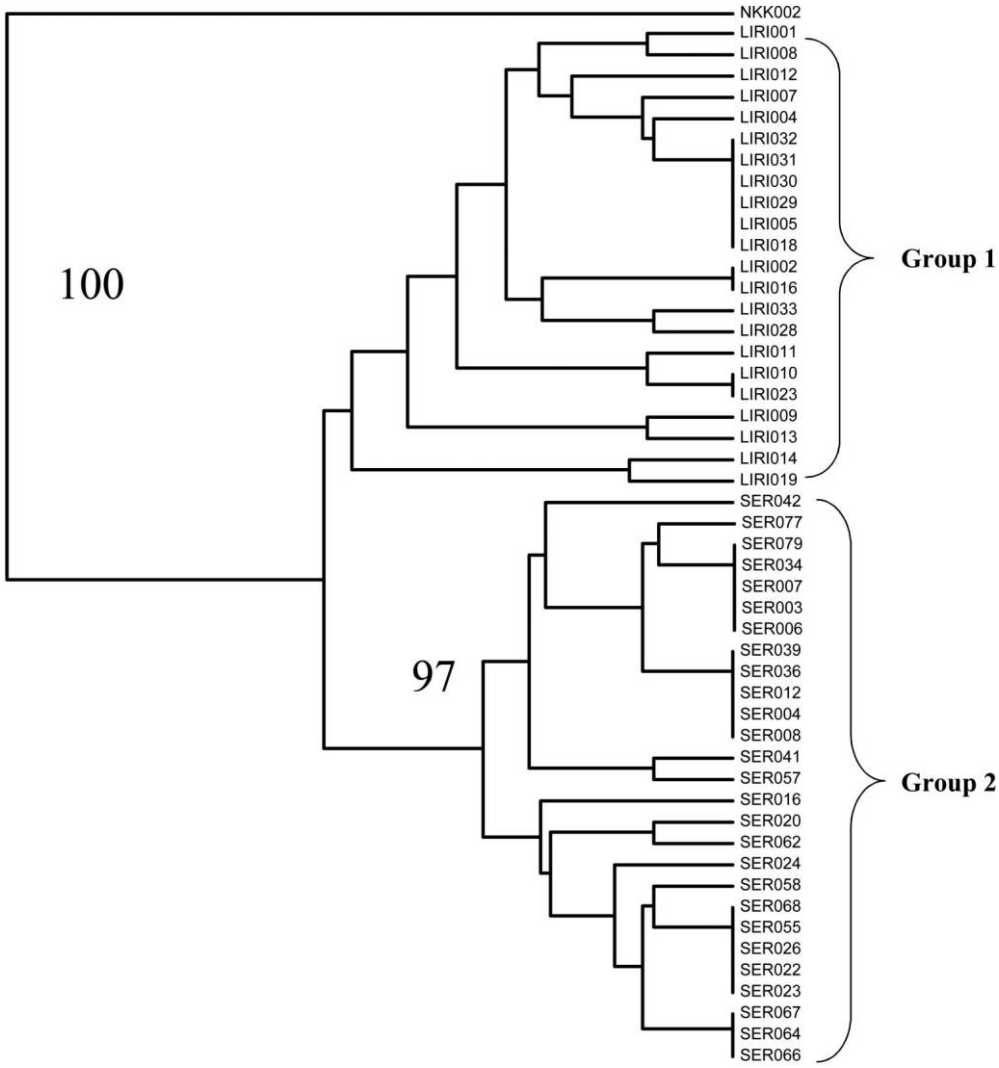

Figure 1. Dendrogram generated by an unweighted arithmetic average as the clustering method, showing the similarities between trypanosome multilocus genotypes. Bootstrap values based on 100 replicates are shown for the main nodes. Groups 1 and 2 comprise the Tororo and Soroti genotypes, respectively.

than in Soroti. Despite the apparently more advanced disease presentation in the Tororo focus, there was no significant difference in the duration of illness as reported by patients. There were differences in age and sex composition in Tororo and Soroti; however, these factors were not significantly ($P > .3$) associated with infection stage.

Characterization of Tororo and Soroti foci by differences in plasma IFN-γ and IL-6 concentrations. The plasma concentrations of a panel of cytokines involved in the inflammatory response and its regulation were then measured (figure 2). The concentrations of IFN-γ in Tororo and of IL-6, IL-10, IL1-β, and TGF-β in both Soroti and Tororo were elevated in relation to control levels in patients with HAT, and furthermore there were significant differences in the concentrations of IFN-γ ($P < .0001$, unpaired t test) and IL-6 ($P < .05$) between the 2 foci. TNF-α concentration was not significantly increased in either patient group. Because blood samples from a subset of Tororo and Soroti patients had been used for parasite genotyping (figure 1), a further analysis was carried out in which the plasma cytokine levels of only genotyped infections were measured. The results from this analysis were entirely consistent with those from the full Tororo and Soroti study cohorts (table 3). For comparison, the relevant plasma cytokine data for Malawi patients published in MacLean et al. [5] is also presented in table 3.

Because the Tororo and Soroti groups differed in age and sex structure (table 2), these factors were tested for effects on IFN-γ and IL-6 concentrations. Although age had no significance as an explanatory variable, female patients from Soroti showed a significant increase in plasma IFN-γ concentration (for female patients with HAT, geometric mean [95% confidence interval {CI}] concentration of 78.2 (60.9–102.5) pg/mL; for male patients with HAT, geometric mean [95% CI] concentration of

Table 2. Characteristics of patients with sleeping sickness recruited in Tororo and Soroti Districts.

Characteristic	All patients with HAT (n = 144)	Soroti patients (n = 115)	Tororo patients (n = 29)
Age, median (range), years	22 (2–85)	20 (2–85)	25 (15–39)
Female	56	58.6	44.8
Packed cell volume, mean ± SE, %	29.7±0.5	29.5±0.6	30.7±0.9
Temperature, median (IQR), °C	36.8 (36.4–37.2)	36.8 (36.4–37.0)	37.4 (36.8–38.0)[a]
Late stage (meningoencephalitic)	75.7	72.4	93.5[b]
GCS <12			
All cases	13.3	6.9	37.9[c]
Meningoencephalitic cases only	17.1	9.7	40.7[c]
Parasitemia, median (IQR)[d]	0.5 (0–3)	0.4 (0–1)	1.5 (0–16.5)
Reported duration of illness, median (IQR), days	56 (22–86)	47 (21–67)	62 (30–102)
Lymphadenopathy	41	43.1	33.3
Hepatomegaly	4.1	1.0	14.8[b]
Splenomegaly	15.2	10.4	31.0[b]
Ethnic composition, Bantu; Eastern Nilo Saharan; Western Nilo Saharan	6.9; 83.3; 9.8	0.8; 99.2; 0	31; 20.7; 48.3

NOTE. Data are percentage of patients, unless otherwise specified. GCS, Glasgow Coma Score; HAT, human African trypanosomiasis; IQR, interquartile range.

[a] Significantly increased over Soroti ($P < .001$, unpaired t test).
[b] Significantly higher frequency in Tororo ($P < .001$, χ^2 test).
[c] Significantly higher frequency in Tororo ($P < .05$, Fisher's exact test).
[d] Parasites per 10 fields (at ×400) of wet film.

41.7 (30.8–56.2) pg/mL; $P < .05$, unpaired t test). No similar sex difference was evident in the Tororo patients. Because of the apparent effect of sex on IFN-γ levels in Soroti, cytokine data were reanalyzed using age- and sex-matched pairs of case patients. Each of the 29 Tororo patients was paired with a Soroti patient of the same sex and within 5 years of the same age. Where >1 patient met these criteria, the pair with the closest diagnosis date was selected. In this subset of matched cases, IFN-γ and IL-6 concentrations continued to differ between the groups (for Tororo, geometric mean [95% CI] IFN-γ concentration of 262.4 (179.5–383.7) pg/mL; for Soroti, geometric mean [95% CI] IFN-γ concentration of 68.0 [48.9–94.6] pg/mL; $P < .001$, paired t test) (for Tororo, geometric mean [95% CI] IL-6 concentration of 76.7 [47.5–123.9] pg/mL; for Soroti, geometric mean [95% CI] IL-6 concentration of 27.9 [16.3–44.7] pg/mL; $P < .01$, paired t test).

Independence of IFN-γ response from subject ethnicity. Analysis of plasma IFN-γ levels in the aggregated Tororo and Soroti patient groups suggested that ethnolinguistic group was a significant determinant of response ($F_{2,137} = 12.6$; $P < .0001$), with the lowest concentrations in Eastern Nilo Saharan patients (table 4). However 99% of the Soroti subjects (table 2) were of Eastern Nilo Saharan background, and thus the apparent influence of ethnicity on IFN-γ could be confounded by the predominance of this one ethnic group. This interpretation is supported by the observation that, when only patients from the Tororo focus ($n = 29$) were analyzed, ethnicity had no effect on plasma IFN-γ concentration (table 4).

As a further test for the effects of ethnicity on IFN-γ concentration, the 6 Eastern Nilo Saharan subjects from the Tororo focus were age and sex matched with 6 Eastern Nilo Saharan subjects from Soroti. In this subset of case patients, there continued to be a significant difference in plasma IFN-γ concentration between the groups (for Soroti, geometric mean [95% CI] of 60.3 [27.1–134.2] pg/mL; for Tororo, geometric mean [95% CI] of 365.0 [221.4–544.6] pg/mL; $P < .05$, paired t test). Plasma IL-6 concentration did not vary according to subject ethnicity.

Association of CNS IFN-γ concentration with moderate and severe coma. Patients with trypanosomiasis from Tororo and Soroti did not exhibit any significant difference in CSF IFN-γ concentration (table 5). Because there was an increased frequency of patients with moderate (GCS of <12) and severe impairment of consciousness (GCS of <8) in Tororo, the relationship between CSF IFN-γ concentration and GCS was investigated. Although there was no significant effect of GCS on CSF IFN-γ in Soroti, in Tororo, patients with moderate ($n = 5$) and severe ($n = 3$) impairment of consciousness exhibited a significantly higher concentration of IFN-γ in CSF (table 5) than patients with a GCS of 12–14 (mild impairment of consciousness, $n = 6$) and 15 (normal, $n = 15$).

DISCUSSION

HAT caused by *T. brucei rhodesiense* presents a spectrum of clinical profiles, from acute infection rapidly progressing to the lethal meningoencephalitic stage to more chronic infections that,

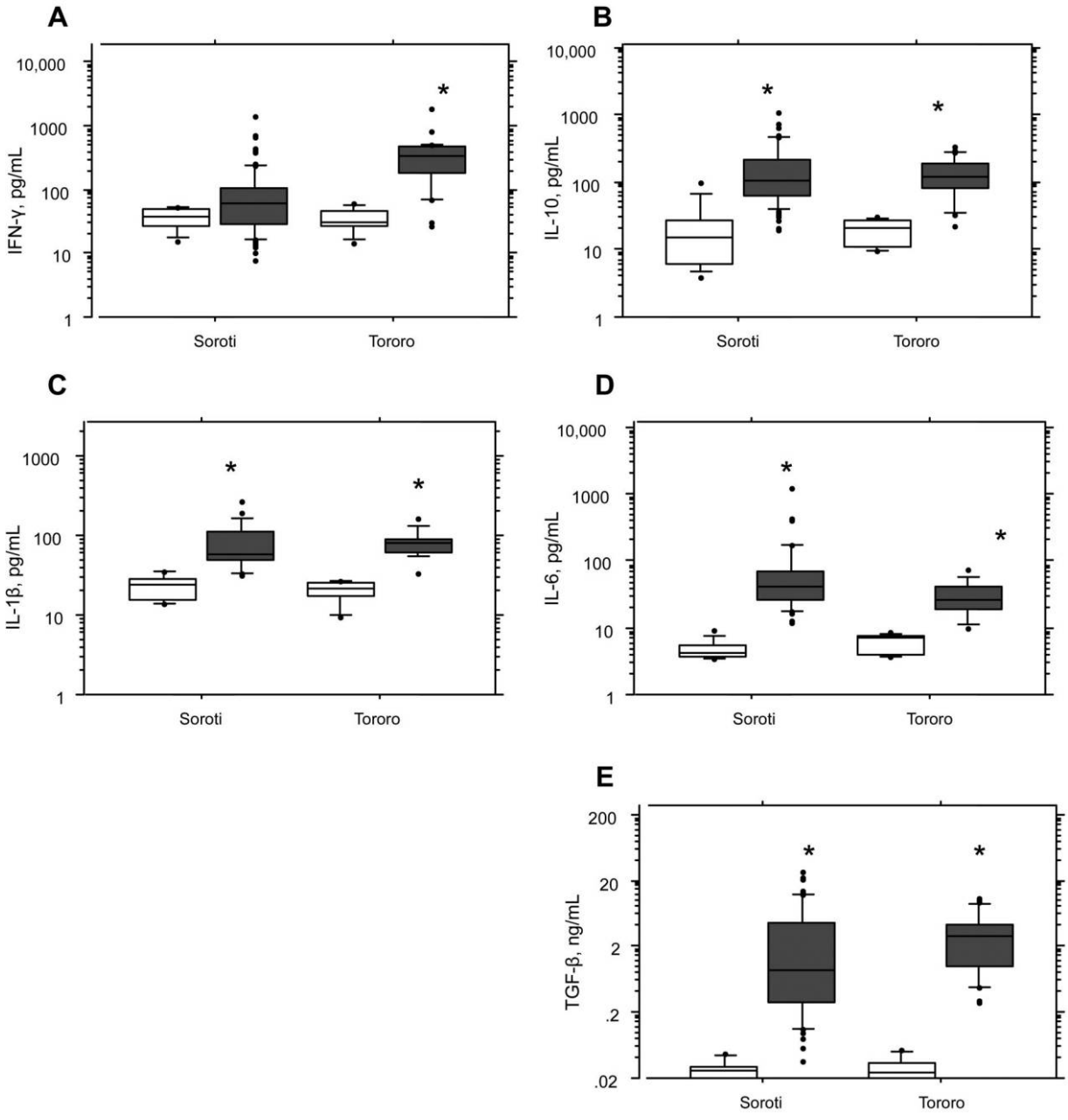

Figure 2. Plasma concentrations of interferon (IFN)–γ *(A),* interleukin (IL)–10 *(B),* IL-1β *(C),* IL-6 *(D),* and transforming growth factor (TGF)–β *(E)* in patients *(gray boxes)* and control subjects *(white boxes)* in the Tororo and Soroti foci. Boxes show the median and 25th and 75th percentiles; whiskers show 10th and 90th percentiles. Dots represent outliers. *Significantly increased over control ($P < .0001$, unpaired *t* test on log-transformed data).

similar to *T. brucei gambiense* infection, take several months or more to progress to CNS infection [4]. It has been proposed that such differences in disease tempo may be controlled by either parasite genotype or host genotype or by a combination of both [5]. Spatial genetic variation of parasite populations certainly does occur [26], but its relationship to pathology has hitherto been unclear.

In 1998, the first cases of a new epidemic of sleeping sickness emerged in Soroti District [11], some 150 km north of the historic Tororo focus, and between 2002 and 2003 we conducted a cross-sectional survey of cases in both foci. Analysis of clinical histories indicated a difference in disease virulence between the 2 foci, with Tororo subjects showing increased progression to the CNS infection stage. Although this could have been the result of a longer period of illness before admission, patient interview data indicated no significant difference in duration of disease between Tororo and Soroti. This suggests that the more advanced progression in Tororo is not simply the result of a longer duration of disease. The difference in clinical profile between these spatially distinct disease foci allowed us to test the hypoth-

Table 3. Plasma cytokine concentration (geometric mean [95% confidence interval]) in patients with human African trypanosomiasis (HAT) with genotyped parasite infections.

Cytokine	Soroti (genotype 1) ($n = 27$)	Tororo (genotype 2) ($n = 22$)	Malawi HAT cases reported in [5]
IFN-γ, pg/mL[a]	74.4 (48.9–114.4)	254.6 (156.0–411.6)	56.8 (34.1–93.7)
IL-6, pg/mL[b]	79.8 (58.5–108.9)	51.9 (37.7–68.7)	121.5 (73.7–200.3)
IL-10, pg/mL[a]	115.6 (83.9–159.2)	130.3 (90.9–186.8)	134.3 (90.0–194.4)
IL-1β, pg/mL[a]	76.7 (54.6–107.8)	79.8 (58.5–108.9)	82.2 (50.9–132.9)
TGF-β, ng/mL[c]	1.58 (0.86–2.92)	2.51 (1.28–4.91)	6.0 (2.7–13.8)

NOTE. IFN, interferon; IL, interleukin; TGF, transforming growth factor.

[a] Tororo was significantly increased over Soroti and previously reported Malawi [5] values ($P < .001$, unpaired t test).

[b] Soroti was significantly increased over Tororo ($P < .05$, unpaired t test); Malawi was significantly increased over Soroti and Tororo ($P < .05$, Tukey post-hoc test).

[c] Soroti and Tororo were significantly lower than previously reported Malawi value ($P < .001$).

esis that disease progression—and thus virulence—in sleeping sickness is determined by parasite genotype. On the basis of the difference in allele type and multilocus genotype, we have shown that the geographically separated *T. brucei* populations in Soroti and Tororo, isolated at the same time and from the same host type, are genetically distinct, providing evidence of a lack of gene flow between these populations. This conclusion was supported by a moderate genetic distance between the populations. Thus, we demonstrate for the first time that distinct parasite genotypes circulate in spatially separated foci and are associated with differences in virulence as measured by progression to and severity of the CNS infection stage.

It has previously been demonstrated in experimental HAT models that pathology is associated with the systemic [27] and CNS production [10] of inflammatory cytokines. We therefore investigated the relationship between systemic cytokine response and difference in clinical presentation between patients in Tororo and Soroti. There was a generalized up-regulation of plasma IL-1β, TGF-β, and IL-10 in all patients, consistent with the finding of previous studies of *T. brucei rhodesiense* [16] and *T. brucei gambiense* [28, 29]. However, IFN-γ and IL-6 were detected at consistently different concentrations in the plasma of Tororo subjects compared with Soroti subjects, after controlling for differences in age, sex, and ethnicity between the 2 foci. IFN-γ concentration was higher in Tororo patients, whereas IL-6 concentration was higher in Soroti patients. Studies in the mouse model of infection have demonstrated that IFN-γ plays a pivotal role in the host response to trypanosomiasis. It is detected in splenic T cells within 24 h of infection [30], reaches higher plasma concentrations with laboratory trypanosome strains of increasing virulence [7], and appears to be derived from both the innate cellular immune system [31] and adaptive responses by T cells to variant surface glycoprotein epitopes [32], trypanin [33], and other undefined parasite antigens. IFN-γ contributes to pathogenesis through macrophage activation and resulting nitric oxide and TNF-α production [27, 34, 35]. However, IFN-γ appears to be essential for the control of parasitemia in mouse models [36–38]. For the present study, of particular relevance is the recent finding in the mouse model that IFN-γ is a regulator of the blood-brain barrier, which trypanosomes must cross to initiate CNS infection [6]. Mice genetically manipulated to be deficient for IFN-γ and the IFN-γ receptor all showed impaired and delayed invasion of the brain parenchyma, with an accumulation of parasites between the endothelial and parenchymal basement membranes. If these findings also apply to human disease, individuals responding with high levels of IFN-γ would be expected to show increased progression to CNS infection, and this is the case in the Tororo focus in this study. Patients with HAT in Tororo also showed a higher frequency of moderate and severe impairment of consciousness (as measured by GCS), compared with those in Soroti. In mouse models, up-regulation of IFN-γ production in the brain paren-

Table 4. Plasma interferon (IFN)–γ levels and patient ethnicity.

Ethnicity	All patients	Tororo patients only
Bantu	183.0 (92.2–365.0) [$n = 10$]	242.0 (121.5–483.2) [$n = 9$]
Western Nilo Saharan	288.6 (148.4–555.6) [$n = 14$]	288.6 (148.4–555.6) [$n = 14$]
Eastern Nilo Saharan	64.1 (52.4–78.3) [$n = 120$][a]	270.4 (119.1–620.2) [$n = 6$]

NOTE. Data are geometric mean (95% confidence interval) IFN-γ levels, in picograms per milliliter.

[a] Significantly reduced compared with Western Nilo Saharan and Bantu ($P < .05$).

Table 5. Cerebrospinal fluid (CSF) interferon (IFN)–γ levels and Glasgow Coma Score (GCS) in Tororo and Soroti patients.

Score	Tororo	Soroti
All patients	19.6 (13.3–29.1)	17.8 (14.7–21.5)
GCS of 12-15[a]	14.8 (9.0–24.5) [n = 21]	17.4 (14.2–21.4) [n = 107]
GCS of <12[b]	32.0 (21.1–48.4) [n = 8][c]	22.6 (14.1–35.8) [n = 8]

NOTE. Data are geometric mean (95% confidence interval) IFN-γ levels, in picograms per milliliter.

[a] GCS of 15 is normal; GCS of 12–14 is mild impairment of consciousness.

[b] GCS of<12 is moderate impairment of consciousness; GCS of 8 and lower is coma.

[c] Significantly higher than in patients with a GCS of 12–15 ($P < .01$, unpaired t test).

chyma is associated with the onset and increasing severity of neuroinflammation [10, 39], leading to the neurological sequelae of sleeping sickness. When the level of IFN-γ in the CSF of patients was investigated, it was found that, in Tororo, patients with sleeping sickness with a GCS of <12 exhibited significantly higher levels of CSF IFN-γ. Thus, we provide the first clinical evidence of an involvement of IFN-γ in the initiation and severity of meningoencephalitic sleeping sickness.

The significance of the higher concentration of plasma IL-6 in Soroti patients is not clear. IL-6 is a multifunctional cytokine with proinflammatory characteristics but also acts as a neuroprotective factor [40]. Although IL-6 has previously been shown to be expressed at high levels both in the peripheral circulation and in the CNS during experimental mouse [10, 41] and human [16] trypanosomiasis, the functional relationship between IL-6 and the pathogenesis of HAT requires further investigation.

Plasma IL-10, IL-1β, and TGF-β were elevated at similar levels in both Tororo and Soroti. In a previous study contrasting mild and severe HAT in Malawi and Uganda, respectively, it was proposed that higher levels of plasma TGF-β observed in mild cases may point to a role of this cytokine in regulating inflammatory pathology [5]. In the present study, TGF-β was not related to disease severity. However, when compared with the cytokine responses reported in MacLean et al. [5], plasma TGF-β levels in both Tororo and Soroti patients were lower than those in patients with HAT in Malawi, while at the same time plasma IFN-γ levels in Tororo patients were significantly higher than those in Malawi patients, which were not significantly different from the plasma IFN-γ concentrations in Soroti patients. Thus, while the variations in disease severity we describe within Uganda appear to be related to differences in the IFN-γ response to infection, at larger spatial scales—such as those encompassing the mild and severe HAT symptoms of the southern and northern areas of East Africa, respectively—additional involvement of inflammatory pathology and its regulation by TGF-β may be involved.

Field clinical studies of HAT are limited to cross-sectional design by ethical and logistical constraints; therefore, it is not possible to confirm causality or mechanism in the relationship between plasma and CSF cytokines and disease progression. The detailed dissection of the host response to the Tororo and Soroti parasite genotypes and the mechanistic role played by IFN-γ in promoting CNS invasion and neuropathology now requires study in refined experimental mouse models [10] in which the invasion of the CNS and the progression of CNS pathology may be measured.

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