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BRIEF REPORT

Augmented Zika and Dengue Neutralizing Antibodies Are Associated With Guillain-Barré Syndrome

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The role of neutralizing antibodies in Zika-induced Guillain-Barré syndrome (GBS) has not yet been investigated. We conducted a case-control study using sera from the 2016 Zika epidemic in Colombia to determine the neutralizing antibody activity against Zika virus (ZIKV) and dengue virus serotype 2 (DENV2). We observed increased neutralizing antibody titers against DENV2 in ZIKV-infected individuals compared with uninfected controls and higher titers to both ZIKV and DENV2 in ZIKV-infected patients diagnosed with GBS compared with non-GBS ZIKV-infected controls. These data suggest that high neutralizing antibody titers to DENV and to ZIKV are associated with GBS during ZIKV infection.

Keywords. Guillain-Barré syndrome; neutralizing antibody; flavivirus; Zika; dengue.

Zika virus (ZIKV) is a flavivirus spread mainly by the *Aedes aegypti* mosquito. In 2015–2016, an epidemic of Zika in the Americas was accompanied by severe neurologic complications including microcephaly in babies born to mothers infected with ZIKV during pregnancy and Guillain-Barré syndrome (GBS) in adults [1]. GBS is a disorder of the peripheral nervous system often triggered by a preceding viral or bacterial infection or vaccination [2]. Although the exact cause of most GBS cases remains unknown, several studies have demonstrated that for some pathogens, such as *Campylobacter jejuni*, an infection-induced antibody cross-reacts with the ganglioside surface components of peripheral nerves [2]. Although the mechanism whereby Zika is associated with GBS has not been clearly elucidated, it is likely that there is a similar pathogenesis. As many vaccines currently under development for Zika are designed to elicit protective titers of neutralizing antibodies, it is critical to define the role of ZIKV antibodies in the development of GBS.

During the 2015–2016 Zika epidemic in Colombia, there was a simultaneous increase in the number of neuroinflammatory disorders reported [3]. Specifically, there was an increase in GBS cases in individuals found to be ZIKV positive (ZIKV$^+$) by reverse-transcription polymerase chain reaction, lending support to the role of ZIKV infection in GBS pathogenesis [3]. To investigate the relationship between ZIKV infection and GBS, anti-ZIKV neutralizing antibodies were assayed in plasma samples obtained from ZIKV-infected patients and controls collected during the 2016 outbreak in Barranquilla, Colombia.

METHODS

Ethics Statement

This study was approved by the ethics committee of the Universidad El Bosque, and a nonhuman subjects determination was made by the George Washington University Institutional Review Board for analysis of de-identified data. All participants received written informed consent.

Participants and Setting

Adult patients with a clinical diagnosis of Zika and Zika-related GBS were referred to this study from the Atlántico Department and Bolívar Department, Colombia, while asymptomatic participants from Bogotá, Cundinamarca Department, a mountainous region without endemic ZIKV transmission, were enrolled as Zika-negative controls.

Case and Control Definitions

Zika-Related Guillain-Barré Syndrome Case

A Zika-related GBS (ZGBS) case was defined as a participant with clinically diagnosed ZIKV infection, confirmed by serologic analysis as described below, and GBS, as diagnosed and reported by a local neurologist. The Brighton criteria for the level of GBS diagnosis certainty was determined if documentation was available [4].

Zika-Positive Control

Participants with clinical symptoms of ZIKV infection and serological ZIKV confirmation (ZIKV$^+$ control) were matched by age and sex using simple stratified random sampling from patients from the Atlántico and Bolívar departments with clinical ZIKV infection.

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Zika-Negative Control

A Zika-negative control (ZIKV$^-$) was defined as an asymptomatic participant confirmed to be negative for ZIKV infection by serology.

Design

Each ZGBS case was age- and sex- matched to 2 ZIKV$^+$ controls and 1 ZIKV$^-$ control by simple random sampling within age and sex strata. All patients with a clinical diagnosis of ZIKV or ZGBS completed a brief symptom questionnaire prior to blood sample collection. A retrospective chart review was performed for cases of ZGBS cases where the medical records were available.

Serologic ZIKV Infection Determination

Participants were considered to be positive for a ZIKV infection if they fulfilled the following diagnostic criteria: ZIKV nonstructural protein 1 (NS1) antibody positive by the previously described Zika NS1 blockade-of-binding assay [5] or a reciprocal 50% neutralizing titer (NT_{50}) against ZIKV strain H/PF/2013 that was at least 2-fold greater than the NT_{50} titer against dengue virus serotype 2 (DENV2) 16681.

Reporter Virus Particle Neutralization Assays

Neutralization of ZIKV H/PF/2013 and DENV2 16681 by plasma samples was measured using a reporter virus particle assay as described previously [6]. In brief, heat-inactivated plasma was serially diluted 5-fold from 1:50 and incubated with 100 µL of virus for 1 hour at 37°C, after which 50 µL of target Vero cells (400 000 cells/mL) was added. Input virus dilution was calculated from titration experiments to ensure sufficient luciferase output within the linear portion of the titration curve. Cell-only and virus-only controls were included on each plate, and all serum samples (and virus only) were run in triplicate. After a 48-hour incubation, luciferase activity was measured, and neutralization curves were calculated by averaging luciferase units from triplicates, subtracting cell-only control background and calculating the percentage difference in serum samples to virus-only controls. Data was fit by nonlinear regression using the asymmetric 5-parameter logistic function in GraphPad Prism. The 50%, 80%, and 90% neutralizing titers (NT_{50}, NT_{80}, and NT_{90}, respectively) were defined as the reciprocal serum dilution resulting in a 50%, 80%, or 90% reduction in infectivity.

Statistical Analysis

Nonparametric Mann–Whitney *U* tests were performed to determine if there were differences in reciprocal dilutions between ZGBS and ZIKV$^+$ groups for neutralization of DENV and ZIKV, respectively. Samples with no neutralization at a dilution of 1:50 were assigned a titer of 49 for statistical analysis. The differences between the mean reciprocal dilution vectors for neutralization of DENV2 and ZIKV in these groups (ZGBS and ZIKV$^+$) were further assessed with Hotelling T^2 test and graphically with 95% probability confidence ellipses. Spearman rank correlation was used to determine the association between neutralization of DENV and ZIKV for each group. Statistical analyses were conducted using SAS software, version 9.4 (SAS Institute, Cary, North Carolina), and tests were considered statistically significant with a *P* value ≤.05.

RESULTS

The clinical and serological factors associated with ZGBS were studied in 23 patients with a clinical diagnosis of Zika and GBS in Barranquilla, Colombia, from December 2015 through May 2016. Six participants were excluded from further analysis because their clinical Zika diagnosis was not serologically confirmed. Seventeen ZGBS cases, 34 age- and sex-matched ZIKV$^+$ controls, and 17 age- and sex-matched ZIKV$^-$controls were included in the current analysis. The ZGBS cases were adults with median age of 49 years, and 47% were male (Supplementary Table 1). Two patients reported a history of a previous suspected DENV infection, and 2 patients of a suspected prior chikungunya virus infection. All patients reported viral symptoms during ZIKV infection including arthralgias (94%), fever (88%), and myalgias (88%). The median time from onset of ZIKV symptoms to neurologic symptoms was 10 days (interquartile range [IQR], 7–19; Supplementary Table 1). Access to medical records allowed Brighton criteria GBS classification in 8 of the 17 patients, demonstrating certainty of diagnosis level 1 (based on both nerve conduction studies and cerebrospinal fluid [CSF] analysis) in 18% of cases, level 2 in 18% of cases based on either nerve conduction studies or CSF analysis, and level 3 (based on clinical features) in 12% of cases [4]. One patient was diagnosed with Miller–Fisher syndrome (Supplementary Table 1). Two patients demonstrated demyelination and axonal involvement based on nerve conductions studies.

The most common neurologic symptoms were lower extremity weakness (100%), inability to walk (88%), and paresthesias (100%). The great majority of patients were cared for in the intensive care unit (88%). Half the patients had difficulty breathing, and 38% had respiratory failure requiring intubation. Most patients were treated with intravenous immunoglobulin (63%) or plasmapheresis (25%), and none were treated with steroids. The median duration of hospitalization was 11 days (IQR, 7–24 days), with a median of 9 days (IQR, 5–13 days) in the intensive care unit. One patient died, one-fourth had a full recovery, and 63% reported chronic morbidities including upper and lower extremity weakness, facial tremors, and sensory alterations.

The relationship between antibody responses to ZIKV infection and a clinical diagnosis of GBS was assessed by comparing neutralizing antibody titers between the ZGBS cases and the ZIKV$^+$ and ZIKV$^-$ controls. Because DENV2 recently circulated in Colombia, plasma neutralizing antibody titers against both

Case/Control	Age	Sex	Days from Zika symptom onset to sample draw	Median days to sample draw	DENV2 16680 NT_{50}	DENV2 16680 NT_{80}	DENV2 16680 NT_{90}	ZIKVH/PF NT_{50}	ZIKVH/PF NT_{80}	ZIKVH/PF NT_{90}
GBS POSITIVE	22	M	120		4464	1577	809	9174	4211	2440
	22	M	137		1680	786	505	5990	2588	1486
	32	F	19		132716	43852	23158	286725	115102	64033
	34	F	17		69459	10085	3229	215122	62746	26743
	35	M	30		117540	26841	10438	143053	30164	11209
	38	F	15		106494	31028	13843	1628613	305336	106118
	39	F	55		12182	5528	3167	23051	10014	5747
	40	F	14		195125	87154	51088	121528	35051	15150
	45	F	30	23	74221	33779	27625	37971	14089	7508
	46	M	14		38939	12732	7289	372419	56306	17768
	48	F	29		29850	18817	13748	22686	9957	5752
	48	M	30		103159	36812	27969	195874	57355	25425
	49	F	79		47725	15309	8627	3005	1007	519
	50	F	13		186916	46154	21426	509996	119161	45612
	56	F	23		23309	5499	2207	4525	1231	516
	59	M	10		16791	3348	1280	22231	9494	5302
	88	M	20		31581	10605	6581	15437	4581	2090
ZIKA POSITIVE	15	F	26		62	<50	<50	19452	6104	3183
	18	M	9		1291	524	290	3001	1411	797
	18	M	66		283776	58674	34524	54888	20516	10917
	20	F	8		<50	<50	<50	16277	4410	2257
	20	F	16		2842	1198	967	4526	2067	1258
	21	M	20		1295	647	404	7652	3036	1598
	22	M	11		9477	3233	1709	1071	351	179
	23	M	13		12476	5316	2958	5096	956	372
	26	F	85		7090	2424	1501	29014	9351	5226
	29	F	76		3012	923	459	38083	13088	6543
	30	M	21		93250	37718	21516	41250	10055	6557
	30	F	7		28888	8134	3533	18867	5340	3866
	39	F	5		44797	21184	12351	169474	58392	29451
	40	F	24		5041	1807	1004	9547	2313	1002
	42	M	32		155	61	<50	19654	4889	2020
	43	F	25		5445	2266	1283	3751	1010	436
	44	F	37	26	11078	3535	2216	126872	41558	20211
	45	M	32		12822	4950	3070	12119	5741	4049
	45	M	11		9212	3327	1742	5606	2509	1454
	46	M	29		8048	4191	2723	109021	18314	5500
	46	F	51		70359	24072	12795	237402	146111	128080
	46	F	28		6201	3013	1984	7393	2706	1444
	48	F	53		31252	15538	9750	2039	651	375
	48	F	21		2560	1288	1083	2019	979	804
	49	F	32		1754	810	524	7776	2729	1540
	50	F	13		5353	1984	1021	6137	2897	1783
	51	F	78		359	105	58	1337	395	143
	51	F	63		5800	1743	1122	10826	3239	1536
	55	M	27		12328	4151	2395	6096	2271	1162
	55	F	13		9597	3341	2021	5648	2472	1561
	62	F	13		20644	5520	2486	3725	1409	797
	64	M	50		1359	369	182	2546	962	544
	66	M	8		1967	799	476	9553	2586	1540
	67	M	65		12516	4400	2301	140761	26712	10782
ZIKA NEGATIVE	18	M	-		393	236	204	187	60	<50
	18	M	-		851	395	232	170	<50	<50
	19	M	-		<50	<50	<50	<50	<50	<50
	20	F	-		340	133	100	<50	<50	<50
	23	F	-		1020	298	148	<50	<50	<50
	31	F	-		3751	772	320	222	<50	<50
	39	M	-		58	<50	<50	54	<50	<50
	40	F	-		582	254	206	116	<50	<50
	40	F	-		1250	177	85	173	<50	<50
	41	F	-		787	297	181	<50	<50	<50
	45	F	-		6986	2868	1585	91	<50	<50
	47	M	-		3263	977	495	164	50	<50
	49	F	-		1464	626	331	241	<50	<50
	50	F	-		1137	535	324	371	86	<50
	50	F	-		1450	681	393	<50	<50	<50
	55	M	-		8354	3946	2290	233	68	<50
	50	M	-		1584	1168	1074	<50	<50	<50

KEY

Reciprocal plasma dilutions

- <300
- 300-999
- 1000-4999
- 5000-10000
- >10000

Figure 1. Neutralizing antibody titers to Zika virus (ZIKV) and dengue virus serotype 2 (DENV2) in cases and controls. Age and sex are listed for each case-control participant as well as the time from reported Zika symptoms to date of sampling in days. Reciprocal 50%, 80%, and 90% neutralizing antibody titers to ZIKV strain H/PF/2013 and DENV2 strain 16681 are reported and shaded according to potency as indicated in the key. Abbreviations: DENV2, dengue virus serotype 2; GBS, Guillain-Barré syndrome; NT_{50}, 50% neutralizing titer; NT_{80}, 80% neutralizing titer; NT_{90}, 90% neutralizing titer; ZIKV, Zika virus.

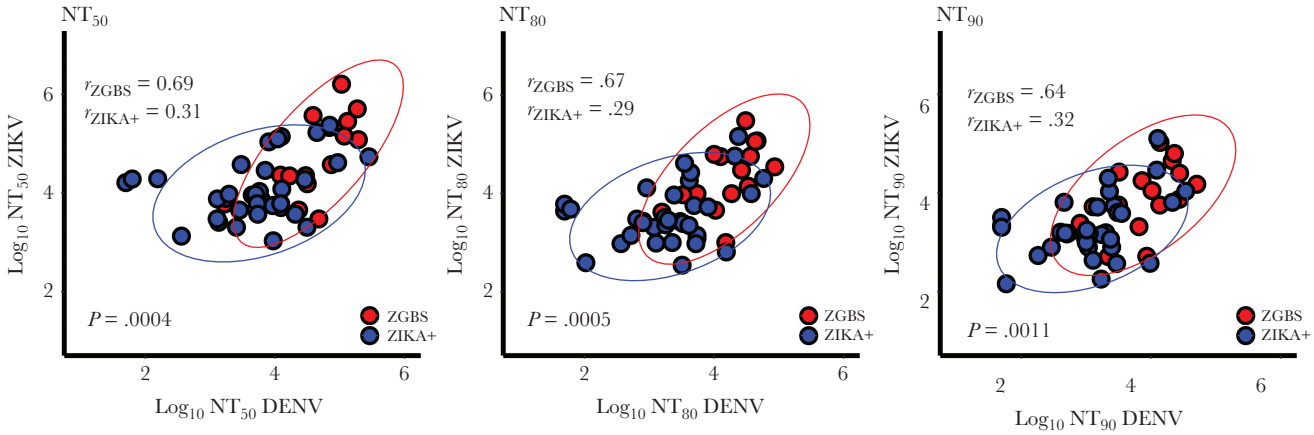

Figure 2. Differences in Zika virus (ZIKV) and dengue virus serotype 2 (DENV2) neutralizing antibody titers between Zika-related Guillain-Barré syndrome (ZGBS) cases and Zika-positive controls. Spearman rank correlation coefficient is provided for each group. The 95% confidence ellipses are indicated for each population and significant *P* values indicate differences between these 2 populations (Hotelling T^2 test). Abbreviations: DENV, dengue virus; NT_{50}, 50% neutralizing titer; NT_{80}, 80% neutralizing titer; NT_{90}, 90% neutralizing titer; ZGBS, Zika-related Guillain-Barré syndrome; ZIKV, Zika virus; ZIKV+, Zika virus positive.

ZIKV H/PF/2013 and DENV2 16681 for all cases and controls were measured, and calculated reciprocal plasma NT_{50}, NT_{80}, and NT_{90} (Figure 1) were reported. We found that mean reciprocal titers against ZIKV were significantly elevated in the ZGBS cases compared with ZIKV+ controls when comparing NT_{50} values (212 788 vs 33 485; $P = .0052$), NT_{80} values (49 317 vs 11 986; $P = .0038$), or NT_{90} values (20 201 vs 7617; $P = .0043$) (Supplementary Table 3). Four of the 17 patients had reciprocal ZIKV NT_{50} <10 000, but 3 of these patients had the longest time interval between onset of disease and sampling (79–137 days). We observed a trend toward lower NT_{80} with increasing days post–Zika infection in the ZGBS group. When comparing ZGBS cases to ZIKV+ controls, we found significantly elevated titers against DENV2 as well (Supplementary Table 4).

As expected, there was a significant correlation between ZIKV and DENV2 neutralizing antibody titers in all ZIKV-infected individuals (both ZGBS and ZIKV+ groups). This correlation was stronger within ZGBS cases (NT_{50}: $r = 0.69$, $P = .002$; NT_{80}: $r = 0.67$, $P = .003$) compared with ZIKV+ controls (NT_{50}: $r = 0.31$, $P = .077$; NT_{80}: $r = 0.29$, $P = .095$) (Supplementary Table 5). These data are summarized by graphing ZIKV vs DENV2 neutralizing antibody titers for each patient, along with 95% confidence ellipses (Figure 2). These ellipses illustrate that there is a statistical difference between the ZGBS cases and the ZIKV+ controls and that ZGBS is associated with elevated neutralizing antibody titers not only to ZIKV but also to DENV2.

DISCUSSION

The clinical and demographic characteristics of ZGBS cases from Barranquilla, Colombia, are in accordance with other studies [3, 7–12]. GBS may occur with rapid onset of both motor and sensory neurologic symptoms following symptomatic ZIKV infection. These cases reported here tended to be severe, with most patients (88%) admitted to intensive care units and more than one-third requiring mechanical ventilation.

The higher neutralizing antibody titers found in the ZGBS cases compared to ZIKV+ controls provide evidence of a correlation between these titers and the development of GBS in these patients, though not causation. This finding could represent an indirect effect that results from high virus load. The time course between the onset of Zika-like symptoms and the development of neurologic symptoms is sufficient for antibody production and would be compatible with the role of an adaptive immune response in this process; however, this observation does not prove that adaptive immunity causes GBS. The onset of neurologic symptoms occurred in median of 10 days after the initial onset of viral symptoms, comparable with other GBS cohorts from Colombia [3, 9, 11].

Higher titers of anti-DENV2 antibodies in ZIKV-infected participants compared to uninfected participants provides more evidence for the observation that DENV B cells are activated after ZIKV infection. This is in agreement with a previous reports such as an analysis of samples from Brazil where neutralizing antibody titers to ZIKV and DENV1 were boosted after ZIKV infection [13]. Confirmation of the role of DENV neutralizing antibodies in ZIKV infection was recently reported in a study of longitudinal B-cell responses to ZIKV after previous DENV infection. It was observed that both ZIKV and DENV neutralizing antibodies are boosted following ZIKV infection but that they derive from distinct B-cell populations and that the anamnestic dengue response occurs first, followed by a de novo ZIKV response [14]. It is possible that the development of GBS is related to molecular mimicry, where virus specific antibodies (either ZIKV or DENV) cross-react with nerve cells, but it is also possible this association between neutralizing antibodies and GBS results from indirect effects such

as high virus load or high immune activation. This cross-sectional analysis cannot fully resolve that issue. Further research is needed to characterize the specific antibody populations responsible for ZGBS, and this information will be critical for Zika vaccine development.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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