Supplementary material

ZIKV and DENV infection diagnosis

Detection of IgM against ZIKV and DENV was performed using indirect immunofluorescent assay (IFA) on the blood samples from patients in both GBS and CTR1 groups. Briefly, ZIKV [PF13-251013-18] isolated during the epidemic in French Polynesia[1] and DENV [D1-Hawaii 1944 reference strain] were independently inoculated onto Vero cells (African Green Monkey kidney cells). One week later, ZIKV- or DENV-infected cells were fixed on microscope slides and exposed to sera, and the presence of anti-ZIKV or anti-DENV IgM was revealed by incubation with goat anti-Human IgM antibody, FITC conjugate (Novex, Life technologies).

Detection of IgG against ZIKV and each the four DENV serotypes was performed on blood samples from patients in GBS, CTR1 and CTR2 groups using a recombinant-antigen based SHERPAxMap microsphere immune assay (MIA) adapted from Beck et al.[2] and detailed in Vanhomwegen J.[3] Briefly, sera were diluted 1/400 and incubated with a mix of microspheres coupled with either DENV-1, -2, -3,- -4 or ZIKV recombinant antigens (E protein domain III) produced in Drosophila S2 expression system. Detection of anti-DENV-1,-2,-3,-4 or anti-ZIKV IgG was performed using a Biotin-SP-conjugated Goat Anti-Human IgG antibody (Jackson ImmunoResearch) and streptavidin, R-phycoerythrin conjugate (SAPE, Life Technologies). The median fluorescence intensity was read on a MagPix instrument (Bio-Rad Laboratories). The cut-off of the MIA was determined by ROC curve analysis for all the antigens using positive and negative control sera.

Detection of neutralizing antibodies against ZIKV and each of the four DENV serotypes was performed for patients in GBS (latest blood sample) and CTR1 groups. Briefly, Vero cells cultured on 96-well plates were inoculated with serial dilutions of each serum previously incubated with titrated ZIKV [PF13-251013-18], DENV-1 [PF15-080108-88], DENV-2 [PF96-300896-243/158], DENV-3 [PF90-300190-30/56] or DENV-4 [PF09-290509-104]. One week later, infected cells were detected by ELISA using primary mouse pan-flavivirus E mAb 4G2 which reacts with ZIKV E protein[4] and a secondary goat anti-mouse IgG HRP-conjugated antibody (Santa Cruz). The neutralizing antibody titer was defined as the inverse of the latest serum dilution that inhibited the virus.

Reactivity of sera with ZIKV by Western blot

ZIKV [PF13-251013-18][1] was used to infect BHK21 and Vero cells with a MOI of 1 for 1hr at 37°C. Fresh medium was added and after 48h of incubation, the supernatant was removed, the cells were washed three times with PBS and collected in RIPA buffer. The cell extract was centrifuged 10 min at 4000g and the supernatant was kept at -80°C.

ZIKV cell extracts were mixed with LDS buffer and was or not heated 10 min at 95°C. Non-infected BHK21 and Vero cells extracts were used as control. Viral proteins recognised by patients' antibodies were detected by Western blot after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of ZIKV cell extracts. After transfer, the nitrocellulose strips were saturated in PBS (pH 7.2) containing 5% dried milk (PBSM) for 1 h at room temperature (RT). The GBS patient's sera were diluted 1:1000 in PBSM containing Tween 20 (0·1%). Pan-flavivirus monoclonal antibody 4G2 was used as control and was diluted 1:1000. They were incubated with the strips for 1 hr at RT. After washings, peroxidase-conjugated goat anti-human IgM and IgG (Cappel) (diluted 1:3000) or anti-mouse antibody IgG(H+L) (BioRad) were added and incubated for 1 h. After 3 washes in PBS, strips

were incubated with peroxidase chemiluminescent substrate (Immobilon®, Sigma) using standard conditions and detected according to the manufacturer's recommendations.

Immunosuppression test for exploration of molecular mimicry mechanisms

Molecular mimicry was evaluated using a previously published method[5]. The serum from the patient showing highest reactivity towards GA1 without showing reactivity towards any other glycolipid (n°20 on Suppl. Table 2) was first diluted at 1:2000 with normal saline; then 5 ml aliquots were incubated overnight with 100 µg, 300 µg, and 600 µg of purified GA1 before repeating the Western blot analysis, using an aliquot at the initial dilution as control. Immunosuppression was also tested with sera n°6 after incubation with 300 µg of GA1.

Combinatorial microarray

A combinatorial microarray screening method that is based on a refinement and miniaturisation of our previous published combinatorial glycoarray assay[6] was used to assess ZIKV GBS sera for anti-complex antibodies. Lipid microarrays were fabricated in-house using a non-contact, piezoelectric dispensing system (S3 flexarrayer, Scienion, Germany). All lipids (GM1, GM2, GA1, GD1a, GD1b, Phospatidylserine (PS), Cholesterol (Chol), Sulphatide (Sulph) and Galactocerebroside (GalC)) were obtained from Sigma (Poole, UK), with the exception of GQ1b and CTH (Matreya, Pleasant Gap, USA), and SGPG and LM1, which were kindly gifted by Prof. R. Yu (Georgia Regents University, Augusta, USA). Working solutions of each lipid was prepared at a concentration of 200µg/ml in methanol and heteromeric lipids were prepared by mixing equal volumes of working solution, with a total concentration of 200µg/ml. Microarray platforms were made by adhesion of low fluorescence PVDF membrane (Millipore, Nottingham, UK) on to glass microscope slides. Each slide was printed with 16 individual arrays. For each array, approximately 500pl (100pg/spot) of lipid solution was applied at predetermined locations, in a grid-like pattern. As all lipids were printed in duplicate, arrays were designed with a line of symmetry, diagonally from top left to bottom right, with all single lipids printed in the first row and column. The location of all heteromeric complexes are identified in the array grid, as a 1:1 ratio of the two different single lipids printed in the first position of each row and column. Each array contained 91 different lipid targets (13 single lipids and 78 heteromeric complexes) in duplicate.

Sera Testing

Due to the limited availability of patient samples, only 41 sera at the initial time point and 27 of the 42 patients sera at 3 months were screened by combinatorial array. Microarray slides were blocked in 2% Bovine Serum Albumin (BSA, Europa Bioproducts, Ely, UK) in PBS on a rocker platform, for 1 hour at room temperature. After which, microarray slides were placed in a FAST frame device containing a 16-well incubation chamber (Maine Manufacturing, Sanford, USA), thereby enabling the isolation of each of the 16 subarrays and corresponding samples, per slide. All patient and control sera were diluted 1:50 in 1% BSA/PBS and 100µl was added per sub-array for 1 hour at 4˚C. Serum samples were aspirated from each well and washed twice with 1% BSA/PBS prior to removal from the FAST frame, after which microscope slides were washed twice, *en masse* in 1% BSA/PBS for 15 mins, on a rocker platform. Antibody-lipid interactions were identified using Alexafluor-647 conjugated anti- human IgG (Jackson ImmunoResearch Laboratories, West Grove USA) and Alexafluor-555 conjugated anti-human IgM (Life Technologies, Eugene, USA) isotype specific antibodies. Array slides were place in the FAST frame device once more and 100µl of secondary detection antibodies were applied at 4µg/ml in 1% BSA/PBS to each well, allowing identification of

both isotypes binding events, in each array. Slides were incubated for 1hour at 4˚C, followed by washing twice *en masse* in 1%BSA/PBS for 30mins followed by 5 mins in PBS and a further 5 mins in deionised water, before air drying.

Array imaging and quantification

Arrays were visualised with a FLAIR scanner (Sensovation, Radolfzell, Germany) and quantified using ProScanArray Express software (Perkin Elmer, Seer Green, UK). Median fluorescent intensity signals were calculated for each lipid target, with the local background signal subtracted. Mean intensity values were calculated for duplicate spots. Samples were tested on 3 separate occasions and the mean was calculated. Each of the 88 sera samples, were tested against 91 different targets, resulting in the measurement of 8008 individual interactions for each antibody isotype. For ease of comparison, data was visually displayed as heat maps (TM4-MeV MultiExperiment Viewer v4.66 software), in which the rainbow scale was used to assign a colour to each interaction, which indicated the intensity of the antibody binding for that target. Hierarchical clustering was performed with Pearson's correlation, to group sera with similar binding profiles. In order to establish the normal range of naturally occurring anti-glycolipid antibodies in this discrete population, the 95th percentile of the healthy controls was calculated for each target. These values were used as the threshold of positivity when screening the GBS cohort. As a result 5 % of all controls were identified as positive for each target (specificity=95%). Fisher's exact test was used for comparison of proportionality.

Results

Amongst the anti-glycolipid IgG antibody repertoire, complex dependent GM2:LM1 binding was observed in 4 sera (3 patients and 1 control; sensitivity=7.3%, specificity= 95%), in which no binding was observed with the single component lipids (Suppl Figure 4). Whilst not significant, it is interesting to note that the one control sample had a GM2:LM1 binding intensity approximately one-third of the average intensity observed in the patient's serum (1205 IU vs 3820 IU). Eight patient samples had strong IgG binding to GA1, as a single glycolipid (sensitivity= 19.5%, specificity 95%). Whilst not a significant target alone, when GA1 was in complex with sulphatide, antibody binding was enhanced, reaching a sensitivity of 46.3% and specificity of 95% (p=0.001), of which, 13 samples were complex dependent, in which antibody binding intensities were below the positivity threshold for the individual components (GA1 and sulphatide). In addition, a significant number of patient sera were positive for GA1 in complex with cholesterol and/or phosphatidylserine (48.8%), however many were of low binding intensity, despite being above the threshold. One patient sample had strong GM1 single (2867IU) and complex reactivity which also reacted with GA1. One patient sample contained antibodies strongly reactive against single GD1a (1278 IU), but only in the 3 month sample. This interaction was attenuated in complexes containing other large glycolipid molecules (namely GD1b, GQ1b, SGPG, LM1 and CTH). The binding profile of IgM antibodies in patient and control sera was very similar, in which binding to single and complexes of GA1 was frequently observed. Three of the 41 patient samples taken at the initial time point, demonstrated IgM binding to single and complexes of GM1 (sensitivity=7.3%, specificity= 95%), one of which also had weak binding to GD1b. While heteromeric GA1:sulphatide, GA1:cholesterol and GA1:phosphatidylserine were all significant targets in this screen, it is interesting to note the absence of single and complex targets (for example GM1, GD1a, GD1a:GD1b) frequently associated with the AMAN variant of GBS in other studies.

References

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