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EPSTEIN-BARR-NEGATIVE MS: A TRUE PHENOMENON?

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Epstein-Barr virus (EBV) infection is associated with MS; up to 3.3% people with MS are EBV nuclear antigen-1 (EBNA1)-seronegative compared with 6.0% controls.¹ EBV serology is complex, and multiple antigens are required to assess seropositive status.^{2,3} We examined a cohort of seemingly EBV-negative patients with clinically isolated syndrome (CIS). The size of the population enrolled in the International CIS study allowed us to examine the largest population of seemingly EBV-negative patients with CIS gathered to date.

Methods. The International CIS study is a collaborative study across 33 centers. Inclusion and exclusion criteria and methods for sample and data collection have previously been described.⁴ Immunoglobulin G (IgG) reactivity against EBNA1 was initially evaluated using commercially available ELISA (ETI-EBNA-G, Diasorin, Italy) according to the manufacturer's instructions.

Samples with anti-EBNA1-IgG reactivity less than the manufacturer's cutoff value (CoV) (<20 AU/mL) for the ETI-EBNA-G ELISA (screen-negative) were tested using well-validated in-house ELISAs based on multiepitope peptides of EBNA1 and virus capsid antigen (VCA) using previously described methods.^{5,6} The CoV for the in-house ELISA was the mean OD450 value of 4 truly EBNA1- and VCA-negative sera plus twice the SD (mean + 2 SD). OD450 values were normalized against the CoV, and values >1.0 were considered positive. Samples demonstrating negative or borderline (0.8–1.2) results against both EBV antigens were investigated using an EBV-specific immunoblot.⁵

Results. Patient details are provided in the table. The CIS cohort has previously been described in detail.⁴ Forty-one (3.9%) of 1,047 patients were screen-negative. Age, sex, time to serum sampling and clinically definite MS (CDMS) and follow-up duration were not different to the whole cohort. Screen-negative patients were less likely to be CSF oligoclonal band (OCB)-positive (48.8% vs 74.3%; $p = 0.0009$, Fisher exact test) and less likely to be smokers ($p = 0.01$). Anti-cytomegalovirus (CMV)-IgG⁴-negative patients were not less likely to be OCB-negative (52% vs 48%).

Of the screen-negative samples, 5/41 had reactivity <1.0 for anti-EBNA1-IgG and 2/41 <1.0 for anti-VCA-IgG and 2/41 had reactivity <1.0 against both EBV antigens. When values obtained from the 2 EBNA1 assays were compared, they demonstrated a correlation coefficient (r_{Spearman}) of 0.57 after 3 outliers with reactivity <CoV of the ETI-EBNA-G ELISA, but high reactivity on the in-house ELISA were excluded (figure, A). A Bland-Altman plot demonstrated no evidence of systemic bias and reasonable agreement between the 2 assays (figure, B). Of the 2 samples with low reactivity to both EBV antigens, 1 showed no reactivity on immunoblot. This patient was OCB-positive and developed CDMS during follow-up.

Discussion. Only 1 of 1,047 patients (<0.01%) was truly EBV-negative. Previous literature demonstrates a strong association between MS and EBV infection; in keeping with this, 41 patients (3.9%) showed no EBNA1-reactivity on initial screen. More detailed testing revealed a much higher rate of EBV seropositivity in CIS and MS patients than previously described.

There is significant antigenic diversity of anti-EBV-IgG responses in EBV carriers and patients with EBV-related disease.^{2,3} Serologic testing against multiple antigens is recommended to accurately define EBV status when examining the link between EBV infection and other diseases. In studies examining MS risk and EBV infection, the rate of EBV positivity in the population is highly dependent on the method used to determine EBV serostatus.⁷

ELISA against 2 different EBV antigens provides a screening method; immunoblot on apparently seronegative samples increases sensitivity. The ETI-EBNA-G ELISA was validated for Food and Drug Administration's approval against a cell-based EBNA1-anticomplement immunofluorescence. The CoV for this ELISA is likely set relatively high, giving borderline values a negative interpretation. The in-house ELISA that we used utilizes the same peptide antigen, but was validated against purified EBNA1, increasing sensitivity and explaining the correlation between EBNA1 titers in the 2 ELISAs. Some EBNA1-IgG-negative sera are positive against other EBV antigens.

The fact that patients who were EBNA1-screen-negative were less likely to have OCBs provides an avenue for future research; there is likely a biological link

Supplemental data
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Table Details of the 41 patients with CIS who had undetectable anti-EBNA1-IgG levels on initial screening

	EBNA1-screen-negative cohort	Entire CIS cohort ^a	p Value
No. of patients, n	41	1,047	
Age, y, mean (SD)	33.3 (10.1)	32.9	NS
Sex (F:M)	27:14 (65.9% F)	714:333 (68.2% F)	NS
Duration of follow-up, d, median (IQR)	1,483 (988–2,138)	1,574 (1,042–2,330)	NS
OCB-positive, n (%)	20 (48.8)	778 (74.3)	0.0009
CMV positive, n (%)	25 (61.0)	546 (52.1)	NS
EDSS at CIS (where available), median (IQR) ^b	2.0 (1.0–2.0)	2.0 (1.0–2.5)	NS
EDSS at last follow-up, median (range) ^c	1.0 (0–2.0)	1.5 (1.0–2.0)	NS
Converted to CDMS, n (%)	23 (56.1)	623 (59.5)	NS
Median time to conversion, d (IQR)	339 (166–552)	421 (212–853)	NS
T2 lesion number at baseline			
0–1	3 (7.3)	151 (14.4)	NS
2–9	18 (43.9)	438 (41.8)	
>9	20 (48.8)	458 (43.7)	
Gadolinium-enhancing lesions at baseline, n (%) ^d	8 (25.8)	366 (45)	0.060
No. of gadolinium-enhancing lesions at baseline, median (IQR) ^e	0 (0–1)	0 (0–1)	NS
Serum vitamin D3, nmol/L, median (IQR)	47.9 (37.1–66.6)	49.3 (32.2–72.5)	NS
Cotinine level >14 ng/mL, n (%)	6 (14.6)	350 (33.7)	0.010

Abbreviations: CDMS = clinically definite MS; CIS = clinically isolated syndrome; EBNA1 = Epstein-Barr virus nuclear antigen-1; IQR = interquartile range; CMV = cytomegalovirus; EDSS = Expanded Disability Status Scale; NS = not significant; OCB = CSF oligoclonal band.

^a Includes 41 patients with undetectable EBNA1.

^b EDSS available on 34/41 patients in the undetectable EBNA1 group and 925 in the entire cohort.

^c Follow-up EDSS available on 26/41 patients in the undetectable EBNA1 group and 736 in the entire cohort.

^d Data on the presence of gadolinium-enhancing lesions available on 31/41 in the undetectable EBNA1 group and 809 in the entire cohort.

^e Data on the number of gadolinium-enhancing lesions available on 30/41 in the undetectable EBNA1 group and 755 in the entire cohort.

between lower EBV immunoreactivity and OCB production. However, in our earlier study, while OCB predicted a second event, lower EBV reactivity did not⁴—the relationship therefore is more complex than a simple linear or threshold effect. EBNA1-seropositivity occurs later than VCA seroconversion; low titers of anti-EBNA1-IgG may reflect recent EBV infection which has not yet triggered downstream biological events. It may be that patients who are EBNA1-screen-negative have a lower overall level of immune response, resulting in negative OCBs. However, the same relationship is not seen in the CMV-seronegative population, hinting at an EBV-specific link.

With only 1 of 1,047 patients in our large international cohort testing negative for EBV across multiple antigens and 2 platforms, it seems that while it is possible to be truly EBV seronegative and develop MS, this is extremely rare. It seems likely that this indicates a role for EBV in MS development, and further research is needed to examine this further.

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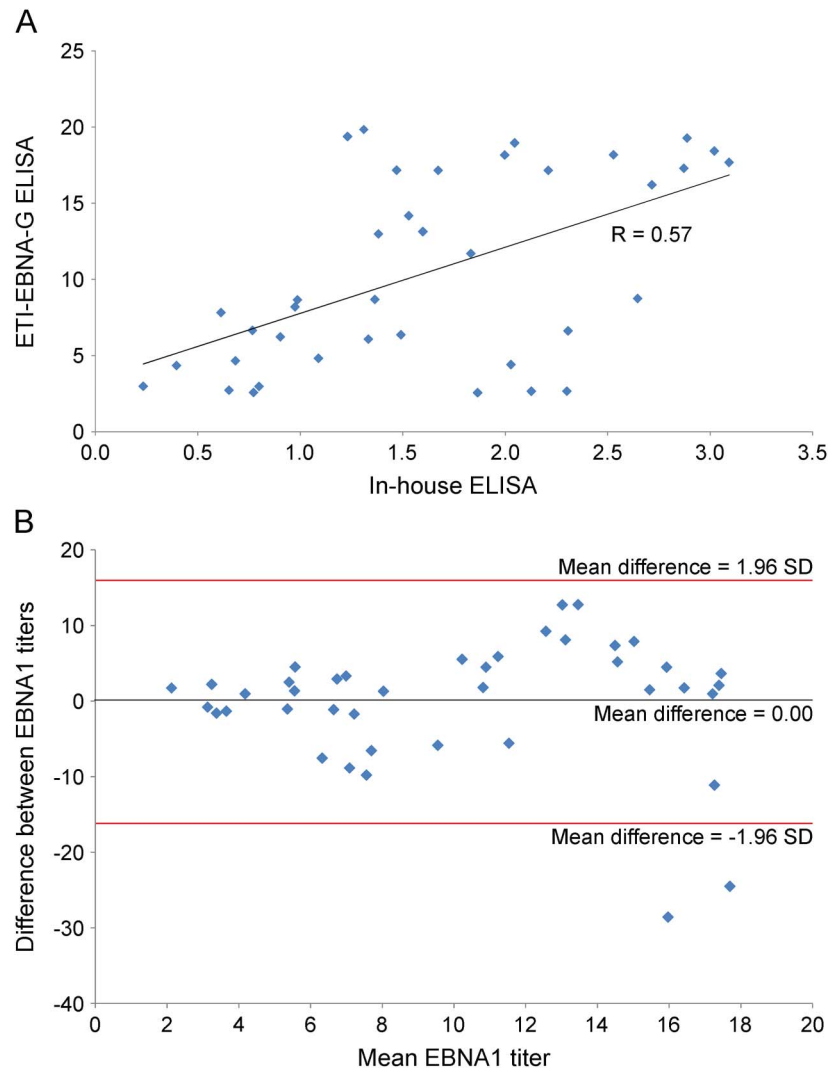
Full list of investigators and centers can be found in the coinvestigators list at Neurology.org/nn.

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Author contributions: R.D. analyzed the data, performed the statistical analysis, drafted the manuscript, and produced the final version. J.K. led the international CIS study, arranged sample analysis, and collated all data prior to analysis. J.M. performed the EBV ELISA and immunoblot. G.G. conceived the study and provided intellectual guidance. All authors revised the draft manuscript and approved the final version.

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Figure Scatter graph and Bland-Altman plot demonstrating correlation and agreement between the 2 EBNA1 assays



(A) Scatter graph demonstrating correlation between the 2 Epstein-Barr virus nuclear antigen-1 (EBNA1) assays. (B) Bland-Altman plot demonstrating reasonable agreement between the 2 assays. Given the different arbitrary scales used to report assay results, the in-house ELISA values have been multiplied by the ratio of the mean values of each ELISA to allow them to be reported on the same scale.

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