Clinica Chimica Acta 558 (2024) 119679

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Case report

Hook-effect in MAGLUMI immunoassay for serum anti-GAD antibodies in neurological disorders: When "wrong" matrix is the right choice

G. Musso [a,b,*], M. Zoccarato [c], N. Gallo [b], M. Plebani [a,b], D. Basso [a,b]

[a] Department of Medicine-DIMED, University of Padova, Padova, Italy
[b] Laboratory Medicine Unit, University-Hospital of Padova, Padova, Italy
[c] Neurology Unit, Ospedale Sant'Antonio, University-Hospital of Padova, Padova, Italy

ARTICLE INFO

Keywords:
Hook-effect
Immunoassays
Chemiluminescence
Analytical interference
Autoantibodies
Anti-GAD
Neurological disorders

ABSTRACT

Antibodies against glutamic acid decarboxylase (anti-GAD) are a valuable diagnostic tool to detect severe autoimmune conditions as type 1 diabetes mellitus (T1DM) and anti-GAD related neurological disorders, having the latter more often anti-GAD concentrations in serum multiple times higher than in the former. Automated immunoassays, either with ELISA or chemiluminescent technology, are validated for diagnostic use in serum with analytical ranges suitable for T1DM diagnosis. In a patient presenting with a suspected autoimmune ataxia, anti-GAD testing on an automated chemiluminescent immunoassay (CLIA) resulted in slightly abnormal concentrations in serum (39.2 KIU/L) and very high concentrations in CSF (>280 KIU/L), thus prompting to proceed to serum dilutions to exclude a false negative result and a misdiagnosis. Different dilutions of serum resulted in nonlinear concentrations with endpoint result of 276,500 KIU/L at dilution 1:1000. CSF dilution was instead linear with endpoint result of 4050 KIU/L. In this case report we found that anti-GAD testing in CSF was essential to establish the clinical diagnosis and to suspect hook-effect in serum due to the excess of autoantibodies in this severe autoimmune condition.

1. Introduction

Antibodies against glutamic acid decarboxylase (anti-GAD) have a key diagnostic value for type 1 diabetes mellitus (T1DM) [1] but are associated also with severe neurological autoimmune disorders related to an altered GAD-mediated synthesis of γ-aminobutyric acid (GABA) from glutamate [2], such as stiff-person syndrome (SPS), cerebellar ataxia, epilepsy and limbic encephalitis [3], where anti-GAD serum concentrations may be found even a hundred folds higher than those in diabetes [4].

Commonly used immunoassays are ELISA (more widely used) and chemiluminescence (CLIA) that are certified for in vitro diagnostic (IVD) use for serum only and not for other matrices, and whose measuring ranges are optimized for T1DM diagnosis [2]. Nevertheless, a diagnosis of a possible neurological anti-GAD related disorder is usually established when a consistent clinical phenotype is supported by very high levels of serum autoantibodies, though a definite and internationally accepted decisional level for serum anti-GAD concentration is still lacking in the neurological setting [2].Cerebrospinal fluid (CSF) is a valuable matrix when suspecting an autoimmune neurological disorder [5] and different clinical algorithms [2,3] suggest testing for CSF also when considering anti-GAD disease. Following these approach, analytical performances of a chemiluminescence testing on CSF were previously evaluated by us and proposed to be acceptable for routine clinical use [6].

Immunoassays are a pillar in the clinical laboratory work-up to detect and quantify autoantibodies; the principle of antigen–antibody interactions enables high analytical sensitivity and specificity, nevertheless different interferences might affect the measurement of any analyte, whether depending or not on the measurand itself [7]. Among the most common causes of inaccurate quantification, mainly false-negative results, the hook-effect or prozone effect is of particular interest for autoantibodies and laboratory professionals should be sufficiently trained to suspect and detect this event [8]. The hook-effect is caused by an excess of antigen concentration in the patient sample that saturate the antigen-binding sites of the capture and/or detection antibody [9], therefore, when plotting the curve of the assay output signal against antigen concentration, as it increases above the limit of the assay saturation the curve paradoxical decline with a "hook-like" shape [8,9]. Hook-effect of serum anti-GAD in a case of neurological disorder has

* Corresponding author at: Department of Medicine-DIMED, University of Padova, via Giustiniani, 2 35128 Padova, Italy.
E-mail address: giulia.musso@unipd.it (G. Musso).

https://doi.org/10.1016/j.cca.2024.119679
Received 15 February 2024; Received in revised form 26 March 2024; Accepted 17 April 2024
Available online 18 April 2024

already been reported for a single-step radioimmunoassay, where also the measurement range was developed for T1DM [8]; two-step solid-phase immunoassays, such as ELISA and CLIA, are generally designed to lower the risk of prozone effect by means of washing protocols or sequential incubations [8,9], therefore a false-negative result is usually not common, as long as the commercial immunoassay is employed for the intended diagnostic use.

2. Case presentation

2.1. Patient

A woman aged 74 years old underwent neurological examination for progressive limitation of the ability to walk due to instability. One year before she had a vertigo attack with vomiting and subsequent fall. Later she developed dysarthric speech without swallowing impairment. 4 months before the visit she fell again with pelvis fracture. Magnetic resonance imaging (MRI) conducted one month before the visit mild diffuse atrophy of the brain, especially of the cerebellum. Previous medical history included psoriasis, osteoporosis, breast cancer treated with quadrantectomy and intra-operatory radiotherapy; chronic therapy: low-dose aspirin.

Neurologic examination revealed a cerebellar syndrome consisting of an ataxic gate, requiring support for walking. The speech was notably dysarthric, the finger-to-nose and the heel-to-shin test were impaired bilaterally, and she had diplopy and upbeat nystagmus in every gaze direction.

2.2. Laboratory work-up

A complete routine blood work up showed normal complete blood count (CBC), normal glucose, no impairment in renal nor hepatic function, no inflammatory signs, normal immunoglobulins concentrations (IgG, IgA, IgM); no autoantibodies of systemic autoimmune diseases were found, with the exception of low concentrations of anti-Ro52 antibodies.

A lumbar puncture was performed: CSF had a normal cell count (4 WBC/μL), mainly CD4 + lymphocytes at flow cytometry analysis (Navios Ex, Beckman Coulter, USA); glucose, total protein, and lactic acid concentrations were normal; oligoclonal bands were found in the CSF, IgG index was 0.685 (reference 0 – 0.7).

Specific neurological antibodies against neuronal surface antigens (indirect immunofluorescence on fixed cell-based assay, Autoimmune Encephalitis Mosaic 6, Euroimmun, Germany) and onconeural antibodies (immunoblotting, Paraneoplastic Neurological Syndromes-12, Euroimmun, Germany) were negative in both serum and CSF, except for anti-GAD with a strong positive result in both matrices.

Anti-GAD quantification was performed on an automated CLIA system (MAGLUMI 2000 Plus by Snibe, China) with the assay kit MAGLUMI GAD65 lot number 069,220,211 having 2 calibrators (calibrator 1: 9348 RLU, 13.7 KIU/L; calibrator 2: 229,026 RLU, 169.3 KIU/L), with a measuring range of 1.0–280.0 KIU/L and a reference range for serum of < 17 KIU/L. Serum anti GAD concentration was 39.2 KIU/L, CSF concentration was > 280 KIU/L. Given the high concentration in CSF, an antigen excess in serum was suspected, so serum and CSF samples were then diluted with saline solution as standard laboratory procedure. Serum was diluted at 1:10, 1:50, 1:100 and 1:1000 with nonlinear results of relative light units (RLU): increasing as dilution proceeded until 1:100 and then falling abruptly (Fig. 1). At the endpoint dilution of 1:1000 anti-GAD concentration was 276,500 KIU/L.

CSF was diluted at 1:10 and 1:100 only due to low sample volume, with linear RLU results (Fig. 2). At the endpoint dilution of 1:100 anti-GAD concentration in CSF was 4050 KIU/L.

To exclude a possible effect of heterophyilic antibodies, serum was also treated in heterophyilic blocking tube (HBT, Scantibodies, USA), and tested again resulting in 41.4 KIU/L for the whole specimen and 207.9 KIU/L for dilution 1:10.

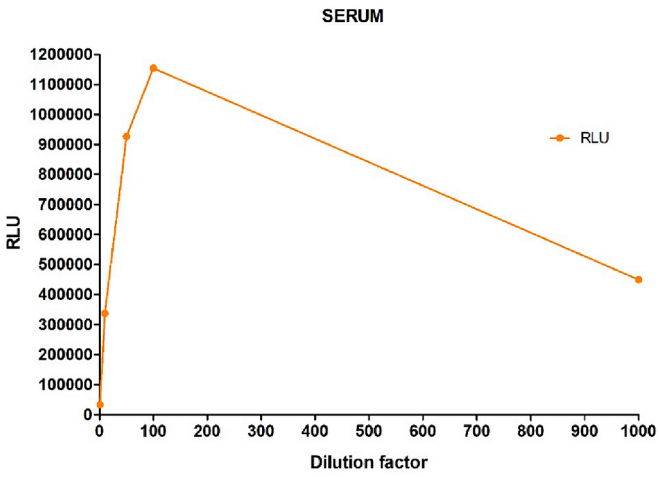

Fig. 1. Relative light units (RLU) of serum anti-GAD at different dilution factors.

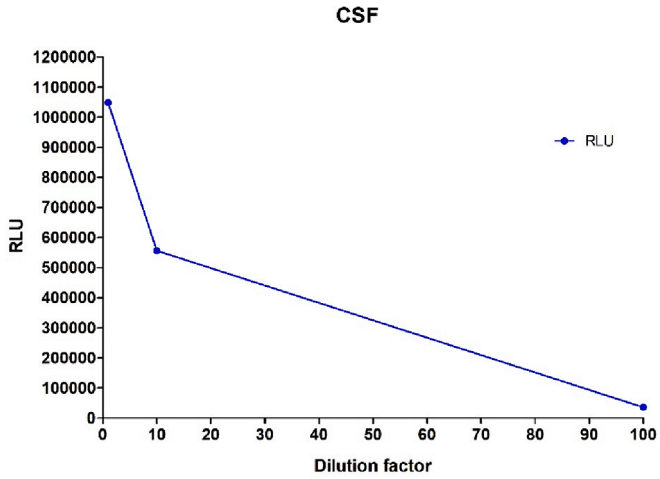

Fig. 2. Relative light units (RLU) of cerebrospinal fluid (CSF) anti-GAD at different dilution factors.

Final concentrations in KIU/L for both matrices and complete dilutions are reported in Table 1.

The patient was eventually diagnosed with anti-GAD cerebellar ataxia.

3. Discussion

To our knowledge, this is the first report of hook-effect in the automated CLIA anti-GAD immunoassay: although generally exempted from hook effect, especially when compared to one-step immunoassays such as radioimmunoassay, chemiluminescent two-step immunoassays might also encounter this effect [8], particularly when measuring autoantibodies if an extreme antigen excess is due to a severe autoimmune condition.

The results of this case report highlighted a mismatch between antibody concentrations in CSF and serum, that led us to hypothesize a hook-effect that was demonstrated with a dilution protocol. This case might support the pragmatic approach that the appropriate matrix for anti-GAD neurological disorders might be CSF, that do not suffer from antigen excess effect because of the relative lower intrathecal concentration of autoantibodies. For this reason, more efforts should be directed towards the validation of IVD assays for CSF use, a goal that is

Table 1
Measured and calculated (measured * dilution factor) concentrations of anti-GAD in serum and cerebrospinal fluid (CSF). HBT: heterophylic blocking tube.

	Serum		Serum + HBT		CSF	
Dilution	Measured KIU/L	Calculated KIU/L	Measured KIU/L	Calculated KIU/L	Measured KIU/L	Calculated KIU/L
UNDILUTED	39.2		41.4		>280	
1:10	222	2220	207.9	2079	>280	>280
1:50	>280	>280				
1:100	>280	>280			40.5	4050
1:1000	276.5	276500				

currently far from achievement, leaving CSF as a "wrong" matrix to test. Additionally, measuring ranges for serum anti-GAD immunoassays, whether based on ELISA or CLIA technology, should also be optimized for autoimmune neurological disorders: the magnitude of the hook-effect in our case is of particular note when comparing the undiluted serum anti-GAD result of 39.2 KIU/L to the endpoint dilution of 276,500 KIU/L, thus highlighting that the antibody concentration relevantly exceeded the measuring range of this immunoassay, event that might be expected in a pathological context characterized by an extraordinary high antibody concentration. Despite our relative experience with this specialized diagnostic, we were surprised by this finding, that was not considered in our previous work on the analytical validation of this assay for CSF [6], for which the measuring range showed to be fairly fitted for the purpose and the hypothesis of a possible hook-effect on the combined serum samples was not undertaken. Although rare, with an estimated prevalence of 1–2 cases per million [2], the anti-GAD neurological disease is severe and a major source of permanent disability [2,10], and a recent case involving a world-famous celebrity has raised public awareness on this concern [11], which might lead to a progressive increase in the number of requests for anti-GAD testing and a concurrent finding of other cases of extremely high antibody concentrations in serum with a possible hook-effect.

A debated key point in this field is a fixed decisional level for anti-GAD serum concentration to support the diagnosis, where clinical presentation is essential but not sufficient to establish an "anti-GAD" related disorder. Updated neurological guidelines mention "high concentrations" in serum but fail to provide a unique value, with most recent studies proposing a possible cut point of > 10000 KIU/L with ELISA methods [12]. Moreover, while in our case report the patient was diagnosed with anti-GAD cerebellar ataxia, an additional issue would be defining concentration ranges specific for the different anti-GAD neurological phenotypes, but until now a clear discrimination between the disorders has not been found [12]. It is also worth mentioning that T1DM is a more common disease than anti-GAD neurological syndromes [13], so a coincidental finding of serum anti-GAD levels above reference range is not unlikely and is *per se* ambiguous if T1DM cannot reliably be excluded.

Different techniques enable the detection of anti-GAD, including qualitative techniques as immunoblotting and indirect immunofluorescence on tissue-based assays [2], that generally have a lower sensitivity than quantitative methods [2]. To prevent a paradoxical request from clinicians of a confirmatory test with a qualitative instead than with a quantitative method, to ensure the detection of "really high concentrations" of anti-GAD [12], laboratory professionals should carefully work together with expert clinical neurologists in the field to develop testing algorithms the include CSF testing and appropriate decisional limits for anti-GAD related disorders.

The analytical phase is of all the total testing process under utmost control from clinical laboratory professionals and a constant concern is paid to the thorough validation of analytical results in the diagnostic laboratory [14]. Recognition of an analyte-dependent interfering effect as the hook-effect, that may occur for any analyte at extremely high concentrations [7], is essential for laboratory professionals, that should suspect it in case of discordant results or upon interaction with the clinicians [7] and should apply appropriate investigation algorithms [15]. Immunoassays were a breakthrough innovation at their first appearance in 1959 and their pivotal role in the modern laboratory medicine is continuously strengthened by technological innovation [16]: in this scenario, the clinical reasoning and a critical thinking are essential tools to beware of unpredicted analytical interferences, and, most importantly, to "convert results in clinical information" [17].

CRediT authorship contribution statement

G. Musso: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **M. Zoccarato:** Writing – review & editing, Methodology, Investigation, Conceptualization. **N. Gallo:** Writing – review & editing, Investigation, Data curation, Conceptualization. **M. Plebani:** Writing – review & editing, Supervision. **D. Basso:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

The authors thank Maria Grazia Marzellan for valuable technical support.

References

[1] W.E. Winter, D.A. Schatz, Autoimmune markers in diabetes, Clin. Chem. 57 (2011) 168–175, https://doi.org/10.1373/clinchem.2010.148205.
[2] F. Graus, A. Saiz, J. Dalmau, GAD antibodies in neurological disorders - insights and challenges, Nat. Rev. Neurol. 16 (2020) 353–365, https://doi.org/10.1038/s41582-020-0359-x.
[3] M.C. Dalakas, Stiff-person syndrome and GAD antibody-spectrum disorders: GABAergic neuronal excitability, immunopathogenesis and update on antibody therapies, Neurotherapeutics 19 (2022) 832–847, https://doi.org/10.1007/s13311-022-01188-w.
[4] A. Saiz, Y. Blanco, L. Sabater, F. González, L. Bataller, R. Casamitjana, L. Ramió-Torrentà, F. Graus, Spectrum of neurological syndromes associated with glutamic acid decarboxylase antibodies: diagnostic clues for this association, Brain 131 (2008) 2553–2563, https://doi.org/10.1093/brain/awn183.
[5] T. Blinder, J. Lewerenz, Cerebrospinal fluid findings in patients with autoimmune encephalitis-A systematic analysis, Front. Neurol. 10 (2019) 804, https://doi.org/10.3389/fneur.2019.00804.
[6] G. Musso, M. Zoccarato, N. Gallo, A. Padoan, C. Cosma, L. Zuliani, P. De Gaspari, E. Pegoraro, M. Plebani, D. Basso, Analytical evaluation of a GAD65 antibodies chemiluminescence immunoassay for CSF in neurological syndromes, Clin. Chem. Lab. Med. 61 (2023) 1802–1807, https://doi.org/10.1515/cclm-2023-0072.
[7] G. Ward, A. Simpson, L. Boscato, P.E. Hickman, The investigation of interferences in immunoassay, Clin. Biochem. 50 (2017) 1306–1311, https://doi.org/10.1016/j.clinbiochem.2017.08.015.
[8] J.F. Jacobs, R.G. van der Molen, X. Bossuyt, J. Damoiseaux, Antigen excess in modern immunoassays: to anticipate on the unexpected, Autoimmun. Rev. 14 (2015) 160–167, https://doi.org/10.1016/j.autrev.2014.10.018.
[9] C. Selby, Interference in immunoassay, Ann. Clin. Biochem. 36 (1999) 704–721, https://doi.org/10.1177/000456329903600603.

[10] J.F. Baizabal-Carvallo, The neurological syndromes associated with glutamic acid decarboxylase antibodies, J. Autoimmun. 101 (2019) 35–47, https://doi.org/10.1016/j.jaut.2019.04.007.
[11] A. Elsalti, M. Darkhabani, M.A. Alrifaai, N. Mahroum, Celebrities and medical awareness-the case of celine dion and stiff-person syndrome, Int. J. Environ. Res. Public. Health. 20 (2023) 1936, https://doi.org/10.3390/ijerph20031936.
[12] A. Muñoz-Lopetegi, M.A.A.M. de Bruijn, S. Boukhrissi, A.E.M. Bastiaansen, M.M.P. Nagtzaam, E.S.P. Hulsenboom, A.J.W. Boon, R.F. Neuteboom, J.M. de Vries, P.A.E. Sillevis Smitt, M.W.J. Schreurs, M.J. Titulaer, Neurologic syndromes related to anti-GAD65: Clinical and serologic response to treatment, Neurol. Neuroimmunol. Neuroinflamm. 7 (2020) e696. doi: 10.1212/NXI.0000000000000696. Erratum in: Neurol. Neuroimmunol. Neuroinflamm. 7 (2020) e733. Erratum in: Neurol. Neuroimmunol. Neuroinflamm. 11 (2024) e200215.
[13] Gbd, Diabetes Collaborators, Global, regional, and national burden of diabetes from 1990 to 2021, with projections of prevalence to 2050: a systematic analysis for the global burden of disease study 2021, Lancet 402 (2023) (2021) 203–234, https://doi.org/10.1016/S0140-6736(23)01301-6. Erratum. In: Lancet 402 (2023) 1132.
[14] M. Plebani, Analytical quality: an unfinished journey, Clin. Chem. Lab. Med. 56 (2018) 357–359, https://doi.org/10.1515/cclm-2017-0717.
[15] M. Zaninotto, M. Plebani, Understanding and managing interferences in clinical laboratory assays: the role of laboratory professionals, Clin. Chem. Lab. Med. 58 (2020) 350–356, https://doi.org/10.1515/cclm-2019-0898. Erratum. In: Clin Chem Lab Med. 59 (2021) 1005.
[16] A.H. Wu, A selected history and future of immunoassay development and applications in clinical chemistry, Clin. Chim. Acta 369 (2006) 119–124, https://doi.org/10.1016/j.cca.2006.02.045.
[17] M. Plebani, M. Laposata, G. Lippi, A manifesto for the future of laboratory medicine professionals, Clin. Chim. Acta 489 (2019) 49–52, https://doi.org/10.1016/j.cca.2018.11.021.