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ORIGINAL ARTICLE

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EBER in situ hybridization in subcutaneous aluminum granulomas/lymphoid hyperplasia: A diagnostic clue to differentiate injection-associated lymphoid hyperplasia from other forms of pseudolymphomas and cutaneous lymphomas

Verena G. Frings[1] | Sabine Roth[2] | Andreas Rosenwald[2] | Matthias Goebeler[1] | Eva Geissinger[2,3] | Marion Wobser[1]

[1]Department of Dermatology, Venereology and Allergology, University Hospital Würzburg, Würzburg, Germany

[2]Institute of Pathology and Comprehensive Cancer Centre Mainfranken, University Würzburg, Würzburg, Germany

[3]Pathology Ingolstadt, Ingolstadt, Germany

Correspondence
Verena G. Frings, Department of Dermatology, Venereology and Allergology, University Hospital Würzburg, Josef-Schneider-Straße 2, 97080 Würzburg, Germany.
Email: frings_v@ukw.de

Abstract

Background: Subcutaneous vaccination or desensitization may induce persistent nodules at the injection sites. Without the knowledge of prior injection, histopathological work-up may be challenging.

Objective: Aim of this study was to contribute to the histopathological work-up of unclear subcutaneous nodules, especially their differentiation from cutaneous lymphoma.

Methods: We retrospectively reviewed clinical data and histopathological slides of four patients with subcutaneous nodules, which were suspected to suffer from cutaneous T- or B-cell lymphoma. Sections of these cases and 12 negative controls were stained with hematoxylin and eosin and a standardized immunohistochemical panel of B- and T-cell markers including EBER in situ hybridization as well as electron microscopy.

Results: In all cases, large histiocytes with granular cytoplasm compatible with intracellular aluminum hydroxide were present. EBER in situ hybridization revealed positive staining of these granular histiocytes while staining was absent in negative controls.

Limitations: Post hoc completion of medical history revealed that vaccination or specific immunotherapy had been applied before at the biopsy site in only three out of four patients; one patient was lost to follow-up.

Conclusion: EBER in situ hybridization is an adjunctive tool to differentiate aluminum-induced granuloma/lymphoid hyperplasia from other forms of pseudolymphoma and cutaneous B- or T-cell lymphomas.

KEYWORDS
aluminum granuloma, EBER in situ hybridization, lymphoid hyperplasia, pseudolymphoma, RNA probe

1 | INTRODUCTION

Aluminum hydroxide is widely used as an effective adjuvant agent for enhancing and prolonging the immune response to the injected antigen in vaccines and in solutions for allergen desensitization.[1-3] Adverse effects of such adjuvant-augmented vaccinations and desensitization are usually benign and self-limiting, mostly consisting of a superficial inflammatory reaction presenting with tender erythema at the site of injection.[4] In 20% to 30% of patients transient palpable subcutaneous nodules have been observed, which may last for several weeks.[5-7] A prolonged immunologic interaction with the administered adjuvant-antigen preparation as well as a foreign-body reaction to the adjuvant aluminum hydroxide[7-11] have been postulated to explain the appearance of these injection site nodules.[12] In some rare cases (0.5% to 6%) subcutaneous granulomas at injection sites even persist for several years after vaccination or desensitization.[5,6,13,14] These nodules are usually painful and pruritic and therefore may worry patients, who therefore seek medical advice.[5] In addition to a perpetuate foreign-body reaction,[6,15] a delayed hypersensitivity to aluminum hydroxide has been postulated as the cause for the persisting immune response.[5,6,16]

Without the knowledge of prior injection at the respective site, histopathological work-up of these subcutaneous nodules can be challenging, as a wide range of histopathological patterns may be present, thus often leading to misdiagnosis. In this regard, histopathological examination may reveal either a histiocytic foreign body reaction showing a granulomatous inflammation with or without necrosis or a prominent lymphoid hyperplasia with a various amount of large mononuclear histiocytes.[6,7,11,17-22] Especially concerning the latter, clear-cut differentiation of such pseudolymphomatous infiltrates from true cutaneous lymphoma presents a major obstacle for the pathologist. Especially if germinal centers are present,[23] discrimination from primary cutaneous B-cell lymphomas (marginal zone lymphoma or follicular lymphoma) requires further immunohistochemical as well as molecular work-up incorporating close clinicopathological correlation.[24,25] Likewise, in cases of deep subcutaneous lymphoid infiltrates, panniculitis-like T-cell lymphoma has to be ruled out.

We herein delineate EBER in situ hybridization as an adjunctive discriminative tool to differentiate aluminum-induced granuloma/lymphoid hyperplasia from cutaneous B- or T-cell lymphoma. In a case series of four patients with aluminum granuloma, we show positive results of EBER in situ hybridization, which were not present in further inflammatory and granulomatous skin disorders serving as negative controls. Positive staining of aluminum by EBER in situ hybridization turned out to be especially helpful in those cases in which a clinical history of prior vaccination at the biopsy site cannot be taken. Recognizing the positive cell type (histiocytes) and cytoplasmic positivity in the EBER in situ hybridization helps in the etiological differential diagnosis (e.g. *Borrelia burgdorferi*-induced lymphadenosis cutis benigna) and prevents misdiagnosis of aluminum-associated skin immune reactions as cutaneous lymphoma but also helps to differentiate it from true EBV-induced lymphoproliferations.

2 | MATERIALS AND METHODS

2.1 | Case selection and data assessment

Within a retrospective setting, we reviewed histopathological slides of four patients (at least 18 years of age) who underwent biopsies of persisting cutaneous lesions at the upper extremities. In addition, we collected detailed clinical data of the patients with respect to any previous injection at the biopsy site at time of presentation (patient 1) or by post hoc telephone calls (patients 2-4). According to the referring physicians, all patients had previously been suspected to suffer from cutaneous T- or B-cell lymphoma based on either clinical and/or histopathological grounds. Histological slides and/or patients were referred to our institution: in three cases (patient 2-4), the samples were externally taken and sent for second opinion to the Institute of Pathology. In one case (patient 1), the patient was seen at the out-patient clinic for second opinion on suspected cutaneous lymphoma without prior histopathological investigation of skin lesions. In addition to history and clinical examination, this patient underwent biopsies and subsequent histopathological work-up of skin lesions at the Department of Dermatology. All included patients had received skin biopsies between 2013 and 2018. We investigated the following clinical parameters: age and sex; type of prior injection at the biopsy site; delay between injection and onset of skin symptoms; anatomic site and symptoms of cutaneous lesions (see Table 1).

2.2 | Morphological studies

All cases were reviewed by at least three pathologists or dermatopathologists (E.G., M.W., A.R.) with sections stained with hematoxylin and eosin and a standardized panel of immunohistochemical stainings of B- and T-cell markers (CD3, CD5, CD7, CD4, CD8, CD20, CD10, BCL6, BCL2, immunoglobulin heavy and light chains such as IgA, IgG, IgG4, IgM, Ki67) and further immunohistochemical stainings such as CD68 for histiocytes. Immunohistochemical staining of skin biopsy specimens was performed on formalin-fixed specimens with a standard avidin biotin immunoperoxidase procedure.

For detection of Epstein-Barr Virus (EBV)-encoded RNA the EBER 1 DNP Probe was used (Roche, USA). For EBER in situ hybridization VENTANA BenchMark Ultra was used with ISH iVIEW Blue Plus Detection Kit (Roche, USA). Histological slides from various other granulomatous reactions such as cutaneous sarcoidosis (n = 5), granulomatous cheilitis (n = 2), granuloma annulare (n = 2), and foreign body granuloma (n = 3) being retrieved from the histopathological archive of the Department of Dermatology served as control group for EBER in situ hybridization.

For electron microscopy, tissue was first formalin-fixed and paraffin-embedded (FFPE) and then dewaxed and rehydrated, post-fixed in osmium tetroxide, dehydrated, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate. Electron micrographs were obtained using a Zeiss EM 900 electron

TABLE 1 Patients' characteristics

Case	Sex	Year of birth	Year of biopsy	Site of biopsy	Mode of injection	Time interval between injection and chronic swelling	Suspected external clinical or histopathological diagnosis	EBER positivity
1	F	1991	2013	Upper arm	Specific immunotherapy against pollen (trade name ALLERBIO Vitesse)	NA	Cutaneous marginal zone lymphoma	✓
2	F	1977	2018	Upper arm	Specific immunotherapy against pollen (preparation/trend name not known)	8 years	Cutaneous lymphoma	✓
3	F	1995	2017	Upper arm	Vaccination (hepatitis B, trend name not known)	2 years	Cutaneous lymphoma	✓
4 A	F	1971	2014	Upper arm	NA	NA	Subcutaneous panniculitis-like T-cell lymphoma	✓
4 B			2015	Upper arm	NA	NA	Subcutaneous panniculitis-like T-cell lymphoma	✓
4 C			2015	NA	NA	NA	Subcutaneous panniculitis-like T-cell lymphoma	✓

Abbreviations: F, female; NA, not available.

microscope using a 2K-CCD camera and Image SP (Professional) Software of TRS.

3 | RESULTS

3.1 | Clinical characteristics

The clinical data of the four analyzed patients are summarized in Table 1. In all four cases, persistent subcutaneous swellings or nodules were present at the time of biopsy on one or both upper arms. All patients were female and between 22 and 43 years of age at time of biopsy. In most cases, the lesions were asymptomatic while in one patient (case 1) the nodules were painful on pressure. Further investigation revealed that in two patients repetitive subcutaneous injections in the upper arms for the purpose of desensitization had preceded the occurrence of the subcutaneous nodules (cases 1 and 2). In patient 3, nodules occurred after a subcutaneous vaccination for hepatitis B in the upper arm. With respect to patient 4, who underwent repetitive biopsies (case 4 A-C) of subcutaneous nodules with external diagnosis of subcutaneous panniculitis-like T-cell lymphoma or lupus panniculitis, no data on putative prior injection at the site of biopsied nodules could be obtained since the patient was lost to follow-up (Table 1).

3.2 | Histopathological findings

3.2.1 | Case 1

Histopathology of case 1 presents with a dense lymphoid infiltrate with follicles in the deep dermal layer extending to the subcutaneous tissue. Some eosinophilic granulocytes and plasma cells with mature cytomorphology are intermingled. Further immunohistochemical processing shows that the lymphocytic infiltrate is composed both of CD3– and CD5– positive T– and CD20– positive B-lymphocytes with the latter building up the lymph follicles. Scattered CD138– positive plasma cells are mainly found in the interfollicular zone; however, show a polytypic expression of kappa and lambda light chains. Expression of immunoglobulin heavy chains was regular with only single IgA-positive plasma cells and a regular distribution of IgG4. Lymphocytes with blastic cytomorphology are lacking. Additionally, there is an extensive population of large histiocytes with a peculiar fine-granular cytoplasm. Some necrobiotic zones can be seen. EBER in situ hybridization highlights the histiocytic population within the lymphoid infiltrate. Higher magnification reveals that the positive blue signal corresponds to the granular cytoplasm of the histiocytes with some nuclear overlay (Figure 1). Electron microscopic examinations show cytoplasmic filamentary or crystalline structures in these histiocytes corresponding to storage of aluminum crystals (Figure 2).

3.2.2 | Case 2

Histopathology reveals prominent lymph follicles with predominantly sharp demarcation being localized in the dermal and subcutaneous layers. No atypical blasts can be detected. Mast cells and plasmacytoid cells are loosely intermingled. In addition, there are areas of large histiocytes with fine-granular intracellular material. In the further immunohistochemical work-up, CD20 stains the prominent B-cell follicles. Immunohistochemistry for the immunoglobulin light chains kappa and lambda shows a polytypic expression pattern for plasma cells at the periphery of the germinal centers. The proliferative activity (Ki67) is physiologically high in the germinal centers and not remarkably increased in the interfollicular zone. EBER in situ hybridization unravels

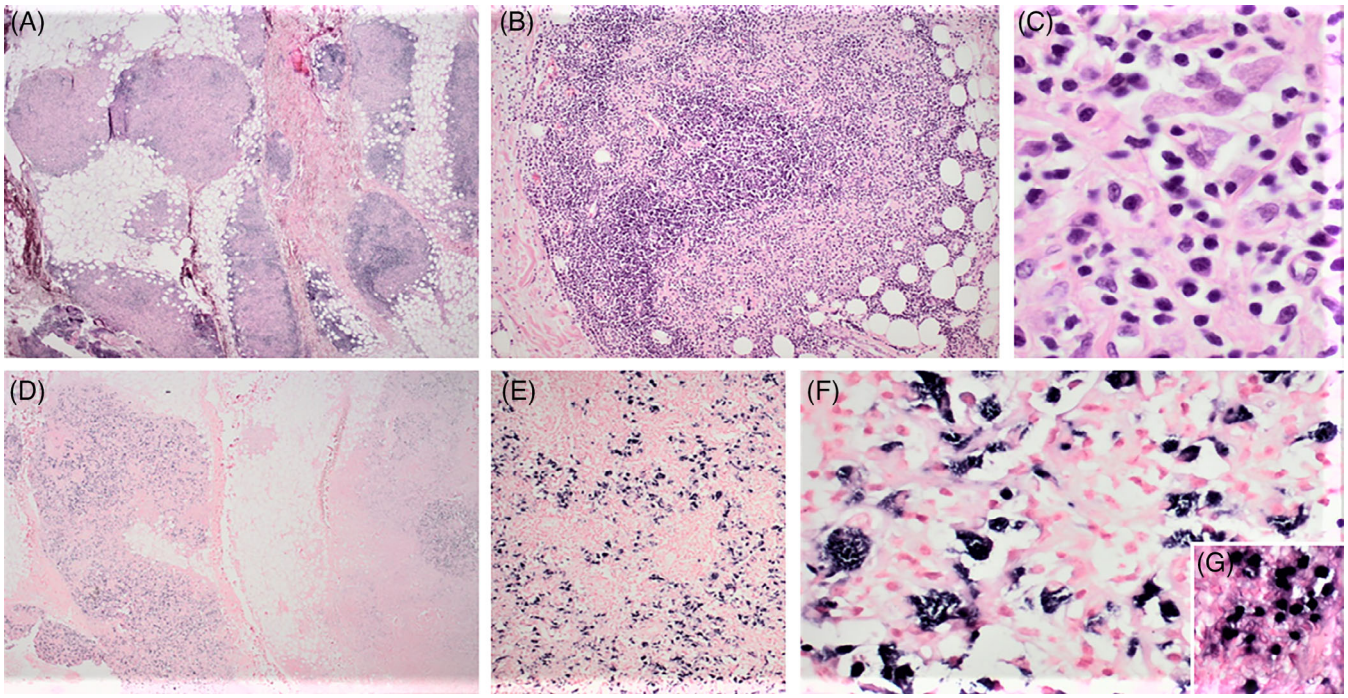

FIGURE 1 Aluminum granuloma. Histomorphology exemplified by case 1. (A-C) H&E staining. Lymph follicles with sharp demarcation in the subcutaneous layer; ×100. Additionally to small and medium-sized lymphocytes, eosinophilic granulocytes and abundant plasma cells, there are numerous large-sized histiocytes; ×200. These histiocytes show a fine granular cytoplasm; ×400. (D-F) EBER in situ hybridization. There is a striking reaction of the unusual histiocyte population, with signals that do mainly appear to be cytoplasmatic, but may also correspond to a nuclear overlay. (G) Nuclear positivity of EBER in situ hybridization in EBV-associated tonsillitis as positive control

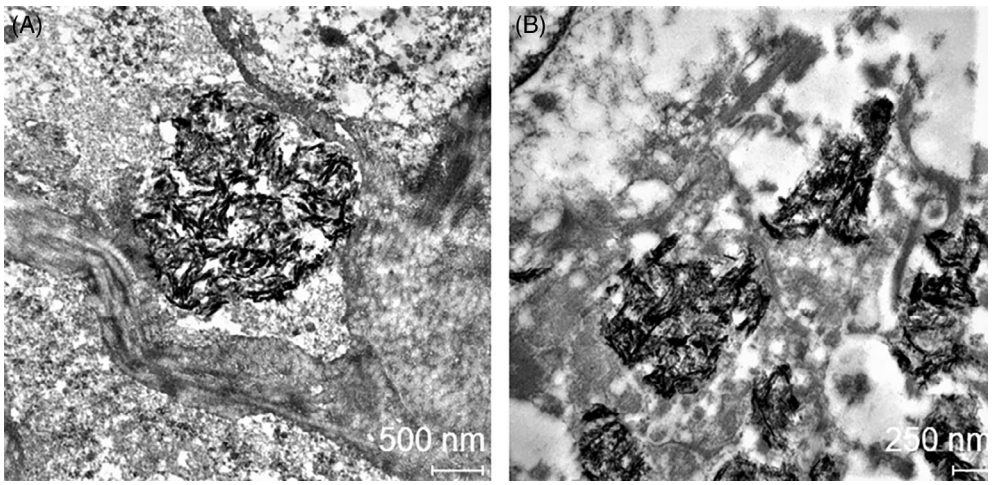

FIGURE 2 Aluminum granuloma. Electron microscopy exemplified by case 1. (A, B) In the cytoplasm of the histiocytes there are interwoven, filamentary or crystalline structures that correspond to the EBER positive signals

a positive reaction (Supporting Information, Figure S1) with a cytoplasmic staining pattern sparing the nucleus or with some nuclear overlay.

3.2.3 | Case 3

Numerous lymph follicles of variable size with reactive germinal centers can be found in the dermis and subcutis. In addition to small and medium-sized lymphocytes, eosinophilic granulocytes and abundant plasma cells, there are numerous large-sized histiocytes with a fine granular cytoplasm. Further immunohistochemistry shows a mixed population of CD5-positive T-cells with a diffuse pattern and lymph follicles made up of CD20-positive B-cells. Numerous plasma cells are detected especially at the periphery of the lymph follicles, however, not exhibiting any light chain restriction as assessed by staining for kappa and lambda light chains of immunoglobulins. Atypical blasts cannot be detected. Ki67-staining shows physiological high proliferative activity in the germinal centers and no elevated proliferative activity in the interfollicular zone. EBER in situ hybridization reveals positivity of the histiocyte population corresponding to a cytoplasmic

reaction without nuclear staining signal. Electron microscopy detects interwoven, filamentary or crystalline structures that colocalize with the EBER-positive signals within the cytoplasm of large histiocytes (Figure S1).

3.2.4 | Case 4

The three biopsies which were obtained during a time course of 12 months show similar histopathological pattern. Dense lymphoid infiltrates extend into the deep dermis and subcutaneous tissue whereby classic rimming around adipocytes is not present. The lymphoid infiltrates consist of small to medium-sized lymphoid cells, histiocytes and plasma cells. Additional immunohistochemical stainings show that the lymphoid infiltrates consist of CD20-positive B-cells building up small, scattered lymph follicles and intermingled CD3-positive T-cells. There is no evidence of an aberrant immune phenotype by analysis of kappa and lambda light chains. There are no atypical CD30-positive blasts. The proliferation activity (Ki67) reflects physiological conditions. A clonally expanded T-cell population cannot be detected by clonality analysis of TCRγ gene rearrangement. EBER in situ hybridization shows a very strong, but cytoplasmic reactivity corresponding to the histiocyte population (Figure S1).

Taking together, cutaneous lymphoma was ruled out in all cases.

As negative control for EBER in situ hybridization served slides of inflammatory granulomatous diseases being characterized by the presence of large histiocytes, namely sarcoidosis of the skin (n = 5), granulomatous cheilitis (n = 2), annular granuloma (n = 2) and foreign body granuloma (n = 3). All of these analyzed cases were negative for EBER in situ hybridization (Figure S2).

4 | DISCUSSION

Aluminum hydroxide is widely used as an adjuvant in vaccines and in allergen immunotherapy, but little is known about its mechanism of action. In animal models, subcutaneously administered aluminum hydroxide elicits a delayed hypersensitivity response histopathologically characterized by a granulomatous reaction associated with necrosis.[15] When aluminum hydroxide is absorbed to an antigen such as tetanus toxoid, the injected compound generates a prominent lymphoid hyperplasia suggesting a specific cellular immune response to the adsorbed antigen.[26] In patients, subcutaneous nodules as a result of prior aluminum-based immunization at the injection site thus show diverse histopathological patterns ranging from histiocyte-rich to lymphocyte-dominated infiltrates. Especially the latter may be misdiagnosed as skin lymphoma,[6-8,10,11,13,17-19,22] a clinically relevant issue that built up the major motivation for this study.

Especially in consultant cases of such equivocal lymphoma-suspicious infiltrates, relevant clinical data with respect to prior vaccination at the site of biopsy are often lacking. Hence, any method, which directly highlights aluminum hydroxide within the tissue, would add an integral adjunctive diagnostic tool to better differentiate true lymphoma from pseudolymphoma due to aluminum-based vaccination strategies. Additionally, differentiation from pseudolymphomas of other etiology (e.g. *B. burgdorferi*-induced lymphadenosis cutis benigna) and—not irrelevant—from true EBV-induced lymphoproliferations by recognizing the positive cell type (histiocytes) and cytoplasmic positivity in the EBER in situ hybridization would prove very useful.

In previous studies, aluminum crystals have been detected within subcutaneous injection-site granulomas by electron microscopy,[7,21] X-ray microanalysis,[7,17,19-21] and atomic absorption spectrophotometry.[7,17]

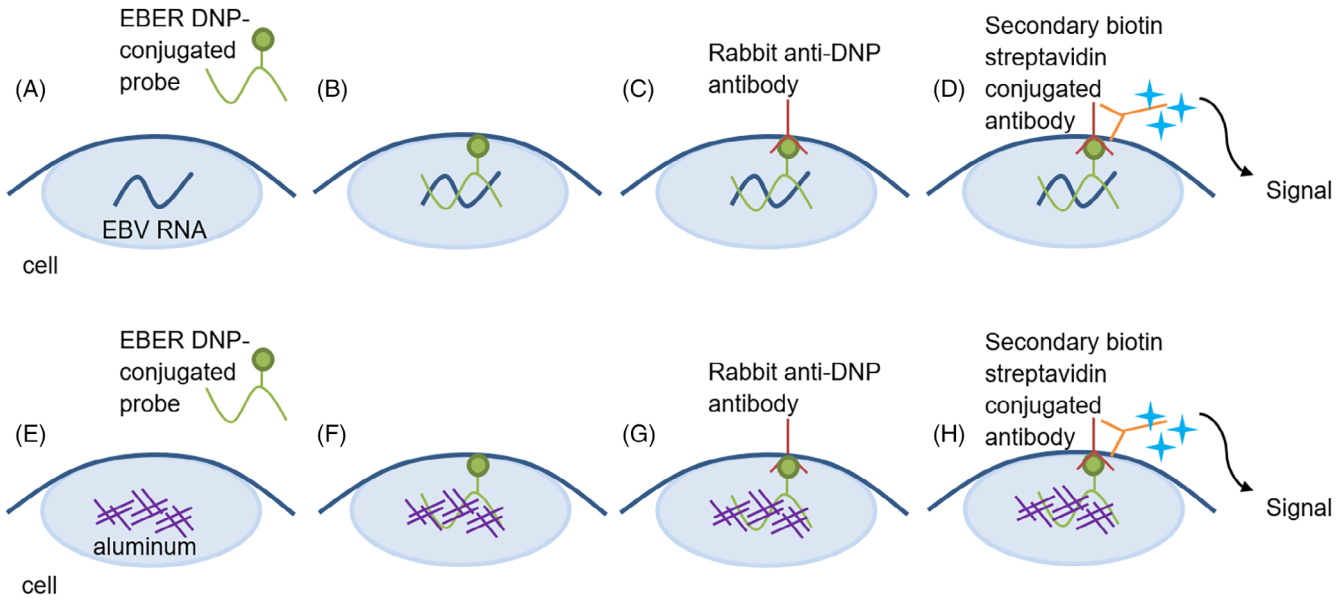

FIGURE 3 EBER in situ hybridization detects aluminum structures. (A-D) Steps involved in EBER in situ hybridization using the INFORM EBER probe (Ventana, Roche, USA) and the ISH iVIEW Blue Plus Detection Kit (Ventana); (E-H) concordant steps in detecting aluminum crystalline structure

Additionally, fluorescent labels for the detection of aluminum can be used, such as morin (2′,3,4′,5,7-pentahydroxyflavone) and lumogallion (4-chloro-3-(2,4-dihydroxyphenylazo)-2-hydroxybenzene-1-sulphonic acid).[27] All of these sophisticated visualization methods are, however, cost- and time-intensive and not readily available for the pathologist within the daily routine.

For this reason we herein delineated that an otherwise commonly used diagnostic method (without major limitations in expense or availability), namely RNA in situ hybridization of EBER, discloses additional properties in highlighting the presence of aluminum hydroxide within the tissue with high sensitivity. Hence, simple RNA in situ hybridization with EBER as shown in this study may dissolve the above-depicted dilemma about aluminum detection on post-vaccination pseudolymphomas in order to better discern these cases from true skin lymphomas. It is also very likely that any other commercially available RNA in situ hybridization will produce the positive signal seen in aluminum granulomas shown herein.

The exact mechanism of how the visualization of aluminum by EBER in situ hybridization works within the tissue is up to now unknown. The work of Lari et al[28] investigated the interaction of the aluminum ion with two different synthetic RNAs, poly(rA) and poly(rU), through a detailed thermodynamic and kinetic study. As both free ribonucleotides and polymerized single-stranded RNA chains, ribonucleotides are highly charged with phosphate, and this system is extremely vulnerable to disruption by a large number of electrostatic forces, and primarily by cationic metals such as aluminum. Aluminum strongly binds to single-stranded poly(rA) and poly (rU) at acidic and neutral pH, interacting with the phosphate and the base nitrogen groups, thus inducing a notable alteration of the polynucleotide secondary structure. However, the interaction with phosphate seems to prevail in the case of double-stranded [poly(rA)]. The results of Lari et al indicate that aluminum strongly interacts with single and duplex RNA structures[28] and, thus, explains why EBER in situ hybridization (and assumable any other RNA in situ hybridization method) by the above-mentioned mechanisms produces (false) positive staining results (Figure 3). Of note, the pattern is not nuclear—as would be awaited from specific detection of EBV-RNA components—but show a cytoplasmic hue of aluminum-loaded histiocytes corresponding to the granular cytoplasm on hematoxylin-eosin staining.

One of the major challenges of dermatopathologists is the differentiation of pseudolymphoma from neoplastic lymphoid infiltrates. In addition to meticulous histopathological, immunohistochemical and even molecular work-up of respective biopsies, close clinicopathological correlation represents the mainstay to reach a definitive diagnosis. Although *B. burgdorferi* infection,[29] viral infections,[30,31] medication,[32,33] or tattoos[34] can be identified as causative factors of pseudolymphomas, their pathogenesis remains enigmatic in many cases[24] and a clear differentiation from true lymphoma may remain uncertain. Especially in the case of prior injections (vaccinations, desensitizations) at the biopsy site, histopathological work-up may be difficult if data on the clinical history are lacking and the causative link therefore missed.[8,10] A plethora of immune preparations for injection is based on the adjuvant aluminum. Owing to the fact that aluminum strongly binds to synthetic RNA, its presence in tissue can be readily detected by in situ RNA hybridization methods such as used in EBER in situ hybridization. Taking these facts together, the results of this study reveal a helpful adjunctive method to differentiate pseudolymphoma due to aluminum-based injections from other forms of pseudolymphomas and especially from true lymphoma.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

V.G.F., E.G., and M.W. have made substantial contributions to conception and design. V.G.F. and S.R. have acquired the data. V.G.F. has analyzed as well as interpreted the data, and drafted the manuscript. A.R., M.G., E.G., and M.W. have critically revised the manuscript. All authors have given final approval of the version to be published.

DATA AVAILABILITY STATEMENT

Research data are not shared.

ORCID

Verena G. Frings https://orcid.org/0000-0002-8256-7961

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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