when indirect immunofluorescence test is combined with antigen-specific assay. The new developed more sensitive and specific methods for ANCA detection in AAV, which may replace the need for a combined analysis with IFT and ELISA in the future, should be evaluated in multicentre studies. ANCA testing can be improved by restricting the use of the tests to clinical situations with a rather high pretest probability for AAV, and carefully searching for conditions such as drug exposures and infections that are known to be associated with the occurrence of ANCA, whether the vasculitis is present or not. Therefore, a rational strategy for ANCA testing is needed and the international consensus statement on testing of ANCA should urgently be revised.

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Further reading


Csernok Elena
Medical University Luebeck, Department of Rheumatology, Klinikum Bad Bramstedt, Germany.
Correspondence: Csernok Elena,
Department of Rheumatology, Medical University Luebeck, Oskar-Alexanderstr, 26, 24576 Klinikum Bad Bramstedt, Germany.
csernok@klinikumbb.de
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L29. Relevance of anti-LAMP-2 in vasculitis: Why the controversy

Introduction

The description of autoantibodies to neutrophil cytoplasmic components antigens (ANCA) [1] and the identification of myeloperoxidase (MPO) and proteinase-3 (PR3) as their principal targets has [2,3] revolutionised understanding of small-vessel vasculitis. Assays for ANCA are central components of the diagnosis and led naturally to the term ANCA-associated vasculitis (AAV) to describe granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA) [4]. Despite uniform treatment recommendations based on multinational prospective randomised controlled trials, relapses are common and mortality remains high [5], emphasising the need for therapies targeted to the underlying immunopathogenesis and disease activity.

Clinical and experimental studies provide overwhelming evidence for the involvement of autoantibodies to MPO and PR3 in the pathogenesis of AAV (reviewed in [6]). Despite this, there is compelling evidence that autoimmunity to MPO and PR3 is not the complete answer: ANCA and conventional assays for antibodies to MPO or PR3 are negative in at least 10% of patients with apparently identical disease. More importantly, neither the presence nor the titre of antibodies to MPO and PR3 correlate with disease activity during long term follow up [6] which suggests additional factors are needed for injury. The clinical data are supported by experimental studies. Thus the in vitro effects of antibodies to MPO and PR3 depend critically on the simultaneous use of TNF-α or LPS; and anti-MPO antibodies in rodent models similarly require additional triggers such as cytokines, LPS or binding of small amounts of anti-GBM antibodies to induce severe injury (reviewed in [7–9]).

These uncertainties together with the fact that neither MPO nor PR3 are expressed by endothelium prompted the original systematic search for autoantibodies to glomerular and neutrophil membrane proteins that led to the original discovery of autoantibodies to human lysosome associated membrane glycoprotein 2 (hLAMP-2) in AAV [10]. Our subsequent studies demonstrated a very high prevalence of antibodies to hLAMP-2 in those patients presenting with AAV and provided evidence for their pathogenicity [11,12] but the findings have been challenged [13], leaving the role of anti-hLAMP-2 antibodies controversial [14,15]. Here we summarise current data on antibodies to hLAMP-2 in AAV, highlighting areas of consensus and disagreement. Finally we consider the evidence for the pathogenicity derived from rodent studies.

Autoantibodies to hLAMP-2 and their measurement

LAMP-2 is a heavily glycosylated type 2 membrane protein with critical roles in lysosomal integrity and cellular homeostasis more generally (reviewed in [16]). It has three domains: an 11 amino acid cytoplasmic domain responsible for lysosomal targeting; a 24 amino acid transmembrane domain; and a heavily glycosylated 324 amino acid luminal (or extracellular) domain that is the invariable target for the patients’ autoantibodies. Carbohydrate moiety accounts for 50 to 75% of the molecular mass of the extracellular domain with the extent of glycosylation depending both on cell type and activation state.
Despite its name, LAMP-2 is also found on the cell surface [17] and traffics from there to lysosomes with the relative proportions within the cell and on its surface again varying in cell type and activation dependent manner. Intracellular LAMP-2 has central roles in autophagy, intracellular cholesterol transport and antigen presentation [18]. The function of cell surface LAMP-2 is less well characterised but includes cell and bacterial adhesion and possibly receptor-mediated endocytosis. Regardless of its function, LAMP-2 on the surface of microvascular endothelium, neutrophils and monocytes is freely accessible and binds anti-LAMP-2 antibodies [11,12]. Anti-hLAMP-2 antibodies were first identified in patients with AAV by their ability to bind hLAMP-2 purified from human glomeruli and neutrophils which had molecular masses of 130 kDa and 170 and 80-110 kDa respectively [10], reflecting differences in their glycosylation [19,20]. Subsequent analysis showed that the most abundant autoantibodies recognised epitopes on the peptide backbone of the luminal domain that in almost all cases were equally accessible in naturally glycosylated and unglycosylated antigen. However in rare patients, the autoantibodies recognised glomerular hLAMP-2 but not neutrophil hLAMP-2, presumably because its more complex carbohydrate pattern. Conversely, exceptional patients’ antibodies only recognise glycosylated hLAMP-2.

Further mapping showed the IgG autoantibodies bind to two common epitopes, designated P41-49 and P331-341 based on their position in the amino acid sequence [11]. However, these are not the only epitopes but merely the most common ones. A very striking feature of the P41-49 epitope is that its amino acid sequence is 100% homologous (and 90% identical) to a sequence in FimH, a bacterial adhesin expressed by type 1 fimbriated bacteria, such as *Escherichia coli*, and is critical for their pathogenicity. Furthermore, the autoantibodies to hLAMP-2 cross-react with FimH, and rats immunised with FimH develop anti-LAMP-2 antibodies synthesis, confirming molecular mimicry between the two molecules [11]. The implications of this mimicry will be discussed later.

Currently, there are no anti-hLAMP-2 antibody assays sufficiently robust for general use largely because of the difficulty of producing suitable antigen in large enough quantities. The native antigen as used in the original study is appropriately glycosylated but can only be purified in small amounts. Recombinant hLAMP-2 expressed in *E. coli* is easy to produce and contains all the peptide epitopes but is relatively unstable. Mammalian LAMP-2 is more stable but less easy to generate in large quantities and has the added complication that glycosylation varies greatly depending on the expression vector and cell type used and is often aberrant when (transient) transgenic expression is driven by powerful promoters [11–13,19,21–23].

To circumvent these problems, we developed an indirect immunofluorescence assay using hamster Idd1 cells stably transfected with full-length hLAMP-2A with a point mutation in the cytoplasmic tail that prevents its retrieval from the plasma membrane. This leads to its unique expression on the cell surface and provides a cell line that can be used to quantify antibodies to hLAMP-2 by IIF without permeabilization of the cells [11,12].

**Autoantibodies to hLAMP-2 antibodies in ANCA-associated vasculitis**

Antibodies to hLAMP-2 were originally discovered in a group of 15 patients presenting with pIFNGN in the context of AAV, 13 of whom (87%) had detectable IgG autoantibodies in Western blots [10]. Their prevalence was similarly high in two subsequent much larger studies: 93% in a cohort of 84 patients from Vienna and Aberdeen (UK) [11]; and 89%, 91% and 80% in three new cohorts from Vienna, Cambridge (UK) and Groningen (Netherlands) [12]. Notably, anti-hLAMP-2 antibodies were also detected in 7 of 8 ANCA negative patients in the various cohorts [11]. By contrast, antibodies to hLAMP-2 present in less than 7% in patients with AAV in remission; and were not detected in healthy controls [10–12]. In the two latter studies, anti-hLAMP-2 antibodies were assayed using at least three independent assays: ELISA and Western blotting using unglycosylated recombinant antigen and indirect immunofluorescence on stably transfected Idd1 cells. There was close agreement between results from the three assays with a combined concordance rate of over 80%. Anti-hLAMP-2 antibodies were not judged to be present unless at least two assays were positive and in practice all three were positive in over three quarters of the positive sera [12]. Thus, we can be confident of our conclusion that the frequency of anti-hLAMP-2 antibodies is between 80% and 90% in untreated patients presenting with AAV in six separate European cohorts from three different countries [10–12].

The most important factors responsible for the remarkable consistency of the results are probably the use of multiple assays for anti-hLAMP-2 antibodies together with stringent patient selection criteria to ensure that the patients had truly active AAV but were not subject to the potentially confounding effects of therapy. Accordingly, the initial studies were restricted to untreated patients presenting with biopsy proven pIFNGN in the context of AAV. In retrospect, this was fortunate because, once treatment is started, anti-hLAMP-2 autoantibodies become undetectable much more rapidly than anti-MPO and anti-PR3 ANCA. Indeed, assays for them became negative within one month of starting treatment in 42 of 43 patients and, in the absence of clinical relapse, remained so for the subsequent 11 months [12]. Although surprising, rapid treatment-induced disappearance of pathogenic autoantibodies to plasma membrane proteins is well-recognised [24], and there is also a precedent for it in the case of anti-MPO ANCA in Eosinophilic Granulomatosis with Polyangiitis (EGPA - Churg-Strauss Syndrome) [25]. However, it contrasts results with the
classical ANCA that commonly persist during clinical remission in other forms of AAV.

One consequence of the rapid effect of treatment is that the frequency of anti-hLAMP-2 antibodies is significantly less in patients presenting with AAV after immunosuppressive therapy had been started [12]. Anti-hLAMP-2 antibodies were again detectable during clinical relapses of disease activity in 16 of 28 (57%) patients and the evolution from negative to positive anti-hLAMP-2 assays has been documented [12]. These data suggest that anti-hLAMP-2 antibodies correlate with disease activity and this idea is supported by a retrospective study of 273 consecutive sera from 44 patients from the Aberdeen vasculitis service followed for 2 years. Anti-hLAMP-2 ELISA were positive in sera from 44 of 52 (85%) of sera taken when disease was clinically active either at presentation or relapse and only 12 of 214 (6%) sera in those judged clinically inactive compared to 64% of conventional ANCA assays. Currently, the INTRICATE Consortium (http://www.intricate.eu/) is conducting a multicentre prospective observational study to determine whether anti-hLAMP-2 antibodies really do faithfully reflect disease activity.

The only other published study of anti-hLAMP-2 antibodies in AAV comes from University of North Carolina Kidney Center (UNC) [13] and superficially the results appear very different. The overall frequency of antibodies to hLAMP-2 was 21% in those with AAV but, in contrast to the Vienna studies, this combines results from individuals from presentation and during follow up and those with active and quiescent disease. The study included two AAV cohorts: 103 individuals from UNC and 226 from the Massachusetts General Hospital, Boston. Disease activity was scored according to an established clinical index (BVAS) [26] and the patients were segregated into two groups: 58 in remission with BVAS scores of 0; and 45 (including those newly presenting) with BVAS scores greater than 0 and defined as having active disease. The level of activity thus differs from the patients studied by Kain et al. in which those at presentation or in clinical relapse generally had BVAS scores between 10 and 15 [12].

Comparisons are further complicated by the use of different assays. The UNC sera were analysed by ELISA and Western blots with recombinant HEK cell expressed hLAMP-2 as substrate and by IIF. Boston sera were assayed by an ELISA using a commercially available polypeptide spanning just over a quarter of the sequence of the hLAMP-2 luminal domain. This assay resulted in very low IgG binding and assay validation data with known anti-hLAMP-2 antibody positive and negative sera were not reported. Consequently, it is impossible to know whether the differences in IgG binding reflect the presence of authentic antibodies to hLAMP-2 or resulted from non-specific binding. Analysis of the UNC group in more detail emphasises the differences in patient selection and the lack of conformity between the assays used. Only 15 of the sera were from newly presenting patients and thus suitable for testing the Vienna groups central proposition – and some of these were already on treatment. Using an ELISA validated by positive and negative control sera provided by the Vienna group, 7 (47%) of these were positive for antibodies to hLAMP-2 which is much higher than the 4% incidence in healthy controls (2 of 52: $P = 0.0002$, Fisher’s Exact Test). However none of these positive sera were positive when tested by Western blotting and indirect immunofluorescence assays using recombinant HEK cell expressed hLAMP-2 in this study; but the positive control sera from Vienna were also negative in these assays making negative results impossible to interpret [13].

**Pathogenicity of antibodies and autoantibodies to LAMP-2 in rodents**

Rodent models have been used to address the critical issue whether antibodies to LAMP-2 can be pathogenic both by passive immunisation with IgG containing antibodies to hLAMP-2 or a peptide derived from it [11], and by active immunisation to induce anti-LAMP-2 antibodies [11]. Kain et al. raised a high titre antiserum to hLAMP-2 by immunising rabbits with recombinant hLAMP-2. IgG purified from it bound human hLAMP-2 and rat LAMP-2 from liver, kidney and neutrophils by IIF. WKY rats injected intravenously with this IgG had circulating anti-hLAMP-2 antibodies whose concentration decreased rapidly over the subsequent 24 hours. Rabbit IgG was readily detected bound to glomerular endothelium 2 hours after injection but not at later time points. The injection induced glomerulonephritis, evidenced by dipstick positive haematuria, severe proteinuria and pifNGN with crescents in around 25% of glomeruli [11]. None of these effects were seen in rats injected with normal rabbit globulin.

Roth et al. also reported passive immunisation studies by injecting WKY rats with IgG prepared from a rabbit immunised with a 9-mer synthetic peptide corresponding to the hLAMP-2 epitope P$_{41...49}$ identified by Kain et al. [11,13]. IgG from this serum bound to the peptide immunogen but data were not presented to show it bound to rat LAMP-2 (rLAMP-2). This is important as only six of the nine amino acids in the immunising peptide are conserved between human and rat LAMP-2. This antiserum caused no disease after injection into WKY rats but assays for circulating anti-rat LAMP-2 antibodies and early binding to glomerular endothelium were not performed. Consequently robust evidence that the anti-hLAMP-2 antibodies bound strongly to native rat LAMP-2 are essential before the results of a negative study such as this can be interpreted. Active immunisation to induce anti-LAMP-2 antibodies provides a more stringent test of pathogenicity. The cross-reactivity between FimH and hLAMP-2 provided opportunity to test the pathogenicity of anti-LAMP-2 antibodies because the common hLAMP-2/FimH epitope recognised by patients’ autoantibodies is partially conserved in rLAMP-2. WKY rats immunised with
recombinant FimH developed antibodies to FimH and eight of the 10 studied developed antibodies that reacted with rat and human LAMP-2. The sera bound to the shared P41-49 peptide by dot blot and affinity purified IgG to P41-49 from these sera bound to human glomerular endothelium by IIF. Immunoelectron microscopy confirmed the binding and showed the antibodies to P41-49 bound the same structures within cells as a monoclonal antibody to hLAMP-2. The immunised rats had positive ANCA assays using rat neutrophils and developed piFNGN. This supports the results of the passive immunisation experiments and confirms by a different strategy that autoimmunity to LAMP-2 and presumptively anti-LAMP-2 antibodies can be pathogenic and cause piFNGN in rats. These studies demonstrate that immunization with FimH induces antibodies to rat and human LAMP-2 accompanied by the development pauci-immune FNGN and proves the molecular mimicry between the two molecules – at least under these experimental conditions. It raises however the question whether natural infection with fimbriated bacteria could induce AAV in the same way. Two sets of clinical data are consistent with this: Kain et al. [11] reported that 9 of 13 consecutive patients presenting with AAV had had a microbiologically proven infection with a fimbriated organism within the preceding 3 months; and Roth et al. reported that 12% of a sample of 105 patients with UTIs had positive assays for LAMP-2 in their ELISA [13]. A suitably powered prospective multicentre study is currently performed by the INTRICATE Consortium (http://www.intricate.eu/) to determine whether infections with type 1 fimbriated bacteria induce antibodies to hLAMP-2 in man and, if so, to ascertain their association with AAV.

Conclusion

There is still insufficient data to know whether autoantibodies to hLAMP-2 are important for the pathogenesis of AAV or will have practical utility in its management. However, there is much greater agreement between results from the published studies than would be anticipated from the strength of the controversy. Both groups find highly significant increase in the frequency of anti-hLAMP-2 antibodies at presentation (albeit with different frequencies); and both agree that, in contrast to conventional ANCA, assays for hLAMP-2 antibodies are negative when patients are in remission. A critical difference is whether they reflect disease activity: the Vienna group find a correlation with clinical relapse whilst the UNC group find no correlation with disease activity defined as BVAS greater than 0. Again the contradiction may not be real, because anti-hLAMP-2 antibodies become negative within a month of starting effective treatment well before BVAS scores return to normal. More data from prospective studies are needed to resolve these issues and this needs generally applicable assays, which are currently sorely missing.

Disclosure of interest: the author declares that he has no conflicts of interest concerning this article.

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[19] Lee N, Wang WC, Fukuda M. Granulocytic differentiation of HL-60 cells is enhanced by a monoclonal antibody to hLAMP-2. The immunised rats had positive ANCA assays using rat neutrophils and developed piFNGN. This supports the results of the passive immunisation experiments and confirms by a different strategy that autoimmunity to LAMP-2 and presumptively anti-LAMP-2 antibodies can be pathogenic and cause piFNGN in rats. These studies demonstrate that immunization with FimH induces antibodies to rat and human LAMP-2 accompanied by the development pauci-immune FNGN and proves the molecular mimicry between the two molecules – at least under these experimental conditions. It raises however the question whether natural infection with fimbriated bacteria could induce AAV in the same way. Two sets of clinical data are consistent with this: Kain et al. [11] reported that 9 of 13 consecutive patients presenting with AAV had had a microbiologically proven infection with a fimbriated organism within the preceding 3 months; and Roth et al. reported that 12% of a sample of 105 patients with UTIs had positive assays for LAMP-2 in their ELISA [13]. A suitably powered prospective multicentre study is currently performed by the INTRICATE Consortium (http://www.intricate.eu/) to determine whether infections with type 1 fimbriated bacteria induce antibodies to hLAMP-2 in man and, if so, to ascertain their association with AAV.

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L30. Assessment of vasculitis extent and severity

Introduction

Various criteria have been proposed to date to classify vasculitis. The diameter of the vessels involved is the main parameter upon which classification criteria are based, while additional parameters are the presence of granulomata and of antineutrophil cytoplasmic antibodies (ANCA). For the purpose of classification, small vessels are considered those whose caliber is inferior to 50 μm, medium vessels those with a caliber of 50 to 150 μm, and large vessels those with a caliber superior to 150 μm. Large-vessel vasculitides typically include giant cell arteritis (GCA) and Takayasu arteritis (TAK). Medium-vessel vasculitis is classically represented by polyarteritis nodosa (PAN), while ANCA-associated vasculitides (AAV) affect medium to small vessels. Herein, we reviewed the evidence on assessment of disease extent and severity in the main types of vasculitides affecting large, medium and small vessels, respectively.

Large-vessel vasculitides

Clinical criteria to assess TAK were developed back in 1994 at the National Institute of Health (NIH) by Kerr et al. and still go by the name of Kerr or NIH criteria. These criteria encompass the following four parameters:
- systemic features (fever, musculoskeletal manifestations...);
- a raised erythrocyte sedimentation rate (ESR);
- manifestations of vascular ischemia (decreased/absent pulses, claudication, carotodynia, asymmetric blood pressure readings in the limbs);
- typical angiographic features (long, smooth stenoses). These criteria were developed from a population of 60 Takayasu patients followed up prospectively, of whom those with active disease received aortograms every 4 to 6 months. According to these criteria, active disease is defined by the presence of at least two of the above items. An analysis by the authors, however, revealed that their main drawback was a quite poor sensitivity, with 61% of patients judged as having “inactive” disease incurring progression of angiographic lesions.

More recent tools to assess disease activity of TAK are the Disease Extent Index.Takayasu (DEI.TAK) and the Indian Takayasu Activity Score (ITAS), which are both derived from the Birmingham vasculitis activity score (BVAS), the ITAS being a simplified version of the DEI.TAK. Both tools consider the presence of clinical features in organ-based domains, inflammatory markers (ESR and C-reactive protein) and physician global assessment (active/persistent/inactive). Clinical features must be specific for vasculitis and are scored as absent or present; for clinical features to be considered present, they must be new or have recently worsened. Disease is considered active if at least one organ system scores positive, while inflammatory markers and physician global assessment do not count per se toward assessment of disease activity. As these tools are remarkably similar, they show a high degree of correlation. A comparative analysis also demonstrated a high concordance between ITAS and Kerr indices in a population of patients with TAK. However, 31% of clinically active patients were in remission according to the DEI.TAK; conversely, the DEI.TAK was positive in 18% of patients felt to be clinically inactive. No specific tool has been yet designed to assess disease activity of GCA, although the Kerr and ITAS have empirically been used to do so in patients with large-vessel GCA. OMERACT 2011 has acknowledged the limitations inherent in the Kerr and ITAS indices and recognized the need to develop finer-tuned assessment tools.

In clinical practice and trials alike imaging procedures are the mainstay to assess large-vessel vasculitis. Color-Doppler sonography (CDS), magnetic resonance (MR) combined with MR angiography (MRA) and contrast-enhanced computerized tomography (CT) combined with CT angiography (CTA) can visualize both the vessel wall and the lumen of large vessels. All these techniques are able to demonstrate early inflammatory signs (vessel wall thickening and mural inflammation) as well as late complications (stenoses and aneurysms). 18F-Fluorodeoxyglucose (FDG) positron emission tomography (PET) is able to detect increased FDG uptake by metabolically active cells, including inflammatory cells infiltrating the vessel wall in vasculitis, while digital subtraction angiography (DSA) is useful to demonstrate luminal changes.