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Anti-KS: Identification of Autoantibodies to Asparaginyl-Transfer RNA Synthetase Associated with Interstitial Lung Disease

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Autoantibodies to five of the aminoacyl-transfer RNA (tRNA) synthetases have been described, and each is associated with a syndrome of inflammatory myopathy with interstitial lung disease (ILD) and arthritis. Serum KS, from a patient with ILD and inflammatory arthritis without evidence of myositis, immunoprecipitated a tRNA that was distinct from that precipitated by any described anti-synthetase or other reported tRNA-related Abs, along with a protein of 65 kDa. KS serum and IgG fraction each showed significant (88%) inhibition of asparaginyl-tRNA synthetase (AsnRS) activity, but not of any of the other 19 aminoacyl-tRNA synthetase activities. Among 884 patients with connective tissue diseases tested, only two other sera were found to immunoprecipitate tRNAs and proteins of identical gel mobility. These two and KS showed identical immunodiffusion lines using HeLa cell extract. The new sera significantly inhibited AsnRS without significant effects on other synthetases tested. Both patients had ILD but neither had evidence of myositis. These data strongly suggest that these three sera have autoantibodies to AsnRS, representing a sixth anti-synthetase. Anti-KS was more closely associated with ILD than with myositis. Further study of this Abs might prove useful in dissecting the stimuli responsible for the genesis of anti-synthetase autoantibodies. The Journal of Immunology, 1999, 162: 2315–2320.

Autoantibodies directed against aminoacyl-transfer RNA (tRNA)3 synthetases can be found in ~25–35% of patients with the chronic, inflammatory muscle disorders polymyositis (PM) and dermatomyositis (DM) (1). Each member of this family of enzymes catalyzes the formation of an aminoacyl-tRNA from a specific amino acid and its cognate tRNAs. Autoantibodies to five of these synthetases (histidyl-, threonyl-, alanyl-, isoleucyl-, and glycyl-tRNA synthetases) have been identified in patients with PM and DM (1–5). Among these “anti-synthetase Abs,” anti-histidyl tRNA synthetase (anti-Jo-1) is the most common, found in 20–30% of such patients (6–10). Anti-threonyl tRNA synthetase (anti-PL-7) and anti-alanyl tRNA synthetase (anti-PL-12) Abs are less common, found in 3–4% of all patients with PM/DM (3, 4, 10–12), while autoantibodies to isoleucyl-tRNA synthetase (anti-OJ) and glycyl-tRNA synthetase (anti-EJ) are the least common, occurring in < 2% (5, 13, 14). Isoleucyl-tRNA synthetase is the only one of these synthetase autoantigens that is a component of the multienzyme synthetase complex, and some anti-OJ sera also react with other components of the synthetase complex, but such additional reactivity does not change the immunoprecipitation (IPP) picture of anti-OJ. Thus, excluding the nine synthetase activities that are part of the complex and the four other described anti-synthetases, seven aminoacyl-tRNA synthetases exist for which autoantibodies have not been described, as determined by IPP of tRNA. The reason for this selectivity for certain synthetases is not known. With the exception mentioned for anti-OJ, it is extremely rare for a patient to have more than one anti-synthetase (15).

Anti-Jo-1 and other anti-synthetases have each been associated with a similar syndrome marked by myositis with a high frequency of interstitial lung disease (ILD) (50–80%) and arthritis (50–90%) (7–10, 16, 17), as well as an increase when compared with the overall myositis population in Raynaud’s phenomenon (60%), fever with exacerbations (80%), and the skin lesion of the fingers referred to as mechanic’s hands (70%) (8). Other associations, such as an increase in sicca and sclerodactyly, have been observed by some investigators (10). Although the similarities between patients with different anti-synthetases are most striking, certain differences have been observed that must be considered preliminary due to the small number of patients with non-Jo-1 anti-synthetases reported. One important difference is that patients with anti-PL-12 are more likely than anti-Jo-1 patients to have ILD and/or arthritis either without myositis or with subclinical signs of muscle disease. Absence of significant myositis over the full course of patients with anti-Jo-1 is rare (< 5%), although it may occur. Clinically significant myositis was seen in 60% of U.S. patients with anti-PL-12 (12, 18), whereas none of six Japanese patients with anti-
PL-12 Abs fulfilled criteria for myositis (19). In the limited number of patients thus far observed, 2 of 10 anti-OJ patients had ILC without detectable myositis and one had ILC with subclinical myositis.

Most sera with any of the five reported anti-synthetases specifically inhibit the aminoacylation of the respective tRNAs, indicating inhibition of the enzymatic function of the synthetase (2, 4–6, 11). For example, anti-Jo-1 serum, IgG fraction, and affinity-purified IgG inhibit histidyl-tRNA synthetase activity and not that of other synthetases (6). Only occasional anti-synthetase sera have been exceptions, i.e., did not inhibit (12, 13). Such inhibition is not consistently seen with animal antisera raised against synthetases and suggests that autoantibodies target an active site of the enzyme (20). The identification of the previous five anti-synthetases was initially based on the demonstration that several sera that shared the same Ab and immunoprecipitated the same tRNAs could inhibit the same synthetase enzyme and not others (2–5, 14). Later, other methods were used to support these identifications, such as demonstration for anti-Jo-1 and anti-EJ of reaction with enzymatically active recombining protein (21, 22).

In this report, we describe a novel autoantibody that immunoprecipitates tRNA and provide evidence that it reacts with asparaginyl-tRNA (AsnRS) synthetase.

Materials and Methods

Sera

Serum samples were obtained from 884 patients with connective tissue diseases followed in clinics at Keio University in Tokyo and Kyoto University in Kyoto, Japan. These included 114 with PM/DM, 592 with SLE, 220 with systemic sclerosis, 56 with rheumatoid arthritis, and 102 patients with ILC who had no evidence of myositis and did not meet criteria for other conditions. Stored sera known to contain autoantibodies against synthetases for histidine, threonine, alanine, glycine, and isoleucine were used as controls.

IPP

IPP from HeLa cell extracts was performed as previously described (5, 9). A total of 10 μl of patient sera was mixed with 2 mg of protein A-Sepharose CL-4B (Pharmacia Biotech AB, Uppsala, Sweden) in 500 μl of IPP buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% Nonidet P-40) and incubated with end-over-end rotation (Labquake shaker; Lab Industries, Berkeley, CA) for 2 h at 4°C. The IgG-coated Sepharose was washed four times in 500 μl of NET-2 buffer using 10-s spins in a microfuge tube and was resuspended in 400 μl of NET-2 buffer.

For analysis of RNAs, this suspension was incubated with 100 μl of extracts, derived from 6 × 10⁶ cells, on the rotator for 2 h at 4°C. The Ag-bound Sepharose was then collected with a 10-s centrifugation in the microfuge, washed four times with NET-2 buffer, and were resupend in 300 μl of NET-2 buffer. To extract bound RNAs, 30 μl of 3.0 M sodium acetate, 30 μl of 10% SDS, 2 μl of carrier yeast tRNA (10 mg/ml), Sigma, St. Louis, MO) and 300 μl of phenol/chloroform/isoamyl alcohol (50:50:1, containing 0.1% 8-hydroxyquinoline) were added to the Sepharose beads. After agitation in a Vortex mixer and spinning for 1 min, RNAs were recovered in the aqueous phase after ethanol precipitation and dissolved in 30 μl of NET-2 buffer. To extract bound RNAs, 30 μl of 3.0 M sodium acetate, 30 μl of 10% SDS, 2 μl of carrier yeast tRNA (10 mg/ml), Sigma, St. Louis, MO) and 300 μl of phenol/chloroform/isoamyl alcohol (50:50:1, containing 0.1% 8-hydroxyquinoline) were added to the Sepharose beads. After agitation in a Vortex mixer and spinning for 1 min, RNAs were recovered in the aqueous phase after ethanol precipitation and dissolved in 30 μl of NET-2 buffer. For protein studies, Ab-coated Sepharose was mixed with 400 μl of [35S]methionine-labeled HeLa extract derived from 2 × 10⁶ cells, and rotated at 4°C for 2 h. After four washes with IPP buffer, the Sepharose was resuspended in SDS-sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, 0.005% bromphenol blue). After heating (90°C for 5 min), the proteins were fractionated by SDS-10% PAGE gels, enhanced with 0.5 M sodium salicylate, and dried. Labeled proteins were analyzed by autoradiography.

Aminoacylation

Aminoacylation inhibition reactions were performed as described previously with minor modification (5, 23). First, 6 ml of HeLa cell extract diluted 1:10 in Tris-buffered saline (TBS) were incubated at 37°C for 10 min followed by dilution of serum for 2 h at 4°C. This was combined with 17 ml reaction solution (50 mM Tris-HCl, pH 7.5, 0.02 M NaCl, 0.01 M MgSO₄, 1 mM DTT, containing 8 units of yeast tRNA), 3 ml of [14C]-aspartagine or [14H]amino acid, and 1 ml of 200 mM cold amino acid. Then, 10 ml aliquots were tested at 10 min and 20 min, spotted onto filter paper treated with 5% TCA, then washed five times with 5% TCA, then with ethanol, then dried for autoradiography. Results of inhibition testing was defined as the percent inhibition of the average activity seen with the normals included in that experiment: % inhibition = [(average cpm with normal serum) − (cpm with test serum)] × 100/(average cpm with normal serum). Inhibition of >50% compared with the activity with normal serum was considered significant. In previous studies, although nonspecific effects on aminoacylation reactions by serum were common, nonspecific inhibition was usually <25% and inhibition >50% reliably reflected specific Ab effects (5, 6, 11, 12, 23).

To test the AsnRS activity of purified KS antigen, the procedure was similar except that 6 ml of purified Ag was substituted for the HeLa extract and Tris-buffered saline was substituted for serum.

Purification of the KS Ag

Affinity chromatography was performed as previously described (23). The KS Ag was purified from HeLa cell extracts. IgG fraction was purified from 20 ml of KS serum using DEAE. KS IgG was coupled to Affi-gel (Bio-Rad Laboratories) hydroxysuccinimamide-agarose in 0.1 M bicarbonate buffer, pH 8.3, with >90% coupling. The immunoabsorbent was washed extensively, including with the intended eluting agent (3 M MgCl₂). Later experiments were performed using a second column prepared in a similar manner. After HeLa extract was applied to the column in excess of adsorbing capacity, the column was extensively washed with 0.5 M NaCl in 0.05 M Tris buffer, pH 7.2, with 0.01 M sodium azide and 0.1 mM PMSF and was eluted with 3 M MgCl₂.

ELISA

ELISA was performed as previously described (6, 11). The immunoaffinity-purified KS Ag was diluted 80-fold in PBS for application to the microtiter well. Wells were blocked with 0.2% BSA in PBS. Sera were diluted 1:100 for testing with 2% BSA in PBS with 0.05% Tween 20. An affinity-purified goat anti-human IgG conjugated to alkaline phosphatase (Sigma) was used with p-nitrophenyl phosphate substrate.

Other

Ouchterlony double immuno-diffusion was performed as described previously using HeLa cell extract as Ag (9). The IgG fraction of the patient sera was purified by DEAE chromatography.

Cases

Case 1. In April of 1979 (at age 36), patient KS developed a nonproductive cough and shortness of breath. Chest radiography showed interstitial fibrosis, and pulmonary function testing revealed a restrictive pattern. A diagnosis of ILD was made, and prednisolone 40 mg/day was begun, resulting in dramatic improvement of respiratory symptoms. In the autumn of 1980, inflammatory arthritis developed, which was treated with aspirin. In 1988, she was admitted to Keio University Hospital because of worsening polyarthritis and pulmonary hypertension due to fibrosis of both lower lung fields. No muscle weakness was found, and the creatine kinase level was normal (49 IUL). Neither Raynaud’s phenomenon nor elevation of creatine kinase occurred at any point in her course.

Case 2. In 1990 (at age 61), patient NI noticed slight dyspnea on exertion. In 1991, her chest radiograph showed bilateral interstitial fibrosis in the lower lung fields, but she did not develop any other symptoms. The following year, a diagnosis of usual interstitial pneumonitis was made on the basis of open lung biopsy. She did not have any muscle weakness, elevation of the creatine kinase level, arthritis, or Raynaud’s phenomenon.

Case 3. In 1966 (at age 44), patient KN was found to have a reticular pattern on her chest radiograph, but was followed up without treatment. In 1968, she noticed dyspnea on exertion and fatigability. In 1983, at age 61, she developed Raynaud’s phenomenon. At age 64, open lung biopsy was performed, with histology showing usual interstitial pneumonitis, but she did not satisfy criteria for any connective tissue diseases.
Results

Identification of a new tRNA-related Ab

Serum of patient KS was found to immunoprecipitate a strong predominant nucleic acid band of tRNA size, accompanied by a faster band (Fig. 1, lane 6). This gel pattern of tRNAs was clearly distinguishable from the pattern of tRNAs precipitated by the five described anti-synthetases (shown in Fig. 1 for five) or that associated with other identified tRNA-related autoantibodies. The predominant band was faster in migration than the Jo-1 RNA and slower than the four major PL-12 bands. The additional faster band was almost identical in migration to the slowest band of the EJ RNAs. This serum also immunoprecipitated a very strong protein band from \[^{35}S\]methionine-labeled HeLa cell extracts (Fig. 2, lane 6) migrating at 65 kDa that was clearly different from the bands immunoprecipitated by sera with the described anti-synthetases (shown in Fig. 2 for five). A second, much fainter band was seen at 63 kDa.

Serum KS showed a line by immunodiffusion against HeLa cell extract that was nonidentical with that of anti-PL-7, anti-PL-12, and anti-EJ and quite different from Jo-1 because the KS line was present but the Jo-1 line was not appreciated clearly (Fig. 3A).

Of 884 patients with connective tissue diseases or ILD and controls, sera from two other patients with ILD (KN and NI) immunoprecipitated tRNA bands that were identical to those of serum KS each time they were analyzed (Fig. 1, lanes 7 and 8). Both of these sera immunoprecipitated strong 65-kDa protein bands that were also identical to that of serum KS, along with a faint 63-kDa band (Fig. 2, lanes 7 and 8). Furthermore, by immunodiffusion against HeLa cell extracts, a line of immunologic identity was seen between serum KS, serum NI, and serum KN, confirming the presence of the same autoantibody in each serum by immunodiffusion against HeLa cell extracts.
between serum KS, serum NI, and serum KN, confirming the presence of the same autoantibody in each serum (Fig. 3B). Thus, anti-KS Abs were found in 0.34% of 884 patients with connective tissue disease and 2.94% of 102 patients with ILD.

None of these three sera immunoprecipitated any RNA after deproteinization of the HeLa cell extracts, whereas control anti-PL-12 sera (lanes 7 and 8) consistently precipitated the PL-12 tRNA pattern from the deproteinized extract.

Identification of the KS Ag

IPP of a unique tRNA and a strong protein band by anti-KS suggested the possibility that it was a new anti-synthetase. This was assessed by testing KS serum for the ability to inhibit each of the 20 aminoacyl-tRNA synthetases in turn. The enzyme source, HeLa cell extract, was preincubated with serum, at a 1:100 final concentration in the reaction mixture, before using the extract in an in vitro aminoacylation assay. Significant (>50%) inhibition of AsnRS was seen, with inhibition of 88% of the activity seen when normal serum was added, but there was no significant inhibitory effect on other aminoacyl-tRNA synthetases (range 0–29%) (Table I). To further demonstrate that the inhibition of AsnRS resulted from Abs, the purified IgG fraction of KS serum was tested at a concentration of 0.6 mg/ml in the final reaction mixture. IgG showed similar inhibition of AsnRS by 88% at 20 min compared with the activity in the absence of IgG, whereas there was no significant inhibition of other synthetases (<0–16%). Normal control serum and anti-KS-negative myositis serum showed no significant inhibition of AsnRS, although sera with other anti-synthetases inhibited their respective enzymes. Normal IgG inhibited only 14%.

In view of these results, sera KN and NI were similarly tested and also showed specific inhibition of AsnRS compared with the activity with normal serum (96 and 98%, respectively). There was no significant inhibition of any of the 16 other synthetases tested (KN range 0–9%; NI range 0–27%). IgG from these sera also showed specific inhibition (87 and 64%, respectively) at a concentration of 0.3 mg/ml in the final reaction mixture.

The KS Ag was purified from HeLa cell extract by immunofinity chromatography using KS prototype serum. When 80-fold diluted KS Ag was tested against KS serum and NI and KN IgG by ELISA, all showed activity above controls (OD 1.19, 0.811, and 1.027, respectively, vs 0.227–0.574 for normal or other anti-synthetase sera), indicating that the affinity-purified material was active antigenically. This Ag preparation was tested for AsnRS enzymatic activity in an asparagine aminoacylation reaction, and it was found to be highly active (8,059 cpm with KS Ag vs 126 cpm without enzyme at 10 min), confirming that KS Ag is AsnRS.

Discussion

In the present study, a novel autoantibody was described directed at AsnRS, the sixth in a series of autoantibodies to aminoacyl-tRNA synthetases. The evidence for its identification was similar
to that provided for other anti-synthetases, including the IPP of a distinctive set of restricted tRNAs differing from those precipitated by other anti-synthetases, a protein of a size consistent with that expected of the synthetase, and specific inhibition of the enzyme target by IgG from each patient that shows the Ab, without inhibiting other synthetases. In addition, immunoaffinity-purified KS Ag shows enzymatic activity as AsnRS. Although the background in the ELISA was high, all three sera showed higher binding with the Ag. These findings show human AsnRS to be ~65 kDa, similar in size to other forms of AsnRS that have been characterized (bacterial AsnRS at 53 kDa and yeast AsnRS at 51 kDa, and Brugia malayi at 63 kDa (24–26)). Recently Hartline et al. tested our prototype serum KS against a recombinant form of human AsnRS, and the serum demonstrated reactivity, providing further proof of the identification of the KS Ag as AsnRS (M. Hartline, et al., personal communication).

Aminoacyl-tRNA synthetases are divided into class I and class II synthetases based on several properties shared by members of the class, including: sequence motifs (signature sequences); molecular structures (Rossman dinucleotide binding fold and parallel β-sheet regions for class I vs extensive anti-parallel β-sheet regions for class II); and the site of initial aminoacylation (class I at the 2’ OH of the terminal ribose vs class II at the 3’ OH of the terminal ribose) (27, 28). Among higher eukaryotes, nine synthetase activities, most of which are class I enzymes, are associated into a multienzymecomplex. Including AsnRS, five of six synthetase Ags are class I aminoacyl tRNA synthetases, each found free and uncomplexed in the cell cytoplasm. Anti-OJ sera immuno precipitate the full multienzyme complex with nine synthetase activities, but most anti-OJ sera react primarily with isoleucyl-tRNA synthetase, a class I synthetase (13). A very small number of sera have anti-OJ by IPP but appear to be equally or more strongly reactive with leucyl-tRNA synthetase, another class I synthetase. However, anti-OJ is one of the least common anti-synthetases, and thus, most anti-synthetase Abs, and most anti-synthetase sera, react with uncomplexed class II synthetases. The reason for this preference is unknown. Possibly, such Ags can be expressed on the surface or presented more easily.

Six synthetases remain that have not been found to be Ags by IPP and are not in the multienzyme complex. This, along with the fact that anti-Jo-1 is more common than all other anti-synthetases together, clearly indicates that synthetases are not randomly targeted. If Abs to these other six synthetases occur, they must be extremely rare. Thousands of myositis and connective tissue disease sera, and hundreds of ILD sera, have been tested by us and others by IPP (18, 19). This would detect tRNAs precipitated by anti-synthetases, but unidentified Abs that immunoprecipitate tRNA are very uncommon. However, if autoantibodies to these synthetases existed that did not immunoprecipitate tRNA, which can be seen with some animal antiserum to synthetases, they may not have been detected.

All three patients with anti-KS autoantibodies had ILD, some with other associated features of connective tissue disease including Raynaud’s phenomenon and arthritis, but none with any evidence of myositis or myopathy such as weakness or elevated creatine kinase. Each of the five previous anti-synthetases were first identified as myositis-associated autoantibodies and then found to be associated with ILD. However, as noted above, a small number of patients may have ILD without clinical evidence of myositis, and this is more common with some anti-synthetases (anti-PL-12 and, from available evidence, anti-OJ) (18). In a recent report, none of six Japanese patients who had anti-PL-12 Abs fulfilled criteria for myositis (19). In this aspect, anti-KS appears to resemble anti-PL-12 more than anti-Jo-1. Also, like anti-PL-12, anti-KS may prove to be associated with myositis in other populations. The features that these three patients had can be considered to be within the spectrum of the “anti-synthetase syndrome” that has been associated with other anti-synthetases. ILD is one of the major features of the anti-synthetase syndrome, and Raynaud’s phenomenon and arthritis, as seen in some anti-KS patients, are also felt to be part of the syndrome. The syndrome associated with anti-KS may be one end of the spectrum of anti-synthetase patients. This highlights the clinical importance of looking for such Abs in patients with ILD even if no signs of myositis or of connective tissue diseases are present.

This group of autoantibodies is unique in having a combination of three characteristics: 1) they are directed at functionally related enzymes (performing the same function for different amino acids); 2) they are associated with a similar syndrome; and 3) they are mutually exclusive. Anti-KS Abs seem to follow this pattern. No previously studied anti-synthetase serum has had evidence of Abs to AsnRS, and none of the three anti-AsnRS sera reported here showed signs of reaction with other synthetases. The mechanism for this picture remains unknown. Several possible mechanisms have been proposed, such as similar interaction with myositis-inducing viruses (through complexes with tRNA-like structures on viral genomes (2) or anti-idiotypic mechanisms (4, 29)) or a similar pattern of surface expression. However, these proposed mechanisms remain speculative, and further studies could provide important clues for understanding the possible mechanisms for the development of these Abs. Study of these Abs may provide insight into the etiologic and pathogenetic mechanisms of myositis and ILD.

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References


