AUTOANTIBODIES AGAINST ALANYL-tRNA SYNTHETASE AND $tRNA^{Ala}$ COEXIST AND ARE ASSOCIATED WITH MYOSITIS

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Autoantibodies directed against cellular antigens are frequently found in the connective tissue diseases (1, 2). In myositis, an inflammatory condition of muscle, the antigens are often cytoplasmic rather than nuclear components (3, 4). We recently identified two such antigens as aminoacyl-tRNA synthetases; anti-Jo-1 antibody, found in 25–30% of myositis sera (2, 4), reacts with histidyl-tRNA synthetase (3), and PL-7 antibody, found in 5% of cases, is directed against threonyl-tRNA synthetase (5). Both antibodies immunoprecipitate ribonucleoprotein complexes containing the enzyme and its transfer RNA; they recognize the protein free of RNA, but do not recognize the RNA alone. Here we describe a third myositis-related autoantibody, PL-12, that inhibits alanyl-tRNA synthetase. Both the enzyme and $tRNA^{Ala}$ are immunoprecipitated, but in this case, distinct sets of antibodies react with each component independently. Furthermore, antibody against the synthetase fails to recognize the enzyme bound to its cognate tRNA. We discuss the possibility that one antibody may be an antiidiotype to the other.

Materials and Methods

Sera. This study is based on sera shown by ourselves and others to immunoprecipitate tRNA from extracts of ^{32}P-labeled HeLa cells. Serum samples (patients 3–6) were provided by Drs. J. Keene, J. A. Hardin, J. A. Steitz, and R. Reddy. Serum Ha (6) and serum LL (7), a sample of the tRNA-1 specificity (8), are included among the sera reported here. IgG was prepared by chromatography through DEAE-Sephadex A50 (Pharmacia Fine Chemicals, Piscataway, NJ), as described previously (5).

Immunoprecipitation of RNA and Protein. HeLa cells labeled with [^{35}S]methionine or [^{32}P]phosphate (NEX 054; New England Nuclear, Boston, MA) were harvested, washed, and lysed in buffer A (5, 9). Immunoprecipitations were conducted as described previously (5), using IgGSORB (Tufts-New England Medical Center, Boston, MA) to precipitate immune complexes made by incubating cell extract with patients' IgG. Proteins dissociated from the precipitate were resolved by electrophoresis in 15% SDS-polyacrylamide gels and detected by fluorography (10, 11). Immunoprecipitated RNA was resolved in 7 M urea/10% polyacrylamide gels. Immunoblotting was performed according to the procedure of Towbin et al. (12).

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TABLE I
Clinical Features of Six Patients with PL-12 Antibody

Patient	Sex	Age (yr)	Clinical notes
1	Female	60	8-yr history of myositis, Raynaud's phenomenon, sicca syndrome, sclerodactyly, arthritis, and pulmonary fibrosis
2	Female	60	2-yr history of myositis, Raynaud's phenomenon, sicca syndrome, arthritis, and pulmonary fibrosis; preceded by mastectomy and chemotherapy for carcinoma 6 yr earlier
3	Female	48	Fatal pulmonary fibrosis preceded by a 16-mo history of myositis, Raynaud's phenomenon, and swollen fingers
4	Male	60	8-yr history of myositis, Raynaud's phenomenon, and pulmonary fibrosis
5	Female	35	5-yr history of adult Still's disease and widespread tenosynovitis (7, 8)
6	Female	—	SLE (6)

Aminoacylation of tRNA. Standard reactions (25 μl) contained 90 μg calf liver tRNA, a HeLa cell extract containing charging enzymes, one ^{3}H-labeled amino acid (or [^{35}S]-methionine or [^{35}S]cysteine), IgG as specified, and other components as described previously (3, 5). Aliquots (5 μl) were removed for determination of cold TCA-insoluble radioactivity. In some experiments, the charging enzyme extract was preabsorbed with PL-12 IgG bound to protein A–Sepharose (5) to deplete it of PL-12 antigens before incubation. To examine the amino acid acceptance of PL-12 RNA, PL-12 IgG was incubated with a fourfold excess (by weight) of HeLa cell cytoplasmic RNA, and absorbed to protein A–Sepharose. RNA was isolated from the washed immunoprecipitate by phenol and chloroform extraction followed by ethanol precipitation.

Removal of Anti-tRNA Antibody. Calf liver tRNA (Boehringer-Mannheim Diagnostics, Houston, TX) was bound to cyanogen bromide–activated Sepharose 4 B (1.25 μmol ligand per milliliter of gel) using the procedure recommended by the manufacturer (Pharmacia Fine Chemicals). Any unreacted sites were blocked with Tris base. ~25% of the tRNA was bound. Control adsorbants were prepared in the same way by omitting tRNA or substituting heat-denatured calf thymus DNA (Sigma Chemical Co., St. Louis, MO). Antibody was eluted from the adsorbant with 20 mM triethylamine, pH 12.4

Results

Antibody Frequency and Clinical Association. Six examples of the autoantibody specificity under study were recognized by the identical patterns of tRNA and protein bands they gave on immunoprecipitation. Subsequently, a precipitin line was detected by counterimmunoelectrophoresis (13), and the specificity was named PL-12. Four of the six examples were derived from patients with a syndrome of myositis, pulmonary fibrosis, and Raynaud's phenomenon; the fifth and sixth patients has episodes of arthritis and fever (Table I). In our British series of patients with myositis, the frequency of the antibody was ~3% (data not shown).

PL-12 IgG Recognizes Two Antigens. All six PL-12 isolates precipitated an [^{35}S]methionine-labeled protein of 110 kD (Fig. 1*A*). In two cases, the identity of the immunoprecipitated polypeptides was confirmed by comparing their partial proteolysis products; identical peptide patterns were obtained (Fig. 1*C*). The protein remained precipitable after digestion of the cell extract with ribonuclease (Fig. 1*A*) under conditions that destroyed the precipitability of both U1-

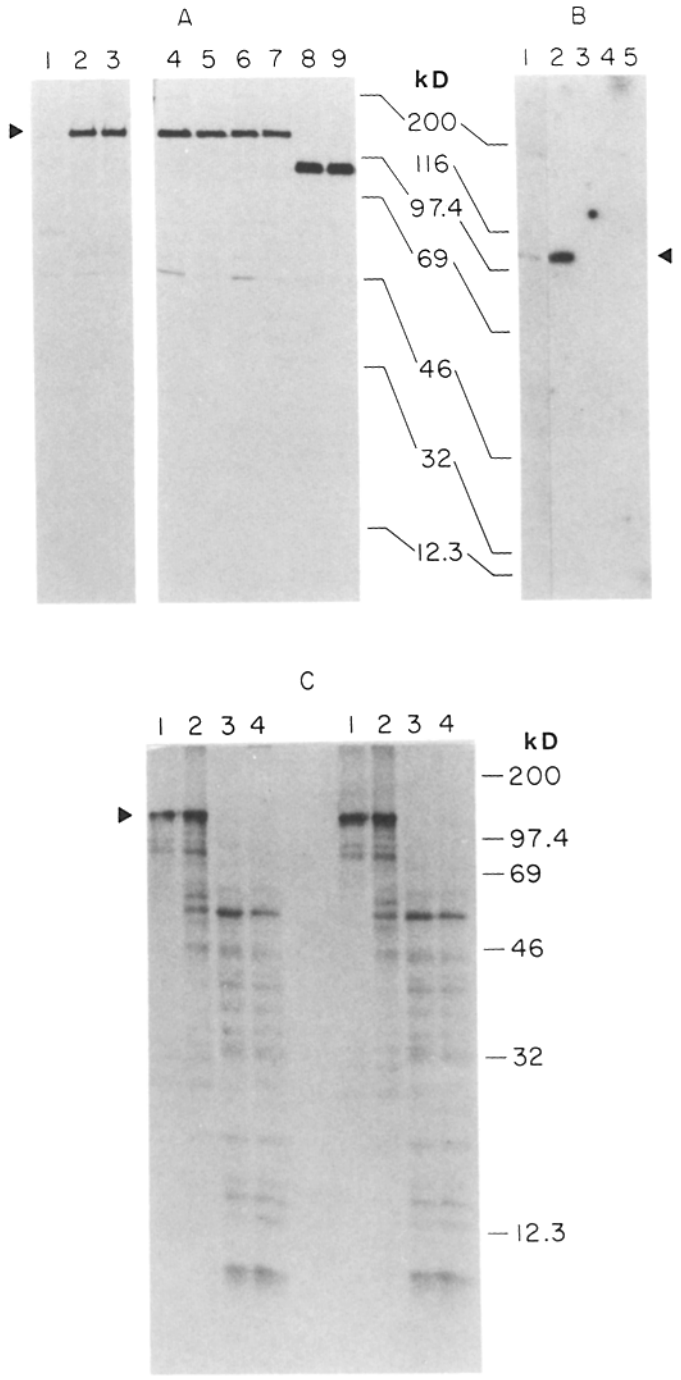

FIGURE 1. Polypeptides recognized by PL-12 antibodies. (*A*) Protein precipitated from [^{35}S]-methionine-labeled HeLa cell extract by normal IgG (lane *1*), PL-12 IgG (lanes *2–7*), and PL-7 IgG (lanes *8* and *9*). The extract was pretreated with RNase at 0.5 mg/ml for 5 min at 37°C for lanes *4*, *6*, and *8*. PL-12 IgG from patients 4, 3, 2, and 1 were used in lanes *2*, *3*, *4* and *5*, and *6* and *7*. (*B*) Immunoblot (from a 10% gel) reacted with serum 1. Lane *2*, HeLa cell extract; lanes *3–5*, three PL-12 IgG isolates; lane *1*, ^{35}S-labeled PL-12 protein, not reacted with serum 1 or ^{125}I–protein A. (*C*) Partial proteolysis of PL-12 protein precipitated by sera 4 (*left*) and 5 (*right*), digested with 0, 5, 50, and 500 ng (lanes 1–4) *S. aureus* V8 protease (4). Positions of molecular weight markers are shown.

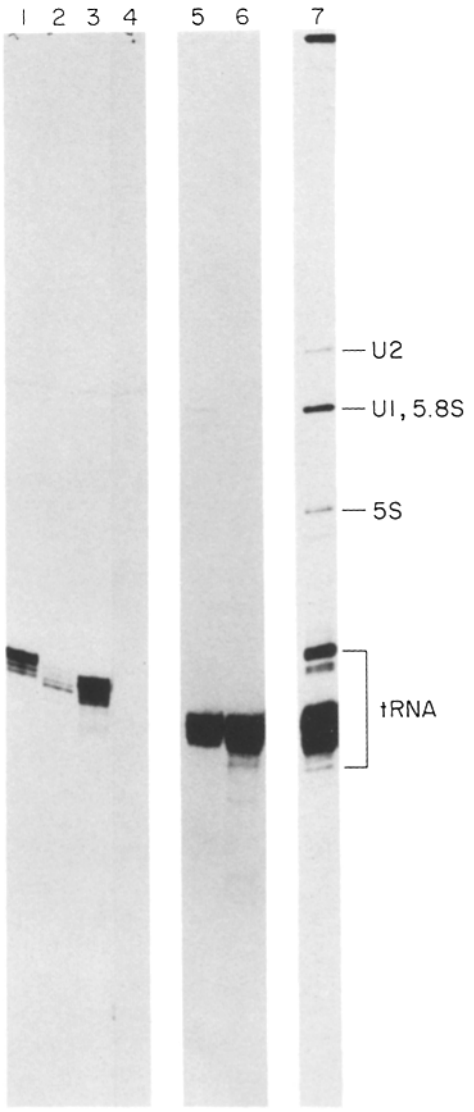

FIGURE 2. RNA recognized by PL-12 antibody. RNA was precipitated from ^{32}P-labeled HeLa cell extracts by PL-7, Jo-1, PL-12, and control IgG (lanes *1–4*). Lanes *5* and *6*, PL-12 precipitates from standard and deproteinized extracts, respectively; lane 7, extract RNA as marker.

RNA by anti-RNP (3) and of $tRNA^{Ala}$ by PL-12 antibody (not shown). The protein was also detected by immunoblotting after transfer to nitrocellulose paper and probing with one of the PL-12 isolates (Fig. 1*B*). These results show that the 110 kD protein is recognized directly by the antibody.

Immunoprecipitates from ^{32}P-labeled extracts revealed several bands of RNA in the tRNA size range (Fig. 2). The exact banding pattern varied slightly from experiment to experiment, but was simplified when freshly prepared extracts were used, suggesting that some of the complexity might be due to partial

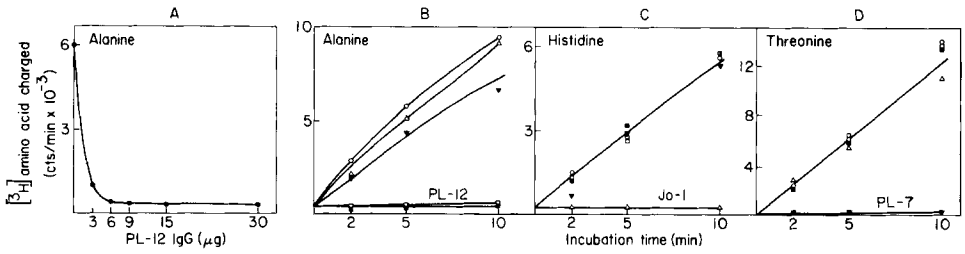

FIGURE 3. Inhibition of tRNA charging by PL-12 antibodies. (*A*) Effect of increasing amount of PL-12 IgG on aminoacylation with [^{3}H]alanine. Incubation was for 10 min. (*B–D*) Kinetics of charging of [^{3}H]alanine, [^{3}H]histidine, and [^{3}H]threonine, respectively, in the presence of two isolates of PL-12 IgG (□, ■) Jo-1 IgG (△), PL-7 IgG (▼), or no IgG (○).

TABLE II
Aminoacylation in PL-12-depleted Extracts

Amino acid	[^{3}H]Amino acids charged (cpm)	
	Control	PL-12-depleted
Alanine	1,550	70
Lysine	3,360	3,440
Tryptophan	3,090	3,110

A HeLa cell extract, pretreated with PL-12 IgG bound to protein A-Sepharose to deplete PL-12 antigens, was assayed for aminoacyl-tRNA synthetase activities with added calf liver tRNA. All 20 amino acids were tested; only alanyl-tRNA synthetase was removed.

degradation of the RNA. The PL-12 tRNAs were electrophoretically distinct from $tRNA^{His}$ and $tRNA^{Thr}$ precipitated by anti-Jo-1 and PL-7 antibodies. The PL-12 tRNA remained precipitable when extracts were deproteinized with pronase and phenol (Fig. 2) before reaction with antibody. The six PL-12 IgGs gave the same banding pattern, and all were able to precipitate naked tRNA, in contrast to the otherwise comparable Jo-1 and PL-7 antibodies studied previously (3, 5). To exclude the possibility that RNA precipitation was due to incomplete removal of protein, ^{35}S-labeled cell extracts were deproteinized by phenol extraction; no protein was detected in the immunoprecipitate (not shown). We also considered the possibility that the IgG samples might contain free protein antigen that could bind to RNA and account for the precipitation of naked RNA; however, no antigen was detected in the IgG samples by immunoblotting (Fig. 1*B*). We conclude that PL-12 antibodies react with both the 110 kD protein and with tRNA species.

Identity of the Protein Antigen. By analogy with the Jo-1 and PL-7 specificities, which both react with charging enzymes (3, 5), we examined the ability of PL-12 IgG to inhibit the aminoacylation of tRNA. As shown in Fig. 3, the addition of increasing amounts of PL-12 IgG led to a progressive decrease in the rate of tRNA charging with alanine. No other amino acids were affected, as exemplified here with histidine and threonine. All four of the PL-12 isolates tested specifically inhibited alanine charging. To exclude the possibility that the block was due to the binding of antibody to $tRNA^{Ala}$ or to the addition of a nonspecific inhibitor, we carried out the depletion experiment shown in Table II. When the charging

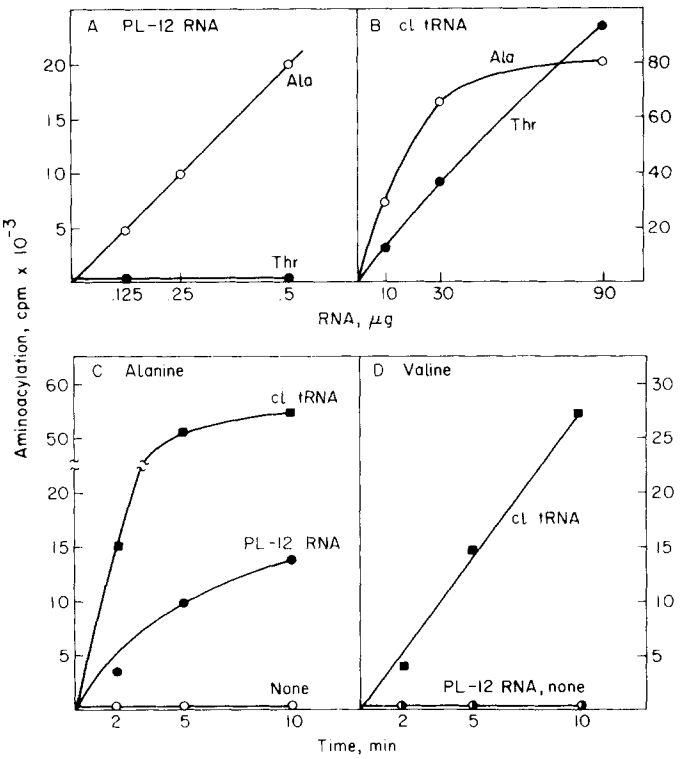

FIGURE 4. Charging of RNA immunoprecipitated by PL-12 antibody. (*A* and *B*) Titration of PL-12 and calf liver tRNA, respectively, into aminoacylation reactions containing [^{3}H]-alanine (○) or [^{3}H]threonine (●). (*C* and *D*) Kinetics of aminoacylation with [^{3}H]alanine and [^{3}H]valine, respectively, comparing reactions with PL-12 RNA (●), calf liver tRNA (■), and no tRNA (○). All 20 amino acids were tested in this way; only alanine was charged.

extract was treated with PL-12 IgG immobilized on protein A–Sepharose before addition of tRNA and the other components of the reaction mixture, subsequent aminoacylation with alanine was again greatly reduced, whereas charging with the other 19 amino acids was unaffected. The protein antigen is therefore alanyl-tRNA synthetase.

Identity of the RNA Antigen. The fingerprint of the PL-12 RNA (not shown) was complex and consistent with the presence of more than one species. We therefore turned to aminoacylation reactions with the RNA immunoprecipitated by PL-12 IgG to make an identification. As shown in Fig. 4*A*, PL-12 tRNA could be charged efficiently with alanine but not with threonine, whereas unfractionated calf liver tRNA accepted both amino acids (Fig. 4*B*). On a weight basis, PL-12 tRNA was charged with alanine ~13-fold more efficiently than calf liver tRNA, and its amino acid acceptor activity exceeded 0.5 mol alanine per mole tRNA. Using each amino acid in turn, we compared the acceptor activity of PL-12 RNA and calf liver tRNA in kinetic experiments like those of Fig. 4, *C* and *D*. With the exception of alanine, no incorporation above background was detected with any of the other amino acids. These results show that PL-12 antibodies recognize $tRNA^{Ala}$. Direct sequence analysis and hybridization to a

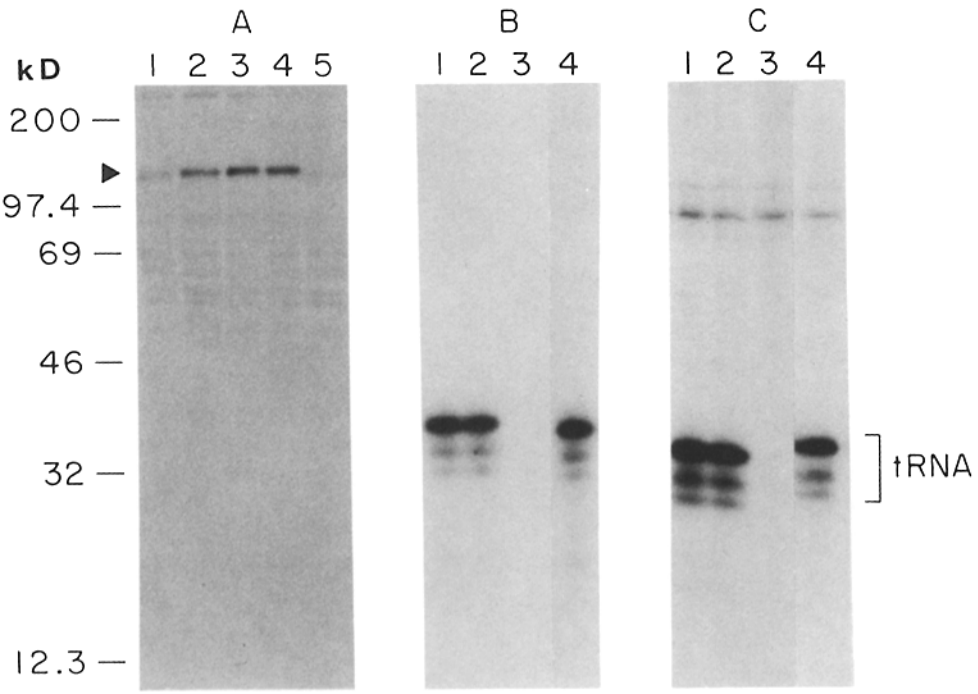

FIGURE 5. Properties of anti-alanyl-tRNA synthetase antibody. (*A*) Immunoprecipitation from ^{35}S-labeled extract with 0.2, 0.9, and 1.8 μg untreated PL-12 IgG (lanes *1–3*); 1.8 μg PL-12 IgG preadsorbed with Sepharose-tRNA (lane *4*); or no IgG (lane *5*). (*B*) Immunoprecipitation of RNA from deproteinized ^{32}P-labeled extract by untreated PL-12 IgG (lane *4*), and by PL-12 IgG preabsorbed with Sepharose (lane *1*), Sepharose-DNA (lane *2*), Sepharose-tRNA (lane *3*). (*C*) As in *B*, except that the ^{32}P-labeled extract was not deproteinized before immunoprecipitation.

cloned *Bombyx mori* $tRNA^{Ala}$ gene (14) confirms that at least two isoaccepting forms of this RNA are present in the immunoprecipitate (C. C. Bunn and M. B. Mathews, manuscript in preparation).

Distinct Antibodies against tRNA and Protein. The data described so far show that PL-12 antibodies are directed at alanyl-tRNA synthetase and its cognate, $tRNA^{Ala}$. These two reactivities coexist in all six isolates of this specificity available to us, implying either that separate antiprotein and anti-tRNA antibodies occur in all cases, or that a single antibody specificity crossreacts with both antigenic moieties.

To distinguish between these two possibilities, PL-12 IgG was incubated with tRNA immobilized on an inert matrix. Antibodies precleared in this way were unable to immunoprecipitate tRNA (Fig. 5*B*), whereas precipitation of the 110 kD protein antigen was unimpaired (Fig. 5*A*). As expected, antibody eluted from the adsorbant efficiently precipitated $tRNA^{Ala}$ (data not shown). No loss of anti-tRNA activity was occasioned by pretreatment of the antibody with Sepharose that had been incubated with calf thymus DNA or without nucleic acid (Fig. 5*B*). To our surprise precleared PL-12 IgG not only failed to recognize naked tRNA but also failed to coprecipitate tRNA with the enzyme from whole cell extracts (Fig. 5*C*). To be sure that excess unlabeled tRNA had not leached from the

tRNA-Sepharose matrix and competed with labeled RNA in this experiment, we conducted control immunoprecipitations in the presence of precleared PL-12 IgG or of a deproteinized extract therefrom. The ability of antibody to precipitate [^{32}P]tRNA was not significantly impaired by prior mixing with preabsorbed PL-12 IgG itself, or with material prepared from it by phenol/chloroform extraction and alcohol precipitation. Similarly, immunoprecipitation by PL-7 antibody was unaffected. These experiments discount the release of substantial amounts of tRNA from the immunoadsorbant. We conclude that, at least in the two cases studied, the PL-12 specificity contains two separate populations of antibodies: one set recognizes alanyl-tRNA synthetase, possibly only in its tRNA-unbound form, while the second set is directed against tRNA^{Ala}.

Discussion

Six human sera of the PL-12 specificity immunoprecipitated a protein of 110 kD, as well as tRNA from HeLa cell extracts. In all cases, the protein and tRNA were recognized independently. The antibodies inhibited aminoacylation with alanine, identifying the protein antigen as alanyl-tRNA synthetase. The immunoprecipitated tRNA efficiently accepted alanine but none of the other amino acids, identifying the RNA antigen as tRNA^{Ala}. After preabsorption with tRNA bound to Sepharose, PL-12 IgG still recognized the protein antigen, but no longer precipitated tRNA from deproteinized RNA, showing that the two components are recognized by separate antibodies. The preabsorbed IgG also failed to coprecipitate tRNA with the protein antigen from whole cell extracts, a finding which may indicate that the enzyme is recognized only in a tRNA-free state.

Antisynthetase antibodies of the Jo-1 and PL-7 specificities are not accompanied by detectable levels of antibody against tRNA, although they coprecipitate tRNA with the enzyme (3, 5). The PL-12 system differs in both respects; its antisynthetase antibodies are unable to precipitate the antigen in tRNA-bound form, while other antibodies in the same sera react directly with tRNA. The absence of coprecipitating tRNA could be attributed to instability of the complex between alanyl-tRNA synthetase and tRNA^{Ala}, but it is not clear why this complex should be less stable than those containing tRNA^{His} or tRNA^{Thr} and their charging enzymes. The instability argument is weakened further by the existence of experimental rabbit antibodies that resemble PL-7 autoantibodies in reacting with threonyl-tRNA synthetase but differ in that they fail to coprecipitate tRNA (5). It is possible, therefore, that anti-alanyl-tRNA synthetase antibody binds the protein in such a way as to preclude binding of tRNA.

These observations are consistent with the idea that one PL-12 antibody is an antiidiotype to the other (15–18) and with the general hypothesis that autoantibodies are antiidiotypes of antiviral antibodies (19). We have argued (20) that the available evidence does not sustain this idea so far as ribonucleoprotein antigens are concerned, but support could be adduced from the complementary activities of PL-12 antibodies reported here. Antiidiotype antibodies, directed at the antigen-combining site of antibodies against tRNA^{Ala}, might themselves mimic tRNA^{Ala} and bind to the charging enzyme in its place. Perhaps it is more likely, in view of the other autoantibodies against aminoacyl-tRNA synthetases

(3, 5), that PL-12 antiprotein antibody incited an antiidiotype response that recognizes $tRNA^{Ala}$. This would explain both the coexistence of two types of antibody in PL-12 sera and the absence of coprecipitating tRNA. In an effort to test this idea, we have attempted to block antibody binding to the synthetase by competition with an excess of tRNA. No inhibition of synthetase immunoprecipitation was observed (unpublished data), but the relative affinities of antibody and tRNA for the synthetase are unknown. In progress are more direct tests, including the determination of the synthetase and antibody binding sites on $tRNA^{Ala}$.

The concurrence of antibodies to alanyl-tRNA synthetase and $tRNA^{Ala}$ in the same serum is also reminiscent of other cases where autoimmune sera contain antibodies to more than one component of a macromolecular complex. For example, lupus sera may contain antibodies reactive with several components of the nucleosome (DNA, poly(ADP-ribose), histones) (21–23), ribosome (proteins and high molecular weight RNA) (2), and small nuclear ribonucleoproteins (snRNP) particles (several proteins with "Sm" or "RNP" reactivity) (24). Antibodies to Ro and La also often occur together, and the Ro and La protein antigens are related in that they bind overlapping sets of RNAs, and may be associated at least transiently (25). An interpretation of these observations is that antibodies are generated against the whole particle or complex, perhaps because a change in one component leads to an antihapten-like response to associated macromolecules. Changes in the nucleic acid moiety may be of particular importance (2, 3). Simultaneous autoimmunity to several components of a complex argues against the hypothesis (26) that autoantibodies are initially raised against foreign immunogens and crossreact merely by chance with host components.

Autoimmunity to aminoacyl-tRNA synthetases is closely associated with myositis (3, 5), particularly with a syndrome of myositis, fibrosing alveolitis, Raynaud's phenomenon, and sicca syndrome (27). Four of the patients with antibodies against alanyl-tRNA synthetase and $tRNA^{Ala}$ were suffering from this condition. In the other two cases, our clinical data are incomplete. Despite their effects in vitro, little evidence suggests that immunoglobulins are able to penetrate living cells, making it seem unlikely that these antibodies have a major pathogenetic effect in vivo. Rather, we believe they may be a clue to etiology, possibly pointing to a viral origin of the disease (3).

Summary

The sera of six patients with autoimmune disease, predominantly myositis with pulmonary fibrosis, contain antibodies of the PL-12 specificity. These autoantibodies react with both protein and RNA components of human cells. The protein has a subunit molecular mass of 110 kD, and the RNA comprises a group of bands in the tRNA size class. Aminoacylation experiments identify the antigens as alanyl-tRNA synthetase and its corresponding tRNAs, $tRNA^{Ala}$. Anti-tRNA antibody can be absorbed out without depleting antisynthetase activity, showing that the antigens are recognized independently by separable antibodies that coexist in these sera. The concurrence of separate antibodies to the two components suggests that the autoimmune response may be mounted against the charging enzyme–tRNA complex. However, the antisynthetase antibody fails to

coprecipitate tRNA with the enzyme, suggesting that the antibody reacts with its target only when it is not complexed with tRNA.

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