

HUMAN ANTI-P68 AUTOANTIBODIES RECOGNIZE
A COMMON EPITOPE OF U1 RNA CONTAINING
SMALL NUCLEAR RIBONUCLEOPROTEIN
AND INFLUENZA B VIRUS

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Autoimmune phenomena are known to occur in a large variety of human diseases (for review see reference 1). Antibodies to self-components (autoantibodies) can be directed against organ-specific or non-organ-specific target structures and can develop transiently or persist chronically. Some are regarded as diagnostic markers for certain diseases, and some are pathogenic or contribute to disease development (reviewed in reference 2).

Several factors, ranging from genetic susceptibility to environmental influences, have been proposed as triggers of autoimmune processes. There is also ample evidence for an important role of various viruses in generation of autoimmunity (3, 4). Autoantibodies to lymphocytes are common after infection with Epstein-Barr, rubella, and measles viruses (5). Patients infected with hepatitis B and delta (6), influenza (7), herpes (8), and coxsackie viruses (3) often develop antibodies to host cell- and organ-specific antigens.

Crossreaction of antibodies with epitopes shared by microbial antigens and host self-components, termed molecular mimicry (4, 9), is one of the mechanisms that has been speculated to initiate development of autoantibodies. Polyclonal and monoclonal antiviral antibodies induced by immunization were frequently observed to crossreact with host proteins (10), and some of the target antigens bearing shared epitopes have been identified (4). An epitope shared by a major human autoantigen and a protein of a human virus, and occurrence of the corresponding antibodies in sera of patients with chronic autoimmune diseases, has, however, not been described so far.

To search for shared epitopes, the autoimmune response to U1-RNA containing small nuclear ribonucleoprotein particles [(U1)snRNP]¹ was used as a model system. Anti-(U1)snRNP autoantibodies are characteristic for certain inflammatory rheumatic diseases (11), and a 68-kD protein (p68) of the (U1)snRNP particle is a major antigenic target (12). The cDNA coding for p68 has been cloned (13-16), and three

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¹ Abbreviations used in this paper: TPBS, 5% Tween-20 in PBS, SnRNP, small nuclear ribonucleoprotein.

major autoantigenic domains have been identified with different fragments of the recombinant p68 protein (17). One of these domains is located between amino acid positions 233 and 276 (17). Subsequent studies with fusion proteins and synthetic peptides confined this autoreactive region to the sequence NH₂-PTRAETREE-RMERKRREKIE-COOH (amino acid position 234–253, subsequently called domain A), and showed that it is recognized by 35% (37/103) of anti-p68 autoimmune sera (H. Guldner et al., unpublished data).

Here, we demonstrate that a subset of human anti-p68/domain A autoantibodies react with a p68 epitope that is present also on the matrix protein M1 of human influenza B viruses, and that autoimmune sera recognize this shared epitope also in the viral sequence context.

Materials and Methods

Human Autoimmune Sera, mAbs, and Influenza Virus Strains. Sera from patients with mixed connective tissue disease (18) and systemic lupus erythematosus (diagnosed according to the criteria of the American Rheumatism Association; reference 19) were obtained from Dr. H.-J. Lakomek, Department of Medicine C, Endocrinology and Rheumatology, University of Düsseldorf, FRG, and Dr. Frederick Miller, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD. Reactivity towards the (U1)snRNP-specific p68 protein was determined by immunoblotting with HeLa nuclear extracts (20) and ELISA with recombinant p68 fusion protein (16).

Anti-p68 mAbs were generous gifts from Dr. R. Lührmann, Institut für Molekularbiologie und Tumorforschung, Universität Marburg, FRG (clone H386); Dr. S. O. Hoch, The Agouron Institute, La Jolla, CA, (clone 2.73); and Dr. D. G. Williams, The Mathilda and Terence Kennedy Institute of Rheumatology, London, UK (clone K8.43).

Influenza strains B/Beijing/1/87 and A/Singapore/6/86 (H1N1) grown in hen eggs were kindly provided by Dr. J. Hinz, Behring-Werke AG, Marburg, FRG.

Immunization of Mice with Domain A Peptide and Fusion Protein. Six and four female 8-wk-old BALB/c mice were immunized with 80–100 µg, i.p. and s.c., respectively, of peptide R1 (see Fig. 1) coupled to palmitic acid (coupling procedure as described in reference 21) or with 50–100 µg of SDS-PAGE-purified MS2 domain A fusion protein. For the first immunization, antigens were emulsified in CFA; for the following immunizations at days 14, 28, and 42, the same amount of antigen emulsified in IFA was used. Blood samples were taken at days 1, 22, 51, 82, 113, 144, and 176.

Competitive Inhibition ELISA with Synthetic Peptides. Peptide R1 spanning domain A (for amino acid sequence, see Fig. 1 a) was adsorbed to Maxisorp microtiter plates (Nunc, Roskilde, Denmark) at a concentration of 2 µg/well in PBS (10 mM Na₂HPO₄, 2 mM KH₂PO₄, 3 mM KCl, 170 mM NaCl, pH 7.4) for 2 h at room temperature, and free binding sites were saturated overnight with 5% Tween-20 in PBS (TPBS). Human autoimmune sera (diluted 1/400 in TPBS) were preincubated with inhibitor peptides (0.05 µg/µl diluted serum; for amino acid sequences, see Fig. 1 a) for 1 h at room temperature, followed by incubation of the serum/peptide mixture in R1 peptide-coated microtiter wells for 45 min. The amount of antibodies bound to solid-phase R1 was measured with goat anti-human IgG coupled to peroxidase (1/4,000 in TPBS; Medac, Hamburg, FRG) and ortho-phenylenediamine as substrate. Absorbance was read at 492 nm in a Titertek Multiscan photometer (Flow Laboratories, Bonn, FRG).

Construction of Recombinant Fusion Proteins. Construction of pEx34a plasmids expressing various subfragments of the p68 protein has been described elsewhere (17).

The expression plasmid coding for part of domain A of the p68 protein (sequence NH₂-PTRAETREERMERKRRE-COOH) was obtained by insertion of a pair of synthetic oligonucleotides (sequence of coding strand: 5'-GATCC CCA ACT CGT GCT GAA ACC CGA GAG GAG CGC ATG GAG AGG AAA AGA CGG GAA TGAT-3') with Bam HI and Xba I sticky ends into vector pEx34a. Single amino acid substitutions in domain A were generated by exchange of the following nucleotide positions in the coding strand of the synthetic oligo-

nucleotide (compare Fig. 2 a): 2, 28/C; 3, 33/A; 4, 40/C; 5, 44/C; 6, 50/T; 7, 51/G; 8, 54/A, 55/G, 56/C. Correct sequence and in-frame insertion was confirmed by dideoxy sequencing using a MS2 oligonucleotide as primer (17).

Expression and Preparation of Recombinant Fusion Proteins. Transformation of recombinant pEx34 plasmids in *Escherichia coli* 2136 cells, induction of protein expression by temperature shift, and preparation of total bacterial extracts containing phage MS2 polymerase/p68 domain A fusion proteins were as described elsewhere (17).

Gel Electrophoresis and Immunoblotting. Total bacterial extracts or influenza virus proteins were solubilized in sample buffer containing 2% SDS and 0.1 M dithiothreitol, separated on 20% (*E. coli* extracts) or 17.5% (influenza virus proteins; 10 µg/lane) SDS-polyacrylamide gels (22) and transferred electrophoretically to nitrocellulose paper (23). Residual protein binding sites were blocked with 10% horse serum in TBS (10 mM Tris-HCl, pH 7.6, 150 mM NaCl) and human autoimmune sera (diluted 1/400), or affinity-purified autoantibodies (see below) were applied in 3% horse serum/TBS. Bound antibodies were visualized with goat anti-human IgG, either coupled to peroxidase (1/2,000) and chloronaphthol substrate or coupled to alkaline phosphatase (1/20,000) and NBT/BCIP substrate.

For inhibition experiments with synthetic peptides, patients' sera or affinity-purified antibodies were preincubated for 1 h with peptide R12 (ERKRR, see Fig. 1) or peptide R4 (REKIE) (10 µg peptide per ml antibody dilution) before incubation of the antibodies on nitrocellulose blots.

Affinity Purification of Antibodies. For affinity purification of antibodies reactive with the shared determinant, *E. coli* cell extracts containing the MS2/p68 domain A fusion protein or total influenza B virus proteins were separated by preparative SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau S. The corresponding proteins (MS2/p68 domain A, 17 kD; M1 matrix protein, 27 kD) were cut as horizontal strips and unblocked binding sites were saturated with 10% horse serum in TBS. Human sera (diluted 1/100) containing anti-ERKRR autoantibodies were incubated for 3 h, and, after washing with TBS/0.1% Tween-20, antibodies were eluted by the pH shock method (24) followed by neutralization and concentration in Centricon microconcentrators (Amicon Corp., Danvers, MA). As control substrate for affinity purification, equivalent amounts of MS2 protein without any fused p68 sequence or M1 matrix protein of influenza A virus were used, respectively.

Results

Fine Mapping of p68 Domain A Autoepitopes. Precise mapping of domain A autoepitope(s) was performed by peptide-based competitive inhibition ELISA (Fig. 1). Peptide R1, spanning the entire length of domain A (Fig. 1 a), was used as solid-phase coupled antigen to measure binding of human anti-domain A autoantibodies after inhibition with truncated versions of R1 (peptides R2-R12; Fig. 1 a). Based on the reaction patterns of 37 domain A-positive sera tested, three groups of sera were identified. Group I sera reacted with peptide R1, and this reaction could not be inhibited by any of the shorter peptides R2-12 (e.g., serum AA), suggesting that both the NH₂- and COOH-terminal amino acid residues of domain A are involved in autoantibody binding. In contrast, reaction of group II sera with R1 was markedly reduced upon preincubation with all peptides containing the ERKRR motif (R1, R2, R6-12), but not with any of the peptides with truncated versions of the ERKRR motif (R3-R5; e.g., serum UG). Thus, group II sera (8 of 37 domain A-positive sera) contain autoantibodies to an epitope located within the sequence ERKRR. The reactivity of group III sera (5 of 37 domain A-positive sera; data not shown) was only partially reduced upon preincubation with shortened ERKRR-containing peptides, suggesting that they contain both group I and II autoantibodies.

Amino Acid Substitutions within Domain A. To investigate if all residues of the ERKRR motif are essential for immunoreactivity, a series of MS2-p68 fusion proteins with

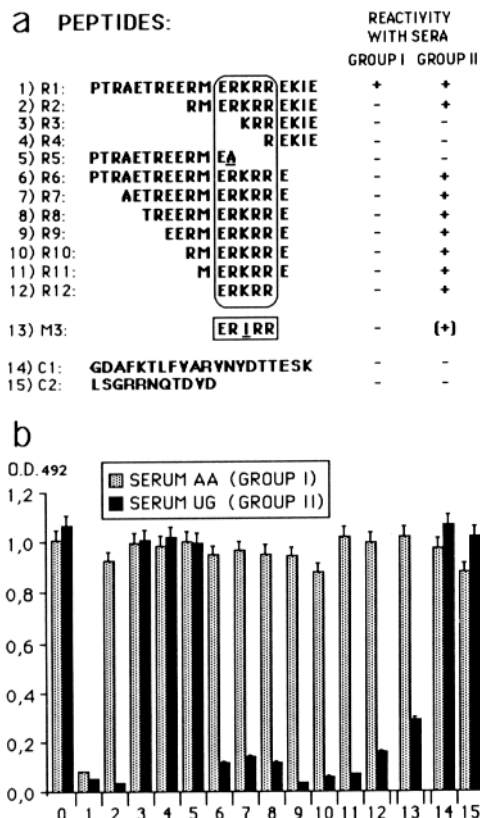


FIGURE 1. Identification of autoreactive epitopes in domain A by competitive inhibition ELISA with truncated peptides as inhibitors for autoantibody-binding to solid-phase coupled R1 peptide. (a) Peptides used for preincubation of human autoimmune sera (numbers correspond to columns in b), and reaction patterns of group I and II sera. (b) ELISA demonstrating the inhibitory effects of the peptides for a representative group I (patient AA with MCTD) and group II serum (patient UG with SLE). Both types of sera (diluted 1/400) showed strong binding to the solid-phase coupled R1 peptide (column 0, without inhibition), which was drastically reduced upon preincubation with the homologous peptide R1 (column 1). No reduction of the signal was observed after preincubation with any of two unrelated control peptides (C1, column 14; and C2, column 15). Preincubation of the sera with truncated peptides R2-12 (columns 2-12) allowed classification of the sera: serum AA (group I, dotted bars) could not be inhibited by any of the truncated peptides R2-12. In contrast, group II serum UG (filled bars) was inhibited by peptides containing the ERKRR motif (R2 and R6-12). Peptides lacking the complete ERKRR sequence (R3, R4, and R5) had no inhibitory effects on group II autoantibodies. Changing the amino acid lysine (K) of the ERKRR motif to isoleucine (I, peptide M3) resulted in significant but, compared with R12, less efficient inhibition of group II sera (compare columns 12 and 13).

single amino acid exchanges in the domain A region were generated and tested by immunoblotting with ERKRR-positive sera (Fig. 2). All examined substitutions within the ERKRR motif abolished immunoreactivity (Fig. 2 b, lanes 4-7), whereas substitutions in the NH₂- or COOH-terminal-flanking regions did not (Fig. 2 b, lanes 2, 3, and 8). Competitive inhibition ELISA with peptide M3 (Fig. 1, column 13) did, however, indicate that changing the amino acid lysine (K) of the ERKRR motif to isoleucine (I) decreases but does not prevent antibody binding. These data suggest that all five amino acids of the ERKRR motif are important for binding of autoantibodies, although the lysine residue is not essential.

Identification of a Shared ERKRR Motif on Influenza B Matrix Protein. Computer-assisted search of the protein sequence data bank (software package of the University of Wisconsin Genetics Computer Group, version 5.3, 1988) revealed the ERKRR motif also within the M1 matrix protein of influenza B viruses (B/Lee/40 and B/Singapore/222/79; amino acid positions 73-77: NH₂-ERKRR-COOH; references 25 and 26). The analogous proteins of influenza A viruses (A/Udorn/72 and A/PR/8/34; references 25 and 27) do not have this motif (amino acid position 73-77: GLQRR). Other viral or bacterial proteins with an ERKRR motif have not been found.

Crossreaction of Human Anti-ERKRR Autoantibodies with Influenza B M1 Protein. To

a AMINO ACID SUBSTITUTIONS:

	2	3	4	5	6	7	8	
MS2- PTRAETREERM				ERKRR				E (1)
	↓	↓	↓	↓	↓	↓	↓	↓
	A	S	AS	SG	S			
REACTIVITY:	+	+	-	-	-	-	+	

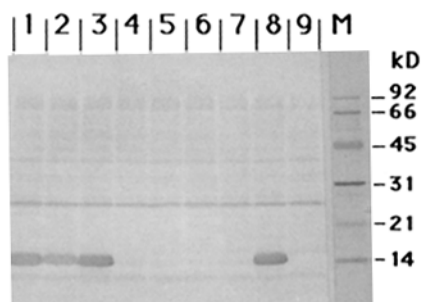
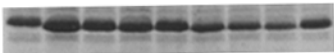
b**c**

FIGURE 2. Effect of amino acid substitutions in the ERKRR motif on immunoreactivity of group II sera. (a) Immunoreaction of group II sera with recombinant MS2 polymerase fusion proteins containing domain A-derived sequences (corresponding to peptide R6; see Fig. 1) with single amino acid substitutions as indicated. (b) Immunoblot of group II serum UB (diluted 1/400) with bacterial protein extracts from *E. coli* expressing the various fusion proteins (lane numbers correspond to numbers in a). Amino acid substitutions within the ERKRR motif abolished immunoreaction of the serum (lanes 4-7), whereas exchanges in the flanking regions did not (lanes 2, 3, and 8). The MS2 polymerase protein (14 kD) was not immunostained (lane 9). M, molecular weight markers. (c) Region of a Coomassie-stained SDS-polyacrylamide gel with the recombinant p68 domain A proteins to demonstrate that similar amounts of fusion proteins have been used for immunoblotting.

investigate if human autoantibodies recognize the ERKRR motif also in the viral sequence context, anti-ERKRR autoimmune sera were incubated on immunoblots with proteins of influenza B virus separated by SDS-PAGE. As shown for a representative example (Fig. 3 a, lane 1), the sera reacted with the M1 protein (27 kD), as well as with a variety of other viral proteins, indicating a previous viral infection or vaccination. Preincubation of the sera with peptide R12 (ERKRR) resulted in reduced staining of the matrix protein (Fig. 3 a, lane 2), suggesting that at least part of the anti-M1 response was due to anti-ERKRR antibodies. The specificity of the inhibition was demonstrated by preincubation of a control pentapeptide R4 (REKIE), which did not reduce the staining intensity (Fig. 3 a, lane 3). To further prove crossreactivity, anti-ERKRR autoantibodies were affinity purified from a truncated MS2 domain A fusion protein (labeled 1 in Fig. 2 a) and incubated on immunoblots with influenza B virus proteins. Strong staining was observed solely with the M1 protein (Fig. 3 a, lane 4), and this was drastically reduced upon preincubation of the purified antibodies with peptide R12 (Fig. 3 a, lane 5) but not with control peptide R4 (Fig. 3 a, lane 6). A reaction of affinity-purified antibodies with M1 protein of influenza A viruses (which do not have the ERKRR-motif) was not observed (Fig. 3 a, lane 7).

In a different set of experiments, patient antibodies were affinity purified from influenza B M1 protein. These antibodies reacted strongly with the domain A peptide R1 in ELISA (Fig. 4, column 1, B), whereas antibodies purified from influenza A M1 protein did not (Fig. 4, column 1, A). Again, anti-ERKRR antibodies were

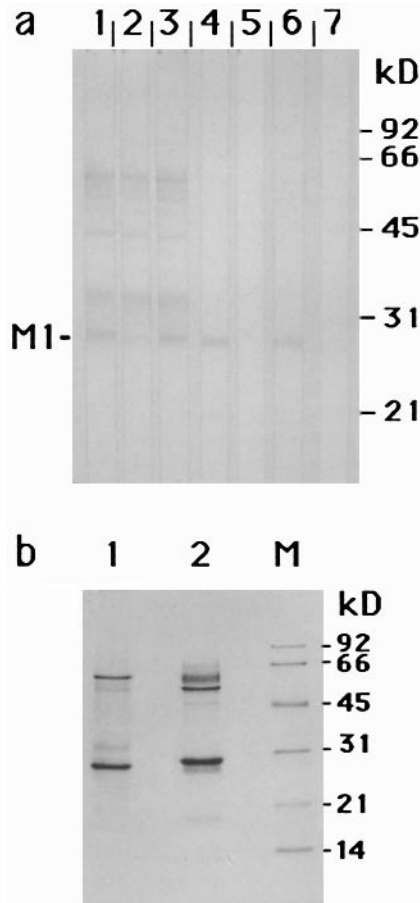


FIGURE 3. Crossreaction of anti-ERKRR autoantibodies with influenza B M1 matrix protein. (a) Immunoblots of influenza B (lanes 1-6) and influenza A proteins (lane 7). (Lanes 1-3) Incubation of ERKRR-positive serum from patient UG (dilution 1/400); lane 1, without preincubation of peptide; lane 2, with preincubation of ERKRR peptide R12; lane 3, with preincubation of control peptide R4 (REKIE). (Lanes 4-7) Incubation of anti-ERKRR autoantibodies from patient UG affinity purified from truncated MS2 domain A fusion protein; lanes 4 and 7, without preincubation of peptide; lane 5, with preincubation of peptide R12; lane 6, with preincubation of control peptide R4. (b) Coomassie Blue-stained SDS gel with influenza B (10 μ g protein; lane 1) and influenza A virus proteins (10 μ g protein; lane 2) used for immunoblotting; M, molecular weight markers.

responsible for crossreaction, since preincubation with the ERKRR peptide reduced the signal by >70% (Fig. 4, column 2, B).

Anti-ERKRR antibodies were not found in >50 healthy individuals tested, nor in anti-p68⁻ sera from patients with various rheumatic diseases and high titers of autoantibodies to other antigens (e.g., Sm, Ro, La, centromere, topoisomerase I, PM-Scl, histones, dsDNA, fibrillarin, Jo-1; data not shown). This demonstrates the specificity of the immune reaction and rules out binding of antibodies unrelated to anti-p68 to the ERKRR epitope.

Taken together, these data establish that a subset of human anti-p68 autoantibodies recognize an epitope shared by the p68 autoantigen and the influenza B virus M1 matrix protein, and that the different sequence context does not interfere with crossreactivity of the antibodies.

Immunoreactivity of Mouse Mono- and Polyclonal Anti-p68 Antibodies. We investigated if anti-ERKRR antibodies can be induced experimentally by immunizing nonautoimmune mice either with domain A peptide R1 or MS2 domain A fusion protein.

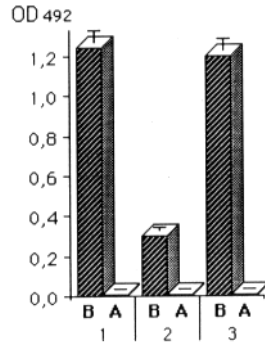


FIGURE 4. Crossreaction of human anti-ERKRR antibodies (from MCTD patient JS) affinity purified from influenza B M1 protein with p68-derived peptide R1 in ELISA. Antibodies purified from influenza B virus M1 protein (bars labeled *B*) gave a strong signal with peptide R1 (1), which was markedly reduced after preincubation with the ERKRR pentapeptide R12 (2), but not with peptide R4 (3). In a control experiment, antibodies purified from influenza A virus M1 protein (bars labeled *A*) were not reactive with the R1 peptide.

Each of the 10 animals immunized developed high titers of antibodies to domain A as revealed by ELISA with peptide R1 and immunoblotting with domain A fusion protein (data not shown). ERKRR-specific antibodies, however, were not detectable either by competitive inhibition ELISA, as described in Fig. 1, or in ELISA with a solid-phase coupled peptide derived from the influenza B M1 protein (amino acid position 66–85: NH₂-FLKPKDQERKRRFITEPLSG-COOH; data not shown). It is possible that induction of anti-ERKRR antibodies depends on a specific conformation not adopted by the synthetic peptide (for a general discussion, see reference 28) and/or on genetically determined factors involved in immune recognition.

Three anti-p68 mAbs from autoimmune mice (29; and D. G. Williams, personal communication) or normal mice immunized with snRNPs (30) failed to show any immunoreactivity with domain A–spanning peptide R1 (data not shown), indicating the absence of antibodies with specificity towards the ERKRR motif.

Discussion

In this study, a sequence motif of five amino acids was identified as an autoepitope recognized by a subset of anti-p68 autoantibodies from (U1)RNP-positive patients' sera. The same motif was found on the influenza B virus M1 matrix protein and was recognized by human anti-p68 autoantibodies also in the viral sequence context. The occurrence in autoimmune sera of antibodies reactive with the shared epitope may be causally related by representing an example of molecular mimicry. This would implicate that influenza B viruses, which are highly prevalent human pathogens, could act as trigger for development of autoantibodies to p68. Recently, a similar hypothesis has been raised for retroviruses (14, 31) based on the identification of a crossreactive epitope on the human p68-autoantigen and p30^{gag} protein of non-human retroviruses. The existence of a common epitope of p68 and a murine retrovirus has been confirmed in an independent study (data not shown). There is, however, so far no evidence for the existence of a protein of a human retrovirus exhibiting such an epitope.

Several features of the influenza viruses render them particularly attractive candidates for contributing to autoimmune processes. First, they are known to interact with lymphocytes (32), which may lead to partial breakdown of self-tolerance; second,

they can cause polyclonal B cell activation (33), which is considered important for autoantibody formation (34); third, influenza viruses impair function of phagocytes (35), which may be followed by delayed clearance of self-antigens (including snRNP particles) released from necrotic cells; fourth, p68 as an RNA-binding protein (36) may interact with influenza virus RNA and thus be secreted in virus-encapsulated form, rendering the extracellular p68 a possible target for B cells; and fifth, a high percentage of influenza virus-infected patients have been described to develop autoantibodies (7).

Based on the identification of multiple autoepitopes recognized by anti-p68 sera, the role of the p68 protein as an immunogen at some stage of the autoimmune process has been suggested (17). In addition to possibly initiating autoimmunity by molecular mimicry, influenza B might promote such an antigen-driven process by release of snRNP as a consequence of virus-induced cell lysis.

It is generally accepted that multiple factors are involved in autoimmunity: genetic predisposition, immune deficiency, and hormonal, environmental, and other influences (37). Therefore, not all individuals infected with influenza B would be expected to develop anti-p68 autoantibodies.

Only a subset of anti-p68 autoimmune sera (13/103) was found to contain anti-ERKRR antibodies. It is, however, conceivable that in some cases crossreacting anti-ERKRR antibodies are present during viral infection only and may later disappear because of viral antigen elimination and low immunogenicity of the shared self-epitope. Nevertheless, the autoimmunization process once started by the crossreacting antibody may continue in an antigen-driven fashion. Consistent with this speculation is the finding of high titers of anti-ERKRR antibodies in early sera of two anti-p68 autoimmune patients that dropped to undetectable levels during progression of the rheumatic disease. In the same patients, the antibody titers to other p68 epitopes remained unchanged (data not shown).

To further investigate the potential involvement of influenza B viruses in autoimmunity, it will be interesting to examine whether ERKRR-specific antibodies commonly occur in patients infected or vaccinated with influenza B viruses. If, as hypothesized, initiation of the autoimmune response by molecular mimicry is occurring, vaccination with inactivated virus and not only infection could lead to autoimmune phenomena, provided other predisposing factors are present. Direct experimental testing of the possible role of influenza B viruses as initiators for the formation of anti-p68, and eventually of other anti-(U1)snRNP autoantibodies, can be performed. In case of presence of the appropriate exogenous and endogenous parameters, infection of animals with influenza B viruses may provide experimental evidence for its possible trigger function in autoimmunity to snRNP.

Summary

Autoantibodies from patients with systemic rheumatic diseases were used to map antigenic sites on the 68-kD autoantigen (p68) associated with (U1)RNA-containing small nuclear ribonucleoprotein (snRNP) particles. With truncated recombinant fusion proteins and synthetic peptides, a subset of anti-p68 autoantibodies was found to recognize the amino acid sequence motif Glu-Arg-Lys-Arg-Arg (ERKRR). To investigate the possible involvement of epitopes shared by microbial antigens and host self-components in initiation of autoimmunity (molecular mimicry), a sequence

data bank was screened for proteins containing an amino acid motif identical or related to ERKRR. The identical motif was found on the M1 matrix protein of influenza B viruses, and affinity-purified human anti-ERKRR autoantibodies recognized this epitope also in the viral amino acid sequence context. The common epitope recognized by human autoantibodies suggests that influenza B virus infection may play a role in initiation of the anti-p68 and anti-(U1)RNP autoimmune response.

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