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Anti-Ki antibodies recognize an epitope homologous with SV40 nuclear localization signal: clinical significance and reactivities in various immunoassays

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Abstract We studied the characteristics of anti-Ki antibodies that react with a synthetic peptide (KILT) which has homology with SV40 large T antigen nuclear localization signal (SV40 T NLS). Immunoreactivity of antibodies to KILT was tested by enzyme-linked immunosorbent assay (ELISA), and this reactivity was compared with results obtained by immunoblotting using purified Ki and ELISA using purified or recombinant Ki antigen. The clinical significance of anti-KILT antibodies in lupus patients was also studied. Twenty percent of anti-Ki sera reacted with KILT, and all sera that reacted with KILT recognized both purified and recombinant Ki antigen in all our assay systems. A significant correlation was observed between reactivities in ELISA using KILT and with these using purified and recombinant Ki. Some sera with low titers by double immunodiffusion (DID) reacted with KILT, whereas high-titer anti-Ki sera showed a tendency to react with Ki antigen in different assays. The prevalence of discoid rash and sicca complex was higher in the anti-KILT-positive group. The amino acid sequence homologous to SV40 T NLS is an immunologically active epitope on the Ki antigen; reactivity to this epitope is associated with characteristic clinical features allied with anti-Ki antibodies in lupus patients.

Key words Anti-Ki antibody · Ki antigen · SV40 large T antigen · Systemic lupus erythematosus (SLE)

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Introduction

An autoantibody to the soluble nuclear antigen Ki was first reported by Tojo et al. in 12% of patients with systemic lupus erythematosus (SLE).[1] In 1986, Bernstein et al. subsequently studied the clinical and biochemical characteristics of the sicca lupus (SL) autoantibody system,[2] originally reported by Harmon et al.,[3] and suggested that the SL system was identical to the Ki system.

We have purified and characterized the Ki antigen as a nonhistone nuclear protein, PI 4.3, consisting of a 32-kDa polypeptide.[4] We have isolated the bovine and human cDNAs coding for Ki antigen (pb-Ki-1 and lh-Ki-10, respectively) using anti-Ki serum as a probe and found that the nucleotide sequence predicts a polypeptide with a molecular weight (MW) of 29.508 kDa with highly hydrophilic and weakly acidic characteristics.[5] Sequencing has recently shown that Ki antigen is identical to PA28γ, a proteosome activator which plays an important role in peptide presentation by major histocompatibility complex (MHC) class I molecules[6–8] and cell cycle regulation.[9–12]

Around amino acid residues 81–91, the Ki cDNA encodes an amino acid sequence resembling the sequence responsible for nuclear localization in SV 40 large T antigen (SV 40 T NLS). The domain that is homologous to SV 40 T NLS was suggested to be highly antigenic, and it has been shown that this sequence is one of the epitopes on Ki antigen which can be recognized by autoantibodies in sera obtained from lupus patients.[13] Using a synthetic peptide that has homology with SV 40 T NLS, only 36.7% of anti-Ki sera (detected by double immunodiffusion, DID) reacted in an enzyme-linked immunosorbent assay (ELISA). The reported frequency of anti-Ki antibodies has varied between 6.7% and 21.4% using different immunoassays for detection.[1,2,4,14] These data suggest that there are several autoantigenic domains on the Ki antigen and that reactivity of these epitopes is dependent on the assay system and antigen preparation.

In this report, we have attempted to clarify whether the epitope homologous to SV 40 T NLS is recognized by anti-

Ki sera in different immunoassays using various antigens. In addition, we have studied the clinical characteristics of lupus patients with anti-Ki antibodies that recognize the epitope homologous to SV 40 T NLS.

Methods

Patients and sera

Fifty-five sera from patients with SLE who met the American College of Rheumatology (formerly, the American Rheumatism Association) classification criteria[15] were selected by positive DID as described below and used to study the reactivities in different immunoassays. In addition, 105 lupus patients who met the criteria were also selected to study the clinical significance of anti-Ki antibodies.

Anti-Ki reference serum was kindly provided by Dr. T. Tojo (Keio University, Tokyo, Japan). By DID, we confirmed the specificity of our own standard sera against the anti-Ki reference serum.

Preparation of purified Ki antigen

Native Ki antigen was purified from rabbit thymus extract by a combination of ammonium sulfate fractionation, anti-Ki Sepharose 4B affinity chromatography, and high-pressure liquid chromatography gel filtration, as previously reported.[4]

Preparation of recombinant Ki antigen

The recombinant Ki fusion protein (bNAX) was made from cDNA pb-Ki-1 encoding bovine retina.[14] Ki antigen was inserted into expression vector pUC8, and this plasmid clone was propagated in *Escherichia coli* (strain DH-1α) in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside), followed by purification as previously reported.[5]

Preparation of synthetic peptide

KILT, a synthetic peptide comprising the amino acids between 79 and 94 of the Ki antigen (H-Leu-Asp-Gly-Pro-Thr-Tyr-Lys-Lys-Arg-Arg-Leu-Asp-Glu-Cys-Glu-Glu-OH), which has homology with SV 40 T NLS, was synthesized by the *t*-butyloxycarbonylaminobenzoic acid-based solid-phase method of Merrifield[13,16] using an ABI 430 A peptide synthesizer (Applied Biosystems, Foster City, CA, USA). The peptide was purified by high performance liquid chromatography (HPLC). The crude peptide was separated on a C18 SG300Å column of 4.6-mm inner diameter ×250-mm length equilibrated in 100% solvent (0.1% trifluoroacetic acid in water) at room temperature. The column was eluted with a linear gradient from 10% to 100% solvent (0.1% trifluoroacetic acid, 70% CH_3CN, 29.9% water) at a flow rate of 1 ml/min. Composition of the purified peptide was tested on a Hitachi 835 High Speed Amino Acid Analyzer (Hitachi, Tokyo, Japan).

Double immunodiffusion

The DID assay was conducted in 0.4% agarose (Sea Kem, Rockland, ME, USA), 0.01% sodium azide in phosphate-buffered saline (PBS; 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.4) as described previously.[4] Precipitation reactions were allowed to develop for 48 h at room temperature.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (IB) were performed under denaturing and reducing conditions according to the method previously described.[4] Horseradish peroxidase-conjugated goat antihuman IgG (Cappel, West Chester, MD, USA) diluted to 1:1000 with PBS containing 0.1% Tween (PBS-Tween) was used as the secondary antibody.

Enzyme-linked immunosorbent assay

To determine the optimal ELISA condition to detect reactivity to KILT, bNAX and purified Ki antigen at various concentrations and dilutions were tested (data not shown). Based on these results, 160 μg/ml KILT, 5 μg/ml bNAX, and 5 μg/ml purified Ki were the antigen concentrations and 1:1000 was the serum dilution.[13]

For the ELISA, antigen diluted in 0.05 M carbonate-bicarbonate buffer (pH 8.5) was added to the wells of an immunoplate II (Nunc, Roskilde, Denmark) and incubated overnight at 4°C. After the coating solution was removed, the plate was washed three times with PBS-Tween. Unoccupied sites were blocked by adding 250 μl 1% bovine serum albumin in PBS-Tween to each well, and the plate was incubated for 24 h at 4°C. The plate was washed five times, 100 μl patient sera diluted 1:1000 was added, and the plate was incubated for 90 min at room temperature. After washing with PBS-Tween, 100 μl alkaline phosphatase-labeled rabbit antihuman IgG (KPL, Gaithersburg, MD, USA) diluted 1:1000 in PBS-Tween was reacted. The plate was washed three times, 100 μl enzyme substrate solution (1 mg/ml *p*-nitrophenyl phosphate in diethanolamine buffer, pH 9.8) was added, and the reaction was allowed to proceed for 1 h at room temperature. Absorbance at 405 nm was then measured.

Positive sera were defined as those having an absorbance greater than 3 SD above the mean of 11 healthy controls.

Other methods

Protein concentrations were determined by the method of Lowry et al.[17] Statistical significance was tested by Fisher's exact probability test and Welch's *t* test.

Results

Immunoreactivity of anti-Ki antibodies in various immunoassays

Immunoreactivity of anti-Ki sera detected by DID was tested by various immunoassay systems, ELISA using KILT, purified Ki, and bNAX and IB using purified Ki (Table 1). The 55 sera tested were all positive for anti-Ki antibody by DID, but no other method completely detected anti-Ki activity. Forty-two of 55 sera (76.3%) were positive by IB using purified-Ki antigen, 40 of 55 reacted in an ELISA using purified Ki, and 36 of 55 reacted by ELISA using bNAX. IB using purified-Ki antigen was the most sensitive, but this was not significantly different from the other methods.

Only 11 of 55 sera reacted with KILT by ELISA, but we made the striking observation that all 11 sera were positive for anti-Ki by all other methods. Additionally, 18 sera that did not react with KILT were positive in all the methods that used the entire Ki antigen, purified Ki, or bNAX. The other 26 sera had a variety of reactivities, 6 sera did not react with any method, and some sera reacted in one or two of these methods (Table 2). These results suggest that the epitopes on Ki antigen are affected by preparation or detection methods.

Table 1. Reactivity of anti-Ki sera detected by DID in other immunoassays

Methods	Number of positive sera	(%)
IB		
Purified Ki	42	76.3
ELISA		
Purified Ki	40	72.3
bNAX	36	65.5
KILT	11	20.0

DID, double immunodiffusion; IB, immunoblotting; LISA, enzyme-linked immunosorbent assay; bNAX, recombinant Ki antigen; KILT, a 16-mer peptide homologous to nuclear localization in SV40 large T antigen

Table 2. Immunoreactive patterns in anti-Ki antibody detection system

Number of sera (%)	KILT (ELISA)	Purified Ki (IB)	Purified Ki (ELISA)	bNAX (ELISA)
11 (20.0)	+	+	+	+
18 (32.7)	−	+	+	+
5 (9.1)	−	+	+	−
3 (5.5)	−	+	−	+
3 (5.5)	−	−	+	+
5 (9.1)	−	+	−	−
3 (5.5)	−	−	+	−
1 (1.8)	−	−	−	+
6 (10.9)	−	−	−	−

Correlation between reactivity with KILT and results in other assays

Because sera reactive with KILT could react with all other assay systems, relationship between the reactivity in ELISA using KILT and other systems was analyzed. First, the relationship between DID titer and reactivities in the ELISA using KILT was compared (Fig. 1). There was a tendency for sera that reacted with KILT to have titers more than 1:64 by DID, but some sera that did not react with KILT had high titers in DID. In addition, two sera with low titers in DID reacted with KILT. These results suggested that reactivity to KILT was not associated with titers of anti-Ki antibodies detected in DID.

When the reactivity with KILT was compared to the ELISA using purified Ki or bNAX, the optical density (OD) in the KILT-ELISA was significantly correlated with ELISA results using purified Ki and bNAX ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 2). However, some sera that had high titers in ELISA using purified Ki and bNAX did not react with KILT. These results indicated that KILT represents one of the antigenic domains of Ki antigen, but Ki antigen has different conserved epitopes reactive with anti-Ki antibodies in lupus sera.

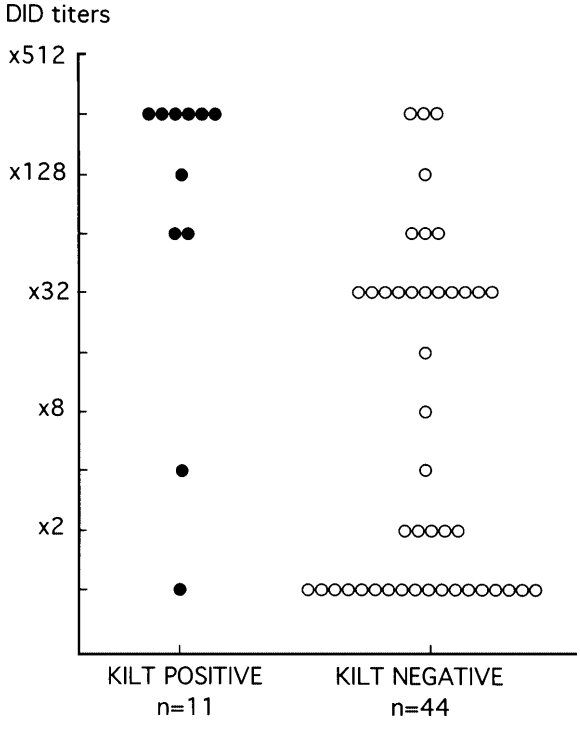

Fig. 1. Relationship between the titer by DID (double immunodiffusion) and reactivities in an enzyme-linked immunosorbent assay (ELISA) using KILT (a 16-mer peptide homologous to nuclear localization in SV40 large T antigen). Titers detected by DID were compared between KILT-positive and KILT-negative groups. Sera that did ($n = 11$) or did not react ($n = 44$) with KILT are indicated by *open* and *closed circles*, respectively

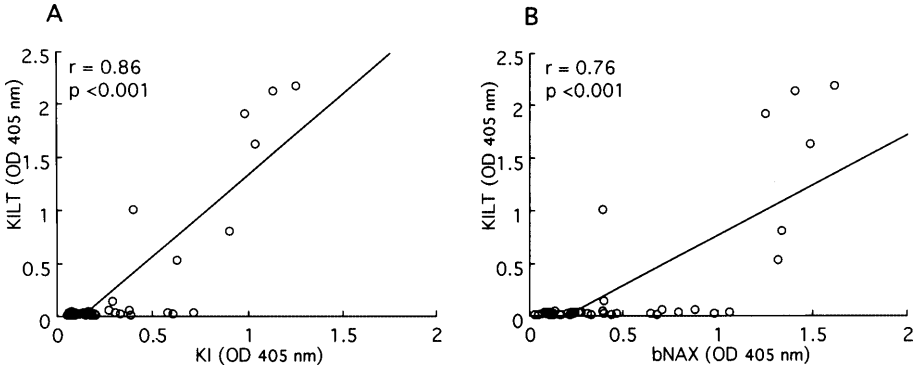

Fig. 2. Comparison of ELISA using KILT, purified Ki, and recombinant Ki (bNAX). To confirm that KILT represents antigenicity of Ki, reactivity to KILT was compared with reactivities to purified Ki and bNAX on ELISA. *x*-axis indicates reactivity to KILT and *y*-axis indicates reactivity to purified Ki (**A**) and bNAX (**B**). Optical density (OD) in the KILT ELISA was significantly correlated with ELISA results using purified Ki and bNAX ($P < 0.001$ and $P < 0.001$, respectively)

Clinical significance of anti-KILT antibodies in lupus patients

The clinical characteristics of patients with antibodies to KILT were studied using lupus patients with and without anti-KILT antibodies. As shown in Table 3, the prevalence of discoid rash and sicca complex was significantly higher in anti-KILT-positive patients than anti-Ki-negative patients (3.6% vs. 27.3%, 15.6% vs. 50.0%, respectively). Also, a tendency toward higher incidence of discoid rash and sicca complex in patients with anti-KILT was also observed compared with anti-Ki-positive patients without antibodies to KILT.

Table 3. Clinical manifestations of systemic lupus erythematosus patients with and without anti-Ki antibody

	anti-Ki negative	anti-Ki positive	
		anti-KILT	
	(n = 79)	Negative (n = 62)	Positive (n = 19)
Death	3.6	0.0	10.0
Drug allergy	27.8	17.2	10.0
Photosensitivity	48.1	62.1	40.0
Noninfectious fever	29.3	23.1	44.4
Malar rash	48.3	51.2	50.0
Discoid rash	3.6	9.8	27.3*
Alopecia	25.5	30.2	25.0
Oral ulcer	17.5	19.0	41.7
Raynoud's disease	32.8	17.1	41.7
Arthralgia	67.8	57.1	50.0
Myositis	10.3	9.8	8.3
Sicca complex	15.6	32.1	50.0*
Lymphadenomathy	14.3	7.0	0.0
Cardiac involvement	5.3	0.0	0.0
Serositis	1.8	4.8	0.0
Internal pneumonitis	3.7	2.3	0.0
Renal involvement	74.5	73.2	58.3
CNS involvement	16.1	11.6	9.1
Leukopenia	72.0	64.3	58.3
Thrombocytopenia	13.5	4.7	16.7
LE cell prep(+)	13.2	29.4	25.0
Hypergammagloblinemia	11.1	18.6	18.2
Hyperuricemia	43.1	41.9	25.0
Hyperlipidemia	68.4	65.0	40.0
RAHA(+)	21.4	13.5	36.4
RF(+)	24.4	18.2	30.0
AntiDNA(+)	86.0	74.4	72.7
AntiU1RNP(+)	37.5	23.7	30.0
AntiSm(+)	10.0	11.1	10.0
AntiSSA(+)	57.1	50.0	71.4
AntiSSB(+)	15.0	11.1	0.0
Hypocomplementemia	65.5	54.8	58.3

Values are percentages
CNS, central nervous system; LE cell prep, lupus erythematosus cell preparation; RAHA, rheumatoid arthritis hemagglutination assay; RF, rheumatoid factor
*Significant differences compared to the group of anti-Ki-negative patients ($P < 0.05$)

Discussion

The frequency of anti-Ki antibodies in lupus patients varies between 7% and 22%.[1,2,4,13,14] The incidence of anti-Ki antibodies in lupus sera was 6.7% by immunoprecipitation,[2] 11.8% by DID,[1] 18.9% by ELISA using recombinant Ki,[14] 21.8% by ELISA using purified Ki,[4] and 16% by ELISA using KILT,[13] suggesting that the frequencies of anti-Ki antibodies depend on the immunoassay system.

In our study, the immunoreactivity of anti-Ki sera (detected by DID) was tested by ELISA using KILT, purified Ki, or bNAX and by IB using purified Ki. Of the 55 sera that were positive for anti-Ki antibody by DID, only 42 (76.3%) reacted by IB using purified-Ki antigen; this was the most sensitive method (see Table 2).

Such discrepancies are well recognized not only in Ki assay systems but also in many other antigen–antibody assay systems. There are several reports showing that 10%–35% of anti-SS-A/Ro precipitin-positive sera do not react with 60-kDa SS-A/Ro by IB.[18–20] Boire et al. studied the reactivity of anti-SS-A/Ro antibodies detected by DID or immunoprecipitation in IB and found that 10% of these sera did not react with 60-kDa SS-A/Ro polypeptide, suggesting that some sera recognize conformational determinants on the antigen.[18] Tsuzaka et al. also studied the reactivities of anti-SS-A/Ro antibodies to native and denatured SS-A/Ro; they showed that 6 of 14 sera (43%) from SLE patients with anti-SS-A/Ro antibodies reactive by immunoprecipitation recognized denatured SS-A/Ro, whereas only a few sera from primary SS or SLE with SS patients with antibodies to native SS-A/Ro reacted with denatured SS-A/Ro by IB (4 of 46 and 1 of 12, respectively).[19] They concluded that antibodies to SS-A/Ro in

patients with SS targeted only the conformational epitopes on the antigen.

Buyon et al.[20] compared ELISA and IB for measurement of anti-SS-A/Ro and anti-SS-B/La antibodies and showed that only 30 of 57 positive sera for SS-A/Ro by ELISA reacted with the 60-kDa SS-A/Ro on IB. In contrast, the reactivity of anti-SS-B/La antibodies on IB was higher than the ELISA and suggested that anti-SS-B/La antibodies reactive with epitopes altered by denaturation may characterize mothers of children with neonatal lupus. These data also suggest that some anti-Ki sera which recognize conformational epitopes on the Ki antigen are reactive in DID but not in IB. In addition, the fact that some anti-Ki sera reactive by IB do not react with Ki antigen on ELISA suggests that some epitopes by Ki antigen are altered on the denaturation, similar to the SS-B/La antigen.[20]

Human autoantibodies appear to recognize functional and highly conserved domains.[21] Because these functional domains often have a specific conformational structure, autoantibodies that react with the native antigen may not react under denaturing conditions.[22] It was recently shown that Ki antigen is identical to PA28γ, a 20S proteosome activator, with an important role in antigen presentation and cell cycle regulation.[6–12] Sakamoto et al. suggested that native Ki antigen was a heptamer (MW, 224 kDa) that consisted of 32-kDa Ki polypeptides based on analysis by HPLC.[4] Tanahashi et al. reported that PA28γ forms a single homopolymer complex, a hexamer, which behaves as a positive allosteric activator binding to the 20S proteasome.[7,23] This finding is interesting because some antibodies to proliferating cell nuclear antigen (PCNA), a target antigen in lupus sera, react with the native three-dimensional form as a trimer by immunoprecipitation but do not react with monomeric PCNA on IB.[24] In addition, we have found that some anti-PCNA sera show lower reactivity to purified trimer PCNA by ELISA than DID (data not shown). It is possible that the conformation of the trimer PCNA is modified when the antigen binds to the plate. The homopolymer structure of the Ki antigen (PA28γ) may explain differences in the reactivities by DID, IB, and ELISA. In addition, the difference of the species of the antigens may also related to the reactivities to anti-Ki antibodies in lupus sera.

Although our results revealed that there are several different epitopes on the Ki antigen which can be altered by antigen preparation or detection methods, all the sera reactive with KILT were positive for Ki in all other assays. In addition, results of the ELISA using KILT were significantly correlated with ELISA results using purified Ki antigen, recombinant Ki, or bNAX, and the titer of anti-Ki antibodies by DID was not critical to obtain reactivity with KILT. Therefore, the epitope with homology to SV 40 NLS is one of the representative epitopes on the Ki antigen to obtain reactivity to anti-Ki sera, although the frequency of antibodies to this epitope is relatively low.

Clinical characteristics of lupus patients with anti-Ki antibodies have been studied by several investigators,[1,2,4,14] but it has not been reported that clinical characteristics are associated with any particular epitope on the Ki antigen. In this study, we have shown that the prevalence of discoid rash and sicca complex was significantly higher in anti-KILT-positive patients compared with anti-Ki-negative patients. It is of interest that Harmon et al. found a strong relationship of this new system with SLE accompanied by Sjögren syndrome.[3] Although other investigators have not found an association between anti-Ki antibodies and sicca complex,[1,4,14] we could show the association of anti-Ki antibodies with sicca complex that was reported as the most characteristic clinical feature in the original report when the reactivity of anti-Ki antibodies focused on the epitope homologous to SV40 T NLS. A longitudinal and multicomparison study using larger numbers of patients and analyses of reactivity to other epitopes on the Ki antigen are needed to more precisely determine the relationship between these antibodies and clinical SLE features such as discoid lupus and sicca complex.

We conclude that immunoreactivity to the amino acid sequence of Ki homologous with SV40 T NLS can be detected by different methods using native and denatured Ki antigens and is associated with sicca complex in addition to discoid rash.

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