Probing antinuclear antibody specificities by peptide phage display libraries

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Abstract

Objective

To uncover the specificities of autoantibodies to nuclear proteins (ANA) in patients with juvenile rheumatoid arthritis (JRA).

Methods

Peptide ligands for ANA were selected by panning random peptide phage display libraries on antibodies binding to HEp-2 cells. Positive phage clones were identified by the immunoscreening technique.

Results

Groups of peptides were identified, some of which share the core motifs of KTTTnPY, RVADnL/I or RnNSPL. Perinuclear and nuclear staining of HEp-2 cells were obtained with patient serum antibodies binding to the phage displaying the core peptide motifs. In contrast, no significant reactivity was seen with the antibodies binding to the wild type phage. Antibodies to the phage displaying peptides containing some of the core motifs were detected more frequently in ANA-positive as compared to ANA-negative JRA patients. Homology search with the selected core motifs revealed a significant homology with a number of human nuclear proteins and proteins from potential infectious agents that could serve as trigger in the breakdown of tolerance.

Conclusion

Panning of phage display libraries on antibodies reacting with cellular structures can lead to the identification of their specificities. Thus, the peptide epitopes reported here constitute additional information that may lead to the development of diagnostic tests and the identification of the parental antigens that initiated the B cell responses in patients with JRA.

Key words

Phage display, peptide library, antinuclear antibodies, JRA.

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Introduction

Antinuclear antibodies (ANA) are found in many patients with juvenile rheumatoid arthritis, in particular in the early onset pauciarticular form of the disease. Notably, the presence of ANA is associated with the high risk of developing iridocyclitis (1-3). Antibodies to specific nuclear proteins, such as DNA topoisomerase II, ribonucleoproteins and histones have been detected in ANA positive JRA patients (4-6). Autoantibodies to the high mobility group (HMG) nucleosomal proteins HMG1, HMG2 and HMG-7 were also detected in the sera of ANA positive patients with JRA (7, 8). In contrast, autoantibodies to DNA and to intracellular antigens such as centromere proteins, scleroderma ScL-70 and Ro are rarely seen in patients with JRA when compared to their high occurrence in patients with other rheumatic diseases (1, 9). Although some ANA parental autoantigens have been identified, very little is known about the antigenic specificities of these autoantibodies at the amino acid level to date.

As an initial approach towards the identification of the target epitopes of ANA, we have used peptide phage display libraries. This strategy can identify ligands for monoclonal antibodies, whether or not the antigens are known (10). By using autoimmune sera, we have demonstrated that common antibody specifities among the patients can be selected (11). Thus, specific B cell responses in patient sera can be traced by the use of random peptide phage display libraries (11-15). In addition, the strategy can identify linear, conformational and mimic epitopes (12, 16). To gain insight into the molecular characteristics of ANA specificities, serum antibodies from a JRA patient with homogenous pattern were captured on slides containing HEp-2 cells, and epitope mapped by a random peptide phage library using a competition assay.

Materials and methods

Patients

Serum samples were obtained from 36 ANA positive JRA patients who fulfilled the diagnostic criteria of the American Rheumatism Association diagnostic criteria (17). ANA were detected in sera diluted 1/32 by indirect immunofluorescence using HEp-2 cells as substrate. Sera from 25 ANA negative JRA patients were included as controls. ANA slides were purchased from Immuno Concepts (Sacramento, CA, USA).

Biopanning experiments

ANA 18-well slides with HEp-2 cells were incubated with 20 µl/well of a 1/32 dilution of a ANA positive serum in phosphate buffered saline (PBS) +1% bovine serum albumin (BSA) for 30 min at room temperature (RT) in a humidifying chamber. The slides were washed 4 times with PBS, rinsed with water and a drop of 20 µl of 10^{11} TU/ml of a 15 mer amplified random peptide library (18) was added to each well and incubated overnight in a humidifying chamber at 4°C. During this step phages displaying ANA specificities would compete with the nuclear antigens to bind the autoantibodies. Following incubation, all drops were collected and incubated with biotin conjugated anti-human Ig, pre-absorbed overnight with fd phage, for 2-3 hours at RT. The mixture was incubated for 10 min on a plate coated with streptavidin, the phage solution was removed and the plate was washed with PBS/0.2% Tween over a period of 1 hour at RT. Streptavidin-binding phages were eluted by incubation with 800 µl of elution buffer (HCl, 0.1M pH 2.2, 1 mg/ml BSA) for 10 min at RT. Eluted phages were removed, neutralised with Tris base and then amplified by infecting logarithmic growing *Escherichia coli* K91 cells. Amplified phages were titrated and then used for biopanning as described above. In some experiments streptavidin coated beads (Dynal, Oslo, Norway) were used. In this case the beads were captured on a magnet and phages were processed as above.

Immunoscreening

Following 3 rounds of biopanning, *E. coli* K91 cells were infected with the selected phages, plated on LB plates containing tetracycline at 50 µg/ml, incubated overnight at 37°C and then transferred to nitrocellulose membranes. The membranes were washed twice in TNT buffer (10 mM Tris HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20) for 15 min and then blocked for 30 min in TNT/20% fetal calf serum (FCS). Following block-

ing, membranes were incubated with ANA positive patient sera diluted 1/500 in TNT/20% FCS buffer for 2 hours at RT with rotation. The membranes were washed 2 times with TNT/0.1% BSA and one time with TNT/0.1% BSA/0.1% NP40 buffer and then incubated with anti-human IgG alkaline phosphatase (AP) conjugate diluted 1/5,000 for 2 hours. Following 3 washes with TNT/0.1% BSA buffer, immuno-reactive clones were detected with the addition of the BCIP/NBT premixed solution (Zymed, San Francisco, CA, USA).

Isolation of the phage particles and sequencing

Positive clones were grown and bacteria cells were pelleted by centrifugation at 8,000 rpm for 10 min. Phage particles contained in the supernatant were precipitated with polyethylene glycol 6,000/NaCl, resuspended in PBS, filtered through a 0.45 mm filter and then stored at 4°C. For DNA preparation, the phages were phenol extracted and processed as described by Sambrook *et al*., (19). Sequencing of the DNA insert-coding peptides was carried out using the Sequenase version II Kit (United States Biochemical, Cleveland, OH, USA).

ELISA experiments with the phage-displayed peptides

Serum antibodies reacting with phage-displayed peptides were detected by enzyme-linked immunosorbent assay (ELISA) as previously described (11). Briefly, 96-well microtiter plates were coated overnight with the phage particles (10^9 TU) in 100 µl PBS buffer. After blocking with 0.5% BSA in PBS buffer for 1 to 2 hours at RT and subsequent washing, the plates were incubated with sera samples (1/100 dilution in PBS/0.2% Tween 20) for 1 hr at 37°C. After additional washing, plates were incubated with anti-human Ig AP conjugate diluted 1/5,000 for 1 hr. The immune complexes were detected by adding *p*-nitrophenyl phosphate as substrate. In the case of the competition assays, various concentration of the phage displaying SKTTTMPYTPGPTAL, APVDSRTYTRVADHI, PGRNNSPLGADSVQS or the wild type phage were added to glass tubes containing 100 µl of the patient serum diluted 1/100 at 4°C overnight. After incubation, the mixtures were added to phage displaying SKTTTMPYTPGPTAL, APVDSRTYTRVADHI or PGRNNSPLGADSVQS-coated microtiter plates. Results were expressed as percentage of inhibition.

Affinity purification of the peptide binding antibodies

Phages (10^{10} TU) were biotinylated with a large molar excess of biotin for 4 hours at RT, and then free biotin molecules were removed by centrifugation using microsep microconcentrators (Filtron Technology Corporation, MA, USA). Biotin-conjugated phages were incubated overnight at 4°C with 10 µl patient serum diluted at 1/100 in serum buffer. Following incubation, the mixtures were incubated with streptavidin coated beads (Dynal, Oslo). The beads were captured on a magnet, washed 3 times with PBS buffer and then antibody-binding phages were acid eluted in 50 µl and neutralised with Tris base (1 µl). Twenty µl of the eluted antibodies were incubated with HEp-2 cells at RT for 30 min. Following washing with PBS, the cell binding antibodies were detected with FITC-conjugated anti-human IgG.

Statistics

Differences between the numbers of patients and controls with increased antibody reactivity were tested for significance with an unpaired t-test.

Results

Biopanning on HEp-2 cells

To test if the ANA peptide specificities could be determined by the peptide phage libraries, ANA in the serum of a patient with early onset polyarticular JRA and early onset iridocyclitis were captured on HEp-2 cells. After capturing, the ANA specificities were determined by the use of a 15 mer random peptide phage library using a competition assay as illustrated in Figure 1. Fol-

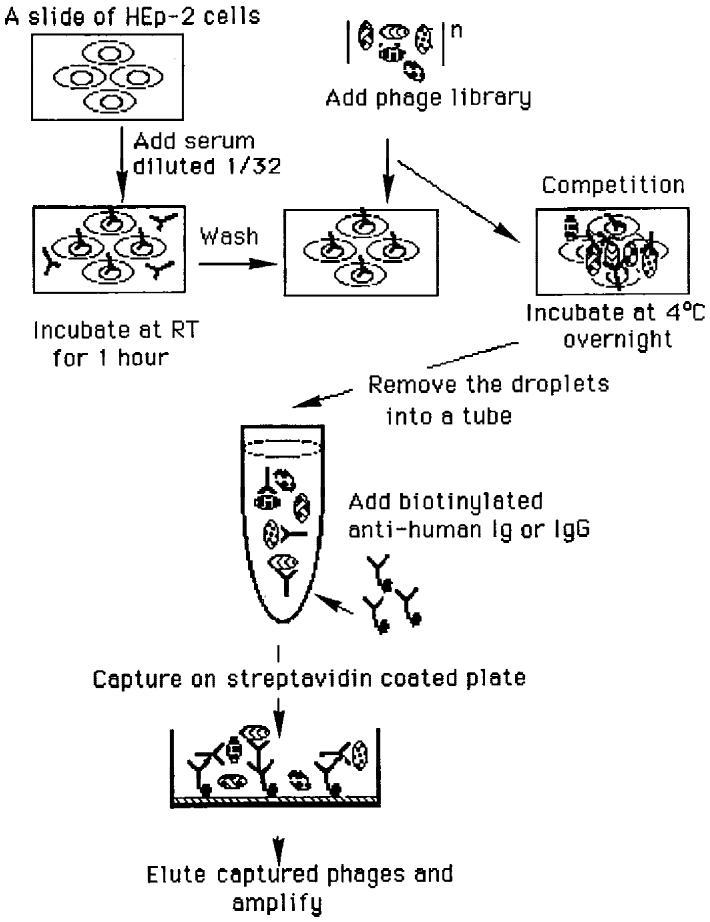

Fig. 1. Schematic illustration of the biopanning experiments.

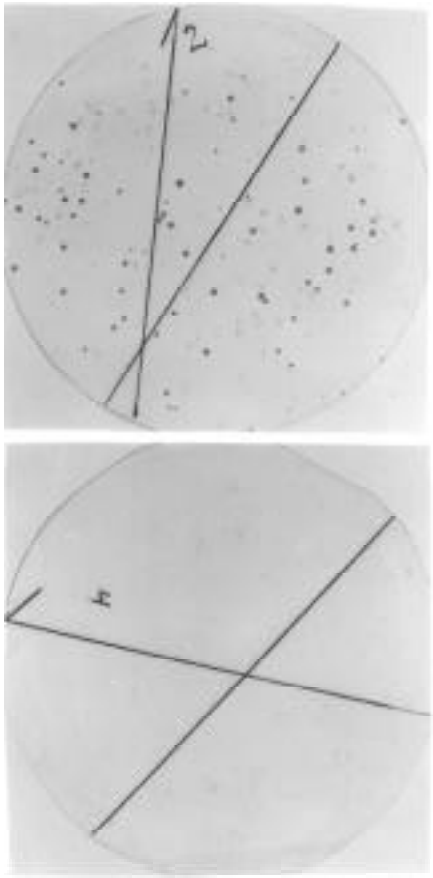

Fig. 2. Immunoscreening. (**A**) Reactivity of the patient serum with random selected phage clones. *E. coli* K91 cells were infected with the selected phages from the third round of biopanning, plated on LB plates, transferred to nitrocellulose membranes and then immuno-screened with patient serum 1/100 as described in *Materials and methods*. Some positive clones are indicated by arrows. (**B**) Reactivity of the patient serum with random non serum selected phage clones. Conditions are as in A.

Table I. Sequences of 71 phage-displayed peptides which are positive in the immunoscreening test (Fig. 2A).

A	Freq[a]	B	Freq[a]
SKTTTMPYTPGPTAL	12	KNAASERGSFTDLKS	4
SLNTAD**SKTTTFPA**R	6	MAYVPPYEMIVRELK	3
TKTSN**KTTTTP**FLGP	3	QTRGFPSVERGYLSP	3
DSARNTF**KTTTAPY**S	2	GTYKAGNQHNHVKYG	2
HKTTTSPDYTTAVGT	2	MQTPPGNLENAVETL	2
QKTTTRPYPNLQIDP	1	ATNMKMSNMHTLIGA	1
GLEGQD**TKTITF**DVS	1	PGRMTDNSGPQRFSA	1
		LTNSNAPSITVQGLE	1
APVDSRTYT**RVADHI**	2	AVSNRPRLKDTTYRL	1
SPSDAKSHV**RVADLI**	2	NPQPNISLSQRGYPQ	1
DYANPRNTP**RVADNL**	1	KSQPNQTNNYHLLRT	1
RIADTIHANNSRHIL	1	EVLTNRPIDKTKAIS	1
		MGTVLLPNGEFSIEN	1
PG**RNNSPL**GADSVQS	10	MSVPQTTYSKITELI	1
VQFKES**NRLNSPL**AL	3	MTQMPTNHAMQLILE	1
EIDGYNNQFKK**SPLP**	1		

[a]The number of times each sequence occurred in the 71 phages is given in the frequency column.

lowing 3 rounds of panning, *E. coli* cells were infected with the selected phages, plated on LB plates with tetracycline/IPTG, and then incubated overnight at 37°C. The reactivity of the phage clones with the patient serum was tested by an immunoscreening technique. This method allows the direct identification of binding phages following a transfer to nitrocellulose membrane filters (20). As shown in Figure 2A, a high proportion of the selected phages reacted with the patient serum. In contrast, no positive reactivity of the patient serum was seen with random phages which were only selected on HEp-2 cells (without serum) under the same experimental conditions (Fig. 2B). This data would indicate successful panning experiments.

The structural characteristics of the peptides displayed by the positive phage clones

To investigate the structural characteristic of the peptides reacting with the patient serum, peptide sequences derived from 71 random positive clones were determined (Table I). These peptide sequences were arranged into 2 groups, A and B. The peptides in group A showed core motifs (indicated in bold letters), whereas group B consisted of peptides with no obvious homology. None of the selected peptides were seen when the patient serum was omitted from the panning experiment (data not shown). Interestingly, the peptides containing the consensus (KTTTnP and RVADnL/I) were also selected when the panning experiments were performed with a pool sera from ANA positive JRA patients, suggesting potential common ANA specificities. In addition, it is worth noting that the RVADnL core motif was also selected from a hexamer library (data not shown).

To confirm that the selection of the phages in group A were due to the reactivity of ANA to the peptide displayed by the phages, the reactivity of phages displaying SKTTTMPYTPGPTAL, APVDSRTYTRVADHI or PGRNNSPLGADSVQS was analysed by competition experiments (Fig. 3A). In competitive ELISA, the reactivity of the patient serum to the phage-coated plates was inhibited by the phage itself, but not with the wild type phage. These results would indicate that the ANA do bind to the peptides displayed by the tested phages. To see whether the binding of patient serum to the phage-displayed peptides reflected the existence of conventional antibody-antigen reaction, titration experiments were performed. In these experiments patient serum was serially diluted and then tested for binding (Fig. 3B). Typical antibody titration curves were found.

Patient antibodies binding to the selected peptides stain HEp-2 cells.

The term “antinuclear antibodies” normally refers to a diverse group of autoantibodies which are directed against antigens normally, but not exclusively, found in the nucleus. To assess whether the selected core motifs are present in

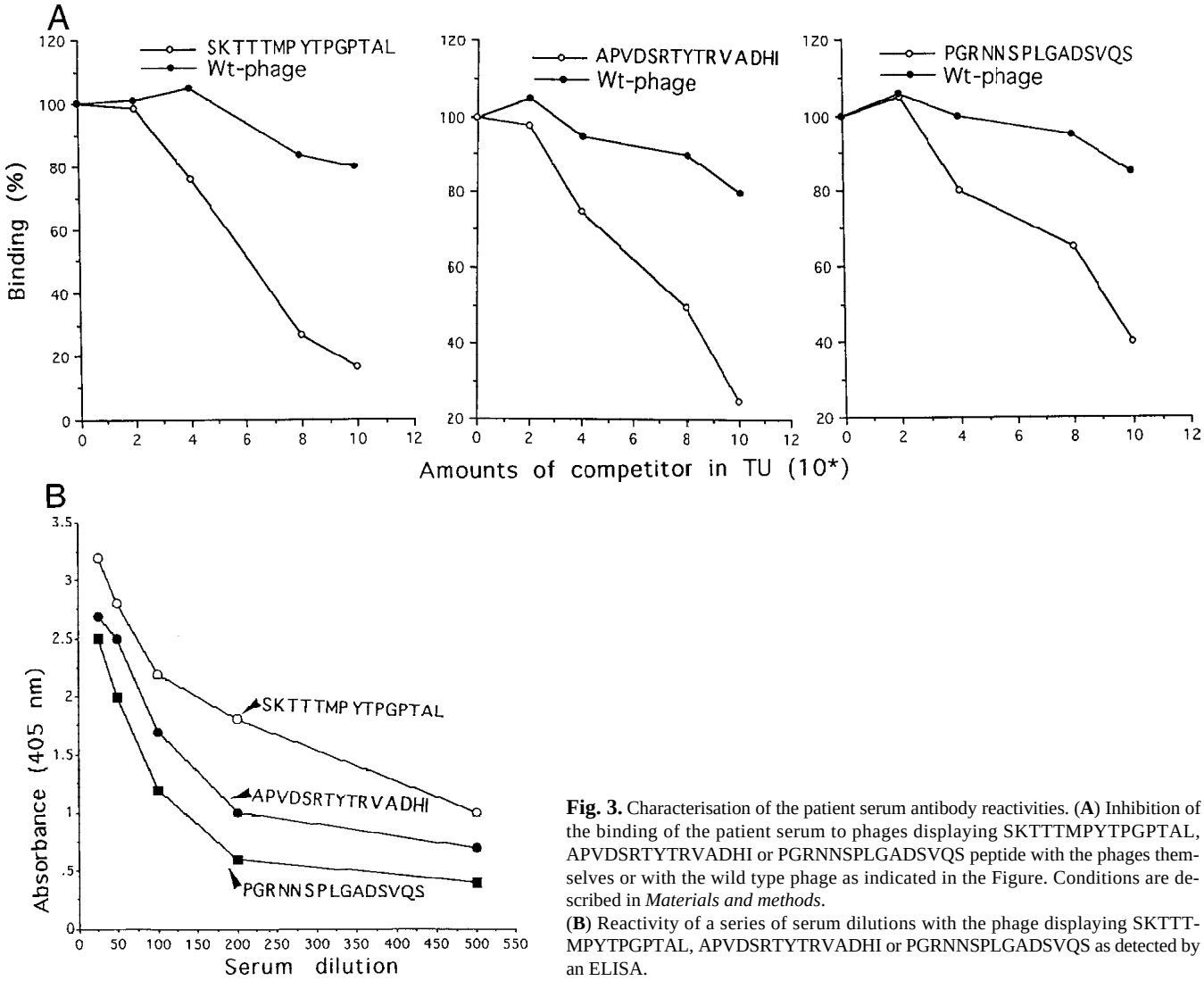

Fig. 3. Characterisation of the patient serum antibody reactivities. (**A**) Inhibition of the binding of the patient serum to phages displaying SKTTTMPYTPGPTAL, APVDSRTYTRVADHI or PGRNNSPLGADSVQS peptide with the phages themselves or with the wild type phage as indicated in the Figure. Conditions are described in *Materials and methods*.
(**B**) Reactivity of a series of serum dilutions with the phage displaying SKTTTMPYTPGPTAL, APVDSRTYTRVADHI or PGRNNSPLGADSVQS as detected by an ELISA.

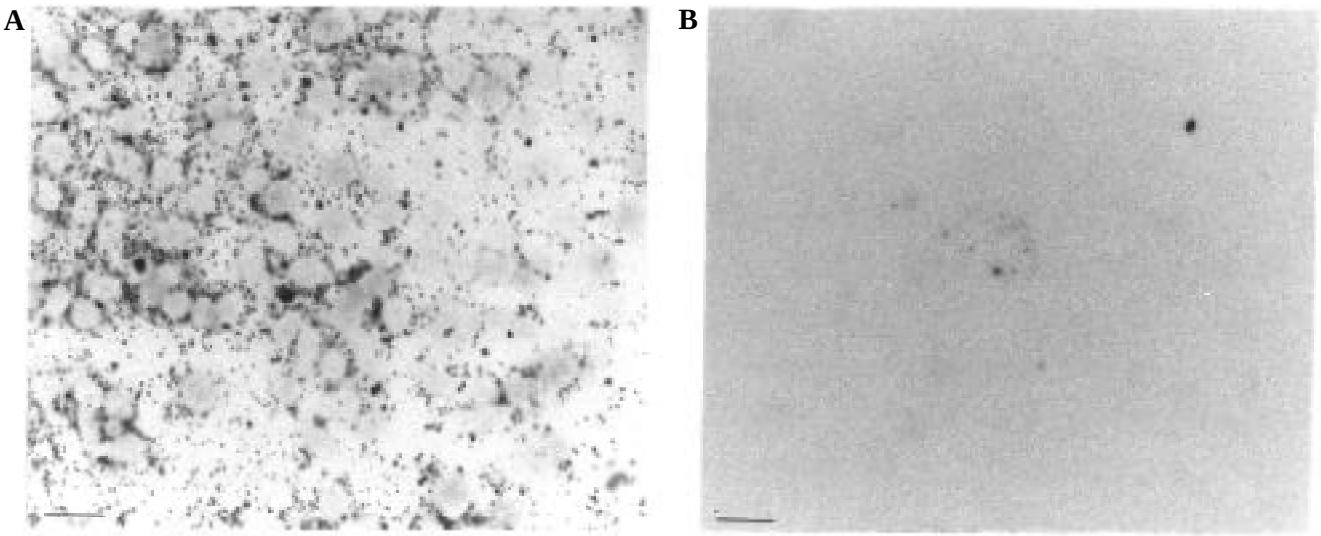

Fig. 4. Immunostaining of HEp-2 cells with the affinity purified serum autoantibodies. Serum antibodies binding to phage displaying APVDSRTYTRVADHI peptide or the wild type phage were affinity purified and used to stain HEp-2 cells as described in the *Materials and methods* section. (**A**) Cells stained with the APVDSRTYTRVADH peptide binding antibodies. (**B**) Cells stained with the wild type phage binding antibodies. Bars represent 25 μM.

HEp-2 cell proteins, the peptide-binding antibodies were affinity purified and then used to stain HEp-2 cells (Fig. 4). A distinct punctuate fluorescence of the nuclear membrane was obtained with serum antibodies binding to the phage displaying the APVDSRTYTRVADHI peptide (Fig. 4A). Some cells showed nuclear particulates, while others showed fluorecence at the outer edge of the nuclear membrane. A weak staining of the nucleus was obtained with the antibodies binding to the phage displaying the SKTTTMPYTPGPTAL or PGRNNSPLGADSVQS peptide (data not shown). In contrast, no significant reactivity was seen with the antibodies binding to the wild type phage (Fig. 4B). Taken together, these results would indicate that the selected epitopes are more likely to be present in perinuclear and nuclear proteins. During these experiments FITC-conjugated anti-human IgG antibodies were used for the detection. As can be seen, a strong staining was obtained with the antibodies binding to the phage displaying the APVDSRTYTRVADHI peptide. Thus, suggesting that peptides for high affinity IgG antibodies can be selected.

The humoral response of ANA positive and negative patients to some of the peptides containing the core motifs

To investigate the biological relevance of the selected peptides, we have as a first step investigated the humoral reactivity of ANA positive and negative patients to the phage displaying APVDSRTYTRVADHI, SKTTTMPYTPGPTAL, or PGRNNSPLGADSVQS peptide. The relative reactivity of each patient serum to the phage-displayed peptides was obtained by subtracting the absorbency of the serum to a phage-displaying a random peptide (Fig. 5). There were increased APVDSRTYTRVADHI peptide specific antibodies in ANA positive patients (mean=0.63 OD units) compared to ANA negative patients (mean = 0.3 OD units) ($p < 0.0024$). There was also a significant difference in antibody reactivity to the PGRNNSPLGADSVQS peptide in ANA positive patients (mean = 0.51 OD units) when compared to ANA negative patients (mean = 0.31 OD units) ($p < 0.005$). The mean reactivity of ANA positive and negative patients for the SKTTTMPYTPGPTAL peptide was found to be 0.54 and 0.43, respectively. Although the difference is not statistically significant ($p < 0.324$), 7 out of 36 ANA positive patients (19%) reacted strongly with the phage displaying the SKTTTMPYTPGPTAL peptide. Patients with pauciarticular onset JRA and polyarticular onset JRA recognised the tested peptides with similar frequency. Four out of 5 ANA positive JRA patients with iridocyclitis reacted with the peptide sharing the core motifs. Further studies on larger patient groups with iridocyclitis are needed to confirm the clinical relevance of our observation.

Homology between of the selected peptide motifs and proteins found in SwissProt databank

In order to identify potential putative autoantigens, a SwissProt databank search was performed with the peptide core motifs (Table II). The motif KTTTMPY is present in some nuclear proteins such as the centromere DNA-binding protein complex CBF3 subunit and the DNA binding protein RFX3 (21, 22). Homology search with the RVADHI and GRNNSPL motif gave homology with the major centromere autoantigen B and the transcription factor AP1. As shown in Table II, the homology search also led to the identification of a number of human pathogens that could act as putative trigger of B cell activation. This observation would support the notion that some pathogenic microorganisms may contribute to autoimmunity through "molecular mimicry" as discussed elsewhere (23).

Discussion

In this investigation, we have demonstrated that the panning of peptide phage libraries on ANA reacting with HEp-2 cells can lead to the identification of their peptide specificities. Although some of the selected peptides need to be further investigated, the demonstration of a significant humoral reactivity in ANA positive patients when compared to ANA negative patients towards the selected peptides is encouraging.

The ANA selected peptide motifs were

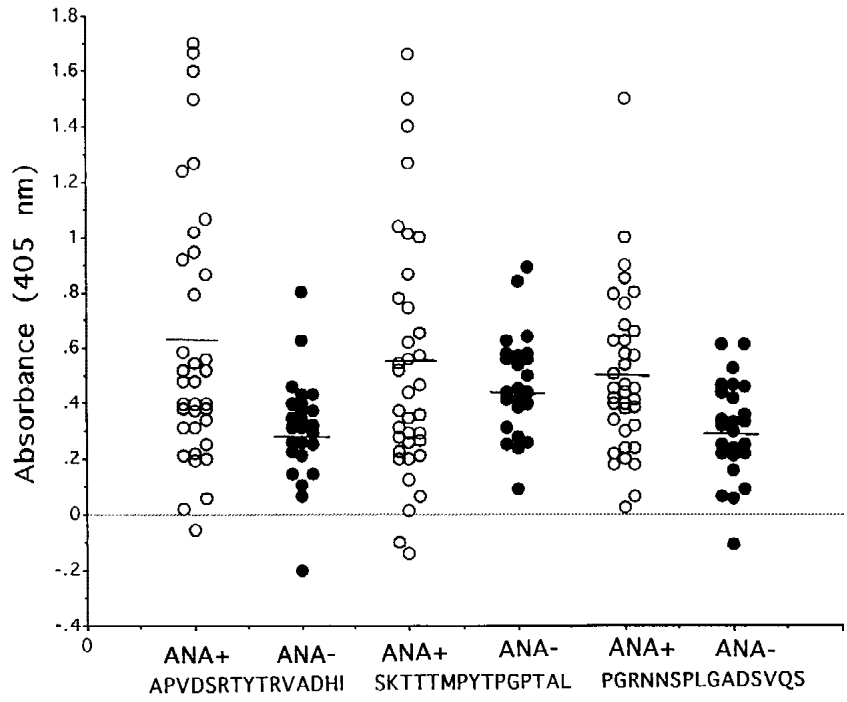

Fig. 5. Reactivity of 36 ANA positive and 25 ANA negative patient sera to phages displaying APVDSRTYTRVADHI, SKTTTMPYTPGPTAL or PGRNNSPLGADSVQS. JRA sera were tested for the phages displaying the peptides by ELISA as described previously (11). The reactivity of each serum was obtained by subtracting its absorbency to a phage displaying a random peptide (KDLPKENRLYAPIST).

Table II. Examples of matches between the peptide core motifs and proteins found in the SwissProt databank.

KTTTnPY	Selected motif	RVADHI	Selected motif	GRNNSPL	Selected motif
.::: ::		::::::		::.::::	
RTTTYPY	DNA-binding RFX3 protein*	RVADHI	ORF8 protein (Archae)	GRSNSPL	Transcription factor (AP-1)*
KTTTnPY	Selected motif	RVADHI	Selected motif	GRNNSPL	Selected motif
::.: ::		:::.::		::.::::	
KTPTDPY	Major capsid protein (HPV)	RVAAHI	APO-2 ligand*	GRGNSPL	Fungal carboxylase
KTTTnPY	Selected motif	RVADHI	Selected motif	GRNNSPL	Selected motif
::: ::		::..::		::.:.::	
ATTTYPY	Centromer DNA-binding complex CBF3*	RVQSHI	Major centromer autoantigen B*	GRVNPPL	Flagellar protein Yersinia E.
KTTTnPY	Selected motif	RVADHI	Selected motif	GRNNSPL	Selected motif
::. ::		::.:::		::...::	
ITTKEPY	Glycoprotein B (HSV)	RVQDLI	DNA helicase (VZV)	GRGAPPL	Capsid protein B (HSV)
				GRNNSPL	Selected motif
				.:...::	
				SRAGAPL	Transcription factor GATA-6*

· = Conserved substitution, * Human Proteins

found in, for example, some human nuclear proteins and proteins from infectious agents. Such agents might contribute to the breakdown of tolerance, with the subsequent generation of autoantibodies to structural proteins. Since the strategy used in this study identifies linear, conformational and mimic epitopes, the identified candidate autoantigens found in SwissProt databank should be considered as promising leads that need further investigation. However, regardless of the nature of the autoantigens, the data presented here prove that panning of peptide phage libraries on autoantibodies reacting with cellular structures can select specific antibody ligands. More important, some of the investigated peptides seem to be present in HEp-2 cells (Fig. 4A). Thus, the new panning protocol described in this paper should be useful for the characterisation of the specificities of autoantibodies reacting with cellular structures. Notably, the present strategy relies upon the competition experiments which are expected to be affected by the number of each individual peptide sequence within the amplified library. If such a number is low, the protocol may lead to the selection of ligand for low affinity antibodies. In principle, the powerful enrichment technique based upon successive rounds of phage growth and selection would also allow the detection of ligands for high affinity antibodies. As shown in Figure 5, some patients showed a strong humoral reactivity to the tested peptides, while others showed a moderate reactivity. These results would suggest that epitopes for high and low affinity antibodies have been selected. The strong immunofluorescence pattern obtained with the antibodies binding to the APVDSRTYTRVADHI peptide (Fig. 4A) would further support the notion that epitopes for high affinity IgG antibodies were selected.

The identification of serological autoantibodies reacting with cellular antigens located in the nucleus or the cytoplasm can be helpful in the classification of patients with rheumatic diseases. In this respect, antibodies to DNA were found in the circulation of the majority of patients with SLE and seem to be specific for the disease (24). Therefore the identification of ANA specificities at the amino acid level may lead to the identification of peptide epitopes that can discriminate between connective tissue diseases with ANA. In this respect, panning of the peptide phage libraries with ANA from patients with SLE identified peptides that are mostly recognized by SLE patients when compared to patients with JRA or RA (unpublished results).

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