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New autoantigens in rheumatoid arthritis (RA): screening 8268 protein arrays with sera from patients with RA

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ABSTRACT

Objective: To identify new IgG autoantibodies in sera from patients with rheumatoid arthritis (RA).

Methods: We tested serum samples from 19 patients with RA with given human leukocyte antigen (HLA)-DR genotypes, from 7 patients with spondylarthropathy, 2 patients with lupus, 4 patients with systemic sclerosis and 10 healthy individuals on 8268 human protein arrays.

Results: We identified four antigens (peptidyl arginine deiminase 4 (PAD4), protein kinase C β 1 (PKC β 1), phosphatidylinositol 4 phosphate 5 kinase type II γ (PIP4K2C) and v raf murine sarcoma viral oncogene homologue B1 catalytic domain (BRAF)) that were recognised almost uniquely by sera from patients with RA on protein arrays. Using purified proteins, we confirmed that PAD4 and BRAF are recognised almost uniquely by patients with RA.

Conclusion: We identified PAD4 and BRAF as RA specific autoantigens.

Two elements are central to the development of rheumatoid arthritis (RA): human leukocyte antigen (HLA)-DR alleles and autoantibodies. HLA-DR alleles can be susceptible (such as DRB1*0401, *0404, *0101, *0102, *1001), neutral (like HLA-DR15) or protective (like HLA-DRB1*07, *08, *0402) towards the development of RA.^{1,2} The maximal risk to develop RA is observed in individuals expressing two susceptibility alleles. The most critical autoantibodies in RA are directed at citrullin residues on proteins such as fibrin, filaggrin and vimentin.³ Non-citrullinated proteins can also be autoantibody targets in RA.⁴

To identify new IgG autoantibodies in RA, we selected sera from patients with RA to screen protein arrays.

PATIENTS AND METHODS

Sera from patients with RA for protein microarray

We studied 19 patients with RA from the rheumatology unit at La Conception Hospital in Marseille, France. All patients with RA fulfilled the American College of Rheumatology (ACR) 1987 revised criteria. Sera were selected from three patients expressing HLA-DRB1*0401 and HLA-DRB1*0404, four patients homozygous for HLA-DRB1*0401, three patients homozygous for HLA-DRB1*0404, two patients expressing HLA-DRB1*0401 and HLA-DR7, two patients expressing HLA-DRB1*0404 and HLA-DR7, three patients homozygous for HLA-DR7 and two patients expressing HLA-DR7/DR9.

Sera from controls for protein microarray

Controls were seven patients with spondylarthropathy (AS) and two patients with systemic lupus erythematosus (SLE) from the rheumatology unit at La Conception Hospital, and four patients with systemic sclerosis (SSc) from a national cohort. A total of 10 healthy controls were recruited at UMR639, Marseille, France. All participants gave informed consent.

Protein microarray

We used Invitrogen ProtoArrays that contain 8268 human proteins (protein content list 4.0).^{5,6} All proteins have been expressed as GST fusion proteins, purified under native conditions and spotted in duplicate on nitrocellulose-coated glass slides. (<http://orf.invitrogen.com>).

Serum profiling assays on protein arrays

Slides were blocked with 1% bovine serum albumin (BSA)/phosphate buffered saline/Tween (PBST). Serum samples were added to arrays. After washing, anti-human IgG conjugated to Alexa Fluor 647 dye was added. Arrays were washed and dried (Partnership, Evry, France).

Data acquisition

Arrays were scanned with a GenePix 4000B Fluorescent Scanner. Data were acquired with GenePix Pro software and processed using ProtoArray Prospector 2.0 (Invitrogen, Carlsbad, California, USA).

Data analysis

A panel of values was calculated for each protein array including the Z score, the Chebyshev Inequality Precision (CIP) value and the coefficient of variation (CV) value. The Z score is the signal value for a given spot minus the mean signal value from all the human proteins on the array, divided by the standard deviation of the signal values for all the human proteins on the array. The CIP value evaluates the signal strength for a given spot relative to all the negative control spots and calculates the probability that the observed signal may come from the negative control distribution. The CV evaluates the similarity between duplicates. A Z score > 3.0, a CIP value < 0.05 and a CV < 0.5 define a positive spot.

Patients with RA and controls for autoantigen validation

Patients with RA and patients with AS were chosen from the Rheumatology Ward at Hopital La

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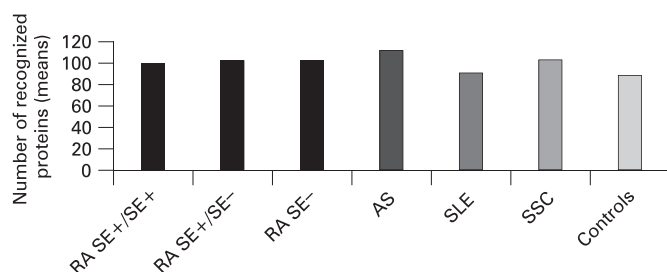


Figure 1 The mean number of proteins recognised by patients with rheumatoid arthritis (RA) expressing two, one or no RA-associated human leukocyte antigen (HLA)-DR alleles, by patients with spondylarthropathy (AS), by patients with systemic lupus erythematosus (SLE), by patients with systemic sclerosis (SSc) and by healthy controls.

Conception, Marseille. Volunteers from UMR639 and the Marseille Blood Transfusion Center staff served as normal controls.

In-house protein binding assay

The *v raf* murine sarcoma viral oncogene homologue B1 catalytic domain (BRAF) catalytic domain and PKC β 1 (protein kinase C β 1) were spotted on nitrocellulose-membrane. After blocking, serum samples were added. After washing, a fluorescently labelled anti-human IgG antibody was added. Positive sera were defined by a value more than twice protein control.

ELISA

Plates were coated with 0.1 μ g/well protein diluted in PBS, pH7.4. Plates were blocked with PBS containing 5% milk. Sera diluted to 1:100 were incubated. After washing with 0.1% Tween 20, peroxidase-conjugated anti-human IgG (Sigma, France) was added. Optical density (OD) was read at 405 nm. Background OD was obtained by adding each serum to a well without protein. Positive sera were defined by an OD value more than twice background OD.⁴

Western blotting

Glutathione S-transferase (GST)-tagged BRAF catalytic domain was separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene fluoride (PVDF) membranes. Blots were incubated with sera of patients and controls followed by peroxidase-conjugated anti-human IgG and read by chemiluminescence (Roche Diagnostics, Meylan, France). GST-tagged BRAF catalytic domain detected by anti-GST antibody defined the positive signal.

Statistical analysis

p Values were calculated using the χ^2 test.

RESULTS

Autoantibody pattern associated with patients with RA

Sera from 19 patients with RA and 23 controls were used to probe arrays containing 8268 human proteins. The presence of autoantibodies bound to each protein was detected by anti-human IgG antibody.

The number of proteins recognised by each patient and control was very similar (fig 1). On average, RA sera bound 101 proteins, AS sera bound 112 proteins, SLE sera bound 91 proteins, SSc sera bound 103 proteins and healthy control sera bound 89 proteins ($p=0.943$, 19 patients with RA vs 23 controls).

Anti-cyclic citrullinated peptide (CCP) positive sera recognised on average 99 proteins. Anti-CCP negative sera recognised on average 109 proteins ($p=0.530$).

Sera from patients with disease duration less than 10 years recognised on average 101 proteins. Sera from patients with disease duration more than 10 years recognised on average 102 proteins ($p=1$).

There was no difference in the number of recognised proteins between patients with RA expressing two, one or no RA associated HLA-DR alleles ($p=1$, SE positive patients expressing at least one SE vs patients that were SE negative).

Identification of specific autoantibodies associated with RA

The number of proteins on the 8268 protein array which are recognised by at least 1, 2, 3...19 patients with RA is shown in fig 2.

Among proteins recognised by many patients with RA, we found three that were not recognised by any control serum. These proteins are peptidyl arginine deiminase 4 (PAD4), PKC β 1 and phosphatidylinositol 4 phosphate 5 kinase type II γ (PIP4K2C).

Indeed, 9/19 patients with RA were positive for PAD4 vs 0/23 controls ($p=0$). Similarly, 6/19 patients with RA were positive for PKC β 1 vs 0/23 controls ($p=0.014$). Finally, 5/19 patients with RA were positive for PIP4K2C vs 0/23 controls ($p=0.032$).

We also identified another potential marker for RA: BRAF. Indeed, 9/19 sera from patients with RA were positive for BRAF vs 0/13 disease controls and only 1/10 healthy individuals ($p=0.004$, 19 patients with RA vs 23 controls).

Autoantigen validation

To confirm the validity of our protein array detection, we used the same proteins in different assays.

We confirmed that PAD4 (by ELISA) and BRAF (by western blot and "in house" protein binding assay) are specific

Table 1 Frequency of positive sera to peptidyl arginine deiminase 4 (PAD4), protein kinase C β 1 (PKC β 1), phosphatidylinositol 4 phosphate 5 kinase type II γ (PIP4K2C) and *v raf* murine sarcoma viral oncogene homologue B1 catalytic domain (BRAF)

	Patients with RA	Patients with AS	Healthy subjects	p Value	Method of validation
PAD4	34/116	0/33	2/60	0	ELISA
BRAF	9/19	0/17	0/17	0	Western blot
	13/50	2/30	3/30	0.025	In house protein binding assay
PKC β 1	34/50	17/30	20/30	0.623	In house protein binding assay
PIP4K2C	9/49	6/33	4/40	0.658	ELISA

p Value vs control groups (patients with AS and healthy subjects). AS, spondylarthropathy; RA, rheumatoid arthritis.

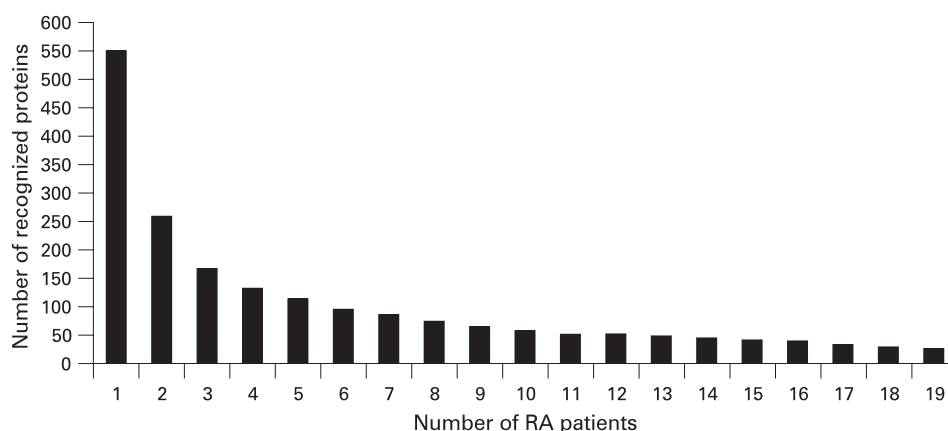


Figure 2 The total number of proteins recognised by at least one patient with rheumatoid arthritis (RA), at least two patients with RA, at least three patients with RA, and so on.

autoantigens in RA but not PIP4K2C (by ELISA) and PKC β 1 (by “in house” protein binding assay) (table 1). Anti-PAD4 and anti-BRAF antibodies were not higher in patients expressing the shared epitope compared to patients that were SE negative (data not shown).

DISCUSSION

By screening protein arrays, we found that on average, sera from patients and controls recognised 100 of the 8268 human proteins expressed on the array. Most of the proteins recognised by sera from patients with RA were also recognised by sera from controls. We found and validated two major autoantigens recognised by patients with RA: BRAF and PAD4.

BRAF encodes a 766 amino acid serine threonine kinase that contains a Raf-like Ras-binding domain, a protein kinase C-conserved region 1 domain and a serine threonine protein kinase catalytic domain. Sera from patients with RA recognised the BRAF catalytic domain, which encompasses amino acids 416 to 766. This stretch displays polymorphism at position 599 where the usual valine residue can be replaced by a glutamate residue, a mutation observed in human cancers and associated with increased kinase activity.⁷ BRAF specific antibodies recognising BRAF and BRAF V599E are detected in the sera of 9% of melanoma patients and 2.5% of controls.⁸ In patients with RA, the BRAF catalytic domain can be used as a diagnosis marker in patients that were CCP negative. Indeed, 33% of patients with RA positive for BRAF catalytic domain were CCP negative.

BRAF is involved in the transduction of mitogenic signals from the cell membrane to the nucleus.⁹ BRAF regulates the mitogen-activated protein kinase (MAPK) signalling pathway, known to be implicated in the production of proinflammatory cytokines.^{10–11} Thus, anti-BRAF antibodies could activate the MAP kinase pathway through BRAF, leading to cytokine production and joint inflammation.

Autoantibodies against PAD4 have already been described in RA.¹² PAD4 converts arginine residues into citrulline. Thus, PAD4 is not only involved in the generation of citrullinated epitopes, it is in itself a target for RA specific antibodies.^{13–15} This might just indicate that PAD4 self-citrullinates and is thus recognised by RA specific anti-citrulline antibodies. Indeed, we found a positive correlation between anti-PAD4 and anti-CCP antibodies. However, 19% of patients that were anti-CCP negative with RA are positive for anti-PAD4. Furthermore, we

checked that the PAD4 protein used in our assay is not citrullinated, using the Senshu antibody (citrulline detection kit, Upstate Temecula, California, USA; data not shown).

Alternatively, finding simultaneously antibodies to PAD4 and citrulline in most patients with RA may indicate that the event that initiates anti-citrullin immunisation may involve a complex associating PAD4 and a target protein undergoing citrullination. This event might be citrullination of fibrinogen deposits by PAD4.

Finally, it is also possible that autoantibodies directed to PAD4 stimulate their target, thus increasing the load in citrullinated protein, which in its turn may trigger the development of anti-citrullin immunisation.

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Competing interests: None declared.

Ethics approval: Ethics approval was obtained from the Ethics Committee, Marseille, France.

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