Peptidyl arginine deiminase immunization induces anticitrullinated protein antibodies in mice with particular MHC types

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Autoantibodies to citrullinated proteins (ACPAs) are present in two-thirds of patients with rheumatoid arthritis (RA). ACPAs are produced in the absence of identified T cell responses for each citrullinated protein. Peptidyl arginine deiminase 4 (PAD4), which binds proteins and citrullinates them, is the target of autoantibodies in early RA. This suggests a model for the emergence of ACPAs in the absence of detectable T cells specific for citrullinated antigens: ACPAs could arise because PADS are recognized by T cells, which help the production of autoantibodies to proteins bound by PADS, according to a “hapten/carrier” model. Here, we tested this model in normal mice. C3H are healthy mice whose IEβk chain is highly homologous to the β1 chain HLA-DRB1*04:01, the allele most strongly associated with RA in humans. C3H mice immunized with PADS developed antibodies and T cells to PAD and IgG antibodies to citrullinated fibrinogen peptides, in the absence of a T cell response to fibrinogen. To analyze the MHC background effect on hapten/carrier immunization, we immunized DBA/2 mice (whose IEβd chain is similar to that of HLA-DRB1*04:02, an HLA-DR4 subtype not associated with RA). DBA/2 mice failed to develop antibodies to citrullinated fibrinogen peptides. Thus, T cell immunization to PAD proteins may trigger ACPAs through a hapten/carrier mechanism. This may constitute the basis for a new mouse model of ACPA-positive RA.

ACPAs are unknown. Here, we propose a model to explain the presence and development of autoantibodies to citrullinated proteins (ACPAs) are highly associated with rheumatoid arthritis (RA). The mechanisms leading to the production of ACPAs are unknown. Here, we propose a model to explain the emergence of anticitrullinated protein autoantibodies in RA. Indeed, we could trigger the development of anticitrullinated fibrinogen autoantibodies in normal mice by immunization with peptidyl arginine deiminase (PAD), most likely through a hapten/carrier mechanism in which the carrier is the PAD enzyme that performs citrullination and the hapten is any protein bearing citrullination (hence bound) by PAD. Our results allow us to understand the birth of anticitrullin autoimmunity.

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Significance

The authors declare no conflict of interest.

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In C3H mice immunized with murine PAD2, the average background OD was 0.06, and the average test OD was 1 after the first immunization, 1.4 after the second immunization, 1.8 after the third immunization, and 1.7 after the fourth immunization (Fig. 2B).

In C3H mice immunized with human PAD2, the average background OD was 0.06, and the average test OD was 0.8 after the first immunization, 0.9 after the second immunization, 1.5 after the third immunization, and 1.6 after the fourth immunization (Fig. 2B).
In C3H mice immunized with human PAD4, the average background OD was 0.07, and the average test OD was 1.1 after the first immunization, 1.8 after the second immunization, 1.6 after the third immunization, and 1.3 after the fourth immunization (Fig. 2B).

The presence of anti-PAD antibodies was confirmed by titration assays for 12 C3H mice immunized with PADs (Fig. S1).

**T Cell Responses to PADs in C3H Mice Immunized with PADs.** Spleen and lymph nodes were obtained 65 d after immunizations with murine PAD2 or human PAD2 or human PAD4 or PBS. T cell proliferation to PADs, native or citrullinated fibrinogen, or phytohemagglutinin (PHA) was evaluated by bromodeoxyuridine incorporation. Positive responses were defined by OD values higher than twice the background OD obtained for cells cultured without antigen.

T cell responses to PADs were detected in 24/28 C3H mice immunized with PADs versus 4/20 C3H mice immunized with PBS (Fisher’s test, \( P = 6 \times 10^{-6} \)) (Fig. 3).

Indeed, 11/13 C3H mice immunized with murine PAD2, 4/4 C3H mice immunized with human PAD2, and 9/11 C3H mice immunized with human PAD4 developed immunizing PAD-specific T cell responses (Fig. 3B). No T cell response was detected for native or citrullinated fibrinogen in mice immunized with PADs or PBS. Among the four mice immunized with PBS who responded to PADs, two recognized murine PAD2, one recognized human PAD2, and one recognized murine PAD2, human PAD2, and human PAD4 (Fig. 3).

**Antibodies to Citrullinated Peptides from Human Fibrinogen in C3H Mice Immunized with PADs.** To test whether anticitrullinated peptide antibodies were produced in C3H mice after PAD immunization, we screened sera from 28 mice immunized with murine PAD2 or human PAD2 or human PAD4 and sera from 20 mice immunized with PBS with 46 citrullinated peptides from the alpha, beta, and gamma chain of human fibrinogen. These
peptides were 17 15-mers from the alpha chain of human fibrinogen (locus NP_000499) with 50–100% identity with their murine counterparts, 28 15-mers from the beta chain of human fibrinogen (locus NP_005132) with 70–100% identity with their murine counterparts, and one 15-mer from the gamma chain of human fibrinogen (locus NP_068656.2) with 100% identity with its murine counterpart.

We identified four major citrullinated epitopes: peptides 4C, 5C, 6C, and 8C (Fig. 4). These peptides encompassing residues 420–479 of the beta chain of human fibrinogen were recognized by 8/28 sera from mice immunized with PADs versus 0/20 sera from mice immunized by PBS (Fisher’s test, \(P = 0.014\)).

The presence of anticitrullinated peptide antibodies was confirmed by titration assays for six C3H mice immunized with PADs (Figs. S2–S4).

To check for the presence of citrullin residue-specific antibodies, we then screened the same sera from 28 mice immunized with murine PAD2 or human PAD2 or human PAD4 and from 20 mice immunized with PBS, with peptides 4, 5, 6, and 8 under their native and citrullinated form (Fig. 5).

IgG responses to native peptides were detected in 10/28 mice immunized with PADs versus 2/20 mice immunized with PBS (Fisher’s test, \(P = 0.05\)) (Fig. 5).

Citrullinated peptide-specific IgG responses were detected in 6/28 mice immunized with PADs versus 0/20 mice immunized with PBS (Fisher’s test, \(P = 0.03\)) (Fig. 5).

Peptide 8C was the most interesting because it was only recognized under its citrullinated form by the sera from 4/28 mice immunized with murine PAD2 or human PAD2 or human PAD4 (Fig. 5).

Influence of MHC Background on Anticitrullinated Peptide Immunization.

To test whether polymorphism of the I\(\text{E}\)\(\beta\) chain influences the development of T cell responses to PAD and antibody responses to citrullinated peptides from fibrinogen, we immunized mice expressing an I\(\text{E}\)\(\beta\) allele other than I\(\text{E}\)\(\beta\)k with PAD.

We chose DBA2 mice whose I\(\text{E}\)\(\beta\) chain is similar to that of non-RA–associated HLA-DRB1*0402 (16) (Fig. S5). IgG responses to PADs were analyzed by ELISA. Sera were diluted at 1/40. DBA/2 mice were immunized with murine PAD2 or human PAD4 proteins, which had given the highest anti-PAD antibody titers in C3H mice after PAD immunization.

We detected IgG responses to PADs in 10/10 DBA/2 mice immunized with murine PAD2 and human PAD4 and 0/9 DBA/2 mice immunized with PBS (Fisher’s test, \(P = 1 \times 10^{-5}\)) (Fig. 6).

In DBA/2 mice immunized with PBS, the average background OD was 0.06, and the average test OD was 0.06 when adding each serum to a well with murine PAD2 or human PAD4 (Fig. 6).

Citrullinated peptide-specific IgG responses were detected in 6/28 mice immunized with PADs versus 0/20 mice immunized with PBS (Fisher’s test, \(P = 0.03\)) (Fig. 5).
In DBA/2 mice immunized with murine PAD2, the average background OD was 0.05, and the average test OD was 0.27 after the first immunization, 0.77 after the second immunization, 0.7 after the third immunization, and 0.8 after the fourth immunization (Fig. 6B).

In DBA/2 mice immunized with human PAD4, the background OD was 0.06, and the average test OD was 0.44 after the first immunization, 0.47 after the second immunization, 0.88 after the third immunization, and 0.93 after the fourth immunization (Fig. 6B).

The presence of anti-PAD antibodies was confirmed by titration assays for 10 DBA/2 mice immunized with PADs (Fig. S6).

T cell responses to PADs were detected in 8/10 DBA/2 mice immunized with murine PAD2 and human PAD4 and 3/9 DBA/2 mice immunized with PBS (Fisher’s test, \( P = 0.07 \)) (Fig. 7).

Indeed, in DBA/2 mice immunized with PBS, the average background OD was 0.15 in cells cultured without antigen, and the average test ODs were 0.2 in cells cultured with murine PAD2 or human PAD4, 0.14 in cells cultured with native or citrullinated fibrinogen, and 0.35 in cells cultured with PHA (Fig. 7).

In DBA/2 mice immunized with murine PAD2, the average background OD was 0.18 in cells cultured without antigen, and the test ODs were 0.53 obtained in cells cultured with human PAD4, 0.18 in cells cultured with human PAD2, 0.17 in cells cultured with native or citrullinated fibrinogen, and 0.6 in cells cultured with PHA (Fig. 7).

In DBA/2 mice immunized with human PAD4, the average background OD was 0.18 in cells cultured without antigen, and the test OD was 0.57 in cells cultured with murine PAD2, 0.17 in cells cultured with native or citrullinated fibrinogen, and 0.61 in cells cultured with PHA (Fig. 7).

T cell responses (as indicated by test ODs) to murine PAD2 were lower in DBA/2 mice immunized with murine PAD2 than in C3H mice immunized with murine PAD2. T cell responses (as indicated by test ODs) to human PAD4 were similar in DBA/2 and C3H mice immunized with human PAD4. No T cell responses were detected in native or citrullinated fibrinogen in DBA/2 mice immunized with PADs or PBS. T cell responses to PHA (as indicated by test ODs) were lower in DBA/2 than in C3H mice. DBA/2 mice are known to have the lowest PHA responsiveness of all classical mouse strains, whereas C3H are among the low responders (17).

Finally, we screened sera from mice immunized with PBS or murine PAD2 or human PAD4 on the 46 citrullinated peptides from the alpha, beta, and gamma chain of human fibrinogen with 50–100% homology with their murine counterparts. No IgG response to citrullinated fibrinogen peptides was detected in any of the DBA/2 mice immunized with PADs or PBS (Fig. 8). The lack of anticitrullinated peptide antibodies was confirmed by titration assays for seven DBA/2 mice immunized with PADs (Figs. S2–S4).

Discussion

In this study, we tested whether immunization to PAD proteins triggers ACPAs through a hapten/carrier mechanism in which the carrier is PAD, which performs citrullination, and the hapten is any protein being citrullinated by PAD.

Indeed, the sera of two-thirds of patients with RA contain anticitrullinated peptide autoantibodies. These IgG antibodies recognize citrullin residues on different proteins. ACPAs are central to the development of RA. However, the mechanisms leading to the production of ACPAs are unknown.

A tentative model proposes that cigarette smoking may induce citrullination of proteins in the lungs. HLA-DRB1 alleles associated with RA would then bind citrullinated peptides and present them to helper T cells. This mechanism would explain anticitrullinated protein immunization in patients whose HLA-DRB1 molecules express the shared epitope. However, this model has serious limitations.
i) RA may occur in nonsmokers.

ii) IgG antibodies against citrullinated proteins usually develop in the absence of identified helper T cell response.

iii) The preferential binding of citrullinated peptides to shared epitope-positive HLA-DRB1 alleles has never been properly demonstrated. Indeed, the article that was supposed to have demonstrated the point studied the binding of one unique citrullinated vimentin peptide to three shared epitope-positive and five shared epitope-negative HLA-DRB1 alleles (11). Conversely, thorough studies of the binding of hundreds of peptides to shared epitope-positive or -negative HLA-DRB1 alleles did not show preferential binding of citrullinated peptides to shared epitope-positive alleles, compared with their native counterparts (12–14).

Thus, the identity of the antigen(s) whose recognition by T cells can help the development of IgG autoantibodies directed at citrullinated proteins is still unknown.

We focused on PAD2 and PAD4 as candidate antigens in anticitrullinated protein immunization for several reasons:

i) Anti-PAD4 IgG autoantibodies are detectable during the preclinical phase of RA in one-third of patients and are associated with ACPA positivity (18–24).

ii) PAD2 and PAD4 expressed in the RA synovium (25) are involved in the citrullination of fibrin, the major synovial autoantigen in RA (10).

iii) PADI4, the gene encoding PAD4, is a susceptibility locus for RA in Asians and in some, but not every, Western populations...
Increased susceptibility to develop RA might be due to higher stability of PAD4 transcripts and thus higher expression of PAD4 (27).

To test whether we could induce ACPAs by immunizing normal mice with PADs, we used C3H mice whose I€βk chain is similar to that of RA-associated HLA-DRBI*04:01. We immunized C3H mice with four PAD proteins (murine and human PAD2 and PAD4) and obtained IgG and T cell responses to PAD after immunization with murine and human PAD2 or with human PAD4.

No T cell response was observed to native or citrullinated fibrinogen in C3H mice immunized with PADs.

To detect anticitrullinated protein antibodies, we used 46 citrullinated peptides from human fibrinogen with 50–100% homology with their murine counterparts. Among these 46 peptides, four were preferentially recognized by sera from primed mice. These peptides are located in the C-terminal domain of human beta fibrinogen (amino acids 420–479). IgG responses to citrullinated fibrinogen peptides were only detected in C3H mice immunized with murine or human PAD2 or human PAD4.
C3H/HeNHsd and DBA/2 mice were purchased from Envigo Laboratories. Murine PAD2 and PAD4 and human PAD2 and PAD4 proteins were expressed in baculovirus and purified. Their activity and autocitrullination status were tested before immunization.

**Citrullinated Proteins.** Citrullinated fibrinogen, the most likely target antigen of ACPA, expressed in the synovial tissue of RA patients, allows ACPA detection with the same efficiency as the anticyclic peptide antigen kits (anti-CCP2) in RA. Citrullinated human fibrinogen (Calbiochem and Oxford Bio-medical Research) was obtained after incubation in 1 M Tris-HCl (pH 7.4), 100 mM CaCl2, 50 mM DTT buffer at a concentration of 1 mg/mL with rabbit PAD2 (Sigma Aldrich), for 2 h at 37 °C. Noncitrullinated proteins were treated identically, except that water was added instead of rabbit PAD2.

**Immunization Protocols.** C3H or DBA/2 mice were immunized s.c. with 100 μg of murine or human PAD2, or murine or human PAD4, or PBS in CFA. Three booster injections with 100 μg of PADs or PBS in IFA were given s.c. 15, 35, and 55 d later.

**Detection of Anti-PAD Antibodies.** Sera from primed mice were obtained at days 15, 35, 55, and 65 and tested by ELISA. Plates were coated overnight with PADs. Plates were blocked with 2% BSA. Sera were incubated. After washing with Tween 20, peroxidase-conjugated antimurine IgG was added. The OD was read at 405 nm. Background OD was obtained by adding each serum to a well without protein. Positive sera were defined as an OD value higher than twice the background OD.

**Synthetic Peptides.** Each peptide was synthesized using a solid-phase system (Neosystem) and purified. We started with 46 citrullinated peptides from the alpha, beta, and gamma chain of human fibrinogen and used them for ELISAs in 67 mice. We tested 17 15-mers from the alpha chain of human fibrinogen (locus NP_000499) with 50–100% identity with their murine counterparts, 28 15-mers from the beta chain of human fibrinogen (locus NP_005132) with 70–100% identity with their murine counterparts, and one 15-mer from the gamma chain of human fibrinogen (locus NP_068656.2) with 100% identity with its murine counterpart. Peptides 4C, 5C, 6C, and 8C from the beta chain of human fibrinogen were the targets of all of the anticitrullinated fibrinogen antibody responses. They are 93–100% identical to their murine counterpart. They encompass residues 420–479 of the beta chain of human fibrinogen (locus NP_005132). The arginine version of these four peptides was used to confirm citrullinated peptide specificity of anticitrullinated fibrinogen antibodies.

**Detection of Anticitrullinated Peptide Antibodies.** Sera from primed mice were obtained at days 55 and 65 and tested for anticitrullinated peptide antibodies by ELISA. Plates were coated with human fibrinogen peptides under citrullinated and native forms. After blocking, sera from primed mice were incubated with peptides. After washing, peroxidase-conjugated antiamurine IgG was added. The OD was read at 405 nm. Background OD was obtained by adding each serum to a well without peptide. Positive sera were defined as an OD value higher than twice the background OD.

**T Cell Proliferation Assays.** Spleen and lymph nodes were obtained at day 65 postimmunization and used in antigen-specific proliferation assays. T cell responses to PADs, native or citrullinated fibrinogen, or PHA were evaluated using the colorimetric bromodeoxyuridine kit (Roche Diagnostic) (31). Background OD was obtained for cells cultured without antigen. Positive responses were defined as an OD value higher than twice the background OD.

**Statistical Analysis.** Comparisons between groups were performed using Fisher’s exact test.

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Arnoux et al.


