Immunity to Citrullinated Proteins in Rheumatoid Arthritis

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Annu. Rev. Immunol. 2008. 26:651–75

First published online as a Review in Advance on January 2, 2008

The *Annual Review of Immunology* is online at immunol.annualreviews.org

This article's doi: 10.1146/annurev.immunol.26.021607.090244

0732-0582/08/0423-0651$20.00

Key Words

genes, environment, autoimmunity, smoking, HLA-DR

Abstract

Antibodies to citrullinated proteins (ACPA), i.e., to peptides post-translationally modified by the conversion of arginine to citrulline, are specific serological markers for rheumatoid arthritis (RA). Studies on anticitrulline immunity, summarized in this review, demonstrate that the criterion-based syndrome RA should be subdivided into at least two distinct subsets (ACPA-positive and ACPA-negative disease). A new etiological model is proposed for ACPA-positive RA, built on MHC class II–dependent activation of adaptive immunity. Fundamentals of this model include the following: (*a*) ACPA antedate onset of arthritis; (*b*) ACPA may aggravate arthritis in rodents; (*c*) ACPA are triggered in the context of genes that confer susceptibility to RA (HLA-DRB1 SE) and by environmental agents triggering RA (smoking or bacterial stimuli); (*d*) ACPA may complex with citrullinated proteins present in target tissue as part of a multistep process for arthritis development. The model provides a new basis for molecular studies on the pathogenesis of ACPA-positive arthritis.

RA: rheumatoid arthritis

RF: rheumatoid factors

MHC: major histocompatibility complex

ACPA: antibodies to citrullinated protein antigens

CII: collagen type II

BACKGROUND

Rheumatoid arthritis (RA) is a disease defined by criteria (1) that have been useful in harmonizing clinical trials and clinical practice but that are not based on what is now known about its etiology or pathogenesis. It follows that any studies on the molecular pathogenesis of arthritis as defined by these criteria should consider the possibility that the findings are relevant only for a subset of RA. RA is often denoted an "autoimmune" disease, largely based on the presence of rheumatoid factors (RF) (2), one of the seven classification criteria. However, the presence of RF is not specific for RA but is rather a general consequence of immune activation in the context of immune complex formation (3, 4); no experimental studies have demonstrated any proarthritogenic effects of RF. However, other data favor a role for adaptive immunity and possibly for autoimmune reactions in the disease. Such features are the genetic linkage to MHC class II genes (5, 6), the pattern of immune cells and MHC class II molecules in inflamed synovium (7), and the presence of activated T and B cells in the joint (7–9). Taken together, these observations underscore a pathogenetic role for MHC class II–dependent immune activation in RA (7, 10). The nature of such specific immune reactions has, however, been surprisingly difficult to define.

In recent years, advances in research and therapy within the field of cytokine regulation and cytokine-directed therapy have largely dominated the research field of RA (11–14), illustrating how therapeutic progress is possible even though the role of adaptive immunity in this disease is not fully understood. In parallel with this progress, there has also been a major development in the field of immunity focused on antibody reactivity to proteins modified by citrullination, i.e., an enzyme-mediated post-translational modification of peptidylarginine to peptidylcitrulline. Notably, epidemiological and genetic studies of RA in relation to the anticitrulline immunity have redefined RA phenotypes, demonstrating major differences in genetic and environmental risk factors, and thus probably in molecular pathogenesis too, between RA patients with and without the presence of antibodies to citrullinated proteins (ACPA). The implication for immunological studies of RA is that meaningful molecular studies on RA, particularly when dealing with adaptive immunity, should no longer be performed in patients with "RA" but rather in subsets of patients, grouped according to serology as well as to genetic, environmental, and clinical determinants.

A short summary of studies from many groups over the past 10 years tells us that (*a*) antibodies to citrullinated proteins can be found in approximately 60% of RA patients (15–19). These antibodies are highly specific for RA, i.e., they exist in around 2% of normal populations (15) and are also quite rare in other inflammatory conditions (15). (*b*) The occurrence of ACPA is seen several years before onset of disease (20–23), and very few patients with RA develop ACPA after onset of their symptoms (24, 25). (*c*) The occurrence of ACPA is closely linked to the presence of MHC class II alleles that predispose for RA (26, 27); most notably, the association of HLA-DRB1 alleles is seen exclusively for the ACPA-positive subset of disease but not for the ACPA-negative variant (26, 27). (*d*) Immunity toward citrullinated self proteins contributes to arthritis in rodents. The two experiments supporting this conclusion demonstrated that transfer of monoclonal antibodies to citrullinated fibrinogen can enhance arthritis development in mice at the same time as tolerization against citrullinated antigens diminishes arthritis severity (28), and that immunization with the citrullinated self antigen type II collagen (CII) leads to a more severe arthritis than immunization with the same noncitrullinated protein (29).

These studies have renewed interest in the field of adaptive immunity in RA by focusing on a defined subset of the disease. This review summarizes this research and suggests potential directions for research into the etiology of selected RA subpopulations. By dissecting the

syndrome now called RA, we might be able to devise immunotherapies specifically adapted to individual arthritis subpopulations.

CITRULLINATION, A POSTTRANSLATIONAL PROTEIN MODIFICATION OF LARGELY UNKNOWN SIGNIFICANCE

Deimination of the charged peptidyl arginine to the neutral peptidylcitrulline is an enzymatic process in mammals mediated by a series of enzymes denoted peptidyl arginine deiminases (PADs) (30, 31). The activity of these enzymes is dependent on high concentrations of calcium, and deimination can occur intracellularly in conjunction with apoptosis (32) as well as extracellularly given high enough Ca^{2+} concentrations (33). In addition, certain proteins present in the stratum corneum of outer epidermis (34) and in the CNS in conjunction with astrocytes appear to be constitutively citrullinated (35, 36).

The precise physiological function of citrullination is incompletely understood. From the fact that the deimination changes the charge of critical residues of a protein, it is known that citrullination often makes proteins more prone to degradation by proteolytic enzymes (33) (**Figure 1**).

PAD: peptidyl arginine deiminase

An interesting recent finding is that proteins undergoing processing in antigen-presenting cells (APCs) can be citrullinated before presentation to T cells (37). The functional consequence of this intriguing observation is not yet understood but warrants further consideration. In human disease, increased citrullination was first demonstrated to take place in lining and sublining cells in the joints of patients with RA (32). Subsequently, citrullinated proteins were detected by immunohistochemistry in a number of inflamed tissues, including arthritic joints in several different forms of arthritis (38), lungs (26, 39), extra-articular inflammatory sites in RA (39), human brain (40), and inflamed muscle as well as inflamed lymphoid organs (41). No selectivity of citrullination for certain arginine-containing proteins has been demonstrated to date, and citrullination has been observed in many different synovial proteins, including fibrinogen (42), vimentin (43, 44), and CII (M. Hermansson, unpublished observation); furthermore, α-enolase (45) has been demonstrated to colocalize with citrullination in conjunction with joint inflammation.

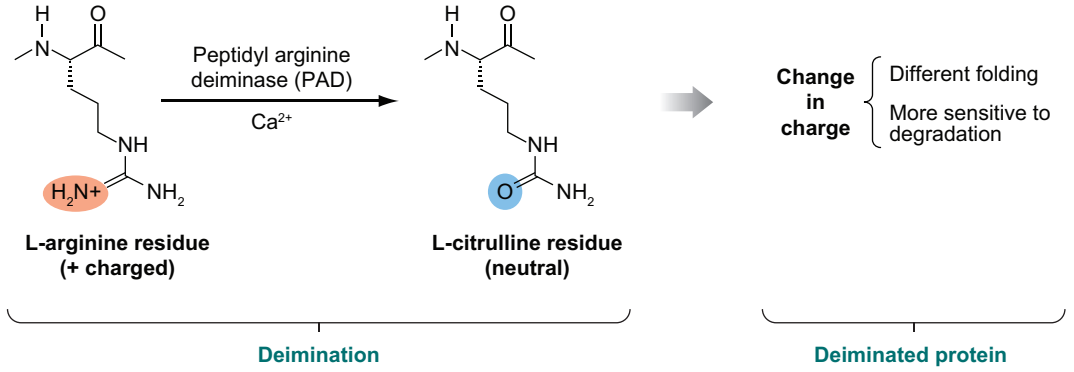

Figure 1

Deimination of peptidylarginine to peptidylcitrulline is a posttranslational process, also known as citrullination, driven by the calcium-dependent enzyme peptidyl arginine deiminase (PAD). The enzymatic conversion results in the loss of one positive charge for every arginine residue converted to a neutral citrulline. This causes changes in intra- and intermolecular interactions, which could lead to altered protein folding, enhanced degradation by proteases, and exposure of cryptic epitopes.

CCP: cyclic citrullinated peptides

IMMUNITY TO CITRULLINATED PROTEIN ANTIGENS IN RA AND RELATED CONDITIONS

The first demonstration that antibodies in RA patients display a preferential reactivity with proteins modified by citrullination came from work by two groups, in France and in the Netherlands. Guy Serre's group in Toulouse investigated the molecular basis of antibodies that reacted with keratin in the skin (45) and with perinuclear cellular structures (46), which were both used in RA diagnostics, and showed that filaggrin was the common target of these antibodies (47, 48). Walther van Venrooij's group in Nijmegen subsequently demonstrated that the filaggrin-specific reactivity of RA sera is dependent on deimination ("citrullination") of arginine residues in filaggrin-derived peptides (49). This failure to see reactivity against recombinant filaggrin produced for evaluation in potential immunoassays for RA led to the recognition that the reactivity was dependent on posttranslational citrullination (49). Initially, these findings were mainly used to develop better diagnostic markers for RA, and several assays were developed that increased the specificity and sensitivity for RA. The assays most widely used were those with cyclic citrullinated peptides as substrates for the detection of antibodies (15, 18, 50).

The initial studies on ACPA reactivity were performed on in vivo citrullinated filaggrin molecules, and then on intact fibrinogen where arginines had been deiminated in vitro using PAD (51). Investigations on larger groups of RA patients showed comparable reactivity patterns when cyclic citrullinated peptides (CCP), citrullinated filaggrin (16), or fibrinogen (52) were used as substrates for assays focusing on IgG antibody reactivities. Further studies have demonstrated RA-specific reactivities against a wide range of citrullinated peptides and proteins. Reactivity to vimentin was originally observed as the so-called anti-Sa reactivity (53–55), and subsequent assays using a mutated variant of vimentin showed reactivities similar to, or with even better sensitivity than, those toward CCP (56) and fibrinogen (44). Reactivities to citrullinated α-enolase represent one recent observation (57), of interest because α-enolase is widely expressed in RA joints and, in immunohistochemical analysis, it colocalizes with citrulline staining. The antibody reactivity to citrullinated CII represents a specific challenge because CII is the major structural protein in hyaline cartilage and because native CII is the classic autoantigen used to provoke polyarthritis in rodents (58, 59). The relatively low frequency of antibody reactivity to native CII in sera of patients with RA has argued against a pathogenetic role of anticollagen immunity in human disease (60), although there are some reports of higher frequencies of immunity against native CII in joints of patients with RA (61, 62). The current demonstration of a high frequency of antibodies against the citrullinated variants of CII in sera of RA patients, especially certain citrullinated peptides (63), provides a new angle on collagen immunity in RA.

The extent to which reactivities of single antibodies are directed toward private epitopes on different citrullinated peptides or proteins and the extent to which they react with public epitopes common to many citrullinated peptides/proteins are incompletely understood, as is the isotype distribution of the ACPA (see Reference 64 for an initial description).

The most remarkable features of the data emerging from investigations of different ACPA are the high specificity for RA and the fact that they define a distinct subset of RA. Most of these studies have been performed using CCP-based assays. Typically, 50%–70% of early RA patients are anti-CCP positive (18, 19), and the phenotype is thereafter very stable, i.e., very few patients shift from being anti-CCP positive to being anti-CCP negative or vice versa, even after treatment with disease-modifying antirheumatic drugs

(24, 25). This qualitative phenotypic stability is also seen after treatment with TNF-blocking agents, although ACPA concentrations remain stable in some (65, 66), but not in all (67–69), studies. Comparatively few individuals (typically approximately 2%) in a population of healthy controls are positive for anti-CCP antibodies. In contrast to RF, anti-CCP antibodies are rather specific for RA. Thus, only relatively few patients with systemic inflammatory diseases, such as systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), Sjögren's syndrome, or myositis, have anti-CCP antibodies; most investigators have reported fewer than 10% of such patients to be anti-CCP positive. When patients with these diagnoses are subgrouped according to anti-CCP phenotype, those with detectable anti-CCP antibodies often present with RA-like features (including polyarthritic disease, erosions, RF, and HLA association) or can be classified as RA in addition to other diagnoses. This ambiguity has also been described for patients with psoriatic arthritis (70–73), juvenile idiopathic arthritis (74–77), SLE (78), and MCTD (79).

These data suggest a potential new classification of arthritis, as some patients with polyarthritis and concomitant features of other systemic rheumatic conditions might well share etiologic features with ACPA-positive classical RA. Further studies of common genetic and environmental determinants for ACPA-positive arthritides may suggest a new ACPA-related classification that has a wider inclusion than only those patients who fulfill today's classification criteria for RA (1).

EVIDENCE THAT ACPA-POSITIVE AND ACPA-NEGATIVE RA CONSTITUTE TWO CLINICALLY AND GENETICALLY DISTINCT SUBSETS OF RA

One of the more exciting features of current work on ACPA in RA is the evidence that ACPA-positive and ACPA-negative RA constitute two distinct subsets of this criterion-based disease. The first confirmation is purely clinical in the sense that ACPA-positive RA patients have a disease course considerably more severe than that of ACPA-negative patients, irrespective of treatment (24, 25, 80, 81). In particular, erosiveness is highly linked to an ACPA-positive status (81–83). A second verification comes from treatment, where a recently published study demonstrated how methotrexate induced remission in ACPA-positive early arthritis patients but had no effect in ACPA-negative subjects (84).

HLA-DRB1 SE: HLA-DRB1 shared epitope

The most compelling data from a pathophysiological perspective, however, demonstrate large differences concerning susceptibility genes for ACPA-positive and ACPA-negative disease. By the late 1970s, the MHC class II genes (5, 6, 85) were already identified as the major genetic risk factor for RA. Numerous studies on the association between HLA class II genes, in particular the HLA-DRB1* shared epitope (SE) alleles and RA, have provided a strong rationale for MHC class II–dependent T cell activation and thus for adaptive immunity having a pathogenic role in RA. This association also differentiated RA from other MHC class II–associated arthritic diseases such as ankylosing spondylitis, psoriatic arthritis, and reactive arthritis (86).

The genetic association of RA to HLA-DRB1 SE has recently been shown to be entirely confined to the ACPA-positive subset (87, 88). This finding indicates that the implication of MHC class II–dependent T cell activation in the pathogenesis should be limited to ACPA-positive RA and to anticitrulline immunity (see below). In contrast, two studies suggested that ACPA-negative RA may be associated with HLA-DRB1*03, an allele not previously associated with disease susceptibility in the unstratified RA population (64, 89). Following this initial demonstration of a genetic distinction between ACPA-positive and ACPA-negative RA for HLA-DR genes, a second major genetic risk factor for RA, the

polymorphism in PTPN22 gene, was also shown to be associated only with the ACPA-positive disease (90–92). Other genetic risk factors, in particular variations in the interferon regulating factor 5 (IRF-5) (93) but also polymorphisms in a newly identified risk gene in the C-type lectin complex (94), were associated exclusively with ACPA-negative disease (93).

Taken together, the descriptive studies of disease course and genetic linkages strongly indicate that ACPA reactivity splits RA into two major and clinically relevant subsets of disease. Thus ACPA-positive and ACPA-negative RA should be treated as separate entities when studying the molecular pathophysiology of RA.

EVIDENCE THAT ANTICITRULLINE IMMUNITY CAN CONTRIBUTE TO DEVELOPMENT OF ARTHRITIS

The key issue related to anticitrulline immunity is whether the observed autoantibodies are causally related to the disease, consequences of the disease, or just epiphenomena. Two groups of experiments suggest that anticitrulline immunity may contribute to arthritis. That ACPA may precede clinical RA by years was first demonstrated from studies of blood repositories in northern Sweden, where individuals who subsequently developed arthritis had donated blood samples several years before onset of disease. Anti-CCP antibodies were demonstrated up to nine years before clinical onset of RA, and increased frequencies of higher concentrations of antibodies were seen as the individuals approached onset of disease (22). These studies were well in line with a series of earlier investigations from Kimmo Aho and associates in Finland; these authors had shown that rheumatoid factors (95) as well as antikeratin (20) and antifilaggrin antibodies (21) preceded RA development. An independent study from the Netherlands further corroborated the finding that most individuals who would develop ACPA-positive RA had already developed their autoantibodies before disease onset (23). Little is yet known about the evolution of ACPA epitope specificities during arthritis development, information that might shed light on the pathogenic role of different ACPAs. However, the time sequence of ACPA development pre-RA and, in particular, the finding that very few patients develop ACPA after disease onset, provide indirect evidence for the contribution of anticitrulline immunity, or some other concomitant immunity, in the pathogenesis of ACPA-positive RA. Studies of immunoglobulin isotypes and IgG subclasses in early and long-standing RA also indicate that the isotype repertoire is fully developed by the time of arthritis development, but shows a sustained presence of IgM anti-CCP, interpreted as a continuous activation of ACPA-reactive B cells (64).

The second line of evidence in support of a direct pathogenic role for anticitrulline immunity comes from experimental animal models. In the mouse, Kristine Kuhn and collaborators found that transfer of monoclonal antibodies to citrullinated fibrinogen enhanced a mild arthritis that was initiated with anti-CII antibody transfer (28). However, antibodies to citrullinated fibrinogen alone were not able to cause arthritis in naive animals with no joint lesions. Furthermore, mice immunized with native CII developed anticitrulline immunity, i.e., reactivity to native as well as citrullinated CII, and administration of citrullinated peptides in a tolerogenic protocol ameliorated the collagen-induced arthritis (CIA). These latter data thus suggested that citrullination of collagen and/or additional proteins might be involved in CIA.

In two different reports in the rat, citrullination was shown to enable otherwise nonimmunogenic self molecules to trigger autoantibody production against albumin, CII (29), and fibrinogen (96). Neither the immunity to citrullinated albumin (29) nor to fibrinogen (96) was able to cause arthritis, whereas the immunity to citrullinated CII enhanced

arthritis in Lewis 1AV1 rats (29). Taken together, these papers document how citrullination may change the immunogenicity of self antigens and suggest that immunity to some citrullinated proteins may contribute to arthritis development. More detailed studies are needed to determine which cells, specificities, and mechanisms are involved in this process (see below).

WHEN AND HOW IS ANTICITRULLINE IMMUNITY TRIGGERED?

The data summarized above indicate that antibodies to autoantigens that have been modified by citrullination may contribute to arthritis. As citrullination is a common process in many physiological events, including apoptosis and inflammation, the rate-limiting steps appear to be whether and when immunity is triggered against these modified proteins and which epitopes of which proteins are being recognized.

Most information on how genes and environmental determinants interact in increasing risk for ACPA-negative as well as ACPA-positive RA comes from case control studies in which population-based RA cohorts were compared with healthy individuals from the same population. The most striking finding from these studies overall is the dichotomy between ACPA-positive and ACPA-negative RA concerning both genetic and environmental determinants.

Thus, as noted above, both the major genes, i.e., HLA-DRB1 SE and PTPN22 alleles that predispose for RA, were shown to be risk factors for ACPA-positive disease, but not for ACPA-negative disease. For HLA-DRB1, the susceptibility alleles DRB1*0401 and *0404 were also closely linked to frequencies as well as levels of ACPA, measured with the CCP assay (87, 88), indicating not only that these genes were susceptibility factors for the disease, but also that they directly influenced ACPA production. The PTPN22 codes for a tyrosine phosphatase that is present in many cells, and the allelic difference influences susceptibility (97) by altering the threshold for activation of PTPN22-expressing cells. This is true not only for T cells but also for a number of other cell types (98). The PTPN22 1858 T polymorphism (620W) is specifically associated with anti-CCP-positive RA in many (91, 99, 100), but not in all (101), studies.

Recently, a striking gene-gene interaction was demonstrated between HLA-DRB1 SE alleles and the susceptibility allele of the PTPN22 gene, where the combination of two SE alleles and the 620W allele of PTPN22 increased the risk for ACPA-positive RA 20-fold compared to individuals with none of these genetic risk factors (92). These data indicated that both molecular pathways involving MHC class II–dependent T cell activation and tyrosine phosphatase–mediated cell activation are of pathogenic importance in ACPA-positive RA. More recently, additional genetic determinants, notably one gene in the TRAF1-C5 region, have also been shown to associate with ACPA-positive RA (102), but not with ACPA-negative disease (103). Although the susceptibility gene(s) has not yet been definitely identified, its possible relation to TRAF1- or C5-mediated functions makes it an additional determinant influencing a pathway related to adaptive immunity and antibody-mediated effector functions.

Smoking, long known to be the major environmental factor in increased risk for RA (104–107), was recently demonstrated to be a significant influence on RF^+ disease (104, 106, 108–110). Smoking used to be considered as an unspecific risk factor, of interest mainly from a public health perspective, rather than a reasonably well-defined trigger of RA, and studies of its actions could help elucidate the molecular pathology of RA. Our investigations showed a striking gene-environment interaction between smoking and the presence of the HLA-DRB1 SE risk alleles as risk factors first for RF^+ disease (110), and then even more pronounced for ACPA-positive RA (26) (**Figure 2**). The relative risk of developing

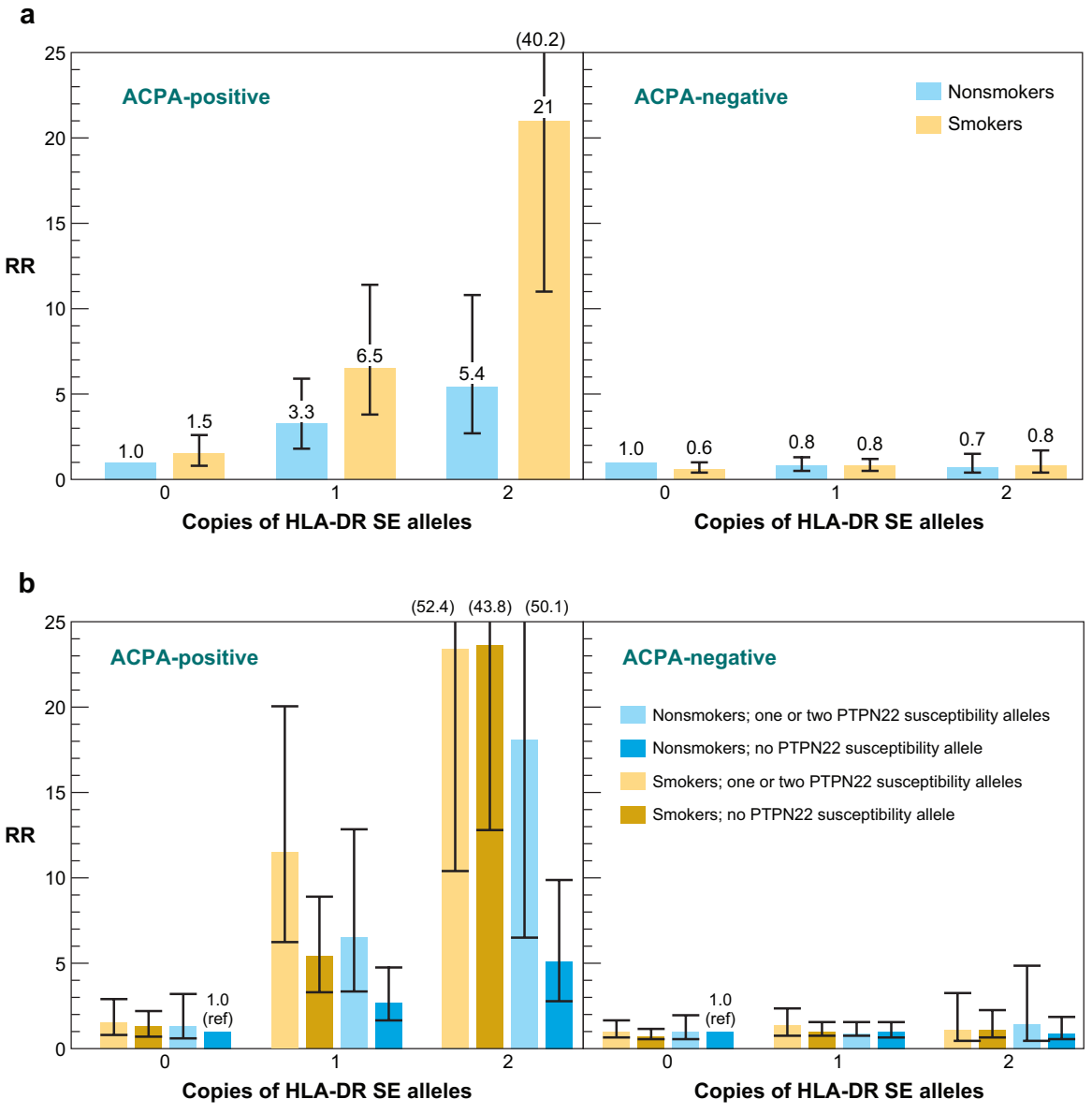

Figure 2

(*a*) A major gene-environment interaction between HLA-DR SE and smoking is present in the ACPA-positive RA subpopulation (*left*) but not in the ACPA-negative RA subpopulation (*right*). Smoking only confers an increased risk of developing ACPA-positive RA, as does possessing a single copy of the HLA-SE allele or, even more so, two copies of the HLA-SE alleles (RR, relative risk). (Original data published in Reference 26.) (*b*) The two panels demonstrate the combined effects of the three risk factors (HLA-DRB1 SE, PTPN22 620W allele, and smoking) in ACPA-positive RA (*left*) and the complete absence of effect of any of these three factors in ACPA-negative RA (*right*). (Original data published in Reference 92.)

ACPA-positive RA was over 20 times higher for smokers carrying two copies of the HLA-DRB1 SE alleles than for nonsmokers with no SE alleles. On the other hand, no increased risk was discerned for development of ACPA-negative RA (26). This gene-environment interaction has subsequently been replicated both in a Dutch study of ACPA-positive and ACPA-negative cases only (27) and in a Danish case control (111) study, which found even higher risk ratios for the combination of smoking and HLA-DRB1 SE than in our original study. Notably, in our continued studies in the Swedish cohorts, we extended the data for gene-environment interactions between smoking and HLA-DRB1 by showing the dose-dependency of smoking, with a combined relative risk of ACPA-positive RA of close to 50 times higher in heavy smokers carrying two copies of HLA-DRB1 SE alleles than in nonsmokers lacking these variations (112). However, no interaction was found between the PTN22 risk alleles and smoking (92). In a recently published study from North America, a more mixed picture emerged on the interaction between smoking and HLA-DRB1 SE, indicating that other genes or environmental factors may also influence the risk of RA development (113).

The studies described above provide one of the most striking examples of gene-environment interaction in the risk of developing a specific diagnosis, where this risk is strictly confined to an immunologically defined subgroup of patients. These observations provide an obvious basis for further molecular studies that would explain the observed effects of genes and environment as well as provide a more general insight into the pathogenesis of ACPA-positive RA. As a first step, we initiated studies to determine if and how smoking may influence citrullination of proteins in lungs. We showed initially that smoking is associated with an increased presence of citrullinated proteins in bronchoalveolar lavage (BAL) cells from healthy smokers and smokers with pulmonary inflammation (26). Subsequently, we extended this study to demonstrate that this increased citrullination may be due to increased expression of PADs, in particular PAD2 in BAL cells from smokers (D. Makrygiannikos, M. Sköld, L. Klareskog, and A. Catrina, submitted for publication).

We then proposed the following model for how immunity to citrullinated proteins might be triggered by smoking and how the gene-environment interactions might be explained (26, 114): Smoking may cause PAD activation and subsequent citrullination in lungs, at the same time as components in smoke act as unspecific adjuvants activating APCs in the pulmonary compartment. In individuals carrying immune response genes that predispose to a strong immune reaction against certain citrullinated peptides, and where other genetic variants are also present (such as the PTPN22-coded tyrosine phosphatase), an immune response with antibody production to citrullinated proteins might be triggered. The validity of this model was further strengthened by a report in a mouse model that immunity to a citrullinated vimentin peptide may be restricted by HLA-DRB1 SE alleles (115).

A POSSIBLE ETIOLOGIC MODEL FOR ARTHRITIS THAT INVOLVES CITRULLINATION AND IMMUNITY TO CITRULLINATED PROTEINS

The studies described above provide a framework for molecular studies of adaptive immunity in RA. In the remainder of this review, we use this framework to discuss a potential model for an etiology of ACPA-positive RA that could perhaps be extended to other cases of ACPA-positive arthritis. Notably, most of the issues related to specificity and regulation of anticitrulline immunity have still been incompletely investigated. The epidemiologic and longitudinal studies referred to above have also produced data to suggest the existence of different stages in a multistep process that leads to ACPA-positive RA. An outline of this model that we discuss below is provided in **Figure 3**.

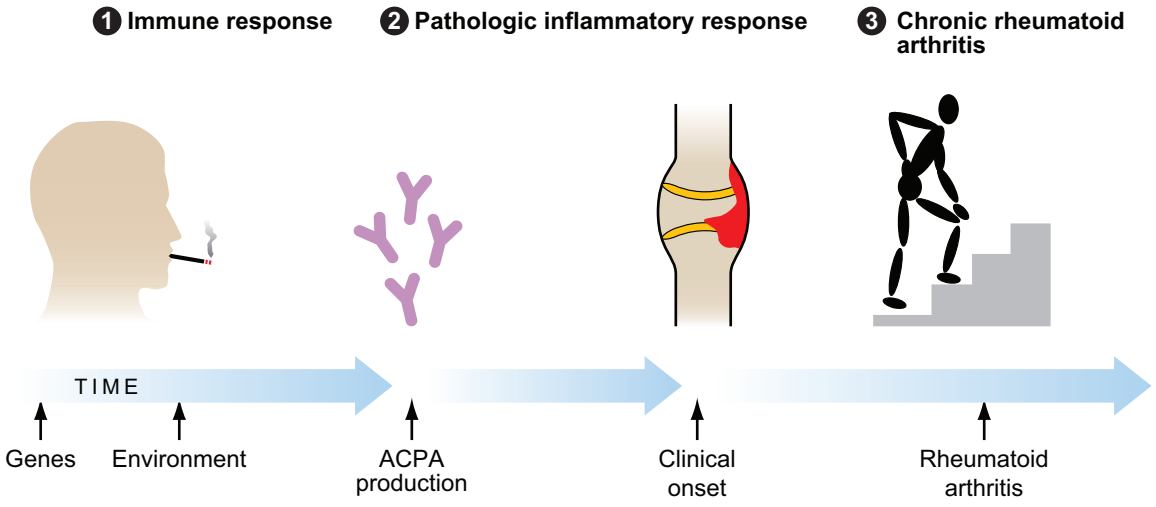

Figure 3

A three-stage etiologic model for the development of ACPA-positive RA. Stage 1, the immune response: Environmental risk factors, such as smoking, may induce citrullination of proteins in the lungs. An altered antigen uptake, processing, and presentation of citrullinated antigens could, in genetically susceptible individuals (i.e., HLA-DR SE positive), lead to the production of ACPA. Stage 2, the pathologic inflammatory response: An unspecific arthritis, accompanied by citrullination of proteins in the joints, develops at a later stage. Recruitment of ACPA from the circulation results in the formation of immune complexes. Stage 3, chronic RA: The generation of citrullinated proteins, the influx of immune cells, and the production of cytokines and autoantibodies, as a result of the immune complex formation, perpetuate the joint inflammation into chronic RA.

Stage 1: Citrullination and Triggering of Immunity to Citrullinated Proteins

Key to understanding anticitrulline autoimmunity in arthritis is the determination of conditions that trigger the occurrence of (*a*) citrullination and (*b*) immunity to citrullinated proteins. Activation of PADs and citrullination occurs in inflammatory conditions as well as in apoptosis, and many different cells including macrophages and neutrophils can express PADs (30) (**Figures 4** and **5**). Active immunization with citrullinated self proteins can trigger antibodies directed to citrullinated as well as to the corresponding noncitrullinated proteins.

The conditions required to trigger immunity to citrullinated proteins in a more physiological context are less well known. From the relatively rare occurrence of anticitrulline antibodies in healthy individuals (human as well as mouse) and the frequent occurrence of citrullination already in utero (116) and in inflammation (41), the brake of tolerance to citrullinated proteins should be a relatively rare event. In many respects, the occurrence of immunity to citrullinated proteins can be compared with immunity to molecules exposed to the immune system during apoptosis. Here, immunity toward DNA and other constituents of apoptotic blebs may be triggered under certain relatively rare conditions, where both genetic and environmental factors are important (117). We know from studies described above in humans that at least some anticitrulline immunity may be preferentially triggered in the context of certain MHC class II genetic variants, but also other genetic factors should be studied, preferably in a context where anticitrulline immunity can be investigated per se and independently of its possible later involvement in RA pathogenesis.

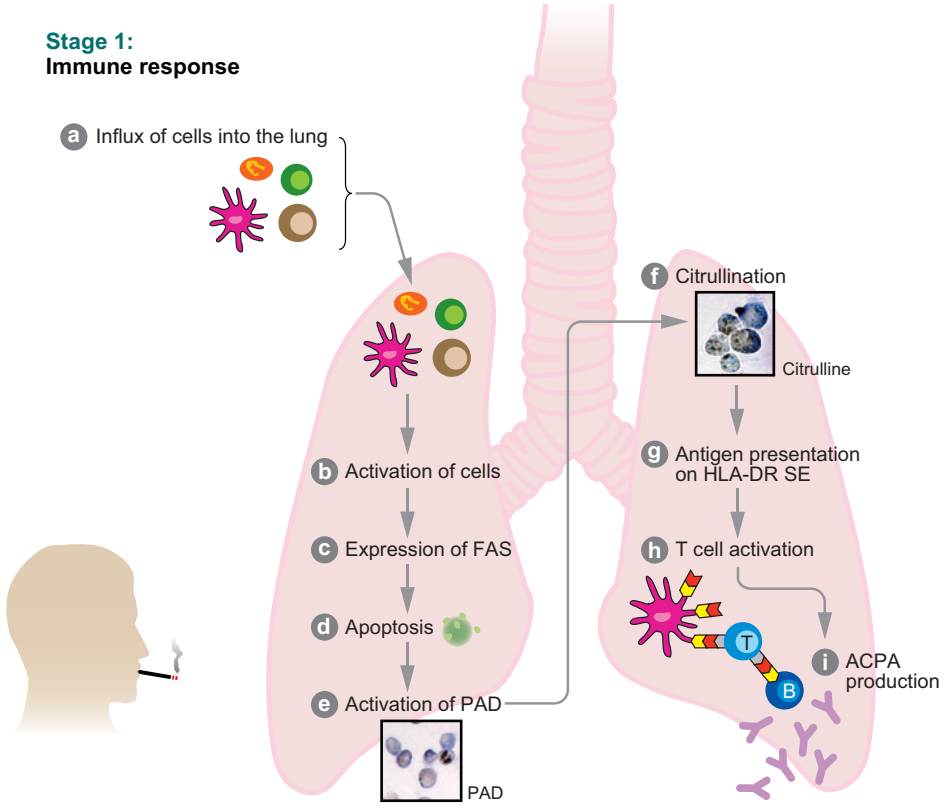

Figure 4

Stage 1 in the etiological model for the development of ACPA-positive RA: the immune response. (*a*) Heavy cigarette smoking stimulates an influx of cells into the lungs. (*b*) Toxic components in the smoke activate the cells and (*c*, *d*) render them more prone to apoptosis. (*e*) PAD becomes activated during the apoptotic process and (*f*) deiminates proteins present in the lungs. (*g*) In genetically predisposed individuals, such as those carrying the HLA-DR SE alleles, presentation of citrullinated peptides or other neo-epitopes from citrullinated proteins could (*h*) activate autoreactive T cells, which in turn could induce B cell help and (*i*) stimulate the production of ACPA.

Functional polymorphisms in genes coding for the PADs are obvious candidates, and a polymorphism in the *PADI4* gene has been reported to increase risk for RA in Japanese (118) and Korean (119) populations, but probably not in Western European populations (120–123). It is, however, not yet formally proven that this polymorphism affects anticitrulline immunity; other possible molecules in pathways related to the citrullination may have variations that predispose to the disease.

Of particular interest is whether anti-PAD can be triggered in conjunction with citrullination. A few small studies suggest the existence of anti-PAD2 and anti-PAD4 antibodies in RA (124). This line of research warrants further attention, particularly given its attractive parallels to the situation in celiac disease, in which the complex of transglutaminase and gluten modified by this enzyme constitutes the immunogenic protein complex (125). In the ACPA parallel, the entire complex of PAD and a citrullinated antigen may constitute the nonself antigen complex.

The possible association between citrullination in lungs and smoking—and the possible triggering of anticitrulline immunity in this context—is a challenging concept. But

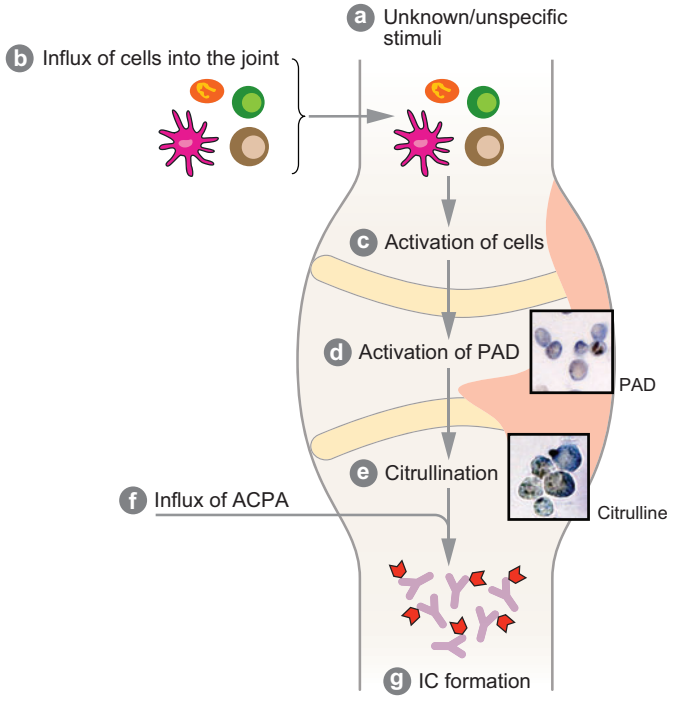

Figure 5

Stage 2 in the etiological model for the development of ACPA-positive RA: the pathologic inflammatory response. (*a*) A second, joint-specific inflammatory event is initiated by an unknown and unspecific stimulus, for example, infection or trauma. (*b*) Inflammatory cells are recruited to the joint, (*c*) activated by the unknown "trigger," and in this inflammatory milieu (*d*) PAD becomes activated and (*e*) deiminates proteins present in the joint. (*f*) Circulating ACPA enter the joint, bind to the citrullinated proteins, and (*g*) form immune complexes (IC).

the model should not be limited to smoking. We might instead consider inflammation in lungs driven by various exposures as a potential trigger of citrullination within the airways and development of anticitrulline immunity. Silica dust and mineral oil exposure have both been linked to an increased risk for ACPA-positive RA (L. Klareskog, J. Rönnelid, K. Lundberg, L. Padyukov, and L. Alfredsson, unpublished results). Several other factors, including air pollutants and maybe also charcoal as in Caplan's syndrome (126), are also possible agents. The fact that IgA anticitrulline immunity is seen early during development of an anticitrulline immune response (64) indicates that immunity triggered from mucosal surfaces such as the lungs may be involved in triggering of anticitrulline immunity (**Figure 4**).

The nonequal importance of genetic variations in the PADI4 gene for risk of RA in Asian and in Caucasian populations (118–123) is an indication of possible multiple genetic and environmental factors that lead to the production of ACPA.

Another trigger of citrullination and citrulline immunity could be infections. Although many pathogens are suggested to be involved in triggering of RA, no solid evidence exists linking infectious agents to RA. The discovery of ACPA provides new opportunities to reinvestigate this issue. Pratesi and colleagues, for example, have recently shown reactivity to citrullinated Epstein-Barr nuclear antigen-1 in RA patients (127). Another pathogen of interest is *Porphyromonas gingivalis*, the causal agent of adult periodontitis, a disease with many features in common with RA, of which the most striking is an HLA-SE association (128, 129). Given that *P. gingivalis* is the only bacterium known to express a functional PAD enzyme, one could hypothesize that infection by *P. gingivalis* could induce local citrullination and subsequent citrulline-immunity in susceptible individuals, which could lead to the development of RA in a fashion similar to that described for smoking.

The seroconversion of a healthy individual from an ACPA-negative to an ACPA-positive state is quantitatively and "risk-wise" the most important step in the series of events that may lead to RA. Thus, for a healthy individual who is positive for ACPA, measured with an anti-CCP test, the risk of developing RA in the future is 100-fold greater than that for the general population. This risk is further increased to over 100-fold if HLA-DRB1 and PTPN22 genes are taken into account (100).

Little is known to date about the specificity, as well as affinity maturation, of antibodies during the pre-RA stage of development and whether there is a dynamic in the

fine specificity and levels of antibodies during this time. It is of prime importance that such features be investigated, similar to the way studies were conducted in lupus (130). As described above, even less is known for T cell reactivities.

Stage 2: Development of Unspecific Arthritis and the Possible Further Progression to RA

A major feature in the proposed RA scenario is that antibodies to citrullinated proteins occur before onset of disease, and that the occurrence of these antibodies is associated with substantially increased risk of developing RA, even though most individuals with these antibodies do not develop disease. This observation is also compatible with the mouse data described above, in which transfer of anticitrullinated fibrinogen antibodies alone was not able to cause arthritis in naive mice but could enhance arthritis in mice with a low-grade arthritis caused by other means (28).

Antibodies as well as T cells typically cause inflammation at sites where the autoantigen(s) is present. No citrullination has yet been demonstrated in normal, uninflamed joints or other uninflamed tissues (41) except at sites that are relatively inaccessible for the immune system in the stratum corneum (34) or in the CNS (35). A number of different unspecific proinflammatory stimuli appear to cause citrullination in joints, including trauma and postinfectious events (D. Makryannikos, L. Klareskog, A. Catrina, unpublished observations). This suggests that some autoantibodies that are triggered outside the joints, e.g., in the lungs, may bind to autoantigens in joints after an unspecific "second strike" has caused joint inflammation and citrullination. Such a scenario is compatible with observations in both human and mouse: ACPA-positive patients with unspecific mild arthritis are much more likely to develop chronic and long-lasting arthritis than are patients without these antibodies (17, 131). This clinical experience can be compared to the mice that were subjected to mild CIA and that developed more severe disease after transfer of antibodies to citrullinated fibrinogen (28). Antibodies reacting with citrullinated proteins in the joint may thus enhance arthritis development in human as in mouse. This scenario would favor a three-stage development of ACPA-positive RA: the formation of antibodies, followed by the actual development and chronicity of arthritis (see **Figure 6**).

The hypothesis that immunity to citrullinated proteins in joints may contribute to development of arthritis raises a fundamental question: Why does immunity to citrullinated but ubiquitously expressed proteins such as fibrinogen cause arthritis but not inflammation at other sites?

Two tentative explanations can be offered: First, the main target molecule for the autoimmune attack may indeed be tissue specific, whereas reactivities to other proteins are epiphenomena, partly due to cross-reactivities. This possibility is nicely illustrated in the putative case of CII immunity and in reports that citrullinated CII is recognized by antibodies from RA patients. This line of reasoning would reactivate the issue of collagen- and cartilage autoimmunity in RA and provide new relevance to the CIA model and collagen autoimmunity. All of this supports calls for greater emphasis in this line of research.

The second assumption is that immunity to citrullinated proteins such as fibrinogen, vimentin, and α-enolase, which appear to be present in many sites of inflammation, can cause or enhance arthritis. In this scenario, we have to describe how immunity to such common proteins can cause a tissue-specific inflammation in joints. This problem might be parallel to how antibodies to the ubiquitous protein glucose phosphate isomerase (GPI) were shown to cause tissue-specific joint inflammation (132) and to how an initially T cell–driven pathology subsequently became driven by antibodies. A possible explanation for this tissue specificity (133) is that certain immune complexes containing antibodies

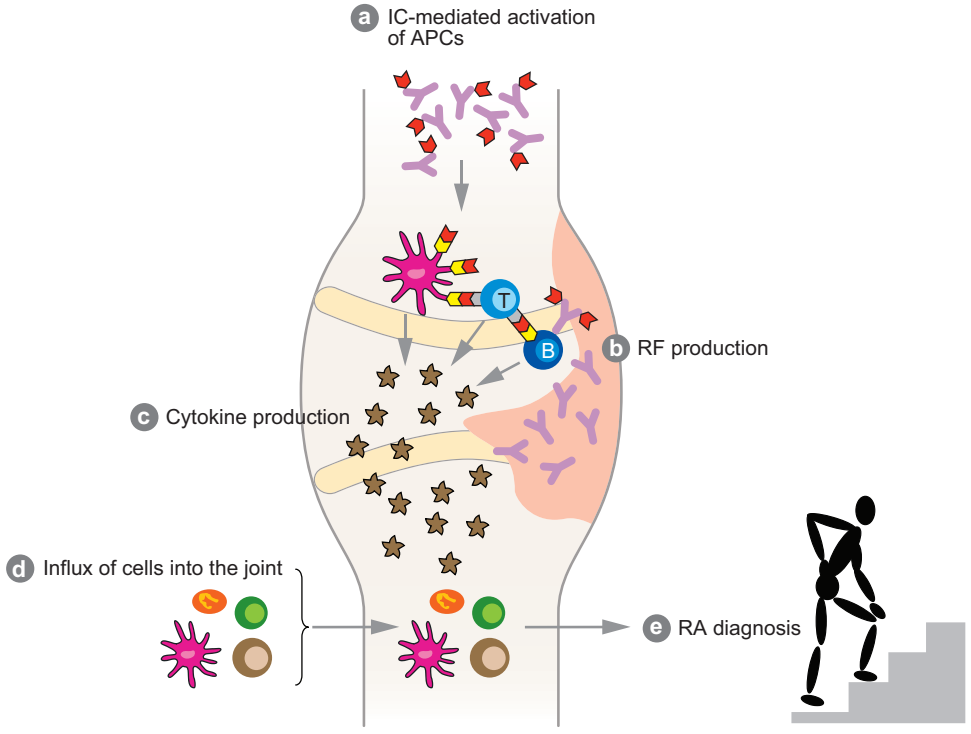

Figure 6

Stage 3 in the etiological model for the development of ACPA-positive RA: the chronic RA. (*a*) Immune complexes of ACPA and citrullinated proteins further stimulate antigen-presenting cells (APCs), by binding to complement and Fc receptors (not shown). Activated APCs present more citrullinated antigens, activate more T and B cells, increase the ACPA production but also (*b*) RF production. (*c*) Increased production of proinflammatory cytokines, including TNF, IL-1, and IL-6, in turn recruits more immune cells into the joint (*d*), perpetuating the inflammatory process. Activation of PAD generates more citrullinated proteins, establishing a vicious cycle that ultimately leads to (*e*) the development of chronic RA.

to ubiquitous self molecules may preferentially accumulate in joint tissue. If also valid in human, such a mechanism would provide an option for antibodies to citrullinated forms of ubiquitous proteins to preferentially accumulate in joints and thereby contribute to arthritis development or perpetuation. Further comparative studies between anti-GPI-induced and ACPA-induced arthritis in rodents may show the extent to which lessons from anti-GPI-induced arthritis could help us understand ACPA-associated joint inflammation.

Stage 3: Chronicity of Joint Inflammation and Fulfillment of the Diagnostic Criteria for RA

Most cases of joint inflammation in human, such as synovitis in conjunction with trauma and postinfectious events, are transient and do not lead to chronic disease or to permanent joint damage. There have been some systematic studies of joint inflammation in a broad setting, i.e., including a very broad set of individuals with joint inflammation in early arthritis clinics. Strikingly, these studies demonstrate that most unspecific arthritides

resume spontaneously (134). Patients with early arthritis also demonstrate huge differences in prognosis depending on initial ACPA status (17, 131, 135), as ACPA-positive subjects were more likely to develop RA, whereas ACPA-negative individuals with a clinically comparable picture often went into spontaneous remission. These data are of major clinical interest, as they allow a new clinically relevant classification of patients with early unspecific arthritis. In a biological context, these data support the notion that anticitrulline immunity may indeed enhance development of unspecific and otherwise transient arthritis into a chronic disease. Notably, chronicity (more than six weeks of disease) is an inherent part of the classification criteria for RA (1). It is therefore feasible that a capacity to enhance and/or prolong an otherwise transient arthritis may provide a causative role of anticitrulline immunity.

CONCLUDING REMARKS AND RESEARCH DIRECTIONS

The principal message we hope to convey in this review is how knowledge gained from genetic epidemiology and longitudinal studies of the syndrome called RA can be used to provide a new basis for molecular studies of this disease. The lessons learned are as follows:

- RA should be divided into at least two very different subsets, where multiple genetic and environmental determinants distribute dichotomously between the ACPA-positive and ACPA-negative variants of RA. Fundamentally different molecular pathologies must also be assumed for these two variants of disease.
- Analysis of gene-gene and gene-environment interaction can provide precise leads to further molecular studies on etiology as well as therapy of distinct subgroups of arthritis. In the case of ACPA-positive RA, these studies point to the contribution of MHC class II– and T cell–dependent adaptive immunity involving immune reactions toward proteins modified by citrullination.
- Longitudinal analyses, from pre-disease states to late disease, help in elucidating potential distinct breakpoints, where progression to disease may depend on different determinants in each of these stages.
- Future studies of ACPA-positive arthritis should be separate from ACPA-negative RA subjects, as discussed above. To be complete, however, ACPA-positive subjects with RA-like features, but who have other primary diagnoses (70–79), should also be included.

Notably, these lessons have been derived using information from only a very limited part of the mammalian genome, from a very limited set of antibody reactivities, and from limited data on environmental exposures. With new information on genetic variants over the entire genome now burgeoning (102, 136), as well as that from systems detecting multiple antibody reactivities (137), further insight into the molecular pathways involved in various subgroups of RA can be expected. This new information should be incorporated into our models and experimental systems.

The implication is thus that any type of experiment aimed at understanding molecular events in RA, in particular when related to adaptive immunity, should be performed using biological specimens or patients' clinical data for which we have precise knowledge of the profile of ACPA status, genetic determinants, and, if possible, pre-existing environmental triggers, and for which the investigated patients have been subgrouped accordingly.

If these measures are taken, the possibilities will be better than ever before to define arthritis-specific immune reactions that take place at distinct time periods in disease development. This ability, in turn, should enable

us to apply the experience garnered from different animal models to the more precise study of pathogenesis and therapies in different groups of RA patients. We conclude that studies on citrullination and immunity against citrullinated proteins, taken together, may furnish critical knowledge that will allow us to understand and modulate adaptive immunity in newly defined sets of arthritis, including a major subset of RA that is characterized by the presence of antibodies to citrulline-modified proteins.

SUMMARY POINTS

1. Antibodies to citrullinated protein antigens (ACPA) constitute a relatively specific diagnostic tool for RA, as they are present in approximately 60% of an early RA cohort and in approximately 2% of the general population.
2. ACPA-positive and ACPA-negative RA constitute two very different subpopulations of RA concerning the role of major susceptibility genes (HLA-DRB1 and PTPN22) and major environmental risk factors (smoking) as well as clinical course. These two subpopulations, which most likely also have different molecular pathogenesis, should be treated as separate entities in further immunological studies of RA.
3. Antibodies to citrulline-modified proteins may be causatively involved in the development of arthritis. Support for this proposal comes from data showing the emergence of ACPA before onset of disease and from animal model studies showing that immunity against citrulline-modified self molecules, such as fibrinogen or collagen type II, can enhance development of arthritis.
4. A new model has been proposed to explain the contributions from genetics and environment (smoking) in causing ACPA and the onset of arthritis. This model includes citrullination induced by environmental agents such as smoking, induction of ACPA in individuals with RA susceptibility genes (including HLA-DRB1 SE), and citrullination of molecules in target tissues.
5. The new model provides a framework for studies of adaptive immunity and of interactions between innate and adaptive immunity in RA. The data and the model emphasize the need to use data on genetic features, on environmental exposures, and on clinical course to subdivide RA in subpopulations before embarking on molecular studies on pathogenesis and treatment.

FUTURE ISSUES

1. The evidence from genetic epidemiology demonstrating the presence of at least two very distinct subsets of RA should be used to define relevant subsets of arthritis. Hypotheses on etiology in the different subsets of RA, should be tested in molecular immunology using information from genetic epidemiology to properly subdivide patients.
2. Different animal models for arthritis should be used to study different subsets of RA, where these subsets are defined by their genetic, environmental, and clinical characteristics.

3. Prospective studies on the evolution of citrullination, anticitrulline immunity, and arthritis are needed to determine the different stages and breakpoints, initiation of ACPA, initiation of arthritis, and progression into chronic RA.
4. Further therapeutic and preventive studies can be directed toward molecular pathways defined from increased knowledge on citrullination and anti-citrulline immunity.

DISCLOSURE STATEMENT

The authors do not declare any conflicts of interest. The studies from the authors' laboratories and clinics were supported from multiple and noncommercial sources, including the Swedish Research Council, the Swedish Council for Working Life and Social Research, King Gustaf V's 80-Year Foundation, the Swedish Rheumatism Association, the Söderberg Foundation, FAMRI (Flight Attendants Medical Research Academy), the insurance company AFA, and the EU-supported project AutoCure. These sponsors had no influence on the writing of this manuscript.

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Annual Review of Immunology
Volume 26, 2008

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