Historical observations contributing insights on etiopathogenesis of rheumatoid arthritis and role of rheumatoid factor

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When studies on rheumatoid arthritis (RA) that were made many decades ago and could be considered "historical" in nature are analyzed in the context of recent observations, important insights on RA and on the function of rheumatoid factor (RF) become apparent. RF in the role of antibody to immune complexes (ICs) appears to be involved in activation of the complement system and in the production of chemotactic and inflammatory mediators, creating a condition that can be sustained and reinitiated. In the synovial cavity, a state of nonresolving inflammation is produced with the formation of citrullinated protein antigen–antibody complexes or other forms of ICs. This is followed by a second wave of IC production in the form of RF acting as antibody reactive with the initial ICs. Both of these processes are associated with complement consumption and production of inflammatory mediators. We present a model of an initiation phase of RA that might represent an example of repetitive formation of ICs and complement-mediated inflammation. Targeting therapy at this phase of RA to break the cycles of recurrent inflammation might be a novel approach to aid in further control of the disease.

Introduction

Recent progress in the treatment of rheumatoid arthritis (RA) with the use of biologics such as anti-TNF supplemented with other drugs such as immunomodulatory agents has resulted in significant improvements in prognosis and in the quality of life in a majority of patients (Maini et al., 1998; Feldmann and Maini, 2003). Unfortunately, there remain an estimated 30–40% of patients who do not respond favorably to such treatment, and only few enjoy complete remission (Feldmann and Maini, 2003). Another important progress in RA is the identification of a group of cellular proteins that induce autoantibody responses, and the measurement of antibodies to citrullinated and other altered proteins has become a highly useful addition to the diagnostic repertoire in clinical practice (Simon et al., 1993; Schellekens et al., 1998, 2000). The two major diagnostic tests for RA, rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs), have approximately equal sensitivity and specificity for RA, but they are autoantibodies to entirely different target antigens. RF is believed to constitute antibody to immune complexes (ICs) where the antigen portion may in large part consist of citrullinated peptides of arginine-containing cellular proteins that have been modified by deimination. With these and other important advances, we believe that there could be some clues that might help us to develop a better understanding of etiopathogenetic events in RA, with the hope that this might contribute to finding new directions in treatment. In this review, we have reexamined earlier clinical and experimental studies on RA and analyzed them in the light of recent observations. We include analyses showing that several "historical" publications are contributing insights on many aspects of RA, and with a perspective focusing on the initial phase of RA pathogenesis, new approaches to therapy might be recruited to control and treat this serious illness.

RF as antibody to ICs

In 1957, an interesting observation was reported from the laboratory of Henry G. Kunkel of The Rockefeller University in New York. Using analytical ultracentrifugation, he and his associates identified proteins of high molecular weight (sedimentation rate of 22S) in sera of patients with RA (Franklin et al., 1957). The 22S component was a complex of 19S material (RF) and lower molecular weight material, one of which was 7S gamma globulin. The 22S peak could be completely removed by absorption with altered gamma globulin that had been denatured with mild heating. The following year, the same laboratory used a variety of ICs including human serum albumin (HSA)–rabbit anti-HSA, human IgG-rabbit anti–human IgG, and OVA-rabbit anti-OVA to show that all

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Abbreviations used: ACPA, anti-citrullinated protein antibody; DFP, diisopropylfluorophosphate; EBER, EBV-encoded small RNA; HSA, human serum albumin; IC, immune complex; MAC, membrane attack complex; OA, osteoarthritis; PAD, peptidyl arginine deiminase; RA, rheumatoid arthritis; RANA, RA nuclear antigen; RF, rheumatoid factor; RFLS, RF-like substance; SBE, subacute bacterial endocarditis; SDAI, simplified disease activity index; SpA, spondyloarthritis; SS, Sjögren's syndrome.

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of these ICs were able to remove RF from RA serum as efficiently as heat-denatured 7S IgG (Edelman et al., 1958).

The differing components of the ICs were the antigens, but the common component was the rabbit gamma globulin in these complexes. From these studies, the investigators were able to make the tentative conclusion that the determinants on the ICs that were reacting with RF were in the antibody and not the antigen components of the ICs. They cautiously stated that "a search for the other portion (antigen) of the hypothetical antigen–antibody complex giving rise to the RF might yield considerable further information" (Edelman et al., 1958). These observations and others described in more detail in their papers clearly point to the gamma globulin component of ICs as the target of RF, but the state of scientific technology at that time was inadequate to the task of identifying the antigen portion of such ICs.

Shortly after these two studies, the same laboratory made a study of patients with subacute bacterial endocarditis (SBE), a bacterial infection of heart valves as a consequence of repeated prior bouts of streptococcal pharyngitis leading to rheumatic heart disease. 50% (22/44) of these patients were positive for RF (Williams and Kunkel, 1962). In a way, this finding could have been confounding because RFs appeared not to be specific for RA. In contrast, in line with the preceding studies that had shown that ICs of different antigenic specificities were related to RF production, the finding in SBE would support what was at that time a novel hypothesis, that ICs themselves might be the immunogens inducing RF production.

A key study at this time by Abruzzo and Christian (1961) elucidated many features of RF production that have helped in understanding the observations just described. Rabbits were given intensive and prolonged immunization with killed *Escherichia coli* or *Bacillus subtilis*, and all animals that received *E. coli* injections developed what they called RF-like substance (RFLS), whereas with *B. subtilis*, a weaker antigen, only animals that developed bacterial agglutinins in high titer did so. They postulated that ICs formed during the course of immunization exposed "new" antigenic determinants on the gamma globulin molecule, stimulating production of RFLS, and that the titer of RFLS paralleled the duration of immunization and the intensity of the immune response to bacterial antigens. They also referred to earlier studies that RF in humans had been identified in nonrheumatoid conditions including leprosy, kala azar, syphilis, pulmonary tuberculosis, chronic liver disease, and sarcoidosis that were related to sustained immunological responses to microorganisms or other antigens. Interestingly, they raised the possibility that the old question of an infectious etiology for RA might come from a clearer understanding of RF.

Several other studies have emphasized that prolonged or intensive immunization in experimental animals are important in the induction of RF-like responses. In addition, the nature of the antigen can play a role. Repetitively arranged epitopes, such as are present in vesicular stomatitis virus, can induce RF production in mice after a single immunization (Fehr et al., 1997). This was postulated to be caused by efficient cross-linking of B cell receptors to induce T cell–independent IgM response. Of further interest were studies in which ICs of collagen II–anti–collagen II (Holmdahl et al., 1986) and ICs from synovial fluid of RA patients (Abedi-Valugerdi et al., 1995) were used to immunize mice, and in both of these studies, IgG RFs were produced in high titer. IgG RFs can form large complexes, including self-aggregating complexes, and interact with inflammatory cells (Mannik, 1992), but this class of RFs has not been used in clinical diagnostics, perhaps because of the long history and familiarity with IgM RF as a diagnostic tool. A comprehensive review of the prevalence of RFs in different clinical conditions that are associated with microbial infections has highlighted the importance of factors such as the requirement of a constant source of antigen to elicit the production of RF (Newkirk, 2002).

Citrullinated protein antigens as key players in the pathogenesis of RA

Although there are many different antigens that could be the "antigen" component of ICs driving RF production, citrullinated proteins might be a major player and the sought-after antigen mentioned in the Kunkel papers of the late 1950s. The forerunners of anti-citrullinated protein autoantibodies in RA were first described in 1964 (Nienhuis and Mandema, 1964), using scrapings of buccal mucosa cells as target substrates in immunofluorescence and called antiperinuclear factors. The observation did not gain a following perhaps because it was difficult to replicate, as only about 5% of buccal mucosa cell donors had cells containing a high number of perinuclear material (Schellekens et al., 1998). Subsequently, anti-keratin antibodies, as detected on rat esophagus sections, were described in RA (Young et al., 1979), and they turned out to be identical to antiperinuclear factor (Aho et al., 1993; Youinou and Serre, 1995).

Studies by Simon et al. (1993) and Schellekens et al. (1998) elucidated the perinuclear factor as filaggrin, a cytokeratin filament–aggregating protein that undergoes deimination, a posttranslational modification, during the terminal differentiation of squamous cells. This process is based on activation of intracellular enzymes called peptidyl arginine deiminases (PADs) that deiminate arginine-containing proteins.

Importantly, the resultant altered proteins are the targets of autoantibody responses. As will be discussed further, a process called homocitrullination has been shown to deiminate lysine-containing proteins to produce carbamylated proteins that are also immunogenic, and antibodies to carbamylated proteins (anti-CarP) are now an additional diagnostic marker for RA (Shi et al., 2011).

The major citrullinated antigens are both intracellular and extracellular proteins and include the α and β chains of fibrin or fibrinogen, vimentin, fibronectin, α-enolase, and collagen type II (Masson-Bessière et al., 2001; Vossenaar et al., 2004; Chang et al., 2005; Kinloch et al., 2005; Uysal et

al., 2009). The majority of studies have been focused on demonstrating the presence of citrullinated proteins in synovial tissue, but citrullinated α-enolase antigen and antibody to α-enolase, as well as PAD4 and PAD2 deiminating enzymes are present in synovial fluids from patients with RA, spondyloarthritis (SpA), and osteoarthritis (OA; Kinloch et al., 2008). Free citrullinated α-enolase was detected in all disease samples, and of interest, the mean levels were highest in RA, lower in SpA, and lowest in OA. PAD2 enzyme was detected in 18/20 RA patients, 16/20 SpA patients, and 0/20 OA patients. The striking finding was the presence of antibodies to α-enolase in 12/20 RA samples, none in SpA, and 1/20 in OA. An important question is the origin of α-enolase, which in the presence of antibody might be expected to exist in the form of ICs leading to binding of complement and activation of inflammatory mediators. Because the study was performed on effusions from knee joints, the origin of α-enolase and its subsequent citrullination could either be from synovial tissue secretions or from the circulation. As will be discussed later (see next section), neutrophils and other cells in the blood are most likely to be the sources of citrullinated antigens in the study cited.

To facilitate detection of ACPAs, a cyclic citrullinated peptide was synthesized as a surrogate for citrullinated proteins, although such citrullinated peptides might not exist in vivo. ACPAs have been detected in early RA (Paimela et al., 1992) and in archived sera from blood donors (Rantapää-Dahlqvist et al., 2003; Nielen et al., 2004). In the latter groups of subjects, multiple serum samples were available from healthy subjects who subsequently developed RA, making it possible to ascertain that ACPAs and IgM-, IgG-, and IgA-RFs could be identified in these individuals at a median time of 2.5 yr before clinical onset of joint disease. A more detailed study of such cohorts of blood donors (Shi et al., 2014) showed that the precise median time points when ACPA, anti-carbamylated fibrinogen, and IgM RF were detected before the appearance of overt clinical symptoms were 6 (range 3–10), 7 (4–10), and 2 (1–5) yr, respectively. Indeed, similar observations were made before by Aho et al. (Aho et al., 1991, 1993), who reported that RF and anti-keratin antibodies (i.e., ACPAs) antedated the clinical manifestations of RA. Worthy of special interest was that IgM-RF appeared later and not at the same time as the other two antibodies. This would be expected if IgM-RF was indeed antibody to ICs stimulated by prior appearance of antigens in the form of citrullinated and/or carbamylated protein antigen–antibody complexes. Another feature of interest in this study was that the number of different antibody specificities increased in many subjects as they approached the time clinical diagnosis was made, implying that there might be a threshold of inflammatory burden required for conversion to clinically active RA. Similar increases in the number of autoantibodies of different specificities before clinical manifestations have been described for type-1 diabetes and systemic lupus erythematosus. Other aspects of immune responses to citrullinated proteins including cellular immunity have been addressed in recent reviews (Wegner et al., 2010; Klareskog et al., 2013). Of note, ACPAs also occur in other diseases than RA, but at much lower frequencies (Abdel Fattah et al., 2009; Lima and Santiago, 2010; Singh et al., 2011).

IC-mediated activation of complement and generation of nonresolving inflammation

The identification of autoantigens such as citrullinated and carbamylated proteins and the induction of autoantibodies to these antigens followed by the appearance of RFs that are antibodies to ICs strongly suggest that the pathogenesis of RA is intricately involved with immune responses. Some of the earliest studies to examine this issue were directed at looking for complement activation in synovial fluid where antigen–antibody reactions with subsequent binding of complement might be expected to activate the complement cascade, resulting in production of different complement fragments with inflammatory properties. Complexes of immunoglobulin consisting primarily of IgG were identified that sedimented over a wide range of molecular weights (7S to 30S), and this group of patients with RA had markedly lower complement levels and lowered C1q and C3 in synovial fluid (Winchester et al., 1970). In the light of our current knowledge of properties of IgG RFs (Mannik, 1992), aggregates of IgG-RFs themselves or with non-RF IgGs might be capable of binding and activating complement, and this was what the authors postulated.

Other studies have confirmed the observation of C1q and C3 breakdown products in synovial fluid from RA patients (Moxley and Ruddy, 1985; Sjöholm et al., 1986). Complement activation in the synovial cavity has been shown to progress to completion in the complement cascade with the demonstration that the chemotactic complement fragment C5a and its more stable metabolite C5a *des Arg* are both elevated (Jose et al., 1990). The C5a fragments are highly chemotactic for neutrophils and play a major role in the large numbers of neutrophils migrating to inflammatory RA synovial cavities (Yamamoto et al., 1996). In addition, the other portion of activated C5 consisting of the C5b–C9 complex, also called the membrane attack complex (MAC), may play another important role in RA pathogenesis by damaging neutrophil membrane integrity with other dire consequences.

IC activation of complement appears to be a multiple hit condition in the synovial cavity. The first hit would come from a variety of ICs, some of which would consist of altered cellular antigens like citrullinated proteins and carbamylated proteins and their respective autoantibodies (De Rycke et al., 2005; de Hair et al., 2014). In addition, there are other antigen–antibody systems such as nuclear histones and nucleoproteins and their respective antibodies, part of the array of antinuclear antibodies commonly associated with rheumatic autoimmune diseases and previously shown to be present in RA synovial fluid and serum (Robitaille et al., 1973; Aitcheson et al., 1980). However, our hypothesis is

that the first hit would be ICs formed between infecting microbes combining with their respective antibodies. This first line of ICs would initiate complement activation and release anaphylatoxins like C3a, C5a, and other inflammatory mediators. In addition, and perhaps unique to RA and a few other diseases, we postulate that the same ICs would be recognized as antigens by RF, producing new ICs in the form of IC–RF molecular aggregates and reinitiating the cycle of complement binding and activation, leading to exacerbation and sustained inflammation in the synovial cavity. Evidence for RF–IC reactions that bind and activate complement have been abundantly documented (Tanimoto et al., 1975; Reyes et al., 1983; Zilberfarb et al., 1985) and reviewed in detail (Okroj et al., 2007). Of relevance in this context was the observation that ACPAs from RA patients activated both the classical and alternative complement pathways (Trouw et al., 2009). The first wave of IC-mediated complement activation as responses to infectious microbes would be followed by RF binding to citrullinated protein–anti-citrullinated protein ICs to start the second and subsequent rounds of complement activation. Recent clinical studies have shown that in RA patients, RF more than ACPA is directly related to and determines both progression of structural damage in the joints and disease activity (Aletaha et al., 2015). This conclusion was based on observations that patients with RF exhibit high disease activity, independent of presence or absence of ACPA (Aletaha et al., 2015). Baseline data were analyzed from multinational clinical trials that were designed to study the outcome of clinical trials with different therapeutic regimens: early RA with rituximab or methotrexate (Fig. 1, A and B) and established RA with golimumab or placebo (Fig. 1, C and D). In the left columns of Fig. 1, patients were matched in terms of ACPA titers and duration of disease to determine the relationship of RF with the simplified disease activity index (SDAI). Fig. 1 (A and C) shows that patients with RF had higher SDAI than those without RF in both groups and that this observation was independent of disease duration. When patients were analyzed in an analogous manner, but with matching RF titers to determine the potential contribution of ACPAs to disease activity (Fig. 1, B and D), patients with ACPAs had significantly lower disease activity. The totality of these data showing increased disease activity in patients with RF is fairly definitive, given that the patients came from a large number of clinical centers in different countries and were in both early and late stages of RA. It is important to note that this study of Aletaha et al. (2015) was not directed at determining the response of RA patients to different therapeutic regimens. The results depicted in Fig. 1 used patient data before their participation in the different clinical drug trials. Moreover, the fact that RF is highly associated with progression of joint damage has also been shown in a separate analysis (Aletaha et al., 2013); finally, disease activity has been known to be a major driver of joint damage.

As described previously, RFs in RA as well as in several other disease conditions are capable of activating the complement system (Tanimoto et al., 1975; Reyes et al., 1983; Zilberfarb et al., 1985; Okroj et al., 2007), and the observation that IgM-RF is more closely associated with pathogenicity may in part be related to its high complement activating property (Sabharwal et al., 1982). In line with these findings, RF-positive RA patients express higher levels of proinflammatory cytokines, especially TNF but also partly interleukin-6, than RF-negative patients, with little influence of ACPA (Sokolove et al., 2014). Indeed, progression of erosions but not joint space narrowing is related to the presence of RF (Aletaha et al., 2013), suggesting that ICs that involve RF may drive osteoclastogenesis. This is in line with findings that ICs activate macrophages to produce excessive amounts of TNF, presumably via interaction with Fc receptors (Debets et al., 1988; Mathsson et al., 2006; Aringer and Smolen, 2012). TNF, in turn, amplifies osteoclastogenesis, especially in the presence of receptor activator of NF-κB ligand (RANKL; Lam et al., 2000; Binder et al., 2013). All of these observations together are compatible with our view that disease activity and joint damage in RA are determined by ICs that include RF, with ACPA-containing ICs serving as the major target of RFs.

The origin of RF has not been clarified in complete detail. However, RFs belong to the family of natural autoantibodies that originate mostly in B-1b B cells (Hardy et al., 1987; Hardy, 2006). This population of B cells may be regulated differently than other populations and may be subject to a higher degree of plasticity.

Indeed, patients with RA in whom disease is well controlled may experience a significant reduction of RF levels and even turn seronegative, an observation that is more rarely made for ACPA (Böhler et al., 2013). Nevertheless, it is conceivable that in RA, RFs may also be produced by other B cell populations (Natvig et al., 1990).

Nonresolving inflammation is a concept advanced by Nathan and Ding (2010), whose underlying thesis is a state of perpetual inflammation when the host reaction to an initial inflammatory stimulus induces a response caused by damaged tissue that propagates and perpetuates the inflammation. This concept has been widely accepted by investigators in infectious diseases such as tuberculosis, leprosy, and kala azar and in noninfectious diseases such as atherosclerosis, obesity, cancer, and other conditions. Like Nathan and Ding (2010), we believe that the clinical and experimental observations in RA fit well with the concept of nonresolving inflammation or perpetuation of inflammation by the accrued tissue damage from host response and, importantly, might elucidate the inflammatory pathways involved in bringing about repeated cycles of antigenic stimulation and antibody responses with the associated pathological consequences (Smolen et al., 2009).

The inflamed RA joint has special characteristics. The knee joint has synovium typical of chronic infection with extensive infiltration by macrophages, lymphocytes, and synovial proliferation but few neutrophils. In contrast, synovial fluid is chockfull of neutrophils, sometimes comprising 90% of total leukocyte counts of 50,000 cells/μl. It was noted

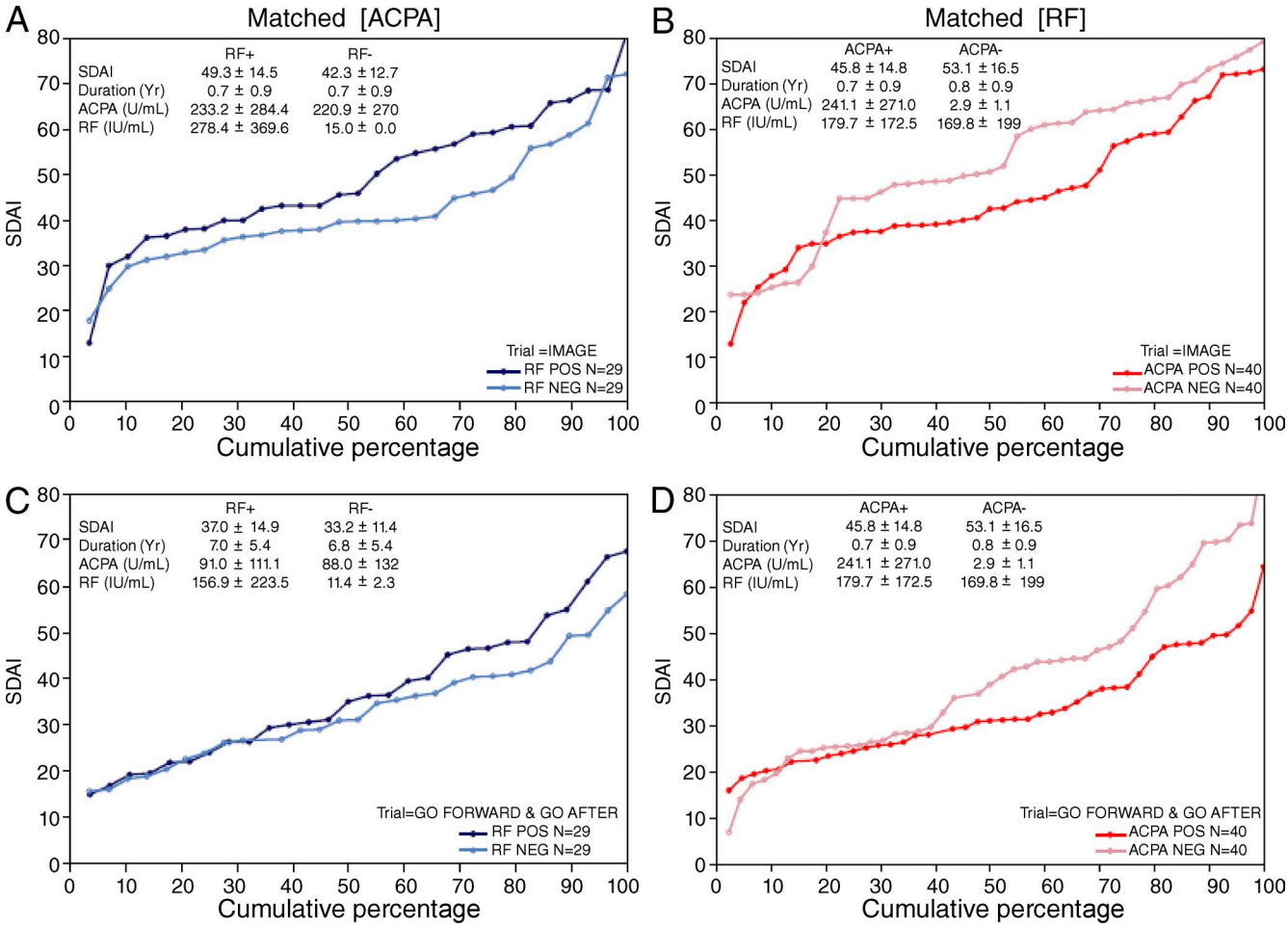

Figure 1. **Association of ACPA or RF with disease activity according to the SDAI.** Probability plots of levels of disease activity according to SDAI. (A and C) Distributions of baseline SDAI values by RF status (low titer vs. high titer) in patients matched for ACPA levels and disease duration: higher disease activity in RF-positive patients overall, P = 0.0067. (B and D) Distributions of baseline SDAI values by ACPA status (low titer vs. high titer) in patients matched for RF levels and disease duration of RA: higher disease activity in ACPA-negative patients, P = 0.045. Data were analyzed from patients before they received clinical trial drugs: the IMAGE trial on patients with early RA (A and B) and on long-standing and early RA in the GO-FORWARD+GO-AFTER trials (C and D). See text and Aletaha et al. (2015) for details. Statistically, the Greedy matching algorithms and the Mahalanobis distance calculations were applied in these assessments (adapted from Aletaha et al. [2015]; reproduced with permission from the authors).

that these effusions often remained constant in volume and in differential cell type. This led some investigators to think that these polymorphonuclear cells were being sequestered in the synovial cavity in a relatively adynamic state. It prompted Hollingsworth et al. (1967) to perform a study in which they followed the disappearance of labeled diisopropylfluorophosphate (DFP) injected into the knee. The DFP marker would attach to leukocyte membranes, and its disappearance over time would give an indication of leukocyte turnover. To their surprise, the loss of labeled granulocytes was rapid, and they calculated that the granulocyte half-life in knee synovial cavities was ∼4 h. They calculated that in an effusion of 30 ml containing 25,000 granulocytes/µl, the daily breakdown could exceed a billion cells. The preponderance of neutrophils in RA synovial fluids has been confirmed by many other investigators, and some experiments have shown that this could be active migration related to chemotactic factors (Norberg et al., 1983).

In the synovial cavity, C5a is the major neutrophil chemoattractant factor, and the state of neutrophilia is augmented by C4a, which is a chemotaxis inhibitor for monocytes (Yamamoto et al., 1996), thus allowing preferential accumulation of neutrophils. The combination of these factors results in leukocyte infiltration patterns in synovial tissue and joint fluid, which are distinctly different. The role of IC-mediated inflammation in the synovial cavity by way of complement activation is of even greater impact on pathogenesis because of recent informative studies showing that RA synovial fluid cells contain a broad range of citrullinated cellular proteins of different molecular weights (Romero et al., 2013; Zhou et

al., 2015). This hypercitrullination could be induced by two immune-mediated membranolytic pathways, one related to perforin released by lymphokine-activated killer cells and the other by the C5b-C9 MAC, both of which cause loss of cell membrane integrity by producing pores in cell membranes. In synovial fluid, there is a paucity of mononuclear cells, and membranolytic activity is probably related more to the function of MACs than to lymphokine-activated killer cells. The crucial relationship of these studies to the neutrophilia in synovial fluid induced by C5a is that neutrophils have intrinsic cytosolic PADs that are activated after influx of extracellular fluid calcium, which is of higher molarity than that in the intracellular milieu (Romero et al., 2013; Zhou et al., 2015). Hypercitrullination could then occur in the cellular proteins of neutrophils to produce an abundance of antigens to stimulate antibody responses.

This would in turn produce more IC formation and more complement activation, creating recurring cycles of nonresolving inflammation. This situation could be further aggravated by production of RF creating another tier of ICs and inflammatory mediator formation. It raises the possibility that some "sequestered" sites like synovial cavities and gingival crevices provide environments conducive for certain immunochemical reactions to take place, like recurring cycles of IC formation and complement activation. Other such sequestered sites of nonresolving inflammation and, interestingly, associated also with RF formation are probably the verrucae of diseased heart valves in SBE, the tubercles of granulomatous tissues in sarcoidosis and tuberculosis, and other granulomatous lesions seen in sarcoidosis, leprosy, and tertiary syphilis.

Microbial infections as triggers initiating RA

The possibility that EBV infection might be involved in causation of RA arose from studies to identify diagnostic autoantibodies in Sjögren's syndrome (SS). In a subset of SS called SS-RA or SS associated with RA (Bloch et al., 1965), a precipitating antibody to an antigen in solubilized extract of B lymphocyte cell line WiL2 was detected (Alspaugh et al., 1976) and later shown to be specific to RA and not to SS (Alspaugh and Tan, 1976). The WiL2 cell line was an EBV-infected B lymphocyte cell line, and further studies showed that other EBV-infected B lymphocyte cell lines contained the RA-reactive antigen, but extracts of EBV-negative B cell lines were not reactive, nor were T cell lines (Alspaugh et al., 1978). The RA-reactive antigen could be induced in naive B cell lines by transformation of cultured B lymphocytes with EBV (Alspaugh et al., 1978). It was postulated that because EBV has been implicated as an etiologic agent in diverse diseases like Burkitt's lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis, host responses must play an important role if it were to be involved in the etiology of RA (Tan, 1979).

Subsequent studies by many investigators added important information on specific host responses to EBV and the identity of the RA precipitin, which in view of its immunohistochemical localization was called RA nuclear antigen (RANA). The frequency of anti-RANA in RA sera was as high as 93–94% in RF-positive RA in some studies (Catalano et al., 1979; Ng et al., 1980), and further important findings were that anti-RANA, by either immunoprecipitation or immunohistochemistry, was significantly higher in titer and frequency in RA sera than normal sera and sera from many different controls (Catalano et al., 1979, 1980; Ng et al., 1980; Alspaugh et al., 1981; Ferrell et al., 1981).

The identity of RANA is likely to be a peptide encoded by a nucleotide sequence region in EBV called IR3, which expresses part of EBV nuclear antigen 1 (EBNA1) with long stretches of glycine-alanine repeats (Baron and Strominger, 1978; Billings et al., 1983; Hennessy and Kieff, 1983; Rhodes et al., 1985; Venables, 1988; Venables et al., 1988).

Elevated frequencies of EBV-infected cells have been found in blood of RA patients (Tosato et al., 1984), in cells isolated from saliva, in synovial fluid cells, and in bone marrow cells (Newkirk et al., 1994; Blaschke et al., 2000; Magnusson et al., 2010). As evidence that EBVs in these cells were capable of inducing cell transformation, a spontaneously growing fibroblastoid cell line called DSEK was established from RA synovium that expressed EBV proteins EBNA1 and 2, early antigen (EA), VCA, and latent membrane protein 1, as well as the capacity to produce of several cytokines, IL-10, basic fibroblast growth factor (bFGF), and TGFβ1 (Koide et al., 1997). IL-10 levels have been reported to be elevated in RA synovial fluid (Cush et al., 1995).

Recently, new EBV detection methods have provided convincing support for EBV infection in RA synovium. EBV-encoded small RNAs (EBER1 and 2) are expressed in virtually all EBV-infected tumors and in lymphoid tissues of infectious mononucleosis (Chang et al., 1992; Fan and Gulley, 2001; Iwakiri and Takada, 2010; Iwakiri, 2014). Another EBV-encoded marker, BZLF1, is a transcription activator expressed during the lytic phase of EBV infection associated with viral replication (Scotet et al., 1996; Wen et al., 2007). EBER1 and 2 have been detected in synovial tissues with prominent lymphocytic proliferation and at the apex of villus projections and also more frequently in RA patients refractory to treatment (Takei et al., 1997; Chiu et al., 2013). EBV-DNA–positive individuals detected by PCR of synovial tissue extracts were at 5.47-fold higher risk of presenting with RA than EBV-negative individuals (Saal et al., 1999). The concurrent detection of EBV-DNA, EBERs, CMV, and parvovirus B19 in RA synovium has also been reported (Mehraein et al., 2004).

The presence of lytic EBV infections in RA synovial tissues was analyzed in a rigorous study by Takeda et al. (2000), who used several different methods to detect EBV-DNA and EBV-encoded RNA and proteins in synovial tissues of 32 patients with RA and 30 control patients with OA. By PCR, EBV-DNA was detected in 15 of the 32 (45%) samples from RA patients but in none of the 30 OA

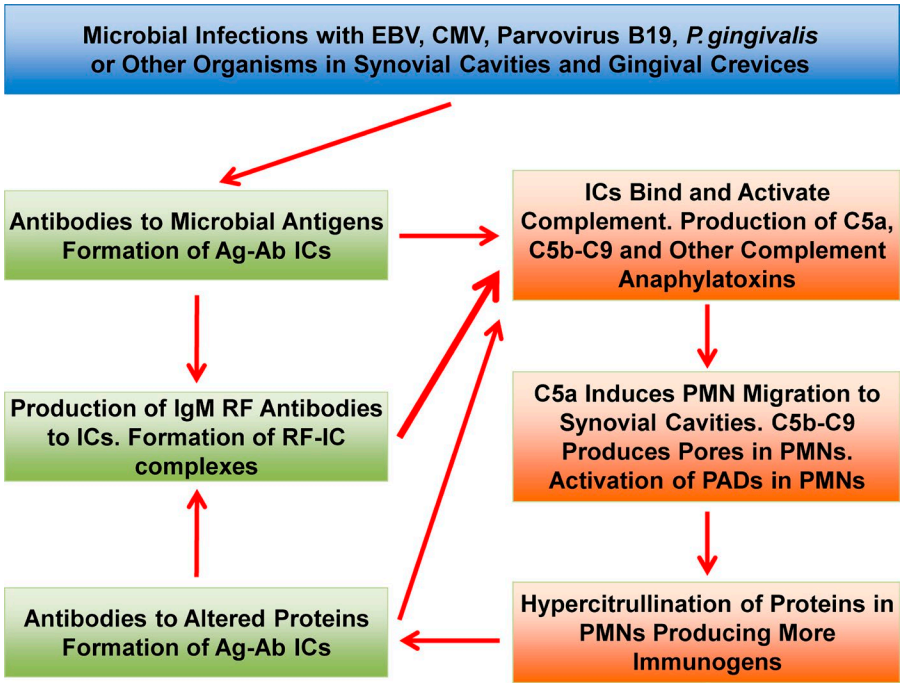

Figure 2. **A model for the early phase of RA.** Infectious organisms such as EBV in synovial tissue cycle between latent and lytic states, producing viral antigens that stimulate antibody responses and form ICs. ICs composed of EBV antigens and antibodies bind and activate the complement cascade, producing C5a anaphylatoxin and C5b-C9 MAC. C5a is a chemoattractant for neutrophil/PMN migration to the synovial cavity, and MAC induces pores in neutrophil cell membranes, causing influx of extracellular calcium and activating intrinsic neutrophil deiminating enzymes (PADs) to produce altered proteins such as citrullinated α-enolase. These and other altered cellular proteins induce new antibodies and form more ICs that bind and activate complement to repeat the inflammatory cycle described. The continuing production of antigens by EBV and by deiminating enzymes sustains a state of IC formation stimulating production of IgM RF, which binds to these ICs, reinitiating complement fixation and activation. The pivotal role of RF in perpetuating cycles of inflammation is depicted as a thicker arrow. This model points to certain targets where therapeutic intervention might break these cycles of nonresolving inflammation.

patients. Using an EBV-positive cell line, Raji, which contained a known number of EBV copies per cell as reference, they calculated that 9 of the 15 EBV-DNA–positive tissues had more than one EBV copy/1,000 cells and 6 contained one copy/1,000–5,000 cells. These results indicated that in RA synovium, the nine patients with more than one copy of EBV-DNA/1,000 cells had >100 times the EBV-DNA present in peripheral blood mononuclear cells of EBV-seropositive individuals, as reported previously in other studies (Saito et al., 1989; Wagner et al., 1992). Five of these nine patients were also positive for EBER, by RNA in-situ hybridization. Of special interest because of its association with the lytic phase of infection and virus replication, all of these nine samples were positive in immunohistochemistry for the lytic phase protein BZLF1. The investigators were impressed by the large numbers of BZLF1-positive specimens because in a previous study (Koide et al., 1997), they found that even in a spontaneous EBV-producer fibroblast cell line derived from culture of RA synovium, not more than 5% of cells expressed BZLF1. They stated that their study (Takeda et al., 2000) showed that there was an extremely high EBV load in the synovial tissue of RA patients as well as ongoing active EBV infection. These recent studies give strong evidence that EBV infection, both latent and lytic, are present in RA synovial tissue (Scotet et al., 1996; Takei et al., 1997; Saal et al., 1999; Takeda et al., 2000; Mehraein et al., 2004; Wen et al., 2007; Iwakiri and Takada, 2010; Chiu et al., 2013).

A hypothetical model for the initiation phase of RA and rational approaches to therapy

The targeted biological therapies introduced over the last two decades directed against cytokines, B cells, or costimulatory molecules have allowed unprecedented treatment responses. However, they (a) still do not allow 30–40% of patients to reach the desired therapeutic effect and (b) do not cure the disease, as stopping therapy is usually followed by reactivation of RA (Smolen et al., 2013). Therefore, other approaches may have to be sought that, on the one hand, tackle the autoimmune response and its early consequences rather than pathways downstream of that response and, on the other hand, focus at potential etiologic factors that might give leads to the possibility of a curative therapy. We present in Fig. 2 what we postulate could be several etiological and pathogenic mechanisms that might be involved in early RA. We are not addressing the cellular aspects of the immune response but have focused mainly on the idea that in early RA, antigen–antibody ICs and complement-mediated inflammation are the driving forces contributing to the development of full-blown disease. The cellular aspects of the immune response in RA might be intertwined with the initiation phase or succeed it, but this aspect of RA is not in the scope of this paper. We also advance the notion that this model of RA points to several approaches to therapy that are based on intervention of pathogenic pathways relevant to early RA and may continue to play a role in the later stages of the disease.

Microbial organisms, including EBV, CMV, parvovirus B19, and *Porphyromonas gingivalis* have been implicated as triggers that initiate the onset of RA (Fig. 2), and the evidence for EBV involvement has been discussed. The left half of the figure shows the three major categories of ICs in synovial fluid, binding of microbial antigens with antibodies, binding of altered autologous proteins with antibodies, and binding of IgM RF with the first two categories of ICs.

A key feature in this initial phase of RA is the fixation of complement by ICs and the activation of the complement cascade with formation of anaphylatoxins and other inflammatory mediators shown on the right half of Fig. 2. C5a is a strong chemoattractant for neutrophils, and C5b-C9 MAC causes damage to cell membrane integrity by making pores that allow ingress of extracellular fluids. This raises levels of intracellular calcium, causing activation of intrinsic PADs in neutrophils and producing hypercitrullination of neutrophil proteins (Romero et al., 2013; Zhou et al., 2015). These antigens stimulate the production of antibodies that are detected as ACPAs, ICs are formed that fix complement, and the process of complement-mediated inflammation is repeated. Importantly, as depicted in Fig. 2, both citrullinated protein– and microbial antigen–associated antigen–antibody complexes have the capacity of stimulating RF production, and the binding of RF to these ICs reinitiates complement fixation and activation. This hypothesis conforms well with the concept of a situation of nonresolving inflammation (Nathan and Ding, 2010).

The proposed model of the initiation of RA raises certain possibilities for novel therapeutic intervention of the disease process. Two important objectives could be obtained by reducing calcium concentration in the synovial cavity, the first being prevention of complement activation and the second being inhibition of deiminating PAD enzymes in invading neutrophils, both of which are calcium dependent. This could be achieved by treatment with chelating agents like EDTA, a therapeutic modality that has been used in a National Institutes of Health–funded multi-institutional study for the treatment of postmyocardial infarction patients (Clarke et al., 1956; Born et al., 2013; Lamas et al., 2013; Avila et al., 2014; Peguero et al., 2014; Sidhu et al., 2014). The objective of these studies was to produce transient hypocalcemia with the intention of decalcifying atherosclerotic plaques (Avila et al., 2014). Of interest is that intravenous EDTA therapy has also been used in RA, the rationale here being directed at normalization of presumptive abnormal turnover rates of collagen and elastin in inflamed synovial membranes and correction of this connective tissue metabolism defect by stimulation of parathyroid gland function (Leipzig et al., 1970). Although several good clinical responses were claimed, there have been few reports of such trials since then (Bamonti et al., 2011). We propose that EDTA therapy should be revisited in RA but with different rationales in mind: the objective of EDTA therapy would be to reduce calcium concentrations in the synovial cavity to neutralize IC-induced activation of complement and deiminating enzymes. Because the activation of these pathogenic pathways is in the synovial cavity, EDTA therapy should be targeted exclusively to those sites in order to deliver adequate doses and avoid side effects related to systemic intravenous therapy. We are cognizant of the fact that intra-articular therapy is more arduous than oral therapy, but if targeted therapy at inflamed joints is justified, formulations of EDTA feasible for percutaneous application at such sites could conceivably be devised. There is the possibility that in the case of polyarthritis, targeted therapy of a limited number of joints and reducing but not eliminating the total inflammation burden might be of benefit. Another approach to mitigating the inflammation in RA joints would be to target therapy at the complement system at various points along the activated complement cascade. There have been excellent review articles on modulation of complement as potential treatment for autoimmune and other diseases (Kalli et al., 1994; Holers, 2003; Wagner and Frank, 2010). Because C5a may be playing an important role in attracting neutrophils carrying potential citrullinated protein antigens to the joints, this complement anaphylatoxin would be a logical target. One such therapeutic agent would be an anti-C5a monoclonal antibody called eculizumab (Soliris), which has been successfully used in the treatment of paroxysmal nocturnal hemoglobinuria (Dmytrijuk et al., 2008) and has also been shown to prevent collagen-induced arthritis in experimental animals (Wang et al., 1995), but it may be effective in only a subset of patients, namely those with high synovial complement consumption. Also, many other drugs targeting complement components and receptors are in clinical trials (Kalli et al., 1994; Holers, 2003; Wagner and Frank, 2010; Woodruff et al., 2011), and some of these may be effective in RA. As in the case of calcium chelators, it would be important to focus therapy at the joints. The basis for targeting synovial cavity over systemic therapy might be further rationalized by clinical observations that, in spite of highly active complement consumption in the joints, complement levels in peripheral blood are normal or even slightly elevated given the nature of complement components as acute phase reactants, indicating that IC complement–mediated inflammation is taking place primarily in the synovial cavity.

We have described studies showing that there is high expression of EBV biomarkers in RA synovium, including EBV-DNA, EBERs, latent membrane protein 1, and lytic phase–associated EBV protein BZLF1, which altogether denotes that there might be an ongoing dynamic cycling between the latent and lytic phases of EBV infection. BZLF1 is a transcriptional activator that initiates the disruption of latency by triggering signaling cascades leading to viral replication and virion production (Scotet et al., 1996; Takeda et al., 2000; Wen et al., 2007). BZLF1 is also highly immunogenic and elicits robust $CD4^+$ and $CD8^+$ T cell responses, and $CD8^+$ T cells can confer protection from EBV-related lymphoproliferative disease in experimental animals (Scotet et al., 1996). BZLF1 is currently a major target of vaccine de-

velopment for EBV-related conditions like infectious mononucleosis, posttransplant lymphoproliferative disease, and nasopharyngeal carcinoma (Hartlage et al., 2015). RA would be another condition deserving trials with BZLF1 vaccines to determine whether the cycle of latent to lytic infection can be disrupted. This would be especially desirable in chronic remitting RA resistant to current therapeutic modalities, where there is a higher expression of EBV markers in synovial tissue (Takei et al., 1997; Takeda et al., 2000; Iwakiri and Takada, 2010; Chiu et al., 2013).

Conclusion

We wish to emphasize that we have focused only on early RA, which studies from the past and present suggest is initially a multifaceted IC disease. The ICs exist as ICs composed of microbial (such as but not limited to EBV) antigens and antibodies, ICs composed of altered autologous proteins (such as but not limited to citrullinated proteins) and antibodies, and ICs composed of one or the other of the two classes of ICs described above complexed with RF. We have described how interactions between and among these entities associated with activation of complement in synovial cavities could lead to continuous reinitiation of inflammation. This model allows us to consider some therapeutic approaches that are targeted at breaking up these repeating cycles of immune reactions leading to inflammation. There are undoubtedly other target sites for therapeutic intervention that we have not described. There is a good possibility that intervening at some junctions in these intertwining inflammatory pathways may add new treatments to the current therapeutic armamentarium.

It is of interest to reexamine the forerunner studies that led to identification of RF. Erik Waaler was doing "routine work with complement fixation reactions" (Waaler, 1940) when he noticed that serum from a patient with RA caused agglutination, not hemolysis, of antibody-sensitized sheep red cells. In a series of carefully controlled studies, he came to the conclusion that antibody coating of the red cells as well as a globulin fraction in RA serum were necessary components in the agglutination phenomenon. He called the component in RA serum agglutination-activating factor. However, he noted that some control sera also showed this activity, although with lower intensity reactions than RA sera. When he settled on a method to classify intensity of agglutinating activity, he found that two thirds of the RA patients showed no difference from cases in the control group. He concluded that "the reaction can therefore not be of any diagnostic value." We consider this conclusion may be minimizing the significance of his observations because on examination of Table 5 in his article, we notice that in terms of agglutination-activating factor characterized as "slight to marked," 27/77 (35.0%) of RA patients were positive compared with 10/202 (4.9%) of his controls. The finding, which challenged Waaler and made him arrive at his conclusion, was likely to be the high percentage of RA patients 50/77 (64.9%) that showed "insignificant reaction." Several years after this study, Rose et al. (1948), using a somewhat similar sheep red cell agglutination technique, showed that in 27 RA patients with active joint disease, 23 (85.1%) had high serum titers of agglutination activating factor, whereas in 16 RA cases in remission, 11 (68.7%) had insignificant reactions and only 5 (31.2%) had positive reactions. In the study by Waaler (1940), the RA patients he studied were described as under treatment at a rheumatology clinic without any other characterization, and it might be possible that many of them might have been in remission and therefore showed "insignificant reactions." After these forerunner studies, several other studies have confirmed the utility of RF in clinical diagnosis of RA (Plotz and Singer, 1956; Vaughan, 1956; Milgrom et al., 1964).

The studies just described led to a series of publications on the immunochemical reactivities of RF (see section on RF as antibody to ICs above). What were most thought provoking were the observations and hypotheses of The Rockefeller University Kunkel group (Franklin et al., 1957; Edelman et al., 1958) that IgM-RF, presumably bound to ICs and sedimenting as 22S complexes in analytical ultracentrifugation, could be detected in some RA sera. They suggested that identification of the RF-targeted ICs might "yield considerable further information." This prompted us to pursue this line of investigation with the specific objective of attempting to determine what RF in the role of antibody to ICs might be playing in the etiopathogenesis of RA. This notion was in part stimulated by our earlier studies in systemic autoimmune diseases that suggested that autoantibody induction might be antigen driven (Tan, 1989; Fritsch et al., 2002), a feature which was also detected in cancer as serum autoantibodies to oncogenic proteins such as c-myc or to tumor suppressors such as mutated p53 (Tan and Zhang, 2008). Therefore, identification of the antigen component of ICs in RF–IC complexes might contribute information on cellular or tissue proteins participating in RA pathogenesis.

The IC, which turned out to be most helpful in elucidating this question, was described in a paper (Nienhuis and Mandema, 1964) in which immunofluorescent microscopy was used to identify antibodies in RA sera that reacted with perinuclear factors in buccal mucosa cell scrapings of healthy individuals. Subsequent studies by many other investigators (Young et al., 1979; Aho et al., 1993; Simon et al., 1993; Youinou and Serre, 1995; Schellekens et al., 1998) showed that the antigen was filaggrin, a cytokeratin filament-aggregating protein. Its antigenicity was related to deimination of amino acid arginine to produce the citrullinated form of the protein. A compelling reason for focusing our studies on citrullinated protein antigen–antibody complexes was in part related to the very high incidence (70–82%) of such antibodies in RA sera (Schellekens et al., 2000; van Venrooij and Zendman, 2008). In autoimmune conditions in general, the frequency of autoantibodies is usually in the range of 15–40% (Tan, 1989), and this high frequency of ACPAs suggested that this antigen–antibody system could be playing a major role in immune reactions in RA.

The next question that had to be addressed was where citrullination was taking place and how immune-mediated inflammatory reactions were initiated in RA patients. The answers to these questions appeared to fall logically into place when one considers that RA is an inflammatory condition of joints, that complement-mediated inflammation is highly activated in synovial cavities, that citrullinated proteins as a source of antigens have been shown to be produced in abundance by PAD2 and PAD4 enzymes in invading neutrophils, and that because of repeated cycling of antigen–antibody binding between citrullinated proteins and antibodies and between RF and preformed ICs, a state of nonresolving inflammation might be taking place, as depicted in Fig. 2. The involvement of EBV as one of many infectious agents that might initiate this cascade of inflammation has been strongly supported by recent findings of both latent and lytic phase EBV antigens in synovial tissues, suggesting that certain microbial antigens (EBV being one) might be in a state of continuous antigen presentation to immune cells. In addition, it would be important not to think of RF only as an important diagnostic tool in RA, but also to seriously consider the various ways RF as antibody to ICs might be involved in perpetuation of complement-mediated inflammation. We present this model of the initiation phase of RA as a starting point for further analysis and input by investigators in the field so that we might arrive at a better understanding of the challenging and complex nature of this disease.

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