Commentary

Citrullinated proteins: sparks that may ignite the fire in rheumatoid arthritis

Erik R Vossenaar and Walther J van Venrooij

Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands

Corresponding author: Erik R Vossenaar (e-mail: e.vossenaar@ncmls.kun.nl)

Received: 28 Jan 2004 Revisions requested: 15 Mar 2004 Revisions received: 5 Apr 2004 Accepted: 5 Apr 2004 Published: 19 Apr 2004

Arthritis Res Ther 2004, **6**:107-111 (DOI 10.1186/ar1184)

Abstract

Antibodies directed to citrullinated proteins (e.g. anti-CCP [cyclic citrullinated peptide] antibodies) are highly specific for rheumatoid arthritis (RA). These antibodies are produced at the site of inflammation in RA, and therefore citrullinated antigens are also expected to be present in the inflamed synovium. We discuss literature showing that the presence of citrullinated proteins in the synovium is not specific for RA. The RA-specific antibodies are therefore most likely the result of an abnormal immune response that specifically occurs in RA patients. It was recently shown that presence of anti-CCP antibodies precedes the onset of clinical symptoms of RA by years. It thus appears that it may take years for initial events that cause the generation of anti-CCP antibodies to develop into full-blown disease.

Keywords: anti-CCP autoantibodies, citrullination, peptidylarginine deiminase, rheumatoid arthritis

Introduction

Autoantibodies directed to citrullinated proteins (e.g. anti-CCP [cyclic citrullinated peptide] antibodies) are specific serological markers for rheumatoid arthritis (RA). These antibodies (for review [1]) are detected in approximately 80% of RA patients at a specificity of 98% [2–4]. Moreover, the antibodies are often present in the early stages of the disease and are predictive of disease outcome [5,6]. It was recently shown that several RA-associated genetic factors may be functionally linked to RA via modulation of the production of citrullinated proteins or the antibodies directed at them [7]. Taken together, it appears that autoreactivity against citrullinated proteins might be involved in the disease process in RA.

If such a functional relationship exists, then the antibodies and the antigens are expected to be present at the site of inflammation. This is clearly the case. Anti-citrullinated protein antibodies are produced locally in the synovium, as indicated by their 1.4-fold higher proportion of total IgG in synovial fluid than in serum (our unpublished data). Furthermore, Masson-Bessière and coworkers [8] showed that the antibodies comprised a 7.5-fold higher proportion of IgG in synovial tissue than in serum. These findings suggest diffusion of the locally produced antibodies from the synovium to the periphery. The presence of anti-CCP antibody producing plasma cells in the synovium is indicative of an antigen-driven maturation of CCP-specific B cells at the site of inflammation in RA [8,9]. It is therefore likely that citrullinated proteins are present in the inflamed RA synovium. This raises several questions that are discussed here.

When does citrullination occur?

Citrullination is the post-translational conversion of arginine residues to citrulline residues by peptidylarginine deiminase enzymes (PADs; EC 3.5.3.15; for review [10]). Five isotypes of PAD have been described in mammals. Based on the tissue-specific expression of the enzymes, PAD2 and PAD4 are the most relevant to RA because they are expressed by certain leucocytes. Normally, PAD enzymes are present intracellularly as inactive enzymes [11]. Calcium ions are required for activation of the PAD enzymes, but the intracellular calcium concentration in normal cells (10^{-7} mol/l) is much lower than the threshold calcium concentration for PAD activity (approximately

CCP = cyclic citrullinated peptide; HLA = human leukocyte antigen; PAD = peptidylarginine deiminase; RA = rheumatoid arthritis.

10^{-5} mol/l; our unpublished observations and data from Takahara and coworkers [12]). During cell death, however, the integrity of the plasma membrane is lost [13,14], causing influx of calcium from the extracellular space and subsequent activation of intracellular PAD [11,15–18]. Alternatively, PAD enzymes may leak out of the dying cells, become activated (the extracellular calcium concentration is approximately 10^{-3} mol/l, which is sufficient for PAD activity [12]), and cause citrullination of extracellular proteins.

Which citrullinated proteins are likely to be found in the RA synovium?

Many cells in the inflamed synovium have fragmented DNA, which is generally considered a sign of apoptosis. Nevertheless, these cells lack an apoptosis-specific morphology, and the presence of large numbers of apoptotic cells in the inflamed synovium is therefore still debated [19]. This discrepancy could be explained by the possible occurrence of impaired apoptosis. It may be that the apoptotic process is halted or that apoptotic cells switch to necrosis. In both cases, PAD enzymes may become activated by raised intracellular calcium levels, as probably is the case during the terminal differentiation of keratinocytes [20], a process that exhibits many parallels with halted apoptosis. We recently found that vimentin is citrullinated in dying human macrophages [11]. Because macrophages are abundant in the RA synovium, it is not surprising that citrullinated vimentin is present in the synovium as well. Indeed, the Sa antigen, which was recently identified as citrullinated vimentin [21], can be detected in pannus tissue [22]. The Sa antigen is specifically targeted by autoantibodies in RA sera (for review [23]).

In the inflamed synovium, oxygen metabolism is in disequilibrium. This leads on one hand to sites with oxygen excess (and subsequent generation of reactive oxygen species) and on the other hand to sites of hypoxia, which can cause synovial tissue microinfarctions [24,25]. At these sites, plaques containing extravascular fibrin are commonly found. Masson-Bèssiere and coworkers [26] have shown that such deposits contain citrullinated proteins. One of these proteins could be identified as citrullinated fibrin, which is efficiently recognized by autoantibodies present in RA sera.

Although the presence of citrullinated histones in the inflamed synovium has not (yet) been reported, it is not unlikely that they are present. Citrullination of histones has been described *ex vivo* during calcium-ionophore induced apoptosis of granulocytes [17,18]. Large numbers of granulocytes are present in the inflamed synovium, especially in the synovial fluid. Because granulocytes have a lifespan of only about 3 days, they will die in large numbers at the site of inflammation, which may trigger histone citrullination.

In summary, although many different citrullinated proteins may be present in the RA synovium, thus far three proteins can be considered as candidate autoantigens in RA: citrullinated fibrin, citrullinated vimentin and citrullinated histones.

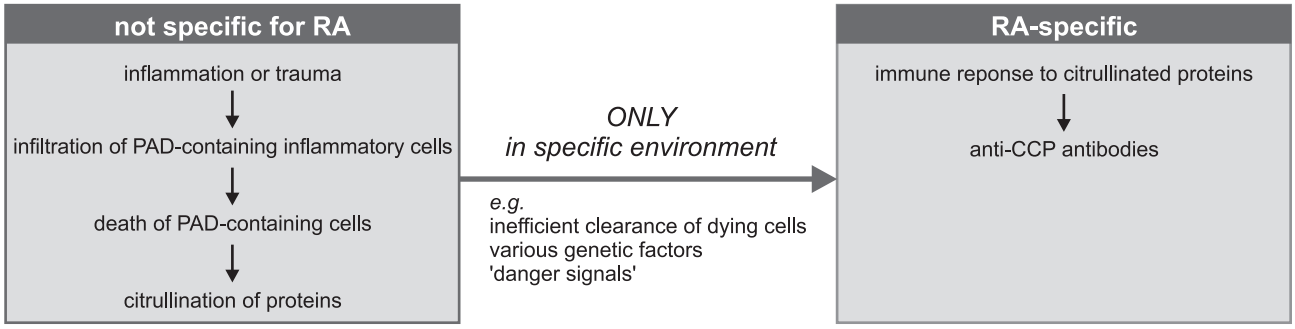
Is the occurrence of synovial citrullinated proteins a phenomenon specific to RA?

Two possible explanations may be considered for the high specificity of autoantibodies directed to citrullinated antigens for RA. One possible explanation is that there is an RA-specific overexpression of citrullinated antigens in the rheumatoid synovium that leads to an immune response. Alternatively, presence of citrullinated proteins may be a common phenomenon in any inflamed (synovial) tissue but RA patients may have an abnormal humoral response to them.

The first possibility is supported by the finding of genetic polymorphisms in the PAD4 gene [27]. One haplotype of PAD4 was associated with susceptibility for RA. Although it was not shown whether the PAD4 encoded by the susceptible haplotype exhibits an altered enzymatic function, it was shown that the RA-susceptible haplotype increases PAD4 mRNA stability. In theory, this could result in more PAD4 enzyme being produced and subsequently lead to increased citrullination of proteins and a higher chance of developing anti-CCP antibodies [7,27]. Unfortunately, the increased presence of citrullinated proteins was not investigated in that study.

Some studies dealing with the second possibility have recently been published. Masson-Bèssiere and coworkers [26] showed that various citrullinated proteins are present in the synovial tissue of RA patients. They could be detected in the cytoplasm of various mononuclear cells as well as in deposits of extravascular fibrin (as described above). Although no synovial tissue specimens of non-RA patients were initially investigated, a recent follow up of that study [28] showed that citrullinated fibrin may also be present in synovial tissue of patients with other forms of joint inflammation. By contrast, Baeten and colleagues [29] found citrulline staining in about half of the RA patients studied but in none of the control individuals. However, the staining pattern they observed was quite different from that in the study conducted by Masson-Bèssiere and coworkers [26]. The immunohistochemical staining was observed in a few, widely dispersed cells but not in extracellular structures. The explanation for this discrepancy is probably that the antibody used in the study by Baeten and colleagues was developed for the detection of free L-citrulline and not for the detection of citrullinated proteins [30]. This is illustrated by the fact that the staining could be completely abolished by competition with free L-citrulline [29]. In addition, staining patterns similar to the ones described by Baeten and coworkers

Figure 1



Schematic representation of the processes that may lead to the citrullination of proteins (left panel) and the immune response to these proteins (right panel). As discussed in the text (under the headings 'When does citrullination occur?' and 'Which citrullinated proteins are likely to be found in the RA synovium?'), peptidylarginine deiminase (PAD)-containing inflammatory cells infiltrate the site of inflammation. At the onset of death of these cells, the intracellular calcium concentration is raised, thereby causing activation of PAD enzymes and consequently citrullination of proteins. This process is not specific for rheumatoid arthritis (RA; see section entitled 'Is the occurrence of synovial citrullinated proteins a phenomenon specific to RA?'). A combination of impaired clearance of dying cells [42], presence of 'danger signals' [41] and genetic factors may blend into a susceptible environment in which the presence of citrullinated proteins leads to an immune response (see section entitled 'Why are anti-citrullinated protein antibodies so specific for RA?'). As the result of this RA-specific immune response, RA-specific anti-CCP (cyclic citrullinated peptide) antibodies are produced.

[29] were observed in a separate study after staining with an irrelevant control antibody (rabbit anti-FITC) [31]. Based on cellular morphology and colocalization with CD38, it appeared most likely that rheumatoid factor-producing plasma cells are detected by this antibody rather than citrullinated proteins [31].

Finally, data from our studies, obtained using several types of anti-citrullinated protein antibodies, suggest that citrullinated proteins can be detected in RA patients but also in control individuals (such as patients with osteoarthritis or reactive arthritis; our unpublished data, and data from Smeets and coworkers [31]). Presence of citrullinated antigens in synovial tissue was also not associated with the presence of anti-CCP autoantibodies in serum or synovial fluid. These results thus suggest that the presence of citrullinated proteins in the synovium is not specific for RA. This conclusion is supported by our recent observation that, also in mouse models of arthritis, various synovial proteins are citrullinated during inflammation [32]. This suggests that citrullination (of synovial proteins) is not an RA-specific phenomenon, but rather it is an inflammation-related process.

Why are anti-citrullinated protein antibodies so specific for RA?

Although arthritis affected mice express citrullinated proteins (as discussed above), they do not produce anti-citrullinated protein antibodies [32]. This suggests that the presence of these antibodies in RA patients is probably the result of an abnormal but specific humoral response to citrullinated proteins. The anti-citrullinated protein antibody subclass distribution (predominantly IgG_1 [1,33]) is indicative of a T-cell dependent antibody production and thus suggests human leukocyte antigen (HLA) involvement [34]. It has been known for more than 25 years that certain HLA haplotypes (e.g. HLA-DR4 [HLA-DRB1*0401 and *0404]) confer a genetic predisposition to RA [35]. It was recently shown that citrullinated peptides can be bound much more efficiently by DR4 molecules than by corresponding non-citrullinated peptides [36]. This citrulline-specific interaction might be the basis of a citrulline-specific immune response. In experiments with HLA-DR4 transgenic mice, proliferation and activation of T cells could be induced by citrullinated peptides but not by the corresponding arginine-containing peptides [36]. It is known that there is a strong correlation between HLA-DR4 status and anti-CCP antibody positivity in RA patients [37]. The requirement for HLA-DR4 in order to develop anti-CCP antibodies is not absolute, but this may be attributed to the possibility that other HLA genes (e.g. DQ) may have a similar preference for certain citrullinated peptides [38]. It thus appears that genetic factors such as HLA-DR4 are involved in the process that determines whether anti-citrullinated protein antibodies are made.

It may take years for the sparks to ignite the fire

It was recently shown that anti-CCP antibodies are present very early in the disease, and that their presence predicts future progression to RA [5]. Even more so, the antibodies can be detected in serum many years before onset of the first symptoms of arthritis [39,40]. Although the initial events that lead to the anti-citrullinated protein immune response are unknown, we believe that in principle every trauma or infection that causes death of

PAD-expressing cells may be enough to initiate citrullination of proteins. Only in a susceptible individual [7] and in a susceptible environment [41] will the presence of these citrullinated proteins lead to an immune response and to the generation of anti-CCP antibodies. After this initial spark, some unnoticed symptoms may smolder for years until they become more severe, as the first clinical symptoms of RA appear. As discussed previously [1,7], anti-CCP antibodies may contribute to perpetuation of joint inflammation and thereby to chronicity and severity of RA.

Conclusion

Recent literature on anti-citrullinated protein antibodies and on citrullinated antigens suggests their involvement in the disease process of RA. The presence of citrullinated antigens is not specific for RA and should probably be considered the consequence of the inflammatory process (Fig. 1). Based on current knowledge, citrullinated fibrin, vimentin and histones are the most likely candidate antigens in RA.

Competing interests

None declared.

References

1. Vossenaar ER, van Venrooij WJ: **Anti-CCP antibodies, a specific marker for (early) rheumatoid arthritis.** *Clin Applied Immunol Rev* 2004, **4:**239-262.
2. van Venrooij WJ, Hazes JM, Visser H: **Anticitrullinated protein/peptide antibody and its role in the diagnosis and prognosis of early rheumatoid arthritis.** *Neth J Med* 2002, **60:** 383-388.
3. Vasishta A: **Diagnosing early-onset rheumatoid arthritis: the role of anti-CCP antibodies.** *Am Clin Lab* 2002, **21:**34-36.
4. Pinheiro GC, Scheinberg MA, Aparecida-da Silva M, Maciel S: **Anti-cyclic citrullinated peptide antibodies in advanced rheumatoid arthritis.** *Ann Intern Med* 2003, **139:**234-235.
5. van Gaalen FA, Linn-Rasker SP, van Venrooij WJ, de Jong BA, Breedveld FC, Verweij CL, Toes REM, Huizinga TW: **In undifferentiated arthritis, autoantibodies to cyclic citrullinated peptides (CCP) predict progression to rheumatoid arthritis: a prospective cohort study.** *Arthritis Rheum* 2004, **50:**709-715.
6. Meyer O, Labarre C, Dougados M, Goupille P, Cantagrel A, Dubois A, Nicaise-Roland P, Sibilia J, Combe B: **Anticitrullinated protein/peptide antibody assays in early rheumatoid arthritis for predicting five year radiographic damage.** *Ann Rheum Dis* 2003, **62:**120-126.
7. Vossenaar ER, Zendman AJW, van Venrooij WJ: **Citrullination, a possible functional link between susceptibility genes and rheumatoid arthritis.** *Arthritis Res Ther* 2004, **6:**3-7.
8. Masson-Bessière C, Sebbag M, Durieux JJ, Nogueira L, Vincent C, Girbal-Neuhauser E, Durroux R, Cantagrel A, Serre G: **In the rheumatoid pannus, anti-filaggrin autoantibodies are produced by local plasma cells and constitute a higher proportion of IgG than in synovial fluid and serum.** *Clin Exp Immunol* 2000, **119:**544-552.
9. Reparon-Schuijt CC, van Esch WJ, van Kooten C, Schellekens GA, de Jong BA, van Venrooij WJ, Breedveld FC, Verweij CL: **Secretion of anti-citrulline-containing peptide antibody by B lymphocytes in rheumatoid arthritis.** *Arthritis Rheum* 2001, **44:** 41-47.
10. Vossenaar ER, Zendman AJW, van Venrooij WJ, Pruijn G: **PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease.** *Bioessays* 2003, **25:**1106-1118.
11. Vossenaar ER, Radstake TR, van der Heijden A, van Mansum WAM, Dieteren C, de Rooij DJ, Barrera P, Zendman AJW, van Venrooij WJ: **Expression and activity of citrullinating PAD enzymes in monocytes and macrophages.** *Ann Rheum Dis* 2004, **63:**373-381.
12. Takahara H, Okamoto H, Sugawara K: **Calcium-dependent properties of peptidylarginine deiminase from rabbit skeletal muscle.** *Agric Biol Chem* 1986, **50:**2899-2904.
13. Schwab BL, Guerini D, Didszun C, Bano D, Ferrando-May E, Fava E, Tam J, Xu D, Xanthoudakis S, Nicholson DW, Carafoli E, Nicotera P: **Cleavage of plasma membrane calcium pumps by caspases: a link between apoptosis and necrosis.** *Cell Death Differ* 2002, **9:**818-831.
14. Tombal B, Denmeade SR, Gillis JM, Isaacs JT: **A supramicromolar elevation of intracellular free calcium ($[Ca^{2+}]_i$) is consistently required to induce the execution phase of apoptosis.** *Cell Death Differ* 2002, **9:**561-573.
15. Asaga H, Yamada M, Senshu T: **Selective deimination of vimentin in calcium ionophore-induced apoptosis of mouse peritoneal macrophages.** *Biochem Biophys Res Commun* 1998, **243:**641-646.
16. Mizoguchi M, Manabe M, Kawamura Y, Kondo Y, Ishidoh K, Kominami E, Watanabe K, Asaga H, Senshu T, Ogawa H: **Deimination of 70-kD nuclear protein during epidermal apoptotic events *in vitro.*** *J Histochem Cytochem* 1998, **46:**1303-1309.
17. Hagiwara T, Nakashima K, Hirano H, Senshu T, Yamada M: **Deimination of arginine residues in nucleophosmin/B23 and histones in HL-60 granulocytes.** *Biochem Biophys Res Commun* 2002, **290:**979-983.
18. Nakashima K, Hagiwara T, Yamada M: **Nuclear localization of peptidylarginine deiminase V and histone deimination in granulocytes.** *J Biol Chem* 2002, **277:**49562-49568.
19. Tak PP, Firestein GS: **Apoptosis in rheumatoid arthritis.** In *Apoptosis and inflammation*. Edited by Winkler JD. Basel, Switzerland: Birkhauser Verlag; 1999:149-162.
20. Senshu T, Kan S, Ogawa H, Manabe M, Asaga H: **Preferential deimination of keratin K1 and filaggrin during the terminal differentiation of human epidermis.** *Biochem Biophys Res Commun* 1996, **225:**712-719.
21. Vossenaar ER, Despres N, Lapointe E, van der Heijden A, Lora M, Senshu T, van Venrooij WJ, Menard HA: **Rheumatoid arthritis specific anti-Sa antibodies target citrullinated vimentin.** *Arthritis Res Ther* 2004, **6:**R142-R150.
22. Despres N, Boire G, Lopez-Longo FJ, Menard HA: **The Sa system: a novel antigen-antibody system specific for rheumatoid arthritis.** *J Rheumatol* 1994, **21:**1027-1033.
23. Menard HA, Lapointe E, Rochdi MD, Zhou ZJ: **Insights into rheumatoid arthritis derived from the Sa immune system.** *Arthritis Res* 2000, **2:**429-432.
24. McCarthy DJ, Cheung HS: **Origin and significance of rice bodies in synovial fluid.** *Lancet* 1982, **2:**715-716.
25. Weinberg JB, Pippen AM, Greenberg CS: **Extravascular fibrin formation and dissolution in synovial tissue of patients with osteoarthritis and rheumatoid arthritis.** *Arthritis Rheum* 1991, **34:**996-1005.
26. Masson-Bessière C, Sebbag M, Girbal-Neuhauser E, Nogueira L, Vincent C, Senshu T, Serre G: **The major synovial targets of the rheumatoid arthritis-specific antifilaggrin autoantibodies are deiminated forms of the alpha- and beta-chains of fibrin.** *J Immunol* 2001, **166:**4177-4184.
27. Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, Nagasaki M, Nakayama-Hamada M, Kawaida R, Ono M, Ohtsuki M, Furukawa H, Yoshino S, Yukioka M, Tohma S, Matsubara T, Wakitani S, Teshima R, Nishioka Y, Sekine A, Iida A, Takahashi A, Tsunoda T, Nakamura Y, Yamamoto K: **Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis.** *Nat Genet* 2003, **34:**395-402.
28. Chapuy-Regaud S, Sebbag M, Baeten D, Clavel C, de Keyser F, Serre G: **The presence of deiminated fibrin in the synovial membrane is not specific for rheumatoid arthritis [abstract].** *Arthritis Res Ther* 2004, **Suppl 1:**S8.
29. Baeten D, Peene I, Union A, Meheus L, Sebbag M, Serre G, Veys EM, de Keyser F: **Specific presence of intracellular citrullinated proteins in rheumatoid arthritis synovium: relevance to antifilaggrin autoantibodies.** *Arthritis Rheum* 2001, **44:**2255-2262.
30. Zdanski CJ, Prazma J, Petrusz P, Grossman G, Raynor E, Smith TL, Pillsbury HC: **Nitric oxide synthase is an active enzyme in the spiral ganglion cells of the rat cochlea.** *Hear Res* 1994, **79:**39-47.

31. Smeets TJ, Vossenaar ER, van Venrooij WJ, Tak PP: **Is expression of intracellular citrullinated proteins in synovial tissue specific for rheumatoid arthritis? Comment on the article by Baeten *et al.*** *Arthritis Rheum* 2002, **46:**2824-2826.
32. Vossenaar ER, Nijenhuis S, van Helsen MM, van der Heijden A, Senshu T, van den Berg WB, van Venrooij WJ, Joosten LA: **Citrullination of synovial proteins in murine models of rheumatoid arthritis.** *Arthritis Rheum* 2003, **48:**2489-2500.
33. Chapuy-Regaud S, Nogueira L, Clavel C, Sebbag M, Vincent C, Serre G: **Subclass distribution of IgG autoantibodies to deiminated fibrinogen in rheumatoid arthritis [abstract].** *Arthritis Res Ther* 2003, **Suppl 1:**S2.
34. Meulenbroek AJ, Zeijlemaker WP: *Human IgG Subclasses: Useful Diagnostic Markers for Immunocompetence,* 2nd ed. Amsterdam, The Netherlands: CLB; 2000.
35. Silman AJ, Pearson JE: **Epidemiology and genetics of rheumatoid arthritis.** *Arthritis Res* 2002, **Suppl 3:**S265-S272.
36. Hill JA, Southwood S, Sette A, Jevnikar AM, Bell DA, Cairns E: **Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule.** *J Immunol* 2003, **171:**538-541.
37. Goldbach-Mansky R, Lee J, McCoy A, Hoxworth J, Yarboro C, Smolen JS, Steiner G, Rosen A, Zhang C, Menard HA, Zhou ZJ, Palosuo T, van Venrooij WJ, Wilder RL, Klippel JH, Schumacher HRJ, El-Gabalawy HS: **Rheumatoid arthritis associated autoantibodies in patients with synovitis of recent onset.** *Arthritis Res* 2000, **2:**236-243.
38. Zanelli E, Breedveld FC, de Vries RR: **HLA class II association with rheumatoid arthritis: facts and interpretations.** *Hum Immunol* 2000, **61:**1254-1261.
39. Rantapää-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, Sundin U, van Venrooij WJ: **Antibodies against citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis.** *Arthritis Rheum* 2003, **48:**2741-2749.
40. Nielen MMJ, van Schaardenburg D, Reesink HWR, van de Stadt RJ, van der Horst-Bruinsma IE, de Koning MHM, Habibuw MR, Vandenbroucke JP, Dijkmans BA: **Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors.** *Arthritis Rheum* 2004, **50:**380-386.
41. Matzinger P: **The danger model: a renewed sense of self.** *Science* 2002, **296:**301-305.
42. Rodenburg RJ, Raats JM, Pruijn GJ, van Venrooij WJ: **Cell death: a trigger of autoimmunity?** *Bioessays* 2000, **22:**627-636.