Rheumatoid factors

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Publication date
2020
Document Version
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GENERAL DISCUSSION

Rheumatoid arthritis and rheumatoid factors

Considering that rheumatoid factors (RFs) were discovered more than 80 years ago and have been investigated ever since, it is remarkable how much about these autoantibodies remains unknown. It is clear that RFs are associated with the disease rheumatoid arthritis (RA), from which they derive their name, but how RFs arise and what role they play in pathophysiology is still a matter of investigation and debate. On a more practical level, there are issues concerning how RFs are measured and what their clinical value is in diagnosing RA, predicting disease course, and how this affects treatment decisions. The research projects described in this thesis aimed to provide more insight into the mechanistic properties of the rheumatoid factor response and improve the clinical value of its measurement. We expected the results generated in the pursuit of one aim would also benefit the other. A better understanding of the molecular binding mechanisms of RFs may provide clues about their pathogenic potential and thereby identify clinically relevant differences between RF responses found in RA patients and those found in other diseases or in healthy donors. The other way around, differences in RF reactivity between cohorts of patients with different diseases or disease outcomes may signal underlying mechanistically and pathophysiologically important characteristics of different RF responses.

We investigated the RF response from different angles in this thesis. We looked at the RFs themselves, their targets and their associations with clinical outcomes. We also examined potential interactions with other autoantibodies, specifically anti-citrullinated protein antibodies (ACPAs).

Rheumatoid factor measurements: problems and opportunities

Since measurement of RFs is a major item in the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria of RA one would expect that there is consensus on how to measure RFs. This is not the case. There are many different commercially available assays to measure RFs and these differ significantly in mechanism of the test and in the targets (human IgG versus rabbit IgG) they use for the RFs to bind to. The classification criteria do not specify which RF tests or targets should be used, and regarding quantification of RF levels they refer to the upper limit of normal (ULN) for the individual laboratories and assays (1). As we showed in **chapter 3**, the fact that RF

assays are standardized nor harmonized can lead to important differences in results between tests, both in a qualitative (positive versus negative) and quantitative (levels) fashion. This problem also affects ACPA assays, although to a lesser degree (2). For patients, discrepancies between different assays can mean the difference between being classified as having RA or not. Which RF assay should then be used? This is not an easy question to answer, since there are no recent - and very few older - studies comparing different assays side by side on performance in diagnosing RA and predicting disease course (3). Even so, it would be advantageous to perform these side by side studies to standardize the RF assays used in the clinic, in particular to make sure that results used for research can be compared between studies. Until such standardization has occurred, researchers reporting RF data should state the mechanism and target of the assays used to generate their data, as has been advocated before (4, 5). Currently this information is widely omitted in papers. Although it is not yet possible to point out the best RF assay, the most appropriate *target* to use is human IgG, instead of the rabbit IgG currently used in many assays as our data suggest human IgG may be the more sensitive target in the arthralgia phase of RA (6). Furthermore, an individual RF clone we studied in **chapter 3** specifically bound human but not rabbit IgG. This RF clone binds to a region on IgG-Fc that is an important target in pre-clinical as well as clinical RA populations (as we showed in **chapter 5**). However, earlier studies have presented conflicting data about which target is superior (3, 5, 7, 8).

RF assays are considered to be as sensitive as ACPA assays for diagnosing RA, but less specific. This lack of specificity, caused by the presence of RFs in other diseases and in a small proportion of the healthy population, may at least partly be due to the lack of a robust standardized RF assay that uses the most clinically relevant targets. There are at least two ways to standardize RF testing. The first option is to compare existing RF tests, preferably in studies with international collaboration. A problem is that most patients referred to clinics have already undergone RF testing, creating a biased research population. This issue could be resolved with a recruiting approach as is currently used for the Clinically Suspect Arthralgia (CSA) cohort at the Leiden University Medical Center. In the Leiden area general practitioners are encouraged to refer patients early and discouraged from first-line testing of RF and ACPA. This approach has resulted in the large

majority of patients being referred with unknown RF and ACPA status (9). This creates an opportunity to compare the performance of different RF assays in a relevant population and select the most optimal assay. A second option is to create a better RF assay to replace all currently used assays. To investigate the feasibility of such a superior RF assay was one of the aims of this thesis. We attempted to improve RF measurements by determining whether relevant, RA-related RFs can be discriminated from non-relevant RFs in healthy individuals. We showed in **chapter 8** that there are no clear differences between RF clones derived from RA patients and healthy individuals. The genes that are used to encode for RFs are not different in the two groups and we found no significant differences in mutation frequency or affinity. The platform we created in **chapter 5** with the engineered IgG targets enabled us to compare not just individual RF clones, but the complete polyclonal RF responses present in patient groups and healthy donors. This made it possible to identify patterns of RF reactivity associated with arthralgia patients who do not develop arthritis and patterns that distinguish between RA patients and patients with primary Sjögren's syndrome (pSS). Our engineered IgG targets will hopefully be the basis of a new and improved RF assay that will replace the diverse mix of RF assays currently used in clinics around the world.

The pathophysiological role of RFs and other autoantibodies

Although it is still unclear how RFs originate, two possibilities seem attractive. RFs could arise from the natural antibodies that are present in all individuals and that most likely perform house-keeping functions such as removing immune complexes (IC) and debris from dead cells, and also provide an innate first line of defense against invading pathogens (10, 11). The fact that RFs are also found in healthy individuals supports this hypothesis. From our own experiments in **chapter 5** we have learned that whether RFs are identified in samples of healthy donors (HDs) is greatly influenced by the cut-off set for the RF assay. Since many RF assays have a relatively high cut-off level to maintain a reasonable specificity, presence of RFs in HDs as measured by conventional RF assays may have been underestimated.

A second possibility is that RFs arise independently from natural antibodies through a conventional antigen driven immune response. The antigen in question could be a viral or bacterial antigen that induces an antibody

response that subsequently cross-reacts with IgG, or the antigen could be IgG itself altered through changes in e.g. glycosylation of the Fc domain or other structural or conformational changes that expose Fc epitopes (12). Certain HLA-types associated with RA show enhanced binding and presentation of citrullinated peptides (13). This may play a role in overcoming tolerance against autoantigens. Whether enhanced HLA-binding of altered IgG-Fc peptides plays a role in inducing RF responses has yet to be determined.

ACPA IgGs have been shown to have altered galactose content in their Fc domains (14) and we investigated in **chapter 6** whether this makes them better targets for RFs than non-ACPA IgGs. We found no clear differences in binding strength between IgGs with various levels of galactosylation, suggesting that this specific modification of the IgG target glycans is not associated with better RF binding.

An appealing idea that only recently gained interest is the possibility of synergy between ACPAs and RFs. Clinically, this synergy is suggested by the fact that patients who have both antibodies on average fare worse than those who have just one (15-17). RFs should be able to bind to the Fc domains of ACPA IgGs even though they are probably not intrinsically better targets than non-ACPA IgGs, as we showed in **chapter 6**. However, two reasons may make ACPAs preferential targets over non-ACPA IgGs in the setting of RA. One is availability: the proportion of ACPAs of total IgG present in the inflamed cartilage tissue of the joints of RA patients may be much higher than at other sites (18). The other reason has to do with the fact that IgM-RFs are generally of low affinity, as we showed in **chapter 8** (19). In the joint space of an RA patient, an abundance of citrullinated proteins will lead to clusters of ACPA IgGs binding to their targets. Such clusters of Fc domains in close proximity are then ideal targets for IgM-RFs to bind to with low affinity, but high total avidity. Unpublished data suggest that IgM-RFs in turn may increase the total avidity of ACPA-citrullinated protein-complexes by cross-linking the ACPA IgGs, after they have bound to citrullinated proteins (20). Complexes containing both RF and ACPAs could be more potent in inducing and maintaining an auto-inflammatory response in the joint than complexes containing either antibody alone. Studies showed that more pro-inflammatory TNF-alpha is produced by macrophages in response to such complexes and binding of complement

is enhanced (17, 21, 22). However, this mechanism is not as straightforward as one might think. A contrasting possibility would be that RFs, by binding to the ACPA IgG Fc domains would instead block the parts of the ACPA Fc domains that interact with Fc receptors. Such a blocking mechanism was suggested for complement binding in a study where RFs allegedly blocked the complement binding sites of therapeutic anti-CD20 antibodies (23). However, in **chapter 5** we showed that this is probably not the case for the Fc receptor binding site. With recombinant IgG targets we were able to show that RFs most likely do not target the Fc receptor binding site, leaving it available to interact with Fc receptors and induce pro-inflammatory signals. Moreover, complement activation could in fact be enhanced in two ways: the crosslinking of ACPA IgGs by RFs generates ICs for C1q to bind to and the IgM-RFs themselves are likely potent activators of the complement pathway since IgMs are considered better targets than IgG for complement activation, due to their pentameric or hexameric structure. Since IgM-RFs are especially large molecules it is still possible that their presence in ICs also has a blocking effect on interactions with cell-bound Fc receptors and C1q molecules through steric hindrance. More research is needed to determine the cumulative effects of the interactions between RF-ACPA ICs and effector molecules.

How does the RF response mature into a more pathogenic autoantibody response during the natural history of RA development? Our data suggest that two mechanisms of antibody response maturation, epitope spreading and isotype switching, go hand in hand in RF responses. In **chapter 4** we found that RF responses with a broader reactivity, targeting all four IgG subclasses –and therefore likely more different epitopes– were associated with a much higher frequency of IgA-RF responses and higher levels of IgA-RF. As explained in **chapter 2,** the evolution of the RF responses in the development of RA may go through a phase of epitope selection, followed by epitope spreading, if indeed the pathological RFs arise from a pool of natural IgM-antibodies. In this scenario, a natural IgM response would first evolve into an RF response through increased specificity against IgG, and thereby loss of reactivity against foreign antigens. Subsequently, this true RF response may increase the number of IgG epitopes it reacts with in the course of the development of RA. Such a broader RF response may be capable of forming larger ICs by incorporating multiple RF molecules and IgG from different subclasses. This would then accelerate damage in

inflamed joints through increased complement activation and increased production of pro-inflammatory cytokines through Fc receptor crosslinking on macrophages. It has been shown before that epitope spreading is also a feature of the ACPA response. Both RFs and ACPAs have been shown to be present years before clinical RA becomes apparent and the ACPA response, starting as a restricted response targeting one or two antigens, slowly progresses to higher levels and reactivity against multiple epitopes (24, 25). It has not been studied yet whether once RA has developed, the epitope spreading of the RF stops, as appears to be the case for the ACPA response (24). The most recent data indicate that in double positive patients the RF response and ACPA response develop in parallel by different mechanisms, with the RFs as a rapid but short-lived innate-like response, driven by T cell independent stimuli and the ACPA response as a more long-lived T cell dependent response with more affinity maturation (26, 27). A model proposed by Malmström characterizes the ACPA response as an RA-specific immune reaction and the RF response as an immune reaction shared by several auto-immune diseases (27). Although the presence of conventionally measured RFs in several auto-immune diseases supports this model, our data comparing RF responses in RA and pSS in **chapter 5** suggest that RA-specific RF responses exist. An interesting future research subject could be to compare autoimmune related RF responses to RF responses in non-autoimmune diseases. **Chapter 8** shows that in contrast to RA- and healthy donor derived RF clones, lymphoma and Hepatitis C virus associated RF clones do show skewed gene usage and it would be interesting to determine whether this translates into lymphoma- or virus-specific RF reactivity patterns.

Concluding remarks and suggestions for future research

This thesis describes our research on the dissection of the RF response to learn about its pathophysiological properties and improve the clinical value of its measurement. We show that by determining where RFs bind their target a more specific characterization of RF responses is feasible. Our new methodology improves our understanding of the pathophysiological consequences of RFs, by showing that the Fc receptor binding region in RF-IgG ICs remains accessible. It has the potential to eventually replace existing RF assays by a new RF assay that more specifically measures RA-related RFs. Follow-up research projects should establish whether our methodology has prognostic value for patients at risk of RA and can

contribute in predicting response to therapy in RA patients by comparing RF reactivity patterns for responders and non-responders. Since differential expression of disease-associated RFs has also been suggested in pSS (28) it may also provide insight into the pathophysiological role of RFs in other auto-immune diseases and during bacterial or viral infection. The reactivity patterns that we describe in **chapter 5** are still limited and engineering additional IgG targets may provide even more useful information.

If our new methodology would result in more specific RF assays with a lower cut-off it could help to answer another old, but still not settled, question on RF and ACPA: What comes first? (29, 30) If ACPAs arise first, followed by RFs, this would strengthen the hypothesis that RFs act as a secondary, amplifying autoantibody response. Right now, if RFs are detected later than ACPAs, it remains unclear whether this is just because the cut-off of the RF assays is too high.

Furthermore, it would be interesting to investigate whether RF reactivity patterns are different for 'ACPA-induced' RF responses and RF responses induced by other auto-antibodies or even by antigens such as tetanus toxoid (31). This may show whether an amplifying role of RFs is restricted to RA, or also present in other auto-immune diseases.

A meaningful future approach to studying autoantibodies in RA would be to follow the evolution of multiple antibody systems in a patient in parallel. Earlier studies have looked at presence of RFs and ACPAs in longitudinal samples, but it could prove even more insightful to compare epitope spreading, changes in affinity and glycosylation and isotype switching in parallel for RFs, ACPAs, antibodies directed against carbamylated antigens (anti-CarPs) and anti-hinge antibodies. Are changes in Fc glycosylation for example followed by changes in RF reactivity patterns? Do we see more affinity maturation of the RF response in patients that also have ACPAs compared to ACPA negative individuals? The RF affinity data that we collected for **chapter 8** are almost all from studies performed before the introduction of ACPA testing, so this question is also open for future research.

In conclusion, we showed that "the" rheumatoid factor does not exist. RF responses represent different mixtures of anti-IgG autoantibodies targeting

various IgG epitopes. Determining reactivity patterns can help to explain the pathophysiological consequences of RFs and provides clinical value for patients and physicians by helping assess the risk of developing RA. 80 years after their discovery, the new methodology of characterizing RFs presented here should provide a stepping stone to finally understand the role of RFs and related autoantibodies in rheumatoid arthritis.

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