Heat-shock proteins as antigens in autoimmunity

Willem van Eden,* Els J. M. Hogervorst,* Marca H. M. Wauben,* Ruurd van der Zee,† and Claire, J. P. Boog*

*Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, University of Utrecht, Yalelaan 1, 8508 TD Utrecht, The Netherlands and †Department of Bacteriology, National Institute for Public Health and Environmental Hygiene, P.O. Box 1, Bilthoven, The Netherlands

Introduction

Recognition of self is currently regarded to be a normal and essential element of a proper immune response [1]. Autoimmunity, however, is due to deviations from the normal functioning of the immune system, where recognition of self components leads to disease with signs of a destructive response targeted at self antigens. In most diseases supposedly due to autoimmunity the relevant target autoantigens have not yet been defined. In a number of experimental models of autoimmune diseases, immunization with a self antigen does lead to the induction of disease. Under such experimental conditions the immune system has been shown to develop specific responses directed at the autoimmunogen [2]. Furthermore, in some experimental models it has been demonstrated that disease can be induced by the inoculation of selected T cells with specificity for the autoimmunogen. Interestingly enough, it appears that such disease-inducing T cells can be obtained not only from healthy animals after disease induction by active immunization, but also from animals either genetically or otherwise resistant to disease induction. Thus, in the healthy immunological repertoire, T cells with the potential of eliciting autoimmune disease are normally present without leading to disease. The question then is how such cells can exist under normal circumstances without causing disease and it is expected that the study of the mechanisms underlying their safe containment will help the development of specific means to combat autoimmune diseases.

Autoimmune disease can also result from immunization with non-self antigens. In particular, this seems to be the case with arthritis. Heat-killed mycobacteria [adjuvant arthritis (AA)] or cell walls obtained from streptococci (SCW-induced arthritis) induce an inflammatory arthritis which is T cell-mediated. A critical antigen involved in the induction of AA has now been demonstrated to be the 65 kDa heat-shock protein (HSP65) [3]. For several reasons this is of interest. HSPs are particularly well conserved proteins. The mycobacterial HSP65 has extensive sequence homologies with the mammalian endogenous HSP65. Therefore, although the actual target antigens in AA have not been defined exactly, it is possible that recognition of endogenous HSP65 is part of the autoaggressive response in the disease. Be that as it may, responses to HSP65 tend to dominate in AA. Although similar to self antigens, both at the level of B and T cells, recognition of HSPs, including HSP65, is a prominent feature of the immunological response to microbial agents. On the one hand, this may be the consequence of repeated exposure, since these antigens have their homologues in all prokaryotic and eukaryotic parasites. On the other hand, the immune system may have good reasons to focus on such antigens, in order to be able to avoid undesired responsiveness by careful network regulation [4]. Evidence for regulatory recognition of HSP65 epitopes is currently being obtained. Moreover, in accordance with that are the observations that in different models of experimental arthritis, including models without the involvement of bacterial antigens, HSP65 has been found to induce protective immune responses.

In various human conditions of spontaneous autoimmune diseases, immune reactivity to HSP antigens has now been found. Although it is possible that such reactivity is the consequence of the inflammatory responses rather than that it has a causal relationship, the experimental model of AA clearly shows that bacterial immunization may elicit recognition of HSP65 in such a way that disease results.

The model of AA

By the nature of the disease-inducing antigen, which is of microbial origin, AA differs from most other experimental animal models in autoimmunity, where immunization is usually done with an endogenous self antigen presented in an immunogenic form. Since its discovery, AA has been studied as a model for rheumatoid arthritis, because of the histopathological similarities with the human disease [5]. Holoshitz et al. [6] isolated an arthritogenic T cell

Abbreviations used: AA, adjuvant arthritis; HSP, heat-shock protein; Mt, Mycobacterium tuberculosis; MHC, major histocompatibility complex; SCW, streptococcal cell wall.
line, called A2, after in vivo immunization of susceptible Lewis rats with Mycobacterium tuberculosis (Mt) and subsequent culture with repeated restimulations of the cells with Mt in vitro. Under different conditions A2 was found to induce protection against AA. So, apparently the T cell line A2, with specificity for mycobacterial antigens, was composed of T cells with specificity for antigens of critical importance for recognition for the regulatory processes involved in AA. Subsequent subcloning of A2 gave rise to several clones, including clones A2b and A2c [7, 2]. A2b was found to be virulently arthritogenic, while A2c was found to be protective. Having isolated such monoclonal T cell reagents with functional activities in disease in vivo, a search for the nature of the antigens critical to the arthritic processes was undertaken. Although the original A2 cell-line had been found to recognize collagen type II to some extent, the more virulent A2b clone did not [7]. However, when this clone was tested on various crude preparations enriched for cartilage proteoglycans, significant reactivity was seen [8]. So, in addition to an antigen present in mycobacteria, the T cells were recognizing an antigen associated with cartilage.

The inevitable conclusion from this was that these clones were critical to arthritis because of their specificity either for one antigenic epitope shared between mycobacteria and proteoglycans or for two distinct epitopes that were structurally related and present in mycobacteria and proteoglycans [9]. Both situations would be perfect examples of antigenic mimicry, this time identified at the level of the disease regulating agents themselves. The finding of such mimicry was the stimulus to define the epitope seen in mycobacteria by A2b and A2c. A number of recombinant proteins of M. bovis BCG and M. leprae, expressed in Escherichia coli [10] were screened for stimulatory activity on the rat arthritis T cell clones. A remarkably strong reactivity was seen with a 65 kDa protein of M. bovis BCG [3]. Sequencing of the 65 kDa gene revealed that its amino acid sequence was 100% identical to its M. tuberculosis homologue. So, as a result of these studies, the mycobacterial antigen seen by clones A2b and A2c, and having the shared or mimicry epitope, has been identified. In searching for the mimicry epitope(s), fragments of the 65 kDa protein were obtained from deletion mutants after fusion with the β-galactosidase gene. From analysis of the fragments, the area between positions 170 and 234 in the molecule was found to be critical for stimulation of the T cell subclones. Further analysis of peptides showed that both A2b and A2c were specific for an epitope located at positions 180–188. Comparing the sequence of 180–188 (TFGLQLELT) with known protein sequences in cartilage proteoglycans revealed a minor sequence homology, four out of nine identical, with a rat link protein sequence [3]. Whether this resemblance represents the mimicry we were looking for is uncertain, since A2b and A2c did not respond to this link protein nonapeptide. Recently, Van der Zee introduced a modification of the so-called Pepscan-method, which makes this method suitable for T cell epitope mapping and characterization [11]. By the Pepscan method large numbers of peptides can be synthesized simultaneously on polyethylene rods, which had been successfully used for the efficient mapping of continuous B cell epitopes. Since the modification allowed for the recovery of the peptides from these rods, a systematic analysis of our T cell epitope for an epitope located at 180-188. Simultaneously on polyethylene rods, which had been successfully used for the efficient mapping of continuous B cell epitopes. Since the modification allowed for the recovery of the peptides from these rods, a systematic analysis of our T cell epitope became possible. By this method systematic analysis has now been done on sequentially overlapping peptides together with deletion, insertion and substitution peptides. This revealed that only the amino acids at positions 180–186 were critical for stimulation and further analysis of stimulatory substituted peptides in the search for cartilage-associated mimicry epitopes is currently being done (M. H. M. Wauben et al., unpublished work). Alternative possibilities, apart from a mimicry of the 65 kDa protein with a proteoglycan molecule, are, however, suggested by the very nature of the mycobacterial 65 kDa protein itself. It was already observed that antibodies raised against mycobacteria and recognizing the mycobacterial 65 kDa molecule were frequently bound to many other bacterial organisms [12]. By Western blotting it was observed that the cross-reactive antigen in the other bacterial organisms invariably was a 59–65 kDa antigen, which had been known already as the so-called common antigen of Gram-negatives. Based on the extensive sequence homologies of the mycobacterial 65 kDa antigen with a known HSP in E. coli, groEL, the mycobacterial 65 kDa antigen was recognized to be a member of the 65 kDa HSP family [13]. These results explained the cross-reactions of anti-mycobacterial 65 kDa antibodies with other bacterial organisms, since heat-shock proteins are exceptionally well conserved not only throughout prokaryotic organisms, but also eukaryotes and even mammals. Therefore, one should take into account the possibility that the cartilage-associated target molecule in AA is not proteoglycan itself, but a proteoglycan-associated HSP of mammalian origin.
It can be concluded that exposure of rats to a conserved bacterial protein may result in the triggering of T cells exhibiting a specificity relationship with some cartilage-associated antigen. Such T cells may be responsible both for inducing arthritis (A2b) and for down-regulation or control of such a disease process (A2c). Inbred rat strains differ in their relative susceptibility to AA. Although evidence compatible with a role of the major histocompatibility complex (MHC) in AA has been reported [14], the contribution of the MHC cannot be more than a relative one, a situation similar to the susceptibility to, for instance, rheumatoid disease in humans. That additional genetic factors are definitely involved is exemplified, for instance, by the fact that Fisher rats, having an MHC haplotype identical with that of Lewis rats, are notoriously resistant to AA [15]. However, germ-free-bred Fisher rats are again susceptible to AA. From this it can be inferred that resistance is an immunologically-active principle, which can be learned and subsequently memorized by the immune system. Lewis rats develop resistance after having recovered from AA. Re-exposure to Mt, after spontaneous remission of AA, which is the rule in Lewis rats, does not lead to reappearance of disease. Also after inoculation of A2 or A2c into non-irradiated naive recipients of the same strain, resistance to AA was induced. Thus, tolerance to the critical self antigens residing in the joints can be re-established by specific changes in the regulatory repertoire of the animal.

Since T cells with specificity for the 65 kDa HSP seem to be critical in this regulatory repertoire, one may ask whether this antigen can be used for therapeutic purposes in the experimental model. Immunizations with the HSP65 itself have not been seen to lead to arthritis, but interestingly enough resistance against subsequent attempts to induce AA with Mt has been noted after the immunization with HSP65 [3]. Depending on the conditions, such as dose, etc. administration of HSP65 during overt AA may lead to a flare-up of the disease, again demonstrating the critical role of this particular protein in the disease (E. J. M. Hogervorst et al., unpublished work).

Both HSPs and cartilage proteoglycans are evolutionary well-conserved molecules. Therefore, a most relevant question seems to be how general the phenomenon of AA is. Since the antigenic make-up of cartilage in rats and humans will be largely identical, and humans can be similarly exposed to bacterial HSPs, one should also expect that in human beings arthritis can be triggered equally well along the same lines. Alternatively, the same memory responses in the regulatory circuits theoretically could play a role in the maintenance of tolerance for joint-associated antigens, both in rats and man.

**HSPs in immunopathology**

As mentioned above, HSPs are readily seen by the immune system. Their important functional activities and their unprecedented high level of homology with bacterial antigens makes them likely candidates both as target and as trigger in immunopathology. Significant increases in antibody levels against HSP65 have been seen in rheumatoid arthritis [16, 17]. Furthermore, antibodies to HSP90 are found in ankylosing spondylitis and antibodies to ubiquitin, HSP70 and HSP90 are found in SLE (reviewed in [18]). Nevertheless, apart from AA, a direct role of HSP in immunopathology has not yet been demonstrated. However, that responses to bacterial HSP may lead to cross-recognition of autologous HSP has been demonstrated [19]. It was found that CD8-positive T cells raised against bacterial HSP65 peptides, not only lysed peptide-pulsed macrophages, but also lysed stressed macrophages in the absence of exogenously added peptides. One way of causing the critical stress was by infection of macrophage with virus or by stimulation of macrophages with interferon-γ. So, physiological stress seems to allow for class I-restricted presentation of autologous HSP65. This could clearly be a target for T-cell-mediated immunopathology. Of further interest is that a number of groups have now found reactivity with mycobacterial HSP65 in γ/δ T cells. Mouse T cell hybridomas fused with thymocytes of newborn mice expressing the γ/δ T cell receptor have been found to produce interleukin-2 spontaneously, probably by recognizing a cell-membrane-expressed self antigen. Many of these cells were shown subsequently to be stimulated with mycobacterial HSP65, suggesting HSP65 to be a stimulus for thymocyte development in newborn mice [20]. Also human γ/δ cells now have been identified to recognize HSP65, both in peripheral blood of normal individuals [21] and in the synovial compartment of a joint from a rheumatoid arthritis patient [22]. Others have found now that amongst γ/δ cells responding to mycobacterial antigens, it is, however, only a minority of the cells that actually respond to the HSP65 [23]. Nevertheless, the hypothesis that γ/δ cells may recognize endogenous HSP and as a...
result may play a role in the elimination of stressed host cells remains an attractive one, and will be the subject of further research.

Returning now to models of experimental arthritis, several recent findings suggest that HSP65 has general significance in the immunopathology of arthritis. Being an antigen with the potential of triggering the disease in AA, experiments were done to define a possible role of HSP65 in models of arthritis without any mycobacterial involvement. Such a result may play a role in the elimination of stressed host cells remains an attractive one, and will be the subject of further research.

T cell responses to conserved HSP epitopes in humans

The first indications that T cell responses to mycobacterial antigens could have significance for human arthritic diseases were obtained from several observations. T lymphocytes collected from synovial fluids of arthritis patients were found to be responsive to mycobacterial antigens, including the HSP65 of mycobacteria [27, 28]. Furthermore, delayed type hypersensitivity skin testing (the in vitro correlate for measuring T cell responsiveness) with mycobacterial preparations revealed that individuals carrying the MHC allele HLA-DR4 responded more vigorously than those lacking DR4 [29]. HLA-DR4 is found at increased frequency in rheumatoid arthritis. Thus, the genetic susceptibility to rheumatoid arthritis correlates with strong anti-mycobacterial T cell reactivity, which was also found by the analysis of T cell proliferative assays in vitro. The functional relevance of the responding synovial T cells being uncertain, and the evidence from the genetic findings being rather indirect, one should be careful in concluding that mycobacterial antigens have a causal significance in human arthritis. More recent findings, however, have indicated that T cell reactivity to endogenous host HSP antigens can be seen. Depending on the circumstances, such reactivity could well be part of badly controlled autoimmunity. Firstly, anti-mycobacterial T cell lines obtained from healthy humans, were found to recognize peptides comprising epitopes conserved between the bacterial and mammalian HSP65 [30]. Secondly, similar to what was seen in the mouse, human cytotoxic anti-HSP65 T cell lines were found to recognize non-pulsed macrophages [31]. Thirdly, an analysis of human T cell epitopes present on mycobacterial HSP65 has shown that recognition of sequences occurs independently of whether the sequence is located in a conserved area of the molecule or not [32]. Thus, under experimental conditions, T cells with the potential of recognizing the endogenous HSP can be demonstrated. It may now be suggestive of their potential role in disease that, in a case of reactive arthritis caused by Yersinia bacteria, a T cell clone was isolated from the inflamed synovium that recognized the mycobacterial HSP65 and a recombinant human HSP65 in addition to Yersinia itself [33]. So, under pathological conditions the T cells with specificity for the human endogenous HSP homologue are at the site of inflammation. Together with the findings of a selective expression of this HSP65 in arthritic synovium [34, 35], the essential ingre-
dients for autoimmunity with HSPs as critical antigens in human arthritic conditions are there. The ultimate possibility of successfully interfering with the process by immunization with HSP-related antigens is a logical and tantalizing option for the near future.


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