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The Adjuvant Arthritis Experience

Introduction

Adjuvant arthritis (AA) is a frequently used model of experimental arthritis. Because of its histopathology which is reminiscent of RA in humans, AA is used as a model for the development of novel anti-inflammatory drugs. Recently, it has become evident that AA is a typical T cell mediated autoimmune condition. Therefore, novel immunotherapies targeted to T cells can be developed in this model. Analysis of responding T cells in AA have now led to the definition of various antigens with potential relevance to arthritis, including human arthritic conditions. One such antigens defined in AA is the 60kDa heat shock protein. Both T cell vaccination approaches and active antigen immunisations and antigen tolerisation approaches have turned out to be effective in suppressing AA.

HSP60 is the critical antigen in rat adjuvant arthritis

In adjuvant arthritis, disease is induced by substances that have no obvious antigenic relationships with joint-tissues. Originally this experimental model was discovered by Pearson (1956) when he observed the development of arthritis in rats after experimental immunizations using complete Freund's adjuvant containing heat-killed mycobacteria. More recently, other oily adjuvantia, such as pristane (Thompson et al. 1990) and the synthetic lipoidal amine CP 20961 (Chang et al. 1980), have been shown to induce a similar form of arthritis. What these models have in common is an arthritis produced in the absence of immunization with a 'self-antigen'. Although the pathogenic mechanisms involved are probably not identical, they may well be related, since in all three models immunity to hsp antigens is involved, as we will discuss later.

Proof of the role of T cells was obtained by Holoshitz et al. (1983), when they were able to select an arthritogenic T-cell line from cells obtained from mycobacteria-immunized Lewis rats. When administered in sublethally irradiated syngeneic Lewis rats, this T-cell line, called A2, caused an arthritis which was indistinguishable from the actively, i.e. with mycobacteria, induced disease.

Upon cellular cloning of A2, several CD4+ T-helper subclones were obtained. One of these subclones, called A2b, was found to be virulently arthritogenic. This particular sublone A2b was found to proliferate not only in the presence of mycobacterial antigens,

but also in the presence of semi-purified preparations of cartilage proteoglycans (Van Eden et al. 1985). These findings led to the concept of molecular mimicry between a mycobacterial antigen and a cartilage-associated 'self-antigen' as the critical pathogenic mechanism explaining mycobacteria-induced arthritis.

The mycobacterial antigen we have now defined exactly. Molecular cloning of M.bovis BCG genes in *E. coli* resulted in the expression of several mycobacterial proteins, amongst which was a 65 kD M.W. protein. This protein turned out to be the antigen we were looking for. A2 and A2b turned out to respond vigorously, while none of the other clones tested showed any responsiveness (Van Eden et al. 1988). Furthermore, by Western-blotting it became evident that polyclonal or monoclonal antibodies raised against mycobacteria were frequently reactive with this 65 kD molecule, and not exclusively in mycobacteria but also in many other bacterial organisms.

From the sequence homologies of the *M. bovis* 65kD protein with known heat-shock proteins such as the *E. coli* GroEL, the 65 kD of mycobacteria was identified as a heat-shock protein, to be called hsp65 of mycobacteria. By the study of deletion mutants of the 65 kD gene expressing only parts of the molecule and pEX2 expression products consisting of fusion proteins composed of *E.coli* betagalactosidase and various truncated derivatives of the 65 kD protein, a provisional mapping of the epitopes of A2b was obtained in the area located from positions 171 to 234. Finally, by means of several overlapping synthetic peptides, an epitope was defined that was located at positions 180 through 188 that stimulated A2b (Van Eden et al. 1988). The amino acid sequence of this epitope has some limited sequence homology with a rat proteoglycan link protein sequence. Although supportive evidence for the relevance of this homology was obtained, for instance in human T-cell responses, it is uncertain whether this link protein sequence is the target structure in adjuvant arthritis. The homology could also be with an as yet unidentified or sequenced cartilage-associated protein or with another protein type which is present in all cells and therefore all tissues.

Nasal tolerance to hsp peptides suppresses antigen and non-antigen induced arthritis

Recently, the unique relationship of the 180-188 sequence with the arthritic process was further substantiated by tolerising rats for this particular sequence by administering this peptide in the nose (nasal tolerance) or by giving it subcutaneously in PBS at high dosages (high dose tolerance). This procedure was seen to protect the animals from the subsequent induction of arthritis by either mycobacteria in oil or by the non-antigenic synthetic adjuvant avridine (CP20961) (Prakken et al., 1997). Apparently, this single bacterial epitope, which resembles a (sofar not identified) self epitope at the site of inflammation, is capable of inducing regulatory mechanisms of peripheral tolerance. The success of this regimen in suppressing disease, and especially in the case of non-microbially induced disease, seems to support the possibility that this microbial epitope, indeed, has a unique relationship with a disease critical self-antigen in the joint. Apparently, the exposition of such antigen at the mucosal surface of the

nose is already sufficient for setting reactive T cells in a regulatory mode with the capacity to enforce peripheral tolerance, leading to disease resistance.

Conserved hsp60 epitopes induce arthritis suppressive T cells

The identification of mycobacterial hsp60 as a critical antigen in arthritis has led to many studies on the potential of hsp60 in modifying arthritis development. Immunisation experiments in mice and rats using hsp60 proteins or its derivative peptides have never led to induction of arthritis. On the contrary, resistance to arthritis was seen to develop. Prior immunisation of experimental animals with mycobacterial hsp60 has been found to protect against subsequent induction of AA (van Eden et al., 1988; Billingham et al., 1990), streptococcal cell wall arthritis (van den Broek et al., 1989) and avridine (a non-antigenic lipoidal amine) arthritis in rats (Billingham et al., 1990) and also pristane (Thompson et al., 1990) and collagen arthritis (Ito et al., 1991) in mice. Careful analyses of differential T cell responsiveness of whole mycobacteria (AA induction protocol) versus mycobacterial hsp60 (protection protocol) immunised rats have now pointed out that the arthritis inductive capacity of whole mycobacteria does coincide with dominant responses directed to the mimicry epitope 180-188 (Anderton et al., 1994). This is not the case when the hsp60 protein is used for immunisation. Cellular cloning of responsive T cells in the latter protective protocol has revealed clones that recognise sequences conserved between mycobacterial and mammalian hsp60. Adoptive transfer experiments have shown that such T cells, recognising conserved sequences and in particular the mycobacterial 256-265 sequence, were capable of transferring protection against the disease (Anderton et al., 1995). Furthermore, it was demonstrated that such cells had the capacity of responding to heat-shocked spleen cells. Immunisations with conserved peptide 256-265 were also inducing arthritis protection and none of several other peptides containing non-conserved dominant T cell epitopes tested was capable of inducing any protection. Testing of the same 256-265 peptide in a very different arthritis model in Lewis rats, in this case avridine arthritis, revealed that also in a non-bacterially induced model the protective potential of the peptide was present. Protection was also found using the homologous rat (self) peptide. Recent experiments have shown similar protective effects to result from immunisation with mycobacterial hsp70 both in AA and avridine arthritis.

Also for the smaller subunit of the hsp60 complex (GroES of *E. coli* of 10kDa M.W.) its arthritis protective potential has been documented (Ragno et al., 1996).

Suppression in arthritis models is specific for heat-shock proteins

Given the observation that conserved epitopes of hsp's induced resistance to arthritis by setting a focus of T cell reactivity directed to recognition of self homolog (human) hsp, one could easily ask whether other conserved bacterial proteins would have similar arthritis protective capacities. However, in striking contrast with the protection in

autoimmune disease models obtained with hsp's such as hsp60, 70 and 10, we have seen recently that a set of other conserved, but not stress inducible, bacterial proteins, such as superoxide dismutase, glyceraldehyde-3-phosphate-dehydrogenase and aldolase, did not protect in experimental arthritis, despite their good immunogenicity and sequence homologies with their mammalian homologs (Prakken 1996). Therefore, it seems that the protective quality of hsp's is a unique aspect of hsp's, possibly caused by their exquisite behaviour of upregulating local expression under conditions of stress such as existing at sites of inflammation.

HSPs in autoimmune and other inflammatory diseases

In the past the most immunogenic protein of bacteria was known as the 'common antigen of gram negatives'. This protein now has been recognised as being the hsp60 (GroEL of *E. coli*) molecule. Similarly, in parasitic infections, hsp70 and sometimes hsp90 are found to represent other major targets for the humoral immune response. This in itself predicts that the presence of antibodies directed at the major families of heat-shock proteins will be a frequent and normal situation in most individuals and also indicates that immune responses to hsp's are compatible with normal health. Despite the frequent occurrence of hsp antibodies in normal individuals it seems that in the majority of inflammatory diseases raised levels of hsp antibodies can be found (see van Eden and Young, 1996). This has been reported for rheumatoid arthritis (RA), juvenile RA, reactive arthritis, Behcet Disease, SLE, Crohn's Disease, Insulin Dependent Diabetes Mellitus (IDDM) and multiple sclerosis (MS). Significant hsp specific T-cell responses have been observed in RA, JRA (see under), Behcet's Disease, MS and in graft infiltrating lymphocytes during transplant rejection episodes. Raised expression of hsp's in diseased tissues has been documented in Sarcoidosis, SLE, inflammatory liver diseases, chronic gastritis (gastric ulcer), celiac disease, MS, IDDM and atherosclerosis. Despite the general perception that hsp's seem to play a role in different autoimmune diseases, there is no consensus on cause and effect relationships. Evidence in favour hsp's being a trigger leading to autoimmunity, because of their conserved nature, is essentially lacking. A more plausible possibility is that inflammation in general causes raised tissue expression, leading to the generation of hsp specific T- and B-cell responses.

HSPs in human arthritic diseases

Similar to what has been documented in many other autoimmune conditions also rheumatic diseases have been seen to feature the sequelae of immune responses to hsp's. This is most evident from serology studies which have shown the presence of raised levels of hsp60 specific antibodies in patients. Raised expression of hsp in inflamed tissues has been documented for arthritic synovium in both RA and JRA (Boog et al. 1992). In children this raised expression has been seen to be an event that occurred early in the development of disease. Despite earlier reports claiming

also prominent T cell responses to mycobacterial hsp60 in advanced RA, more recent studies have suggested that proliferative T cell responses are,however, more confined to early RA and that in advanced RA such responses are less prominent. Alternatively, responses have been detected in functional assays sensitive for cytokine production, such as in assays measuring the effect of mononuclear cells on cartilage proteoglycan turnover *in vitro* (Wilbrink et al., 1993).

In reactive arthritis and in oligoarticular juvenile rheumatoid arthritis (OA-JRA) T cell responses to hsp60 were again more prominent. In contrast to adult RA, these conditions are characterised by a remitting course of disease development.

In adult RA patients T cell responses to human hsp60 were detectable by culturing the cells in the presence of added IL4. Bacterial hsp60 was found to stimulate cells without added IL4. This suggests a Th2 nature of the T cells responding to the human molecule in particular (Van Roon et al. 1997).

In children with JRA (OA) responses to human hsp60 coincided with raised expression of IL4 (RT-PCR) in the synovial cells. Furthermore, stimulation of both synovial and peripheral blood T cells resulted in a raised CD30 (a possible marker for Th2 cells) expression in activated (CD45RO+) CD4+ and CD8+ T cells.

In addition OA-JRA patient derived hsp60 specific T cell lines were shown to produce IL4 and TGFß (Prakken 1996).

Altogether the data as obtained in human arthritis patients have shown the potential of hsp's to trigger the release of Th2 associated and suppressive cytokines.

HSP's are an intrinsic component of the slow acting antirheumatic drug OM89

In various models of experimental autoimmunity it is known that exposition of the animals to exogenous bacterial flora contributes to resistance against disease induction. Also in adjuvant arthritis germ-free animals were more susceptible than their conventionally reared counterparts (Kohashi et al., 1986). Furthermore, in Fisher rats it was shown that the susceptible germ-free animals became resistant upon gut re-colonisation with *E. coli* bacteria.

In other words, exposure of the immune system to cross-reactive bacterial antigens, such as hsp's, might well stimulate the immune system to resume control over unwanted self-reactive clones. In line with the known contribution of bacterial gut flora to tolerance, it seems best to effectuate such exposure through oral (or nasal) administration of bacterial antigens. Laboratoires-OM (Geneva) is producing a glycoprotein rich extract of *E.coli* (OM-89), which is marketed and used as a slow acting drug for the treatment of RA. It is administered orally and has shown a therapeutic efficacy comparable with that of gold in trials in RA patients (Rosenthal et al., 1991). Recent analyses have revealed that *E.coli* hsp60 (GroEL) and hsp70 (DnaK) are the dominant immunogens present in this material (Vischer & Van Eden 1994; Bloemendal et al. 1997). Furthermore, both in AA and in avridine arthritis in Lewis rats, OM-89 was found to protect against arthritis. Therefore, *E.coli* hsp's, when administered orally, may trigger a T cell regulatory event that contributes to the control of RA, in a way similar to the effect of mycobacterial hsp60 in models of arthritis.

It is therefore possible that the therapeutic potential of hsp's in RA is already exemplified to some extend by the mode of action of OM-89.

Mechanisms by which hsps produce protection in autoimmune arthritis

The early expression of hsp60 in inflamed synovial tissues (stress-response) and the findings of immunological recognition of the protein both at the level of antibodies and T cells especially in remitting arthritic diseases seem to tie in very well with the data obtained in the experimental animal models. The animal experiments have shown that irrespective of the trigger that led to synovial inflammation, prior stimulation of immunity to self-hsp60 using bacterial hsp60 or its conserved peptide raised resistance to subsequent disease induction. Taken together these findings have indicated that T cell recognition of hsp60 (and also hsp10 or hsp70) at the site of inflammation does contribute to the control of the ongoing inflammatory response. The stimulation of such responses by prior immunisation using bacterial hsp or its relevant peptides, therefore is expected to facilitate such T cell mediated regulatory control.

Heat-shock proteins are, despite their conserved nature and therefore antigenic relationship with 'self', immunogenic to an exceptionally high degree. Although the reasons for this are as yet unclear, this phenomenon can be understood in terms of repertoire selection.

Hsp's are well expressed in the thymic medulla, at the sites where positive thymic selection occurs. This will lead to selection of a repertoire of T cells having a receptor that allows low affinity (antagonistic) interactions with self hsp also when it becomes overexpressed at sites of inflammation. High affinity receptors were negatively selected and therefore deleted. During infection with an hsp expressing micro-organism (any bacterium, parasite etc.) the same repertoire will be expanded by recognition of the microbial hsp homolog. As the homolog will be antigenically close to the self hsp, but not identical with it, this recognition will include high affinity interactions. By its nature these high affinity interactions may lead to an agressive (Th1) anti-infectious response. At the same time this repertoire is numerically expanded. On re-encounter of these T cells with self-hsp as expressed in the inflammatory site, the interaction will again be of low affinity and lead to antagonistic T cell regulation. In other words exposition of the immune system to microbial hsp's is expanding a repertoire of potentially anti-inflammatory T cells. The same repertoire expansion can be effectuated by directed stimulation the immune system with hsp proteins or peptides thereof. Given the existing evidence that responses to hsp's comply with peripheral tolerance mechanisms of immune deviation, it is likely that by-stander suppressive mechanisms (secretion of IL4, TGFß) are operational in hsp induced anti-inflammatory mechanisms.

On the other hand it may be that under normal conditions low level expression of self-hsp epitopes by non-professional antigen presenting cells in the periphery or of conserved microbial hsp epitopes in the 'tolerising' gut environment is continuously noted by T cells. This recognition, however, in the absence of co-stimulation or in the otherwise tolerising mode, will drive such T cells into a regulatory phenotype or anergy. Upon subsequent involvement of such cells in autoimmune inflammation,

they will exert regulatory activity, also when they recognise their over-expressed antigen presented by the professional APC. Recently, we have demonstrated that anergic T cells suppressed, in co-culture, proliferative responses of other T cells as soon as the antigen that was used to induce anergy was added to the co-culture (Taams et al. 1998). In the case of heat-shock proteins, thus anergised cells could focus their regulatory activity to sites of inflammation where heat-shock proteins become temporarily over-expressed (by-stander suppression). In infection the activity of such anergic regulators would be outweighted by a dominant frequency of T cells (vigorously) responding to non-conserved microbial hsp epitopes. Depending on the circumstances the pro-inflammatory response will (temporarily) break peripheral tolerance. And indeed, upon immunisation or infection, microbe specific epitopes are more dominantly recognised. The hsp induced protection against autoimmune inflammation is now explained by either the expansion of the self-hsp cross-reactive T cells which assume the anergic phenotype when encountering their antigen on non-professional APCs, or by further imprinting or spreading of the pre-existent anergic state.

Lessons for the development of specific immunotherapy in autoimmunity

As argued above, immunity to bacterial antigens, such as hsp's, may contribute to maintenance of self tolerance as a hedge against autoimmunity. To achieve a lasting restoration of such tolerance in the case of disease, it seems most adequate to target immunotherapy to the reinforcement of natural mechanisms that contribute to such maintenance of self-tolerance. In other words, exposure of the immune system to bacterial antigens, such as hsp's, might well stimulate the immune to resume control over unwanted self-reactive clones. In line with the known contribution of bacterial gut flora to tolerance, it seems best to effectuate such exposure through oral administration of bacterial antigens. Although little support for the effectivity of such an approach can be obtained from work in experimental disease models, sofar from experience in human medicine such support can be obtained. As mentioned above Laboratories-OM (Geneva) has been producing *E.coli* bacterial lysates, containing bacterial hsp's, which are used amongst others for the treatment of RA. For obvious reasons it would be of great interest to analyze such mechanisms at the level of T cell responses in patients treated with this material.

Positive findings of such an analysis would then possibly lead into attempts to develop better defined pharmaceutical compounds such as synthetic peptides. Such peptides could be composed of conserved sequences of bacterial hsp's and be used to stimulate the frequency or activity of T cells with the potential to recognise self-hsp molecules, expressed at sites of inflammation.

On a limited scale first experience with oral administration of a defined hsp peptide has been obtained by Albani (Bonnin and Albani 1998). Based on the sequence homology of an E. coli dnaJ sequence with the so-called 'shared-epitope', a consensus sequence shared by RA associated HLA-DR molecules, the peptide QKRAA was administered to RA patients in an open label clinical trial. Upon analysis of the functional phenotype of responding T cells in these patients it appears that the

oral treatment did effectuate a relative shift from Th1 to Th2 in cells with specificity for the dnaJ peptide.

The manipulation of peripheral tolerance with hsp immunistion may work through the re-inforcing of natural protective mechanisms of T cell regulatory events in inflammation. The approach is broad and can be usefull for the treatment of inflammation as seen in various autoimmune diseases and other inflammatory diseases such as allergy. Furthermore, spin-off may be expected for infection (including vaccines) and cancer. The ubiquitous nature of hsp expression in diseased tissues, may well assure that antigenic spreading as a possible tolerance escape mechanism will not easily take place. This in contrast to alternative more conventional antigen specific approaches.

Effective treatment leading to reduced hsp expression in the tissues will lead to a gradual loss of therapeutical impact as a usefull built-in feed-back mechanism. In other words no therapeutic overshoot.

Conclusion

In the exploration of mechanisms of peripheral tolerance it has become evident that besides control through specific elimination of cells, interactive regulatory effects of antigens do play a role. Work on antagonistic peptides, anergic T cells or cells displaying a regulatory cytokine profile has provided an experimental basis for such effects. Heat-shock proteins can be the example of how such interactive regulatory effects can be targetted to sites of cellular stress. Immunological recognition of heat-shock proteins as seen in association with virtually every inflammatory condition, including allograft rejection and autoimmune diseases, can be central to peripheral tolerance mechanisms meant to control inflammation beyond tissue specific antigenic manipulation (Van Eden et al. 1998).

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