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SUSCEPTIBILITY AND RESISTANCE TO EXPERIMENTAL ADJUVANT ARTHRITIS

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1. ABSTRACT

Autoimmunity is the result of an abnormal immune response against constituents of body tissues. For many years, the study of animal models of human diseases was aimed at defining the factors participating in the autoimmune process. During the past two decades, much of the attention was diverted to another intriguing aspect of animal models: the mechanisms rendering some animal strains autoimmune-susceptible and others resistant. In this report, we focus on one experimental model, adjuvant arthritis (AA) which is inducible in the Lewis rat following stimulation of the immune system by heat-killed mycobacterium and its 65kDa heat shock protein. We describe genetic loci regulating the severity of this disease as well as the contribution of microbial flora and endocrine activity to susceptibility and resistance. In our opinion, a better understanding of the processes underlying susceptibility and resistance to AA is an important step towards the development of new therapeutic approaches to autoimmunity.

2. INTRODUCTION

Adjuvant arthritis (AA) is inducible in genetically susceptible, highly inbred, strains of rats by intracutaneous inoculation of heat killed mycobacteria suspended in Freund's adjuvant. Approximately fourteen days after active induction of disease by immunization with mycobacterial antigens, arthritis becomes overt. Severity increases over a period of 1-2 weeks and gradually diminishes during the subsequent 1-3 weeks. Although the active inflammatory response gradually subsides, the swelling may last for a longer period and irreversible deformities ensue. Upon re-challenge with mycobacterial antigens, animals usually have developed resistance to the disease and will not develop arthritis again. Evidence for the T-cell-mediated autoimmune nature of the disease is demonstrated by successful adoptive transfer of lymphocytes from arthritic rats to naïve rats, and by induction of disease using *in-vitro* selected mycobacteria-reactive T-cell lines (1).

Mice and primates are in general not susceptible to adjuvant arthritis and different rat strains exhibit wide variations in their resistance or susceptibility to disease induction. The genetic factors determining these variations are mostly unknown. Several explanations have been proposed for the marked difference between, for example, the susceptible Lewis and resistant Fischer rats. Some authors have described alterations in the hormonal status due to differences in the hypophyseal-adrenal axis between these strains and suggested an impact of both sex hormones and sex chromosomes on the rates of susceptibility (2, 3). It seems that differential resistance may also be due to variations in the regulation of immunity to exogenous microbial antigens such as those present in the intestinal flora. Acquired resistance is observed after spontaneous remission of adjuvant arthritis in Lewis rats. A second challenge with mycobacteria suspended in oil generally does not result in disease. Immunization with the 65kDa mycobacterial heat shock protein (HSP65) has been found to cause resistance to subsequent induction of AA. Similar observations were made in models in which arthritis was induced with other agents. It is therefore possible that HSP65 elicits T-cell responses to the endogenous-mammalian HSP60 which in turn may confer resistance to arthritis irrespective of the nature of the trigger leading to arthritis.

Adjuvant arthritis was the first animal model for Rheumatoid Arthritis (RA) (1). Many investigators use AA as a model for human disease because the peripheral joint lesions in rats have many of the features that characterize

RA except for spontaneous recovery and some histological differences. In many cases, the full picture of AA includes other specific tissue lesions, such as iridocyclitis, nodular lesions of the skin, genitourinary lesions and diarrhea (1). In some aspects, the disease may resemble reactive forms of arthritis or Behcet's disease. In humans, exposure to high doses of mycobacteria may also lead to transient arthritis (the human counterpart of adjuvant arthritis).

The similarity between the inflammatory processes in AA and in human RA has made this model a useful tool for developing anti-inflammatory drugs for human patients. Thus far, relatively little effort has been invested in deciphering the mechanism of resistance to AA and using it for developing new therapeutic approaches for rheumatoid arthritis. In the present review, we focus on this aspect of AA and discuss genetic, hormonal, environmental and immunologic mechanisms that may account for resistance and susceptibility to AA.

3. FACTORS INVOLVED IN THE DETERMINATION OF SUSCEPTIBLITY AND RESISTANCE TO AA

3.1. Genetics

Several models of arthritis have been devised in an attempt to pinpoint genomic loci regulating autoimmune arthritis. These include arthritis induced in rats by oil (incomplete Freund's adjuvant oil)(4), collagen (5), pristane and avridine (synthetic adjuvants)(6, 7) as well as adjuvant arthritis (8). Evidently, both major histocompatibility complex (MHC) genes as well as genes localized outside the MHC participate in the modulation of arthritis. A genome wide linkage analysis of susceptible Dark Agouti (DA) and resistant Fischer 344 inbred rats yielded several quantitative trait loci (QTLs) associated with the regulation of adjuvant induced arthritis. These loci are *Aia1* which includes the MHC on chromosome 20, *Aia2* and *Aia3* on chromosome 4 (8), *Aia4* on chromosome 15 and *Aia5* on chromosome 10 (9). Fischer 344 genomic regions *Aia1, Aia2, Aia3 and Aia5* contain genes that reduce the severity of mycobacterial adjuvant-induced arthritis when transferred to DA rats (9). These regulatory sites overlap rheumatoid arthritis-susceptibility loci in humans. The arthritis lowering effects of *Aia2* and *Aia3* are sex-influenced and observed only in males and only in females, respectively. Fischer 344 allele at *Aia4* is associated with greater arthritis severity whereas the DA allele at the same site is associated with lower disease severity. The AA regulating loci play a role in the pathogenesis of other autoimmune diseases as well. For example, *Aia1, Aia3* and *Aia 5* overlap genomic regions involved in the regulation of collagen induced arthritis (8) and *Aia1, Aia2* and *Aia3* are implicated in several other autoimmune diseases in rats, including insulin-dependent diabetes mellitus (in diabetic BB rats), thyroiditis and experimental autoimmune uveitis. Furthermore, analysis of conserved autologous sequences among rats, mice and humans suggests that these loci contain candidate genes for several autoimmune diseases in mice and humans (8).

Finally, we should note that although the inbred Lewis strain is known to be highly susceptible to adjuvant induced arthritis and several other autoimmune diseases, there are also reports of resistant Lewis substrains. A recent work on PIA in Lewis rats suggests that a single-nucleotide polymorphism in the *Ncf1* gene, a component of the NADPH oxidase complex, may account for the inconsistent susceptibility (10).

3.2. Germ-free environment

A large body of data supports the hypothesis that bacteria play a pivotal role in both susceptibility and resistance to adjuvant arthritis. For example, Fischer 344 rats are resistant to AA when bred under conventional, non germ-free conditions. However, in a germ free environment they tend to be as susceptible as Lewis rats. When transferred back to the conventional environment or after recolonization with certain bacterial organisms, they acquire resistance (11). Under sterile conditions, not only is the natural immunity curtailed, but acquisition of protection from AA by means of antigen-specific oral tolerance is less effective as well. In a pathogen free facility, Lewis rats fail to develop resistance after recovering from the first episode of arthritis (Cohen IR, personal communication). It therefore seems that exposure of the airways and the gastrointestinal tract to bacterial antigens contributes to the development of tolerance towards bacterial epitopes, resulting in protection from mycobacterial induced arthritis.

The possible role of bacteria can be further tested with the use of oral antibiotics, which allow manipulation of the composition of the intestinal microbial flora. Thus, administration of oral vancomycin to rats after disease induction results in a significant decrease in the clinical course of adjuvant induced arthritis with a concomitant increase in the concentration of the gram negative Escherichia coli in the distal ileum. Co-administration of colistin/tobramycin to prevent the increase in E. coli abrogates the beneficial effect of vancomycin (12).

We should note, however, that the influence of a microbial environment depends on the manner of disease induction. For example, bacterial flora has a negligible impact in oil-induced-arthritis (OIA) in DA rats (Klareskog, personal communication) and a disease enhancing effect in pristane-induced arthritis (PIA) (13).

The mechanisms underlying the immunomodulatory effects of bacteria are yet to be elucidated, although they most probably involve presentation of bacterial epitopes to the host immune system. Nevertheless, it is currently difficult to define the precise identity of such epitopes. Bacterial proteins are considered a major source for antigens but at least part of the effect may be attributed to nucleic acids. A clue for this hypothesis is provided by the observations that unmethylated CpG motifs, which are abundant in bacterial DNA, induce arthritis (when injected directly into joints) and that the injection of heat-killed Mycobacterium tuberculosis (MT) into Lewis rats seems to induce arthritis in a DNA dependent matter (14).

The effects exerted by bacteria invariably succumb to the hormonal and immune system features of

the host. Measurements of plasma corticosterone levels at the onset of arthritis disclose a significant increase in germ-free susceptible Wistar rats (15). This corticosterone response is insufficient to prevent arthritis but may alleviate its manifestations. This observation supports the notion that bacterial flora (and therefore T cell tolerance) is of prime importance in the determination of susceptibility and resistance, whereas the activity of the hypothalamus-pituitary-adrenal (HPA) axis may set the "fine tuning" of disease severity. Indeed, as we discuss below, this led some authors to propose that increased onset and severity of inflammation in AA in Lewis rats is a consequence of an attenuated HPA response to stress.

3.3. Endocrine System Activity

A great deal of work has been carried out in an attempt to define the relationship between the status of susceptibility/resistance to autoimmune diseases and the activity of the HPA axis. In many cases, the hormonal functional capacity was tested by applying various forms of physical, psychological and immunological stress such as acoustic startle response, forced swimming, injection of endotoxin etc. The results of these works disclosed anatomical and functional differences between Lewis (as well as DA and Wistar) and the histocompatible-related Fischer (or BN) strains which may account for their different responses to autoimmune disease induction.

Interestingly, Lewis rats have relatively smaller adrenal and pituitary glands compared to Fischer rats (16, 17). Lewis rats also exhibit abnormal HPA function and blunted circadian profile not only when compared to the resistant Fischer 344 and BN but also to most other inbred strains. Their attenuated response to stress can be attributed both to impaired biosynthesis of CRH in the hypothalamus, and to a low pituitary response to CRH (18). Subsequently, secretion of ACTH from the pituitary glands and release of steroids from the adrenals in response to stress are diminished. When Lewis rats grow old they acquire natural immunity to AA, and this is also reflected by enhancement of the corticosterone response to stress, such that it eventually equals that of Fischer 344 (19). It should be noted that CRH is also secreted peripherally, within inflammatory sites, where it appears to have pro-inflammatory actions (20). Thus, arthritis resistant rat strains express high levels of CRH in the hypothalamus and low levels within the joints, whereas in susceptible rats this ratio is inverted (21).

Recent data from *in vitro* models shed new light on the interactions between the endocrine and immune systems. Cultured cells of hypothalamic origin can produce and respond to pro-inflammatory and anti-inflammatory cytokines, which contribute to the regulation of hypothalamic functions such as sleep patterns, food intake, temperature setting and activation of the HPA axis (22, 23). Hypothalamic cells from Lewis rats respond poorly to pro-inflammatory cytokines, e.g. TNF-alpha, IL-1beta and IL6, as well as to LPS (24). In response to IL-1beta, Fischer hypothalamic cells increase mRNA transcription of CRH and immediate-early genes, which are considered as an indication of neuronal activation of the HPA axis (25). This effect is absent in Lewis cells. When compared to Fischer rats, naïve Lewis cells transcribe more mRNA copies of pro-inflammatory cytokines and their corresponding receptors. The anti-inflammatory IL-10, which was recently found to be a potential inducer of CRH secretion (17), is expressed similarly in both strains following exposure to LPS, whereas the levels of TGF-beta, an inhibitor of CRH, is four times higher in Lewis. Taken together, these observations provide cellular mechanisms for blunted CRH secretion in Lewis rats upon immune challenge.

The HPA axis is strongly associated with the hypothalamus-pituitary-gonadal (HPG) axis. Androgens seem to play a role in the control of HPA axis activity (26), and act in concert with circulating corticosterone (27). However, in females the HPA axis demonstrates increased responsiveness (28), and this has mainly been attributed to estrogen induced reduction of glucocorticoid receptors in the hypothalamus (suppressing corticosterone negative feedback), and direct stimulation of CRH synthesis via estrogen responsive elements in the CRH gene (29, 30). Indeed, administration of estrogen significantly alleviates disease severity and reduces IL-6 levels in both male and female Lewis rats with AA or in DBA mice with CIA (31). Estrogens were also found to be protective against LPS-induced inflammatory responses in both neurons and glial cells (32). Lewis and Fischer female rats have similar plasma estrogen levels following intraperitoneal injection of LPS. However, in Lewis rats there seems to be a defect in the expression and regulation of the estrogen receptor both in the basal state and following an immune challenge (33).

The effect of AA on the endocrine status is certainly not restricted to the HPA/HPG systems, and the production of a vast array of other hormones is also disrupted. For example, there is a decrease in circulating growth hormone and insulin like growth factor I (IGF-I) with a concomitant increase in IGF-I binding protein. Cyclosporine administration renders the arthritic rats less susceptible to both effects, providing an explanation for the beneficial effect of cyclosporine on body weight in AA. (34). Furthermore, the administration of Freund's adjuvant can abolish temporal fluctuations in plasma albumin and beta globulin levels, and decrease the amplitude of daily changes in alpha 1 and alpha 2 globulins. Pretreatment of rats with melatonin effectively prevents mycobacterial adjuvant suppression of this 24-hr rhythm (35).

3.4. The Immune System

3.4.1. Experimental Methods for Induction of resistance to AA

The fact that inoculation of heat-killed, non-infectious MT in complete Freund's adjuvant induces AA, led to the conclusion that bacterial antigens are targets for the innate and foreign immune systems. Two decades ago, a T-cell clone specific for MT antigens and strongly arthritogenic was isolated. Using this cell line, it was possible to transfer arthritis to naïve irradiated recipient rats (36-38). Following the identification of the relevant T-cell epitope as a peptide (aa180-188) within the MT-HSP65 (39-41), intensive studies in both animal models (36, 37, 40, 42) and in humans (43-45) have been carried out in an

attempt to decipher the mechanisms by which heat shock proteins induce AA and modulate its severity. The striking similarity between bacterial and mammalian HSPs in terms of molecular structure and antigenicity (46, 47) provides a possible link between infection and autoimmunity or, alternatively, tolerance to bacteria and protection from disease.

AA cannot be induced in resistant strains of rats (e.g. BN or Fisher) and Lewis rats develop resistance to re-induction of the disease after recovery from arthritis. Likewise, pre-immunization of susceptible rats with the mycobacterial HSP65 leads to resistance to induction of the disease by MT. Although arthritic rats develop vigorous T cell responses to peptide 180-188 of HSP65 following injection of heat killed MT, neither of these molecules is arthritogenic when injected in protein or peptide form, respectively (48-50). This indicates that HSP65 may contain different epitopes, some of which are responsible for its pathogenicity, whereas others confer resistance to disease induction.

Several methods have been applied to confer resistance to or suppression of inflammation in AA susceptible rats. One of the approaches involves nasal administration of synthetic peptides covering the arthritogenic sequence 180-188 (51). A homologous peptide with alanine in the 183 position yields a suppressive effect exceeding that of the wild type epitope. This effect is passively transferred using activated splenocytes (52).

Another approach for AA suppression is based on the concept of "oral tolerance". Oral administration of antigens prior to disease has been shown to induce peripheral tolerance in several experimental autoimmune diseases. The clinical benefit of pretreatment with antigens is generally limited, and therefore an attempt was made to treat AA by oral administration of HSP65 during ongoing disease (53). This manipulation can reduce AA activity significantly, provided that protein breakdown in the gastrointestinal tract is inhibited. Immunization can also be obtained by vaccination of rats with DNA constructs encoding HSP65 or the mammalian HSP60 (54). Evidently, both constructs inhibit AA, with the HSP60 encoding construct showing a stronger effect than the mycobacterial plasmid. Vaccination with overlapping fragments of human HSP60 cDNA was used to probe the regulatory epitope Hu3 (aa31-50), which has the same amino-acid sequence as its rat counterpart (55).

3.4.2. Possible Immunologic Mechanisms for Resistance

The question of the mechanism underlying resistance to or suppression of arthritis is intriguing and challenging. Analysis and definition of the putative protective epitopes in the bacterial and mammalian HSP have become major targets for research, as HSP therapy was also found effective in CIA and in non-antigen induced models of arthritis. Two hypotheses have been proposed thus far to explain this phenomenon. One explanation is that resistance results from a T cell response to epitopes within the heat shock protein while an alternative model suggests a B-cell response with production of specific anti-HSP65 and HSP60 antibodies and activation of the non-specific innate immunity system. It should be noted that both explanations attribute a central role for IL-10 induction in the suppression and prevention of AA, as will be discussed below.

3.4.2.1 T-cell Theory

When discussing possible mechanisms for resistance to AA, one can differentiate between the naturally occurring immunity in the "resistant strains" and experimental immunity in "susceptible strains".

Protection from AA in *resistant* strains, manifested as tolerance to HSP65, is thought to arise from exposure of the gut mucosa to the closely related and highly homologous bacterial GroEl, which is an E. coli protein. However, vaccination of Lewis rats with GroEl has failed thus far to provide resistance, and it is possible that the native bacterial epitopes in the gut are recognized differently from the experimentally injected protein. As mentioned above ("3.2 germ free environment") naïve Fischer rats spontaneously produce T-cells responsive to MT HSP65 only in the conventional microbial environment (56). Such T-cells are cross-reactive and may be primed as a consequence of molecular mimicry, thereby conferring protection from AA in Fischer rats. Therefore, the bacterial population within the intestine and the responses it triggers are crucial in the determination of resistance although there is no simple way to explain why certain strains develop such tolerance while others do not.

Once pre-immunization with HSP65 proved to provide protection of *susceptible* rats against AA and other forms of arthritis, a mechanistic explanation for this acquired resistance was sought. Several putative explanations were then proposed, such as enhanced responsiveness to protective epitopes in HSP65, down-regulation of T-cell responses to the arthritogenic 180-188 epitope, as well as activation of self-HSP60-reactive T-cells. Initial results indicated that HSP65 pre-immunization did not down regulate responses to the AA associated epitope but did enhance responses to several other HSP65 epitopes (57). Of these, cross reactive T-cell recognition of HSP65 and rat HSP60 was limited to a single epitope (256-265). A subsequent work demonstrated that a peptide containing the 256-270 epitope was also able to confer protection from AA as well as other forms of arthritis (58). Likewise, administration of a T cell line specific for this epitope provides protection against AA. During the late phase of disease in Lewis rats, there is diversification of the T-cell responses to include new determinants within HSP65. Thus, at 4 wk after onset of arthritis, T-cell responses to carboxy-terminal determinants are detected (58). Pretreatment with peptides comprising these determinants and their mammalian counterparts induce significant regression of acute inflammatory arthritis. Thus, it is possible to obtain immunity to AA not only by pretreatment of rats with the whole HSP65 but also by using some of its fragments i.e. the arthritogenic peptide 180-188 as well as peptide 256-270 and the carboxy-terminal determinants. Considering the size of HSP65, it is

reasonable that different regions of the same molecule are protective.

In recent years, a new concept has been put forth, stating that in addition to the accepted paradigm that cross reactive T-cell recognition of foreign and self antigens might induce autoimmune disease, cross reactivity between bacterial and self HSP might also support the maintenance of a protective self reactive T-cell population. The observation that self-HSP reactive T-cells can down-regulate inflammation (48-50) also suggests that there are non-pathogenic self-reactive T-cells which apparently escape thymic selection and protect from disease induction.

3.4.2.2. Bcell theory

Resistance to AA may also be explained by the presence of antibodies against HSP65. This concept has emerged from the finding that susceptible rats acquire protection from arthritis by intravenous infusion of immune-globulins derived from resistant strains (59). Rigorous analysis of the epitope specificity of anti-HSP antibodies revealed that natural antibodies to epitopes of the mycobacterial and mammalian HSPs are present in young naïve resistant BN rats, and absent in young susceptible Lewis rats. Naïve Lewis rats produce protective anti-HSP antibodies later in life, as they turn resistant to disease induction (60). The antibodies react with a specific peptide (aa31-46; peptide 6) from the mycobacterial HSP and with its mammalian counterpart (aa61-80; peptide 5). These epitopes are not highly conserved, and do not trigger significant T-cell responses during the course of the disease. Analysis of the primary and tertiary structure of the whole HSP65 molecule revealed that the protective peptides are potential B-cell epitopes found on the outer surface of the molecule. Vaccination of Lewis rats with some of these peptides prior to disease induction resulted in the production of antibodies against the whole molecule as well as resistance to AA. The protective antibodies skew the cytokine profile of mononuclear cells towards an anti-inflammatory response in vitro, and suppression of the disease. The presence of antibodies to the self-HSP peptide 5 in IVIG preparations extracted from healthy individuals (61) implies a similar role for these antibodies in humans.

3.4.2.3. The Role of IL-10

Several studies report that the production of the anti-inflammatory IL-10 cytokine is enhanced following the acquisition of resistance to AA. Examples of experiments in which IL-10 is over-expressed are: priming of T cells with the aa256-270 peptide (62); immunization of rats with HSP70 (63-65); induction of nasal tolerance by HSP65 peptide aa180-188 (52); DNA vaccination against AA (66) and treatment of macrophages with protective anti-HSP65 antibodies (60). This observation highly suggests that IL-10 plays a significant role in the suppression of inflammation in arthritis-resistant animals. Little is known about the cellular mechanisms underlying the induction of IL-10 overproduction. In the case of protective anti HSP antibodies, a concomitant rise in specific IL-10 mRNA levels has been demonstrated, indicating enhancement in promoter activity (60). Preliminary results suggest that binding of the antibodies to a membrane receptor initiates an intracellular process culminating in the binding of transcription factors to a cluster of cis-acting elements upstream to the transcription start site (unpublished data). Whether this molecular explanation applies to the T-cell models of resistance to adjuvant arthritis is yet to be studied.

4. CONCLUSIONS

Autoimmunity is a state of abnormal specific humoral and cellular immune response against constituents of body tissues. Research of human autoimmune diseases has been largely supported by experimental animal models in which the disease has been induced by specific vaccination of mice and rats with the putative self antigen, other target-organ antigens, or by non-specific vaccination such as CFA, as is the case in AA. Most, if not all, of the autoimmune animal models can be induced only in some strains of susceptible animals, while other strains are resistant.

The Lewis rat is susceptible to several autoimmune diseases which cannot be induced in strains such as Fischer or BN. Examples for these diseases are the experimental autoimmune encephalomyelitis (EAE), myasthenia gravis (EAMG), uveitis, myocarditis as well as adjuvant arthritis.

The susceptibility of Lewis rats to autoimmune processes in which both T-cells (e.g. EAE, AA) and B cells (e.g. EAMG) are the pathogenic autoreactive elements aimed at various target antigens and the resistance of other strains to the induction of the same diseases may suggest that the Lewis rat lacks a protective factor that is present in resistant strains.

Traditional research in the field of autoimmunity in animal models of human diseases attempts to define the abnormal factors leading to the autoimmune process. In this review we have focused on a different aspect of the animal models: the differences between the autoimmune-susceptible animals and their resistant counterparts using one experimental model, AA in the Lewis rat.

Our review emphasizes some differences between the susceptible and resistant strains. Specific quantitative trait loci (*Aia1-Aia5*) implicated in the regulation of AA were defined, including both the MHC and genomic loci outside the MHC. We should point out that little is known about the nature of these non-MHC regions, the identity of the putative genes they may harbor and the manner by which these loci affect autoimmunity and inflammation. Germ free environment and reduced corticosterone responses to stress are associated with susceptibility to AA. Nevertheless, there is no simple explanation for the observation that exposure to the same bacterial environment contributes to development of tolerance to CFA only in resistant but not in susceptible strains. T- and B-cell responses to mycobacterial and mammalian HSP epitopes and enhanced anti-inflammatory cytokine secretion are associated with resistance to disease. These responses can produce auto-reactive T-cell clones or antibodies with protective effects. The mechanism

underlying this "beneficial autoimmunity" is currently under investigation.

In conclusion, we suggest that by studying the factors responsible for resistance to induction of AA it is possible to obtain a better understanding of the pathogenesis of autoimmunity as a step towards the development of new therapeutic approaches.

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