Diversification of T Cell Responses to Carboxy-terminal Determinants within the 65-kD Heat-shock Protein Is Involved in Regulation of Autoimmune Arthritis

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Summary

The T cell response to the 65-kD mycobacterial heat-shock protein (Bhsp65) has been implicated in the pathogenesis of autoimmune arthritis. Adjuvant arthritis (AA) induced in the Lewis rat (RT-1l) by injection of Mycobacterium tuberculosis serves as an experimental model for human rheumatoid arthritis (RA). However, the immunological basis of regulation of acute AA, or of susceptibility/resistance to AA is not known. We have defined the specificity of the proliferative T cell responses to Bhsp65 during the course of AA in the Lewis rat. During the early phase of the disease (6–9 d after onset of AA), Lewis rats raised T cell responses to many determinants within Bhsp65, spread throughout the molecule. Importantly, in the late phase of the disease (8–10 wk after onset of AA), there was evidence for diversification of the T cell responses toward Bhsp65 carboxy-terminal determinants (BCTD) (namely, 417–431, 441–455, 465–479, 513–527, and 521–535). Moreover, arthritic rats in the late phase of AA also raised vigorous T cell responses to those carboxy-terminal determinants within self(rat) hsp65 (R hsp65) that correspond in position to the above BCTD. These results suggest that the observed diversification is possibly triggered in vivo by induction of self(R hsp65)-reactive T cells. Interestingly, another strain of rat, the Wistar Kyoto (WKY/NHsd) rat (RT-1l), with the same major histocompatibility complex class II molecules as the Lewis rat, was found to be resistant to AA. In WKY rats, vigorous responses to the BCTD, to which the Lewis rat responded only in the late phase of AA, were observed very early, 10 d after injection of M. tuberculosis. Strikingly, pretreatment with the peptides comprising the set of BCTD, but not its amino-terminal determinants, provided significant protection to naive Lewis rats from subsequent induction of AA. Thus, T cell responses to the BCTD are involved in regulating inflammatory arthritis in the Lewis rat and in conferring resistance to AA in the WKY rat. These results have important implications in understanding the pathogenesis of RA and in devising new immunotherapeutic strategies for this disease.

Rheumatoid arthritis (RA)1 is an autoimmune disease of unknown etiology (1, 2). In the past several years, considerable interest has been generated in the role of the 65-kD mycobacterial heat-shock protein (Bhsp65) in the pathogenesis of autoimmune arthritis both in experimental animals (3, 4) as well as in humans (5–7). In RA patients, an association between T cell responses to Bhsp65 and early stages of joint inflammation has been observed (8–10), suggesting that Bhsp65-reactive T cell responses are involved in the pathogenesis of this disease. Adjuvant arthritis (AA) can be induced in the inbred Lewis rat after immunization with Mycobacterium tuberculosis in oil (11–13). The disease can also be transferred to naive Lewis rats by T cell lines reactive to peptide 180–188 of Bhsp65 (3, 14). Although arthritic Lewis rats develop vigorous T cell responses to native Bhsp65 and to peptide 180–188 of Bhsp65, neither of these is arthritogenic when injected in protein or peptide form, respectively (15, 16). Interestingly, pretreatment with Bhsp65 can protect naive Lewis rats from development of arthritis upon subsequent immunization with
M. tuberculosis (4, 17), suggesting that Bhsp65 contains protective as well as disease-inducing determinants. T cell lines specific for Bhsp65 have also been shown to protect against AA (14, 18). Similarly, Lewis rats afflicted with AA are resistant to reinduction of AA (13). Neither the mechanism of natural regulation of the acute inflammatory phase of AA nor the mechanism of protection from subsequent induction of AA is known. Likewise, the immunological basis of susceptibility or resistance to AA of different rat strains has not been revealed.

In the present study, we have defined the changing pattern of specificity of the T cell responses of Lewis rats to determinants within Bhsps65 during the course of AA. Arthritic Lewis rats in the early and late phase of the disease revealed distinct patterns of T cell responses toward Bhsps65 carboxy-terminal determinants (BCTD). (The phenomenon of spreading of the T cell responses to new determinants within an antigen, after priming with a single determinant of the same antigen, has been previously described as determinant spreading [19]. On the other hand, we have termed the induction of T cell responses to new determinants after priming with the whole, multidentator determinant antigen as diversification [20, 21]). Moreover, arthritic Lewis rats in the late phase of AA also raised significant responses to certain carboxy-terminal determinants within self (rat) hsp65 (R hsp65). (R at hsp60 [22] has been referred to as rat hsp65 in this study to emphasize its relationship with Bhsps65.) These self-determinants correspond in position precisely to that of the BCTD, suggesting that diversification of response to Bhsps65 observed in vitro, might be triggered in vivo by self-hsp65. In contrast with AA-susceptible Lewis rats, MHC class II–identical, Wistar Kyoto (WKY) rats (23, 24) were found to be resistant to induction of AA after immunization with M. tuberculosis. In fact, M. tuberculosis–immunized WKY rats raised early and vigorous responses to the BCTD to which the Lewis rats only respond during the late phase of the disease. Pertinently, pre-treatment of naive Lewis rats with peptides comprising the BCTD, but not its amino-terminal determinants (BNTD), induced significant protection from AA. These results suggest that T cell responses to the BCTD are involved in regulation of acute inflammatory arthritis. Our study suggests one of the immunological bases for natural regulation of acute AA, and of protection (resistance) from development of AA.

Materials and Methods

Animals. Inbred Lewis (RT-1) and Wistar Kyoto (WKY/N Hsd = WKY) rats (23, 24) were found to be resistant to induction of AA after immunization with M. tuberculosis. In fact, M. tuberculosis–immunized WKY rats raised early and vigorous responses to the BCTD to which the Lewis rats only respond during the late phase of the disease. Pertinently, pre-treatment of naive Lewis rats with peptides comprising the BCTD, but not its amino-terminal determinants (BNTD), induced significant protection from AA. These results suggest that T cell responses to the BCTD are involved in regulation of acute inflammatory arthritis. Our study suggests one of the immunological bases for natural regulation of acute AA, and of protection (resistance) from development of AA.

Healthy Organization through Dr. R. van der Zee. The peptides containing amino acid sequences of the Bhsps65 or Rhsp65 (22, 25, 26; the National Biomedical Research Foundation data base) were prepared by three methods: (a) A complete series of overlapping peptides (15-mer with an overlap of 11 amino acid residues) spanning the entire sequence of Bhsps65 was obtained from Chiron M. I-motopes (Clayton, Australia). The peptides were synthesized using the multi-pin peptide synthesis technique using repeated cycles of Fmoc deprotection and amino acid couplings (27). The procedure had been modified so that the peptides could be cleaved from the pins. The terminal amino group of each peptide was acetylated, whereas diketopiperazine was attached at the carboxy terminus. (b) Several peptides of Bhsps65 and R hsp65 were synthesized in the UCLA Peptide Core Laboratory directed by Dr. J. R. R. Jr., using a M. multifile peptide synthesizer (Advanced Chem Tech, 396 M PS, Louisville, KY) as described elsewhere (28). The identity and purity of these peptides were determined by Fast Atomic Bombardment Mass Spectrometry at the UCLA Center for Molecular and Medical Sciences Mass Spectrometry facility. (c) Some peptides were obtained from M. acromolecular Resources (Colorado State University, Fort Collins, CO). These peptides were synthesized according to the method described elsewhere (29). Hen eggwhite lysozyme (HEL) peptide was synthesized according to the method previously described (29).

Induction of an adjuvant arthritis. Inbred male Lewis rats were anesthetized using Halothane (Halcobon Laboratories, River Edge, N.), and then immunized subcutaneously in a hind footpad with 200 μl of M. tuberculosis H37Ra (Difco Laboratories, Detroit, MI) (10 mg/ml) suspended in IFA (Difco) or in mineral oil (Sigma Chemical Co., St. Louis, MO). The bacteria were powdered in a mortar and pestle before suspension in oil. Beginning on day 7 after immunization, the rats were observed daily for clinical signs of arthritis in three of their limbs, excluding the limbs in which immunization was performed. The severity of arthritis was evaluated on the basis of erythema, swelling, and deformity of the joint (30, 31), and graded on a scale of 0 to 4 as follows: 0 = no erythema or swelling, 1 = slight erythema or swelling of the ankle or wrist, 2 = moderate erythema and swelling at the wrist or ankle, 3 = moderate erythema and swelling at the wrist/metacarpals or ankle/metatarsals, 4 = severe erythema and swelling of the forepaw or hind paw (32, 33). Because only the three uninjected limbs were evaluated, the maximum arthritic score for any rat was 12.

Histopathological examination of arthritic joints. The experimental or age- and sex-matched control rats were sacrificed under anesthesia. A hind or front leg was cut above the ankle or the wrist, respectively, with the help of a bone cutter. For histopathological examination, the skin from the entire limb was removed and the limb was immersed in 10% buffered formalin phosphate (Fisher Scientific, Fair Lawn, NJ) for fixation for at least 2 d before decalcification. After decalcification, the sections were cut with a cryotome and then stained with hematoxylin and eosin. The stained sections were studied under the microscope for histopathological changes in the joints as previously described (32, 33).

Lymph Node and Splenic T Cell Proliferation Assay. The draining lymph nodes of rats immunized subcutaneously with M. tuberculosis as described above, were removed and a single cell suspension prepared (29). The debris was allowed to settle, and the cells in the supernatant were washed twice with HBSS (GIBCO BR L, Gaithersburg, MD). These lymph node cells (LNC) were cultured in flat-bottomed 96-well plates at 5 × 10⁶ cells/well in HL-1 serum-free medium (Ventrex Laboratories, Inc., Portland, ME) supplemented with 2 mM L-glutamine, 100 U/ml penicillin G
sodium, and 100 μg/ml streptomycin sulphate, with or without antigen. (Splenic T cell proliferation assays were performed as described for LNC, except for plating SPC at a concentration of 6 \times 10^5 cells/well). For the pin peptides, one or two wells were tested per peptide. Tuberculin purified protein derivative (PPD) (Evans M edical Limited, Horsham, England) was used at a final concentration of 2,000 U/well as a positive control. 1 μCi of [³H]thymidine (International Chemical and Nuclear, Irvine, CA) was added per well for the last 18 h of a 5 d culture. The cells were then harvested on a Printed Filtermat A glass fiber filter (Wallac Oy, Turku, Finland) using a M icro Cell Harvester (Skatron Instruments, Inc., Sterling, VA), and the incorporation of radioactivity was assayed by liquid scintillation counting, using the LKB 1205 Betaplate counter. The results were expressed as either cpm or stimulation index (S.I. = cpm with antigen/cpm with cells in medium alone). For some repeat experiments, HL-1 medium supplemented with 1% (vol/vol) heat-inactivated FCS (Gemini Bio-Products Inc., Calabasas, CA) or X-Vivo 10 serum-free medium (Bio-Wittaker, Walkersville, MD) supplemented with 2% FCS and/or 5 \times 10⁻² M 2-mercaptoethanol (Sigma) was used in place of HL-1 medium. In some of these assays, 2.5-4.5 \times 10^5 cells/well were used instead of 5-6 \times 10^5 cells/well.

Results

Early and Late Arthritic Responses of Lewis Rats. Lewis rats were immunized subcutaneously in a hind footpad with M. tuberculosis in oil, and from 7 d after immunization onwards, were observed daily for clinical signs of arthritis. The induction of arthritis was further confirmed by histopathological examination of joints as described in Materials and Methods.

Two timepoints were chosen for study of proliferative T cell responses to Bhsp65 of LNC of arthritic rats: 6–9 d (early phase; AA) or 8–10 wk (late phase of AA) after onset of clinical signs of arthritis (see Materials and Methods). The rationale for choosing days 6–9 was that once arthritis was evident in the hind and fore paws, it is anticipated that draining LNC in the early and late phases of AA was to determine the dynamics of T cell responses to Bhsp65 during the course of disease.

Response of Lewis Rats to Bhsp65 in the Early Phase of AA. As evident from Fig. 1A, during the early phase of AA, draining LNC of rats raised proliferative T cell responses to several determinants within Bhsp65: 13–27, 33–47, 113–127, 177–191, 189–203, 217–231, 257–271, 277–291, 313–327, 357–371, 385–399, 453–467, and 485–499. Because rats were immunized with M. tbhsp65, the immunogenic determinants shown in Fig. 1A (and in Fig. 1B) represent the dominant and subdominant determinants within Bhsp65. As discussed below, from the viewpoint of arthritis induction, the dominant and subdominant determinants within Bhsp65 are of utmost importance.

Response of Arthritic Lewis Rats to Bhsp65 in the Late Phase of AA. In another series of experiments, the LNC of arthritic Lewis rats in the late phase of the disease were tested in a proliferation assay using pin peptides of Bhsp65. The results of one representative experiment using pooled LNC from four rats are shown in Fig. 1B. The predominant peaks representing the dominant and subdominant determinants within Bhsp65 were found to correspond to the following peptides: 173–187, 179–193, 185–199, 217–231, 237–251, 257–271, 281–295, 309–323, 349–363, 389–403, 417–431, 441–455, 465–479, 489–503, 513–527, and 521–535.

Upon detailed comparison of the profiles of proliferative T cell responses of LNC of arthritic Lewis rats in the early...
Responses to the carboxy-terminal determinants of both Bhsp65 and R hsp65. These results suggest that there is induction of R hsp65-reactive T cells in vivo during the course of AA. W KY rats, with the same MHC class II haplotype as the Lewis rat, are resistant to AA. To determine the role of MHC versus non-MHC genes in determining susceptibility or resistance to AA, we tested the WKY rats, which are of the same MHC class II haplotype as the Lewis rat (23, 24). Strikingly, under the same conditions used for induction of AA in the Lewis rat, age- and sex-matched WKY rats were found to be resistant to AA. Only 3 out of 51

| Peptides | Amino acid sequences |
|----------|----------------------|
| Bhsp 417-431 | LLQAAPTDDELKLEG |
| R hsp 418-432 | --RCI-A---S--PAN |
| Bhsp 441-455 | KVALEAPLQIAFNS |
| R hsp 441-455 | IIR--KI--AMT--K |
| Bhsp 465-479 | KVRNLPAEGHCLNATA |
| R hsp 465-479 | VR--ILQSSSEV--YD-- |
| Bhsp 513-527 | TT--EAVVADKPEKEKA |
| R hsp 512-526 | LL--A--TEI--E-- |
| Bhsp 521-535 | KPKEKASV*PGGDM |
| R hsp 521-535 | T**P--EEKD--M--A-- |

--- = identical residue; * = gap introduced for best alignment.

**Figure 2.** Response of arthritic Lewis rats to peptides containing the COOH-terminal determinants of both Bhsp65 (A) or R hsp65 (B). Arthritis was induced as described in Materials and Methods. 4 wk after the appearance of clinical AA, rats were killed and their spleen cells (SPC) tested in a proliferation assay. The results are expressed as cpm. The results of a representative experiment are shown here. Similar results were obtained in repeat experiments (data not shown). The amino acid sequences of the five peptides comprising the BCTD and of the corresponding R hsp65 peptides are given in Table 2. R response to peptide 177-191 is shown as a positive control. R hsp65 peptide 465-479 could not be tested in this series of experiments.
Importantly, WKY rats were not deficient in raising responses to peptide 177–191, which contains the minimal arthritogenic determinant, 180–188, described for Lewis rats (4), and is cross-reactive with it. These results suggest that despite reactivity to a known (established) arthritogenic determinant within Bhsp65, efficient T cell responses to carboxy-terminal determinants of the same protein were successful in affording protection to WKY rats from development of AA.

Figure 1. The results are expressed as cpm. The T cell responses to the unique carboxy-terminal determinants of Bhsp65 (to which arthritic Lewis rats respond only in the late phase of AA; shown in Fig. 1B) are indicated by arrows. Although the highest proliferative responses correspond to the peptides marked by arrows, comparable or even higher responses also were raised to the adjacent overlapping peptides namely, 417–431, 441–455, 465–479, 513–527, and 521–535 in repeat experiments (data not shown). Responses to the BCTD were observed in repeat experiments in WKY rats tested 10–13 d after M. tuberculosis injection (data not shown).

(5.9%) WKY rats developed mild (grade 1) AA. The remaining WKY rats, observed for more than 18 mo, did not manifest any clinical or histopathological signs of AA (data not shown).

Figure 3. Response of AA-resistant Wistar Kyoto (WKY) rats to Bhsp65 peptides after injection of M. tuberculosis. Rats were immunized with M. tuberculosis subcutaneously and after 10 d, the draining LNC from four rats were pooled and tested in a proliferation assay as described in Fig. 1. The results are expressed as cpm. The T cell responses to the unique carboxy-terminal determinants of Bhsp65 (to which arthritic Lewis rats respond only in the late phase of AA; shown in Fig. 1B) are indicated by arrows. Although the highest proliferative responses correspond to the peptides marked by arrows, comparable or even higher responses also were raised to the adjacent overlapping peptides namely, 417–431, 441–455, 465–479, 513–527, and 521–535 in repeat experiments (data not shown). Responses to the BCTD were observed in repeat experiments in WKY rats tested 10–13 d after M. tuberculosis injection (data not shown).

Early and Vigorous Response to the Carboxy-terminal Determinants of Bhsp65 in AA-resistant WKY Rats after Immunization with M. tuberculosis. To determine the immunological basis of susceptibility/resistance to AA, we tested the T cell responses of WKY rats to peptides of Bhsp65 following injection of M. tuberculosis. Based on the results shown in Fig. 1, we reasoned that susceptibility or resistance of rat strains of the same MHC haplotype to AA might relate primarily to differences in processing and presentation of Bhsp65: efficient and earlier display of the BCTD would ensure protection from AA, whereas lack of or inefficient presentation of these determinants would result in active disease. Alternatively, the resistance to AA of the WKY rat strain could be owing to its inability to process and present the potentially arthritogenic determinant 180–188 within Bhsp65.

The results given in Fig. 3 clearly demonstrate that 10 d after immunization with M. tuberculosis, WKY rats raised T cell responses to several determinants within Bhsp65. Interestingly, unlike Lewis rats in the early phase of AA (see Fig. 1A), WKY rats could raise significant T cell responses to the BCTD; Lewis rats raised T cell responses to the BCTD only in the late phase of AA (see Fig. 1B). Although the highest proliferative responses to Bhsp65 peptides in the carboxy-terminal region correspond to peptides 421–435, 445–459, 469–483, 517–531, and 525–539, WKY rats also raised significant responses to the overlapping peptides immediately preceding these peptides in the pepscan, namely, 417–431, 441–455, 465–479, 513–527, and 521–535 (Fig. 3). In repeat experiments, the proliferative responses to the adjacent overlapping peptides comprising each of these five pairs of peptides either was comparable or the pattern was reversed compared with the above pattern (data not shown). Importantly, WKY rats were not deficient in raising responses to peptide 177–191, which contains the minimal arthritogenic determinant, 180–188, described for Lewis rats (4), and is cross-reactive with it. These results suggest that despite reactivity to a known (established) arthritogenic determinant within Bhsp65, efficient T cell responses to carboxy-terminal determinants of the same protein were successful in affording protection to WKY rats from development of AA.

Pretreatment of naive Lewis rats with peptides containing the BCTD afforded protection from AA. Finally, to determine the physiologic role of diversification of T cell responses to Bhsp65 in AA, we treated naive Lewis rats with an immunogenic combination of five peptides containing the sequence of the BCTD. 5–6 wk later, these rats were then challenged with M. tuberculosis in an attempt to induce AA, and thereafter these rats were observed regularly for clinical signs of AA. The results are given in Fig. 4A. Strikingly, pretreatment with the BCTD afforded significant protection to naive Lewis rats from subsequent induction of AA, whereas age- and sex-matched control Lewis rats treated with an irrelevant HEL peptide exhibited the usual course of the disease. In comparison to control rats, BCTD-treated rats developed much milder arthritis or not at all and, eventually, did not reveal permanent joint deforming. Similar results were obtained in two other similar experiments (data not shown). On the contrary, in another series of experiments in age- and sex-matched Lewis rats using the same protocol except for injection of M. tuberculosis 2 wk (instead of 5–6 wk) after pretreatment with the BCTD, there was no evidence of protection from AA (data not shown). Thus, the duration of the period after pretreatment with the BCTD is the most crucial factor in determining the outcome of the interplay between the BCTD-specific regulatory T cells and the arthritogenic T cells.

In another experiment, pretreatment of naive Lewis rats with peptides comprising certain Bhsp65 amino-terminal determinants (BNTD); namely, 13–27, 33–47, and 121–135, was performed to determine whether peptides from a region other than the carboxy-terminal region of Bhsp65 have any effect on the outcome of AA. The conditions of this experiment were similar to that of the above experiment using pretreatment with BCTD. The control HEL peptide used in the two experiments also was the same. The results of the experiment given in Fig. 4B show that the course of AA in BNTD-treated rats was similar to that of the age- and sex-matched HEL peptide-treated rats. Furthermore, both these groups of rats exhibited disease characteristics similar to that of the control group of HEL peptide-treated rats shown in Fig. 4A. These results demonstrate that the protective effect of Bhsp65 peptides against AA is simply not a general property of just any region of Bhsp65; as shown above, the carboxy-terminal, but not the amino-terminal peptides of Bhsp65 were protective against AA.
with a combination of five Bhsp65 peptides (namely, 417–431, 441–455, 465–479, 513–527, and 521–535) (A) mixed in N,N-Dimethyl-N,N-dioc-tadecyl ammonium chloride (DDA) (GERBU adjuvant; GERBU Biotechnik GmbH, Gaiberg, Germany) (17, 55). Each rat received 100 μg of each of the five peptides mixed together in the same suspension.

Another group of age- and sex-matched control Lewis rats (n = 6) was immunized subcutaneously with HEL peptide 85–96/DDA. From 7 d onwards, rats were examined daily or on alternate days for signs of arthritis. The severity of arthritis in each of the three uninjected paws was graded on a scale from 0 to 4 as described in Materials and Methods, and the highest score achievable in any rat was 12 (32, 33). Rats were observed for up to 61 d after injection of M. tuberculosis. The difference between the arthritic score (mean ± SEM) of experimental (C) and control rats (○) from day 10 through day 47 (after M. tuberculosis injection) was found to be statistically significant (e.g., day 12, P < 0.05; day 17, P < 0.01; day 25, P < 0.01; day 34, P < 0.01, and day 47, P < 0.001, all by Student’s t test). The results of the two groups of rats were also statistically significant when analyzed by nonparametric, Wilcoxon-ranked sum test. Another group of Lewis rats (n = 6) (C) was immunized subcutaneously with a combination of three Bhsp65 amino-terminal peptides (namely, 13–27, 33–47, and 121–135) (B) mixed in DDA. The control group (n = 5) (●) was immunized with HEL peptide 85–96/DDA. The conditions of the experiment were similar to those of the experiment shown in A. Rats were examined for up to 60 d after injection of M. tuberculosis. The difference between the two groups of rats was not statistically significant at any of the timepoints tested.

In summary, the above results demonstrate that induction of T cell responses to the BCTD are involved in providing protection from subsequent induction of AA and, thereby, are also capable of inducing natural regression of acute inflammatory arthritis in vivo.

Discussion

The phenomenon of broadening of the T cell response to other determinants within a particular native antigen, after induction of disease with only a single determinant of the same antigen has previously been described as determinant spreading (19). In this study, we observed a shift in the specificity of T cell responses to new determinants within Bhsp65 after priming with the whole multideterminant antigen (native Bhsp65 as a component of M. tuberculosis), and have termed this diversification of the T cell response (20, 21). Determinant spreading has previously been reported in the diseases murine experimental autoimmune encephalomyelitis (EAE) and insulin-dependent diabetes mellitus (IDDM) (19, 34). In the case of EAE, the disease was induced by a self-peptide, AcL-11, of myelin basic protein (MBP), but during the course of the disease, the T cell response spread to other determinants within MBP, and in other studies (35, 36), to determinants on proteolipid protein (PLP) by intermolecular spreading. Likewise, in the case of spontaneously developed IDDM, there was evidence for both intramolecular (within glutamic acid decarboxylase; GAD65) and intermolecular (to determinants of hsp65, carboxypeptidase H, and insulin) determinant spreading (34). The novel features of our study on diversification of the T cell response in AA compared with the above two examples are the following: first, the observed diversification involved determinants within a foreign (mycobacterial) antigen, Bhsp65; second, both in EAE and IDDM, determinant spreading was implicated in perpetuation of the autoimmune response, whereas in our study we demonstrate that diversification of the T cell response to the carboxy-terminal determinants of Bhsp65 is involved in inducing regulation of inflammatory arthritis or protection from arthritis (regulatory diversification) in the AA-susceptible Lewis or the AA-resistant W KY rat strains, respectively. Furthermore, our results suggest that this diversification might be accentuated by determinants within self(rat) hsp65. In this regard, our study extends a novel dimension to the functional significance of diversification of T cell response in autoimmunity. Here, we describe diversification of T cell responses during the course of an autoimmune disease induced by a foreign antigen. In addition, this model offers us a unique opportunity to analyze relationships between the T cell repertoire directed against pathogenic foreign antigen and the homologous self-antigen. We believe that the principles elucidated in this study are widely applicable to several autoimmune situations in which autoreactivity is induced by molecular mimicry (29, 37, 37a) between a foreign and a self-antigen. Immediate and direct application of this knowledge would be in those autoimmune diseases (e.g., diabetes, Beh-
cet's disease, multiple sclerosis) that might be induced/perpetuated by heat-shock proteins.

In this study, we observed that in the late phase of arthritis in the Lewis rat there was diversification of the T cell responses to include new determinants within the Bhsp65. T cell responses to the BCTD were evident even at 4 wk after onset of AA. Moreover, results of the experiments employing pretreatment with peptides comprising the BCTD clearly demonstrate that BCTD-reactive T cells are indeed responsible for inducing significant regression of acute inflammatory arthritis in the Lewis rat, presumably by down-regulating the activity of disease-inducing effector T cells. Furthermore, the protective effect against AA is not simply a property of peptides derived from any region of Bhsp65; in this study, pretreatment with BCTD but not BNTD brought about protection from subsequent induction of AA. However, the timing of pretreatment with BCTD is a critical factor in determining the final outcome; significant protection from AA in Lewis rats is only achieved if pretreatment is done 5–6 wk before injection of M. tuberculosis; pretreatment 2 wk before induction of AA has no effect on the course of the disease. These results suggest that the appearance of, and manifestation of the effect of, regulatory T cells either during the course of AA or after pretreatment of naive Lewis rats with the BCTD apparently requires 4–6 wk of time. Interestingly, it had been reported earlier that pretreatment of naive Lewis rats with native Bhsp65 or with peptide 180–188 of Bhsp65 induced protection from subsequent induction of AA; again, the protective effect of Bhsp65 or of peptide 180–188 was observed when these antigens were administered 5 wk before induction of AA (4, 16). At this time, we do not know how pretreatment with the BCTD affords protection from AA. It is conceivable that the activity of arthritogenic T cells can be controlled either by cytokines (e.g., by induction of Th2 cells) or through a TCR-centered idiotypic circuit (38). Considering that peptide 180–188 (16) or peptide 256–270 (39) of Bhsp65 also can afford protection from AA, we suggest that protection against AA induced by pretreatment either with peptide 180–188 or 256–270, or by the BCTD might be mediated through parallel or, conceivably, a final common pathway of regulation. The precise mechanisms underlying this regulation are currently under investigation. Considering the size of Bhsp65, it is feasible that different regions of the same molecule are protective, and that this degeneracy in regulation (or multilayered regulation) might be of evolutionary advantage. Additional support for similar redundant regulatory pathways derives from studies using the nonobese diabetic (NOD) mouse: diabetes in this mouse strain can be modulated by administration of any one of several defined antigens (reviewed in reference 40).

The diversification of T cell responses to the carboxy-terminal determinants of hsp65 during the course of AA could be attributed to enhanced processing and presentation of Bhsp65 and/or R hsp65 under the inflammatory conditions (41–43) prevalent during acute AA (Fig. 5). Our results shown in Figs. 2 and 4 suggest that the T cell repertoire shared between Bhsp65 and R hsp65 is involved in diversification of the response as well as in regulation of AA. Thus, the induction of RCTD-reactive T cells in vivo, and their recruitment by cross-reactive BCTD might be a major mechanism underlying the observed diversification of the T cell response to the COOH-terminal determinants of Bhsp65 (Fig. 5, scheme b). However, it is possible that both mechanisms shown in Fig. 5 might operate simultaneously, and that T cell responses to BCTD as well as RCTD could arise even in the absence of significant cross-reactivity between BCTD and RCTD. Which of the above mechanisms is (are) actually involved in the diversification of the T cell response to BCTD and in natural regulation of AA is the subject of current investigations.

In comparison to our results shown in Fig. 1, Anderton et al. (17) (a) observed proliferative responses to relatively fewer dominant/subdominant determinants of Bhsp65 after immunization of Lewis rat with Bhsp65. We believe that these differences are primarily owing to lower sensitivity under their immunization and assay conditions, as well as to possibly subtle differences in the inherent responsiveness of the rat strains used from different sources, as has been reported in the case of EAE (44); (b) did not find any change in the pattern of reactivity at different timepoints after immunization with native Bhsp65. Our interpretation of
these results is that because immunization with Bhsp65 (mixed in the adjuvant, DDA) did not cause AA, the requisite conditions for diversification of T cell responses to Bhsp65 simply did not exist.

We observed that coupled with the diversification of T cell responses during the course of AA in the Lewis rat, the proliferative T cell responses to certain BNTD were significantly downmodulated in the late phase of the disease. At this time, we do not know whether downmodulation of responses to BNTD also might be contributing to induction/perpetuation of arthritis in the Lewis rat and, therefore, to contain the autoimmune reactivity, the responses to these determinants must be downregulated.

We have observed that the WKY rat, with the same MHC class II molecules as the Lewis rat, is resistant to AA. In WKY rats, vigorous responses to the BCTD, to which the Lewis rat responded only in the late phase of AA, were observed very early, 10 d after injection of M. tuberculosis. These results suggest that T cell responses to the BCTD are involved in the downregulation of the acute inflammatory response in the late phase of the disease in AA-susceptible Lewis rat; similarly, T cell responses to the same determinants initiated soon after injection of M. tuberculosis induce effective protection against arthritis in WKY rats. The early and efficient display of the BCTD by M. tuberculosis-immunized WKY rats but not by Lewis rats could be owing to subtle differences in the processing and presentation of Bhsp65 by the APC of WKY and Lewis rats, which in turn could be attributable to the difference in the non-MHC genes of these two rat strains. However, our results do not rule out other factors that also might contribute to the AA resistance of WKY rats, e.g., (a) hormonal and other neurophysiological parameters (45, 46), (b) difference in the RT.6 locus of WKY and Lewis rats (23, 47, 48), and (c) a change in the balance of Th1/Th2 induction (49). In most of the earlier reports on AA, the Fisher F344 rat (RT.1) which is resistant to AA, has been extensively studied and used as a control strain for the Lewis rat (14). It has been reported that the Fisher rat is deficient in generating T cell responses to the arthritogenic determinant, 180–188, of Bhsp65 after injection of M. tuberculosis, and thereby is protected from AA (14). On the contrary, our results show that AA-resistant WKY rats raise vigorous responses to determinant 180–188 (within peptide 177–191) of Bhsp65 upon challenge with M. tuberculosis. Thus, clearly the immunological mechanisms underlying resistance to AA of Fisher and WKY rats are different. In this regard, study of AA in WKY rats would offer novel insights into the pathogenesis and treatment of this disease. Moreover, this rat strain might also be valuable in studying other animal models of autoimmune disease like EAE (49a).

AA can be induced in the Lewis rat by injection of M. tuberculosis; however, the precise autoantigen(s) responsible for AA are not known. Several candidate autoantigens have been proposed (13, 50, 51), but there is no direct evidence so far that any one of these antigens is arthritogenic in Lewis rats. We (29) and others (52–54) have suggested that self-hsp65 (R hsp65) may be a target of T cells primed by Bhsp65. Based on our earlier study on mouse lysozyme as a model self-protein (29), we suggested that newly displayed cryptic determinants within R hsp65 could serve as targets of autoimmune reactivity for T cells primed with the corresponding dominant determinants within Bhsp65. In addition, any self-antigenic determinants within the tissue components of the joints, with the appropriate cross-reactive homology with determinants within self-hsp65, could also serve as targets for the pathogenic T cells. We are currently characterizing the T cell responses to self-hsp65 to evaluate their direct role in the pathogenesis of arthritis.

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References

1. Nepom, G.T., J.A. Hansen, and B.S. Nepom. 1987. The molecular basis for HLA class II associations with rheumatoid arthritis. J. Clin. Immunol. 7:1–7.

2. Lipsky, P.E. 1991. Rheumatoid arthritis. In Harrison’s Principles of Internal Medicine. J.D. Wilson, E. Braunwald, K.J. Isselbacher, R.G. Petersdorf, J.B. Martin, A.S. Fauci, and R.K.
1. Anderton, S.M., R. van der Zee, A. Noordzij, and W. van Eden. 1994. Differential mycobacterial 65-kDa heat shock protein T cell epitope recognition after adjuvant arthritis-inducing or protective immunization protocols. J. Immunol. 152:3656–3664.

2. Lider, O., N. Karin, M. Shinitzky, and I.R. Cohen. 1987. Therapeutic vaccination against adjuvant arthritis using autoimmunity T cells treated with hydrostatic pressure. Proc Natl Acad Sci USA. 84:4577–4580.

3. Lehmann, P.V., T. Forsthuber, A. Miller, and E.E. Sercarz. 1992. Spreading of T cell autoimmunity to cryptic determinants of an autoantigen. Nature (Lond.). 358:155–157.

4. Moudgil, K.D., T.C. Chang, H. Eradat, and E.E. Sercarz. 1994. Identification of determinants within mycobacterial hsp65 involved in induction, perpetuation and recovery from autoimmunity arthritis in the Lewis rat. 12th European Immunology Meeting, June 14–17, Barcelona, Spain. Abstract no. W42/1.

5. Moudgil, K.D., T.C. Chang, H. Eradat, A. Chen, O. Yun, R.S. Gupta, and E.E. Sercarz. 1995. Involvement of diversification of T cell responses to mycobacterial hsp65 (Bhsp65) in inducing remission or protection from adjuvant-induces arthritis. The 9th Int'l. Congress of Immunology, San Francisco, July 23–29. Abstract no. 2367.

6. Van Eden, W., J.E.R. Thole, R. Van der Zee, A. Noordzij, J.D.A. Van Embden, E.J. Hensen, and I.R. Cohen. 1988. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. Nature (Lond.). 331:171–173.

7. Van Eden, W., J.E.R. Thole, R. Van der Zee, A. Noordzij, J.D.A. Van Embden, E.J. Hensen, and I.R. Cohen. 1988. T lymphocytes of rheumatoid arthritis patients show augmented reactivity to a fraction of mycobacteria cross-reactive with cartilage. Lancet. 2:305–309.

8. Pearson, C.M., F.D. Wood, C.M. Pearson, and A. Tanaka. 1969. Capacity of Mycobacterium bovis BCG expressed in K-12. J. Exp. Med. 1315 Moudgil et al.

9. Trentham, D.E., A.S. Townes, and A.H. Kang. 1977. Autoimmune arthritis. The 9th Int'l. Congress of Immunology, San Francisco, July 23–29. Abstract no. 2367.

10. Trentham, D.E., A.S. Townes, and A.H. Kang. 1977. Autoimmune arthritis. The 9th Int'l. Congress of Immunology, San Francisco, July 23–29. Abstract no. 2367.
prevent the onset of diabetes, as a result of interaction between the immune system and 65-kD heat shock protein (hsp65) from mycobacteria.}

34. Kaufman, D.L., M. Clare-Salzler, J. Tian, T. Forsthuber, G.S.P. Ting, P. Robison, M.A. Atkinson, E.E. Sercarz, A.J. Tobin, and P.V. Lehmann. 1993. Mechanisms of autoimmune arthritis. Curr. Opin. Immunol. 15:203–208.

35. McRae, B.L., C.L. Vanderlugt, M.C. Dal Canto, and S.D. Miller. 1995. Functional evidence for epitope spreading in the relapsing pathogenesis of experimental autoimmune encephalomyelitis. J. Exp. Med. 181:943–952.

36. Cross, A.H., V.K. Tsuchiy, and C.S. Raine. 1993. Development of reactivity to new myelin antigens during chronic relapsing autoimmune demyelination. Curr. Top. Microbiol. Immunol. 145:127–135.

37a. Wucherpfennig, K.W., J.L. Strominger. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. Curr. Opin. Immunol. 8:695–705.

38. Cohen, I.R. 1991. Autoimmunity to chaperonins in the pathogenesis of arthritis and diabetes. Annu. Rev. Immunol. 9:567–589.

39. Anderton, S.M., R. van der Zee, B. Prakken, A. Noordzij, and W. van Eden. 1993. Activation of T cells recognizing self 60-kD heat shock protein can protect against experimental arthritis. J. Exp. Med. 181:943–952.

40. W. van Eden. 1991. Heat-shock proteins as immunogenic adjuvants. Adv. Immunol. 47:47–59.

41. Lehmann, P.V., E.E. Sercarz, T. Forsthuber, C.M. Dayan, and G. Gammon. 1993. Determinant spreading and the dynamics of the autoimmune T-cell repertoire. Immunol. Today. 14:203–208.

42. Odenacker, G., and J. Van Damme. 1994. Cytokine-regulated proteases in autoimmune diseases. Immunol. Today. 15:103–107.

43. Moudgil, K.D., and E.E. Sercarz. 1994. The T cell repertoire of experimental autoimmune encephalomyelitis. J. Neuroimmunol. 54:145–146.

44. Gould, K.E., J.A. Stepaniak, and R.H. Swanborg. 1994. Variable susceptibility of Lewis rats to experimental autoimmune encephalomyelitis. J. Neuroimmunol. 54:145–146.

45. Cools, A.R., N.Y. Rots, B. Ellenbroek, and E.R. de Kloet. 1993. Bimodal shape of individual variation in behaviour of Wistar rats: the overall outcome of a fundamentally different make-up and reactivity of the brain, the endocrinological and the immunological system. Experientia. 28:100–105.

46. Sternberg, E.M., W.S. Youn, D.P. Bernardini, A.E. Calogero, G.P. Chrousos, P.W. Gold, and R.L. Wilder. 1989. A central nervous system defect in biosynthesis of corticotropin-releasing hormone is associated with susceptibility to streptococcal cell wall-induced arthritis in Lewis rats. Proc. Natl. Acad. Sci. USA. 86:4771–4775.

47. Fowell, D., and D. Mason. 1993. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes: characterization of the CD4+ T cell subset that inhibits this autoimmune potential. J. Exp. Med. 177:627–636.

48. Greiner, D.L., J.P. Mordue, E.S. Handler, M. Angelillo, N. Nakamura, and A.A. Rossini. 1987. Depletion of RT6.1+ T lymphocytes induces diabetes in resistant Biobreeding/Wistar rats. J. Exp. Med. 166:461–475.

49. Scott, B., R. Liblau, D. De Ferrari, L. Ogata, A.J. Caton, H.O. McDavitt, and D. Lo. 1994. A role for non-MHC genetic polymorphism in susceptibility to spontaneous autoimmunity. Immunology. 71:73–83.

49a. Stevens, D.B., E.E. Sercarz, and K.D. Moudgil. 1997. Biphasic EAE, rather than protection against EAE, in Lewis rat recipients of RT1l congenic Wistar Kyoto rat spleen cells. J. Allergy Clin. Immunol. 99:5262 (Abstr.).

50. Ferre, U., A. Schulmeister, J. Mollenhauer, K. Brune, and H. Bang. 1994. A constitutive 65 kDa chondrocyte protein as a target antigen in adjuvant arthritis in Lewis rats. Autoimmunity. 17:233–239.

51. van den Broek, M.F., W.B. van den Berg, O.J. Arntz, and L.B.A. van de Putte. 1988. Reaction of bacterium-primed murine T cells to cartilage components: a clue for the pathogenesis of arthritis? Curr. Opin. Immunol. 7:29–34.

52. Koga, T., A. Wachtler, P. DeBry, M.E. Munk, B. Schoel, and S.H.E. Kaufmann. 1989. T cells against a bacterial heat shock protein recognize stressed macrophages. Science (Wash. D.C.) 245:1112–1115.

53. van Eden, W. 1991. Heat-shock proteins as immunogenic bacterial antigens with the potential to induce and regulate autoimmune arthritis. Immunol. Rev. 121:5–28.

54. Anderton, S.M., R. van der Zee, and J.A. Goodacre. 1993. Inflammation activates self hsp60-specific T cells. Eur. J. Immunol. 23:33–38.

55. Snippe, H., and C.H. Kraaijveld. 1989. The immunoadjuvant dimethyldioctadecylammonium bromide. In Immunological Adjuvants and Vaccines. G. Gregoriadis, A.C. Allison, and G. Poste, editors. Plenum Press, New York. 47–59.