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The T Cells Specific for the Carboxyl-Terminal Determinants of Self (Rat) Heat-Shock Protein 65 Escape Tolerance Induction and Are Involved in Regulation of Autoimmune Arthritis

Malarvizhi Durai,* Radhey S. Gupta,† and Kamal D. Moudgil 2*

Immunization of Lewis rats with heat-killed Mycobacterium tuberculosis H37Ra leads to development of polyarthritis (adjuvant-induced arthritis; AA) that shares several features with human rheumatoid arthritis (RA). Immune response to the 65-kDa mycobacterial heat-shock protein (Bhsp65) is believed to be involved in induction of AA as well as in experimental modulation of this disease. However, the understanding of several critical aspects of the pathogenesis of AA in the Lewis rat has severely been hampered by the lack of information both regarding the level as well as epitope specificity of tolerance to the mammalian self (rat) homologue of Bhsp65, 65-kDa rat heat-shock protein (Rhs65), and about the functional attributes of the T cell repertoire specific for this self protein. In this study, we established that tolerance to Rhs65 in the Lewis rat is incomplete, and that the residual T cells primed upon challenge with this self hsp65 are disease regulating in nature. We also have defined the T cell epitopes in the C-terminal region within Rhs65 that contribute predominantly to the immune reactivity as well as the AA-protective effect of this self protein. Furthermore, the T cells primed by peptides comprising these C-terminal determinants can be efficiently restimulated by the naturally generated epitopes from endogenous Rhs65, suggesting that self hsp65 might also be involved in natural remission from acute AA. These novel first experimental insights into the self hsp65-directed regulatory T cell repertoire in AA would help develop better immunotherapeutic approaches for autoimmune arthritis. The Journal of Immunology, 2004, 172: 2795–2802.

R

eumatoid arthritis (RA) is a chronic systemic autoimmune disease of unknown etiology that primarily affects the articular joints. Lewis rats (RT.1) challenged with heat-killed Mycobacterium tuberculosis H37Ra (Mt) develop polyarthritis (adjuvant-induced arthritis; AA) that has several features resembling human RA (1, 2). Studies in the AA model as well as analysis of peripheral blood- and synovial fluid-derived T cells of RA patients suggest that T cell responses to the 65-kDa mycobacterial heat-shock protein (Bhsp65) are involved in the pathogenesis of autoimmune arthritis (3–9). Bhsp65 contains both arthritogenic and disease-regulating T cell epitopes (4, 6, 10–14). However, the precise mechanisms by which Bhsp65-specific T cells initiate or propagate arthritis in the Lewis rat, or afford them protection from subsequent induction of disease following recovery from acute arthritis are not yet fully defined.

Efficient induction of self tolerance involving deletion and/or inactivation of potentially self-reactive T cells in the thymus is critical for prevention of autoimmunity (15–17). In addition, the mature T cell repertoire possesses T cell subsets that are capable of controlling the activity of autoreactive T cells (18, 19). Defining the Ag/epitope specificities of potentially self-directed T cell repertoire is critical for understanding the induction as well as regulation of an autoimmune disease. In the case of AA in the Lewis rat, mammalian (rat) hsp65 (Rhs65) is the self homologue of the disease-related mycobacterial Ag, Bhsp65 (Rat hsp60 (Rhs60)) (20) has been referred to as Rhs65 to match Bhsp65. Enigmatically, however, there is barely any information either regarding the level as well as epitope specificity of the T cell tolerance to, or about the functional attributes of the T cell repertoire directed against, Rhs65 in the Lewis rat. This hiatus has severely hindered advances in defining several critical aspects of the pathogenesis of AA in the Lewis rat.

There is a sizable literature in AA on the Bhsp65-directed T cell repertoire and the role of this foreign Ag in experimental modulation of arthritis in the Lewis rat (4, 6, 10–13, 21–25). However, because heat-shock proteins are highly conserved in nature, many aspects of the immune reactivity ascribed to Bhsp65 in AA in various studies might in fact have their origin within the T cell repertoire directed against the self hsp65, Rhs65. Furthermore, the observations by others (12, 26) and us (6) showing that certain peptides of Bhsp65 that have the ability to downmodulate the course of AA in the Lewis rat are crossreactive with the corresponding synthetic peptide sequences of Rhs65, and that DNA vaccination of Lewis rats with human hsp60 (Hhs60) affords protection from subsequent AA (25, 27, 28), have indirectly suggested...
that T cells against self hsp65 (in this case Rhs65) may be involved in regulation of AA. However, until now, no study has directly demonstrated experimentally the role of Rhs65 per se in immune modulation of AA.

In this study, we describe that Lewis rats are not tolerant to Rhs65, and thereby, possess the T cell repertoire specific for Rhs65. Furthermore, pretreatment of naive Lewis rats with native Rhs65 affords significant protection against subsequent AA. Interestingly, both the T cell response to native Rhs65 and the AA-protective effect of this self hsp65 in the Lewis rat is attributable primarily to the C-terminal epitopes of the protein. The T cells primed by the C-terminal determinants of Rhs65 can adaptively transfer protection against subsequent AA. Moreover, these T cells can be efficiently restimulated by epitopes generated from endogenous self hsp65 within heat-stressed APC. These results, along with those showing that artritic Lewis rats spontaneously raise T cell response both to self hsp65 and to its C-terminal epitopes, provide the rationale for one of the mechanisms of self hsp65-mediated regulation of acute AA. This is the first report defining both the state of tolerance to Rhs65 in the Lewis rat and the precise regulatory epitopes for AA within this self Ag. These novel insights would help develop better Ag-specific immunotherapeutic approaches for human RA.

Materials and Methods

Rats

Inbred male Lewis (LEW/SnNhsd) (RT1^b) rats (5–8 wk old, 150–200 g) were procured from Harlan Sprague Dawley (Indianapolis, IN), and then maintained in the vivarium facility of the University of Maryland School of Medicine (Baltimore, MD). All procedures performed on these animals were in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Ages/mitogen/adjuvant

1) Heat-killed Mtb was obtained from Difco Laboratories (Detroit, MI). 2) Recombinant Rhs65 (rat hsp60 [Rhs60] (20) has been referred to as Rhs65 to match Bhsp65), was expressed and purified from pTrcHisA-transformed E. coli BL21 cells (20, 29). Briefly, Rhs65 was exposed by culturing transformed E. coli BL21 cells followed by isopropyl-D-thiogalactopyranoside (0.4 mM) (Sigma-Aldrich, St. Louis, MO) induction. The histidine-tagged recombinant protein was purified by using ProBond nickel resin (InVitrogen, Carlsbad, CA). Any endotoxin contaminating the recombinant protein was removed by using Acticlean Etox Kit (Sterigene Bioseparations, Carlsbad, CA). The identity of the purified protein was confirmed on Western blot by using a mouse anti-hsp60 mAb (clone: HK1-1, StressGen Biotechnologies, Victoria, Canada) that is reactive with Rhs65 but not with bacterial hsp65. 3) Synthetic peptides of Rhs65 (20) were procured from Macromolecular Resources and Global Peptide Services (both at Fort Collins, CO). Rhs65 carboxyl-terminal determinants (RCTD), a mixture of 5 C-terminal peptides of Rhs65 (namely, 418–432, 441–453, 465–479, 512–526, and 521–535) was prepared by pooling equal volume of each of the five peptides (stock concentration, 5 mg/ml for each peptide) to a final concentration of 1 mg/ml for each peptide. 4) Purified protein derivative (PPD) was purchased from Mycos Research (Fort Collins, CO), whereas hen eggwhite lysozyme (HEL), Con A, and IFA were obtained from Sigma-Aldrich.

Induction and evaluation of AA

Lewis rats were immunized s.c. with 200 μl (2 mg/rat) of Mtb-mineral oil suspension at the base of the tail and then observed regularly for clinical signs of arthritis like erythema, swelling, and induration (6). The severity of arthritis in each paw was graded on a scale from 0 to 4; the total score for each paw was 4 and the maximum score for each rat was 16 (6).

Lymph node cell (LNC) proliferation assay

Lewis rats were immunized either with Mtb-mineral oil suspension (2 mg/rat) or with a protein Ag (200 μg/rat) emulsified in IFA (IFA was used instead of CFA to avoid any concurrent contribution of Bhs65 of Mtb within CFA to priming of the T cells). On day 15 (for Mtb-immunized rats) or on day 9 (for protein Ag-immunized rats) after injection, the draining LNC (inguinal, para-aortic, and popliteal) of these rats were harvested and tested in a proliferation assay at 2.5 × 10^−5−5 × 10^5 cells/well in HL-1 serum-free medium (BioWhittaker, Walkersville, MD) in the presence or absence of Ag as described in detail elsewhere (6). Con A or PPD was used as a positive control, whereas HEL served as a negative control. The results were expressed either as cpm or as a stimulation index (S.I. = ratio of cpm in the presence of Ag and cpm of cells in medium only).

Pretreatment of naive Lewis rats with Rhs65 before induction of AA

Lewis rats were challenged s.c. at the base of the tail with Rhs65 (200 μg/rat) in IFA. The control group of Lewis rats received either PBS/IFA or HEL/IFA. On day 9, all rats were injected with Mtb (2 mg/rat) s.c. in mineral oil for the induction of AA. Thereafter, all rats were observed regularly for signs of arthritis. The severity of the disease was graded as described above (6).

Adaptive transfer of RCTD-primed LNC into naive Lewis rats before induction of AA

A group of Lewis rats was immunized s.c. at the base of the tail with RCTD (100 μg of each peptide per rat) in IFA. The control groups of Lewis rats received either PBS/IFA or HEL/IFA. On day 9, draining LNC of these rats were harvested separately and then restimulated with Con A (2.5 μg/ml) in culture (3 × 10^5 cells/ml) for 48 h in complete RPMI 1640 (RPMI 1640 medium supplemented with heat-inactivated FCS (10%) (Life Technologies), penicillin-streptomycin (1%), and 5 × 10^−5 M 2-ME). Thereafter, cells were collected, washed thoroughly, and resuspended in PBS. This cell suspension (1 × 10^5 cells) was injected i.v. into naive recipient Lewis rats (5–6 wk old). On the same day, the recipient rats received Mtb (2 mg/rat) s.c. for induction of AA, and all rats were then observed regularly for signs of arthritis. The severity of the disease was graded as described above.

Testing the cytokine profile of RCTD-primed T cells

Lewis rats were challenged s.c. at the base of the tail with RCTD (100 μg of each peptide per rat) emulsified in IFA. On day 9, the draining LNC were harvested and plated in a 96-well plate as for a proliferation assay described above. These cells were restimulated in vitro either with RCTD (test Ag) or HEL (irrelevant Ag control) for 48 h. Thereafter, the culture supernatants were collected and tested in ELISA for IFN-γ, IL-10, TGF-β, and TNF-α using the commercially available reagents (Biosource International, Camarillo, CA or R&D Systems, Minneapolis, MN) following the manufacturer’s protocol. The lower detection limit for IFN-γ, IL-10, TGF-β, and TNF-α was 13, 5, 31.2, and 4 pg/ml, respectively.

Preparation, heat-shock treatment, and use of the APC in a proliferation assay

APC comprised mostly of macrophages and dendritic cells were prepared from spleen of a naive Lewis rat following the procedure described elsewhere (30) but with minor modifications. The identity of the APC was confirmed by flow cytometry after staining with mouse anti-rat CD11b/c Ab (BD PharMingen, San Diego, CA). One aliquot of cells was subjected to heat-shock by incubating it at 43 ± 1°C for 20 min in a water bath (heat-stressed APC; experimental group), whereas the other aliquot of cells was kept at room temperature (unstressed APC; control). Thereafter, fresh complete RPMI 1640 medium was added to both aliquots of cells, and the cells were then incubated at 37°C for 1 h (recovery period) in a CO₂ incubator. The viability of APC was checked by trypan blue staining. Immunofluorescence assay was performed to determine the cellular expression for Rhs65 in heat-stressed/unstressed APC by staining with mouse anti-hsp60 mAb (reactive with Rhs65) followed by FITC-labeled goat anti-mouse IgG. Staining with isotype-matched Abs served as control. The staining of cells was examined under a fluorescence microscope (Nikon Instruments, Melville, NY) and photographed. LNC proliferation assay using heat-stressed APC was performed essentially as described above but with the difference that LNC (5 × 10^5 cells/well) were cultured with different concentrations of heat-stressed (experimental) or unstressed (control) APC without addition of exogenous Ag (unless indicated). The results were expressed as ∆ cpm (cpm with heat-stressed APC – cpm with unstressed APC).
Results

Lewis rats possess T cells potentially reactive against self (rat) hsp65 (Rhs65): tolerance to Rhs65 is incomplete

To study the T cell repertoire potentially directed against the self protein, Rhs65, in the Lewis rat, a group of Lewis rats was challenged s.c. with Rhs65 in IFA (IFA was used instead of CFA in this and other experiments in the study to avoid any concurrent contribution of Bhsps65 of Mtb within CFA to priming of T cells) and on day 9, the proliferative response of the draining LNC was tested using native Rhs65 as the recall Ag. HEL and Con A served as negative and positive control, respectively. The results (Fig. 1) show that Lewis rats immunized with Rhs65 raised a vigorous T cell response to Rhs65. However, there was no recall response to the irrelevant Ag, HEL. These results demonstrate that T cells against Rhs65 exist in the mature T cell repertoire of the Lewis rat and therefore, Lewis rats are not fully tolerant to this self Ag.

Pretreatment of naive Lewis rats with Rhs65 affords protection against subsequent AA

To determine whether priming of the T cells potentially specific for Rhs65 has any effect on the course of subsequent AA, a group each of naive Lewis rats was immunized with Rhs65 in IFA (experimental group), PBS/IFA (control group), or HEL/IFA (control group). After 2 wk, all rats were injected s.c. with Mtb for the induction of AA and then observed regularly for clinical signs of arthritis. Interestingly, Lewis rats pretreated with Rhs65 developed significantly much less severe AA compared with those treated with PBS/IFA (Fig. 2A) or HEL/IFA (Fig. 2B). These results demonstrate that activation of the Rhs65-specific T cells before Mtb injection affords significant protection against AA.

Defining the T cell epitopes contributing to the Rhs65-directed T cell response in the Lewis rat

To define the epitope(s) of Rhs65 that is (are) involved in inducing immune response to the native Ag as well as protection against AA, Lewis rats were immunized with Rhs65/IFA and on day 9, the draining LNC were tested in a proliferation assay using partially overlapping peptides spanning the entire length of the Rhs65 protein. HEL was used as an irrelevant Ag control, whereas Con A served as the positive control. Our results (Fig. 3) show that Lewis rats immunized with Rhs65 raised significant T cell responses to many epitopes within the C-terminal region of Rhs65 namely, 418–432, 441–455, 465–479, 512–526, and 521–535, and to the peptide mixture containing these five RCTD. The response to RCTD mixture was higher than that to any of the individual peptides tested. In comparison to RCTD, peptide 180–188 (p180–188) gave a borderline response, whereas p213–227 and p357–371 recalled responses that were barely above the cut-off value (S.I. of 2). These results demonstrate that T cell response against Rhs65 was directed predominantly to RCTD within the region 418–535, and therefore, these epitopes contribute significantly to the immune reactivity to, and probably also to the observed AA-regulatory effect of, Rhs65 described above.

The T cells specific for the C-terminal epitopes of Rhs65 can adoptively transfer protection against AA

To further define the functional characteristics of the RCTD-reactive T cells, a group each of Lewis rats were challenged with RCTD/IFA (experimental donor group), PBS/IFA (control donor group), or HEL/IFA (control donor group). After 8 days, the draining LNC of these rats were harvested separately and activated in vitro with Con A for 48 h before i.v. transfer into naive Lewis recipients. On the day of cell transfer, all recipient Lewis rats were immunized with Mtb s.c. for induction of AA, and then examined regularly for clinical signs of AA. Interestingly, Lewis rats that had received RCTD-primed LNC were significantly protected from
The epitope specificity of Rhsp65-primed T cells in the Lewis rat. Lewis rats (n = 4) were immunized s.c. at the base of the tail with naive Rhsp65 (200 μg/rat) in IFA. On day 9, the draining LNC were tested in a proliferation assay using overlapping peptides of Rhsp65. Also included in the assay were HEL (negative control) and Con A (positive control). The results are expressed as a S.I. (mean ± SEM). A proliferative response with S.I. value higher than 2 was considered to be positive. S.I. for Rhsp65 (immunogen) and Con A was 61 and 189, respectively. The cpm value for Medium (= LNC in medium alone) was 1,560. RCTD, a mixture of 5 C-terminal peptides of Rhsp65 (namely 418–432, 441–455, 465–479, 512–526, and 521–535) was prepared by pooling equal volume of each of the five peptides (stock concentration, 5 mg/ml each) giving a final concentration of 1 mg/ml for each peptide in the mixture.

FIGURE 3. The epitope specificity of Rhsp65-primed T cells in the Lewis rat. Lewis rats (n = 4) were immunized s.c. at the base of the tail with naive Rhsp65 (200 μg/rat) in IFA. On day 9, the draining LNC were tested in a proliferation assay using overlapping peptides of Rhsp65. Also included in the assay were HEL (negative control) and Con A (positive control). The results are expressed as a S.I. (mean ± SEM). A proliferative response with S.I. value higher than 2 was considered to be positive. S.I. for Rhsp65 (immunogen) and Con A was 61 and 189, respectively. The cpm value for Medium (= LNC in medium alone) was 1,560. RCTD, a mixture of 5 C-terminal peptides of Rhsp65 (namely 418–432, 441–455, 465–479, 512–526, and 521–535) was prepared by pooling equal volume of each of the five peptides (stock concentration, 5 mg/ml each) giving a final concentration of 1 mg/ml for each peptide in the mixture.

The cytokine profile of T cells reactive against C-terminal epitopes of Rhsp65 is predominantly of Th1 type

To further characterize the functional attributes of RCTD-specific T cells that are potentially disease (AA)-regulating in nature, the cytokine secretion profile of these T cells was studied. The draining LNC of RCTD-immunized Lewis rats were restimulated in vitro with either RCTD or HEL for 48 h. The results (Fig. 5) show that RCTD-primed LNC secreted much higher levels of IFN-γ compared with IL-10. The levels of TGF-β and TNF-α were much lower compared with that of IFN-γ and IL-10. The ratio of IFN-γ to IL-10 was 34.1. These results suggest that the suppression of AA by passive (adoptive) transfer of RCTD-primed T cells described above is apparently mediated by one or more pathways involving IFN-γ and IL-10. The precise mechanism of downmodulation of AA by RCTD-reactive T cells is currently under investigation in our laboratory.

Arthritic Lewis rats spontaneously raise T cell response to Rhsp65/RCTD during the course of AA

Considering that the expression of heat-shock proteins is up-regulated under inflammation and other types of stress, we tested whether arthritic Lewis rats spontaneously (in the absence of any challenge with exogenous Rhs65) develop T cell response to endogenous Rhs65, the self homologue of Bhsp65. Lewis rats were immunized with Mb for induction of AA and thereafter, rats were sacrificed on day 15 (just preceding the peak phase of AA) after Mb challenge. Naive Lewis rats and PBS/IFA-treated rats served as controls. The draining LNC of these rats were tested in a proliferation assay using Rhsp65, RCTD, and Bhsp65 as recall test Ags, and PPD was used as a positive control. The results show that arthritic Lewis rats raised a potent T cell response both to the native Rhs65 and RCTD (Fig. 6) besides Bhsp65, whereas naive Lewis rats and Lewis rats immunized with PBS/IFA did not give any response to these Ags (data not shown). Thus, spontaneous induction of T cell response to Rhs65/RCTD in Lewis rats was associated with inflammatory arthritis. Furthermore, taken together with the results described above that Rhs65/RCTD-primed T cells can afford protection against AA, these results suggest that this naturally emerging T cell response to Rhs65/RCTD during the course of AA might be involved in downregulation of the course of acute AA in the Lewis rat.

The epitopes naturally generated from endogenous Rhs65 within heat-stressed APC can efficiently restimulate RCTD-primed T cells

Stress proteins are up-regulated during heat-shock and other forms of stress. Therefore, we reasoned that the cellular expression of endogenous self hs65 (Rhs65) in naive APC should be inducible by heat-shock, and that this Rhs65 could then be processed by the stressed APC for presentation to the appropriate Ag-primed T

FIGURE 4. Modulation of AA in the Lewis rat by adoptive transfer of RCTD-primed LNC before Mb injection. Two groups of naive Lewis rats were immunized with RCTD (100 μg of each peptide per rat) in IFA (experimental donor rats). The control groups of donor Lewis rats were injected with either PBS/IFA or HEL/IFA. On day 9 postinjection, the draining LNC of each group of rats were harvested separately, and then cultured with Con A for 48 h. A total of 1 × 10^6 RCTD-primed LNC (A) each (A and B) were injected i.v. into two separate groups of naive Lewis rats (n = 3–4) (experimental recipient group), whereas the control recipient Lewis rats (n = 3–4) received 1 × 10^6 PBS/IFA-primed (●) (A) or HEL/IFA-primed (○) (B) LNC. All recipient rats were then injected s.c. with Mb for induction of AA and thereafter, observed regularly for clinical signs of AA. The difference in the mean arthritic score of experimental and control recipient groups was statistically significant from day 10 through day 33 (p value ranged between <0.0002 and <0.05 by Student’s t test) (A), and from day 11 through day 26 (p < 0.00005 to <0.05) (B). The difference between the experimental and control groups was also statistically significant (p < 0.025 (A) and p < 0.05 (B)) as tested by Wilcoxon rank sum test. Similar results were obtained in repeat experiments.
The results are expressed as fold increase in cytokine secretion of RCTD-primed T cells in recall response to RCTD and HEL was statistically significant (*, p < 0.05 by Student’s t test) for IFN-γ as well as IL-10. The ratio of IFN-γ to IL-10 was 34.1.

cells. We tested this hypothesis using naive APC (splenic macrophages and dendritic cells of naive Lewis rats) that were either heat-stressed or unstressed. First, we demonstrated by immunohistochemistry that the expression of Rhsp65 was indeed up-regulated significantly in heat-stressed APC compared with unstressed APC (data not shown). Second, we tested the ability of heat-stressed APC to restimulate RCTD-primed T cells of Lewis rats. For this purpose, RCTD-primed LNC of Lewis rats were cocultured with either heat-stressed or unstressed APC without addition of any exogenous Ag. Naive LNC or HEL-primed LNC cultured with the same preparation of APC served as control. The results (Fig. 7) show that RCTD-primed T cells gave a significantly higher level of proliferative recall response with heat-stressed APC compared to that with unstressed APC, and the level of response was significantly higher than that obtained with Naive LNC or HEL-primed LNC; each of the latter controls gave comparable levels of response with heat-stressed and unstressed APC. However, all three groups of LNC gave comparable level of response to Con A, and in addition, RCTD/HEL-primed LNC gave a vigorous in vitro recall response to the respective Ag (data not shown). These results demonstrate that the heat-stressed APC could present to RCTD-primed T cells the naturally generated self epitopes from endogenous Rhsp65 following its up-regulated cellular expression, and that these epitopes mimicked the synthetic RCTD peptides. Furthermore, these results also provide the rationale for the observed spontaneous induction of T cell response to Rhsp65 and RCTD during the course of acute AA in Lewis rats described above.

**Discussion**

In this study, we demonstrated that Lewis rats are not tolerant to Rhsp65, and that the T cell response of Lewis rats challenged with native Rhsp65 is directed primarily to the epitopes within the C-terminal region (amino acid residues 418–535) of Rhsp65. Intriguingly, pretreatment of Lewis rats with Rhsp65 affords protection from subsequent AA. This protection is attributable primarily to the above-mentioned RCTD, as also demonstrated by the ability of RCTD-primed T cells to adoptively transfer protection against AA. Thus, the T cells against RCTD escape tolerance induction, and are involved in regulation of AA. Our results show that RCTD are naturally generated from the whole (native) Rhsp65 and thereby, represent immunodominant epitopes of this self protein; yet, the T cells potentially directed against RCTD have not been rendered...
tolerant. We suggest that one or more of the mechanisms described earlier by others (31–39) and us (40, 41) might contribute to incomplete tolerance to Rhs65, particularly RCTD in the Lewis rat. However, the subsets of self Ag-specific T cells that evaded thymic tolerance in the studies based on various animal models of autoimmunity (31–37) were involved in induction of autoimmune disease, whereas in this study, RCTD-directed T cells that escaped tolerance induction revealed immunoregulatory properties. Further studies on thymic expression of Rhs65 and the MHC binding of RCTD peptides would clarify the mechanisms involved in inefficient tolerance to this self protein.

We observed that Lewis rats pretreated with Rhs65 developed much less severe disease after Mtb injection compared with control rats. Considering that RCTD are naturally generated from native Rhs65, and that adoptive transfer of RCTD-primed T cells can afford protection to the naive Lewis rats against AA, the down-modulation of AA by pretreatment with Rhs65 is attributable to priming of the RCTD-specific T cells. Thus, we have defined for the first time the T cell epitopes within Rhs65 that have immunoregulatory properties. Our results further show that these RCTD-reactive T cells secreted relatively much higher levels of IFN-γ than IL-10, with only a marginal secretion of TGF-β and TNF-α. These results suggest that IFN-γ might play a predominant role in RCTD-mediated suppression of AA along with some contribution from IL-10. The T cells of similar cytokine profile but of different Ag specificity have been invoked in regulation of AA by other investigators as well (25, 26). We speculate that the anti-arthritis effect of IFN-γ might involve one or more of the following: induction of apoptosis in the target pathogenic T cells, inhibition of proliferation of pathogenic T cells, alteration in the activity of the APC, effect on migration/recruitment of T cells into the target organ, etc. (42, 43). Interestingly, in an earlier study by other investigators, it was reported that the AA-protective clone A2c secreted a higher level of IFN-γ than the pathogenic A2b clone (44). Furthermore, injection of IFN-γ to Lewis rats immediately following induction of disease afforded protection from AA, whereas challenge with the same cytokine just before Mtb injection exacerbated AA (44). In recent years, a gradually increasing realization has emerged regarding the dual role of IFN-γ: a disease-inducing/aggravating and a disease-regulating/protective activity (42–48). In this context, cytokine-mediated regulation of autoimmune arthritis and other disorders would need to be examined and interpreted beyond the simple cross-regulatory function of Th1 and Th2 cytokines (48).

The results of this study have provided direct evidence for the protective effect of self hsp65 (Rhs65) and its C-terminal epitopes (RCTD) in AA. In an earlier study, involvement of self hsp65 in modulation of AA was inferred only indirectly while elaborating the AA-protective effect of peptide 256–270 of mycobacterial hsp65 (M256–270) which is crossreactive with rat 256–270 (R256–270), and pretreatment with M256–270 but not R256–270 affords protection against AA (12). Our results show that RCTD but not R256–270 represent the naturally processed epitopes of Rhs65. In this context, the AA-protective effect of pretreatment of Lewis rats with Rhs65 observed in our study is attributable primarily to RCTD. However, considering that our analysis of T cell epitopes within Rhs65 is based on a relatively limited set of peptides, and that it was performed before induction of AA, it is likely that other yet undefined epitopes of Rhs65 besides RCTD might also contribute to immune reactivity to Rhs65 as well as to its immunoregulatory role in AA. Such an induction or enhancement of response to T and B cell epitopes of Hhs60 and Bhs65, respectively has been described by other investigators (14, 25). Furthermore, mycobacterial hsp65 within Mtb might also recruit, though crossreactivity, T cells already primed by Rhs65/RCTD and thereby contribute to attenuation of the disease process (6, 12, 21–25). In addition, CD4+ CD25+ regulatory T cells (19, 49) might also contribute either directly or indirectly to the observed Rhs65/RCTD-mediated protection against AA. Finally, besides T cell responses to Rhs65, Abs to Rhs65/Bhs65 may also contribute to recovery from acute arthritis. In fact, Abs against Rhs65 and one of the homologous epitopes within self (mammalian) hsp65 have been shown to induce protection against AA (14). This Ab-mediated protection was attributed in part to enhanced production of anti-inflammatory cytokine, IL-10 (14).

Interestingly, we observed that arthritic Lewis rats spontaneously raise T cell response to native Rhs65 as well as to RCTD. The spontaneous priming of Rhs65-reactive T cells in vivo is supported by the in vitro results showing 1) increased expression of Rhs65 in naive heat-stressed APC compared with unstimulated APC, and 2) efficient restimulation of the RCTD-primed T cells by heat-stressed but not unstimulated APC without addition of any exogenous Ag. These results demonstrate that the self epitopes presented by MHC molecules on the surface of heat-stressed APC display “crossreactivity” or “mimicry” with the synthetic peptides comprising RCTD. We suggest that these self epitopes include Rhs65-derived RCTD. We further suggest that the APC in the arthritic joints might also have increased expression of self hsp65, and that processing of self hsp65 in vivo could reveal T cell epitopes (e.g., RCTD equivalents) that might be relevant to the regulation of autoimmune arthritis. In fact, an increased level of expression of mRNA of Rhs60 in ankle joint synovial membrane of arthritic Lewis rats (50), and the expression of self hsp60 in the inflamed pannus of arthritic joints of mice (51) and that of patients with RA and juvenile chronic arthritis (JCA) (52, 53) has been observed by other investigators. Moreover, an interesting correlation between the proliferative T cell response to self hsp60 (Hs60) and clinical outcome in patients with juvenile RA has been observed (54).

The regulatory role of Hhs60 in arthritis is further supported by studies showing inhibition of AA in the Lewis rat by DNA vaccine encoding this protein (25, 27, 28). However, the precise T cell epitopes within the native Hhs60 protein that are involved in regulation of AA have yet to be defined. Although Hhs60 and Rhs60 (=Rhs65) are mammalian homologues, Hhs60 is not truly a self Ag for the Lewis rat. Hhs60 and Rhs65 have a high level of amino acid sequence similarity (≈97.8% homology; 12 of 547 aa residues are different) but the amino acid stretch 418–535 comprising the C-terminal determinants of Rhs65 (RCTD) has the least percent homology (95.8%; 5 aa different) with the corresponding C-terminal region of Hhs60 compared with other paired stretches within these proteins. It is known from studies using other model Ags that changes at even one or a few critical amino acid residue(s) can significantly alter the processing and presentation of epitope (55), and that an epitope at identical position within two homologous proteins can be differentially processed such that the determinant is cryptic in one but dominant in the other protein (56–58). Therefore, despite having certain stretches of total amino acid homology, the two mammalian proteins, Hhs60 and Rhs65, might be differentially processed and presented, leading to quantitative and/or qualitative differences in the naturally generated T cell epitopes from these proteins. Therefore, the modulation of AA by Hhs60 and Rhs65 might involve different epitopes as well as distinct effector mechanisms. Nevertheless, the regulatory role in arthritis of Hhs60 and Rhs65 lends support to the idea of immunological homunculus (59, 60).
In summary, our study based on truly self (rat) mammalian hsp65 (Rhs65) is the first one to document both the state of incomplete tolerance to Rhs65 in the Lewis rat and the role of this self protein in regulation of AA. In addition, this study has highly lighted the contribution of defined T cell epitopes (in this case, the C-terminal terminators of Rhs65) in mediating the AA-protective effect of self hsp65. These results provide novel insights into the pathogenesis of autoimmune arthritis that would help develop better strategies for Ag-specific immunotherapy of human RA.

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References


