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Heat Shock Protein 65-Reactive T Cells Are Involved in the Pathogenesis of Non-Antigenic Dimethyl Dioctadecyl Ammonium Bromide-Induced Arthritis

Md. Younus Mia, Malavizhi Durai, Hong Ro Kim, and Kamal D. Moudgil

Dimethyl dioctadecyl ammonium bromide (DDA) (C₃₈H₈₀NBr) is a nonantigenic lipoid material. DDA-induced arthritis (DIA) in the Lewis (LEW) (RT.1) rat is a new experimental model for human rheumatoid arthritis (RA). DIA is a T cell-mediated autoimmune disease. However, the precise self/foreign Ags associated with the disease process in DIA are not yet known. We observed that LEW rats with DIA spontaneously raised a vigorous T cell response both to 65-kDa self (rat) heat shock protein (Rhs65) and mycobacterial hsp65 (Bhsp65), but not to another arthritis-related Ag, bovine collagen type II. The T cell response to Rhs65 was focused predominantly on determinant regions 120–134 and 213–227 of the self protein. Interestingly, pretreatment of adult LEW rats using either a mixture of peptides 120–134 and 213–227 of Rhs65 or a low nonarthritogenic dose of DDA induced protection against subsequent DIA. Intriguingly, the protection induced by the latter was associated with spontaneous priming of T cells specific for peptide 213–227 of Rhs65. Similarly, LEW rats neonatally tolerized against either Rhs65 or Bhsp65 were significantly protected from subsequently induced DIA at adult stage, showing the disease-modulating attribute of the hsp65-specific T cells. Taken together, the above findings demonstrate that the hsp65-directed T cell repertoire is of significance in the pathogenesis of autoimmune arthritis induced by nonantigenic DDA. Like other animal models of RA involving hsp65, these first insights into the disease-associated Ags in the DIA model would pave the way for further understanding of the immunological aspects of induction and regulation of RA.

Several experimental models of arthritis have been developed to study the pathogenesis of human rheumatoid arthritis (RA). Among the well-studied animal models of polyarthritis are those inducible following challenge either with a microbial product (1–6) or with a defined Ag (7–10), and arthritis of spontaneous origin (11, 12). In addition, various nonantigenic material-induced arthritis models (e.g., arthritis induced in rodents by pristane (13, 14), Avridine (15), dimethyl dioctadecyl ammonium bromide (DDA) (16, 17), mineral oil or IFA (18), squalene (19), synthetic muramyl dipeptide (20), synthetic oligonucleotide (21), etc.) have also contributed to understanding of the disease process in RA. Besides serving as a resource for screening of potential antiarthritic agents for therapeutic purposes, the latter category of experimental models is increasingly being studied for obtaining insights into both immunological (14, 22, 23) and genetic aspects of autoimmune arthritis (24). In this context, it is important to study the immunopathogenesis of arthritis induced by nonantigenic compounds.

DDA (C₃₈H₈₀NBr) is a cationic surface-acting compound with potent adjuvant activity (25). It can elicit an inflammatory reaction characterized by an influx of polymorphonuclear cells and mononuclear phagocytes at the injection site (26), and stimulation of the phagocytic system (27). Furthermore, DDA injection into mice enhances delayed-type hypersensitivity response and induction of type I IFN (28). Interestingly, a single intradermal (i.d.) injection of DDA alone can induce severe arthritis in susceptible strains of rats (e.g., Lewis (LEW) and Dark Agouti rats) (16, 17). DDA-induced arthritis (DIA) is a self-limiting disease with spontaneous recovery from acute arthritis occurring within a period of 6–7 wk. Furthermore, this disease can be adoptively transferred to naïve syngeneic rats by lymph node cells (LNC), but not sera of arthritic rats (17). Because DDA is a nonantigenic chemical compound, defining the Ag specificity of the T cell repertoires that might be associated with the pathogenesis of DIA is critical in unraveling the autoimmune process in DIA. This in turn would contribute to further understanding of the immunological mechanisms involved in RA.

The 65-kDa mycobacterial heat shock protein (Bhsp65) has been invoked in the pathogenesis of arthritis induced by antigenic (e.g., Mycobacterium tuberculosis, H₃₇Ra) (4, 5, 29, 30) as well as nonantigenic (e.g., pristane) (14, 22, 31) materials. Similarly, the mammalian homologues of Bhsp65, self (rat) hsp65 (Rhs65), and human hsp60 have been shown to possess disease-regulating properties, as tested in the adjuvant arthritis (AA) model (32, 33). Considering that DDA can induce an inflammatory reaction, we reasoned that Rhs65, whose expression is up-regulated by
inflammation, might serve as one of the endogenous Ags for priming of the T cell response in DIA. Moreover, in view of the spontaneous recovery from DIA in the LEW rat, we also invoked the T cell response to Rhs65 in regulation of acute DIA. Furthermore, because hsp65s are highly conserved, we envisioned that rats with DIA might also develop T cell responses to Bhs65 in part owing to cross-reactivity between Rhs65 and Bhs65 (32).

In this study, we examined the above propositions in LEW rats with DIA, and also tested in parallel the immune response of these rats to another putative arthritis-related Ag, bovine type II collagen (CII). Our results demonstrate that LEW rats with DIA spontaneously raised T cell response to Rhs65 as well as Bhs65, but not to CII, and that the kinetics of the Rhs65/Bhs65-directed T cell response followed that of the clinical severity of arthritis. Furthermore, the T cell response to Rhs65 was characterized by a prominent reactivity to determinants 120–134 and 213–227 of the protein. Interestingly, pretreatment of naive adult LEW rats with peptides 120–134 and 213–227 of Rhs65 led to a significant protection from subsequent DIA, and a DIA-protective immunization of LEW rats with a low dose of DDA was also associated with induction of T cell response to peptide 213–227 of Rhs65. In addition, neonatal tolerization of LEW rats against Rhs65/Bhs65 afforded them protection against subsequently induced DIA at adult stage. Thus, the T cell repertoire directed against Rhs65 and Bhs65 is involved in the pathogenesis of arthritis induced by nonantigenic DDA.

Materials and Methods

Animals

Inbred male LEW rats (RT.1) (5–8 wk old, 120–200 g body weight) were purchased from Harlan Sprague Dawley, and were maintained in the animal care facility of the University of Maryland School of Medicine. Newborn LEW pups were obtained by breeding 8- to 10-wk-old male and female LEW rats in the vivarium facility. All experimental procedures performed on rats were in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Ags and adjuvants

DDA (C5H4N2Br), hen eggwhite lysozyme (HEL), native bovine CII, and Con A were purchased from Sigma-Aldrich, whereas IFA was obtained from Invitrogen Life Technologies.

Preparation of Rhs65, Bhs65, and denatured CII. Rhs65 (32, 34) was prepared from pTrcHisA-transfected BL21 cells expressing the appropriate cDNA, whereas Bhs65 (35, 36) was prepared from pTrcHisA-transformed BL21 (DE3) pLysS cells (Novagen) transformed by the vector pET236-GroEL2 (Colorado State University). The sequences of the cDNA for these two recombinant proteins were confirmed by DNA sequencing analysis at the Biopolymer Core Facility, University of Maryland School of Medicine. The expressed histidine-tagged recombinant proteins were purified using a nickel column (Invitrogen Life Technologies). Any endotoxin contaminating the recombinant proteins was removed using Acetate Etox kit (Sterogene Bioseparations), followed by testing in the limulus assay using the Limulus Amebocyte kit (BioWhittaker). Native bovine CII was dissolved in 0.1% acetic acid and then dialyzed against PBS. Thereafter, the denatured collagen was prepared by heating the solution of native CII in dissolved in 0.1% acetic acid and then dialyzed against PBS. Thereafter, Macromolecular Resources and Global Peptide Services.

Preparation of Ag-adjuvant emulsion. Equal volumes (1:1, v/v) of IFA and protein/peptide solution (1–2 mg/ml) were mixed in two glass syringes fitted with a three-way connector (Baxter Healthcare) to prepare a stable emulsion. The emulsified Ag (50–100 μg/rat) was injected into rats either i.d. at the base of the tail of adult rats (for immunization) or i.p. to neonatal rats (for tolerization), as needed.

Induction and evaluation of DIA

DDA was added to IFA slowly with continuous mixing, and then injected (2 mg/rat) i.d. at the base of the tail of adult LEW rats. Beginning 7 days after DDA injection, rats were observed regularly for the appearance of signs of arthritis (redness, swelling, etc.) in the fore paws and hind paws.

The severity of arthritis in each paw was graded on a scale of 0–4, as described elsewhere (17). The maximum attainable arthritis score per rat was 16. In a typical course of DIA, different phases of the disease corresponded to days post-DIA injection, as follows: day 5 (before onset), day 13 (onset), day 22 (peak), and day 32 (recovery phase).

LNC proliferation assay

The draining lymph nodes (inguinal and para-aortic) were harvested either from LEW rats afflicted with DIA at defined time points after DDA injection or from Ag-immunized rats on day 9 after injection. A single cell suspension of LNC was prepared and washed three times with HBBS (Sigma-Aldrich) (5). LNC were then cultured in a 96-well plate (2.5 ×103 to 5 ×105 cells/well) in HL-1 serum-free medium (BioWhittaker) supplemented with l-glutamine (1%, v/v), penicillin-streptomycin (1%, v/v), and 5 ×10–3 M 2-ME with or without protein/peptide Ag (25–75 μg/ml final concentration) for 5 days in a CO2 incubator (5% CO2, 95% air). Con A (2.5 μg/ml final concentration) was used as a positive control. [3H]Thymidine (International Chemical and Nuclear) (1 μCi/well) was added for the last 18 h of a 5-day culture, and thereafter, the cells were harvested on a Printed Filtermat A glass fiber filter (Wallac) using a TOMTEC microplate cell harvester (PerkinElmer). The radioactivity incorporated in the DNA of cells was counted in a Wallac microbeta liquid scintillation counter (PerkinElmer). The results were expressed either as cpm or as a stimulation index (SI = ratio of cpm in the presence of Ag and cpm without Ag (cells in medium only)). A proliferative response with SI value of 3.0 and above was considered positive.

Testing the cytokine profile of Ag-reactive T cells

For cytokine assay, the draining LNC of LEW rats with DIA or LEW rats pretreated with a mixture of Rhs65 peptides 120–134 and 213–227 before induction of DIA were plated in a 96-well plate as for a proliferation assay described above. These cells were restimulated in vitro with the appropriate Ag for 48 h (32). Thereafter, the culture supernatants were collected and tested in ELISA for IFN-γ using a commercially available kit (BioSource International), following the manufacturer’s instructions. The results of the assay were expressed as pg/mL. The detection limit for IFN-γ was 15 pg/ml.

Neonatal tolerization of rats with native Rhs65/Bhs65

Newborn LEW pups were given two i.p. injections of either Rhs65/IFA or Bhs65/IFA (total 100 μg/pup each) (experimental groups) following the method described elsewhere (36, 38). The control littermates similarly received two injections each of either PBS/IFA or HEL/IFA. The first injection was given within 24 h, and the second within 72 h of birth. At the age of 4 wk, the same rats received an arthritogenic challenge (2 mg/rat) of DDA. All rats were then observed regularly for clinical signs of arthritis, and the severity of arthritis was graded as described above.

Measurement of the level and isotype of serum Abs

High-binding capacity 96-well ELISA plate (Greiner Bio-One) was coated with 100 μl/well Ag (100 ng/ml) in PBS (pH 7.2) overnight at 4°C. The nonspecific binding activity of the wells was blocked with 200 μl/well 10% BSA in PBST (PBS containing 0.5% Tween 20, v/v) for 2 h at room temperature. The test serum was added to the wells at the appropriate dilution and incubated for 1 h at room temperature. Following rinsing of wells five times with PBST, the plate-bound total Abs were detected by HRP-conjugated affinity-purified goat anti-rat Ig (IgG, IgM, and IgA)-specific polyclonal Ab (1:1000) (BD Pharmingen), whereas anti-rat IgM (1:1000) and anti-rat IgG (1:2000) (both from Zymed Laboratories) were used separately to measure the isotype-specific Ab activity. For cytokine assay, the draining LNC of LEW rats with DIA or LEW rats pretreated with a mixture of Rhs65 peptides 120–134 and 213–227 before induction of DIA were plated in a 96-well plate as for a proliferation assay described above. These cells were restimulated in vitro with the appropriate Ag for 48 h (32). Thereafter, the culture supernatants were collected and tested in ELISA for IFN-γ using a commercially available kit (BioSource International), following the manufacturer’s instructions. The results of the assay were expressed as pg/mL. The detection limit for IFN-γ was 15 pg/ml.

Statistical analysis

One- or two-tailed Student’s t test was performed assuming equal or unequal variance as appropriate for the data. Initially, F test was done to determine whether the variance was equal or unequal, and then an appropriate Student’s t test was used. Wilcoxon rank sum test was also used for the analysis of data of some experiments. Values of p ≤ 0.05 were considered significant.
Results

The severity of DIA in the LEW rat is dose dependent

To determine the influence on the severity of disease of the dose of DDA injected, a group each of naive LEW rats was challenged with 0.5, 1, or 2 mg of DDA per rat. Thereafter, all rats were observed and scored regularly for signs of arthritis. A single injection of DDA/IFA (2 mg/rat) induced arthritis in LEW rats (Table I), and the severity of the disease was significantly (p < 0.0001) higher than that induced by 1 mg of DDA per rat. However, a low dose of DDA (0.5 mg/rat) failed to induce any clinical signs of arthritis. Thus, DDA, a nonantigenic chemical, per se could induce polyarthritis in the LEW rat. Furthermore, the severity of the disease correlated with the dose of DDA used.

LEW rats with DIA spontaneously raise T cell response to Rhsp65

Considering the nonantigenic composition of DDA, and that DIA can be adoptively transferred to naive syngeneic recipients through LNC of arthritic rats (17), we tested whether LEW rats with DIA developed a T cell response to the endogenous self homologue

![Image](FIGURE 1. LEW rats with DIA raise T cell response to native Rhsp65, but not CII. LEW rats (n = 5) were challenged with DDA/IFA (A) (unless indicated otherwise, the dose of DDA used in this and other experiments in this study for the induction of disease was 2 mg/rat), and thereafter, the draining LNC of these rats were harvested at defined time points after immunization. A control group of LEW rats (n = 3) was immunized with PBS/IFA (B), and after day 13 (corresponding to the day of onset of disease in DDA-immunized rats), the draining LNC of these rats were harvested. LNC of the two groups of rats were tested in a proliferation assay using Rhsp65 and type II collagen (collagen II or CII) as recall Ags, as described in Materials and Methods. HEL was used as an irrelevant Ag control. These Ags were tested in this and other experiments at optimal concentrations determined in previous pilot experiments. The results were expressed as SI. In A, the difference in SI values for Rhsp65 at days 5 and 13 (*, p < 0.01), and that at days 5 and 22 (or at day 5 vs day 32) (**, p < 0.0005; Student’s t test) was statistically significant. Also significant (†, p < 0.01; ††, p < 0.04) was the difference in the level of response to Rhsp65 at day 22 compared with that at day 13 or 32. Moreover, SI values for both CII and HEL were significantly lower than that of Rhsp65 (p < 0.0001-p < 0.05) at each of the time points tested. (Denatured CII and native CII gave similar results (data not shown); the data using denatured CII are shown in this and other figures). SI values for Con A at day 13 each were 52 (A) and 46 (B). The background medium (cells in medium without Ag) cpm values were as follows: A, 1420 (day 5), 1610 (day 13), 2033 (day 22), and 3580 (day 32); B, 1580. Similar results were obtained in repeat experiments.

![Image](FIGURE 2. The kinetics of the T cell response to Rhsp65 peptides of LEW rats with DIA. LEW rats were challenged with DDA/IFA (2 mg/rat), and thereafter, their LNC were harvested at different phases of DIA (day 13, onset (A); day 22, peak (B); and day 32, recovery phase (C) of the disease). These LNC were tested in a proliferation assay using a series of overlapping peptides spanning the entire amino acid sequence of Rhsp65. Also used in the assay were native Rhsp65 (positive control) and HEL (negative control). The results were expressed as an SI (mean ± SD) of four to eight samples, and an SI value of ≥3.0 was considered a positive response. The SI values for Con A were as follows: 52 (day 13), 45 (day 22), and 52 (day 32). The medium cpm values were 1949 (day 13), 1717 (day 22), and 1826 (day 32). The above results were confirmed in a repeat experiment.)
(Rhsp65) of the arthritis-related Ag, Bhsp65 (4, 5, 22, 29, 30). For this purpose, the draining LNC harvested from arthritic rats at different phases of the disease were tested in a proliferation assay using Rhsp65. Also tested in the assay was another well-known arthriogen, CII, as control. The results (Fig. 1) show that rats immunized with DDA/IFA raised T cell response to native Rhsp65, and that a positive response was detectable even before the onset of clinical disease as measured 5 days after DDA injection. The level of T cell response to Rhsp65 was found to increase with progression of arthritis, with the highest response observed at day 22, followed by a slight decline at day 32 (Fig. 1A). In comparison, control LEW rats challenged with PBS/IFA failed to give a T cell response to Rhsp65 (Fig. 1B). In contrast to a positive response to Rhsp65, LEW rats with DIA had no T cell reactivity to CII at any of the time points tested (Fig. 1A), and their level of T cell responses to CII was comparable to that of control LEW rats immunized with PBS/IFA (Fig. 1B). These results suggest that the Rhsp65-directed T cell repertoire is spontaneously primed during the course of DIA.

Epitope specificity of the Rhsp65-directed T cell response of arthritic LEW rats with DIA

To identify the antigenic determinants that might contribute to the T cell response to Rhsp65 observed during the course of DIA, we tested the LNC harvested from arthritic rats (at onset (day 13), peak (day 22), and recovery (day 32) phase of DIA, as described in Materials and Methods) in a proliferation assay using a series of Rhsp65 peptides spanning the amino acid sequence of the whole protein. The results (Fig. 2) reveal that LEW rats with DIA raised T cell response to multiple epitopes within Rhsp65, and peptides 120–134 and 213–227 gave consistently higher T cell response compared with other peptides. The overall kinetics of the T cell response to peptides 120–134 and 213–227 of LEW rats with DIA (Fig. 3) was similar to that of the T cell response to Rhsp65 in these rats (Fig. 1).

LEW rats with DIA raised Ab response to Rhsp65, but not CII

Considering that LEW rats with DIA developed T cell response to Rhsp65 (but not CII), we tested whether these rats also developed an Ab response to Rhsp65. Sera of arthritic rats collected at different phases of the disease were tested in ELISA for total Ig as well as IgG and IgM titers against Rhsp65 and CII. Preimmune sera (day 0) served as a control. The results show that LEW rats with DIA developed an Ab response to Rhsp65 (Fig. 4A), but not CII (Fig. 4B). Furthermore, the serum Ig levels against Rhsp65 at days 13, 22, and 32 each were higher compared with those at days 0 and 5. However, the difference was statistically significant (p < 0.05) only for the day 32 anti-Rhsp65 Ig level compared with that of days 0/5. The generation of anti-Rhsp65 Abs in DIA further demonstrates the spontaneous induction of immune response to Rhsp65 following injection of DIA to LEW rats.

Modulation of DIA by neonatal tolerization of the Rhsp65-specific T cell repertoire in the LEW rat

To determine the functional significance of the self hsp65-directed T cells in DIA, a group of newborn LEW pups was tolerized with Rhsp65, and after 4 wk, these rats were given an arthritogenic (2 mg/rat) challenge with DDA. The control LEW littermates were either tolerized with PBS/IFA, followed by DDA challenge 4 wk later, or were left untreated (naive) at the neonatal stage, but immunized with DDA at 4 wk of age. Thereafter, all rats were observed regularly for signs of arthritis. The results (Fig. 5) show that
there was a significant decrease in the severity of DIA in Rhsp65-tolerized LEW rats compared with that of either PBS/IFA-tolerized or untreated (naive) rats. These results demonstrate that neonatal tolerization of the Rhsp65-specific T cells of LEW rats could significantly down-modulate the course of DIA induced at adult stage, and thereby, suggest that the Rhsp65-directed T cell repertoire is involved in the pathogenesis of DIA.

Pretreatment of naive adult LEW rats either with a mixture of peptides 120–134 and 213–227 of Rhsp65 or with a nonarthritogenic low dose of DDA affords protection against subsequent DIA

As described above, LEW rats with DIA raised significant T cell response to two peptides of Rhsp65, namely 120–134 and 213–227, at the peak of disease just preceding the recovery phase of DIA. To further assess the physiological significance of this T cell response, we determined the influence on the course of DIA in the LEW rat of pretreatment with a peptide mixture (containing equimolar amounts of peptides 120–134 and 213–227 emulsified in IFA) 7 days before injection of an arthritogenic dose (2 mg/rat) of DDA. Interestingly, LEW rats challenged with this peptide mixture showed a significant reduction in the severity of subsequent DIA compared with control rats (Fig. 6A). Similar results were obtained in a repeat experiment, including additional controls consisting of naive LEW rats injected with DDA (to determine the level of suppressive effect of IFA treatment), and LEW rats pretreated with a control peptide of mouse lysozyme (ML) in IFA (Fig. 6B). Although IFA treatment slightly reduced the severity of subsequently induced DIA, the difference between the arthritic scores of Rhsp65 peptide/IFA-treated and PBS/IFA-treated rats (or ML peptide-treated group) was statistically significant ($p < 0.05$). However, LEW rats pretreated either with PBS/IFA or with ML peptide/IFA revealed a comparable profile of clinical arthritis. The above results suggest that the T cells specific for the two self-determinants of Rhsp65, namely 120–134 and 213–227, are of significance in the pathogenesis of DIA.

In a preliminary study, we tested the cytokine profile of Rhsp65 peptide-specific cytokine response in control LEW rats having DIA and experimental LEW rats pretreated with Rhsp65 peptides

FIGURE 5. Neonatal tolerization of LEW rats with Rhsp65 suppresses the induction of DIA at adult stage. Rhsp65 emulsified in IFA was administered to newborn LEW rats ($n = 6$) in two i.p. injections within 72 h of birth, as described in Materials and Methods. Age-matched littersmates (controls) ($n = 6$ each) either received two i.p. injections of PBS/IFA or were left untreated (naive). After 4 wk, all rats were challenged with DDA/IFA (2 mg/rat), and then observed and scored regularly for signs of arthritis. The severity of arthritis of Rhsp65-tolerized rats was significantly lower than that of the PBS/IFA-treated rats from day 18 through day 27 ($p < 0.05$; Student’s $t$ test); similarly, the Rhsp65-treated group and naive group showed a significant difference in the disease severity from day 13 through day 27 (except at day 15). The difference in the severity of the disease between PBS/IFA-treated and Rhsp65-tolerized rats ($p < 0.05$), and that between untreated (naive) and Rhsp65-tolerized group ($p < 0.05$) was also significant by Wilcoxon rank sum test. However, as expected, the severity of the disease in PBS/IFA-treated and naive groups of LEW rats was comparable ($p > 0.05$) throughout the disease course.

FIGURE 6. Pretreatment of naive LEW rats either with Rhsp65 peptides or with a nonarthritogenic low dose of DDA affords protection from subsequent induction of DIA. A, LEW rats were immunized either with a mixture of two Rhsp65 peptides (peptides 120–134 and 213–227; 0.1 mg/ rat each) in IFA (n = 7) or with PBS/IFA (n = 4). Another group of LEW rats (n = 4) was challenged with a low dose of DDA (0.1 mg/rat) in IFA. (The day of preimmunization is marked by an arrow). After 7 days (= day 0) of pretreatment, all rats were injected with an arthritogenic (2 mg/rat) dose of DDA and then observed regularly for signs of arthritis. Arthritic scores of Rhsp65 peptide-treated as well as low dose DDA-treated group of rats each were significantly lower ($p < 0.05$) than that of the PBS/IFA-treated group from day 18 through day 29, as tested by Student’s $t$ test. The difference in the severity of the disease in each of these experimental groups compared with control rats was also significant ($p < 0.05$) by Wilcoxon rank sum test. B, In another set of experiments similar to that described in A above, naive LEW rats challenged with DDA (naive control) and LEW rats pretreated with peptide 105–119 (ML) in IFA before induction of DIA served as additional controls for the two groups of LEW rats under testing: an experimental group consisting of rats pretreated with a mixture of Rhsp65 peptides 120–134 and 213–227 in IFA, and a control group of rats immunized with PBS/IFA. All rats were observed regularly for signs of arthritis. The severity of the disease in ML peptide- vs PBS-treated rats was comparable ($p > 0.05$). However, each of these two groups had significantly ($p < 0.05$) higher severity of the disease compared with rats pretreated with Rhsp65 peptides. C, The cytokine profile of Rhsp65 peptide-reactive T cells: the LNC of arthritic or peptide-protected LEW rats pretreated with Rhsp65 peptides. The LNC of arthritic or peptide-protected LEW rats pretreated with Rhsp65 peptides.
120–134 and 213–227 before DDA challenge. The results showed that the control arthritic rats raised a vigorous IFN-γ response to the two recall Rhsp65 peptides tested, whereas the test (pretreated) group of rats showed significantly reduced levels of IFN-γ (Fig. 6C). These results suggest that a significant decrease in IFN-γ induced by Rhsp65 peptide pretreatment might be one of the mechanisms by which the severity of the disease is dramatically reduced in peptide-protected rats. At this time, we do not know the precise mechanism of reduction of IFN-γ. This issue is currently under investigation in our laboratory.

In another set of experiments, we tested whether immunization of naive adult LEW rats with a low, nonarthritogenic dose of DDA could attenuate the course of subsequently induced disease using arthritogenic dose of DDA. Although DDA per se is a nonantigenic material, we reasoned that DDA might behave like other Ags/arthritogens (39, 40) such that initial exposure of rats to the arthritogen could significantly modulate the immune response to (or induction of disease by) subsequent challenge with the same Ag. The results (Fig. 6A) show that administration to naive LEW rats of a low dose (0.1 mg/rat) of DDA 7 days before induction of DIA by a higher dose (2 mg/rat) of DDA resulted in a significant suppression of arthritis.

**LEW rats treated with a nonarthritogenic dose of DDA spontaneously developed T cell response to peptide 213–227 of Rhsp65**

Taking together the results described above (Fig. 6), we reasoned that the protection against DIA offered by a low dose of DDA might involve induction of T cell response to one or both of the Rhsp65 peptides, namely 120–134 and 213–227. To examine this proposition, a group of LEW rats was immunized with a low dose (0.1 mg/rat) of DDA, and 7 days later, the draining LNC of these rats were tested in a proliferation assay using peptides 120–134 and 213–227 of Rhsp65. The results (Fig. 7) show that the challenge of LEW rats with a nonarthritogenic dose of DDA led to induction of a significant T cell response to peptide 213–227 as well as to native Rhsp65. However, as expected, LNC of naive LEW rats failed to respond to peptide 213–227 (data not shown). These results suggest that the protective effect against DIA of a low dose DDA challenge was associated with a significant induction of T cell response to one of the immunomodulatory peptides (peptide 213–227) of Rhsp65.

**Spontaneously induced as well as deliberately primed T cells against self hsp65 (Rhsp65) in the LEW rat are cross-reactive with the foreign, mycobacterial homologue of self hsp65, Bhsp65**

Rhsp65 has 48% amino acid sequence homology with Bhsp65 (36). Therefore, we tested whether the spontaneously primed T cells against Rhsp65 during the course of DIA in the LEW rat could be recalled in vitro with Bhsp65. As shown in Fig. 8Aa, LNC of LEW rats with DIA gave a positive response not only to Rhsp65, but also to Bhsp65 at each of the time points tested. Overall, the kinetics of the T cell response to Bhsp65 was quite similar to that observed for Rhsp65, except for a difference (*, p < 0.05) in the level of response to the two Ags at day 13 after DDA challenge. In comparison, PBS/IFA-treated control rats failed to respond to these two Ags (Fig. 8Ab).

The cross-reactivity of the Rhsp65-primed T cells against Bhsp65 is directly validated by the results shown in Fig. 8B: LEW rats challenged with Rhsp65 raised a potent T cell response to the immunogen, and these T cells could be efficiently restimulated in vitro by Bhsp65, and vice versa. In the case of both Rhsp65 and Bhsp65 as immunogens, the T cell recall response to these two homologous proteins was significantly (p < 0.05) higher than that to the control recombinant Ag, ML (Fig. 8B). Besides raise T cell response to Bhsp65, LEW rats with DIA also showed Ab reactivity to Bhsp65 in the late phase of the disease (Fig. 8C). The overall pattern of Ab response to Bhsp65 was similar to that for anti-Rhsp65 Abs (Fig. 4A). The above results demonstrate that the T cells primed by Rhsp65 in LEW rats are cross-reactive with Bhsp65, and therefore, the spontaneously induced T cell response to Bhsp65 observed during the course of DIA in the LEW rat could be attributed in part to the cross-reactivity between homologous Rhsp65 and Bhsp65.

**Neonatal tolerization of LEW rats with Bhsp65 suppressed the course of subsequent DIA**

Taking into consideration the observed T cell response to Bhsp65 in LEW rats with DIA (Fig. 8A), the cross-reactivity between Rhsp65 and Bhsp65 (Fig. 8B), and the modulation of DIA by neonatal tolerization with Rhsp65 (Fig. 5), we further tested whether T cells reactive against Bhsp65 had any disease-modulating effect in DIA. The results show that the severity of arthritis in Bhsp65-tolerized rats was significantly (p < 0.05) reduced compared with that of either PBS/IFA-tolerized or untreated (naive) rats (Fig. 8D). These results demonstrate that the subsets of T cells directed against Rhsp65 and Bhsp65 were not only cross-reactive, but also possessed a similar functional attribute in regard to suppression of DIA.

**Discussion**

DIA can be adoptively transferred to naive LEW rats using LNC, but not sera of diseased LEW rats (17). Thus, the induction of DIA most likely is primarily dependent on T cell-mediated immune mechanisms. DDA is a lipoid material that lacks any known arthritogenic Ag. Considering that DDA can induce inflammation, we hypothesized that endogenous self hsp65 (Rhsp65), whose expression is enhanced under conditions like heat stress and inflammation (32, 41), might be involved in the spontaneous priming of the T cells during the course of autoimmune arthritis triggered by DDA injection. The choice of self hsp65 as a disease-associated Ag was also favored by the vast literature documenting the role of its foreign homologue, Bhsp65, in the pathogenesis of arthritis in
been described only in murine PIA. Therefore, we have compared the Ag-specific immunological aspects of the disease have to date but it is quite different from PIA in mice (16–18, 40). However, pristane-induced arthritis (PIA) and oil-induced arthritis in the rat, dience/severity of arthritis, etc.), DIA more closely resembles the observed T cell response to the foreign mycobacterial Ag, Bhsp65, in DIA is not clear. We attribute this T cell response to molecular epitope spreading (43). Moreover, the precise origin of arthritis following the injection of a low nonarthritogenic dose of DDA is associated with induction of a potent T cell response to the immunomodulatory peptide 213–227 of Rhsp65; and 5) both the kinetics of the T cell responses in DIA as well as the down-modulation of DIA by induction of immune response to Rhsp65 during the course of DIA are supported by a similar finding in mice afflicted with PIA (22). Mice with PIA develop both T cell and Ab response against self (murine) hsp58 perhaps was not tested for this effect (22). It is likely that besides Rhsp65, one or more of the other self Ags (e.g., Ig-binding protein (Bip), proteoglycan cartilage glycoprotein (gp39), chondrocyte protein 65 (CH65), self hsp70/hsp90, etc.) that have been implicated in arthritis in experimental models as well as patients with RA might also contribute to the disease process in DIA. In addition, DDA has adjuvant properties, and it can induce inflammation (25–28). In this regard, it remains to be determined whether the T cell response to Rhsp65 observed during DIA represents the early primary disease-related immune response or whether it is one of the later responses arising owing to intermolecular epitope spreading (43). Moreover, the precise origin of the observed T cell response to the foreign mycobacterial Ag, Bhsp65, in DIA is not clear. We attribute this T cell response to

**FIGURE 8.** A, LEW rats with DIA also revealed T cell reactivity against Bhsp65. A cohort of LEW rats was challenged with DDA/IFA (2 mg/rat) (a), and thereafter, the draining LNC were harvested from subgroups of these rats at different days postinjection. A control group of LEW rats was immunized with PBS/IFA (b), and after day 13, their draining LNC were harvested. LNC of all rats were tested in a proliferation assay using Rhsp65 and Bhsp65 as recall Ags. The results of four to eight samples are expressed as an SI (mean ± SD). In group a, the response to the two proteins at different time points tested was comparable except for day 13 (\(p < 0.05\)). SI value for Con A was 52 (a) and 46 (b). The SI value for both Rhsp65 (\(p < 0.0005\)) and Bhsp65 (\(p < 0.0005\)) at day 13 (a) was significantly higher than that of control rats (b) at the corresponding time point tested. The background medium cpm values were as follows: a, 1420 (day 5), 1610 (day 13), 2033 (day 22), and 3580 (day 32); b, 1580. B, The T cells primed by Rhsp65 are cross-reactive with Bhsp65, and vice versa. LEW rats (\(n = 5\)) were immunized s.c. either with Rhsp65/IFA (left panel) or with Bhsp65/IFA (right panel), and 9 days later, their draining LNC were harvested and tested separately in a proliferation assay using Rhsp65, Bhsp65, and ML as recall Ags. The results were expressed as a SI. LNC cultured with Con A gave an SI value of 89 for Rhsp65-immunized rats and 92 for Bhsp65-immunized rats. The medium control gave cpm values of 1,653 for Rhsp65-immunized rats, and 1,045 for Bhsp65-immunized rats. (\(*, p < 0.05\) compared with the proliferative response against the control recombinant Ag, ML.) C, Ab response to Bhsp65 of LEW rats with DIA. LEW rats were immunized with DDA/IFA (2 mg/rat), as described in Aa. Blood samples were collected from these rats at defined time points postinjection, and the sera obtained were tested in ELISA for IgG, IgM, and total Ig (IgG, IgM, and IgA) against Bhsp65. The results are shown as OD at 450 nm (mean ± SEM). The level of both total Ig and IgG against Bhsp65 on day 32 was significantly (\(p < 0.05–0.001\)) higher than that at day 0 as well as at each of the other time points tested. D, LEW rats neonatally tolerized against Bhsp65 are protected from subsequent DIA. Bhsp65/IFA was administered i.p. into newborn LEW pups in two injections within 72 h of birth, as described in Materials and Methods. The control littersmates either received two i.p. injections of PBS/IFA or were left untreated (naive). After wk 4, DDA/IFA (2 mg/rat) was injected i.d. to the same rats, and then these rats were observed and scored regularly for signs of arthritis. The difference in the severity of disease in naive vs Bhsp65-treated rats (\(p < 0.05\)) and that between PBS/IFA-treated vs Bhsp65/IFA-treated rats (\(p < 0.05\)) was statistically significant, as analyzed by Wilcoxon rank sum test. However, as expected, the difference between naive and PBS/IFA-treated group of rats was not significant.

Various animal models of this disease (4, 5, 14, 22, 29, 30, 42). Our proposition regarding the involvement of Rhsp65-directed T cell repertoire in DIA in the LEW rat is supported by the following observations from this study: 1) the T cell response to Rhsp65 is spontaneously induced in the LEW rat following injection of DDA, and the level of the Rhsp65-directed T cell response correlates with that of the clinical course of DIA; 2) neonatal tolerization of the Rhsp65-directed T cells of the LEW rat leads to significant protection against DIA induced in the same rats at the adult stage; 3) pretreatment of LEW rats with peptides 120–134 and 213–227 of Rhsp65 results in a significant suppression of DIA; 4) the protection against DIA induced in the LEW rat following challenge with a low nonarthritogenic dose of DDA is associated with induction of a potent T cell response to the immunomodulatory peptide 213–227 of Rhsp65; and 5) both the kinetics of the T cell responses in DIA as well as the down-modulation of DIA by a well-studied arthritis-related Ag, Bhsp65, mirror that of the cross-reactive self hsp65 (Rhsp65).

Considering the clinical features of arthritis following the injection of an arthritogenic material (e.g., the day of onset, the incidence/severity of arthritis, etc.), DIA more closely resembles pristane-induced arthritis (PIA) and oil-induced arthritis in the rat, but it is quite different from PIA in mice (16–18, 40). However, the Ag-specific immunological aspects of the disease have to date been described only in murine PIA. Therefore, we have compared our results of immune response to hsp65 in DIA with murine PIA in addition to that with AA. Our results showing the spontaneous induction of immune response to Rhsp65 during the course of DIA are supported by a similar finding in mice afflicted with PIA (22). Mice with PIA develop both T cell and Ab response against self (murine) hsp58 (22). However, there are some important differences in the two models of arthritis. Our study has highlighted the immunomodulatory effect on DIA of tolerization with native self hsp65 (Rhsp65), and of pretreatment with peptides 120–134 and 213–227 of Rhsp65 in DIA. In contrast, in murine PIA, a self hsp58 peptide test failed to modulate the course of disease, and native hsp58 perhaps was not tested for this effect (22).
Bhsp65 in part to the cross-reactivity between Rhs65 and Bhsp65, and to the exposure of rats to environmental microbial agents posses-
sing hsp65 (44–46). Besides Ag-specific T cell response, mechanisms of innate immunity (47, 48) activated following DDA challenge and adjuvant-induced proinflammatory responses (16) might also contribute to the induction/regulation of DIA in the LEW rat. Unlike hsp65, rats with DIA did not raise a T cell re-
sponse to bovine CII. One likely explanation is that there is rather poor response of rodents to autologous CII vs heterologous CII (49). For this reason, there may be insufficient priming of autolo-
gous CII-reactive T cells during DIA, resulting in poor cross-re-
activity with bovine CII.

DIA is a self-limiting disease, but the immunological basis of recovery from acute disease is not yet clear. The results of exper-
iments demonstrating that pretreatment of naive LEW rats with a combination of peptides 120–134 and 213–227 of Rhs65 leads to a significant suppression of the subsequently induced DIA suggest the immunoregulatory role in this disease of self-determinants within Rhs65 in vivo. This proposition is further supported by the finding that the challenge of naive LEW rats with a low nonar-
thritogenic dose of DDA leads not only to protection against sub-
sequent disease triggered by injection of a higher arthritogenic dose of DDA, but also to spontaneous induction of T cell response to one of the above-mentioned peptides of Rhs65, peptide 213–
227. However, considering that our pepscan consists of a limited number of Rhs65 peptides, it is likely that other yet undefined epitopes within Rhs65, which might be similar to that within human hsp60 (32, 33), might also contribute to regulation of acute DIA. The above results are supported by the observations in the AA model (32, 33, 50) and those in patients with juvenile chronic arthritis (51) regarding the role of self mammalian hsp65 in reg-
ulation of arthritis. In addition, LEW rats with DIA develop an Ab response to Rhs65. It is likely that these anti-Rhs65 Abs might contribute in part to regulation of DIA in vivo. This suggestion is based on the finding in the AA model showing that Abs to Bhsp65 are protective rather than being pathogenic (30). The mechanisms by which Rhs65 or its peptides down-modulate the course of DIA, it is likely that these anti-Rhs65 Abs might contribute in part to regulation of DIA in vivo. This suggestion is based on the finding in the AA model showing that Abs to Rhs65 are protective rather than being pathogenic (30). The mechanisms by which Rhs65 or its peptides down-modulate the course of DIA are not fully defined. Our preliminary studies suggest that a sig-
ificant decrease in IFN-γ might be one of the mechanisms by
which the severity of the disease is dramatically reduced upon pretreatment of LEW rats with Rhs65 peptides. The precise mechanism of down-modulation of IFN-γ is currently under in-
vestigation in our laboratory.

The results of this study along with those in other models of arthritis (4, 5, 14, 22, 52, 53) reveal that there is significantly much more information and experimental evidence about the disease-regulating epitopes within Bhsp65 than that for the disease-induc-
ing epitopes of the protein. Among these animal models, the ar-
thrithmetic epitope (region 180–188) within Bhsp65 has been defined only for the AA model in the LEW rat (4). Enigmatically, however, neither native Bhsp65 nor peptide 180–188 per se is arthritogenic in the LEW rat, although AA is inducible in native syngeneic recipients by adoptive transfer of peptide 180–188-spe-
cific T cell line A2 or a T cell clone A2b (3, 4). However, the generation and maintenance of functional arthriticogenic rat T cell clones have been rather a difficult task (54), and until now, there is no other reported arthriticogenic T cell clone besides A2b that can induce AA in the LEW rat. A similar situation prevails in the PIA model. Mice having PIA develop T cell response to Bhsp65, and the pretreatment of mice with Bhsp65 or its peptide 261–271 can induce protection against disease (14, 22, 31). However, direct demonstration of the pathogenicity of T cells specific for Bhsp65 in PIA has not yet been achieved. For the above-mentioned rea-
sons, considering the difficulties in establishing the arthritogenicity of Bhsp65 per se or of peptides of Bhsp65 in other animal models of arthritis, further studies using some new approaches would be needed to document the pathogenic role of Rhs65/Bhsp65 in DIA. Nevertheless, the suppression of arthritis by tolerization of T cells against Bhsp65 (in AA, PIA, streptococcal cell wall-induced arthritis, oil-induced arthritis, etc.) (4, 22, 42, 52, 53) or against Bhsp65 peptide 180–188 (or the cross-reactive longer peptide 176–190) (in AA and Avidrine-induced arthritis) (23, 55) has been regarded as an evidence for the role of these Ags in the disease process. In this context, we have also shown in this study that tolerization with Rhs65/Bhsp65 or pretreatment with peptides 120–134 and 213–227 of Rhs65 can lead to significant protection against subsequent DIA. Therefore, our results suggest that hsp65-
directed T cell repertoire plays a critical role in the pathogenesis of DIA. Additional comparative studies are needed to delineate the interrelationships between the disease-related T cell effector mech-
anisms directed against self and mycobacterial hsp65 in various animal models of nonantigenic material-induced arthritis.

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References
terminants within the 65-kD heat-shock protein has been involved in regulation of au-


