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*J Immunol* 2002; 168:6463-6469; doi: 10.4049/jimmunol.168.12.6463
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Resistance to Adjuvant Arthritis Is Due to Protective Antibodies Against Heat Shock Protein Surface Epitopes and the Induction of IL-10 Secretion

Rina Ulmansky,* Cyril J. Cohen,† Fanny Szafer,* Eli Moallem,* Zvi G. Fridlender,* Yechezkel Kashi,† and Yaakov Naparstek2*

Adjuvant arthritis (AA) is an experimental model of autoimmune arthritis that can be induced in susceptible strains of rats such as inbred Lewis upon immunization with CFA. AA cannot be induced in resistant strains like Brown-Norway or in Lewis rats after recovery from arthritis. We have previously shown that resistance to AA is due to the presence of natural as well as acquired anti-heat shock protein (HSP) Abs. In this work we have studied the fine specificity of the protective anti-HSP Abs by analysis of their interaction with a panel of overlapping peptides covering the whole HSP molecule. We found that arthritis-susceptible rats lack Abs to a small number of defined epitopes of the mycobacterial HSP65. These Abs are found naturally in resistant strains and are acquired by Lewis rats after recovery from the disease. Active vaccination of Lewis rats with the protective epitopes as well as passive vaccination with these Abs induced suppression of arthritis. Incubation of murine and human mononuclear cells with the protective Abs induced secretion of IL-10. Analysis of the primary and tertiary structure of the whole Mycobacterium tuberculosis HSP65 molecule indicated that the protective epitopes are B cell epitopes with nonconserved amino acid sequences found on the outer surface of the molecule. We conclude that HSP, the Ag that contains the pathogenic T cell epitopes in AA, also contains protective B cell epitopes exposed on its surface, and that natural and acquired resistance to AA is associated with the ability to respond to these epitopes. The Journal of Immunology, 2002, 168: 6463–6469.

A djuvant arthritis (AA)3 is an experimental model of autoimmune arthritis that can be induced in susceptible inbred strains of rats such as Lewis or Wistar strains upon immunization with heat-killed Mycobacterium tuberculosis (MT) in CFA (1, 2). The disease cannot be induced in resistant strains of rats (e.g., Brown-Norway (BN), Fisher) (3, 4), and Lewis rats develop resistance to reinduction of the disease after recovery from arthritis (5, 6).

The role of the 65-kDa heat shock protein (HSP)65 of the MT (7, 8) in the pathogenesis of autoimmune arthritis, both in experimental animals (9, 10) and in humans (11–13), has been extensively investigated in the past. AA can be passively transferred by cellular and humoral immune responses. Resistant BN rats have Abs to certain epitopes of the mycobacterial HSP65 that are not found in the young arthritis-susceptible Lewis rats. These Abs are also present in old naive Lewis rats and are acquired by young Lewis rats after recovery from the disease.

We have previously shown that resistance to AA is due to the presence of Abs against HSP65 and can be transferred to susceptible rats by i.v. infusion of these Igs derived from resistant strains (25). In this study we have analyzed the fine epitope specificity of the anti-HSP Abs of arthritis-susceptible and -resistant rats. We found that resistant BN rats have natural Abs to certain epitopes of the mycobacterial HSP65 that are not found in the young arthritis-susceptible Lewis rats. These Abs are also present in old naive Lewis rats and are acquired by young Lewis rats after recovery from the disease.

Vaccination of Lewis rats with some of these peptide epitopes before disease induction resulted in the production of Abs against the whole molecule as well as resistance to disease induction. These Abs were shown to skew the cytokine profile of mononuclear cells toward an anti-inflammatory response in vitro and to suppress disease upon in vivo treatment.

Analysis of the primary and tertiary structure of the whole HSP65 molecule indicated that the protective peptides are potential B cell epitopes found on the outer surface of the molecule.

Materials and Methods

Animals

Female inbred Lewis rats were obtained from Harlan Laboratories (Jerusalem, Israel). Female BN rats were obtained from Harlan Sprague Dawley (Indianapolis, IN).

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1 This work was partially funded by the Riva Foundation and by the Adolfo and Evelyn Blum Endowment Fund. Y.N. is the incumbent of the Leiferman Chair for Rheumatology.

2 Address correspondence and reprint requests to Dr. Yaakov Naparstek, Clinical Immunology and Allergy Unit, Hadassah University Hospital, P.O. Box 1200, Jerusalem 91120, Israel. E-mail address: yakovn@cc.huji.ac.il

3 Abbreviations used in this paper: AA, adjuvant arthritis; HSP, heat shock protein; RT, room temperature; 3D, three-dimensional; MT, Mycobacterium tuberculosis.
Ags and Abs

Recombinant HSP65 of MT was a kind gift from Dr. M. Singh (World Health Organization Recombinant Protein Bank, Braunschweig, Germany). Recombinant mammalian HSP60 was purchased from StressGen Biotech (Victoria, British Columbia, Canada). Synthetic 16-mer peptides overlapping by 10 amino acids of HSP65 were a kind gift from Dr. L. Adorni (Roche Milano Ricerche, Milan, Italy) (26). Synthetic 20-mer peptides overlapping by five amino acids of the mammalian HSP60 were a kind gift from Dr. I. Cohen (Weizmann Institute, Rehovot, Israel) (27). Goat anti-rat IgG conjugated to alkaline phosphatase was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). R83D is an unrelated control rat mAb.

Induction and clinical assessment of AA

Lewis rats were injected s.c. at the base of the tail with 1 mg of MT H37Rv (Difco, Detroit, MI) in CFA (Difco). Severity of arthritis (arthritis index) was assessed every other day by a blinded observer as follows: 0, no arthritis; 1, redness of the joint; 2, redness and swelling of the joint. The ankle and tarsal-metatarsal joints of each paw were scored. A maximum score of 16 could be obtained.

Dot blot assay

Ags were dissolved in PBS and samples of 1 μg were adsorbed on nitrocellulose paper (Sigma-Aldrich, St. Louis, MO). The paper was air-dried and incubated with 1% BSA (Sigma-Aldrich) in PBS for 20 min to block nonspecific binding. The samples were then washed in 0.05% PBS-Tween 20 and incubated with rat sera diluted 1/100 in 1% BSA for 90 min at room temperature (RT). The membranes were washed and incubated with goat anti-rat Ab conjugated to alkaline phosphatase diluted 1/1000 in 1% BSA for 90 min at RT. After washing the color reaction was developed by adding a mixture of 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium (Sigma-Fast; Sigma-Aldrich). The reaction was stopped by the addition of tap water.

ELISA

Flat-bottom 96-well plates (Corning Glass, Corning, NY) were coated overnight with mammalian HSP60 or mycobacterial HSP65 (10 μg/ml) in carbonate buffer (pH 9.6) at 4°C. After extensive washing with 0.05% PBS-Tween 20 and incubated with rat sera diluted 1/100 in 1% BSA for 60 min at RT. HSP peptides were attached to plates pretreated with glutaraldehyde according to Kasprzyk et al. (28). Briefly, plates were treated with 100 μl/well of 5% (w/v) glutaraldehyde in PBS for 1 h at RT. Plates were washed thoroughly with PBS and peptides (1 μg/100 μl) were added to each well and incubated overnight at 4°C. Plates were shaken dry and blocked with 1% BSA. Plates coated with either whole HSP or peptides were washed again and incubated with rat sera diluted 1/100 in 0.01% PBS-Tween 20 for 90 min at RT. After washing the plates were incubated with goat anti-rat IgG conjugated to alkaline phosphatase for 60 min at RT. The presence of Abs was detected by addition of the substrate p-phenylphosphate (p-NPP; Chemicon, Temecula, CA) to the plates. OD was measured photometrically at 405 nm by an ELISA reader (Microwell System; Organon Teknika, Turnhout, Belgium).

Competitive inhibition was performed by incubation of the Abs with various concentrations (1, 10, and 100 μg/ml) of the inhibitors for 1 h and then tested for the residual binding by ELISA.

Modulation of AA by mycobacterial and mammalian HSP peptides

Mycobacterial and mammalian HSP-derived peptides were tested for their ability to modulate the incidence and severity of AA in Lewis rats. Rats were vaccinated i.p. three times at weekly intervals with 100 μg of each peptide in PBS before induction of AA. Control rats were treated with PBS. Rats were bled for testing Ab presence before injection of MT and 30 days later.

Purification of polyclonal anti-peptide 6 Abs

Naive Lewis rats were vaccinated i.p. three times with 100 μg peptide 6 in PBS at weekly intervals. Rats were bled and their sera (diluted 1/100) were tested for Abs to peptide 6 and to HSP65 by ELISA (as described in Materials and Methods). Polyclonal rat anti-peptide 6 Abs were purified on a 5-ml column of Hitrap NHS-activated Sepharose (Amersham Biosciences, Uppsala, Sweden) to which we coupled peptide 6 covalently, according to the manufacturer’s instructions. Rat serum (1/5 in PBS) was applied on the column and the flow-through was collected. Bound Abs were eluted with 0.1 M glycine-HCl (pH 2.5). Fractions of 1 ml were collected and immediately neutralized with 30 μl 2 M Tris (pH 12) to avoid denaturation. Protein concentration was measured by absorbance at 280 nm, and the protein-rich fractions were dialyzed against PBS and concentrated to 1 mg/ml.

Modulation of AA by anti-peptide 6 polyclonal Abs

Lewis rats were immunized with CFA to induce AA and treated with 10 μg polyclonal anti-peptide 6 Igs, naive Lewis rats polyclonal Igs, or an unrelated rat mAb (R83D). Control rats were treated with PBS. The Abs were injected i.v. on the day of disease induction and i.p. the day after.

Cytokine secretion by murine and human PBMC in vitro

PBMC from naive Lewis rats and human volunteers were collected with heparin, layered on top of a Ficol-Paque solution (Amersham Biosciences), and centrifuged for 25 min at 1800 rpm. PBMC were collected and washed with PBS. A total of 2 × 10⁶ rat or human cells were incubated in 1 ml DMEM or RPMI consecutively (Beit-Haemek, Beit-Haemek, Israel) in a 24-well tissue culture dish (Nunc, Roskilde, Denmark) with 10 ng/ml LPS (Sigma-Aldrich), 10 μg/ml polyclonal anti-peptide 6 Ab, or 10 μg/ml naive Lewis rat Igs, or without Ag (control), at 37°C and 5% CO₂.

After 24 h the supernatants were collected and kept frozen. Rat and human TNF-α and IL-10 were measured by ELISA (Quantikine M; R&D Systems, Minneapolis, MN).

Structure analysis

RasMoL v. 2.6 program and the known three-dimensional (3D) structure of the GroEL molecule (Escherichia coli HSP60; Brookhaven Protein Data Bank no. 1AON) (29) were used to analyze the position of the relevant epitopes. As the crystal structure of MT HSP65 has not been solved yet, we used a 3D model for the tertiary structure of MT HSP65 based on the crystal structure of the E. coli GroEL (Brookhaven Protein Data Bank no. 1GRL) (30) as template. This model was built by programs for comparative protein modeling (31–33).

Results

The binding of rat Igs to the whole MT HSP65 and its derived peptides

We have previously shown that Igs from naive AA-resistant rats (i.e., BN or Fisher) and from Lewis rats that recovered from AA (post-AA Lewis rats) are able to prevent the induction of AA in naive Lewis rats and that this is due to a subpopulation of anti-HSP65 Abs. To further analyze the fine specificity of this reaction we tested the binding of Igs from these rats with the whole molecule of the mycobacterial HSP65, as well as with a panel of overlapping peptides covering the whole molecule.

We found that Igs from arthritis-resistant rats (i.e., 6- to 8-wk-old BN rats, 9-mo-old Lewis rats, and post-AA Lewis rats) reacted strongly with the whole MT HSP65. Igs derived from arthritis-susceptible naive young (up to 4 mo) Lewis rats did not bind to this molecule in the native or heat-denatured form (Table I).

To define the epitopes recognized by the antibacterial HSP65 Abs we tested the interaction of Igs from these rats with a panel of 16-mer overlapping peptides of the MT HSP65 by dot blot and ELISA tests.

Only 10 of the 90 peptides tested were recognized by the Igs tested (Table I). Igs from 6-wk-old naive Lewis rats reacted with two peptides (40 and 63). Four-month-old naive Lewis rats acquire Abs against two additional peptides (36 and 45), and 9-mo-old naive Lewis rats acquire, in addition, Abs to peptides 6, 7, and 31, as well as to the whole HSP65 molecule. The Ab profile of the old Lewis rats is similar to that found in young naive BN rats, which react in addition with peptide 59. Lewis rats immunized with CFA (post-AA) reacted in addition with peptides 21 and 84. Although Igs derived from naive young Lewis rats interact with some of the peptides of this molecule, they do not recognize the whole HSP65 molecule, and they do not have any effect on susceptibility to AA.

As recognition of the whole HSP65 was acquired together with binding to peptides 6, 7, and 31, we have tested the ability of peptides 6 and 31 (at 1, 10, and 100 μg/ml) to inhibit the binding...
inhibition ELISA. We found that 100 of Igs from CFA-immunized Lewis rats to HSP65 by a competitive binding to HSP65 was tested by ELISA on HSP65-coated plates as detailed in Materials and Methods.

Table I. Binding of rat Igs to HSP65 peptides

<table>
<thead>
<tr>
<th>HSP Peptide</th>
<th>Rat Strain</th>
<th>Disease Susceptibility</th>
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<tbody>
<tr>
<td></td>
<td>Naive Lew-6w</td>
<td>8/10</td>
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<tr>
<td></td>
<td>Naive Lew-4m</td>
<td>3/3</td>
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<tr>
<td></td>
<td>Naive Lew-9m</td>
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<tr>
<td></td>
<td>Naive BN-6w</td>
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</tr>
<tr>
<td></td>
<td>Lew post-AA</td>
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</tbody>
</table>

* Binding was tested by ELISA on peptide/protein-coated plates. –, OD < 0.15; +, OD = 0.16–0.45; ++, OD = 0.46–0.75; +++, OD > 0.75.

The binding of rat Igs to mammalian HSP60 and its derived peptides

It has been previously shown that the mycobacterial HSP65 can trigger self-HSP-reactive T cells with disease-suppressive regulatory potential (24). To analyze the anti-mammalian self-HSP60 Ab repertoire of these rats we tested Igs derived from naive and post-AA Lewis rats as well as from naive BN rats for their reactivity with the whole mammalian HSP60 by ELISA. We found that naive young Lewis rats do not have anti-mammalian HSP60 Abs, whereas 9-mo-old Lewis rats, young BN rats, and post-AA Lewis rats had significant binding to the self-HSP molecule (Table II).

The Igs were then tested for their binding specificity to 38 synthetic 20-mer peptides of the mammalian HSP60. We found that Igs derived from BN, old naive Lewis rats, and post-AA Lewis rats, but not from young naive Lewis rats, reacted with only two mammalian peptides: peptide M5 (aa 61–80) and peptide M30 (aa 436–455) (Table II).

Vaccination of Lewis rats with HSP-derived peptides

To test whether active vaccination with HSP peptides recognized by protective Igs can prevent AA induction, we vaccinated Lewis rats with the MT HSP65-derived peptides 6, 7, 21, 31, 36, 45, and 84 and with the mammalian HSP60-derived peptide M5 recognized by Abs from resistant Lewis rats. Control vaccination was performed with the following nonreactive mycobacterial HSP65 peptides: peptides 26 (aa 151–166), 28 (aa 163–178), and 70 (aa 415–430).

We found that vaccination of rats with the bacterial peptides 6 and 7 and the mammalian peptide M5 resulted in a significant suppression of disease severity (Fig. 2). Vaccination with these protective peptides also resulted in the production of Abs against peptide 6 as well as against the whole mycobacterial HSP65 molecule (data not shown). Neither protection nor anti-HSP response was observed after vaccination with mycobacterial peptide 31 as well as with the rest of the peptides.

The effect of treatment with rat anti-peptide 6 Abs on AA severity

As active vaccination with peptide 6 induced anti-peptide 6 Abs and suppressed the severity of AA we tested the effect of passive treatment with these polyclonal Abs on AA. Lewis rats were immunized with CFA to induce AA and were treated concomitantly i.v. with 10 μg of Lewis rat polyclonal anti-peptide 6 Abs, naive Lewis rat polyclonal Igs, or an unrelated rat mAb (R83D). The same Ab treatment was given i.p. the following day. Control rats received PBS.

Table II. Binding of rat Igs to mammalian HSP60 peptides

<table>
<thead>
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<th>HSP Peptide</th>
<th>Rat Strain</th>
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</thead>
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<td></td>
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<tr>
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<td>Naive Lew-9m</td>
<td>0/7</td>
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<td></td>
<td>Naive BN-6w</td>
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<td></td>
<td>Lew post-AA</td>
<td>0/10</td>
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</tbody>
</table>

* Binding was tested by ELISA on peptide/protein-coated plates. –, OD < 0.15; +, OD = 0.16–0.45; ++, OD = 0.46–0.75; +++, OD > 0.75.

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We found that treatment with anti-peptide 6 Abs reduced arthritis severity by 44% on day 22 and by 64% on day 29 (Fig. 3). Treatment with Igs from naive Lewis rats also reduced the severity of the disease. However, this effect was not significant statistically. The rat mAb had no effect on the severity of AA.

**The effect of anti-peptide 6 Abs on cytokine secretion by murine PBMC**

To test whether the protective effect of anti-peptide 6 Abs is due to their effect on inflammatory cytokines we analyzed the effect of these Abs on cytokine secretion in vitro. PBMC from naive Lewis rats and human volunteers were incubated for 24 h at 37°C with LPS (10 ng/ml), naive Lewis rat polyclonal Igs (10 μg/ml), or polyclonal anti-peptide 6 Abs (10 μg/ml). Cells in medium without Ag served as control. Supernatants were collected to measure the secretion of IL-10 and TNF-α.

As can be seen in Table III, the anti-peptide 6 Abs induced at least a 6-fold higher secretion of IL-10 and a 3.7-fold higher secretion of TNF-α by rat PBMC compared with the control. The increase in secretion of human IL-10 and TNF-α was 50.3 and 7.4, respectively.

The effect was specific to the anti-peptide 6 Abs, as Igs from naive Lewis rats did not show a similar effect (data not shown).

**Analysis of the peptide location on the 3D structure of the HSP65**

Fig. 4 shows the location of bacterial peptides 6 and 31 on a model of the mycobacterial HSP65 based on the structure of the *E. coli* GroEL monomer with a space filling and secondary structure representations (29). As can be seen in Fig. 4, the linear peptides 6 and 31 are completely exposed on the outer surface of the HSP molecule.

**Discussion**

In the present study we have shown that MT HSP65, the Ag that contains the T cell epitopes that induce AA, also contains B cell epitopes that can vaccinate against the disease. The identification of these protective epitopes resulted from the analysis of the anti-MT HSP65 Ab repertoire of arthritis-resistant vs arthritis-susceptible rats in the AA model.

We have found that Abs from arthritis-resistant rats bind to the whole MT HSP65 as well as to several HSP peptides, which are not recognized by Abs from arthritis-susceptible rats.

Vaccination of the susceptible rats with some of these peptides resulted in the production of anti-HSP65 Abs and induced resistance to arthritis. The Abs were found to induce IL-10 secretion in vitro and to suppress arthritis upon in vivo inoculation to naive susceptible rats.

Structural analysis of these protective HSP epitopes revealed that they are potential B cell epitopes, mainly located on the outer surface of the HSP molecule.

It has been previously reported that the T cell response to bacterial HSP shows determinant spreading (22). In the present data we show that there is a clear B cell determinant spreading as well, and this spreading can occur also naturally, namely without intentional vaccination. The B cell epitopes, as we will discuss later, are different from the T cell epitopes.

Abs derived from young naive Lewis rats bound to two bacterial peptides, whereas Abs from 9-mo-old Lewis rats reacted with five additional peptides as well as with the whole molecule. Immunization with CFA resulted in recognition of three additional epitopes. The Ab repertoire of the naive young BN rats was similar to that of naive old Lewis rats.

Although we refer to all the anti-HSP peptide Abs found in naive old Lewis rats and in naive young BN rats as natural Abs, it is possible that they are indeed a response to the exposure of these rats to environmental microorganisms (as “natural” Abs may always be) (34), and that the epitope spreading in response to these pathogens occurs in the BN rat earlier and reaches a higher titer than in the Lewis rat. Lewis rats have to be immunized with CFA to mimic the natural response of the BN rats. The similarity of the Ab repertoire of the naive BN rats to that of the immunized Lewis rats supports this possibility.
The T cell response of the Lewis rat to the bacterial HSP65 has been thoroughly studied. It has been shown that in the early postimmunization stages the Lewis T cells respond to several determinants found in the N terminus as well as in the carboxyl terminus of the molecule, whereas later a shift to recognition of carboxyl-terminal epitopes develops (22). The B cell epitopes, in contrast, were found all along the molecule without any predilection for either the carboxyl or the N terminus of the molecule. This is not surprising, as in the tertiary structure of the molecule the carboxyl- and the N-terminal sites are located in the same domains.

A comparison between the published dominant T cell epitopes upon immunization with MT (22) and the B cell epitopes described here in naive Lewis rats did not reveal common epitope recognition. On the contrary, the lack of natural Abs to certain peptide epitopes like 6, 7, or 31 in the naive Lewis rat is associated with an early T cell response to these epitopes upon immunization, whereas the presence of Abs to peptides like 40 and 63 is associated with lack of an early T cell response. Based on these correlations we suggest that the presence of natural Abs to certain epitopes may actually inhibit T cell response to them, whereas the lack of Abs enables the T cells to respond to these epitopes. For example, AA-susceptible Lewis rats lack natural Abs to the bacterial peptide 31 and can therefore develop a T cell response to this peptide; these pathogenic T cells may induce arthritis.

The nature of the B cell epitopes and the correlation between recognition of certain epitopes and the whole molecule can be better understood from the primary and tertiary structure analysis of the molecule. Based on the primary structure of MT HSP65, we looked for potential B cell epitopes composed of nine amino acids or more that include neither proline (P), which can cause secondary structure molecular bending, nor any short-chain residue (gly-}

### Table III. The effect of anti-peptide 6 Abs on cytokine secretion by murine and human PBMC

<table>
<thead>
<tr>
<th>PBMC</th>
<th>Cytokine</th>
<th>Cytokine Secretion (pg/ml)</th>
<th>Cytokine Secretion Index</th>
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<td></td>
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<tr>
<td></td>
<td>TNF-α</td>
<td>378</td>
<td>2410</td>
</tr>
</tbody>
</table>

* PBMC from naive Lewis rats and healthy human volunteers were incubated (for 24 h at 37°C, 5% CO₂) in DMEM with LPS (10 ng/ml), naive Lewis rat lgs (10 μg/ml), or Lewis rat polyclonal anti-peptide 6 lgs (10 μg/ml). Cells in medium without Ab served as control.

* Cytokine secretion to the medium was measured by ELISA.

* The ratio of IL-10 and TNF-α secretion upon incubation with anti-peptide 6 to control medium. Index = (IL-10 with anti-peptide 6/TNF-α with anti-peptide 6/TNF-α with medium).

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A comparison between the published dominant T cell epitopes upon immunization with MT (22) and the B cell epitopes described here in naive Lewis rats did not reveal common epitope recognition. On the contrary, the lack of natural Abs to certain peptide epitopes like 6, 7, or 31 in the naive Lewis rat is associated with an early T cell response to these epitopes upon immunization, whereas the presence of Abs to peptides like 40 and 63 is associated with lack of an early T cell response. Based on these correlations we suggest that the presence of natural Abs to certain epitopes may actually inhibit T cell response to them, whereas the lack of Abs enables the T cells to respond to these epitopes. For example, AA-susceptible Lewis rats lack natural Abs to the bacterial peptide 31 and can therefore develop a T cell response to this peptide; these pathogenic T cells may induce arthritis.

The nature of the B cell epitopes and the correlation between recognition of certain epitopes and the whole molecule can be better understood from the primary and tertiary structure analysis of the molecule. Based on the primary structure of MT HSP65, we looked for potential B cell epitopes composed of nine amino acids or more that include neither proline (P), which can cause secondary structure molecular bending, nor any short-chain residue (gly-}
murine Abs are relatively conserved (data not shown). There is also ~50% amino acid identity between the mycobacterial HSP65 and the mammalian HSP60 (36). Despite this homology, most of the bacterial peptides that were found to be recognized by the anti-MT HSP65 Abs did not share high homology with the mammalian HSP. This may be due to the tolerance to self that protects the rats from developing an autoimmune autoantibody response to their own HSP60.

Analysis of the anti-self (rat) HSP Ab repertoire indeed showed that there is a limited number of self-HSP molecule epitopes recognized by the rat IgGs. Naive young Lewis rats did not respond to any self-HSP peptide or to the whole molecule. BN and post-AA young Lewis rats that reacted with 8 bacterial HSP epitopes or to the whole molecule. BN and post-AA young Lewis rats did not respond to any self-HSP or to the whole molecule. BN and post-AA young Lewis rats did not respond to any self-HSP molecule, whereas 9-mo-old Lewis rats developed these Abs to the synthetic peptides that were found to be recognized by the anti-MT HSP65 Abs. The response of old rats also became resistant to induction of arthritis.

As we have shown in this work, there was a clear correlation between disease resistance and the presence of anti-HSP Abs. Young naive Lewis rats did not have detectable Abs against the HSP molecule, whereas 9-mo-old Lewis rats developed these Abs in a significant titer. Parallel to the development of the anti-HSP response the old rats also became resistant to induction of arthritis.

Young Lewis rats acquired both the Abs and disease resistance after immunization with CFA or with certain HSP epitopes, and the naturally resistant BN rats had anti-HSP Abs spontaneously, without the need for immunization.

The mechanism of disease resistance induced by the natural as well as the induced anti-HSP Abs has not yet been clarified. It is possible that the Abs against the MT HSP inhibit the early steps of induction of pathogenic T cells to the peptide by intervening in the Ag processing or the T cell recognition of the pathogenic epitopes. Alternatively, they may prevent the effector steps of the pathogenic response by binding to the self-HSP cross-reacting target Ag.

Expression of the mammalian (or self) HSP is up-regulated in inflamed synovia of rats with AA (37), and cross-reactive immune response has been found between the mycobacterial HSP65 and endogenous self-HSP60 at the T cell level (13, 38). As the anti-self-HSP Abs were found only in the resistant rats, it is possible that Abs that cross-react with the self-HSP may hide it from the pathogenic T cells and thus act as protective Abs. However, the preliminary results showing that the vaccination suppresses murine collagen arthritis as well suggests that the mechanism is not directly related to the induction of the disease by the HSP Ag.

Another possibility is that Abs against the HSP molecule suppress inflammation by inhibiting the proinflammatory effect of the HSP on the innate immune system. Mycobacterial HSP65 has been shown to induce release of proinflammatory cytokines from human monocytic cells (39), and the mammalian HSP60 has been shown to synergize with IFN-γ and to promote proinflammatory cytokines like IL-12 and IL-15 (40). Analysis of the effect of the protective Abs on cytokine secretion by murine and human mononuclear cells showed that the Abs induce secretion of both IL-10 and TNF-α, and that the increase in IL-10 secretion by murine and human cells was 1.6- to 6.8-fold greater than that of TNF-α. The increase in IL-10 secretion in the inflammatory site can skew the local cytokine profile from an inflammatory to an

### Table IV. Potential B cell epitopes in MT HSP65

<table>
<thead>
<tr>
<th>Name</th>
<th>Potential Epitope</th>
<th>Sequence of the Peptide</th>
<th>Experimental Peptide Matching</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>35–43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>G-RNVVLEKKW-G</td>
<td>6 and 7</td>
</tr>
<tr>
<td>B</td>
<td>123–132&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A-VEKVTETLLE-G</td>
<td>21</td>
</tr>
<tr>
<td>C</td>
<td>135–143&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A-KEVETKEQI-A</td>
<td>21</td>
</tr>
<tr>
<td>D</td>
<td>319–332&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A-RRVVVTKETVG-E-G</td>
<td>None</td>
</tr>
<tr>
<td>E</td>
<td>357–367</td>
<td>S-DYDRELGGERL-A</td>
<td>59</td>
</tr>
<tr>
<td>F</td>
<td>383–396&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A-TEVELLEKPHRLED-A</td>
<td>63</td>
</tr>
<tr>
<td>G</td>
<td>183–195&lt;sup&gt;a&lt;/sup&gt;</td>
<td>G-LQELTEGMRFDK-G</td>
<td>31</td>
</tr>
<tr>
<td>H</td>
<td>250–270&lt;sup&gt;a&lt;/sup&gt;</td>
<td>S-TLVNKRIGTFK-S</td>
<td>45</td>
</tr>
</tbody>
</table>

<sup>a</sup> Underlined residues fit the epitopes that were identified as binding anti-MT Abs.

<sup>b</sup> Peptides found to bind to Abs from CFA-immunized Lewis rats.

<sup>c</sup> Epitopes with a minimal length of nine residues, which are located between molecular bends (proline) and/or molecular spacers (glycine, serine, alanine).

<sup>d</sup> Epitopes that contain at most one molecular spacer (glycine) with a minimal length of 12 residues.

#### Table V. Amino acid comparison of MT HSP65 peptides and the rat homolog HSP60 peptides<sup>a</sup>

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Residues in MT HSP65 (aa)</th>
<th>Residues in Mammalian HSP60 (aa)</th>
<th>HSP</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>31–46</td>
<td>56–71</td>
<td>HSP65 (MT)</td>
<td>GPKGR NVVLEKKWGA P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HSP60 (rat)</td>
<td>GPKGR TVIEQWSWS P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Consensus</td>
<td>GPKGR -V-E-WG- P</td>
</tr>
<tr>
<td>7</td>
<td>37–52</td>
<td>62–77</td>
<td>HSP65 (MT)</td>
<td>VVLEKKWGA PTITNG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HSP60 (rat)</td>
<td>VIEQWSWS PKVTKDG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Consensus</td>
<td>V-E-WG- P-T-DG</td>
</tr>
</tbody>
</table>

<sup>a</sup> Computer programs Pileup and Pretty (Genetic Computer Group, Wisconsin package, version 9.0) were used to compare the MT HSP and the mammalian HSP sequences. Sequence ID: HSP65, P06806; HSP60, P19227.
anti-inflammatory response and thus explain the mechanism of protection against inflammation by these Abs. The mechanism of induction of the various cytokines by anti-peptide 6 Abs is now under study and the preliminary results show that the effect of the Abs is in the level of mRNA of these cytokines.

It is interesting to notice that one of the two self-HSP60 epitopes is the self-peptide 5, which is the rat homologous epitope of the bacterial protective peptide 6. Moreover, vaccination with the bacterial peptides 6 and 7 and with the mammalian peptide 5 led to the production of antibacterial peptide 6 and antibacterial HSP65 Abs as well as protection against disease induction. Looking at the primary structure of these three peptides leads to the conclusion that they express a common motif (VEWGP), which might be the protective motif of these peptides (Table V).

In summary, we suggest that the humoral immune response to the bacterial HSP is aimed at a limited number of potential B cell epitopes. These linear epitopes are found in nonconserved parts of the molecule. Recognition of B cell epitopes that are exposed on the surface of the molecule leads to binding to the whole molecule and is associated with resistance to induction of arthritis. The mechanism of resistance may be due to a local skewing of the cytokine profile toward an anti-inflammatory response. Resistance to AA occurs naturally in some strains of rats, whereas in others it can be acquired with age or upon immunization with HSP. Vaccination with some of the protecting epitopes can lead both to disease resistance and to the serological profile that is present in the resistant strains.

References