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Activation of T Cells Recognizing an Epitope of Heat-Shock Protein 70 Can Protect Against Rat Adjuvant Arthritis

Shigehisa Tanaka,*† Yuki Kimura,∗ Akio Mitani,‡ Genta Yamamoto,*† Hitoshi Nishimura,* Ralf Spallek,‡ Mahavir Singh,‡ Toshihide Noguchi,† and Yasunobu Yoshikai2∗

We have previously reported that CD4+ T cells recognizing a peptide comprising residues 234–252 of the heat shock protein (HSP)70 of Mycobacterium tuberculosis (M.tb) in the context of RT1.B MHC class II molecule emerged in the peritoneal cavity during the course of Listeria monocytogenes infection in rats and suppressed the inflammatory responses against listerial infection via IL-10 production. We report in this work that pretreatment with peptide 234–252 of HSP70 derived from M.tb suppressed the development of adjuvant arthritis (AA) in Lewis rats induced using heat-killed M.tb. T cells from rats pretreated with peptide 234–252 produced a significant amount of IL-10 in response to the epitope. T cells from rats pretreated with the peptide and immunized with M.tb produced the larger amount of IL-10 in response to the peptide, but only a marginal level of IFN-γ in response to purified protein derivative of M.tb. Administration of anti-IL-10 Ab partly inhibited the suppressive effect of pretreatment with peptide 234–252 on the development of AA. Furthermore, transfer of a T cell line specific for the epitope at the time of AA induction markedly suppressed AA. These findings suggested that T cells recognizing peptide 234–252 may play a regulatory role in inflammation during AA via the production of suppressive cytokines including IL-10. The Journal of Immunology, 1999, 163: 5560–5565.

HSP70, heat-shock proteins (HSPs) are widely distributed in nature and highly conserved among prokaryotes and eukaryotes (reviewed in Ref. 1). They are known to be involved in immune responses to many bacterial and parasitic pathogens (reviewed in Refs. 2–4), and in the pathogenesis of autoimmune diseases (reviewed in Ref. 5). Among the various HSPs, mycobacterial 65-kDa HSP (HSP65) has been identified as an immune dominant target (2, 3). TCRβ+ Th1-type T cells recognizing an epitope of HSP65 have been reported to cause some types of autoimmune diseases in rats and mice (3–6). Adjuvant arthritis (AA) is widely used as an experimental model for rheumatoid arthritis and can be induced in Lewis rats by immunization with heat-killed Mycobacterium tuberculosis (M.tb) suspended in IFA (7, 8). The antigenic epitope recognized by an arthritis-inducing T cell clone, which can induce AA, was identified as residues 180–188 of the mycobacterial HSP65 (3, 9, 10). On the other hand, TCRβ+ T cells recognizing different epitopes of HSP65 were suggested to modulate autoimmune diseases in rats. Pretreatment with 256–270 epitope or 376–408 epitope of mycobacterial HSP65 was reported to suppress AA (11, 12). Furthermore, transfer of T cell lines specific for 256–265 epitope can prevent against AA induction (11). Beech et al. (13) reported that pretreatment with mycobacterial HSP65 protected against pristane-induced arthritis via induction of a Th2-dominant response characterized by IL-4 production. Oral administration of recombinant HSP65 was reported to suppress AA, possibly by induction of Th3 cells capable of producing TGF-β (14). Thus, similarly to T cells recognizing conventional Ags, HSP65-reactive T cells may be classified into several subsets in terms of function, and the nature of the HSP65 epitope may be an important factor to determine whether proliferating CD4+ T cells will differentiate into each T cell subset. In addition to HSP65, pretreatment with mycobacterial recombinant HSP70 was also reported to suppress AA in rats. Kingston et al. (15) demonstrated that s.c. injection of recombinant HSP70 produced inhibitory effects on induction of arthritis by M.tb. However, the mechanisms underlying the suppressive effect and nature of Ag epitopes of HSP70 remain to be determined.

We have previously reported that HSP70-reactive CD4+ T cells appeared in the peritoneal cavity during the course of Listeria monocytogenes infection in rats. The HSP70-reactive CD4+ T cells recognized a peptide comprising residues 234–252 of HSP70 of M.tb in the context of RT1.B MHC class II molecules. The HSP70-reactive CD4+ T cells produced significant amounts of TGF-β1 and IL-10 and suppressed host defense against listerial infection (16). We hypothesized that T cells recognizing the specific epitope of HSP70 may be involved in termination of Th1 cell-mediated excessive inflammation after the battle against L. monocytogenes has been won.

In the present study, we investigated the effects of pretreatment with peptide 234–252 of HSP70 derived from M.tb on the development of AA, a Th1 cell-mediated autoimmune disease. Pretreatment with peptide 234–252 of HSP70 protected against...
AA induction, and the T cells from rats pretreated with peptide 234–252 produced significant amounts of IL-10 in response to the relevant epitope. Administration of a T cell line specific for the epitope at the time of AA induction suppressed AA. These findings may facilitate an insight into vaccine development for human arthritic diseases.

Materials and Methods

Animals

Male Lewis rats were obtained from Charles River (Tokyo, Japan). The rats were 5–6 wk old at the start of each experiment.

Ags and adjuvant

*M.tb* strain H37Ra was obtained from Difco (Detroit, MI). Recombinant HSP70 derived from *M.tb* was kindly provided by GBF-German Research Center for Biotechnology (Braunschweig, Germany). IFA was used as an adjuvant (Difco). PPD (purified protein derivative of *M.tb*) was purchased from the Japanese BCG Association (Tokyo, Japan). Two kinds of synthetic peptides of the HSP70 of *M.tb* were synthesized using a method of simultaneous multiple peptide synthesis (Kurabo, Osaka, Japan): peptide 84–103 and 234–252 representing the amino acid sequence 84–103 and 234–252, respectively, which are at comparable levels to the recombinant HSP70 derived from *M.tb*. These peptides encompass the residues, which can bind to mouse I-A molecules, as assessed by T Sites software (MedImmune, Gaithersburg, MD) (16).

Pretreatment with synthetic peptide and induction of adjuvant arthritis

Synthetic peptides were dissolved in PBS and mixed with an equal volume of IFA. The heat-killed *M.tb* were finely ground in a mortar and pestle and suspended in IFA at a concentration of 5 mg/ml. Rats were pretreated by inoculation of 100 μl of emulsions containing 1 mg/ml synthetic peptide into the base of the tail, and were immunized 7 days later by inoculation of 100 μl of *M.tb*/IFA into the base of the tail. Severity of arthritis was assessed by scoring each paw from 0 to 4 based on degree of swelling, erythema, and deformity of the joints. Thus, the maximum possible arthritis score was 16 (17).

Preparation of cells and cell culture

Lymph node cells (LNC) were derived as pooled inguinal and popliteal LN from rats pretreated with the peptide/IFA 7 days previously or on day 35 after *M.tb*/IFA immunization. The cells were passed over nylon fiber column and purified to >80% CD3+ cells, as assessed by FACS analysis. Triplicate cultures of the enriched T cells (2 × 10^6 cells/ml) were cultured with the various number of mitomycin (MMC)-treated normal syngeneic spleen cells as APC in the presence or absence of Ag in 96-well flat-bottom plates (Falcon, Becton Dickinson, Lincoln Park, NJ) for 72 h at 37°C in a humidified atmosphere of 5% CO2. RPMI 1640 complete culture medium contained 10% FBS (CRL, Victoria, Australia), 100 U/ml penicillin, 100 mg/ml streptomycin, and 10 mM HEPES. An optimal dose of each Ag for the maximal response was added to the culture as follows: 50 μg/ml each peptide and 25 μg/ml PPD. For the last 18 h, triplicate cultures of cells were pulse labeled with [3H]thymidine (1 μCi/well; Amersham Life Science, Amersham, U.K.) before harvesting on glass fiber filters. Incorporation of [3H]thymidine was determined with MicroBeta (Wallac, Turku, Finland).

In vivo administration of anti-IL-10 Ab

Rats were injected i.p. with 250 μg of anti-IL-10 Ab (Biosource, Camarillo, CA) or isotype control Ab (Zymed, South San Francisco, CA) three times every 3 days from the beginning of immunization with *M.tb*/IFA. Five rats per each group were used for the experiment.

T cell lines

RT1.B-restricted T cell line specific for peptide 234–252 of mycobacterial HSP70 was isolated from rats immunized with HSP70. T cells were cultured at 5 × 10^6 cells/ml in culture medium in the presence of 10 μg/ml peptide. After 3 days, viable cells were harvested using a Ficoll-Isopaque gradient and cultured for an additional 4 days in culture medium plus 5% FCS and 5% Con A-activated spleen supernatant. Seven days after initial stimulation, cells were restimulated with irradiated spleen APC and 10 μg/ml peptide. Cell lines were maintained by restimulation every 7 days. Rats were administered 5 × 10^6 T cells i.v. at the time of AA induction with *M.tb*/IFA. The T cell line is of CD4+ TCRαβ+ CD44^high phenotype and produced IL-10 in response to peptide 234–252 or recombinant HSP70. RT1.B-restricted T cell line specific for heat-killed *Listeria* (HKL) was isolated from rats infected with viable *L. monocytogenes* (16). The HKL-specific T cell line is of CD4+ TCRαβ+ CD44^high phenotype and produced IFN-γ, but no IL-10 in response to HKL.

Cytokine assays

Culture supernatants were harvested after 72-h incubation time. IFN-γ, IL-10, IL-4, and TGF-β1 activities in the culture supernatants were measured by ELISA using a rat IFN-γ, IL-10, IL-4 ELISA kits (Bioscore International, Carmarillo, CA), and TGF-β1 human ELISA system (Amer sham Life Science), respectively.

Flow-cytometric analysis

Flow-cytometric analysis was used to determine the phenotype of T cells. Cells were stained with FITC-conjugated anti-CD8a mAb or FITC-TCRγδ mAb or FITC-CD44, PE-conjugated anti-CD4 or PE-conjugated anti TCRβ mAb, and biotin-conjugated anti-CD3 mAb, followed by streptavidin-R660 (Life Technologies, Gaithersburg, MD). The stained cells were analyzed using a FACS flow cytometer (Becton Dickinson, Oxnard, MA).

Statistical analysis

The statistical significance of arthritis score was determined by the generation of Kaplan-Meier cumulative hazard plots and Log-rank (Mantel-Cox) analysis. Other data were analyzed using an ANOVA model controlling for treatment, experiment, and the potential for an experiment by treatment interaction. p < 0.05 was taken as the level of significance. Analyses were completed using StatView 4.5 software (Abacus Concepts, Berkeley, CA) and Power Macintosh 7200 computers (Apple Computer, Cupertino, CA).

Results

**Effects of pretreatment with epitope 234–252 of HSP70 on AA induction**

To determine whether pretreatment with peptide 234–252 of mycobacterial HSP70 has a protective effect against AA induction, we examined the modulation of AA development by pretreatment with peptide 234–252 in IFA (Fig. 1B). Rats were pretreated with 50 μg of recombinant HSP70, 100 μg of 83–103 or 234–252 peptide 7 days before induction with *M.tb*/IFA. Consistent with previous findings (15), pretreatment with whole recombinant HSP70 protected against AA induction (mean maximum score = 3.3) (Fig. 1A). Notably, pretreatment with peptide 234–252 significantly suppressed the development of AA (mean maximum score = 1.6) as compared with the PBS-pretreated group (mean maximum score = 14). Of the nine rats pretreated with peptide 234–252, four did develop clinical signs of arthritis, which were milder than those seen in control rats (p < 0.01). No significant differences in the onset or score of AA were observed in rats pretreated with the control peptide 84–103 (mean maximum score = 15.3).

**In vitro response of T cells from rats pretreated with peptide 234–252**

To determine whether pretreatment of rats with a synthetic peptide could prime for T cell reactivity to the epitope, we examined the proliferative responses of the enriched LN T cells against peptide 234–252 or PPD. The enriched T cells, which were prepared from rats on day 7 after pretreatment with peptide 234–252/IFA or PBS/IFA, were used as responder cells, and peptide 234–252 or PPD, and MMC-treated normal syngeneic spleen cells were used as Ag and APC, respectively. As shown in Fig. 2A, the enriched T cells from rats pretreated with peptide 234–252 showed a significant proliferative response to peptide 234–252 and, to a lesser degree, to PPD.

IL-10, IL-4, TGF-β1, and IFN-γ levels were measured in culture supernatants of the enriched T cells incubated with peptide 234–252 or PPD by ELISA. The enriched T cells from rats treated with peptide 234–252 produced a large amount of IL-10 (Fig. 2B), but no IFN-γ in response to peptide 234–252 (Fig. 2C).
There were no differences in the production of TGF-β1 between the culture supernatants of T cells from PBS-pretreated rats and from peptide-pretreated rats in response to the peptide. IL-4 was not detected in any culture supernatant (data not shown). The T

FIGURE 1. Suppression of AA in Lewis rats by pretreatment with recombinant HSP70 (A) or peptide 234–252 of mycobacterial HSP70 (B). Rats were inoculated s.c. with 50 μg of recombinant HSP70, 100 μg of synthetic peptides or PBS in IFA into the base of the tail 7 days before immunization with 100 μl M.tb/IFA (0.5 mg heat-killed M.tb) via the base of the tail. Three rats were used in each group in one experiment, and three experiments were conducted independently. **, Significantly different from the value for PBS-pretreated rats (n = 9), **, p < 0.01, *, p < 0.05. NS, not significant. C. Peptides 84–103 and 234–252 represent the amino acid sequences 84–103 and 234–252, which are at comparable levels to the recombinant HSP70 of derived from M.tb, respectively, and encompass the residues that can bind to mouse I-A^d molecules, as assessed by T Sites software (MedImmune). The amino acid sequence of M.tb HSP70 identical to rat HSP70 is indicated by an asterisk.
cells from PBS-pretreated or peptide-pretreated rats produced an appreciable level of IFN-γ in response to PPD. These findings suggested that pretreatment with the synthetic peptide primed a T cell response producing IL-10 specific for the 234–252 of HSP70.

In vitro response of T cells from rats pretreated with peptide 234–252 and immunized with M.tb/IFA

To determine whether T cells induced by the peptide 234–256 are activated after immunization with M.tb/IFA, we examined the proliferative responses and cytokine production of the enriched LN T cells from rats pretreated with the peptide/IFA and immunized with M.tb/IFA against peptide 234–252 or PPD. Rats were pretreated with PBS/IFA or the peptide/IFA, and 7 days later, immunized with M.tb/IFA. The enriched T cells, which were prepared from rats on day 35 after immunization with M.tb/IFA, were used as responder cells. As shown in Fig. 3A, the enriched T cells from rats pretreated with peptide 234–252 showed a significant proliferative response to peptide 234–252, while those from the control group pretreated with PBS showed limited blastogenesis in response to the peptide. On the other hand, T cells from rats pretreated with peptide 234–252 appeared to proliferate less in response to PPD than those from control rats.

As shown in Fig. 3, B and C, the enriched T cells from the control group pretreated with PBS and challenged with M.tb/IFA secreted a large amount of IFN-γ in response to PPD, but no IL-10 was produced in response to peptide 234–252. On the other hand, the enriched T cells from rats pretreated with peptide 234–252/IFA and challenged with M.tb/IFA produced a large amount of IL-10, whereas little, if any, amount of IFN-γ was detectable in the culture supernatants in response to peptide 234–252 or PPD. There were no differences in the production of TGF-β1 among the culture supernatants of these T cells, and IL-4 was not detected in any culture supernatant (data not shown). These findings suggested that the peptide-specific T cells producing IL-10, but few arthritogenic Th1 cells producing IFN-γ, were activated in rats pretreated with the peptide/IFA after immunization with M.tb/IFA.

Effect of in vivo administration of anti-IL-10 Ab on the development of AA in rats pretreated with peptide 234–252

Pretreatment with the epitope 234–256 induced an epitope-specific T cell response producing IL-10 and protected against AA. To determine whether IL-10 is involved in the peptide-induced suppression of AA, we examined the effect of in vivo administration of anti-IL-10 Ab on suppression of AA induced by pretreatment with peptide 234–252. As shown in Fig. 4, administration of anti-IL-10 Ab from the beginning of AA induction partly inhibited, but significantly, the suppression of AA in rats pretreated with the peptide. None of the five rats pretreated with the peptide and given control of IgG developed AA, whereas all of the five rats developed clinical signs of arthritis when anti-IL-10 Ab was administered at the beginning of AA induction. Administration of anti-IL-10 Ab at the later stage after immunization with M.tb/IFA had no effect on the peptide-induced suppression of AA development (data not shown).

A T cell line specific for the 234–252 epitope protects against AA

To obtain direct evidence for the protective role of the epitope-specific T cells in AA development, we used the epitope-specific T cell line generated from HSP70-immunized rats and the HK-specific T cell line generated from Listeria-infected rats. We confirmed that the epitope-specific T cell line produced IL-10, but no IFN-γ in response to the peptide, and that the HKL-specific T cell line produced IFN-γ, but no IL-10 in response to HKL. Each of the two T cell lines was administered
Effect of in vivo administration of anti-IL-10 Ab on the development of AA in rats pretreated with peptide 234–252. Rats were inoculated with 100 μg of synthetic peptide/IFA 7 days before immunization with 100 μg \( M.\text{tb} \)/IFA and then injected i.p. with 250 μg of anti-IL-10 Ab or isotype control Ab three times every 3 days from the beginning of immunization with \( M.\text{tb} \)/IFA. Five rats per each group were used for the experiment. The data are representative of two separate experiments. \(*, p < 0.05\).

Discussion

Pretreatment with HSP70 has been reported to protect against the induction of AA (15), but the mechanism underlying the protective effect remains unknown. In this study, we demonstrated that pretreatment with peptide 234–252 of mycobacterial HSP70 could protect against development of AA. This pretreatment primed for the responsiveness of T cells recognizing peptide 234–252 to produce IL-10. Thus, activation of T cells recognizing an epitope in HSP70 is important for prevention of AA through IL-10 production. These findings have significant implications for vaccine development in human arthritic diseases.

Th1 cells secrete IL-2, IFN-\(\gamma\), and TNF-\(\beta\) for the induction of cell-mediated immunity characterized by macrophage activation and CTL induction (18, 19), and are responsible for the pathogenesis of several types of autoimmune diseases including AA (6). Th2 cells uniquely secrete IL-4 not only to induce Ab production, but also to inhibit activities of macrophages and NK cells by shutting down the cytokine synthesis of Th1 cells (18–22). Recently, a unique subpopulation of Th cells termed Th3 cells was reported to play an important role in immune regulation, especially oral tolerance, through TGF-\(\beta\) production (23–25). Beech et al. (13) reported that pretreatment with mycobacterial HSP65 protected against pristane-induced arthritis in CBA/Ig mice via induction of CD4\(^+\) Th2 cells specific for mycobacterial HSP65. Haque et al. (14) reported that oral administration of recombinant HSP65 suppressed AA possibly by induction of Th3 cells producing TGF-\(\beta\). Therefore, it is possible that Th2 and/or Th3 cells may be involved in protection against AA by pretreatment with HSP70. The results of the present study indicated that CD4\(^+\) T cells stimulated by pretreatment with synthetic peptide 234–252 produced IL-10, but not IL-4, in response to the relevant Ag in Lewis rats. IL-10 has been reported to shut down the cytokine synthesis of Th1 cells such as IFN-\(\gamma\) (26–28). In fact, T cells from rats treated with the synthetic peptide and challenged with \( M.\text{tb} \)/IFA produced a lower amount of IFN-\(\gamma\) in response to PPD as compared with controls pretreated with PBS/IFA. It has been reported that daily i.p. injection of murine recombinant rIL-10 caused mice to develop milder collagen-induced arthritis (29). We reported previously that CD4\(^+\) T cells specific for peptide 234–252 emerged during the natural course of rat listeriosis and suppressed host defense against listerial infection probably through production of IL-10 and TGF-\(\beta\) (16). Protection against infection with intracellular parasites such as \( L.\text{monocytogenes} \) is dependent on Th1 response capable of producing IFN-\(\gamma\) (30). Therefore, T cells specific for peptide 234–252 of mycobacterial HSP70 may play a vital role in the down-regulation of aggressive Th1 responses through IL-10 production.

Several mechanisms might explain the suppressive effect of 234–252 peptide-primed T cells on the development of AA through IL-10 production. The first is that IL-10 produced by the peptide-specific T cells may suppress the functions of arthritogenic Th1 cells at the effector phase through shutting down IFN-\(\gamma\) production. However, it is unlikely because administration of anti-IL-10 Ab at the later stage after immunization with \( M.\text{tb} \)/IFA had no effect on suppression of AA development by pretreatment with peptide/IFA. Furthermore, transfer of the peptide-specific T cells at the later stage after immunization had no effect on the development of AA (data not shown). The second is that IL-10 produced by the peptide-specific T cells inhibits the generation of arthritogenic Th1 cells at the induction stage after immunization with \( M.\text{tb} \)/IFA, CD4\(^+\) T cells initially stimulated in the presence of IL-12 and IFN-\(\gamma\) tend to develop into CD4\(^+\) Th1 cells (31). IL-10 is known to exhibit a potent suppressive effect on IL-12 production by dendritic cells and macrophages, besides the suppressive effect on IFN-\(\gamma\) production by NK and T cells (32, 33). Therefore, a high level of IL-10 production at the initial phase of AA induction may inhibit the generation of Th1 cells responsible for AA via suppression of IL-12 and IFN-\(\gamma\) production. Administration of anti-IL-10 Ab from the beginning of immunization inhibited the suppression of AA induced by pretreatment with the HSP70 peptide and transfer of the peptide-specific T cell line at the same time AA induction inhibited the AA development, supporting this mechanism. However, our
results with anti-IL-10 Ab do not necessarily imply that protective mechanisms are mediated by IL-10 produced by the epitope-specific T cells because anti-IL-10 Ab administration only partly inhibited the peptide-induced suppression of AA. It is possible that the amounts of the Ab may not be sufficient to neutralize endogenous IL-10. Alternatively, other suppressive cytokines, including TGF-β2 and IL-13, may be involved in the peptide-induced suppression. Additional experiments are needed to clarify these possibilities.

Anderton et al. (11) proposed that cross-reactivity between bacterial and self HSP65 may be important to maintain a protective self-reactive T cell population, which suppresses excessive Th1 responses, induced by exogenous Ags. Peptide 234–252 of HSP70 used in this study differed from the equivalent rat HSP70 by eight amino acid substitutions and showed 58% amino acid identity. In our preliminary experiments, a T cell line specific for peptide 234–252 responded to the conserved peptides corresponding to the equivalent region of the rat HSP70 homologue, supporting the hypothesis described above. However, additional experiments are needed to clarify this proposal.

There are striking strain differences in susceptibility to AA and listerial infection. Lewis rats are highly susceptible to AA, but resistant to listeriosis, whereas F344 and (F344 × Lewis)F1 rats are resistant to AA induction, but susceptible to listeriosis (34–36). Preliminary experiments revealed that the reactivity of T cells to peptide 234–252 of HSP70 was closely correlated with susceptibility to listeriosis and resistance to AA induction. Activities of macrophages, including cytokine synthesis, were reported to be different between Lewis and F344 rats (37, 38). Thus, it would appear that the different processing activity of APC to generate the fragment epitopes of HSP70 between Lewis and F344 rats may be at least partially responsible for the differences in susceptibility to AA and listeriosis.

In conclusion, we have demonstrated that pretreatment with peptide 234–252 of HSP70 derived from M.tb suppressed the development of AA. T cells from rats pretreated with peptide 234–252 produced IL-10 in response to the relevant epitope and administration of T cells specific for peptide 234–252 significantly protected against AA. These findings suggest that T cells recognizing peptide 234–252 may have a regulatory role in inflammation during AA via the production of suppressive cytokines such as IL-10.

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