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Treatment of Murine Collagen-Induced Arthritis by the Stress Protein BiP Via Interleukin-4–Producing Regulatory T Cells

A Novel Function for an Ancient Protein

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***Objective*. Following the demonstration that the stress protein, BiP, prevented induction of collagen-induced arthritis (CIA) in HLA–DRB*0101$^{+/+}$ (HLA–DR1$^{+/+}$) mice, we investigated the immunotherapeutic ability of BiP to suppress disease during the active phase of CIA in HLA–DR1$^{+/+}$ and DBA/1 mice.**

***Methods*. BiP was administered either subcutaneously or intravenously to DBA/1, HLA–DR1$^{+/+}$, or interleukin-4 (IL-4)–knockout mice at the onset of arthritis. Immune cells were used in adoptive transfer studies or were restimulated in culture with BiP or type II collagen (CII). Proliferation and cytokine release were measured. In addition, serum anti-CII antibodies were measured by enzyme-linked immunosorbent assay. Disease progression was scored using a visual analog scale.**

***Results*. BiP was successful in suppressing established CIA in HLA–DR1$^{+/+}$ and DBA/1 mice. Serum levels of anticollagen IgG antibodies were reduced in BiP-treated mice. T cells from BiP-immunized mice produced Th2 cytokines, in particular, IL-4. Treatment with BiP was also shown to increase the production of CII-specific IL-5, IL-10, and interferon-γ at the termination of the study. Development of severe CIA was prevented by the intravenous transfer of BiP-specific cells at the time of CIA induction in HLA–DR1$^{+/+}$ mice or by transferring BiP-specific cells to DBA/1 mice at the onset of disease. BiP failed to ameliorate the development of CIA in IL-4$^{-/-}$, HLA–DR1$^{+/+}$ mice.**

***Conclusion*. These novel results show that BiP can suppress active CIA by the induction of regulatory cells that act predominantly via IL-4. Thus, BiP is a potential immunotherapeutic agent for the treatment of patients with rheumatoid arthritis.**

Collagen-induced arthritis (CIA) is a well-defined experimental animal model shown to closely resemble rheumatoid arthritis (RA), with which it shares many similar clinical and histopathologic features. CIA develops in susceptible strains of animals after immunization with autologous or homologous type II collagen (CII) in adjuvant (1–3). A strong association between disease susceptibility and specific major histocompatibility complex (MHC) class II molecules in both RA and CIA suggests that CD4+ T cells may be involved in disease development. In support of this notion, administration of blocking monoclonal antibodies to α/β T cell receptor (4,5), CD4 (6), or MHC class II molecules (7) has been proven successful in preventing CIA. Furthermore, CD4$^{-/-}$ mice were shown to be resistant to CIA

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Drs. Corrigall and Bodman-Smith own stock in Immune Regulations. Prof. Panayi has received consulting fees or honoraria (less than $10,000 per year) from Wyeth, Roche, Abbott, and Novartis and owns stock in Immune Regulations.

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induction (8), whereas both HLA–DRB*0101 (HLA–DR1$^{+/+}$)–transgenic and HLA–DRB*0401–transgenic mice are highly susceptible to CIA (9,10).

Previously, we and other investigators have identified the immunoglobulin binding protein, BiP, alternatively known as glucose-regulated protein 78, as a putative autoantigen in RA (11,12). BiP is an endoplasmic reticulum chaperone and stress protein belonging to the Hsp70 family. We previously demonstrated that patients with RA have anti-BiP antibodies in their serum (11). In addition, mice with CIA or pristane-induced arthritis produce anti-BiP antibodies. Furthermore, patients with RA were shown to harbor BiP-specific T cells in synovial fluid, in contrast to that found in other inflamed joints (11). These T cells were observed to have unusual properties, in that they proliferated poorly and produced only low levels of interferon-γ (IFNγ). BiP has no arthritogenic potential, since it could not induce arthritis in the several strains of mice and rats tested. However, intravenously administered BiP prevented induction of CIA in HLA–DR1$^{+/+}$ mice (11). This finding was the first indication that BiP has immunomodulatory properties.

In addition to the disease-modulating role of BiP in CIA, we have described populations of human CD4+ and CD8+ T cells, present in the peripheral blood of normal, healthy individuals, that secrete interleukin-10 (IL-10) in response to BiP stimulation. IL-10–secreting, CD8+ BiP-specific T cells could be cloned. Some of these clones also could produce IL-4 and IL-5 (13). Furthermore, BiP induces the secretion of IL-10 from human monocytes (14). These observations add further support to the notion that BiP has novel immunomodulatory properties.

Considerable data have been accumulated to suggest that a Th1 environment is required for the induction of a proinflammatory anticollagen T cell response and induction of complement-fixing anticollagen antibodies, of the IgG2a isotype, which are essential for the development of CIA (15–17). In contrast, Th2-associated cytokines, such as IL-4 and IL-10, are associated with the remission phase of CIA (18). It can therefore be assumed that by appropriate manipulation of the cytokine environment, disease outcome may be significantly altered. This study was designed to investigate whether BiP could indeed alter the cytokine environment in vivo and, as a consequence, influence the course of CIA as a model of human RA. Our results indicate that intravenously or subcutaneously administered BiP was effective in suppressing active CIA. This protective effect was associated with BiP-specific Th2 cytokine production. Of note, the transfer of BiP-primed cells was able both to prevent the induction of CIA and to ameliorate active CIA, whereas BiP failed to suppress active CIA in IL-4$^{-/-}$, HLA–DR1$^{+/+}$ mice. Thus, we provide both in vitro and in vivo data demonstrating that BiP can induce IL-4–secreting T cells with regulatory functions, suggesting a potential for therapeutic application in patients with RA.

MATERIALS AND METHODS

Animals. HLA–DR1$^{+/+}$–transgenic mice were obtained as breeding pairs from Dr. Dennis Zaller (Merck Laboratories, Rahway, NJ) (19). Both HLA–DR1 transgenes are expressed on the B10.M background and are chimeric molecules, i.e., they contain the first domains of HLA–DRB1*0401 or HLA–DRB1*0101 and the DR α-chain, fused to the second domain which is derived from the murine I-E molecule. This enables the product of these class II transgenes to interact with murine CD4. HLA–DR1$^{+/+}$, IL-4–knockout mice were developed by backcrossing C57BL/6 IL-4$^{-/-}$ mice to HLA–DR1$^{+/+}$ mice for 12 generations prior to intercross. Male DBA/1 mice ages 8–12 weeks were purchased from Harlan Olac (Bicester, UK). Animals were used in accordance with the respective institutional guidelines and national legislation.

Antigens. Recombinant human BiP and β-galactosidase were purified from an *Escherichia coli* expression system as previously described (11). Both proteins were professionally assessed by Associates of Cape Cod (East Falmouth, MA) for lipopolysaccharide contamination, and were found to contain <95 EU/mg. Native CII was purified from bovine cartilage and dissolved at 4 mg/ml in 0.01*M* acetic acid. Bovine serum albumin (BSA) was obtained from Sigma-Aldrich (Poole, UK).

Induction and assessment of arthritis. DBA/1 mice were injected intradermally at the base of the tail with 100 μg of bovine CII emulsified in Freund's complete adjuvant (CFA). A booster injection of 100 μg of CII in Freund's incomplete adjuvant (IFA) was given intradermally 21 days later. Disease was induced at the base of the tail of HLA–DR1$^{+/+}$ mice or IL-4–knockout mice by a single subcutaneous injection with 100 μg of CII in CFA. Clinical signs of arthritis were assessed visually in the wrist and ankle joints 3 times weekly, using a previously described severity scale: 0 = no arthritis; 1 = 1 inflamed digit; 2 = 2 inflamed digits; 3 = more than 2 digits and footpad inflamed; 4 = all digits and footpad inflamed (20). Scoring was conducted under blinded conditions.

Immunization of animals. For short-term cell proliferation and cytokine studies, groups of DBA/1 mice were immunized subcutaneously with either 200 μg of BiP alone or BiP in an emulsion with IFA, at 10 days prior to culling. In addition, either BSA or phosphate buffered saline (PBS) with or without emulsion in IFA was administered as a control.

Treatment with BiP. Various treatment protocols were used in this study. 1) HLA–DR1$^{+/+}$ mice were immunized with varying doses of BiP (ranging from 1 μg to 500 μg) intravenously at the onset of arthritis (either 31 or 28 days after CII injection). 2) DBA/1 mice were immunized with 200 μg of BiP subcutaneously or 10 μg of BiP intravenously at the onset of arthritis (24 days following the first CII injection). 3) IL-4$^{-/-}$,

HLA–DR1$^{+/+}$ mice were immunized with 10 μg BiP intravenously at the onset of arthritis (24 days following CII injection).

Cell-transfer treatment. Two treatment protocols were used in this study. 1) Spleens and lymph nodes from HLA–DR1$^{+/+}$ mice that were treated with 10 μg of BiP or PBS intravenously were collected 1 week after immunization. Cell suspensions (60×10^6 cells per mouse) were then adoptively transferred to naive recipients intravenously on the same day as the CII injection. 2) Spleens and lymph nodes from groups of DBA/1 mice that had been immunized subcutaneously with 200 μg BiP or the control protein, BSA, or intravenously with 10 μg BiP or BSA were collected 12 days after immunization. Cell cultures (2.5×10^6 cells/ml) were set up with 20 μg of the respective protein (BiP or BSA) for 5 days. Cells were then washed and injected intraperitoneally into DBA/1 mice (20×10^6 cells/mouse) that had received the first CII immunization 24 days previously.

Measurement of serum antibody titers. Blood was obtained from HLA–DR1$^{+/+}$ mice at 8 weeks after CII immunization, and sera were analyzed for antibodies reactive with native CII, using a modified enzyme-linked immunosorbent assay (ELISA) that has been previously described (21). Blood samples were obtained from DBA/1 mice at 10 weeks after the first CII immunization, and sera were analyzed for total and isotype antibodies reactive with native CII, using a standard ELISA. Results are expressed as the optical density at 492 nm ($OD_{492\ nm}$).

Isolation and preparation of cells for tissue culture. Spleens and lymph nodes were macerated in culture dishes containing complete media (α-minimum essential medium supplemented with 4 m*M* L-glutamine [Gibco BRL, Grand Island, NY], 100 units/ml benzyl penicillin, 100 μg/ml streptomycin sulfate, 5×10^{-5}*M* 2-mercaptoethanol, 20 m*M* HEPES [all from Sigma-Aldrich], and 0.5% fresh normal mouse serum). To purify T cells from spleen and lymph node populations, a panning method was utilized as previously described (22). A purity of greater than 90% was achieved, as assessed by flow cytometry using anti-CD3ϵ (clone 145-2C11). Irradiated antigen-presenting cells (naive splenocytes, 1,000 rads) were added at a 1:3 ratio to the purified T cells. Cell concentrations were adjusted to 2.5×10^6 cells/ml in all experiments. Cells were stimulated with BiP or CII at various concentrations.

Proliferation and cytokine assays. After 4 days in culture, duplicate or triplicate 100-μl samples were transferred to 96-well, round-bottomed culture plates (Nuclon, Roskilde, Denmark) and pulsed with 0.5 μCi ^{3}H-thymidine per ml (specific activity 25 Ci/mmole; Amersham International, Little Chalfont, UK) and incubated for 6 hours. Cells were harvested onto glass filter mats (Wallac, Milton Keynes, UK). Incorporation of ^{3}H-thymidine was measured using a liquid scintillation counter (Microbeta plus; Wallac). For cytokine analyses, additional 100-μl samples were assessed for IL-4, IL-5, IL-10, and IFNγ secretion using a sensitive cell-based ELISA (23). Linear regression analysis was carried out against known concentrations of recombinant cytokines (BD PharMingen, Oxford, UK).

Statistical analysis. The significance of differences was assessed by Student's *t*-test or chi-square test. *P* values less than 0.05 were considered significant.

RESULTS

Immunogenicity of BiP and up-regulation of Th2 cytokine production. An interesting property of heat-shock proteins (HSPs) is their highly immunogenic nature (24). To investigate the nature of the response stimulated by BiP, DBA/1 mice were immunized subcutaneously with 200 μg BiP either solubilized in PBS or emulsified in IFA. Spleens and lymph nodes were collected 14 days after immunization, and recall responses were assessed in vitro.

A similar level of proliferative response was observed in cultures of splenocytes and lymph node cells obtained from the 2 groups of animals (mean $\pm$ SD 10,156 $\pm$ 500 counts per minute with BiP + PBS and 14,000 $\pm$ 854 cpm with BiP + IFA; $P > 0.05$), indicating that BiP is highly immunogenic in the absence of adjuvant. A low proliferative response was observed when splenocytes from naive animals (5,120 $\pm$ 800 cpm) or animals immunized with the irrelevant antigen, BSA (4,022 $\pm$ 789 cpm), were stimulated with BiP in vitro, suggesting that low numbers of BiP-responsive T cells exist as part of the normal peripheral T cell pool. In addition, splenocytes removed from DBA/1 mice immunized with either BiP or BSA without adjuvant proliferated to a similar extent when restimulated with BSA (2,658 $\pm$ 301 cpm and 2,984 $\pm$ 502 cpm without and with BSA, respectively), indicating that the immunogenic properties of BiP are specific.

To further characterize BiP-responsive cells, BiP-stimulated cytokine production was compared in splenocytes and lymph node cells from groups of mice immunized with BiP, BiP + IFA, or BSA (as control antigen). BiP-stimulated cells from the BSA-immunized control mice produced IFNγ (mean $\pm$ SD 289 $\pm$ 53 pg/ml), but produced only extremely low levels of IL-4 (14 $\pm$ 9 pg/ml), IL-5 (55 $\pm$ 20 pg/ml), and IL-10 (14 $\pm$ 9 pg/ml). Similarly, cells from animals that had been immunized with BiP without adjuvant secreted IFNγ (508 $\pm$ 98 pg/ml), but, in contrast to that in the BSA-immunized animals, significantly higher levels of IL-4 (195 $\pm$ 79 pg/ml), IL-5 (511 $\pm$ 68 pg/ml), and IL-10 (468 $\pm$ 106 pg/ml) were detected ($P < 0.05$). Immunization of mice with BiP in IFA caused a significant increase in the level of secreted IFNγ (1,953 $\pm$ 500 pg/ml; $P < 0.05$), but had little effect on the level of production of the Th2-associated cytokines. Therefore, immunization of mice with BiP appears to induce an antigen-specific, Th2-type response that is independent of additional adjuvant.

To elucidate the origin of these cytokines, further experiments were performed using purified T cells.

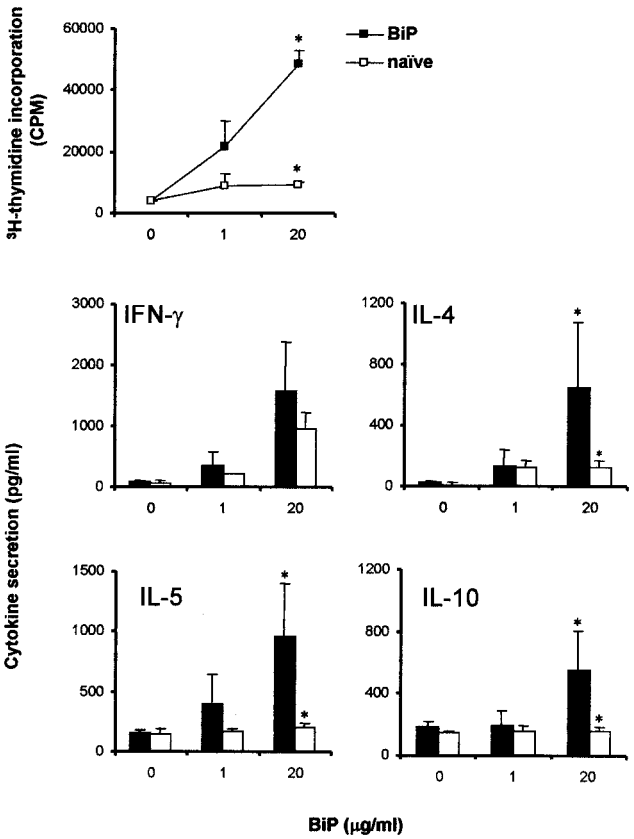

Figure 1. Priming of Th2 cells by soluble BiP. T cells were purified from spleens and lymph nodes removed from mice (n = 4/group) that had been either immunized with 200 μg BiP subcutaneously or left unstimulated (naive). T cells were set up in culture with irradiated antigen-presenting cells (3:1) and stimulated with BiP (1 or 20 μg/ml) for 4 days prior to assessing proliferation by ^{3}H-thymidine incorporation and cytokine secretion (interferon-γ [IFN-γ], interleukin-4 [IL-4], IL-5, and IL-10) by cell-based enzyme-linked immunosorbent assay. Bars show the mean and SD of 3 independent experiments. $* = P < 0.05$ by *t*-test.

Raised levels of IL-4, IL-5, and IL-10 were detected under priming conditions. The level of IFNγ detected in BiP-primed T cells was not significantly higher than the level detected in naive T cells (mean ± SD 1,573 ± 797 pg/ml versus 959 ± 130 pg/ml, respectively). These data indicate a Th2-biased cytokine profile following immunization with BiP (Figure 1). This cytokine response was long lived, as illustrated by the detection of raised levels of IL-4 and IL-10 for up to 6 weeks, at which point the experiment was terminated (production of IL-4 and IL-10 292 ± 45 pg/ml and 244 ± 37 pg/ml, respectively, from BiP-primed cells compared with 41 ± 19 pg/ml and 75 ± 29 pg/ml, respectively, from naive cells; $P < 0.05$).

Effect of parenteral BiP treatment on the course of CIA. BiP administered intravenously can suppress active CIA in HLA–DR1$^{+/+}$ mice in a dose-dependent manner. We have previously shown that 1 mg intravenous BiP can prevent CIA in HLA–DR1$^{+/+}$ mice. In the current experiments, recombinant BiP was tested for its effectiveness in treating established CIA. HLA–DR1$^{+/+}$ mice were intravenously administered 10 μg, 100 μg, or 500 μg of BIP, 40 μg of recombinant β-galactosidase as control protein, or 100 μl of PBS at 31 days after immunization with CII, at the time of appearance of arthritis. As shown in Figure 2a, in relation to that in controls, the higher doses of BiP (100 or 500 μg) did not significantly reduce the severity of arthritis. Interestingly, only the lowest dose of BiP (10 μg) was able to suppress active arthritis.

The differences in mean arthritis severity score were significant by day 39 and remained so throughout the evaluation period ($P < 0.05$) in the mice administered 10 μg BiP as compared with mice given either PBS or β-galactosidase. Based on this finding, we tested a range of lower doses of BIP. As shown in Figure 2b, 1 μg, 5 μg, 10 μg, or 40 μg of BiP administered 28 days after immunization with CII were effective at suppressing the severity of arthritis. These differences were all statistically significant when compared with the control group by day 30 ($P < 0.05$). The differences continued until the end of the 8-week evaluation period. The levels of antibodies to CII were recorded at the end of the therapy studies and were shown to correlate with the severity of arthritis. Mice administered BiP had mean ± SD levels of 20.3 ± 10 units of activity, while mice administered PBS had 46.6 ± 6 units of activity ($P < 0.025$).

Suppression of established CIA in DBA/1 mice by intravenous and subcutaneous administration of BiP. DBA/1 mice were injected with BiP either intravenously or subcutaneously at the onset of arthritis, which, in this case, was 24 days after the first injection of CII. Figure 2c shows that mice given BiP via either route were protected from severe arthritis development as compared with the PBS-treated animals. The differences in mean arthritis severity score were significant at days 38 and 48, both in the subcutaneous and in the intravenous BiP delivery groups when compared with the vehicle (PBS)–treated control ($P < 0.05$), and the differences remained significant throughout the 7-week evaluation period. In addition, a lower incidence of arthritis was recorded among the groups of mice treated with BiP subcutaneously and intravenously (37.5% and 64%, re-

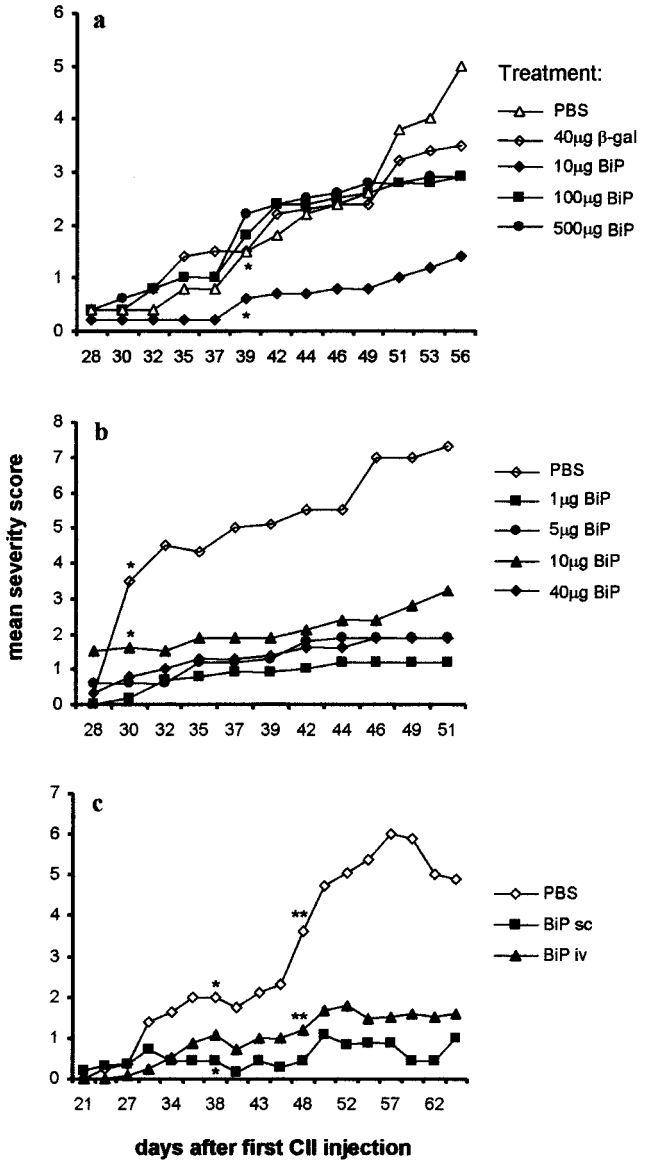

Figure 2. Suppression of active collagen-induced arthritis by BiP in HLA–DR1$^{+/+}$ and DBA/1 mice. **a,** HLA–DR1$^{+/+}$–transgenic mice (n = 10/group) were immunized with type II collagen (CII) emulsified in Freund's complete adjuvant (CFA). At arthritis onset (day 31), groups of mice received 10 μg, 100 μg, or 500 μg BiP or 40 μg β-galactosidase (β-gal) or 100 μl phosphate buffered saline (PBS) intravenously (iv). Disease severity was assessed 3 times a week thereafter. Data are the mean arthritis severity score per group. * = $P < 0.05$ by *t*-test on day 39. **b,** HLA–DR1$^{+/+}$ mice (n = 10/group) were immunized with CII in CFA. At arthritis onset (day 28), groups of mice received 1 μg, 5 μg, 10 μg, or 40 μg of BiP or PBS iv. Data are the mean arthritis severity score per group. * = $P < 0.05$ by *t*-test on day 30. **c,** DBA/1 mice (n = 8/group) were immunized with CII in CFA. At arthritis onset (day 24), groups of mice received 200 μg BiP subcutaneously (sc), 10 μg BiP iv, or PBS. Data in **a–c** are the mean arthritis severity score per group. * = $P < 0.05$ on day 38; ** = $P < 0.05$ on day 48, by *t*-test.

spectively) as compared with that recorded for the control group (100% incidence) by day 70.

Association of BiP treatment of CIA with BiP-specific Th2 cytokine production and altered anti-CII immune responses. We then wanted to determine what cytokines were produced from cells removed from mice that had been treated with BiP and therefore protected from disease development. For this purpose, spleens and draining lymph nodes were collected from each animal, and antigen-specific cytokine secretion was assessed in vitro in DBA/1 mice at the termination of the disease study. Table 1 shows that the amount of secreted IFNγ from cells stimulated with BiP was reduced in the BiP-treated groups compared with the PBS-treated arthritic control group. The levels of BiP-specific IL-5 and IL-10 were invariably low in all groups at this late time point (day 70 after first CII injection). However, the level of BiP-specific IL-4 production was raised in cells removed from both groups of BiP-treated animals (mean ± SD 96 ± 29 pg/ml and 100 ± 35 pg/ml in the

Table 1. BiP- and CII-specific cytokine production from splenocytes and lymph node cells removed from DBA/1 mice that had been treated with BiP or PBS at the onset of collagen-induced arthritis*

Stimulation in vitro, cytokine	Treatment group		
	PBS	Subcutaneous BiP	Intravenous BiP
Media only			
IFNγ	25 ± 12	38 ± 23	69 ± 15
IL-4	14 ± 3	9 ± 3	7 ± 3
IL-5	4 ± 1	6 ± 2	3 ± 1
IL-10	34 ± 6	53 ± 8	50 ± 8
BiP (20 μg/ml)			
IFNγ	1,063 ± 183†	729 ± 139†	822 ± 120‡
IL-4	13 ± 4	96 ± 29‡§	100 ± 35†¶
IL-5	25 ± 2	27 ± 1	29 ± 1
IL-10	69 ± 10‡	97 ± 12‡	84 ± 15‡
CII (40 μg/ml)			
IFNγ	466 ± 94†	1,389 ± 260†§	888 ± 132†§
IL-4	29 ± 8	41 ± 13‡	28 ± 3†
IL-5	12 ± 4	42 ± 13†§	26 ± 9‡§
IL-10	26 ± 6	89 ± 16¶	75 ± 12¶

* Spleens and lymph nodes were removed after the termination of the disease study and cell cultures were set up in the presence of either BiP (20 μg/ml) or type II collagen (CII) (40 μg/ml) or were left unstimulated. Cytokine secretion was assessed after 5 days in culture. The cytokines interferon-γ (IFNγ), interleukin-4 (IL-4), IL-5, and IL-10 were measured. Values are the mean ± SD cytokine secretion (in pg/ml) from each group (n = 8/group). PBS = phosphate buffered saline.

† $P < 0.005$ versus unstimulated cell cultures (media only).

‡ $P < 0.05$ versus unstimulated cell cultures (media only).

§ $P < 0.05$ versus PBS-treated cultures stimulated with the same antigen.

¶ $P < 0.01$ versus PBS-treated cultures stimulated with the same antigen.

subcutaneous BiP– and intravenous BiP–treated groups, respectively) in contrast to the low levels of IL-4 produced from cells obtained from the control arthritic animals (13 ± 4 pg/ml). These differences were statistically significant ($P < 0.05$).

To determine the effect of BiP on the cytokine response to CII, these cell cultures were also assessed for CII-specific cytokine production (Table 1). The amount of secreted IL-4 recorded was low in all cultures. In contrast, an increase in CII-specific IFNγ, IL-5, and IL-10 was observed in the 2 BiP-treated groups compared with the control mice. Serum from these mice were collected at the end of the disease study and analyzed for CII-specific antibodies. Results demonstrated an overall reduction in total IgG CII-specific antibodies in the BiP-treated groups as compared with the control group (mean ± SEM $OD_{492\ nm}$ for total anti-CII IgG 0.52 ± 0.3 in the PBS control group versus 0.36 ± 0.12 and 0.32 ± 0.11 in the subcutaneous and intravenous BiP groups, respectively). In addition, there was an apparent, but nonsignificant, increase in the relative IgG1:IgG2a ratio compared with that in the arthritic control mice (IgG1:IgG2a ratios 0.4, 1.0, and 1.3 in the PBS-, subcutaneous BiP–, and intravenous BiP–treated groups, respectively). These data support the notion that BiP may deviate the CII-specific immune response toward a Th2-type profile.

Protection against CIA following adoptive transfer of splenocytes and lymph node cells from BiP-immunized mice. To test the possibility that BiP may stimulate the induction of BiP-specific Th2 cells that are able to regulate the development of CIA, we undertook cell-transfer experiments. In the first study, HLA–$DR1^{+/+}$ mice were treated intravenously with 10 μg BiP or PBS. The draining lymph node cells and splenocytes were removed 7 days later and then pooled and transferred into naive HLA–$DR1^{+/+}$ mice. These mice were immunized with CII in CFA and observed for the severity of arthritis. Animals receiving transferred cells from BiP-immunized donors had a significant suppression of arthritis by day 46 when compared with animals receiving control cells ($P < 0.05$) (Figure 3a). Levels of antibody to CII were also statistically different between the 2 treatment groups (mean ± SD 61 ± 7 units in the control group compared with 13 ± 3 units in the group receiving cells from BiP-immunized mice; $P < 0.005$).

In the second study, DBA/1 mice were injected subcutaneously with 200 μg of BiP or BSA, or intravenously with 10 μg of BiP or BSA. Spleen and lymph node cells were removed and restimulated with 20 μg of BiP or BSA for 5 days. These cells were predominantly

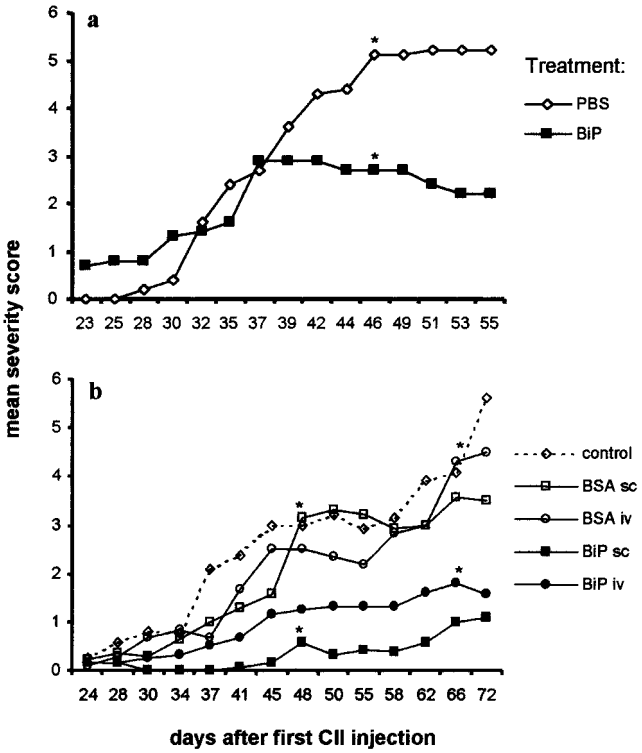

Figure 3. Treatment of collagen-induced arthritis by adoptive transfer of cells from BiP-immunized mice. **a,** HLA–$DR1^{+/+}$–transgenic mice (n = 10/group) were administered 10 μg of BiP or PBS iv. One week later, pooled lymphocytes from the draining lymph nodes and spleens were obtained from the mice and injected iv into naive mice (60×10^6 cells/mouse). On the same day all mice were immunized with CII in CFA. Data are the mean arthritis severity score (scored 3 times a week) in mice given cells treated with either BiP or PBS. $* = P < 0.05$ by *t*-test at day 46. **b,** Spleens and lymph nodes were removed from groups of DBA/1 mice 12 days after they had been immunized with 200 μg BiP sc or control protein bovine serum albumin (BSA) sc, or with 10 μg BiP iv or BSA iv. Cell cultures were set up with 20 μg of the respective protein (BiP or BSA) for 5 days, and 20×10^6 cells/mouse (n = 6 or 7 per group) were then injected intraperitoneally into DBA/1 mice that had been injected with CII in CFA 24 days previously. Data are the mean arthritis severity score (scored 3 times a week). $* = P < 0.05$ on days 48 and 66, by *t*-test. See Figure 2 for other definitions.

CD3+ T cells (60%) as measured by flow cytometry. They were subsequently transferred intraperitoneally to DBA/1 mice that were beginning to show signs of arthritis at day 24. The mice receiving BiP-specific cells subcutaneously had a significant suppression of arthritis by day 48, and a similar significant suppression of arthritis was achieved by day 66 in mice receiving BiP-primed cells intravenously, as compared with mice that had received BSA-primed T cells ($P < 0.05$) (Figure 3b).

Proliferation studies performed on spleen and lymph node cells removed from the mice at the end of

the study showed that a vigorous recall response could be demonstrated in the mice that had received BiP-specific cells (primed subcutaneously or intravenously) compared with those that had received BSA-primed T cells 48 days previously ($P < 0.0001$) (Figure 4). These BiP-primed cells produced IFNγ, IL-10, IL-4, and IL-5 (Figure 4). Taken together, these data show that BiP can prime a population of cells that produce suppressive Th2 cytokines and have potent immunotherapeutic effects on the development of CIA.

Identification of the mechanism of BiP-mediated therapy in CIA. Amelioration of active CIA with BiP was associated with increased production of IL-4 (Table 1). To determine whether IL-4 was essential for the suppression of arthritis by BiP, we used IL-4$^{-/-}$, HLA–DR1$^{+/+}$ mice. We have previously demonstrated that IL-4$^{-/-}$, HLA–DR1$^{+/+}$ mice develop disease with the same kinetics and severity as that exhibited by the IL-4$^{+/+}$, HLA–DR1$^{+/+}$ mice (18). Accordingly, mice were intravenously injected with either 10 μg BiP or PBS 24 days after the first injection of CII in CFA. The mice were then observed for the severity of arthritis. The HLA–DR1$^{+/+}$ wild-type mice showed decreased severity of disease following treatment with BiP compared with control mice receiving PBS. However, BiP was unable to ameliorate the severity of CIA in the IL-4$^{-/-}$, HLA–DR1$^{+/+}$ mice (Figure 5). Moreover, titers of antibody to CII in sera from the IL-4$^{-/-}$ mice treated with BiP were significantly higher than those observed in BiP-treated wild-type mice (mean ± SD 56 ± 7 units in IL-4$^{-/-}$ mice compared with 25 ± 4 units in wild-type mice; $P < 0.05$), whereas anti-CII titers in the wild-type mice treated with PBS were not significantly different (53 ± 5 units) from those observed in the BiP-treated IL-4$^{-/-}$ mice. These data therefore suggest that IL-4 is essential for BiP modulation of arthritis.

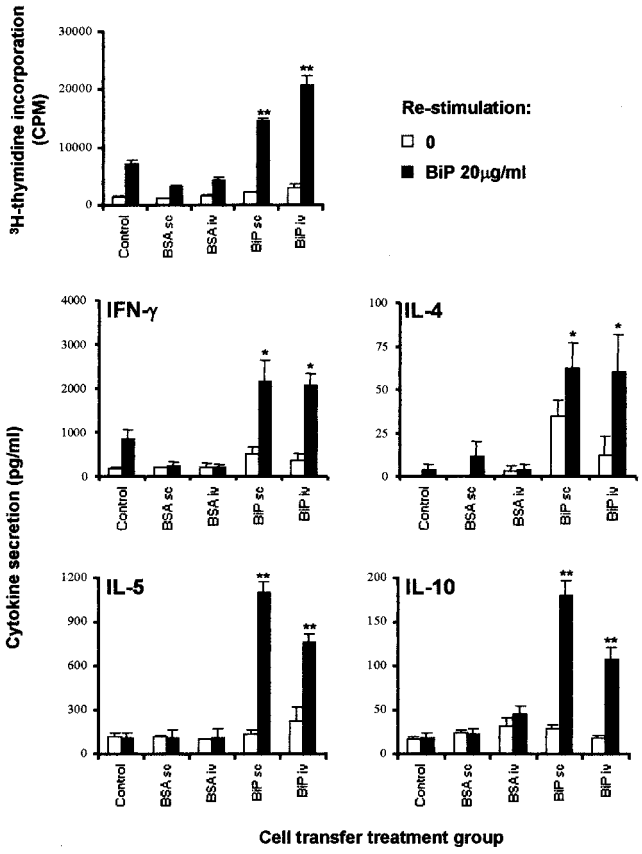

Figure 4. Increased BiP-specific proliferation and production of interferon-γ (IFN-γ), interleukin-4 (IL-4), IL-5, and IL-10 by transfer of BiP-primed cells. Spleens and lymph nodes were removed from all mice after the termination of the cell-transfer therapy study, and cell cultures were set up in the absence or presence of BiP (20 μg/ml). After 5 days in culture, proliferation was assessed by ^{3}H-thymidine incorporation and cytokine secretion was assessed by cell-based enzyme-linked immunosorbent assay. Bars show the mean and SD cytokine secretion in each group (n = 8/group). * = $P < 0.05$; ** = $P < 0.001$ versus culture without BiP restimulation. BSA = bovine serum albumin (see Figure 2 for other definitions).

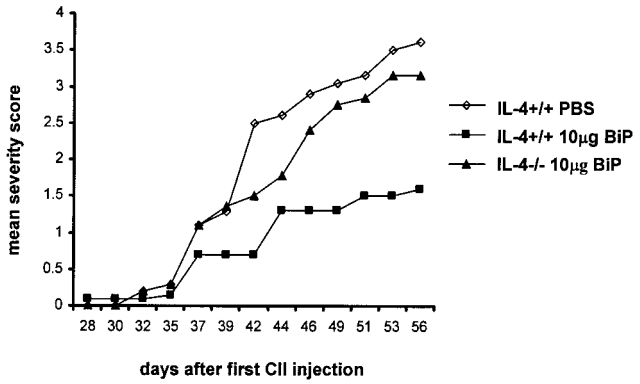

Figure 5. Failure of BiP to suppress active arthritis in interleukin-4 (IL-4)–knockout (IL-4$^{-/-}$), HLA–DR1$^{+/+}$ mice. IL-4$^{-/-}$, HLA–DR1$^{+/+}$ mice (n = 20), developed by backcrossing C57BL/6 IL-4$^{-/-}$ mice to HLA–DR1$^{+/+}$ mice, were immunized with type II collagen (CII) emulsified in Freund's complete adjuvant. At 24 days after immunization, mice were given 10 μg BiP intravenously. Wild-type HLA–DR1$^{+/+}$ mice (n = 20/group) were administered 10 μg BiP or phosphate buffered saline (PBS) intravenously. Mice were assessed for disease development 3 times a week thereafter. Data are the mean arthritis severity score. The value for IL-4$^{-/-}$ mice treated with BiP was not significantly different from that for wild-type mice treated with PBS. Wild-type mice treated with BiP had a significant suppression of arthritis ($P < 0.05$).

DISCUSSION

The studies described herein demonstrate an immunomodulatory role for the stress protein, BiP. BiP

was able to prevent or to treat ongoing CIA in HLA–DR1$^{+/+}$ or DBA/1 mice after intravenous or subcutaneous administration. BiP appears to act by inducing Th2-type cytokines. The therapeutic effect could be transferred by BiP-specific cells. An essential role for one Th2 cytokine, IL-4, was confirmed by the failure of BiP to suppress arthritis in IL-4$^{-/-}$, HLA–DR1$^{+/+}$–transgenic mice.

Immunization of mice with BiP was shown to prime T cells without the requirement of an additional adjuvant, and these T cells secreted high levels of the Th2-associated cytokines IL-4, IL-5, and IL-10. In contrast, although BiP-primed T cells secreted IFNγ, the amount was not significantly higher than that secreted by naive T cells when stimulated with BiP. These results suggest that although IFNγ-secreting BiP-reactive T cells may exist within the peripheral circulation of healthy DBA/1 mice, BiP immunization does not prime such cells. The potent ability of BiP to induce Th2 cytokines was emphasized by the detection of IL-4 and IL-10 for at least 6 weeks after immunization. In light of these properties of BiP, we were interested in its potential ability to regulate the development and treatment of active CIA as a model of a Th1-mediated disease.

One principal purpose of this study was to investigate the therapeutic potential of BiP. When varying doses of BiP were administered intravenously or subcutaneously to DBA/1 mice showing clinical signs of arthritis, there was a significant reduction in both the severity and the incidence of disease. The therapy conferred by administering BiP intravenously was extremely efficient, since a single dose as low as 1 μg was successful in suppressing arthritis.

Because of our results demonstrating that BiP primes a Th2-type response, studies were carried out to assess the role of Th2 cytokines. Analyses performed at the end of the therapy studies demonstrated that BiP-specific IL-4– and, to a lesser extent, IL-10–producing cells were present in DBA/1 mice. This increase in cells with antiinflammatory potential was associated with a concomitant decrease in BiP-specific IFNγ production. These findings implicate a regulatory role for BiP-specific IL-4– and IL-10–producing cells in the treatment of CIA.

Furthermore, treatment with BiP was shown to alter the nature of collagen-specific T and B cell responses, resulting in the up-regulation of antiinflammatory cytokines, a decrease in the total anti-CII IgG level, and an apparent increase in the IgG1:IgG2a anti-CII antibody ratio (representing a reduction in pathogenic, complement-fixing antibodies) at the termination of the study. However, increased production of CII-specific IFNγ was also observed. Recent studies suggest a complex role for IFNγ in CIA. Boissier and colleagues have shown that treatment with neutralizing anti-IFNγ monoclonal antibodies in CIA has differing effects depending on the time of administration (25). Moreover, IFNγ receptor–deficient mice unexpectedly developed rapid-onset arthritis with increased incidence compared with wild-type DBA/1 mice (26). These findings suggest that IFNγ may actually play a role in regulating the autoimmune response and could potentially explain why high levels of IFNγ were detected in T cells removed from BiP-treated mice.

The 2 approaches to the adoptive transfer experiments showed that the mice injected with BiP-primed cells had significantly reduced severity of arthritis. Importantly, with regard to the clinical aspect, BiP-specific cells were able to suppress established disease in DBA/1 mice. The delay in the initiation of arthritis suppression could be explained by the absence of exogenous BiP in the BiP-primed T cells at the onset of disease. Although BiP is ubiquitously expressed, as an intracellular protein under stressful conditions, the transcription of BiP is increased (27) and cell-surface expression and secretion of BiP are seen (Brownlie RJ, et al: unpublished observations). Such conditions are found in the inflamed joint (28) and BiP has been detected in the synovial fluid of patients with inflammatory joint disease (14). The increased availability of extracellular BiP to the immune system in an inflamed joint could act as a target for the transferred BiP-specific cells previously unable to ligate their T cell receptor. It is conceivable that these cells would subsequently be activated to secrete regulatory cytokines that would exert a bystander suppressive effect on the pathogenic, inflammatory cells present in the joint, thereby suppressing arthritis. In support of this contention, a vigorous proliferative response, upon restimulation in vitro, was demonstrated in cells removed from mice that had previously been treated with BiP-primed cells; moreover, high levels of IL-4, IL-5, IL-10, and IFNγ were recorded.

Since there is no antigenic relationship between BiP and the disease-inducing antigen CII, how might BiP regulate the response to CII? It is conceivable that BiP-specific T cells, either primed within the animal or directly transferred, would secrete regulatory cytokines upon activation by up-regulated extracellular BiP found in stressed joints. The secretion of IL-4, IL-5, and IL-10 could exert a bystander suppressive effect on the pathogenic, inflammatory CII-specific cells, thereby resulting in the suppression of arthritis. The finding that BiP fails

to prevent the development of arthritis in IL-$4^{-/-}$, HLA–$DR1^{+/+}$ mice indicates that the regulatory function of BiP is dependent on IL-4 production. However, a contributing role of the antiinflammatory cytokine, IL-10, has not been ruled out and is at present under investigation in our laboratory.

The detection of BiP-responsive, IL-10–secreting CD8+ T cells in the peripheral blood of normal individuals suggests that BiP-responsive T cells may play a homeostatic role in regulating ongoing human immune responses (13). In addition, an alternative mode of immune regulation has been described for BiP. BiP can bind to and stimulate the production of IL-10 from human monocytes (14). Thus, BiP may bind to cells, such as macrophages, via a BiP receptor and generate the production of regulatory cytokines, thus contributing to the regulation of inflammatory processes (14). In support of this contention, unpublished findings from our laboratory suggest that BiP can directly activate murine splenocytes to secrete IL-10.

This study shows unequivocally that the extracellular properties of BiP drive the immune system toward a Th2-type antiinflammatory response. Most other HSPs are effective at broadcasting stress in their local environment, leading to initiation of inflammation (29). In order to maintain homeostasis, however, immunoregulatory HSPs such as BiP may be required to bring about resolution of the inflammatory response, thereby preventing chronic inflammatory damage. We have shown that the therapeutic function of BiP in CIA is dependent on the presence of the archetypal Th2 cytokine, IL-4, and that there is generation of regulatory T cells within this system. Thus, BiP may play a pivotal role in the regulation of immune networks (30). Since CIA is used as a model for human RA, it will be interesting to see whether parenteral administration of BiP has therapeutic potential in this disease.

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