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Peptide-induced nasal tolerance for a mycobacterial heat shock protein 60 T cell epitope in rats suppresses both adjuvant arthritis and nonmicrobially induced experimental arthritis

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ABSTRACT **Adjuvant arthritis (AA) can be induced in Lewis rats by immunization with mycobacterial antigens. Passive transfer of a T cell clone recognizing the 180–188 amino acid sequence in mycobacterial heat shock protein 60 (hsp60) was found to induce AA. In the present study, we investigated whether tolerance was obtained for this AA-associated T cell epitope after intranasal or s.c. administration of a peptide containing this epitope. Two 15-mer peptides containing the mycobacterial hsp60 sequences 176–190 and 211–225 were used; 176–190 contained the T cell epitope 180–188, which was recognized by the arthritogenic T cell clone A2b and was the immunodominant hsp60 T cell epitope after induction of AA, and 211–225 contained a T cell epitope that was recognized both after induction of arthritis with whole *Mycobacterium tuberculosis* and after immunization with mycobacterial hsp60. In rats treated intranasally or subcutaneously with 176–190 and immunized with mycobacterial hsp60, proliferative responses to 176–190 were reduced. Proliferative responses to 211–225 and to whole mycobacterial hsp60 were not affected. AA was inhibited intranasally in the 176–190-treated rats but not in the 211–225-treated rats. Moreover, intranasal 176–190 led to similar arthritis-protective effects in a nonmicrobially induced experimental arthritis (avridine-induced arthritis). Therefore, tolerance for a disease-triggering, microbial cartilage-mimicking epitope may cause resistance to arthritis irrespective of the actual trigger leading to development of the disease.**

Adjuvant arthritis (AA) is an extensively studied form of experimental arthritis resembling rheumatoid arthritis in a number of histopathological aspects. It can be induced in Lewis rats by immunization with mycobacterial antigens. T cell responses to mycobacterial heat shock protein 60 (hsp60) play a role both in the induction of the disease and in the control of disease development. Initially, it was shown that AA could be passively transferred by T cells obtained from diseased rats into syngeneic, disease-free rats (1). Subsequently, passive transfer of a single T cell clone obtained from AA rats recognizing the 180–188 amino acid sequence of mycobacterial hsp60 was found to lead to AA development (2, 3). This T cell clone, called A2b, also responded to cartilage proteoglycan but not to rat hsp60 (4). The clone, therefore, demonstrated the existence of a mimicry relationship between mycobacterial hsp60 and cartilage proteoglycan, which could be a critical factor in the induction of AA.

Several studies in experimental autoimmune models have shown that it is possible to induce antigen-specific T cell tolerance leading to disease resistance (5–9). This is dependent on several factors, such as the use of adjuvants, the route of administration, and the amount of the antigen used (10–12). So far, most evidence comes from the model of experimental autoimmune encephalomyelitis (EAE). EAE is a demyelinating autoimmune disease caused by $CD4^+$ T cells specific for myelin basic protein (MBP) (13). It can be induced in susceptible rats by immunization with MBP in complete Freund's adjuvant. Oral administration of MBP protected rats from developing EAE (5). Protection was transmitted by adoptive transfer of $CD8^+$ T cells, which were found to produce transforming growth factor-β and various amounts of interleukin 4 (IL-4) and IL-10 when stimulated with the relevant antigens (14–17). Encephalitogenic epitopes of MBP have been characterized, and intranasal (i.n.) administration of immunodominant epitopes of MBP has been shown to lead to protection against EAE induction (9, 18).

EAE and related models of experimental autoimmunity are induced with a defined autoantigen in the context of a suitable adjuvant. In AA, however, disease is induced with a complex microbial antigen, of which an antigenic relationship with a potential autoantigen only has been established for the hsp60 180–188 epitope (4). In the present study, we show that nasal tolerance obtained for the hsp60 180–188 amino acid sequence leads to resistance not only against arthritis induced with the complex microbial antigen itself but also against arthritis induced with the nonrelated, nonimmunogenic, synthetic, oily, adjuvant avridine.

MATERIALS AND METHODS

Animals. Male, inbred Lewis rats (RT1 B^1) were obtained from the University of Limburg (Maastricht, The Netherlands). Rats were 6–9 weeks old at the start of each experiment.

Antigens and Adjuvants. Heat-killed *Mycobacterium tuberculosis* (Mt; strain H37Ra) was obtained from Difco. Purified recombinant hsp60 of *Mycobacterium bovis* bacillus Calmette–Guérin (identical to Mt hsp60) was kindly provided by J. D. A. van Embden (National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands). Incomplete Freund's adjuvant (Difco) and dimethyl dioctadecyl ammonium bromide (DDA; Eastman Kodak) were used as adjuvants. DDA was prepared as a 20-mg/ml suspension in PBS

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Abbreviations: AA, adjuvant arthritis; hsp60, heat shock protein 60; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; i.n., intranasally; DDA, dimethyl dioctadecyl ammonium bromide; PLNC, peripheral lymph node cells; Mt, *Mycobacterium tuberculosis*; DTH, delayed-type hypersensitivity.

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and was sonicated/heated to produce a gel that was mixed 1:1 with antigen solution before immunization. The synthetic adjuvant *N*,*N*-dioctadecyl-N^1, N^1-bis[2-hydroxyethyl]propanediamine (CP20961, or avridine) was obtained from Pfizer Diagnostics. Peptides were prepared by automated simultaneous multiple peptide synthesis. Peptides were obtained as COOH-terminal amides. Two 15-mer peptides containing the individual hsp60 sequences 176–190 (176–190) and 211–225 (211–225) were used; 176–190 contains the epitope 180–188 recognized by the arthritogenic T cell clone A2b and is the codominant T cell epitope found after AA and after immunization with mycobacterial hsp60 (19), and 211–225 contains a T cell epitope recognized both after induction of arthritis with Mt and after immunization with mycobacterial hsp60 (19). Thus far, no role for this epitope has been found in disease induction or in protection from disease (20).

Immunizations. Rats were immunized with 50 μg of synthetic peptide in 50 μl of PBS/DDA in each hind footpad (i.e., 100 μg/rat) or with 25 μg of mycobacterial hsp60 in 50 μl of PBS/DDA in each hind footpad (i.e., 50 μg/rat). In some experiments, draining popliteal lymph nodes were removed 14 days after immunization, disaggregated, washed three times, and used as a source of primed lymph node cells. In other experiments, primed lymph node cells derived from pooled inguinal and popliteal lymph nodes and splenocytes from AA rats 42–50 days after Mt immunization were used.

Tissue Culture Reagents. Iscove's modified Dulbecco's medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (all from GIBCO/BRL), and 5×10^{-5} M 2-mercaptoethanol was used as culture medium. Cell populations were washed in Iscove's modified Dulbecco's medium without supplements.

T Cell Proliferation Assays. Peripheral lymph node cells (PLNC) and splenocytes were cultured in triplicate in 200-μl, flat bottom, microtiter wells (Costar) at 2×10^5 cells per well with or without antigen. PLNC and splenocytes were tested for responsiveness to individual peptides and hsp60 at 2 and 20 μg/ml. Con A (2 μg/ml) was used as a positive control for T cell proliferation. Cultures were incubated for 96 h at 37°C in a humidified atmosphere of 5% CO_2. Cultures were pulsed for the final 16 h with [^{3}H]thymidine (Amersham; 0.4 μCi/well, specific activity 1 Ci/mmol), and thymidine uptake was measured using a liquid scintillation β counter. Results are expressed as a mean cpm of triplicate cultures. The magnitude of the proliferative response was expressed as stimulation index (SI): the mean cpm of cells cultured with antigen divided by the mean cpm of cells cultured with medium alone.

Administration of Peptides. Rats were lightly anesthesized under ether, and 100 μg of peptide dissolved in PBS was administered i.n. in a total volume of 10 μl (5 μl per nostril; peptide concentration 10 mg/ml) using a micropipette. This was done on days −15, −10, and −5 preceding the induction of arthritis or immunization with mycobacterial hsp60 (adapted from ref. 6). In a second protocol, 300 μg of peptide dissolved in PBS was administered s.c. (peptide concentration 1 mg/ml). This was done on days −15 and −7 preceding the induction of arthritis or immunization with mycobacterial hsp60 (adapted from ref. 21).

Delayed-Type Hypersensitivity (DTH) Analysis. DTH was measured by injecting 10 μg of peptide in 10 μl of PBS s.c. in the left ear. In the other ear, 10 μl of PBS was injected. Thickness was measured using micrometer calipers. The difference in thickness between the left and right ears was measured for each rat 24 and 48 h after the challenge. The results were expressed as the mean for each experimental group ± SD. DTH responses were determined on day 10 after immunization with peptide in DDA or 5 days after the third and final i.n. administration.

Induction and Clinical Assessment of Experimental Arthritis. AA was induced by a single intradermal injection in the base of the tail with 0.5 mg of Mt suspended in 100 μl of incomplete Freund's adjuvant. Avridine arthritis was induced with 2 mg of avridine in 100 μl of light mineral oil (Sigma). Rats were examined daily for clinical signs of arthritis. Severity of arthritis was assessed by scoring each paw from 0 to 4 based on degree of swelling, erythema, and deformation of the joints. Thus, the maximum score was 16 (22). In most experiments, the weight of individual rats was scored every other day.

Statistical Analysis. Basic descriptive statistics were used to describe the proliferative responses and the characteristics of the different treatment groups. Student's *t* test was used to compare arthritis scores, weight loss, and proliferative responses to antigens between the different groups.

RESULTS

Intranasal and Subcutaneous Administration of Peptides. Rats were exposed to 176–190 by administering the peptide via either the s.c. or i.n. route. Control rats received PBS. The results of the i.n. administration are given in Fig. 1. Rats were given 100 μg of peptide in PBS at days −15, −10, and −10. Five days after the last i.n. administration, rats were immunized with 50 μg of mycobacterial hsp60 in DDA in the footpad, and, 14 days later, proliferative responses to Con A, mycobacterial hsp60, 176–190, and 211–225 in the draining lymph node cells

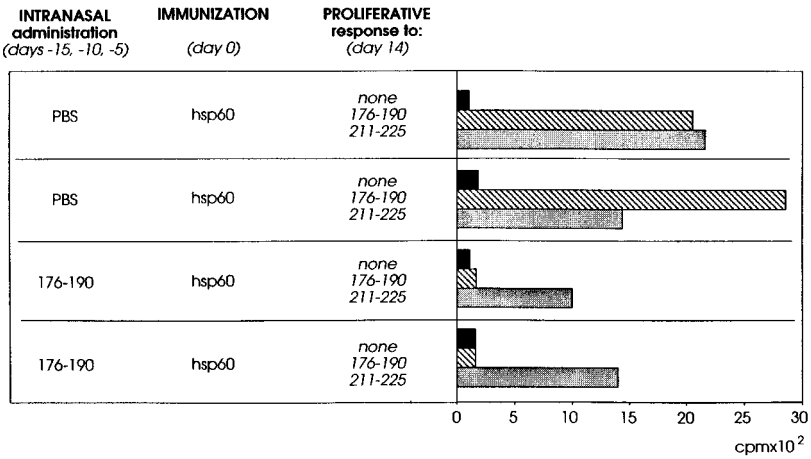

FIG. 1. Modulation of the proliferative response to peptide 176–190 after i.n. administration of PBS (controls) or peptide 176–190. Rats received 100 μg of 176–190 dissolved in 10 μl of PBS or PBS alone i.n. at days −15, −10, and −5. At day 0, rats were immunized with 50 μg of mycobacterial hsp60 in DDA in the hind footpads. PLNC were isolated 14 days after immunization and tested for responses to peptide 176–190 and 211–225.

were measured. The proliferative responses to 176–190 were significantly lower in rats treated i.n. with 176–190 (SI 1.25) than in the control rats (SI 17.5) (Fig. 1). The proliferative responses to 211–225 and to mycobacterial hsp60 and Con A were not affected (data not shown). Subcutaneous administration of 176–190 in PBS led to a similar specific downregulation of the proliferative response to this peptide (SI to 176–190 rats treated with 176–190 s.c. 1.2; SI to 176–190 rats treated with PBS s.c. 4.8). Again, the proliferative responses to 211–225 and to mycobacterial hsp60 and Con A were not affected (data not shown). Thus, i.n. or s.c. administration of hsp60 peptides in PBS was found to lead to specific downregulation of T cell responses as measured in draining lymph nodes after footpad immunization.

Absence of Cellular Immunity After Intranasal Peptide Administration. We next set out to determine whether the i.n. administration of hsp60 peptides led to specific cellular immunity as measured in lymphocyte proliferation and DTH. Peptides 176–190 and 211–225 and PBS were administered i.n. at days −15, −10, and −5 as described above (three rats in each group). At day 0, a lymphocyte proliferation test was performed on PLNC derived from mandibular, inguinal, and popliteal lymph nodes. No proliferative T cell responses to the administered peptides could be measured in lymph node cells derived from rats from the three groups (176–190, 211–225, and PBS groups). Skin tests were performed at day 0 after i.n. peptide administration on days −15, −10, and −5. As a positive control, skin tests also were done at day 10 after immunization with the same peptide in DDA. Four groups were tested: i.n. administration of 176–190 and 211–225 and immunization with the same peptides in DDA. Specific DTH responses both to 176–190 and 211–225 after 24 and 48 h were low or negative in rats pretreated with the same peptides i.n. However, control animals immunized with 176–190 and 211–225 in DDA showed positive DTH reactions after 24 and 48 h (Fig. 2). Thus, i.n. administration of hsp60 peptides in PBS did not lead to specific cellular immunity as measured by either lymphocyte proliferation in cells derived from draining lymph nodes or DTH reactions (23).

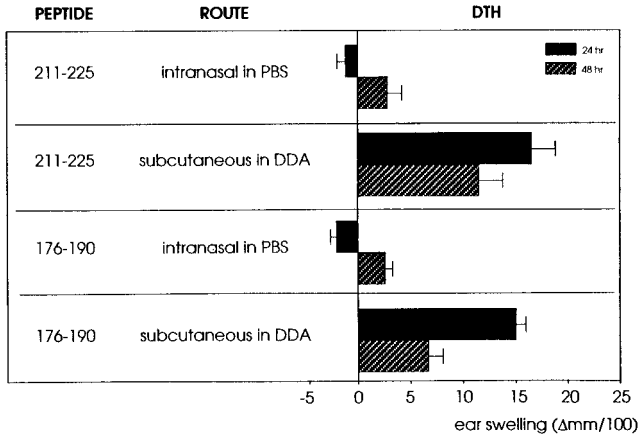

FIG. 2. DTH after i.n. administration or after immunization in DDA with peptide 176–190 or 211–225. Four groups were tested: i.n. administration of 176–190 and 211–225 (100 μg of peptide in 10 μl of PBS) and immunization in the hind footpads with 100 μg of the same peptides in DDA (three rats in each group). Shown are DTH responses in rats after i.n. administration at days −15, −10, and −5 and after immunization at day −10. DTH was measured by injecting 10 μg of peptide in 10 μl of PBS s.c. in the left ear and 10 μl of PBS in the other ear. The difference in thickness between left and right ears was measured (using a micrometer calipers for each animal 24 (black bars) and 48 h (shaded bars) after the challenge. The results were expressed as the mean for each experimental group (mm/100) ± SEM.

Effects of Intranasal Administration of hsp60 Peptides in AA. In subsequent experiments, the effect of i.n. peptide administration was tested in AA. Peptides 176–190 and 211–225 and PBS were given at days −15, −10, and −5 before AA induction. At day 0, AA was induced. Rats exposed to i.n. 176–190 showed a delay in the onset of arthritis (mean day of onset 19.4 compared with 12.8 in control rats) and a lower maximum arthritis score. The maximum mean arthritis score in the 176–190-treated rats was 4.4 compared with a mean maximum arthritis score of 8.6 for the control rats receiving PBS ($P < 0.05$). The maximum mean arthritis score of the 211–225-treated rats was 10.8 ($P < 0.05$) (Fig. 3). Weight curves (a sensitive measure of physical well being) also were analyzed. Mean weight gain at day 25 (day of maximum arthritis score) compared with day 10 (before the onset of arthritis) was +11 g, SEM 2.7 for 176–190-treated rats compared with −8 g, SEM 2.6 for control rats (*t* test $P < 0.05$) (Fig. 4). The findings were confirmed in three subsequent experiments (data not shown).

Surprisingly, the 211–225-treated rats developed a more severe arthritis than the PBS controls (mean maximum arthritis score 10.8 compared with 8.6 in the controls) and showed a more chronic course of disease. At day 40, these rats still had active arthritis and still were losing weight (Figs. 3 and 4). Although the difference in mean maximum arthritis score between 211–225-treated rats and control rats receiving PBS was not significant, this effect was reproduced in a follow-up experiment.

Effects of Intranasal Administration of hsp60 Peptides in Avridine-Induced Arthritis. To see whether the arthritis-suppressive effect of i.n. 176–190 was specific for mycobacteria-induced AA (which is known to feature dominant immune responses to the mycobacterial arthritogenic epitope 180–188), we decided to test the effect of i.n. 176–190 in avridine-induced arthritis. Avridine is a lipoidal amine, which does not contain antigenic components crossreactive with mycobacterial hsp60.

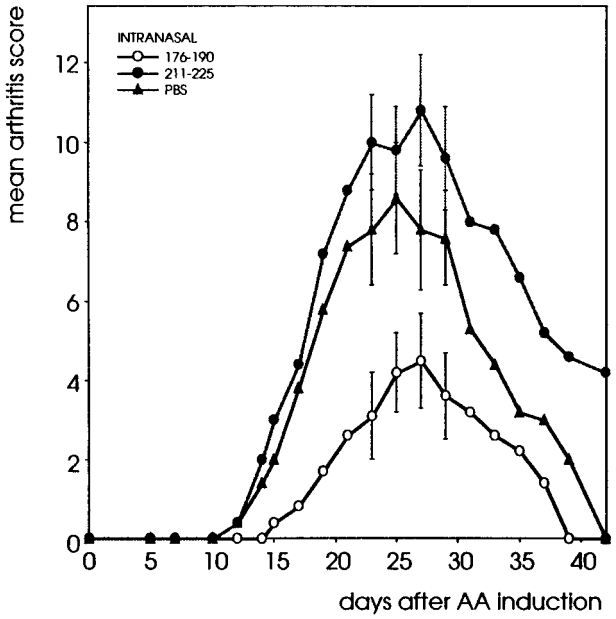

FIG. 3. Modulation of AA development after i.n. administration of peptide 176–190 and 211–225. Rats received 100 μg of peptide (176–190 or 211–225) dissolved in 10 μl of PBS or PBS alone i.n. at days −15, −10, and −5. At day 0, rats were immunized with 0.5 mg of Mt in 100 μl of incomplete Freund's adjuvant to induce AA. Six rats were in each treatment group. Arthritis scores were assessed every other day after Mt immunization. On the Y axis are shown the mean arthritis scores ± SEM (error bars).

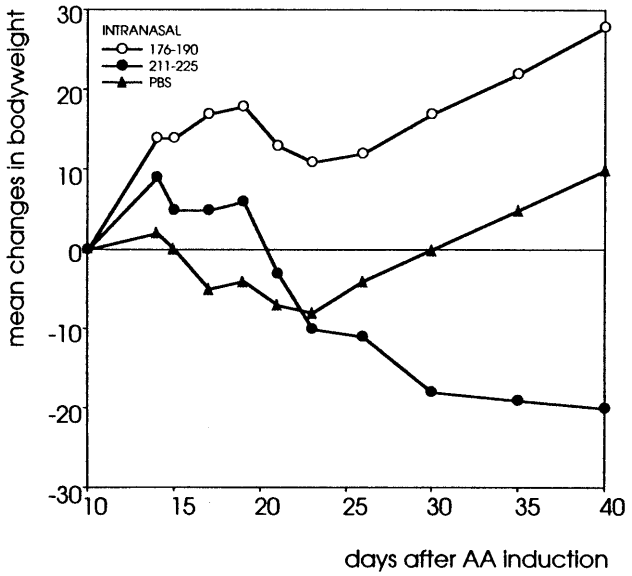

FIG. 4. Mean change in body weight in grams after i.n. administration of peptide 176–190 and 211–225 in AA. Experimental details as in Fig. 2. Body weight was measured every other day. Y axis shows the mean changes of body weight from day 10 after Mt immunization.

Intranasal administration of 176–190 led to similar protective effects in this model (Fig. 5). Onset of arthritis was delayed (mean day of onset 16 compared with 14 in control rats). The mean maximum arthritis score in the 176–190-treated rats was 3.8 ($n = 6$) compared with a mean maximum arthritis score of 9.0 ($n = 6$; $P < 0.05$) for the control rats receiving PBS and 9.2 ($n = 6$) for rats receiving 211–225. Also, 176–190-treated rats showed a more favorable weight curve compared with PBS-treated rats.

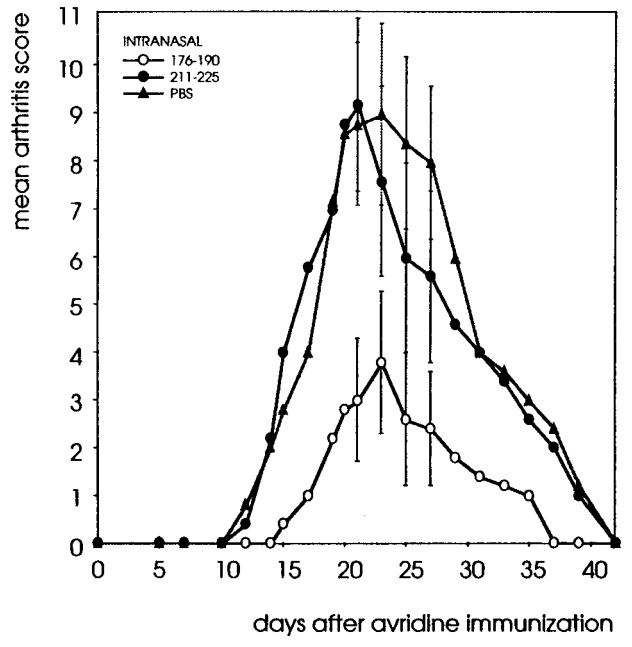

FIG. 5. Modulation of avridine-induced arthritis after i.n. administration of 176–190 and 211–225. Rats received 100 μg of peptide (176–190 or 211–225) dissolved in 10 μl of PBS or PBS alone i.n. at days −15, −10, and −5. At day 0, rats were immunized with 2 mg of avridine in 100 μl of mineral oil injected intradermally at the base of the tail to induce arthritis. Six rats were in each treatment group. Arthritis scores were assessed daily from day 8 after avridine immunization. On the Y axis are shown the mean arthritis scores ± SEM (error bars).

Lymphocyte Proliferation Assays in Arthritic Rats After Intranasal Peptide 176–190. Lymphocyte proliferation assays on primed lymph node cells derived from pooled popliteal and inguinal lymph nodes and splenocytes were performed around day 45 after the induction of arthritis. After AA, 176–190-treated rats had low proliferative responses to 176–190 (mean SI 1.4 compared with mean SI 4.8 in 211–225-treated rats; $P < 0.05$) (Fig. 6). Remarkably, in the group treated with 176–190, only one rat that had suffered from severe arthritis showed clear proliferative responses to 176–190 (SI 3.2; marked with an asterisk). The responses to mycobacterial hsp60, Con A, and Mt were not affected (data not shown).

DISCUSSION

We have shown here that i.n. administration of a synthetic peptide containing a single T cell epitope of mycobacterial hsp60 reduced the severity of AA, a disease induced by an antigen as complex as whole mycobacteria. And, our results showed that this approach also may cause protection against another form of experimental arthritis induced without microbial antigens, avridine-induced arthritis. This indicates that we may have detected a broad arthritis-suppressive mechanism, which can be triggered by the induction of tolerance for a single arthritis-associated T cell epitope. As we have demonstrated, using a tolerizing i.n. route, administration of our hsp60 peptides in PBS did not lead to overt cellular immune responsiveness; we could not measure specific responses in either lymphocyte proliferation or DTH reactions after i.n. administration. On the contrary, both i.n. and s.c. administrations of peptide 176–190 in PBS were found to specifically suppress proliferative responses to this peptide after mycobacterial hsp60 immunization and thus appeared to have tolerized T cells with specificity for this peptide.

As shown here, i.n. tolerance induction to peptide 176–190 had a suppressive effect both in AA and in avridine-induced arthritis, a T cell-mediated form of experimental arthritis that does not involve microbial antigens (24, 25). Peptide 176–190 was selected for these experiments because it contained the mycobacterial hsp60 epitope 180–188, as recognized by arthritogenic T cell clone A2b. Furthermore, the 180–188 sequence has appeared to be the immunodominant hsp60 T cell epitope after induction of arthritis in Lewis rats with whole mycobacteria suspended in mineral oil. Especially relevant in the context of the present observations is that T cell clone A2b was found to crossreact with cartilage proteoglycans (4).

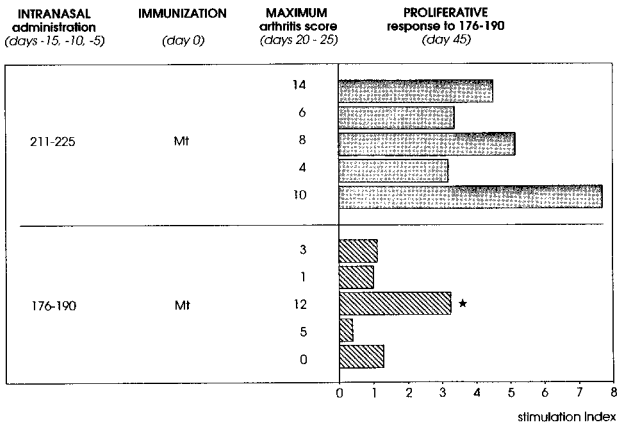

FIG. 6. Lymphocyte proliferative responses on primed lymph node cells from pooled popliteal and inguinal lymph nodes after the induction of AA. Peptide concentration 20 μg/ml; One rat in the 176–190-treated group suffered from severe arthritis (maximum arthritis score 12 at day 21); PLNC derived from this animal showed clear proliferative responses to 176–190 (SI 3.2, marked with an asterisk).

Therefore, our present observations suggest that s.c. or i.n. administration of peptide 176–190 leads to a tolerization, especially including those T cells that recognize this crossreactive epitope in cartilage proteoglycans. Such tolerized T cells well may have exerted a local down-regulatory activity capable of suppressing inflammation in the joint. The finding of modulation of the disease in two unrelated arthritis models indicates that our tolerization procedure using this single arthritis-associated epitope has led to bystander suppression at sites of joint inflammation, which suppressed arthritis in the presence or in the vicinity of the cross-recognized proteoglycan epitope.

The operation of such a bystander type of suppression may provide a suitable explanation for suppression of arthritis induced by a nonspecific oily adjuvant, such as avridine, which has no direct antigenic relationship with 176–190 or its crossreactive proteoglycan epitope. Our findings seem compatible with earlier findings in EAE, in which i.n. induced tolerance to the encephalitogenic epitope on MBP was found to lead to protection against disease induced with spinal cord homogenate, a heterogenic mixture of potential autoantigens (9). However, in contrast to the present findings in avridine-induced arthritis, the spinal cord homogenate used to induce EAE contained the epitope used for prior tolerization. Therefore, suppression of EAE was, in that case, possibly due to local bystander suppression at the site of immunization and not necessarily at the site of central nervous system inflammation. In our case, the latter possibility is unlikely. Although responses to 176–190 were found to be suppressed, draining lymph node responses to the whole mycobacterial hsp60 protein were not suppressed, indicating that responses to other epitopes of mycobacterial hsp60 (as 211–225; Fig. 1) had remained unaffected (19). That such responses appeared to be unaffected by nasal administration of the peptide can be taken as an indication that bystander suppression of responses in the lymph nodes draining the site of immunization (footpad) was minor or absent.

It has been shown that disease-protective peripheral T cell tolerance can be induced in several experimental autoimmune diseases through oral or i.n. administration of an antigen (6, 14, 26, 27). Oral tolerance is now being studied as a therapeutic strategy in human autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (28, 29). Three distinct mechanisms are possibly operational in oral tolerance: clonal deletion, anergy, and active suppression (11, 16, 17, 30, 31). The dosage of the administered antigen appears to determine which mechanism prevails (10, 32–34). High antigen doses lead to clonal deletion or anergy whereas low doses may lead to (bystander) suppression, possibly mediated by $CD8^+$ or $CD4^+$ T cells capable of producing the inhibitory cytokines such as transforming growth factor β and IL-4. It is likely, but not yet proven, that similar mechanisms are operative in i.n. induced tolerance (35, 36). Hoyne and others showed recently that a single i.n. administration of peptide in mice in the dose range we used (100 μg) induced a strong but transient activation of $CD4^+$ cells followed by a state of antigen-specific T cell unresponsiveness. For rats, 100 μg is a relatively low dose of antigen, compatible with the possibility of having induced bystander suppression mechanisms. Furthermore, in initial *in vitro* studies, we have found that, after i.n. administration of peptide 176–190 followed by immunization with Mt, the proliferative responses *in vitro* to Mt in splenocytes were suppressed upon adding peptide 176–190 to the culture (unpublished results), again compatible with bystander suppression as the mechanism.

In mouse EAE, the therapeutic efficacy of tolerization was shown to be enhanced by increased affinity of the peptide for major histocompatibility complex (37, 38). In the Lewis rat, most autoimmune disease-associated $CD4^+$ T cell responses are major histocompatibility complex class II $RT1.B^1$ restricted. By using an *in vitro* major histocompatibility complex–peptide-binding assay peptide 176–190 was found to be a strong $RT1.B^1$ binder (39). Hammer and others determined the peptide-binding specificity of the rheumatoid arthritis-associated HLA-DR molecules DRB1*0401 and DRB1*0404 (40). They have predicted a high selective binding for the rheumatoid arthritis-associated DR4 molecules of mycobacterial hsp60 peptide 181–193. If so (and based on the present findings in the experimental arthritis models), 176–190 and related peptides may have potential for therapeutic application in human rheumatoid arthritis.

The present findings indicate that antigen-specific T cell tolerance induced by i.n. administration of a single microbial epitope may cause resistance to various forms of experimental arthritis through bystander suppression at the site of inflammation. As a consequence, these findings of antigen-specific suppression of disease, even in the absence of an identified self-target antigen, may well create opportunities for the design of novel immunotherapeutic strategies in human autoimmune diseases.

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