Clin Exp Immunol 1997; **110**:72–78

Experimental immunization with anti-rheumatic bacterial extract OM-89 induces T cell responses to heat shock protein (hsp)60 and hsp70; modulation of peripheral immunological tolerance as its possible mode of action in the treatment of rheumatoid arthritis (RA)

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(Accepted for publication 6 June 1997)

SUMMARY

OM-89 is a bacterial (*Escherichia coli*) extract used for oral administration in the treatment of RA. Given the evidence that immunity to bacterial heat shock antigens plays a critical role in the immunomodulation of arthritis and possibly inflammation in general, the purpose of the present studies was to evaluate the presence and immunogenicity of hsp in OM-89. Furthermore, we studied the effects of OM-89 in an experimental arthritis, where hsp are known to have a critical significance in disease development. In rats immunization with OM-89 was found to lead to proliferative T cell responses to hsp60 and hsp70 of both *E. coli* and mycobacterial origin. Conversely, immunization with hsp antigens was also found to induce T cell reactivity specific for OM-89. Based on this and the antigen specificity analysis of specific T cell lines, hsp70 (DnaK) turned out to be one of the major immunogenic constituents of OM-89. Parenteral immunization with OM-89 was found to reduce resistance to adjuvant arthritis (AA), whereas oral administration was found to protect against AA. Given the arthritis-inhibitory effect of oral OM-89 in AA, it is possible that peripheral tolerance is induced at the level of regulatory T cells with specificity for hsp. This may also constitute a mode of action for OM-89 as an arthritis-suppressive oral drug.

Keywords *Escherichia coli* T cells hsp arthritis

INTRODUCTION

OM-89 is a glycoprotein-rich extract of selected *Escherichia coli* strains, which is used as a slow-acting anti-rheumatic drug for oral administration. OM-89 has been shown to improve significantly the clinical parameters of RA without serious side-effects. Despite known effects on both humoral and cellular immune mechanisms, its mode of action is not completely elucidated [1,2].

The presence of *E. coli* hsp60 (GroEL) in OM-89 was postulated [3] and shown already by the staining of OM-89 with hsp60-specific MoAbs [4]. Bacterial hsp were shown to have the capacity of preventing experimental arthritis upon prior immunization. For mycobacterial hsp60 this was demonstrated in mycobacteria-induced adjuvant arthritis (AA) [5], streptococcal cell wall-induced arthritis [6] and avridine (CP20961)-induced arthritis [7] in rats and in pristane [8] and collagen type II-induced arthritis [9] in mice. More recently, also for mycobacterial hsp70, protective immunization leading to arthritis resistance has been demonstrated [10].

Amongst bacterial hsp, the members of the hsp60 and hsp70 families are well represented and evolutionarily most conserved, which means that they have extensive sequence homologies with their mammalian counterparts. Despite their high level of sequence homology with the mammalian homologues, hsp60 and 70 have been found to be very immunogenic proteins, at the level of both B and T cells. Furthermore, synthesis of hsp occurs under conditions of stress—they are also called stress proteins—as is seen to occur in the inflamed synovium [11]. The arthritis-protective effects of hsp immunization are probably related to these characteristics of hsp.

Experiments in AA and avridine-induced arthritis in rats have indicated the possible protective mechanisms leading to arthritis resistance [12]. Transfer studies with selected hsp epitope-specific

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T cell lines and hsp peptide vaccinations have shown that upon hsp immunization T cell responses are induced, which include reactivity directed against self-hsp molecules as expressed by stressed cells. On the basis of this, we have concluded that the arthritis-protective T cells are T cells cross-responsive to self-hsp epitopes, induced by conserved microbial hsp epitopes [13]. As an alternative, in pristane arthritis evidence has been collected for a regulatory effect of mycobacterial hsp60-specific Th2 cells. In that case, control of arthritis was observed in the absence of self-hsp reactivity [14]. Whichever mechanism may prevail, in both cases regulatory responses, possibly of a Th2 nature, are induced. Furthermore, the findings on microbial hsp were in accordance with earlier observations showing that exposure to microbial antigens had a modulating, peripheral tolerance-reinforcing effect in experimental autoimmunity [15].

In humans, immune responses to hsp have been shown to occur in association with many different autoimmune diseases, including arthritic diseases [16,17]. Whether, in these cases, immunity to hsp contributes to disease development, arises as a consequence of inflammatory stress, or is part of (failing) protective mechanisms is unclear [18]. In children with juvenile chronic arthritis (JCA), however, T cell responses to human and mycobacterial hsp60 were found to be associated with the remitting oligo-articular form of the disease [19,20]. Furthermore, responsiveness was seen to increase preceding the actual phases of disease remission. Therefore, in JCA patients, it seemed that T cell responses to conserved self hsp60 epitopes were contributing to arthritis-inhibiting regulatory mechanisms.

In the present study we have investigated the possibility that OM-89 contains bacterial hsp with the potential of activating hsp-specific T cells as a mechanism for its therapeutic efficacy in RA. Previously, OM-89 was tested in different autoimmune disease models. Inflammation was suppressed in AA [21] and avridine-induced arthritis [22]. In the BB rat type I diabetes model, OM-89 showed a delay in the onset of diabetes [23]. In the NZB/W systemic lupus erythematosus (SLE) model oral administration of OM-89 caused a delay of the onset of renal lesions [24]. In rats it was also shown that oral administration of OM-89 increases the migration of $CD8^+$ T cells from the Peyer's patches to the mesenteric lymph nodes [24].

MATERIALS AND METHODS

Rats

Inbred male Lewis rats were obtained from the University of Limburg (Maastricht, The Netherlands). All rats were used at 6–8 weeks of age.

Antigens

The following antigens were used for immunization of the animals or in proliferation assays: heat-killed *Mycobacterium tuberculosis* (Mt) H37 RA (Difco Labs, Detroit, MI), *E. coli* hsp60 (GroEL) and hsp70 (DnaK) (Stressgen Biotechnologies, Victoria, Canada). The recombinant hsp60 of *M. bovis* BCG was cloned and purified as described previously [5]. A purified recombinant preparation of hsp70 (Mt) was obtained from Dr M. Singh (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany) in support of the UNDP/World Bank WHO Special Programme for Research and Training in Tropical Diseases.

OM-89 concentrate (OM-89 conc.) and lyophilisate (OM-89 lyoph.) were provided by OM Laboratories (Geneve, Switzerland). This compound contains mainly acidic glycoproteins of 10–300 kD mol. wt of unknown nature and minimal amounts of lipopolysaccharide (LPS) (< 12 ppm).

Immunizations

Male Lewis rats were immunized in each hind footpad with a mixture of 50 μl of the adjuvant dimethyl dioctadecyl ammonium-bromide (DDA) [25] (Eastman Kodak, Rochester, NY) (10 mg/ml) and 25 μg mycobacterial hsp60, 50 μg mycobacterial hsp70, 50 μg *E. coli* hsp70 or 20 μg OM-89 concentrate in PBS.

Intragastrical administration

OM-89 was administered twice a week intragastrically starting 14 days before AA induction. During the first 2 weeks after AA induction, administration was performed three times a week. One oral dose of 0·5 ml contained 2 mg OM-89 concentrate or lyophilisate in PBS. Control animals received PBS only.

AA induction

Rats were immunized intradermally at the base of the tail with 100 μl of 5 mg/ml Mt (heat-killed *M. tuberculosis*) in Freund's incomplete adjuvant (FIA; Difco Labs). The rats were observed for clinical arthritis and were scored by grading each paw from 0 to 4 based on erythema, swelling and deformity of the joint. 0 represents no signs and 4 = severe arthritis. All four legs were scored, so the highest score achievable was 16 [26].

T cell lines

The OM-89 T cell line was isolated from splenocytes of a rat that had received 700 μg OM-89 concentrate in drinking water daily for 20 days. Mononuclear cells were isolated from the spleen by Ficoll–Isopaque gradient centrifugation. The DnaK T cell line was isolated from popliteal lymph node cells (PLNC) 9 days after footpad immunization with 100 μl of *E. coli* DnaK (1 mg/ml in PBS) mixed 1:1 with DDA (20 mg/ml in PBS). All cells were cultured at 37°C, 5% CO_2 for 3 days at 2×10^6–1×10^7 cells/ml in the presence of 10 μg/ml DnaK or 77 μg/ml OM-89 concentrate in Iscove's modified Dulbecco's medium (IMDM) (GIBCO Labs, Grand Island, NY) supplemented with 2% heat-inactivated rat serum, 1% glutamine, 1% penicillin/streptomycin (GIBCO) and 5×10^{-5} M β-mercaptoethanol (β-ME). Subsequently, the T cell lines were propagated for 4–8 days in IMDM supplemented with 10% fetal calf serum (FCS; Seratech, Griesbach IR, Germany), 10% EL-4 supernatant (as IL-2 source), 1% glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids and 5×10^{-5} β-ME. Restimulation of the lines was performed under the same conditions, but with adding 30 Gy irradiated thymocytes as antigen-presenting cells (APC) (4×10^6 line T cells and 1×10^8 APC).

T cell proliferation assays

For the performance of proliferation assays, mononuclear cells were isolated from spleens or lymph nodes and cultured in IMDM supplemented with 5% FCS, 1% penicillin/streptomycin, 1% glutamine and 5×10^{-5} β-ME. Triplicate cultures of 2×10^5 cells per well (0·2 ml) in flat-bottomed microtitre plates, in the presence or absence of antigens, were made. In proliferation assays for the T cell lines, 2×10^4 cells were added to 1×10^6 30 Gy-irradiated thymocytes in volumes of 0·2 ml/well. ^{3}H-thymidine was added after 3 days of culture, and after an additional 18 h cells were harvested on fibreglass filters. ^{3}H-thymidine incorporation was measured by liquid scintillation counting in an LKB 1205

Betaplate. Stimulation indices (SI) were calculated as the mean ct/min of triplicate samples in the presence of antigen divided by the mean ct/min of triplicate samples in the absence of antigen.

Statistical analysis

Results are expressed as the mean ± s.e.m. The statistical significance ($P < 0{\cdot}05$) of data was evaluated by Student's *t*-test of paired samples.

RESULTS

Lewis rats were immunized in the footpads with OM-89 concentrate (40 μg in DDA). Draining PLNC were isolated 21 days later and tested in a lymphocyte proliferation assay in the presence of mycobacterial hsp60, *E. coli* hsp60, mycobacterial hsp70, *E. coli* hsp70 and OM-89 concentrate. As can be seen in Fig. 1, responses to the antigens were absent or low in the PBS/DDA-immunized control lymphocytes, indicating the absence of mitogenic substances in the hsp preparations and in the bacterial extract OM-89. The cells obtained from OM-89-immunized animals responded to the antigens, indicating that OM-89 had the capacity to prime both mycobacterial and *E. coli* hsp60- and hsp70-specific T cells. Furthermore, when OM-89-immunized rats were immunized with mycobacteria in FIA (AA induction protocol) at day 14 after OM-89 immunization, which was the protocol to induce AA, raised responses to mycobacterial and *E. coli* hsp and OM-89 were detected in OM-89-primed animals compared with the controls that were immunized with mycobacteria only (see Fig. 2).

Immunization of rats with mycobacterial hsp was not found to lead to significant proliferative responses to OM-89 (data not shown). However, upon subsequent immunization with mycobacteria in FIA (AA induction) 2 weeks after hsp60 or hsp70 immunization, animals were found to respond at the level of popliteal lymph node- and spleen-derived mononuclear cells not only to hsp60 and hsp70, but also to OM-89 concentrate. Thus, at the polyclonal T cell level, hsp-expanded T cells were capable of recognizing bacterial hsp antigens in OM-89. Figure 2 shows the popliteal lymph node cell responses in mycobacterial hsp60-primed and PBS-primed control rats. Vigorous proliferative responses were obtained to the priming mycobacterial hsp60, but in addition raised responses were seen to the homologous *E. coli* hsp60 and OM-89 concentrate in the immunized animals, indicating the presence of hsp60 (GroEL) in OM-89. Figure 3 shows the

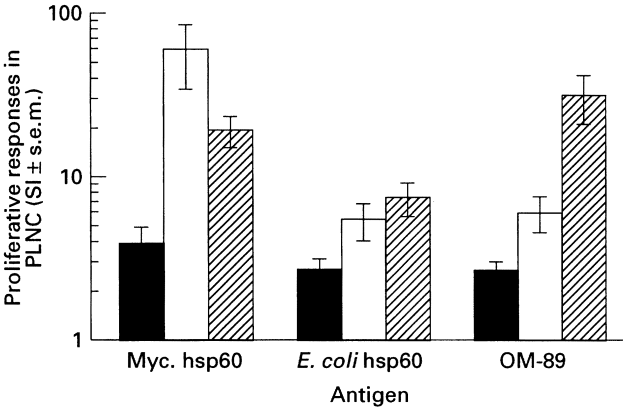

Fig. 2. Proliferative responses to hsp60 (mycobacterial and *Escherichia coli*) and OM-89 in popliteal lymph node cells (PLNC) isolated 35 days after adjuvant arthritis (AA) induction. Immunization of the rats with PBS (■, $n = 4$), mycobacterial hsp60 (□, $n = 4$) or OM-89 (▨, $n = 5$) was performed at day −14 and was followed by *Mycobacterium tuberculosis* immunization at day 0. The results shown are obtained at the optimal antigen concentration of 10 μg/ml for hsp60 and 7·7 μg/ml for OM-89. Background proliferation in unstimulated cultures was 130 + 24 ct/min (mean + s.e.m.) for PBS-immunized rats, 142 + 65 ct/min for Myc. hsp60-immunized rats and 134 + 26 ct/min for OM-89-immunized rats. Differences between PBS controls and OM-89-immunized rats were all significant ($P < 0{\cdot}05$). Differences between PBS controls and hsp60-immunized rats were not all significant (*E. coli* hsp60 and OM-89).

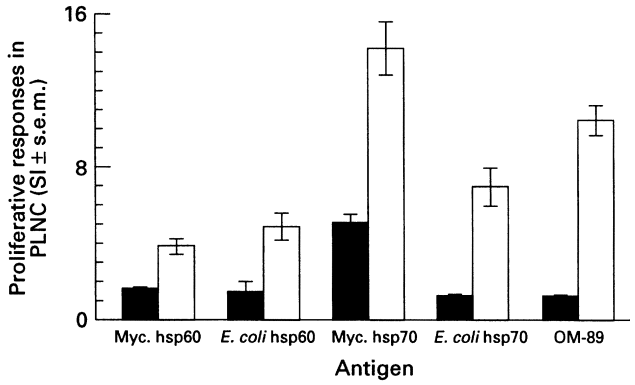

Fig. 1. Proliferative responses to hsp60, hsp70 (mycobacterial and *Escherichia coli*) and OM-89 in popliteal lymph node cells (PLNC) isolated 9 days after immunization with PBS or OM-89. The results shown are obtained at the optimal antigen concentration of 10 μg/ml for hsp60 and hsp70 and 7·7 μg/ml for OM-89. Background proliferation in unstimulated cultures was 4636 + 976 ct/min (mean + s.e.m.) for PBS-immunized rats (■, $n = 3$) and 4128 + 304 ct/min for OM-89-immunized rats (□, $n = 3$). Differences observed between PBS controls and OM-89-immunized rats were all significant ($P < 0{\cdot}05$).

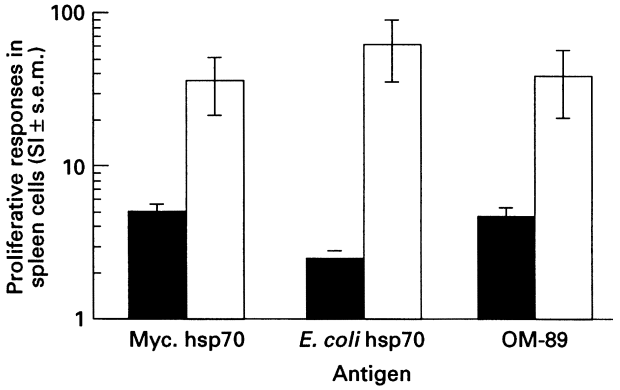

Fig. 3. Proliferative responses to hsp70 (mycobacterial and *Escherichia coli*) and OM-89 of spleen cells isolated 59 days after adjuvant arthritis (AA) induction. Immunization of the rats with PBS (■, $n = 4$) and *E. coli* hsp70 (□, $n = 4$) was performed at day −14 and was followed by *Mycobacterium tuberculosis* immunization at day 0. The results shown are obtained at the optimal antigen concentration of 10 μg/ml for hsp70 and 7·7 μg/ml for OM-89. Background proliferation in unstimulated cultures was 756 + 118 ct/min (mean + s.e.m.) for PBS-immunized rats and 260 + 99 ct/min for *E. coli* hsp70-immunized rats. Differences between PBS controls and hsp70-immunized rats were significant for mycobacterial and *E. coli* hsp70 ($P < 0{\cdot}05$). The data for OM-89 were not significant.

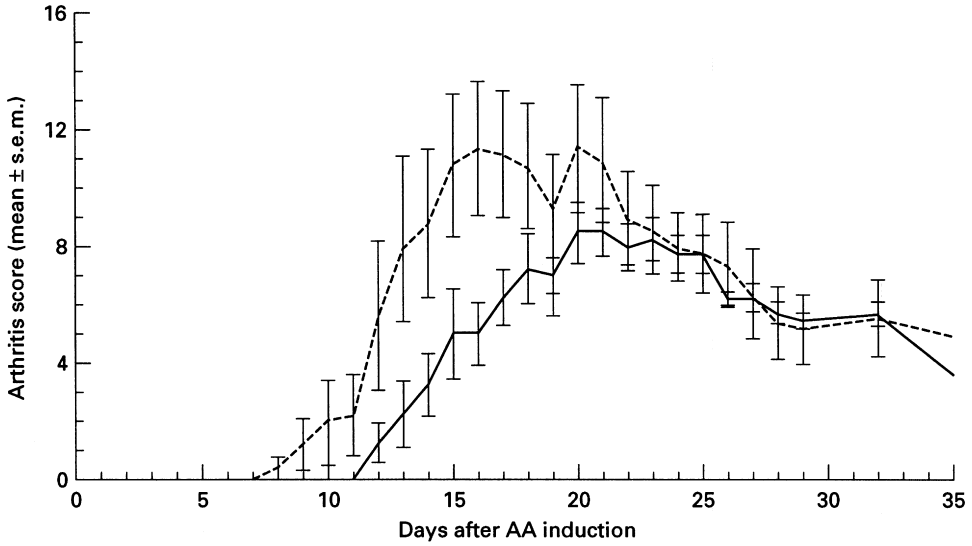

Fig. 4. Effect of parenteral immunization in the hind footpads 14 days before adjuvant arthritis (AA) induction. Rats received 20 μg OM-89 (- - - - -, $n = 5$) or PBS (———, $n = 4$) in 10 mg/ml dimethyl dioctadecyl ammonium-bromide (DDA) in PBS in each hind footpad. At day 0, 5 mg/ml *Mycobacterium tuberculosis* in Freund's incomplete adjuvant (FIA) were injected intradermally at the base of the tail for AA induction. Differences between controls and OM-89-immunized rats were not statistically significant.

spleen cell responses of *E. coli* hsp70 (DnaK)-primed and PBS-primed control rats. In the DnaK-primed rats vigorous responses were obtained to all three antigens: the antigen used for immunization *E. coli* hsp70 (DnaK), the mycobacterial hsp70 homologue, and OM-89 concentrate, indicating the presence of hsp70 (DnaK) in OM-89.

In subsequent experiments parenterally OM-89 immunized animals were immunized 14 days later with mycobacteria in FIA in order to induce AA. As shown in Fig. 4, AA was seen to develop in both the OM-89/DDA-immmunized and the PBS/DDA control animals. Arthritis started already at day 7 after immunization in the OM-89 group, whereas the PBS control animals started to develop disease at day 11 after immunization. Furthermore, severity was increased in the OM-89-immunized animals. Although OM-89 immunization had not been found to induce arthritis (not shown), a subsequently induced AA started earlier, and had a more severe course.

Upon oral administration of OM-89, however, an arthritis-protective effect was obtained. Figure 5 shows the results of three distinct oral administration regimens where OM-89 concentrate or

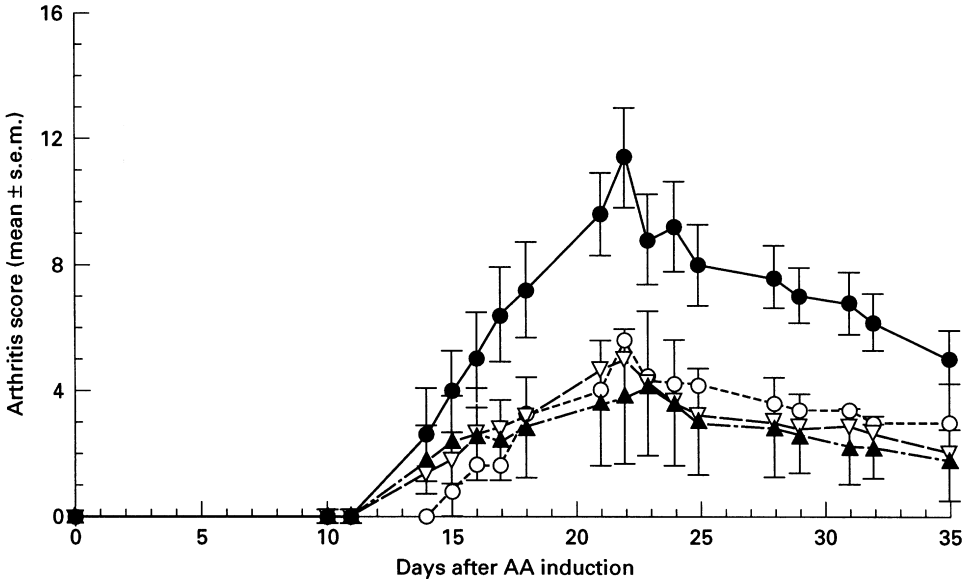

Fig. 5. Effect of oral administration at days −14, −10, −7, −3, 0, 2, 4, 7, 9, 11 and 14 of 2 mg OM-89 concentrate (group 3) or 2 mg OM-89 lyophilisate (dissolved in 0·5 ml PBS) (group 4) on the severity of adjuvant arthritis (AA) development. AA was induced at day 0. Groups 1 and 2 received (orally) PBS or 2 mg OM-89 concentrate on extra days 16, 18, 21, 23, 25, 28, 30, 32 and 35. The differences between PBS control rats and OM-89-treated animals were statistically significant ($P < 0{\cdot}05$). ●, PBS day 35, $n = 5$; ○, 2 mg concentrate day 35, $n = 5$; ▲, 2 mg concentrate day 14, $n = 5$; ▽, 2 mg lyophilisate day 14, $n = 5$.

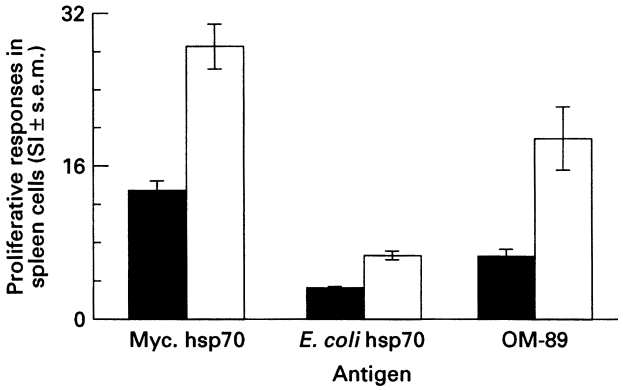

Fig. 6. Proliferative responses to myc. hsp70, *Escherichia coli* hsp70 and OM-89 in spleen cells obtained 35 days after adjuvant arthritis (AA) induction and a regimen of oral OM-89 up to day 14 after AA induction on day 0, as in Fig. 5. Background proliferation in unstimulated cultures was 2729 + 461 ct/min (mean + s.e.m.) for PBS immunized rats (■) and 2456 + 246 ct/min for OM-89-treated rats (□). Differences observed between PBS controls and OM-89-treated rats were all significant ($P < 0{\cdot}05$).

lyophilisate was given intragastrically twice a week. As can be seen in Fig. 5, arthritis development was inhibited ($P < 0{\cdot}05$) in the OM-89-treated animals. There were no differences between the OM-treated groups. Animals treated with OM-89 until day 14 were as protected as animals under continued treatment up to day 35 after AA induction.

In rats treated with oral OM-89, proliferative T cell responses in the spleen were measured 35 days after AA induction. As can be seen in Fig. 6, oral OM-89 stimulated raised proliferative responses to myc. hsp 70, *E. coli* hsp70 and OM-89. In animals treated for a longer period (up to day 35) these responses had disappeared again (not shown).

In order to obtain a more direct proof of the presence of immunogenic hsp T cell epitopes in OM-89, a number of T cell lines were generated using OM-89 or hsp60/70 as an immunogen. In Figs 7 and 8 the antigen specificities of two of such lines are given. Figure 7 shows the responses of a line raised against OM-89 concentrate. Vigorous proliferative responses were seen in the presence of OM-89 concentrate, the *E. coli* hsp70 (DnaK) and the mycobacterial hsp70. Responses to hsp60 were present to a lower extent compared with responses to hsp70, indicating a more

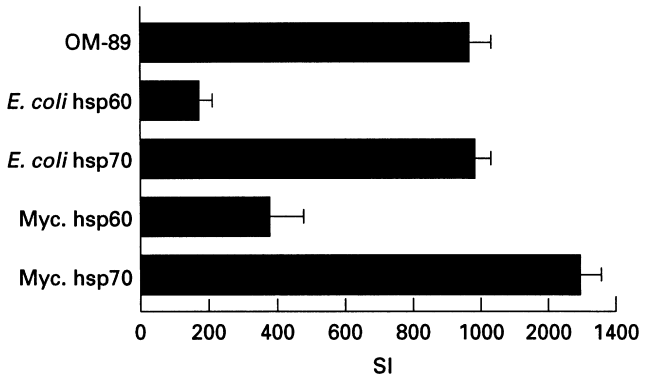

Fig. 7. Proliferative responses of OM-89 T cell line to hsp antigens at a concentration of 10 μg/ml and OM-89 at 20 μg/ml.

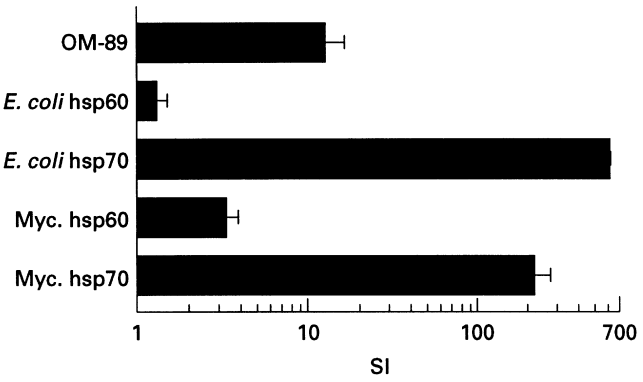

Fig. 8. Proliferative responses of *Escherichia coli* hsp70 T cell line to hsp antigens at a concentration of 10 μg/ml and OM-89 at 20 μg/ml.

abundant presence of hsp70. The capacity of OM-89 to expand hsp70-specific T cell populations is clearly demonstrated by this line. Conversely, as shown in Fig. 8, a line raised against *E. coli* hsp70 (DnaK) recognized, besides the antigen the line was raised against, the homologous mycobacterial hsp70 and OM-89 concentrate. The absence of responses of the DnaK line to *E. coli* (GroEL) and mycobacterial hsp60 indicated the absence of any contaminating hsp70 in these recombinant antigens and the high specificity for the hsp70 protein.

DISCUSSION

The presence of bacterial hsp in OM-89 had been suggested previously, on the basis of the reactivity of some selected hsp60-specific antibodies with OM-89 in Western blots [4]. The present findings have given the evidence that the presence of such hsp can be recognized by specific T cells, that they are capable of priming for raised secondary T cell responses, and finally that besides hsp60, also hsp70 is present in OM-89 concentrate.

As shown in Fig. 1, a single immunization with OM-89 was seen to prime hsp60- and hsp70-specific T cells. The response to *E. coli* hsp was similar to that against mycobacterial hsp, indicating cross-recognition of the mycobacterial homologue, evidently due to sequence homologies present between *E. coli* and mycobacterial hsp. Apparently, most epitopes primed by OM-89 immunization were conserved between *E. coli* and other (myco)bacterial hsp. Immunization with recombinant purified hsp60 or hsp70 was not seen to lead to direct T cell responses to OM-89 (not shown). That priming for OM-89 antigens had taken place was shown indirectly by the fact that upon subsequent immunization with mycobacteria for AA induction, raised responses to OM-89 in the hsp-immunized rats were obtained (Figs 2 and 3). This was especially the case for hsp70-primed animals, indicating the relative abundance of hsp70 in OM-89. An explanation for the fact that the OM-89 by itself was capable of immunizing for hsp60 responses, whereas hsp60-immunized rats without mycobacterial immunization did not respond to OM-89, is that hsp in isolation may induce regulatory (Th2?) cells with a lower proliferative capacity, whereas OM-89 contains additional moieties that direct the hsp-specific T cell responses into a more proliferative (Th1?) mode.

Immunization with DnaK, the *E. coli* hsp70, was seen to prime for the most vigorous OM-89-specific secondary T cell responses (Fig. 3), indicating that hsp70 is, in fact, one of the more dominant antigens of OM-89. This is of interest, since recent

observations have shown that hsp70 immunizations protected against experimental arthritis [10], as was shown previously for hsp60.

The observations made with some T cell lines raised against OM-89 were also indicative of a prominent presence of hsp70 in the material. As shown in Fig. 7, positive responses of this line were obtained in the presence of DnaK (*E. coli* hsp70). In agreement with that, the DnaK-specific cell line responded to OM-89 (Fig. 8).

The now documented presence, in an immunogenic T cell-activating manner, of hsp60 and hsp70 in OM-89 offers possible explanations for the various earlier documented effects of OM-89 in experimental autoimmune diseases, including diabetes [23], arthritis [20] and SLE [23], as immunity to hsp has been described as a common feature occurring in such diseases [27–30].

Parenteral OM-89 immunization did not protect against AA (Fig. 4), despite the fact that it induced hsp-specific proliferative T cell responses. In fact, arthritis was seen to develop even in a more aggressive manner than in the absence of OM-89 immunization. It is possible that we have here a situation similar to what has been documented in the case of mycobacteria. Immunization with whole mycobacteria was seen to induce T cell responses to a number of different T cell epitopes, amongst which the response directed against the arthritogenic 180–188 epitope of hsp60 was dominant. In contrast to this, upon immunization with purified mycobacterial hsp60, in the absence of additional mycobacterial immunogenic substances, the responses directed to other hsp60 epitopes dominated [31]. Along similar lines, one could suppose that, as mentioned above, OM-89 may contain moieties that upon parenteral immunization drive the response into the aggressive, non-protective mode of (Th1) T cell responsiveness.

Oral administration of OM-89, however, was found to induce a relative state of resistance against AA (Fig. 5). Interestingly, a raised level of proliferative T cell responses for hsp70 and OM-89 was noted in such orally treated rats (Fig. 6). In contrast to parenteral administration, oral administration is known to be the preferred route for the induction of tolerance or the induction of a T cell response of a regulatory nature, with the potential of enforcing a dominant form of homeostatic immunological tolerance. For such regulatory activities several cytokines, such as transforming growth factor-beta (TGF-β), but also the Th2-associated cytokines IL-4 and IL-10, have been held responsible [32]. Given the fact that parenteral immunization with OM-89 has now been shown to induce hsp-specific proliferative responses, it is quite possible that oral administration of OM-89 is triggering hsp-responsive T cells in such a way that these cells adopt the Th2 regulatory phenotype. Upon subsequent recognition by such hsp-responsive T cells of the self hsp homologue as expressed on the site of inflammation, the cells may exert their regulatory activity, by causing a bystander suppression of T cells in their immediate vicinity. Transfer experiments using T cells obtained from oral OM-89-treated rats will have to be performed in order to clarify this point.

Various studies, so far, have indicated the potential relationships between immunity to hsp and the development of arthritic diseases, both in experimental animals and in humans. At a mechanistic level several explanations for the existence of such relationships have been postulated, including mimicry relationships between microbial and self (host) hsp molecules, leading to either disease-inducing or disease-protective T cell-mediated immunological events. The present findings on hsp and their immunogenic presence in the anti-arthritic drug OM-89 can be seen as being supportive of this postulated relationship between hsp and arthritic diseases. Moreover, these findings would suggest that hsp may be antigens that can be used for the induction of therapeutic oral tolerance in rheumatoid arthritis.

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