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Presence of hsp65 in bacterial extracts (OM-89): a possible mediator of orally-induced tolerance?

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Abstract. Heat shock proteins (HSP) have been implicated in rodent models of autoimmunity, particularly arthritis, and there is suggestive though inconclusive evidence that they may also play a role in human autoimmune disease. The simplest hypothesis is based on molecular mimicry due to the amino-acid sequence homology between mammalian and microbial HSP. Recently OM-89, an extract of several strains of *Escherichia coli*, has shown some efficacy in the treatment of rheumatoid arthritis (RA) when taken orally. Using species-specific antibodies, we show here that OM-89 contains the 65 kDa HSP (hsp65), while hsp65 was not detected in another bacterial extract containing other microorganisms, including *Staphylococcus aureus* (OM-85). We suggest that if the human homologue of hsp65 is a relevant target antigen in the human disease, the efficacy of the preparation could be due to induction of oral tolerance or to switching the Th1 response towards Th2. Alternatively, even if the human hsp65 is not a target molecule in RA joints, OM-89 may evoke bystander suppression of joint inflammation via induction of TGFβ-secreting effector cells. These hypotheses should be tested in further studies.

Key words. *Escherichia coli* GroEL; hsp65; oral tolerance; rheumatoid arthritis.

Heat shock proteins (HSP) represent a highly conserved group of proteins with homologues in all species examined to date[1]. HSP are usually classified according to their apparent molecular weight, within families containing proteins both constitutively expressed and/or up-regulated under most stressful conditions. HSP function as molecular chaperones[2] and protect cells under stressful conditions. HSP also play pleiotropic roles in the immune system[3]. Members of the hsp65 and hsp70 families are immunodominant antigens in a wide spectrum of bacteria and parasites, inducing both HSP-specific T-cells and antibodies, and members of the hsp70 family may contribute to antigen processing and presentation[4–6].

The interest in HSP in relation to arthritis was initiated by studies of rat strains that develop arthritis when injected with killed mycobacteria suspended in oil. It was observed that the mycobacterial antigen recognized by an arthritogenic T-cell clone derived from such rats was the mycobacterial homologue of hsp65[7]. It subsequently emerged that hsp65 is protective in several rodent models of arthritis[8–10].

The possibility that hsp65 plays a role in human autoimmune diseases such as rheumatoid arthritis (RA) or diabetes remains controversial[11–13]. Some T-cells, even from normal donors, recognise epitopes that are conserved in mycobacterial and human hsp65[11,14], which emphasizes the potential for autoreactivity. There is a report of increased expression of hsp65 in synovial tissue of juvenile RA[15], although expression appeared to be at normal constitutive levels in tissues from adult RA[16], and other reports of elevated levels of antibody to mycobacterial hsp65 in RA[17,18]. Hsp65-responsive T-cells have been found in RA synovial fluid, but it is difficult to determine to what extent they are present in greater numbers than would be expected by chance[19,20].

The bacterial extracts OM-89 (derived from *Escherichia coli*) and OM-85 (derived other microorganisms, including *Staphylococcus aureus*) have been used as immunostimulants[21–23]. In vitro these extracts can activate the production of superoxide anions, the synthesis of intracellular pro-Interleukin-1β (IL-1β), and the induction of a 78 kD doublet, probably corresponding to the glucose-regulated protein grp78 (BiP)[24]. More recently it has been shown that OM-89 may also be active as an immunomodulating agent and some clinical efficacy has been observed in vivo in the treatment of RA[25,26]. The use of OM-89 is particularly interesting as the treatment has no obvious side-effects, and a better understanding of the mechanisms underlying its beneficial effects is of interest to both patients and clinicians.

OM-89 and OM-85 are likely to contain the immunodominant antigens present in the bacteria from which they are derived, such as HSP, and in view of the tentative implication of HSP in the aetiology of RA, the

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presence of HSP in these extracts is of interest. This report deals with the possible presence of HSP – and more specifically, hsp65 – in these extracts. Interestingly, hsp65 was detectable solely in the extract which has proven of benefit in RA, i.e., OM-89.

Material and methods

Bacterial extracts. OM-89 was prepared as a lyophilized extract from soluble components of combined alkalinized fractions from selected *Escherichia coli* strains. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed a continuous spectrum of peptides ranging from 10 to 300 kDa. OM-85 in contrast was prepared from 8 different bacteria species, including *Staphylococcus aureus* and *Klebsiella pneumoniae*[24]. These two extracts are therefore likely to differ in the type and/or the quantity of HSP present, if any. During the course of these experiments, preparations selected for molecular weights ranging from 50 to 100 kDa have also been used. We had hypothesized that this procedure would allow us to concentrate the proteins of interest, i.e., those with molecular weights around 60–65, and would lead to more clear-cut results. However, this was not the case and only the data for the final extracts are presented here. We also did several preliminary dose-response tests. As expected, loading greater concentrations of extract on the gel led to higher levels of hsp65 being detected, while above 300 µg extract we did not obtain adequate penetration of the extracts into the gels. A total of 10 preparations for OM-89 (8 concentrates, 1 of which was neutralized, 1 concentrate after 50–100 kDa fractionation by dialysis and 1 lyophilized) and 5 preparations for OM-85 (4 concentrates, 1 of which neutralized, and 1 lyophilized) was used in these experiments. Results from two representative experiments are shown.

Cells. Heat-shocked human monocytes were used as positive controls for human hsp65. Monocytes were isolated from peripheral blood of healthy donors by gradient centrifugation and purified by adherence[27]. Cells were maintained in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 10% foetal calf serum (FCS) (Gibco) and 1% glutamine (Gibco) (complete medium, CM). Cell cultures were incubated at 37 °C in a humidified incubator (95% air and 5% CO_2).

Exposure to heat shock. Human PBM were cultured in CM at 37 °C for 24 h. Cells were heated in 25 mM HEPES-containing RPMI to 44 °C for 20 min in a water bath, then returned to a 37 °C incubator for 2 h. Sodium dodecyl sulfate extracts of these cells served as the positive control for expression of human hsp65. Mycobacterial hsp65, obtained from WHO (Geneva), was used as the positive control for bacterial hsp65.

Antibodies. The antibodies used were:

1) 5082 (a kind gift from H. Rosenberg, NIH), an antiserum directed against the GroEL of *Escherichia coli*[28]. Antiserum 5082 was raised in rabbits against *Escherichia coli* GroEL.

2) 4B9/89, a monoclonal antibody (mouse IgG2a, ascitic fluid) to human hsp60[16], was developed and described by one of us (G. Rook).

Western blot analysis. Proteins were resolved by reducing polyacrylamide gel electrophoresis according to Laemmli[29], then electrotransferred onto nitrocellulose membranes. The membranes were saturated with casein- or bovine serum albumin (BSA)-containing buffer and hybridized with the appropriate monoclonal antibodies overnight. Antibodies were revealed using anti-rabbit peroxidase (Sigma) for 5082 and anti-mouse peroxidase (Sigma) for 4B9/89. The positive control (mycobacterial hsp65) was incubated either with the same gel as the test conditions, or separately.

Results

Specificity of antibodies. Western blotting of extracts from monocytes exposed to heat shock (HS) (see 'Materials and methods') and the purified mycobacterial hsp65 indicated that the antibodies used were species-specific. No cross-recognition of human and mycobacterial hsp65 was observed (fig. 1).

Along these lines we have previously reported that repeated immunization of mice with conjugated constructs containing the mycobacterial hsp65 induces antibodies that cross-react with hsp65 of other bacteria, but not with the human protein[30].

Recognition of hsp65 in OM-89 but not in OM-85. Analysis of the bacterial extracts OM-89 and OM-85 by Western blotting revealed the presence of bacterial hsp65 as recognized by the Ab 5082 in OM-89,

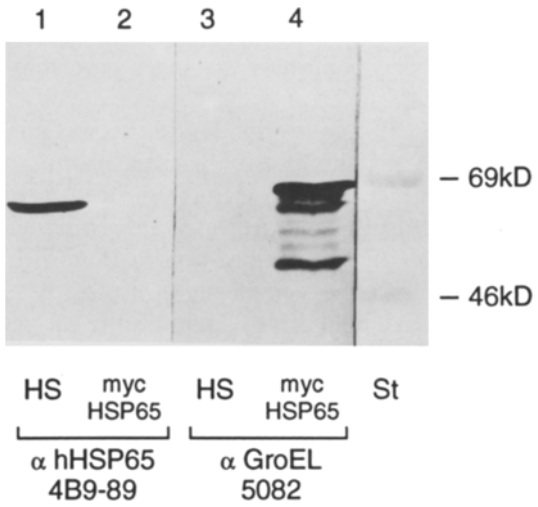

Figure 1. Specificity of the anti-hsp65 antibodies used in this study for the mycobacterial hsp65/GroEL (5082) and the human inducible hsp60–65 (4B9-89).
HS: heat-shocked human monocytes. Note that 5082 also recognizes some degradation products of hsp65.

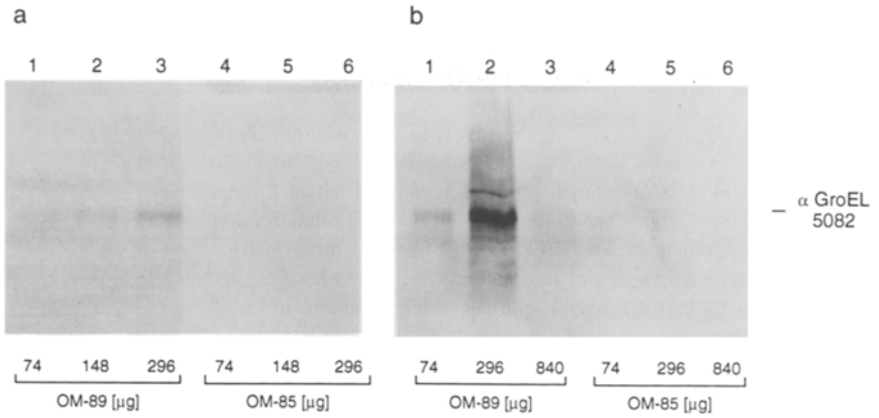

Figure 2. Western blot of bacterial extracts OM-85 and OM-89 with the anti GroEL antibody 5082.
Panels *a* and *b* show similar Western blots with differing concentrations of bacterial extract. Lanes 1–3 OM-89, lanes 4–6 OM-85 on both blots. Staining was as described in 'Materials and methods', with visualization by anti-mouse peroxidase. The apparent quantitative differences in hsp65 expression in the two experiments relate to the fact that in *a*) the positive control was included in the same membrane, while in *b*) it was incubated with the antibody separately, indicating competition between the positive control and the test for antibody binding.

reaching a maximum when 296 µg of protein extract was loaded onto the gel. When higher concentrations of extract were used, the proteins appeared not to enter the gel adequately (fig. 2b, lane 3) thus preventing antibody recognition of hsp65. In contrast, OM-85, which was derived from different bacterial species but not *E. coli*, did not appear to contain an HSP detectable with the polyclonal antibody to the *E. coli* protein at any concentration tested (fig. 2a, lanes 4–6; fig. 2b, lanes 4–6). Furthermore, we confirmed for mycobacterial hsp65 the previously described lack of recognition by 4B9-89[16] (fig. 3).

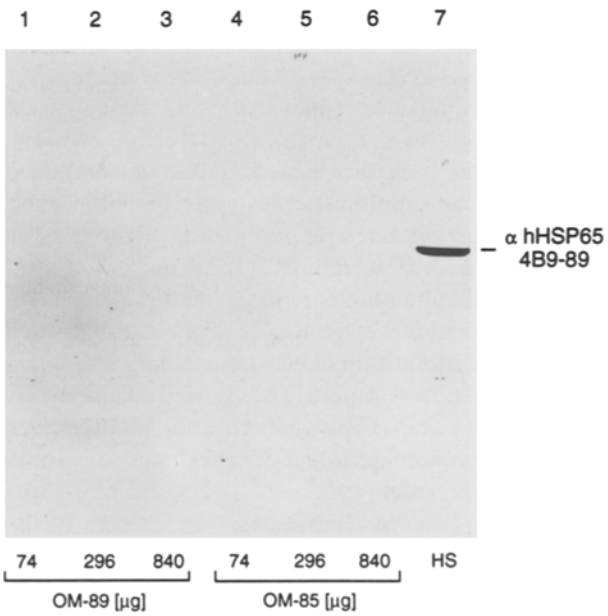

Figure 3. Western blot of bacterial extracts OM-85 and OM-89 with antibody 4B9-89.
Western blot performed as explained in 'Materials and methods'. Visualization of primary antibodies was with anti-rabbit peroxidase. Lanes 1–3 show increasing concentrations of OM-89, lanes 4–6 increasing concentrations of OM-85, lane 7 positive control of heat-shocked monocytes with the antibody against human hsp65 (4B9-89).

Discussion

Our results indicate that OM-89 does contain bacterial hps65, which we demonstrated with the use of species-specific antibodies. Based on this observation, it is plausible to imagine a link between the presence of hsp65 in OM-89 and the protective effects of the drug observed in RA[25,26]. There is some evidence to suggest that OM-89 given orally has a disease-suppressive effect in RA. Interestingly, the effects of OM-89 are only observed within the dose range used in experimental models of oral desensitization, while higher doses do not have an additional effect[31].

The existing literature suggests two types of mechanism by which hsp65 may work. First, the human HSP, or some other autoantigen cross-reactive with the HSP, may be directly involved as a target antigen in RA. This appears to be the case in adjuvant arthrithis in the rat. Animals can be protected from the disease by pre-immunization with the whole protein, or with relevant peptides in aqueous solution or in incomplete Freund's adjuvant[38], and the same is true for the streptococcal cell wall[39] and pristane arthritis models[31]. One possible interpretation of these data is that the pre-immunization drives a Th2 response. Despite the detection of mRNA for IL-2 and IFNγ[33], there is no direct evidence to indicate that Th1 cytokines are the major force responsible for the joint pathology. Indeed, while Th1 cytokines may provide the initial stimulus and participate in perpetuating the cytokine loop, the major cytokines seem to be those produced by macrophages and fibroblasts[34]. Treatment of arthritis with cyclosporin A (a

specific inhibitor of Th1 cells) does appear to provide some relief but only in a few cases[35]. The switch to a Th2-like cytokine profile may thus manifest itself as suppression by antagonizing any Th1-perpetuating factors.

The alternative hypothesis involves 'bystander suppression', and requires the orally administered antigen, or a cross-reactive host antigen, to be present in the inflammatory site but not assume that this antigen is a target of the autoimmune response. This concept is derived from the observation that oral intake of an antigen that is present at an inflammatory site, although not known to be a target of the T-cells mediating that inflammation, can lead to the suppression of the inflammation. The phenomenon may be explained by the orally induced priming of a novel type of T-'suppressor' cells which are able to secrete TGFβ, IL-4 and IL-10[36,37] in the lesions. This may explain the ability of oral type II collagen to prevent pristane arthritis in mice[38], or adjuvant arthritis in rats[39]. Collagen type II is not implicated in these models, but it is inevitably present in the inflamed joints. Similarly the evidence for autoimmunity to type II collagen in human RA is tenuous, yet patients may have benefited from oral intake of this protein[40].

In rats, it has been shown that oral administration of OM-89 induces suppressor cells in the gut and increases intestinal IgA production, mechanisms implicated in induction of oral tolerance[41]. In cases of adjuvant arthritis, use of OM-89 decreases the development of the disease without exhibiting a direct anti-inflammatory effect[42]. Moreover colonization of the gut of germ-free rats by *E. coli* suppressed adjuvant arthritis[43]. At this stage it is not known whether these effects were Th1-to-Th2 switching, bystander effects, or some other form of suppression.

Before any firm conclusions can be drawn, further analyses of other components found within this extract need to be performed. Meanwhile the hypothesis that peptides given orally can elicit a regulatory response capable of decreasing joint inflammation in RA is worth serious consideration. The recent report of relief from symptoms of autoimmune encephalomyelitis in rats by Chen et al.[36] is encouraging and their description of a novel T-cell cytokine profile and may help clarify the situation. Investigation of the pattern of cytokines secreted by T-cells following oral intake of OM-89 may also show this shift to TGFβ production. Alternatively, it may be the antibody response to the *E. coli* hsp65 which is important.

The precise function(s) of HSP and particularly hsp65 in autoimmune diseases are still a matter of debate[44], and it is becoming increasingly apparent that the genetic background of an individual is central not only to the progression of certain autoimmune diseases but also the manner in which they should be treated. We are currently examining the possibility of polymorphism of the regulatory regions of HSP genes mapping to the major histocompatibility complex and their association with diseases.

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