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ORIGINAL ARTICLE

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IgG and IgA antibody titers against human heat-shock protein (hsp60) in sera of rheumatoid arthritis and osteoarthritis patients

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Abstract To learn whether heat-shock proteins (HSP) are involved in the pathogenesis of rheumatoid arthritis (RA), antirecombinant human heat-shock protein 60 (hsp60) IgG and IgA in sera of RA and osteoarthritis (OA) patients were investigated. Only the anti-hsp60 IgG titer of seropositive (RF-positive) patients was found to be elevated. Although RF titers of the sera of seropositive RA patients were increased, there was no correlation between the individual anti-hsp60 IgG titer and the corresponding RF titer. In contrast, all the anti-hsp60 IgA titers of the sera of OA, seronegative RA, and seropositive RA patients were found to be elevated. Among them, only the serum IgA concentration of seropositive RA patients was increased. Thus, it was suggested that the increased anti-hsp60 IgG reflects the pathogenesis of RA and its activity. It was also suggested that the increased anti-hsp60 IgA response reflects an involvement of hsp60 in the pathogenesis of arthritides rather than the pathogenesis of RA.

Key words Heat-shock protein (HSP) · IgA · Rheumatoid arthritis (RA)

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Introduction

Heat-shock proteins (HSP) are highly conserved proteins from bacteria to mammals. Many factors are known to induce HSP, including environmental and pathophysiological stresses.[1–5] In the last decade, HSPs have been thought to be involved in the pathogenesis of rheumatoid arthritis (RA). This was suggested by the fact that T-cell-mediated responses to the mycobacterial 65 kDa heat-shock protein (hsp65) are implicated in the pathogenesis of adjuvant arthritis in rats.[6–9] Correspondingly, a significant increase in IgG antibody titer and enhanced T cell proliferative responses towards hsp65 were found in the sera and synovial infiltrated lymphocytes, respectively,[10–17] of RA patients. However, it has also been reported in recent years that there is no significant difference in anti-hsp65 IgG titer between RA patients and normal adults.[18,19] To date, all the measurements of anti-HSP antibody have been performed using mycobacterial hsp65. Although hsp65 has a 60% homology with human hsp60 in amino acid sequence,[20–22] it is also important to know the antibody titers towards human hsp60. Here, we developed the recombinant human hsp60 and evaluated the antibody titer in the sera from RA patients, osteoarthritis (OA) patients, and normal adults. This study is the first in which the antihuman hsp60 titers in RA and OA have been evaluated.

Materials and methods

Preparation of human recombinant hsp60

The cDNA library was constructed from mRNA from a human histiocytic lymphoma cell, U937. The hsp60 coding DNA was amplified by a polymerase chain reaction with two kinds of primers, UPBam (5′-ACGCTTGCCGCGG ATCCGCAGAAATGCTTCGGTT-3′) and DSP1 (5′-CACAGTTCATTAATAAAGGTAAAGCACTAGT-3′). The resulting gene was ligated to the pUC119 vector and transformed into *E. coli* DH5 aF′ to isolate a clone. A

clone containing the hsp60-coding gene without a leader sequence was isolated and transformed into *E. coli* JM109. pUC119-derived proteins were isolated from the lysate of recombinant *E. coli* strains by a sequential procedure of gel-chromatography (Superdex 200pG, Pharmacia, Uppsala, Sweden) and anion-exchange chromatography (Mono Q HR, Pharmacia). The purified hsp60 showed a single band on SDS–PAGE analysis.

Serum donors

One hundred and fifty-seven patients with established RA and 68 patients with OA attending the rheumatology unit of Matsuyama Red Cross Hospital and Ehime University Hospital in Japan were included in the study (Table 1). Serum samples from 99 normal donors with a similar age distribution to the RA and OA patients were obtained from the Matsuyama Health Check Laboratory.

Rheumatoid factor, serum IgG, and IgA concentrations

Titers of rheumatoid factor and both serum IgG and IgA concentrations were measured by nephelometry (Behring) according to the manual for the clinical laboratory test.

Enzyme-linked immunosorbent assay (ELISA)

Purified hsp60 dissolved in 20 mM borate buffer, pH 8.9, at a concentration of 10 μg/ml, was coated onto an EIA/RIA microplate (Coster, Cambridge, MA, USA) by incubation for 2 h at room temperature. In some experiments, human serum albumin (HSA, Cohn fraction V) was used for the coated antigen. After blocking with gelatin, 0.1 ml 20 mM phosphate-buffered saline, pH 7.2, containing 2 mM EDTA, 0.1% (w/v) gelatin and 0.01% Tween 20, was poured into each well. After the addition of 10 μl 100-fold diluted serum sample into each well, the plate was incubated for 2 h at room temperature. After washing with 20 mM phosphate-buffered saline, pH 7.4, containing 0.01% Tween 20 (PBST), 100 μl of either 2000-fold diluted horse radish peroxidase (HRPO)-conjugated goat antihuman IgG (Jackson Immuno Research, West Grove, PA, USA) or HRPO-conjugated goat antihuman IgA antibody (Zymed, San Francisco, CA, USA) was added to each well and then incubated for 1 h at room temperature. After washing with PBST, 100 μl 0.91 mM 2,2′ azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) solution in 0.1 M citrate buffer, pH 4.1, containing 0.04% H_2O_2, was added. After 30 min incubation at room temperature, 100 μl 0.01% NaN_3 in 0.1 M citric acid was added to stop the reaction. The optical density at 415 nm was measured using a microplate reader (EL 340: Bio-Tek Instruments Inc., Winooski, VT, USA). A serum pool from 500 healthy donors was used as a standard anti-hsp60 antibody solution. Serially diluted standard solutions were included in each assay plate, and the titer of each sample was calculated by comparing it to the standard, which was designated to have anti-hsp60 IgG and IgA titers of 100 units/ml.

Statistical analysis

The significance of the difference of means between groups was tested by Student's *t*-test. Correlations were examined using Spearman's rank correlation method. Statistical analysis was carried out using the StatView J 4.02 software package.

Table 1. Subjects

Subjects	Male	Female	Total	Age (mean)
Normal controls	38	61	99	33–75 (55.1)
OA patients	19	49	68	40–90 (64.6)
RA patients	24	133	157	34–83 (61.0)
Total	81	243	324	33–90 (60.2)

OA, osteoarthritis; RA, rheumatoid arthritis

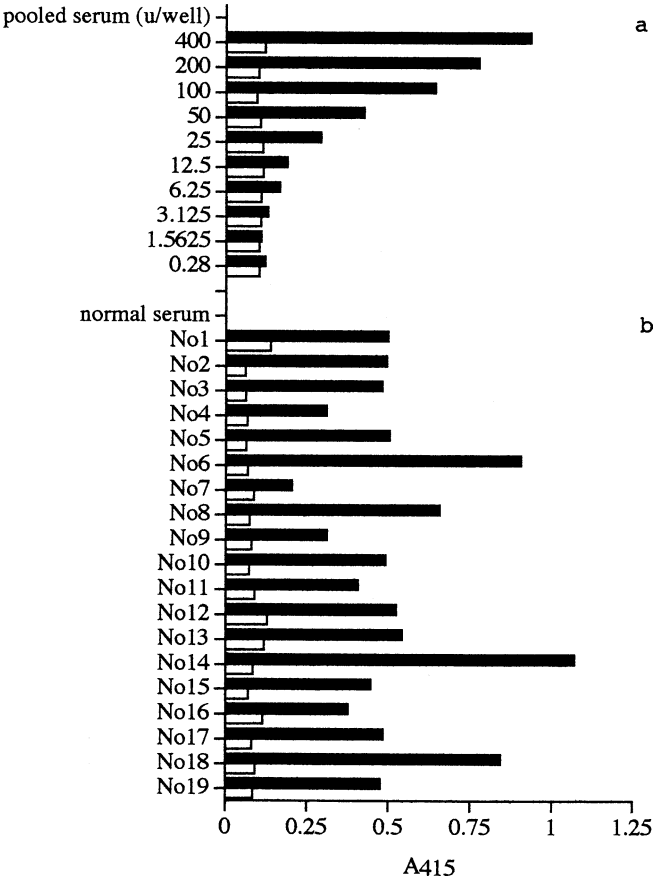

Fig. 1. IgG reactivity of normal human serum towards hsp60 (*solid bar*) and HSA (*open bar*). **a** Titration of the pooled sera from 500 healthy controls. **b** Titration of the 19 individual sera of normal healthy controls

Results

ELISA system

To check the ELISA system for detecting the anti-hsp60 antibody, titration was performed using pooled sera from 500 healthy controls. A dose-dependent binding of IgG to hsp60, but not to HSA, was revealed by the serially diluted pooled serum (Fig. 1a). Nineteen individual sera from healthy controls were examined (Fig. 1b). A higher level of IgG-binding to hsp60 than to HSA was found in all the sera tested. The absorbance at 415 nm of the anti-hsp60 IgG measuring system varied (0.203–1.07), whereas that of anti-HAS was within the background level (0.06–0.137). The anti-hsp60 IgG binding of the sera from two individuals to the coated antigen was dose-dependently inhibited by the presence of soluble hsp60 (Fig. 2). To avoid error in the anti-hsp IgG and IgA titration among plates, a standardization using serially diluted pooled serum was employed in each plate.

Anti-hsp60 IgG and IgA titers, and IgG and IgA concentrations of the sera from normal healthy controls, OA patients, and RA patients

The results of anti-hsp60 IgG and IgA titration and the measurement of serum IgG and IgA concentrations are summarized in Table 2. A significant difference in the mean value of the anti-hsp60 IgG titer was found between RA patients and normal adults ($P = 0.0031$), but not between OA patients and normal adults. The mean values of anti-hsp60 IgA titers of both RA and OA patients were significantly elevated compared with those of normal adults

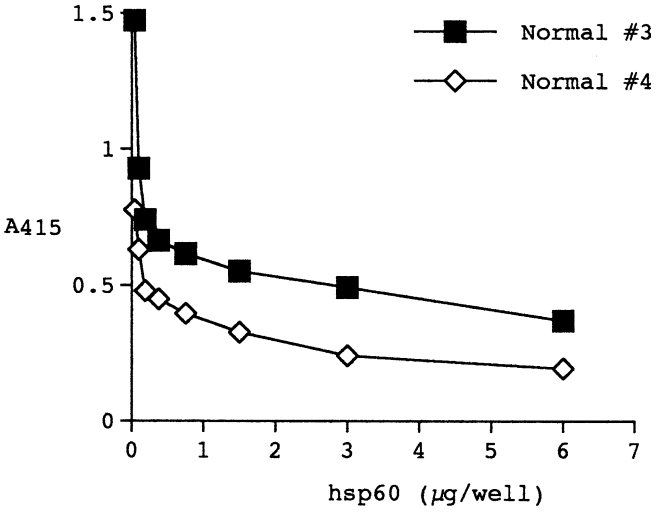

Fig. 2. Inhibition of IgG binding towards hsp60 in the presence of soluble hsp60. Sera of two healthy controls were used

Table 2. Anti-hsp60 IgG and IgA titers and serum IgG and IgA concentrations in normal controls and OA and RA patients

Subjects	Anti-hsp60 IgG[a]	P[b]	IgG conc.[c]	P	anti-hsp60 IgA[a]	P	IgA conc.[c]	P
Normal	116 ± 92	–	1728 ± 321	–	156 ± 144	–	365 ± 149	–
OA	147 ± 221	0.2040	1644 ± 391	0.1630	241 ± 250	0.0061	317 ± 131	0.0511
RA	162 ± 137	0.0031	1885 ± 510	0.0013	292 ± 344	0.0002	408 ± 149	0.1125
RF(−)[d]	130 ± 84	0.4090	1709 ± 460	0.7842	229 ± 148	0.0073	342 ± 224	0.5113
RF(+)[e]	173 ± 149	0.0010	1945 ± 513	0.0009	313 ± 388	0.0002	430 ± 218	0.0187

[a] P values compared with the mean of normal controls
[b] Units ± SD
[c] mg/100 ml serum ± SD
[d] Group of RA patients whose RF was less than or equal to 19 ($n = 40$)
[e] Group of RA patients whose RF was greater than 19 ($n = 117$)

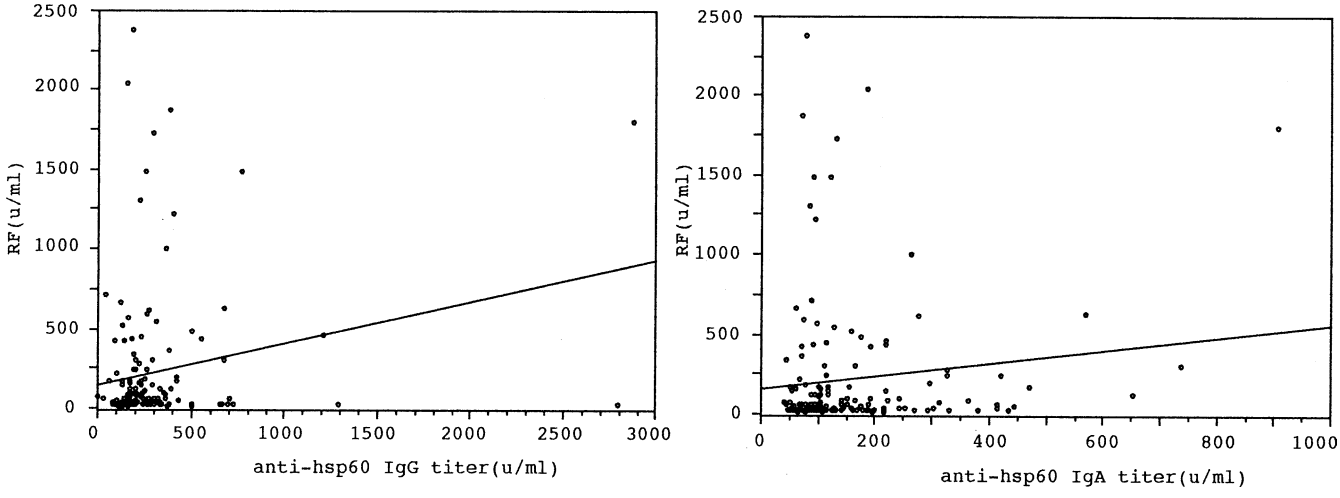

Fig. 3. Correlations between RF titer and anti-hsp60 IgG (*left*) or anti-hsp60 IgA (*right*) titer in sera of RA patients

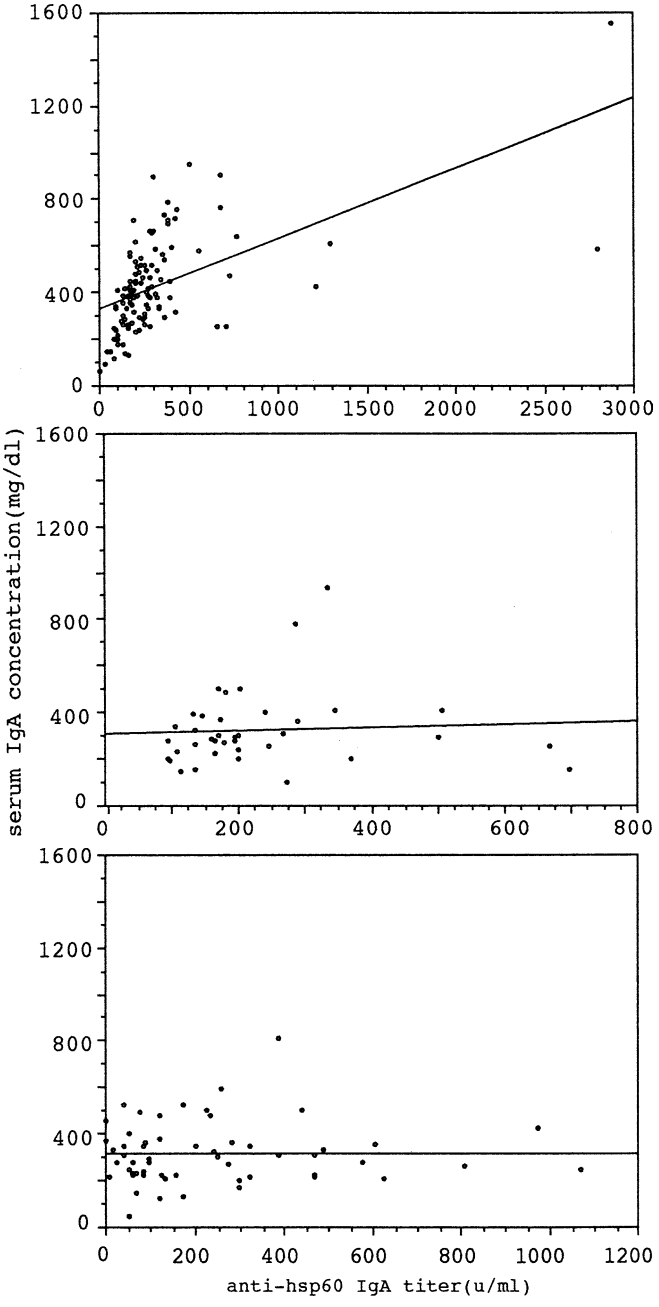

Fig. 4. Correlations between serum IgA concentration and anti-hsp60 IgA antibody titers in seropositive (*upper*; RF > 19), seronegative (*middle*; RF < 19) RA, and OA (*lower*) patients

($P = 0.0002$ and $P = 0.0061$, respectively). Only the mean of serum IgG concentrations of RA patients was significantly elevated compared with that of normal adults. No significant difference in serum IgA concentration among RA and OA patients and normal controls was observed (Table 2).

RA patients were divided into two groups according to their RF titers. The cut-off level of RF was 19. Thus, RA patients whose RF titers were less than or equal to 19 were categorized as seronegative patients (RF[−]), and those whose RF titers were greater than 19 as seropositive patients (RF[+]). As shown in Table 2, all the parameters, both anti-hsp60 IgG and IgA and both serum IgG and IgA, were found to be raised in the RF(+) group, whereas only anti-hsp60 IgA was found to be raised in the RF(−) group.

Correlation between the RF activity and anti-hsp60 titer and Ig concentration

A weak but significant correlation ($r = 0.219$, $P = 0.0067$) between the RF activity and the anti-hsp60 IgA titer was found in RA patients (Fig. 3). In contrast, no correlation was observed between RF activity and anti-hsp60 IgG titer in RA patients ($r = 0.124$, $P = 0.129$). A strong correlation between anti-hsp60 IgA titer and serum IgA concentration was found in the seropositive group ($r = 0.565$, $P = 0.0001$), but not in the seronegative group or in OA patients (Fig. 4). Similarly, a weak but significant correlation between anti-hsp60 IgG titer and serum IgG concentration ($r = 0.264$, $P = 0.0038$) was found in the seropositive group (data not shown).

Discussion

Here, we establish an ELISA system to measure anti-hsp60 IgG and IgA antibody titers. This is the first time the antihuman hsp60 antibody titer of sera of RA and OA patients has been examined. Several studies have evaluated the implication of HSP in autoimmune diseases.[12–14,23–25] However, the conclusions were confusing. In part, this was because of the use of different preparations of mycobacterial hsp65, which has been widely used as an antigen for antibody measurements. Although the amino acid sequence homology between mycobacterial hsp65 and human hsp60 is about 60%,[20–22] it was necessary to evaluate the antihuman hsp60 antibody responses in the strict sense of antigen specificity.

All the sera from 19 normal individuals contained IgG antibody for hsp60 but not for HSA (see Fig. 1). The anti-hsp60 IgG binding to the coated antigen was found to be antigen-specific because it was dose-dependently inhibited by the presence of soluble hsp60 (see Fig. 2). However, it is not known whether the anti-hsp60 IgG observed in normal individuals is induced by a cross-reaction against the HSP of exogenous microorganisms, or by a direct immune response against self hsp60.

As previously reported by Veys and Claessens[26] and Thompson and Asquith,[27] both serum IgG and IgA concentrations in seropositive RA patients were elevated in our study (see Table 2). This elevation may be due either to polyclonal activation or to the presence of RF,[28] or both. It is possible, however, that the increased anti-hsp60 IgG reflects the pathogenesis of RA and its activity because of the lack of a correlation between the anti-hsp60 IgG titer and the RF titer (see Fig. 3). Further, the anti-hsp IgA titer was significantly increased in both OA and RA, regardless of the presence of RF (see Table 2), suggesting the involve-

ment of an anti-hsp60 IgA response in arthritides rather than the pathogenesis of RA.

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