Spontaneous heat shock protein synthesis by alveolar macrophages in interstitial lung disease associated with phagocytosis of eosinophils

B.S. Polla*, S. Kantengwa*, G.J. Gleich**, M. Kondo*, C.M. Reimert*, A.F. Junod†


ABSTRACT: Synthesis of heat shock proteins (HSPs) is induced in all cells and tissues after exposure to elevated temperatures, or a variety of other types of injury, including oxidative injury. We have previously reported that stress proteins are induced in monocytes-macrophages during phagocytosis of red blood cells. Receptor-mediated phagocytosis is associated with activation of the respiratory burst, generation of the lipid mediators of inflammation, and increased production of cytokines. Similar activation events have been described in the alveolar macrophage (AM) during pulmonary fibrosis.

We therefore analysed the pattern of proteins synthesized by human AMs recovered by bronchoalveolar lavage (BAL) in interstitial lung disease, both under basal conditions and after in vitro exposure to heat or hydrogen peroxide (H₂O₂). In two out of the 17 cases studied, we observed a high alveolar eosinophilia (10 and 24%, respectively) and phagocytosis, by the AMs, of eosinophilic material. Whereas exposure to heat or H₂O₂, induced in all AMs the synthesis of the classical HSPs, in these two cases, we found spontaneous synthesis of HSPs and of a 32 kD oxidation-specific stress protein, haem oxygenase (HO). Exposure of AM to purified eosinophil-derived proteins, such as major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), alone or in combination, did not induce stress protein synthesis, further suggesting that phagocytosis is involved in this induction.

Stress protein synthesis by AMs may represent a new cellular marker of pulmonary injury and eosinophilic inflammation, and an autoprotective mechanism against oxidative stress.

Eur Respir J. 1993, 6, 483-488.

Exposure of cells to environmental stresses, such as elevated temperatures or reactive oxygen species (ROS), results in the induction of a specific set of proteins, the heat shock, or stress, proteins (HSPs) (reviews [1, 2]). HSPs are not solely induced by stress, but also during physiological cellular events, such as differentiation, cell cycle, or cell activation [1]. We have investigated the relationships between oxidative injury and the heat shock response [3]. Exogenous hydrogen peroxide (H₂O₂) induces in human monocytes and alveolar macrophages the synthesis of HSPs [3, 4], and HSPs are also synthesized during endogenous generation of ROS by the phagocyte itself. Phagocytosis of sheep erythrocytes by human monocytes and alveolar macrophages (AMs) is associated with the synthesis, by the phagocytic cells, of the classical HSPs, as well as of a 32 kD oxidation-specific stress protein, haem oxygenase (HO); both phagocytosis and generation of ROS are required for this induction [5].

Given the involvement of HSPs in thermotolerance and their role as antigens, HSP synthesis in this context may either serve as an autoprotective mechanism [6], or participate in inflammation and autoimmunity [7]. Indeed, antibodies or T-cells specific for HSPs have been found in a variety of inflammatory and/or autoimmune diseases (review [8]). HSP-specific T-cells appear to be predominant at the site of ongoing inflammation; for such T-cells to play a role in pathology, however, one must assume that host cells at the site of inflammation synthesize HSPs [9].

Interstitial pulmonary fibrosis is characterized by AM activation, by an increased production of cytokines and ROS, and by an ongoing immune response [10, 11]. We investigated the protein synthesis of human AMs recovered by bronchoalveolar lavage (BAL) performed for diagnostic purposes in 17 patients with diffuse interstitial lung disease. Whereas AMs recovered from all patients
synthesized, in vitro, the classical HSPs upon exposure to heat shock or to \( \text{H}_2\text{O}_2 \), in two patients with alveolar eosinophilia, we observed striking spontaneous ex vivo synthesis of HSPs and \( \text{H}_2\text{O}_2 \). We then investigated the effects of purified eosinophil-derived toxic proteins, in particular eosinophil peroxidase (EPO) and major basic protein (MBP), on the stress response of AMs in vitro. The results suggest that phagocytosis of eosinophils is required for stress protein induction.

**Patients**

The clinical characteristics and BAL results of 17 consecutive patients undergoing BAL during the diagnostic work-up of interstitial lung diseases are summarized in Table 1. Transbronchial biopsies were obtained in 10 cases, necropsy was performed in three, and diagnosis relied on clinical presentation and bronchoalveolar lavage alone in four. One patient was excluded from the series because of bacterial contamination of the BAL material. Patients with lung cancer were used as negative controls and for the in vitro experiments with the purified eosinophil proteins [12]. None of the patients had eosinophilia on white blood cell differential.

### Table 1. - Clinical characteristics and BAL results of 17 consecutive patients undergoing BAL during the diagnostic work-up of interstitial lung diseases

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Sex</th>
<th>Age yrs</th>
<th>Diagnosis</th>
<th>Total BAL cells ( \times 10^4 )</th>
<th>Macrophages ( % )</th>
<th>Lymphocytes ( % )</th>
<th>Neutrophils ( % )</th>
<th>Eosinophils ( % )</th>
<th>HSPs on SDS-PAGE</th>
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<tbody>
<tr>
<td>1</td>
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<td>37</td>
<td>HP</td>
<td>48</td>
<td>65</td>
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<td>-</td>
</tr>
<tr>
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<td>F</td>
<td>37</td>
<td>Atypical pneumonia</td>
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<td>98</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>-</td>
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<tr>
<td>3</td>
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<td>33</td>
<td>1.5</td>
<td>0.5</td>
<td>(+)</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>53</td>
<td>IPF</td>
<td>11.7</td>
<td>62</td>
<td>9</td>
<td>5</td>
<td>24</td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>43</td>
<td>CTD</td>
<td>33</td>
<td>57</td>
<td>29</td>
<td>4</td>
<td>10</td>
<td>+</td>
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<tr>
<td>6</td>
<td>M</td>
<td>56</td>
<td>Cardiopathy*</td>
<td>28.8</td>
<td>90</td>
<td>6</td>
<td>1.5</td>
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<tr>
<td>7</td>
<td>M</td>
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<td>8</td>
<td>(+)</td>
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<td>86.5</td>
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<td>5</td>
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<td>41</td>
<td>84</td>
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<td>6</td>
<td>8</td>
<td>+</td>
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<tr>
<td>11</td>
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<td>40</td>
<td>Sarcoidosis</td>
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<td>56</td>
<td>36</td>
<td>3.5</td>
<td>3.5</td>
<td>(+)</td>
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<tr>
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<td>M</td>
<td>26</td>
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<td>30</td>
<td>85</td>
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<td>HP**</td>
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<td>-</td>
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<tr>
<td>15</td>
<td>M</td>
<td>38</td>
<td>****</td>
<td>45</td>
<td>92</td>
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<td>0</td>
<td>2</td>
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</tr>
<tr>
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<td>M</td>
<td>63</td>
<td>IPF</td>
<td>83</td>
<td>20</td>
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<td>-</td>
</tr>
<tr>
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<td>F</td>
<td>74</td>
<td>Actinic fibrosis</td>
<td>83</td>
<td>80</td>
<td>60</td>
<td>6.5</td>
<td>7.5</td>
<td>-</td>
</tr>
</tbody>
</table>

HSPs on SDS-PAGE: comparative appreciation of the amount of HSP70 synthesized by AMs under basal conditions (-: undetectable; (+)+: presence of a faint but detectable band at 70 kD, as for example in case no. 6, see lane 1, fig. 2; +++: amount of HSP70 corresponding to the bands observed at 70 kD in cases no. 4 and 5, see lanes 2 in fig. 3a and b). HSP: heat shock protein; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis; AM: alveolar macrophage; IPF: idiopathic pulmonary fibrosis; HP: hypersensitivity pneumonitis; CTD: connective tissue disease; BOOP: bronchiolitis obliterans with organizing pneumonia. * patient no. 6 died suddenly probably from arrhythmia. ** patient no. 7 died from heart failure; necropsy revealed massive pulmonary embolisms and histology confirmed underlying IPF. *** patient no. 13 was chronically exposed to spores from fungi and endotoxins. A suspected diagnosis of histiocytosis X (7% OKT6 positive cells in bronchoalveolar lavage) was not confirmed on lung biopsy and electron microscopy. ****: patient no. 15 presented with unilateral patchy infiltrates, for which no definitive diagnosis has yet been established.

**Methods**

BAL cells were processed and AMs isolated and purified as described previously [12]. Cells were prepared for microscopy, and stained with May-Grünwald-Giemsa and nonspecific esterase: 200 cells per slide were counted, on two separate preparations, and by two independent workers not involved in the present research. Photographs were taken with a Zeiss Axiophot microscope (x750). Exposure to heat and \( \text{H}_2\text{O}_2 \) were as described previously [4, 13]; cells were then allowed to recover for 2 h at 37°C before labelling. For protein analysis, purified AMs were incubated in RPMI 1640 without methionine (Gibco, Paisley, Scotland), with or without fetal calf serum (FCS) for 2 h, and then labelled with 12 \( \mu \text{Ci·mL}^{-1} \) \( \text{L}^{-3}\text{S}-\text{methionine} \) (Amersham, Buckinghamshire, UK) for 90 min at 37°C, washed twice with phosphate buffered saline and lysed in lysis buffer [14]. Proteins from aliquots corresponding to equal cell numbers were then resolved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in slab gels with 10% acrylamide [14]. Quantitative appraisal of labelled proteins was performed by scanning densitometry of autoradiographs (Genoscan TM laser beam densitometer, Genofit, Geneva, CH).
Exposure to eosinophilic peptides

AMs were isolated as described above and cultured in hydroxyethylpiperazine ethanesulphonic acid (HEPES)-buffered methionine-free medium without FCS. Eosinophil peroxidase (EPO) and major basic protein (MBP) were purified to physical homogeneity from the eosinophils of patients with the hypereosinophilic syndrome as described previously [15,16], and were used at a final concentration of 10 µg·ml⁻¹, either alone or combined with 10⁻⁴ M H₂O₂ and 10⁻¹ M NaCl. The cells were incubated with these compounds for 18 h at 37°C and then labelled with 9 µCi·ml⁻¹³⁵S-methionine for 90 min. Proteins were analysed as described above. Eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) were purified as described and used at 5 and 10 µg·ml⁻¹, respectively [17,18]. Incubation was for 3 h. For peroxidase staining, AM smears were successively immersed in benzidine, benzidine/H₂O₂ solution (1:1) and stained with 10% Giemsa solution (Fluka).

Results and Discussion

Stress proteins include several families of proteins, such as HSPs and HO. In normal cells, these proteins play important roles in protein folding, assembly, and translocation, whereas in stressed cells, these functions are further elicited and are part of the cell’s protective and adaptive mechanisms [1-3]. In two patients (cases no. 4 and 5) with interstitial lung inflammation, we found high alveolar eosinophilia (24 and 10%, respectively), and phagocytosis of eosinophils by the AMs (fig. 1 A and B); there was spontaneous synthesis of high levels of HSPs by these latter cells. In the other cases, including one with 7.5% and two with 8% eosinophils (cases no. 17, 7 and 10), but without phagocytosis of eosinophilic material/eosinophils by the AMs (not shown), protein synthesis was similar to that found in cases without BAL eosinophilia, which suggests that the phagocytic process is required for HSP induction.

Figure 2 shows protein synthesis by AMs from one representative case with low alveolar eosinophilia (case no. 6, 2.5% eosinophils), either under basal conditions (lanes 1 and 4), or after exposure to 45°C for 20 min (lanes 2 and 5), or H₂O₂ (5x10⁻³ M, 120 min) (lanes 3 and 6). AMs were labelled as described, either in the presence (6a) or absence (6b) of 5% fetal calf serum. Exposure to heat shock induced the synthesis of the classical HSPs, 65, 70, 83 and 110 kD (arrows). Exposure to H₂O₂ resulted in similar qualitative induction of HSPs, although quantitatively less important, as is usually observed with oxidative, as compared to thermal, stress [3] (compare lanes 2 and 3, and 5 and 6). There was no effect of serum on HSP synthesis after heat shock. The decrease in overall protein synthesis following exposure to H₂O₂ was, however, more pronounced in the absence of serum, which is probably due to the lack of O₂ radical scavengers normally present in serum.

In several cases, we observed a low level of spontaneous synthesis of HSP70 in AMs from patients without alveolar eosinophilia (see for example fig. 2, lanes 1 and 4), probably related to the stress imposed on the cells by the isolation and culture procedures themselves, as has been described previously for type II pneumocytes [19]. This low grade HSP synthesis ([+]/+ in table 1, last lane (HSPs on SDS-PAGE)) was however not constant, and not comparable to the high levels synthesized either after exposure to heat (fig. 2, lanes 2 and 5), or spontaneously in phagocytosing AMs (fig. 3A; lanes 2 and 3; fig. 3B, lane 2).
Fig. 2. - Protein synthesis by AMs after exposure to heat shock or 
H₂O₂. Autoradiographs of SDS-PAGE (10% polyacrylamide gels) of 
cell lysates of AMs from case no. 6. The cells were labelled either 
in the presence (a) or in the absence (b) of 5% fetal calf serum. Lanes 2 and 5: heat shock; lanes 3 and 6: H₂O₂; lanes 1 and 4: 
controls. Arrows point to the major HSPs (65, 70, 83-90 and 
110 kD). AM: alveolar macrophage; SDS-PAGE: sodium dodecyl 
sulphate polyacrylamide gel electrophoresis; HSP: heat shock protein.

Figure 3 shows the spontaneous synthesis of the classical HSPs (65, 79, 83-90 and 110 kD), and of a 32 kD protein identified as HO, in comparison with the pattern of induction by cadmium or erythrophagocytosis [2, 5] 
by the AMs from cases no. 4 (fig. 3a) and 5 (fig. 3b), as compared to a control case (c) (without interstitial lung disease). With the exception of cases no. 4 and 5, HO 
was not detected under basal conditions or after exposure 
to heat or exogenous H₂O₂ alone.

The levels of HSPs synthesized under basal conditions in 
these two cases was similar to that observed after exposure 
to heat in other cases. Spontaneous synthesis of stress proteins by the AMs may represent a new marker 
for injury to these cells during inflammatory lung dis­
cases. This synthesis is probably related to phagocytosis and the associated generation of high levels of ROS. The fact that in the AMs HO was observed only under 
conditions of ongoing phagocytosis is consistent with our previous observations on the requirements of high intra­
cellular ROS production for this oxidation-specific pro­
tein to be induced. We have previously reported that 
erythrophagocytosis by AMs induces the synthesis of the 
classical HSPs as well as of HO; both phagocytosis and 
generation of oxygen radicals appeared to be required for this induction, since neither activation of the respiratory 
burst by non-phagocytic stimuli nor phagocytosis of in­
ert material (latex beads) had such effects [5].

Fig. 3. - Protein synthesis by AMs from cases no. 4 and 5. 
Autoradiographs of SDS-PAGE (10% polyacrylamide gels) of cell 
lysates of AMs from case no. 4 (A) and 5 (B). In case no. 4, 4a 
and 4b indicate labelling for 90 min (4a) or overnight (4b); in case 
5, 5a and 5b correspond to labelling under basal conditions (5a) or 
after heat shock (5b). Arrows point to the major HSPs and HO (32 
kD). C: control; HO: haeme oxygenase. For further abbreviations 
see legend to figure 2.

Phagocytosis of eosinophils by AMs may represent a 
mechanism for clearing these toxic cells from the alveo­
lar space [20], as has been described for neutrophils [21]. 
We hypothesized that the toxic products of the eosinophil 
itself (MBP, ECP, EDN and EPO) could participate in 
the stressful events responsible for stress protein induc­
tion. Exposure of AMs to the purified eosinophil-derived
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proteins ECP, EDN, MBP and EPO did not induce HSP synthesis, even under conditions in which there was toxicity to the AMs, as assessed by morphological criteria (AM vacuolization) and a decrease in total normal protein synthesis (not shown). In order to verify the uptake of EPO by AMs, peroxidase staining was performed. AMs incubated with MBP became vacuolated but remained peroxidase negative, whereas AMs incubated with the mixture EPO/H2O/NaCl became peroxidase positive, indicating uptake of EPO by AMs. These findings indicate that EPO alone, or in combination with MBP, is not sufficient for the induction of a stress response in human AMs. The spontaneous HSP induction that we observed in some cases of interstitial diseases is, thus, probably mediated by the phagocytosis of eosinophils (including all of their toxic products), rather than by selected eosinophil-derived toxic proteins. The question as to whether the toxic effects of eosinophils and/or their products in other diseases associated with pulmonary or bronchial eosinophilia, such as asthma, also leads to stress protein synthesis by other (non-phagocytic) injured cells (e.g., bronchial epithelial cells) remains to be answered.

The cases presented here do not yet allow us to determine whether HSP synthesis by AMs is, or is not, strictly associated with phagocytosis of eosinophils by this cell, or with a particular type of inflammatory lung disease. It will be necessary to investigate broader groups of patients, with or without eosinophils in BAL, as well as other forms of eosinophilic pneumonitis. In three patients with Carrington's disease (eosinophilic pneumonitis, without eosinophil phagocytosis), no particular HSP expression was found in bronchoalveolar cells using specific anti-HSP70 monoclonal antibodies and electron microscopy (A. Janin et al.; unpublished data), which further stresses the apparent specificity of HSP synthesis in pulmonary inflammation associated with phagocytosis of eosinophils by the AM. In vitro studies testing whether ingestion of senescent eosinophils induces HSP synthesis by the phagocytosing AMs will be required to prove this issue.

HSPs may exert several distinct functions in immunity. They may participate in local inflammatory processes, either by serving as self antigens or by participating in antigen processing [8]. In order, however, for self HSPs to become targets for the immune system, these intracellular proteins must become available for T-cell recognition. This may indeed be the case during inflammation; Jardor et al. [22] suggested cell surface expression of HSP70 on AMs from one patient with interstitial fibrosis. Alternatively, stress proteins may represent an autoreactive mechanism against oxidative injury in the lung. Indeed, stress proteins have a general function in cellular protection from injury [23, 24] and we have previously reported that HSPs have the potential to protect monocytes-macrophages from oxidative injury [4, 13]. Furthermore, HO has a recognized antioxidant potential [25], and heat shock has also been shown to protect cells from cytolysis by tumour necrosis factor [26], a cytokine potentially involved in interstitial lung diseases [11]. Finally, it is possible that the association of spontaneous stress protein synthesis by AMs with alveolar eosinophilia relates to the observation that an increased number of eosinophils in BAL defines a group of patients suffering from pulmonary fibrosis with a poor response to therapy, with corticosteroids [27], and with progressive lung disease [28, 29].

Acknowledgements: The authors are grateful to C. Reynaud and G. Wester for their participation in the early phases of this work.

References
