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# Induction *in vitro* of 72-kD heat shock protein in a continuous culture of rat thyroid cells, FRTL5

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## SUMMARY

In Graves' disease and Hashimoto's thyroiditis, the presence of 72-kD heat shock protein (hsp-72) on thyrocytes has been reported. To clarify the significance of this phenomenon, we induced the antigen in thyroid cell culture *in vitro*. In the FRTL5 rat cell line, which had been heated at 42·5°C or treated with sodium arsenite, expression of hsp-72 was examined with immunoperoxidase staining and immunoprecipitation of the metabolically labelled protein using a specific MoAb. In the cells cultured either with or without thyrotropin (TSH), heat and chemical stresses reproducibly and dose-dependently induced hsp-72 antigen, whereas unstimulated controls had no significant immunoreactivity. Unlike in Graves' retroocular fibroblasts, hydrogen peroxide was not an effective stress in FRTL5, and the induction was not suppressed by methylmercaptoimmidazole and propylthiouracil, nor enhanced by interferon-gamma (IFN-$\gamma$) and tumour necrosis factor-alpha (TNF-$\alpha$). These data could not support the hypotheses that suppression of thyroid autoimmunity by thionamides is due to their modulatory action on hsp-72 expression, or that presence of that antigen in the thyroid tissues affected by autoimmunity is secondary to cytokine secretion from infiltrating immunocytes. On the other hand, coculture experiments of stressed FRTL5 cells and syngeneic Fisher rat splenocytes suggest that aberrantly expressed hsp may activate part of the thyroid-infiltrating lymphocytes and thereby aggravate autoimmune processes. The induction and detection systems of hsp-72 using FRTL5 cells would facilitate future studies, possibly utilizing human materials as well, to explore possible relations between stress proteins and thyroid autoimmunity.

**Keywords** heat shock protein autoimmune thyroid diseases FRTL5 cell line

## INTRODUCTION

Heat shock proteins (hsp) work as intracellular housekeepers or molecular chaperones in normal conditions, and are synthesized in response to various physicochemical or biological assaults to maintain homeostasis of cells [1]. Because of their omnipresence and phylogenically conserved nature, possible roles of hsp in autoimmunity as autoantigens, which may cross-react with bacterial counterparts, have recently drawn much attention [2,3]. In addition, hsp may have another function to regulate immune responses by participating in antigen presentation [4,5].

In the field of thyroidology, Heufelder and colleagues have demonstrated expression of a 72-kD hsp (hsp-72) in the thyroid glands from patients with Graves' disease and Hashimoto's thyroiditis [6], and also in Graves' retroocular fibroblasts [7]. In cultured fibroblasts, they also showed modulatory effects of various cytokines and thionamide antithyroid drugs on abundance of hsp-72 immunoreactivity [7,8]. Is the expression of this protein related to a putative infectious trigger of thyroid autoimmune cascade [9]? Or is it only secondary to the ongoing cellular immunity, where activated lymphocytes and macrophages certainly secrete cytokines [10]? To elucidate further the significance of hsp-72 in thyroid autoimmunity, we attempted to induce and modulate the molecule in a culture of pure thyrocyte population without contamination of fibroblasts or immunocytes, namely the well characterized FRTL5 cell line [11].

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## MATERIALS AND METHODS

### *Materials*

Reagents and culture wares were purchased from the following suppliers: Coon's modification of Ham's F-12 medium, trypsin from bovine pancreas, ethylenediamine tetra-acetate (EDTA), bovine serum albumin (BSA), sodium arsenite, and methyl-

mercaptoimidazole (MMI) from Sigma Chemicals (St Louis, MO); propylthiouracil (PTU), hydrogen peroxide, Triton X-100, SDS, sodium deoxycholate from Nakalai Tesque (Kyoto, Japan); rat recombinant interferon-gamma (IFN-$\gamma$) from Holland Biotechnology bv (Leiden, The Netherlands); mouse recombinant tumour necrosis factor-alpha (TNF-$\alpha$) from Boehringer Mannheim Biochemica (Mannheim, Germany); MoAb to hsp-72 (C92F3A-5 [12], raised against immunogens isolated from human Hela cells and shown to cross-react with various mammalian cell lines, including of rodent origin), from StressGen Biotechnologies (Victoria, Canada); an IgGl mouse MoAb to a human fibroblast antigen propyl-4-hydroxylase, clone 5B5 (used as an isotype-matched control to the specific anti-hsp-72 MoAb C92F3A-5), biotinylated rabbit antibodies to mouse immunoglobulins (biotin-RAM), peroxidase-conjugated streptavidin (Pox-SA), and diaminobenzidine tetrahydrochloride from Dako Reagents (Santa Barbara, CA); RPMI 1640, methionine-free minimal essential medium (MEM), heat-inactivated calf and fetal calf sera, and formalin-fixed *Staphylococcus aureus* (Staph-A) from GIBCO Life Technologies (Grand Island, NY); $^{35}$S-labelled L-methionine (> 37 TBq/mmole) and methyl $^{3}$H-thymidine (249·7 GBq/mmole) from DuPont-NEN Research Products (Boston, MA); Lab-Tek chamber/slides and flaskette/slides from Nunc (Naperville, IL); 24-well culture trays and 12 × 75 mm culture tubes from Corning Glass Works (Corning, NY).

OK-432, an attenuated streptococcal preparation, was a generous gift from Chugai Pharmaceuticals (Osaka, Japan).

*Cell culture*

The thyroid follicular cell line FRTL5 was kindly provided by Dr L. D. Kohn (NIH, Bethesda, MD). As described earlier [13], the cells were maintained in Coon's modified Ham's F-12 supplemented with 5% calf serum and six 'hormones', such as bovine thyrotropin (TSH), insulin, transferrin, somatostatin, hydrocortisone and glycyl-L-histidyl-L-lysine acetate (6H medium). In some experiments, the same medium devoid of TSH (5H medium) was also employed. Cells were detached from culture flasks with 0·05% trypsin and 0·02% EDTA, and seeded in chamber/slides and flaskette/slides for immunostaining, or in 24-well culture trays for metabolic labelling and coculture.

*Induction of hsp-72*

After 5–7 days of culture, FRTL5 cells were heated at 42·5 ± 0·2°C by immersing sealed culture vessels in a water bath for 30 or 60 min, cooled down by changing the medium, and allowed to recover at 37°C in a $CO_2$ incubator for various periods of time. As chemical and bacterial stressors, varying concentrations of sodium arsenite, hydrogen peroxide, IFN-$\gamma$, TNF-$\alpha$, and OK-432 diluted in culture medium were applied for 90 min at 37°C. After terminating the stimuli by rinsing twice with fresh culture medium, cells were further incubated for recovery.

To evaluate modulatory effects of thionamides and cytokines on hsp-72 induction, cells were treated with MMI, PTU, IFN-$\gamma$, or TNF-$\alpha$ for 48 h before challenge by heat or chemical stresses as described above.

*Immunoperoxidase staining*

After removing the plastic 'upper structures' (chambers and flaskettes), cells on slides were rinsed in PBS pH 7·2, air-dried, and fixed in cold acetone for 10 min. The slides rehydrated in PBS were pretreated to reduce non-specific sticking of antibody proteins with 1% BSA in PBS for 10 min at room temperature, incubated serially with MoAb to hsp-72 (dilution 1 : 200), biotin-RAM (1 : 200), and Pox-SA (1 : 150) for 120, 40, and 20 min, respectively. Finally, they were developed in Tris-diaminobenzidine-hydrogen peroxide solution and lightly counterstained with Mayer's haematoxylin [14]. Negative controls included use of the control MoAb 5B5 instead of anti-hsp-72, and omission in turn of the first or second antibody.

*Metabolic labelling and immunoprecipitation*

The whole procedure was a modification of the method employed earlier [15] for quantification of thyroglobulin neosynthesis in FRTL5 cells using a rabbit polyclonal antiserum. After being stressed and allowed to recover for 1 h as described above, FRTL5 cells were washed twice with PBS, preincubated with 250 $\mu$l/well of methionine-free MEM for 1 h, followed by another 2 h incubation with 555 kBq/well of $^{35}$S-methionine in 150 $\mu$l of the same medium for 2 h. After two washes with ice-cold PBS, cells were lysed with 500 $\mu$l of immunoprecipitation buffer (1% Triton X-100, 1% sodium deoxycholate, and 0·1% SDS in 50 mM Tris-buffered saline pH 7·4). The lysate transferred to a microcentrifuge tube (250 $\mu$l/tube) was precleaned by adding 25 $\mu$l of the control antibody (5B5, dilution 1 : 10), incubating for 1 h at room temperature, reacting with 35 $\mu$l of Staph-A at 4°C for 30 min, and centrifuging. The supernatant was transferred to a new tube and incubated with 10 $\mu$l of the anti-hsp-72 MoAb (1 : 10) overnight at 4°C, and reacted with Staph-A as mentioned above. After five washes with the immunoprecipitation buffer, the pellet was resuspended in 500 $\mu$l of 2% SDS solution, mixed with scintillation cocktail (Bray's buffer) and counted in a $\beta$-counter. Immunological specificity of the precipitate was confirmed by autoradiography of samples after SDS–PAGE, which showed no detectable radioactive band other than the one at mol.wt 72 000.

*Coculture of FRTL5 cells and splenic lymphocytes*

We used splenic lymphocytes from Fisher rats, the syngeneic strain from which the FRTL5 cell line originated [11]. Spleens were prepared aseptically from anaesthetized and exsanguinated 4-week-old female rats, which had been reared in specific pathogen-free conditions on standard diet. After mechanical dispersion through fine metal mesh with a rubber policeman, the resultant spleen cell suspension was washed once with RPMI 1640 supplemented with 5% calf serum (washing medium). Following haemolysis with Tris-buffered ammonium chloride pH 7·6, these cells were washed three times with the washing medium, and resuspended at $10^6$ cells/ml in RPMI 1640 supplemented with 15% fetal calf serum, with or without 5 $\mu$g/ml of the anti-hsp-72 or the control 5B5 MoAbs. Just before coculture, FRTL5 cells in 24-well plates, untreated or stressed and allowed to recover overnight, were rinsed twice with PBS. After aspirating PBS, 1 ml/well of splenocyte suspension prepared as above was added and incubated for 7 days. The stimulator FRTL5 cells were not irradiated as ordinary 'mixed lymphocyte reaction' experiments, since that kind of cytostatic treatment may induce hsp-72 by itself [16]. At the end of coculture, non-adherent lymphocytes were recovered by repeated pipetting, transferred to a 12 × 75 mm culture tube,

and pulse-labelled with 74 kBq/tube of $^3$H-thymidine (in 0·1 ml RPMI 1640) for 6 h. The cells were then washed once with ice-cold PBS and twice with 10% trichloric acid. The pellet was dissolved in SDS, mixed with Bray's buffer, and measured in a liquid scintillation counter as described for the metabolic labelling experiments.

*Statistical analysis*

All comparison of numerical data was carried out with analysis of variance of triplicate samples.

## RESULTS

As shown in Fig. 1, heat and arsenite stimulation reproducibly induced hsp-72 on FRTL5 cells, while negative controls and unstimulated cells never showed significant staining. In each positive slide, the intensity of immunostaining varied among the cells. The ratio of positive cells to negative ones did not differ significantly whether 5H or 6H medium was used (data not shown). Overnight recovery resulted in more distinct immunostaining than did 2-h or 4-h periods. When the cells were heated at 42·5°C and allowed to recover overnight, the proportion of positive cells among FRTL5 increased as the heating became longer; less than 1% at 15 min, 3–5% at 30 min, 10–15% at 60 min. A temperature higher than 42·5°C or heating time longer than 60 min caused detachment of cells. Similarly, the chemical stress with sodium arsenite showed a dose-related response; positive cells increased in number as the concentration of the chemical stressor increased from 50 to 100 $\mu$M and up to 200 $\mu$M, whereas at 400 $\mu$M it caused cytotoxic effect. These dose-dependent effects were confirmed quantitatively in metabolic labelling experiments (Table 1). Once induced either thermally or chemically, immunostainable hsp-72 antigenicity persisted for at least 72 h after termination of the stimuli.

Hydrogen peroxide (10 $\mu$M–1 mM), rat IFN-$\gamma$ (10–1000 U/ml), mouse TNF-$\alpha$ (10–1000 U/ml), and a combination of IFN-$\gamma$ and TNF-$\alpha$ had no ability to induce immunostainable hsp-72 on FRTL5 cells. When OK-432 (100 $\mu$g/ml) was used as a stressor, only 1–3% of cells were weakly positive for the hsp-72 staining in three out of five experiments.

MMI (10 $\mu$M–1 mM), PTU (10 $\mu$M–1 mM), IFN-$\gamma$ and TNF-$\alpha$ had no significant effects on thermal and chemical induction of hsp-72 antigen when assessed with immunostaining. As shown in Figs 2 and 3, the quantity of metabolically labelled hsp-72 protein also showed no significant change with the

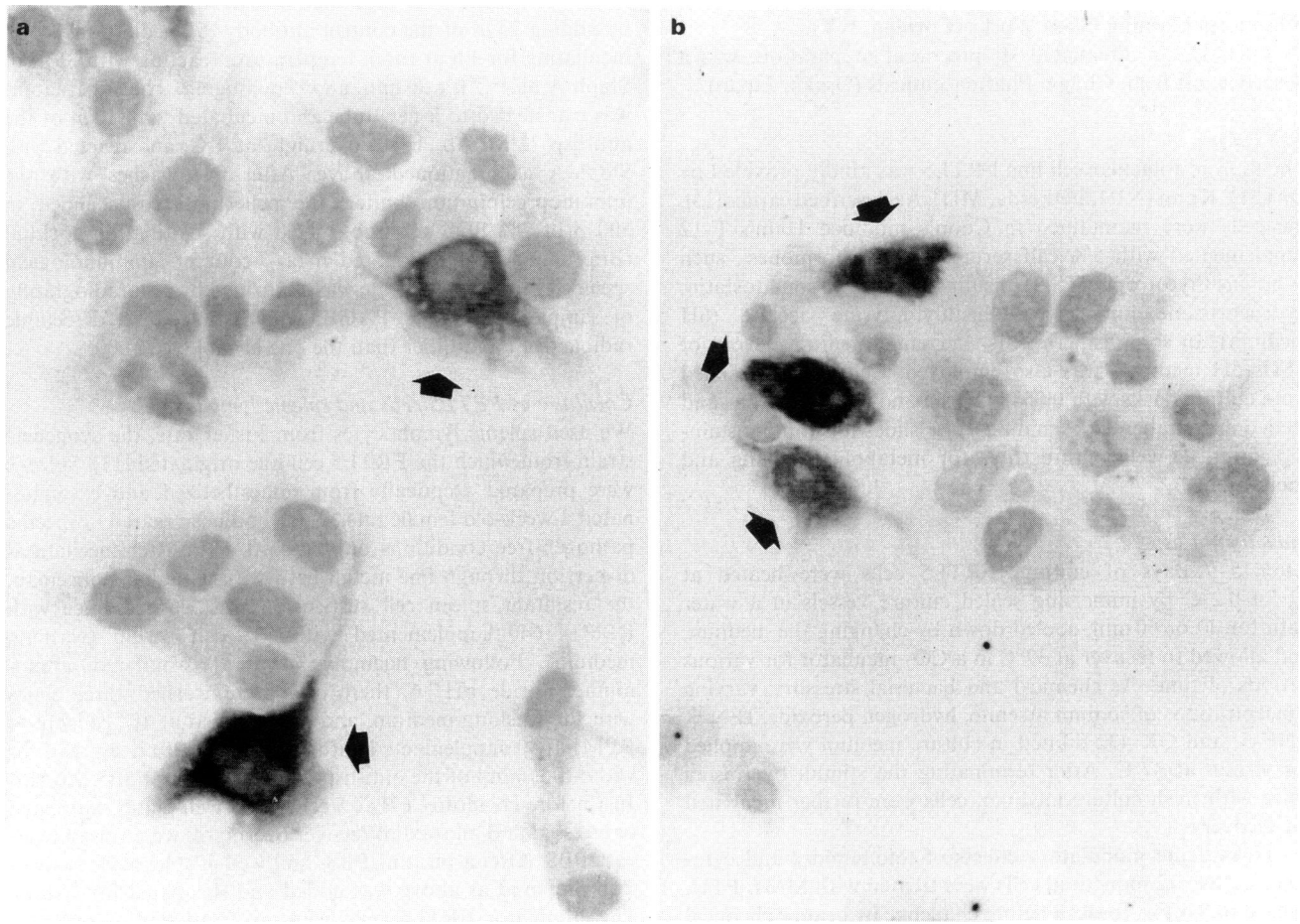


**Fig. 1.** Immunoperoxidase staining of hsp-72 in FRTL5 cells cultured in 6H medium, heated at 42·5°C for 60 min (a) or chemically stressed with 200 $\mu$M sodium arsenite for 90 min (b), and allowed to recover for 16 h. Arrows indicate positive cells with strong cytoplasmic staining. Unstimulated cells (c) and heated cells stained with an irrelevant MoAb (d) showed no significant darkening of the chromogen diaminobenzidine. (Counterstained with haematoxylin, original magnification ×200.)

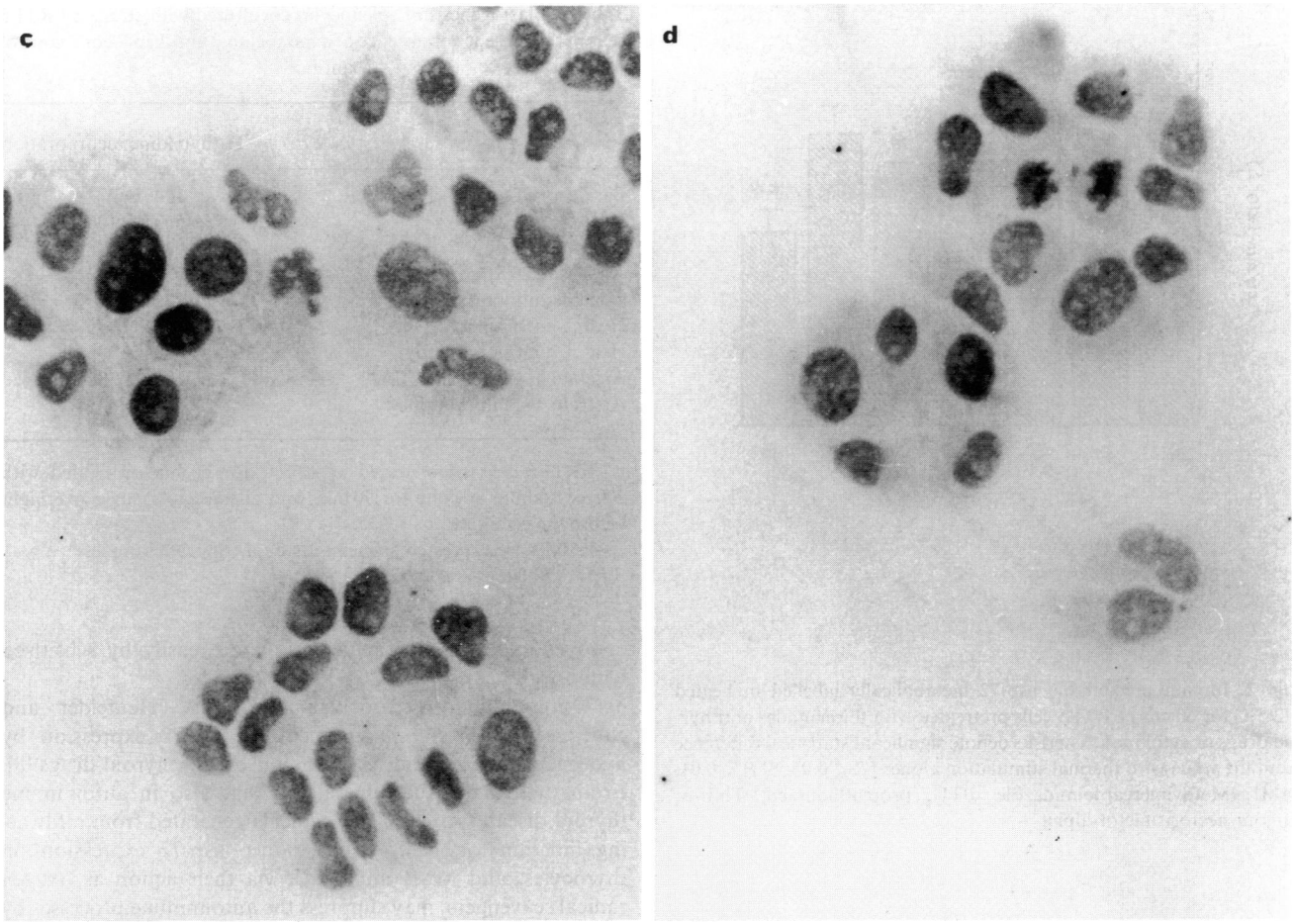


**Fig. 1.** (Continued).

thionamides. However, the quantitative method revealed a slight but significant inhibition by IFN-$\gamma$, and further reduction by combination of IFN-$\gamma$ and TNF-$\alpha$ (Figs 2 and 3).

Table 2 summarizes the blastogenesis of Fisher rat splenocytes in a representative coculture experiment. On four other independent occasions, the lymphocytes cultured with stressed thyrocytes took up several to 10-fold higher amount of thymidine than did the cells cultured with unstressed FRTL5 cells. The neutralizing effect of anti-hsp-72 antibody was also reproduced in all these experiments.

**Table 1.** Immunoprecipitable hsp-72 antigen synthesized by FRTL5 after stresses

| | $^{35}$S-methionine incorporated |
|---|---|
| Basal (no stress) | 6336 ± 242 |
| Heat 15 min | 6558 ± 272 |
| 30 min | 7676 ± 266* |
| 60 min | 9776 ± 406** |
| Arsenite 50 $\mu$M | 6419 ± 340 |
| 100 $\mu$M | 9292 ± 245† |
| 200 $\mu$M | 11 504 ± 485‡ |

The cells were heated at 42·5°C for designated periods or treated with sodium arsenite at indicated concentrations for 90 min.

Results are expressed as mean ± s.d. of triplicate samples.

* $P < 0{\cdot}005$ *versus* 15 min; ** $P < 0{\cdot}001$ *versus* 30 min.

† $P < 0{\cdot}005$ *versus* 50 $\mu$M; ‡ $P < 0{\cdot}001$ *versus* 100 $\mu$M.

## DISCUSSION

With their potential as autoantigens and immune regulators, stress proteins or hsp may be pivotal in the development of autoimmunity in general [2–4]. Therefore, the presence of hsp-72 in the thyroid with Graves' disease and Hashimoto's thyroiditis [6] could be closely related to their autoimmune pathogenesis. Induction experiments of hsp-72 *in vitro* with cultured thyrocytes would promote our understanding of how this aberrant expression takes place and what factors may modulate that expression. The present study showed that the thyroid cell line FRTL5 can respond to heat or chemical stresses by synthesizing hsp-72 as did cells of other lineages, notably fibroblasts [7,12]. The variable immunostainability observed among the cells and relatively low frequency of positive cells could be explained by cell cycle-dependent association of the antigen with other cellular proteins [17]. If hsp-72 antigen has a role for thyroid autoimmunity, the cells with readily detectable epitopes by the specific antibody seemed more relevant to the pathological processes, since these

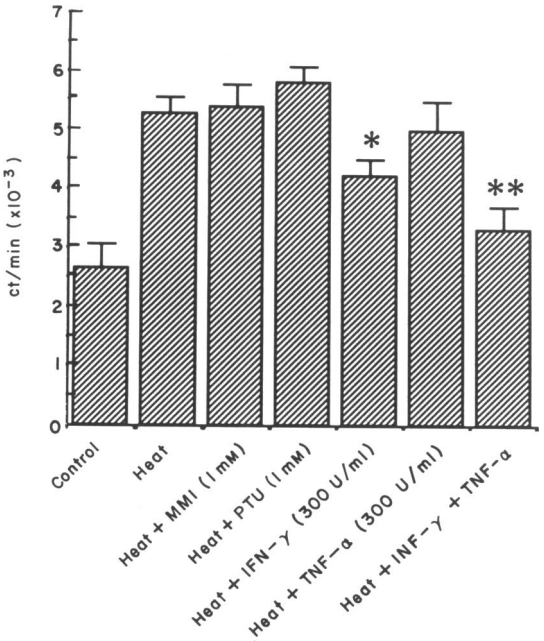


**Fig. 2.** Immunoprecipitable hsp-72 metabolically labelled in heated (42·5°C for 60 min) FRTL5 cells pretreated with thionamides antithyroid drugs or cytokines. Asterisks denote significant statistical difference from the value with thermal stimulation alone: $*P < 0.05$; $**P < 0.01$. MMI, Methylmercaptoimidazole; PTU, propylthiouracil; TNF-α, tumour necrosis factor-alpha.

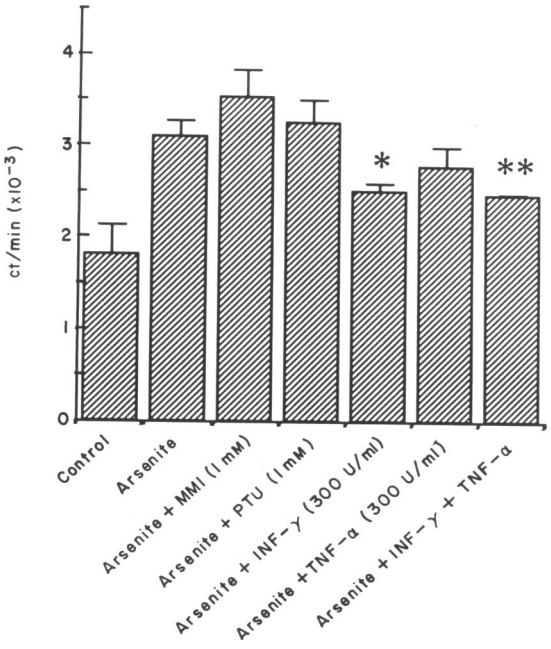


**Fig. 3.** Immunoprecipitable hsp-72 metabolically labelled in arsenite-stimulated (200 μM for 90 min) FRTL5 cells pretreated with thionamides or cytokines. Asterisks denote significant statistical difference from the value with sodium arsenite stimulation alone: $*P < 0.02$; $**P < 0.01$. MMI, Methylmercaptoimidazole; PTU, propylthiouracil; TNF-α, tumour necrosis factor-alpha.

**Table 2.** Blastogenesis of splenocytes cocultured with stressed FRTL5 cells in the presence or absence of a monoclonal anti-hsp-72 or a control antibody

| | $^3$H-thymidine incorporation |
|---|---|
| Basal (no stress) | 1046 ± 277 |
| Heat | 6537 ± 1061* |
| Arsenite | 5760 ± 649* |
| Anti-hsp-72 (5 μg/ml) | 1315 ± 222 |
| Control antibody (5 μg/ml) | 1250 ± 208 |
| Heat + anti-hsp-72 | 1677 ± 335 |
| Heat + control antibody | 7060 ± 996* |
| Arsenite + anti-hsp-72 | 1810 ± 288 |
| Arsenite + control antibody | 6199 ± 801* |

FRTL5 cells were heated at 42·5°C for 60 min or treated with 200 μM sodium arsenite for 90 min, and allowed to recover overnight before the coculture.

Results are expressed as mean ± s.d. of triplicate samples.

*$P < 0.005$ *versus* basal.

epitopes could also be recognized more easily by sensitized lymphocytes.

Using human retroocular fibroblasts, Heufelder and colleagues reported enhancement of hsp-72 expression by hydrogen peroxide and its inhibition by antithyroid drugs [8]. From these data, they postulated that also in autoimmune thyroid diseases, oxygen free radicals generated from infiltrating inflammatory cells could induce hsp-72 expression on thyrocytes, and MMI and PTU, via their action as oxygen radical scavengers, may suppress the autoimmune processes by reducing that expression [8]. Our present study with FRTL5 could not support the hypothesis, since hydrogen peroxide could not induce hsp-72 in the first place. Having their own peroxidase to organize iodine, thyroid follicular cells in general are probably well equipped with subcellular devices to detoxicate oxygen free radicals, and hence resistant to them. In addition, thionamides did not suppress induction of hsp-72 in FRTL5 cells by heat and chemical stresses. Moreover, unlike in human fibroblasts [7], IFN-γ and TNF-α caused neither induction nor upward modulation of hsp-72 in FRTL5 cells. In all these respects, primary culture of Fisher rat thyrocytes showed qualitatively similar results when they were tried parallel to FRTL5 cells (data not shown), suggesting that the observed changes were not peculiar to a special, lined population of thyrocytes. However, like other cellular proteins, regulation of hsp-72 expression may conceivably be different among various species and cell lineages.

The mild reduction of induced hsp-72 immunoreactivity seen in the metabolic labelling may reflect non-specific inhibition of amino acid metabolism by IFN-γ [15] and potentiation of its effect by TNF-α [18]. These data suggest that aberrant expression of hsp-72 in human autoimmune thyroid diseases is not necessarily secondary to production of IFN-γ or TNF-α from infiltrating immunocytes.

What is then the causative agent of aberrant hsp-72 expression on thyroid *in vivo*, if not oxygen free radicals or cytokines? Although our attempt with a streptococcal preparation OK-432 has so far been equivocal, bacterial and/or viral infection seemed the most likely candidate [9]. We may need a

combination of microbes and other factors (cytokines, endotoxins, antibodies, or some unknown biological substances) to reproduce experimentally the immunopathological induction of hsp-72 happening *in vivo*.

Once induced, stress proteins are known to trigger humoral and cellular immune responses by self hsp-reactive lymphocytes [2,9]. In line with these reports in other cell systems, our present study has shown that hsp-induced FRTL5 thyrocytes can initiate blastogenesis of cocultured syngeneic, non-immune Fisher rat splenocytes. Similar events may take place *in vivo* in human autoimmune thyroid diseases, since a spatial correlation was reported between abundance of hsp-72 expression and density of lymphocytic infiltration in Graves' and Hashimoto's diseases [6]. If that assumption holds true beyond species differences, we could say that aberrant hsp-72 on thyrocytes at least exaggerates ongoing autoimmune processes, if not starts them [3].

In future studies, the reproducible induction system of hsp-72 on FRTL5 cells presented above will serve as an experimental model to explore relations between stress proteins and thyroid autoimmunity.

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