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A novel immunocytochemistry technique to measure hsp70 induction in L929 cells exposed to cadmium chloride

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The heat shock response has been suggested as a potential biomarker in toxicology. A vast amount of stimuli have been shown to induce heat shock proteins and new techniques to measure the response are constantly being assessed. In this study we use a novel immunocytochemistry technique to measure heat shock protein 70 (hsp70) induction in L929 cells exposed to cadmium chloride. Hsp70 induction was quantifiably measured using a soluble coloured substrate and qualitatively measured using a coloured substrate that precipitated at the location of hsp70. Using the insoluble coloured substrate hsp70 was identified predominantly within the cytoplasm of control cells. At intermediate cadmium concentrations hsp70 was observed to translocate to the nucleus. At these intermediate concentrations a heterogeneous heat shock response was observed. At lethal concentrations a strong heat shock response was observed with a widespread cellular response. This study demonstrates the potential of this immunocytochemistry technique to measure toxicological effects in cells by identifying the response quantitatively and qualitatively.

Keywords: heat shock proteins, immunocytochemistry, cadmium chloride, toxicity.

Introduction

In vitro toxicity testing uses biochemical, cellular, and physiological responses as diagnostic screening tools in environmental monitoring (Sanders 1990). Ideally, it should be possible for such responses to be used across a broad range of organisms exposed to a variety of stress conditions, to correlate with decreased physiological function or survival and to be easily and economically measured (Sanders 1990). A highly conserved, ubiquitous, primary protective response by the cell, the heat shock response, appears to have great potential as such a biomarker.

The heat shock proteins encompass a group of related proteins that were originally characterized based on their induction after exposure to increasing temperature. Several classes of heat shock proteins have been identified and are grouped according to their apparent molecular weight on one-dimensional SDS-PAGE gels. The majority of heat shock proteins are constitutively found as part of the homeostatic mechanism of the cell. Under normal conditions, they have a chaperonein function in protein assembly, disassembly and transport. Under adverse conditions, the cell ceases the synthesis of normal proteins and concentrates solely on producing heat shock proteins (Gething and Sambrook 1992). Under such conditions the heat shock proteins normal function takes on a defensive role protecting proteins from denaturation and facilitating the removal of

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aggregated proteins. The ubiquitous hsp70 family is the most highly conserved and largest of all the heat shock protein families (Sanders 1993); it also has been the most extensively studied.

Investigators have shown that the heat shock response has potentially significant applications in medicine and toxicology (Welch 1993). Recent studies have suggested a role for the heat shock response in cancer treatment (Vertrees *et al*. 2000) and protection of cardiac and brain cells (Latchman 2001, McCormick *et al*. 2003). The application of the heat shock response to toxicology has proceeded further than in medicine with several assays developed. This may be due to the characteristics of the heat shock response, which lend themselves to toxicity testing.

As a potential measure of toxicity, the heat shock response represents an ideal candidate, occurring as a primary response to stimuli (Bierkens 2000). Heat shock proteins have the advantage over analytical methods of measuring the fraction of a chemical compound that is biologically available (Bierkens 2000).

For the heat shock response to find application in routine toxicity testing it must be shown to be more sensitive than existing toxicity assays, have biological relevance and be applicable to a wide range of organisms. In addition, the heat shock response assay needs to be rapid, reproducible, sensitive, economic and easy to use.

In this paper we investigated the possibility of using a simple *in situ* immunocytochemical assay to measure hsp70 induction in L929 cells exposed to the heavy metal cadmium chloride, a well known toxicant and inducer of heat shock proteins. Hsp70 induction was quantifiably measured using a soluble colored substrate and qualitatively measured using a colored substrate that precipitated at the location of hsp70.

Materials and methods

Chemicals and reagents

All cell culture reagents were cell culture grade and all electrophoresis reagents were electrophoresis grade and were purchased from Sigma Aldrich, Poole, Dorset, U.K. Goat anti-mouse IgG (whole molecule) alkaline phosphatase conjugate, BCIP/NBT tablets (5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium) were purchased from Sigma Aldrich, Poole, Dorset, U.K. Monoclonal anti-hsp70 mouse IgG_1 clone C92F3A-5 was purchased from Stressgen Biotechnologies LTD, Glanford Avenue, Victoria, Canada. All other chemicals were of Analar grade unless otherwise stated and were purchased from BDH Chemicals, Poole, Dorset, UK.

Cell culture

Mouse connective tissue (L929) cells were grown at 37°C in a humidified incubator containing 5% CO_2 and propagated in Dulbecco's modified Eagles medium (pH 7.3) with 10% (v/v) foetal bovine serum. For concentration response experiments the cells were plated into 96 well cell culture plates and incubated at equal densities high enough to ensure exponential growth within 24 h. Exponentially growing cultures were used for all experiments (Doyle *et al.* 1993).

Preparation of test solutions

Cadmium stock solution (100 mg/l $CdCl_2.2½H_20$) was prepared in distilled water and filter sterilized (0.22 μm). The stock solution was further diluted into the appropriate culture medium at a final concentration of 10% stock solution for testing. Metal concentrations were verified by atomic absorption spectroscopy according to standard procedure (APHA 1990). An equivalent volume of distilled water was used as a negative control.

Neutral red assay

In order to gauge the potential of the heat shock response assay as a biomarker in toxicology it was essential to compare it with an established toxicity assay. The neutral red assay was chosen to establish the toxicity data. The neutral red assay was carried out according to the procedure of Borenfreund and Puerner 1984. After 24 h incubation, the toxin amended medium was removed from the 96 well plate and the cells re-incubated for 3 h in 100 μl of a sterile solution of 40 μg/ml neutral red, prepared in Dulbecco's modified Eagles medium (the neutral red is incubated in medium for 24 h to allow crystal formation and the crystals are then removed by centrifugation 1,500 *g* for 10 min). The medium was decanted and the cells washed with 100 μl of 1% formaldehyde/10% calcium chloride solution. The neutral red dye is eluted from the cells using a 1% acetic acid/50% ethanol solution. To increase the efficiency of the elution process the plates were agitated on a plate shaker for 15 min. The optical density of each well was measured on a plate reading spectrophotometer at 492 nm. The optical density was expressed as a percentage of the control well values (Borenfreund and Puerner 1984).

Immunocytochemistry

L929 cells were grown in 24 well plates at 37°C in a 5% CO_2 humidified incubator at a cell density of 4×10^4 cells per ml. The last well received no cells and was used as a reagent blank. After 24 h attachment cells were exposed to increasing cadmium concentrations. The toxin-amended medium was removed and the cells washed twice with PBSA. The cells were fixed using a solution of ethanol and acetone (1:1 v/v) at 4°C for 10 min (Marshall and Kind 1994). The cells were washed three times with PBSA and were subsequently incubated with mouse anti-hsp70 antibody for 2 h (5 μg/ml in PBSA). The cells were washed three times in PBSA. The cells were incubated with goat anti-mouse alkaline phosphatase conjugate secondary antibody for 2 h (5 μg/ml in PBSA). Heat shock protein 70 was either qualitatively assessed by microscopic examination after 30 min incubation using an alkaline phosphatase substrate (nbt/bcip) that precipitates at the site of hsp70, or quantitatively assessed by spectrophotometer at 405 nm after 30 min incubation using an alkaline phosphatase substrate 10 mg p-nitrophenyl phosphate in 10 ml of 10 mM diethanolamine (pH 9.5) containing 0.5 mM $MgCl_2$ that produces a soluble colored end product. To ensure that alkaline phosphatase leakage did not interfere with the assay, a second control plate was assessed. The control plate was prepared in an identical manner to the test plate. However, instead of assessing hsp70 induction the alkaline phosphatase endpoint was used to assess the degree of alkaline phosphatase leakage.

Alkaline phosphatase assay

The protocol used in this study was as described by Connolly *et al*. (1986). The toxin-amended media was removed from the wells by inversion and the cells washed twice with pbsa. Reaction buffer (0.1 M sodium acetate pH 5.5, 0.1% Triton X-100 and 10 mM p-nitrophenyl phosphate) was added to each well (100 μl). The plates were incubated at 37°C in a 5% CO_2 humidified atmosphere for 2 h. The reaction was stopped with the addition of 10 μl of 1N NaOH. The optical density of each well was determined in a plate reader at 405 nm. The optical density of alkaline phosphatase for each test concentration was expressed as a percentage of the control well values and reported as percentage cell mortality (Connolly *et al*. 1986).

Data handling

For the neutral red assay the optical density of the dye for each test concentration was expressed as a percentage of the control well values and reported as percentage cell mortality (Borenfreund and Puerner 1984). Percentage cell mortalities for replicate tests (31 replicate tests) were averaged and plotted against toxin concentrations to generate dose response curves. The median lethal concentration was determined by linear regression (Shopsis and Sathe 1984) and taken as the concentration that resulted in 50% inhibition of cell viability (Trevan 1927). The no effect level was taken as the concentration whose cell mortality did not differ significantly from the control (Rand and Petrocelli 1985) as determined by the student *t*-test ($p = 0.05$). For the immunocytochemistry technique using the soluble colored substrate the optical density of the dye for each test concentration was compared to the control well values and reported as percentage hsp70 induction. Background levels of alkaline phosphatase were measured and subtracted from the optical density for each concentration. Percentage induction of heat shock protein 70 for replicate tests (four replicate tests) were averaged and plotted against toxin concentrations to generate stress response curves. The toxicity data for cadmium chloride was used as a measure to gauge the potential of the heat shock response assay as a biomarker in toxicology. To allow comparison between the neutral red assay and heat shock response assay the LC_{50} was used to compare sensitivities. The relationship between hsp70 induction and cell mortality was investigated using Pearson's rank correlation.

Results

The dose response curve for L929 cells exposed to cadmium chloride showed a typical sigmoidal shape with toxicity increasing in a dose dependent fashion (figure 1, table 1). The median lethal concentration (LC_{50}) was estimated by linear regression to be 2.33 mg/l cadmium chloride. The no effect level (NEL) was estimated using the student *t*-test to be 1.5 mg/l cadmium chloride. At cadmium chloride concentrations above 1.5 mg/l a significant effect on cell viability was demonstrated using the student *t*-test ($p = 0.5$).

The first immunocytochemistry method used a soluble colored enzyme substrate to quantifiably assess hsp70 induction. The heat shock response curve showed a dose dependent increase in hsp70 induction (figure 1, table 2). Heat shock protein induction at the LC_{50} was estimated to be 75%. Hsp70 induction showed a 25% greater response than found when measuring cell mortality. At cadmium chloride concentrations below the LC50 value (2.33 mg/l) a strong correlation between the two endpoints was found ($r = 0.85$) with hsp70 induction increasing with toxicity. However, at concentrations above the LC_{50} there was a rapid decrease in hsp70 accumulation. At concentrations above the LC50 value a negative correlation was found ($r = -0.68$) with hsp70 levels decreasing with increasing toxicity. In order to assess the potential of the heat shock response as a biomarker of sub-lethal toxicity, cadmium concentrations below the no effect level were examined. To allow direct comparison hsp70 induction at the no effect level

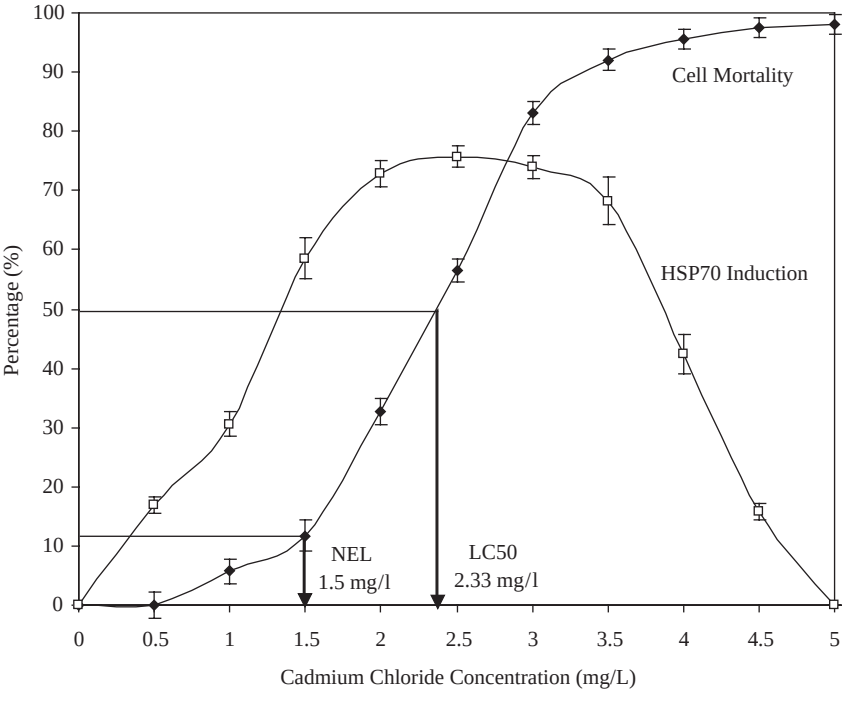

Figure 1. Comparison of cell mortality (●) and hsp70 induction (□) for the mouse connective tissue cell line (L929) to cadmium chloride. Cell mortality can be seen to increase in a dose dependent manner. Hsp70 induction shows a greater response but declines rapidly above the LC_{50} value.

Table 1. L929 cell mortality (%) after 24 h exposure to varying concentrations of cadmium chloride (mg/l).

	Cadmium chloride concentration (mg/l)										
	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5
% Mortality	0	0	6	12	33	57	83	92	96	97	98
SEM (n = 32)	0.00	2.35	1.99	2.60	2.31	2.01	1.95	1.73	1.74	1.61	1.66

was measured. Considerable hsp70 induction was observed at the no effect level (1.5 mg/l). Hsp70 induction at the no effect level was estimated to be 59% (table 2) identifying hsp70 induction at concentrations that were 1.6 times lower than the median lethal concentration. Hsp70 induction at the lowest concentration tested (0.5 mg/l cadmium chloride) showed a significant difference when compared to the control as demonstrated using the student *t*-test ($p = 0.5$). Hsp70 induction at the lowest concentration tested was estimated to be 15%, table 2, identifying hsp70 induction at concentrations that were three times lower than the no effect level.

The second immunocytochemistry method used an insoluble colored enzyme substrate to qualitatively assess the heat shock response. In control cells (0 mg/l cadmium chloride) under normal conditions hsp70 was localized predominantly within the cytoplasm with low levels found in the nucleus (figure 2). However, with increasing toxin concentration hsp70 was observed to translocate to the nucleus. Interestingly at intermediate toxin concentrations (1.5 mg/l cadmium chloride) a heterogeneous heat shock response was observed with some cells displaying a much stronger heat shock response than others (figure 3). At concentrations above the LC_{50} where few cells were alive, a strong heat shock response was observed with all cells inducing hsp70 throughout the cell (figure 4).

Discussion

For the heat shock response to have potential as a biomarker in toxicology it is essential that the heat shock response assay is rapid, reproducible, sensitive, economic, easy to use and suitable for routine application. In our previous study (Gibney *et al.* 2001), we induced heat shock proteins in cell cultures exposed to various agents, separated the proteins using 1-D SDS PAGE and confirmed hsp70 using Western blotting. The assay was extremely time consuming and labour intensive involving several steps each of which introduces the potential for

Table 2. HSP70 induction (%) in L929 cells after 24 h exposure to varying concentrations of cadmium chloride (mg/l).

	Cadmium chloride concentration (mg/l)										
	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5
HSP 70 % Induction	0.00	16.90	30.52	58.56	72.83	75.70	73.87	68.18	42.37	15.85	0.00
SEM (n = 4)	0.00	1.45	2.12	3.50	2.12	1.77	1.97	4.03	3.26	1.45	0.00

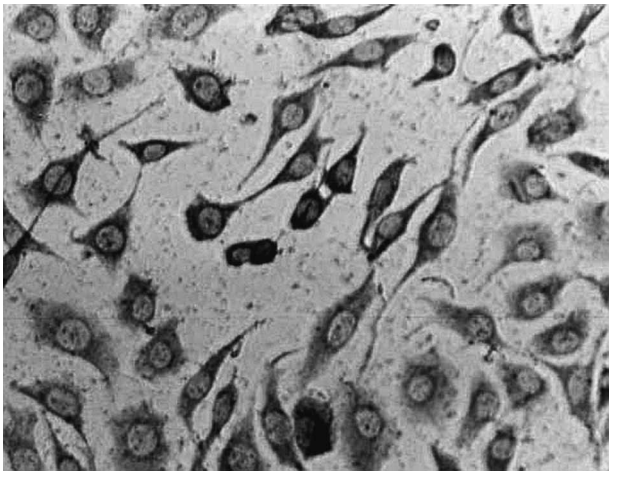

Figure 2. Heat shock protein 70 distribution in the mouse connective tissue cell line (L929). In control cells (0 mg/l cadmium chloride) under normal conditions hsp70 is localized predominantly within the cytoplasm (darker staining) with low levels found in the nucleus.

experimental error. To overcome these problems we developed an ELISA assay to increase the speed and convenience of the assay with the potential for automation. Marshall and Kind (1994) used immunocytochemistry to identify, localize and quantify heat shock proteins in cells. The advantage of immunocytochemistry over ELISA is the *in situ* nature of the technique, requiring no transfer of samples. This has the effect of reducing assay variability. Several investigators have used immunohistochemical techniques to detect heat shock proteins in tissues (Corso *et al.* 1997, George *et al*. 1998, Ding and Candido 2000, Patruno *et al*. 2001). However, few studies (Schlesinger *et al*. 1982, Marshall and Kind 1994, Killimede

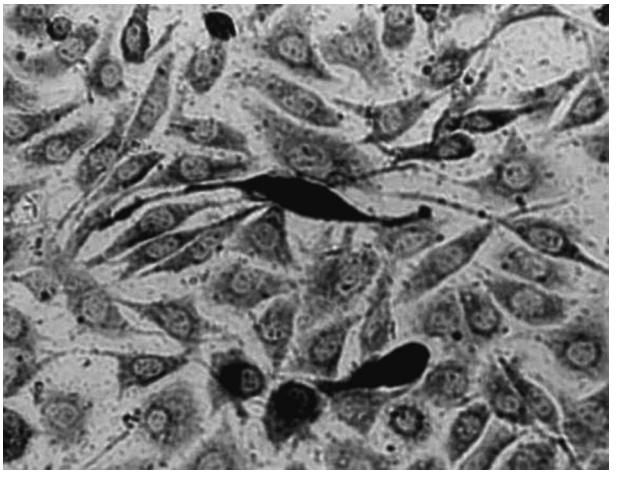

Figure 3. Heat shock protein 70 distribution in the mouse connective tissue cell line (L929) exposed to sub-lethal concentrations of cadmium chloride. At intermediate toxin concentrations (1.5 mg/l cadmium chloride) hsp70 translocates from the cytoplasm to the nucleus. A heterogeneous heat shock response was observed with some cells displaying a much stronger heat shock response than others.

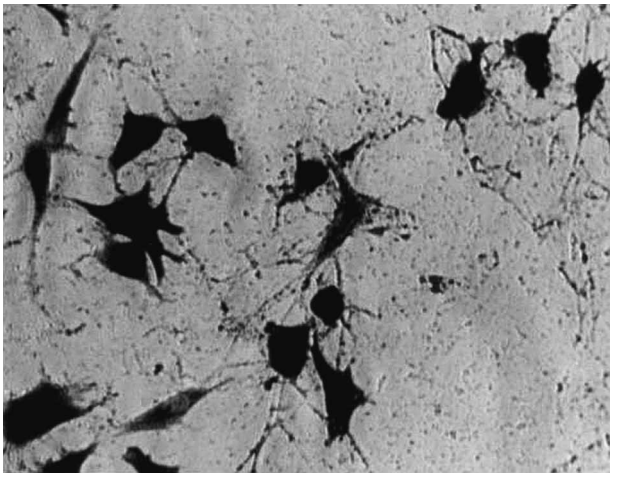

Figure 4. Heat shock protein 70 distribution in the mouse connective tissue cell line (L929) exposed to lethal concentrations of cadmium chloride. At lethal concentrations (3 mg/l cadmium chloride) a strong heat shock response was observed with all cells inducing hsp70 throughout the cell.

and Mothersill 2001, Wheeler *et al*. 2003, Njemini *et al*. 2003) have used immunocytochemical techniques to detect heat shock proteins in cells. In this study two approaches were adopted in order to determine the potential of the immunocytochemistry technique.

Our first approach employs a soluble colored endpoint that allowed the level of hsp70 to be quantitatively determined. L929 cells were exposed to cadmium chloride concentrations ranging from sub lethal to lethal. Using the soluble colored endpoint a dose dependent increase in hsp70 induction was observed (figure 1, table 2) and correlated with increasing cellular toxicity ($r = 0.85$) as determined using the neutral red assay (table 1), demonstrating biological significance. This corresponds to our previous findings (Gibney *et al.* 2001) and those of Fischbach *et al*. (1993), Stringham and Candido (1994) and Ryan and Hightower (1994). At the LC_{50} hsp70 induction was found to have a greater response than that of the neutral red assay (figure 1). This concurs with the findings of Ryan and Hightower (1994) and of Werner and Nagel (1997). The greater response shown by the induction of hsp70 above that of the neutral red assay indicates the heat shock protein response is a more sensitive assay allowing easier assessment of toxicity. However, at concentrations above the LC_{50} there was a rapid decrease in hsp70 accumulation. This may be accounted for by the reduction in cell numbers due to increased cell mortality and denaturation of hsp70 itself (figure 1, table 2). Cell mortality at these concentrations is extreme and outweighs hsp70 induction. A negative correlation ($r = -0.68$) between decreasing hsp70 induction and increasing toxicity was found at cadmium chloride concentrations above the LC_{50}. Fischbach *et al.* (1993) observed a reduced response at higher metal concentrations with excessive metal concentrations resulting in cell lysis. Several investigators have reported heat shock protein induction below a toxins median lethal concentration suggesting the potential to identify sub-lethal toxicity

(Ryan and Hightower 1994, Stringham and Candido 1994, Werner and Nagel 1997). This immunocytochemical approach identified hsp70 induction below the no effect level (1.5 mg/l) and at significant levels (30% above control levels) (table 2). Hsp70 induction at the lowest concentration tested (0.5 mg/l) was significant (15% above control levels) (table 2), demonstrating the sensitivity of the assay at concentrations several fold below the no effect level.

Our second approach using an insoluble colored endpoint that precipitates at the location of hsp70, qualitative data regarding the location and translocation of hsp70 could be ascertained. A single band isolated by the C92 anti-hsp70 monoclonal antibody using Western blotting supports the hypothesis that the results generated using the immunocytochemistry technique are for the inducible form of hsp70. Schlesinger *et al*. (1982) was one of the first to develop antibodies to heat shock proteins and apply them to immunocytochemistry techniques. Using monolayers of chicken embryo fibroblastic cells, they found no antigens present on the cell surface. However, cells fixed with methanol, to allow penetration, showed widespread intense staining. Hsp70 was distributed in both nuclear and cytoplasmic compartments with hsp90 being strictly cytoplasmic. Welch *et al*. (1982) also observed cytoplasmic and nuclear staining in several cell lines using a polyclonal anti-hsp70 antibody. Hsp100 has been observed to associate with the golgi apparatus and may be involved in its catabolic and metabolic functions (Lin *et al.* 1982, Welch *et al*. 1982). In this study, the inducible isoform of hsp70 was observed to be located predominantly within the cytoplasm and to a lesser degree within the nucleus in control samples. Hsp70 was observed to increase dramatically within the nucleus with metal exposure suggesting membrane translocation. This corresponds to the observations of other investigators (Welch *et al*. 1982, Lin *et al*. 1982, Lindquist 1986, Sanders *et al.* 1994, Marshall and Kind 1994, Burkhardt-Holm *et al*. 1998, Killemede and Mothersill 2001) who observed that cytoplasmic hsp70 and hsp60 move into the nucleus in response to heat shock, where they bind to pre-ribosomes and other protein complexes to help protect them from denaturation (Lindquist 1986, Gething and Sambrook 1992) and then returns to the cytoplasm during recovery. Sanders (1993) also noted that hsp70 and hsp60 interact differently with complexes in the nucleus suggesting that they have distinct roles in facilitating repair of different nuclear structures.

In this study using intermediate metal concentrations (1.5 mg/l cadmium chloride) the reaction was heterogeneous with only a portion of the cells showing hsp70. Similar observations have been made with regard to the heterogeneous nature of the heat shock response and have suggested a dependency on cell specific factors in hsp induction (Luce *et al*. 1993, Marshall and Kind 1994). Geetanjali *et al.* (2002) demonstrated that normal rabbit aorta showed normal histology and a homogeneous distribution of hsp70 while rabbit aorta at various stages of experimental atherosclerosis showed a heterogeneous distribution of hsp70. The heterogeneous response identified in this study using immunocytochemistry may account for some of the variability observed when using 1-D SDS-PAGE to demonstrate hsp70 induction. Vedel and Depledge (1995) found a high degree of variability in hsp70 induction of *M. edulis* with coefficients of variation (%CV) ranging from 48 to 66%. They proposed that this variation is masked in other

studies that pool organisms/samples. This study supports their proposal. An increase in hsp70 induction was observed in a dose dependent manner using pooled cells. Stringham and Candido (1994) also demonstrated the heterogeneous nature of the heat shock response.

At concentrations above the LC_{50} value hsp70 induction occurred throughout the cell. Marshall and Kind (1994) also observed this ubiquitous response and suggested that the intracellular distribution of hsp70 depended on the applied stimulus and recovery time. In this study, it was seen that the few viable cells at these lethal concentrations (3 mg/l cadmium chloride) possessed a very strong heat shock response. Killemede and Mothersill (2001) also observed this strong response with only 2–3% of the cells failing to exhibit hsp70. At these lethal concentrations the strength of the heat shock response can be demonstrated; however, the true potential of the heat shock response assay would appear to be at sub-lethal concentrations as demonstrated in this study.

Conclusion

In this study we used a simple *in situ* immunocytochemical assay to measure hsp70 induction in L929 cells exposed to cadmium chloride. Hsp70 induction was quantifiably measured using a soluble colored substrate and quantitatively measured using a colored substrate that precipitated at the location of hsp70. The assay demonstrated the heat shock response to be a sensitive measure of sub-lethal toxicity which concurred with our previous findings (Gibney *et al*. 2001). Hsp70 was observed to translocate from the cytoplasm to the nucleus upon exposure to cadmium chloride. At intermediate concentrations a heterogeneous response was observed. At lethal concentrations a very strong response was observed in the few cells that were still viable. This study demonstrates the potential of this immunocytochemical technique to measure toxicological affects in cells, identifying the response quantitatively and qualitatively. The true potential of this immunocytochemical approach is at sub-lethal concentrations. The ability to identify the location and intensity of hsp70 within the cell will add to our knowledge of heat shock protein function and toxicological pathways.

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