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DEVELOPMENT AND CHARACTERIZATION OF HYDROGEN PEROXIDE-RESISTANT CHINESE HAMSTER OVARY CELL VARIANTS—I

RELATIONSHIP BETWEEN CATALASE ACTIVITY AND THE INDUCTION/STABILITY OF THE OXIDANT-RESISTANT PHENOTYPE

ORAZIO CANTONI,* ANDREA GUIDARELLI, PIERO SESTILI, FERNANDO MANNELLO,†
GIANCARLO GAZZANELLI† and FLAMINO CATTABENI

Istituto di Farmacologia e Farmacognosia and Centro di Farmacologia Oncologica Sperimentale, and †Istituto di Istologia, Università degli Studi di Urbino, 61029 Urbino, Italy

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Abstract—Hydrogen peroxide (H_2O_2)-resistant sublines of Chinese hamster ovary (CHO) cells were isolated by *in vitro* exposure to the oxidant (treatment for 1 hr followed by 3 days of growth in peroxide-free medium). Stepwise increase in low level H_2O_2 concentrations produced variants which were progressively more resistant to the growth inhibitory effect elicited by the oxidant. Removal from H_2O_2 decreased resistance and the curve describing this process was biphasic in nature. In addition, the rate of loss of the H_2O_2-resistant phenotype was more rapid for the toxicity elicited by low concentrations of hydrogen peroxide, compared to that produced by high concentrations. Changes in total cell proteins were found to parallel the variations in sensitivity to the oxidant, since the protein content constantly increased during the adaptation process and decreases upon removal from H_2O_2. Catalase activity did not show large variations in resistant sublines with respect to the parental cell line, and these changes were at least partially related to differences in cell size/amount of total cell proteins of the sublines. In addition, the minor changes observed for catalase activity did not correlate with the degree of resistance to growth inhibition elicited by the oxidant. It may therefore be suggested that the H_2O_2-resistant phenotype of mammalian cells, initially adapted to low—then gradually increased—concentrations of the oxidant, is the result of a complex phenomenon which only partially involves over-expression of catalase.

Exposure of cultured mammalian cells to a low concentration of hydrogen peroxide induces a transient resistance to toxic levels of the oxidant [1]. This effect is similar to the well-described phenomenon of thermotolerance where thermal inactivation is reduced by an initial heat shock [2]. A relationship seems to exist between tolerance to toxicity and the ability of these agents to induce stress proteins. Indeed, exposure of cultured mammalian cells to heat shock as well as to hydrogen peroxide is known to result in the selective and reversible expression of specific polypeptides, while lowering the production of most other cellular protein [1, 3, 4]. Acquired resistance to oxygen free radicals represents a significant problem in the treatment of cancer patients with specific antitumour drugs [5] and may have other important implications since oxygen free radicals are increasingly implicated as mediators of cell injury under a variety of pathological circumstances [6, 7]. Nevertheless, the underlying molecular mechanisms governing cellular resistance to oxygen intermediates remain largely unknown. Recently, attempts have been made to isolate cells resistant to hydrogen peroxide with this specific aim and with the additional purpose to gain insights into the mechanisms involved in cell inactivation.

Spitz and Li [8] have developed H_2O_2-resistant variants of Chinese hamster ovary (CHO†) cells (HA-1) by culturing the cells in progressively higher concentrations of the oxidant. Cell variants obtained in this study were characterized by a very high stability of the oxidant-resistant phenotype, and the degree of resistance was associated with an increased catalase activity. Thus, it would seem that an important mechanism by which cells may acquire resistance to challenge with hydrogen peroxide is through the enhanced expression of the gene coding for one of the enzymes catalysing the inactivation of hydrogen peroxide. In other studies by Spitz and co-workers, cloned cell lines highly resistant to hydrogen peroxide were also found to display an enormous increase in catalase [9–11], reduced glutathione [12, 13], as well as higher glutathione peroxidase activity [10, 11], which also suggests that resistance to hydrogen peroxide and other types of oxidative stress may occur via induction of an increased detoxification system. These data are consistent with previously published work [14–21]. Importantly, however, Spitz and Li [8], in order to isolate resistant variants, have adopted an exposure protocol involving an initial treatment of 2.5×10^5 cells with a high concentration of H_2O_2 (inducing a 0.1% survival, as measured by cloning efficiency assays),

* Corresponding author: Dr Orazio Cantoni, Istituto di Farmacologia e Farmacognosia, Università di Urbino, Via S. Chiara, 27, 61029 Urbino, Italy.

† Abbreviations: CHO, Chinese hamster ovary; ID_{50}, the drug concentration inhibiting cell growth by 50%.

followed by growth for 5 days. The authors reported that, under these conditions, only three to five healthy colonies, over a background of cells unable to form colonies, were detected [8]. Thus, the isolation procedure utilized in this study was based, at least in the initial step, on a selection of cells which may have had an intrinsic resistance to the oxidant.

In the present study, we have attempted to develop hydrogen peroxide resistant cells by using mildly toxic concentrations of the oxidant, which were then progressively increased. In other words, we have attempted to slowly adapt cells, rather than select them on the basis of their intrinsic resistance, to the insult elicited by the oxidant. This procedure allowed us to obtain cell variants characterized by a high level of resistance to hydrogen peroxide and a rapid loss of the oxidant resistant phenotype. Resistance, however, could not be fully explained by the changes in catalase activity.

MATERIALS AND METHODS

Materials. Hydrogen peroxide was purchased as a 30% solution from J. T. Baker Chemicals B.W., (Deventer, The Netherlands). Chemicals and most reagent grade biochemicals were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). McCoy's 5a medium, foetal bovine serum and trypsin were from Gibco (Grand Island, NY, U.S.A.).

Cells and cell culture conditions. CHO cell sensitive and resistant sublines were grown in McCoy's 5a medium supplemented with 10% foetal bovine serum and 1% (v/v) penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified atmosphere containing 5% CO_2 in air, at 37°.

Isolation of hydrogen peroxide-resistant cells. By stepwise incubation with hydrogen peroxide (1 hr treatment followed by 72 hr of growth in drug-free medium) variants from CHO cells have been developed with resistance to the oxidant. Wild type cells were exposed to gradually increasing doses over a 4.5-month period up to a maximum dose of 850 μM H_2O_2 and then grown in the absence of the drug for more than 7 months. The resistant sublines described here, V150, V250, V450, V650 and V850, were named for the final H_2O_2 concentration used for their selection, i.e. 150, 250, 450, 650 and 850 μM, respectively. CHO cells (2×10^6 cells/75 cm^2 flask) were initially treated for 1 hr with 150 μM H_2O_2 (corresponding to 0.75 μmol/cell $\times 10^{-7}$) and then, following rinsing, were allowed to grow for 3 days in fresh culture medium. Under these conditions, cell growth was reduced by about 80%, but only a marginal proportion of the cells underwent lysis and the loss of the reproductive capacity was a transient phenomenon, since the growth rate returned to control levels upon growth for a further 2 days (not shown). At the end of the 3 days post-challenge growth, cells were trypsinized (0.025%), counted and re-plated at the density of 2×10^6 cells/75 cm^2 flask. The above treatment protocol with 150 μM H_2O_2 was repeated four times, i.e. until the cells were able to reach a density of 5–6 $\times 10^6$ cells/flask after the 3 days of post-challenge growth. The concentration of H_2O_2 was then increased to 200 μM and nine cycles of treatment with the oxidant, followed by growth in fresh medium for 3 days, were necessary to induce resistance, i.e. to enable the cells to reach a density of 5–6 $\times 10^6$ cells/flask. Oxidant exposure was continued by treating the cells four times with 250 μM H_2O_2, once with 450 μM H_2O_2, nine times with 650 μM H_2O_2 and seven times with 850 μM H_2O_2. At the end of each cycle cell variants were tested for their sensitivity to the growth inhibitory action elicited by hydrogen peroxide.

V850 cells were cultivated further for up to 7 months and never exposed to the oxidant. Cells were taken at different times and named for the number of days of growth without challenge with H_2O_2. As an example, R127 and R209 cells refer to V850 cells grown for 127 and 209 days, respectively.

Cell growth inhibition studies. Cells (5×10^5) were inoculated into 60 mm tissue culture dishes and allowed to attach. After 6 hr the original medium was removed and replaced with fresh culture medium containing increasing concentrations of hydrogen peroxide. Treatments were for 1 hr. Cells were then allowed to grow for 48 hr and counted with a Coulter counter particle-size analyser. Cell number present in the oxidant-treated dishes was expressed as a function of the number of cells in untreated dishes to assess the influence of hydrogen peroxide on cell replication.

Catalase activity. Cells were seeded at a density of 2×10^6 cells/75 cm^2 flask and harvested after 24 hr of growth. Cell pellets obtained after centrifugation at 400 *g* for 5 min were washed twice and resuspended in saline A. The cell suspensions were then placed on ice and sonicated three times for 15 sec at 20 W using a Branson Sonifier. The resulting homogenates were centrifuged for 5 min at 18,000 *g* at 4°. Catalase activity was assayed spectrophotometrically in the supernatant by the method of Aebi [22] that involves monitoring at 240 nm the disappearance of H_2O_2 (10 mM) in the presence of the cell homogenate. The enzymatic activity was determined by plotting a standard curve constructed with bovine liver catalase and was expressed in Sigma Units (1 Sigma Unit decomposes 1 μmol of H_2O_2 per min at pH 7 at 25°). To exclude the possibility of "matrix interference" (which may result from the presence of substances in the various sublines with the potential of interfering with the assay), refinding experiments were performed by adding bovine liver catalase to known amounts of crude cellular extracts. Refinding of catalase was identical in all cell types.

Assay of protein. The protein content of the cell lysate of CHO cultures was determined by the method of Lowry *et al.* [23].

RESULTS

Growth inhibition induced by H_2O_2 in sensitive and resistant cells

Logarithmically growing sensitive and resistant cells were treated for 60 min with increasing concentrations of H_2O_2 and then assayed for cell number after 48 hr of growth. A typical dose–response pattern of H_2O_2 sensitivity for parental, V150, V250 and V850 cells is presented in Fig. 1. As shown in the figure the adaptation procedure

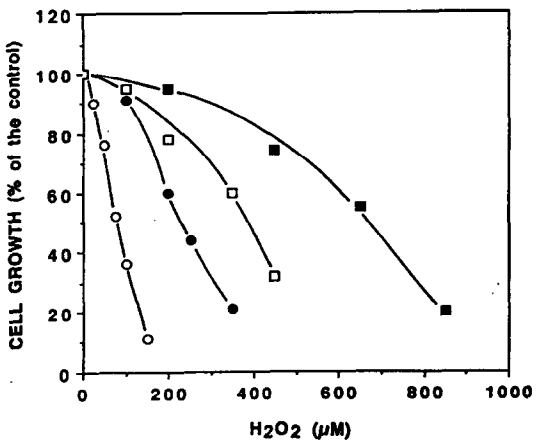

Fig. 1. Growth inhibitory action of hydrogen peroxide in sensitive and resistant cells. Exponentially growing cells resistant to different levels of H_2O_2 were treated for 60 min with increasing concentrations of the oxidant and, after 48 hr of growth, were assayed for cell number as described in the Materials and Methods. Each point is the mean of two determinations (performed in duplicate) with coefficients of variation less than 10%. Key: wild type (○); V150 (●); V250, (□); V850 (■).

Table 1. Induction of the oxidant-resistant phenotype in CHO cells exposed to increasing concentrations of H_2O_2

Subline	Resistance factor*
Wild-type	—
V150	3.1
V250	5.3
V450	5.8
V650	6.2
V850	9.5

* H_2O_2 ID_{50} values were calculated from growth inhibition studies where cells with different levels of resistance to H_2O_2 were exposed to increasing concentrations of the oxidant and then allowed to grow for 48 hr.

Results are expressed as resistance factor, which is defined as the ratio of the ID_{50} of resistant cells to that of the parental cell line.

Each point is the mean of two determinations (performed in duplicate) with coefficients of variation less than 10%.

markedly decreased the sensitivity of the cells to growth inhibition induced by the oxidant. H_2O_2 ID_{50} (the drug concentration inhibiting cell growth by 50%) values were calculated from experiments similar to the ones depicted in Fig. 1 (e.g. obtained with cells with different levels of resistance to H_2O_2) and results, expressed as resistance factor (which is defined as the ratio of the ID_{50} or resistant cells to that of the parental cell line) are shown in Table 1. The ID_{50} of hydrogen peroxide for V850 cells was 700 μM, compared with 75 μM for the parental CHO cell line. The calculated resistance of V850 cells to hydrogen peroxide was 9.5-fold.

Stability of the oxidant-resistant phenotype in V850 cells

V850 cells were incubated for various lengths of time in the absence of hydrogen peroxide and then assayed for their sensitivity to the oxidant. The ID_{50} value was determined and the resistance factor (see above) was expressed as a function of time following removal from H_2O_2. Figure 2A shows that resistance to the oxidant decreased as a function of time and was totally suppressed by approx. 7.5 months of growth in peroxide-free medium. It should also be noted that the curve describing the kinetics of the loss of the oxidant-resistant phenotype displayed a biphasic character. The relative proportion of the total resistance that was suppressed by the fast phase (after 40 days) was approx. 65%. The remaining 35% was lost during the additional 200 days of growth (slow phase). The existence of a rapid loss of resistance (fast phase) was confirmed by growing V650 cells in the absence of the oxidant for 10 or 20 days (Fig. 2A). The analysis of the growth inhibition curves obtained in these studies indicates that also these curves display a biphasic character (not shown) and this would suggest that the oxidant-resistant phenotype is differentially retained under conditions of challenge with low or high concentrations of hydrogen peroxide. We have therefore estimated the ID_{80} values and the calculated resistance factor was related to time of growth in the absence of hydrogen peroxide. Data shown in Fig. 2B indicate that the loss of resistance was once again described by a biphasic curve and the relative proportion of the total resistance that was suppressed by the fast phase (after 40 days) was about 50%. This decreased rate of the loss of the oxidant-resistant phenotype (compared to that shown in Fig. 2A) was confirmed in V650 cells (Fig. 2B). Interestingly, the data presented in Fig. 2B also indicate that, even after 230 days of growth in the absence of hydrogen peroxide, a significant proportion of resistance to challenge with the oxidant was retained (30%).

Protein content of sensitive and resistant cells

Resistant cells were characterized by an increased total protein content. Indeed, V150, V250 and V850 cells had more proteins than wild-type cells ($152 \pm 9.98/10^6$ cells) and the increase (1.46-, 1.56- and 2.33-fold, respectively) was statistically significant (V150, $P < 0.001$; V250, $P < 0.0003$; V850, $P < 0.0001$). Total protein content in R127 cells was significantly lower (1.48-fold), compared with the V850 subline ($P < 0.05$), but still higher than wild-type cells (1.58-fold, $P < 0.005$). Further growth in peroxide-free medium resulted in an additional decrease in total protein content. However, although R209 cells had 1.86 times less proteins than V850 cells ($P < 0.0001$) maintained a protein content higher than wild-type cells (1.25-fold, $P < 0.05$). In order to assess the relationship between the level of total proteins and the degree of resistance, the ID_{50} values for growth inhibition by H_2O_2 in sensitive and resistant cells were plotted against the μg proteins/1 $\times 10^6$ cells in the various sublines. An excellent direct correlation was found either in cells differentially adapted to the oxidant

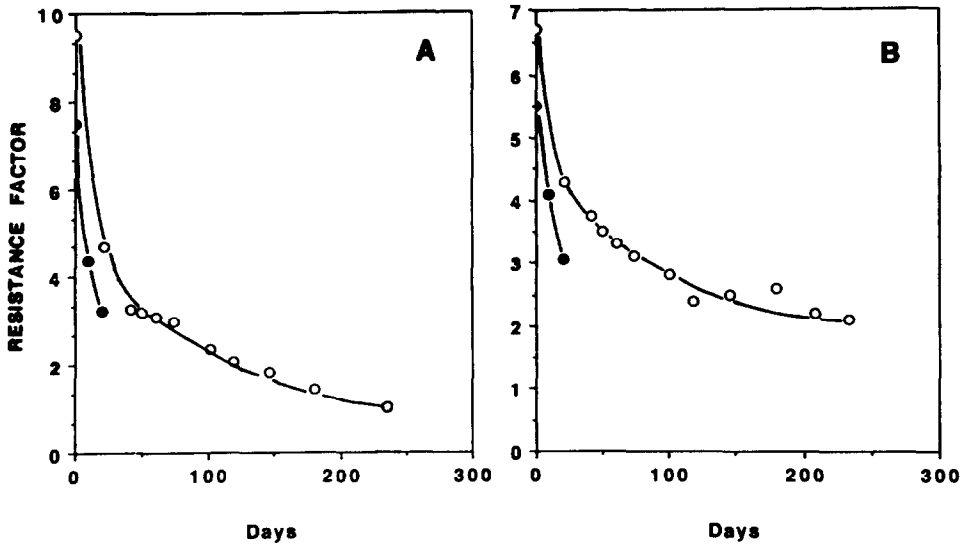

Fig. 2. Loss of the oxidant-resistant phenotype in resistant cells following removal from H_2O_2. ID_{50} (A) and ID_{80} (B) values were calculated from growth inhibition experiments performed in resistant cells challenged with increasing concentrations of H_2O_2 at various time intervals following cessation of the adaptation protocol. Results are expressed as resistance factor. V650 (●) and V850 (○) cells were utilized in these experiments. Each point is the mean of two determinations (performed in duplicate) with coefficients of variations less than 10%.

($r = 0.988$; Fig. 2A) or in V850 cells grown for various lengths of time with no hydrogen peroxide ($r = 0.902$; Fig. 3B).

Catalase activity in sensitive and resistant cells

Data illustrated in Fig. 4A indicate that the adaptation process resulted in a slight increase in the cellular content of this enzyme when the activity was expressed on a per 10^6 cell basis. Indeed, V150, V250 and V850 cells had greater catalase activity than wild-type cells and the increase (1.1-, 1.44- and 1.29-fold, respectively) was statistically significant for both V250 and V850 cells ($P < 0.0001$). Catalase activity was also higher in R127 cells (1.22-fold increase vs wild-type cells; $P < 0.0001$) and returned to control levels in R209 cells. Growth for 127 and 209 days in peroxide-free medium lowered cellular catalase activity and the decrease vs V850 cells (0.95-fold for R127 and 0.8-fold for R209) was statistically significant ($P < 0.01$ and $P < 0.0001$, respectively). Due to the fact that different amounts of proteins were present in wild-type and resistant sublines, results appear markedly different by expressing catalase activity on a per mg protein basis (Fig. 4B). By using this normalization procedure, variants were found to display a lower content of this detoxifying enzyme as compared to wild-type cells (0.75 times for V150 cells, $P < 0.0001$; 0.92 times for V250 cells, $P < 0.01$; 0.51 times for V850 cells, $P < 0.0001$; 0.77 times for R127, $P < 0.0001$; 0.83 times for R209, $P < 0.01$). Catalase activity apparently increased upon growth in peroxide-free medium. The activity was 1.5 and 1.6 times higher in R127 ($P < 0.01$) and R209 ($P < 0.01$) with respect to that found in V850 cells. We have next attempted to correlate catalase

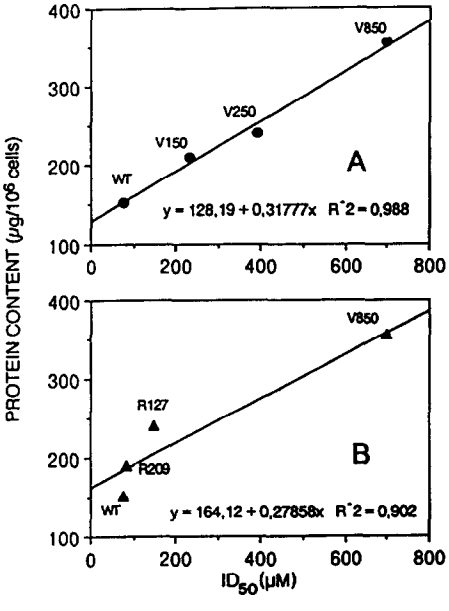

Fig. 3. Correlation between total protein content in sensitive and resistant cells and growth inhibition induced by hydrogen peroxide. Values for total proteins expressed in μg per 10^6 cells have been plotted against the ID_{50} values for growth inhibition by H_2O_2 in sublines obtained during the adaptation process (A) or following growth of V850 cells in the absence of the oxidant for various lengths of time (B).

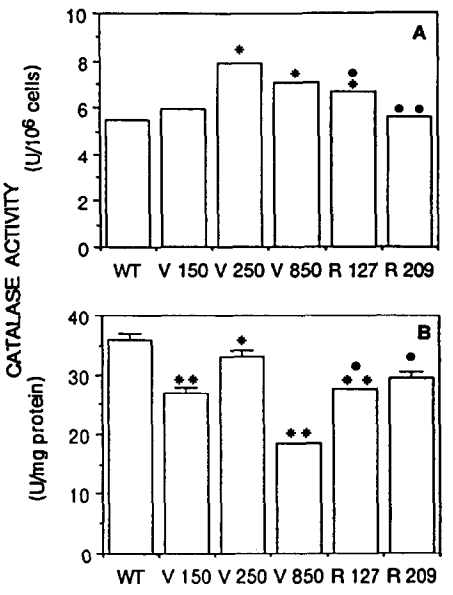

Fig. 4. Catalase levels in sensitive and resistant cells. Catalase activity was determined 1 day after splitting, as described in the Materials and Methods. Values represent the mean ± SEM calculated from 8–16 determinations in separate cultures. Data are expressed both on a per 10^6 cells basis (A) and per mg protein basis (B). (A) Significantly different from wild-type cells: $P < 0.0001$ (*). Significantly different from V850 cells: $P < 0.01$ (•); $P < 0.0001$ (••). (B) Significantly different from wild-type cells: $P < 0.01$ (*); $P < 0.0001$ (**). Significantly different from V850 cells: $P < 0.01$ (•).

activity to the ID_{50} values for growth inhibition by H_2O_2 in sensitive and resistant cells. Data illustrated in Fig. 5A indicate that catalase activity expressed on a per 10^6 cells basis poorly correlates with the degree of resistance ($r = 0.461$) of the sublines obtained at the different stages of the adaptation process. When catalase activity was expressed on a per mg protein basis (Fig. 5B) a paradoxical inverse correlation, although very weak ($r = 0.676$), was apparent. The poor relationship existing between catalase activity and resistance to the oxidant was further assessed by comparing the cytotoxic response of V850, R127, R209 and wild-type cells to hydrogen peroxide with catalase activity expressed on a per 10^6 cell basis (Fig. 5C; $r = 0.668$). As shown in Fig. 5C this was mainly due to the fact that R127 and V850 cells although markedly differing in their level of resistance to the oxidant, displayed very similar catalase activity. It is worth noting that, in analogy to the data shown in Fig. 5B, a paradoxical inverse correlation was found when the enzyme activity was expressed on a per mg protein basis (Fig. 5D; $r = 0.822$).

DISCUSSION

In this paper we have described the isolation and partial characterization of CHO cell variants resistant to hydrogen peroxide. Cells were gradually adapted to an initial mildly toxic level of H_2O_2 and stepwise increase in the oxidant concentration resulted in a slow and progressive increase in the resistance of the cells to the insult generated by hydrogen peroxide (Fig. 1 and Table 1). The H_2O_2-resistant phenotype, however, was highly unstable since growth of the cells in peroxide-free medium for as short as 20–40 days resulted in a marked decrease in the resistance to growth delay induced by the oxidant (Fig. 2). These results differ from those obtained by Spitz and Li [8], whose resistant cells were characterized by a very high stability of the H_2O_2-resistant phenotype. The likely explanation for this discrepancy is that our cells were slowly adapted to H_2O_2 and behave as true variants, whose oxidant-resistant phenotype is strictly dependent on the environment, i.e. the presence of hydrogen peroxide in the extracellular milieu. On the other hand, resistant cells obtained by Spitz and Li [8], as pointed out in the introduction, may have been selected on the basis of their intrinsic resistance to H_2O_2 and therefore may carry genetically stable alterations.

Another important feature of the resistant cells described in this paper is that the rate of the loss of the H_2O_2-resistant phenotype depended upon whether ID_{50} (Fig. 2A) or ID_{80} (Fig. 2B) values were taken into account. This is to say that resistance to concentrations of hydrogen peroxide, resulting in 50%, or less, reduction of cell proliferation, was lost faster than resistance to levels of the oxidant producing a marked (80% or more) inhibition of the reproductive capacity. Although the explanation for the biphasic loss of the H_2O_2-resistant phenotype is not immediately apparent, we may speculate that cells had developed different mechanisms for resistance to low or high concentrations of H_2O_2 and that these mechanisms are characterized by differing degrees of stability. Such a model would be consistent with an interpretation of a dual mechanism of toxicity operating at low or high concentrations of hydrogen peroxide.

Results obtained in this study also demonstrate a correlation between total protein content of the various sublines and resistance to H_2O_2. This correlation was found either when biochemical data were related to the cytotoxic response obtained in cells characterized by different levels of resistance to H_2O_2 (i.e. cells isolated at various steps of the adaptation process, Fig. 3A) or in resistant cells grown for various time intervals in the absence of the oxidant (Fig. 3B). This correlation could either be circumstantial or suggestive of a mechanism according to which cells may become resistant to hydrogen peroxide. There are at least two possibilities which could explain why an increase in total cell proteins might result in protection against oxidative stress. Firstly, an increase in protein content should be paralleled by an enhanced availability of scavenging enzymes and, since these enzymes are located at critical sites, minor variations in their activity within a single cell would greatly enhance the resistance to cell injury caused by the H_2O_2. Secondly, an enhanced amount of proteins should decrease the statistical probability of oxyradicals interacting with sensitive targets. Indeed, an increased cell mass should increase the possibility of

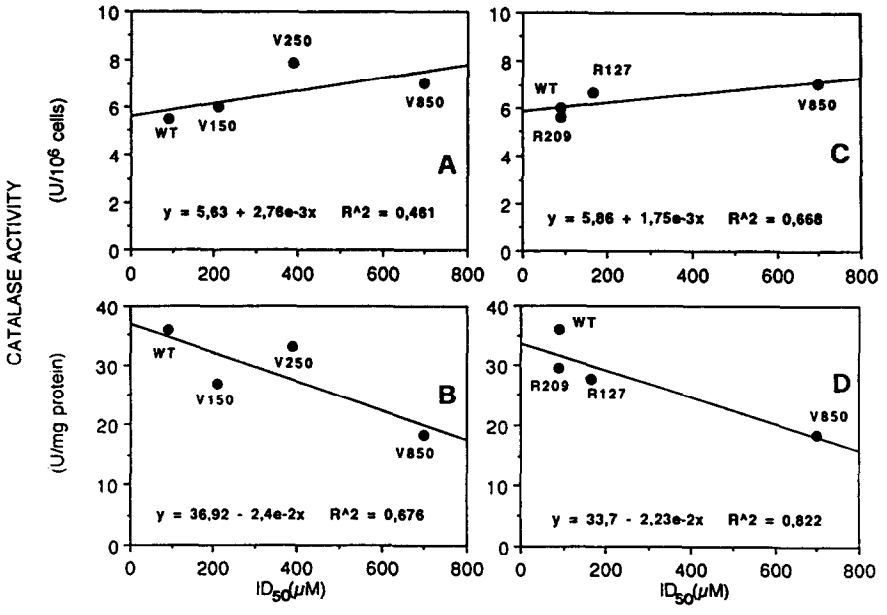

Fig. 5. Correlation between catalase activity and cytoxicity induced by hydrogen peroxide in sensitive and resistant cells. Values for catalase activity expressed on a per 10^6 cells basis (A and C) or per mg proteins basis (B and D) have been plotted against the ID_{50} values for growth inhibition by H_2O_2 in sublines obtained during the adaptation procedure (A and B) or following growth of V850 cells in the absence of the oxidant for various lengths of time (C and D).

the interaction of hydroxyl radicals, and/or other reactive oxygen species derived from H_2O_2, with non-specific/non-critical subcellular components thus decreasing the probability that these radicals may reach critical targets.

Finally, we report that catalase activity, expressed on a per 10^6 cell basis, shows moderate changes in resistant sublines (Fig. 4A) and is actually lower when the data are expressed on a per mg protein basis (Fig. 4B). Obviously, this decrease is only apparent since it is largely due to the marked increase in cellular proteins which accompanied the adaptation process. Indeed, by comparing the results shown in Fig. 4 (catalase activity) with those displayed in Fig. 3A (protein content) it may be inferred that chronic challenge with hydrogen peroxide initially increases catalase activity at a slower rate than total proteins (V150 cells). Then, catalase continues its slow increase whereas total proteins do not (V250) and, finally, the opposite seems to occur (total proteins increase whereas catalase remains unchanged). Thus, the levels of this scavenging enzyme display very limited variations in sensitive and resistant cells and these changes poorly correlate with the degree of resistance to the oxidant (Fig. 5). As an example, V250 cells, although displaying more catalase activity (on a per 10^6 cell basis) than V850 cells, were markedly less resistant to growth inhibition by H_2O_2 (Fig. 5A). A similar observation could be made for R127 and V850 cells (Fig. 5C). Clearly, a lack of correlation exists between catalase activity expressed on a per mg protein basis and resistance to H_2O_2; this inference is best explained by the data shown in Fig. 5B and D indicating that catalase activity (expressed on a per mg protein basis) had a paradoxical inverse relationship with resistance to H_2O_2. Interestingly, catalase activity of V850 was practically half of that of wild-type cells, whereas the variant was about 10 times more resistant to growth inhibition induced by H_2O_2. Taken together, the above results may suggest that the rapid loss of the H_2O_2-resistant phenotype observed in the first 4 months of growth of V850 cells in the absence of hydrogen peroxide, was only marginally, if at all, related to changes in catalase activity. This event appeared more relevant as a determinant for the loss of the oxidant-resistant phenotype observed at longer time intervals. A further conclusion which can be drawn from this set of experimental data is that catalase distribution within the cell must be critical since small changes in the levels of this enzyme on a per cell basis play a protective role, independently on the total protein content. Obviously, care should be taken in future experiments comparing the levels of this enzyme in different cell lines, especially those investigating the molecular basis for resistance to specific antitumour drugs in resistant tumour cell lines.

In conclusion, results presented in this study indicate that mammalian cells can be adapted to high concentrations of hydrogen peroxide with minor alterations in catalase activity. Although these results are in apparent contrast with those obtained by other authors, indicating an association between the hydrogen peroxide-resistant phenotype and catalase over-expression [8–10], we should point out that in our cells resistance may depend upon an increased efficiency of other cell defense mechanisms (e.g.

glutathione peroxidase, etc.), which are currently under investigation. Alternatively, and this is the subject of future research, resistant cells may have developed more efficient mechanisms to recover from the insult generated by hydrogen peroxide.

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