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THE INDUCTION/LOSS OF THE OXIDANT-RESISTANT PHENOTYPE OF CHINESE HAMSTER OVARY (CHO) CELL VARIANTS DOES NOT CORRELATE WITH SENSITIVITY TO DNA SINGLE STRAND BREAKAGE BY HYDROGEN PEROXIDE

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Abstract—Hydrogen peroxide resistant variants of Chinese hamster ovary (CHO) cells characterized by different levels of resistance to growth inhibition induced by the oxidant displayed a decreased susceptibility to the induction of DNA single strand breakage by hydrogen peroxide. Resistance to DNA damage, however, was maximal in cells resistant to killing by low concentrations of H_2O_2, and did not increase further in cells characterized by a much higher resistance to the toxic action of the oxidant. Different sensitivities to the induction of DNA single strand breakage observed in wild type and resistant sublines were related to a decreased susceptibility/differential depletion of H_2O_2, rather than being dependent on different velocities in DNA repair processes. Growth of resistant cells in the absence of H_2O_2 resulted in a rapid loss of resistance to induction of DNA strand scission by H_2O_2. Cells retained resistance to the growth-inhibitory effect of the oxidant under conditions where resistance to the production of DNA single strand breaks was lost. Experiments aimed at elucidating the molecular basis for resistance to DNA damage induction by H_2O_2 have demonstrated that this effect is dependent upon the catalase activity of the specific sublines as well as on their different total protein content.

Key words: hydrogen peroxide; oxidant-resistant phenotype; DNA damage; cytotoxicity

The toxicity of H_2O_2 seems to depend on the formation of hydroxyl radicals (OH·) since scavengers of this reactive oxygen species efficiently prevent the lethal action of H_2O_2 [1–5]. In addition, iron chelators are also capable of preventing cell inactivation induced by the oxidant [4, 5], indicating that the formation of OH· occurs via an iron-catalysed reduction of H_2O_2, the so-called Fenton reaction. At toxic levels, hydrogen peroxide produces DNA SSBs† [3–17] and although these lesions are repaired very rapidly, an association between these two events has been suggested [5, 6]. Experimental evidence, however, is largely circumstantial, and this theory is grounded on the fact that inhibiting the production of DNA single strand breakage with iron chelators or scavengers of hydroxyl radicals can also reduce toxicity [5, 6]. On the other hand, the production of DNA single strand breakage could simply be an epiphenomenon; an alternative interpretation could be that both events are generated by the same radical species and, therefore, by inhibiting their production (iron chelators) or their effects (hydroxyl radical scavengers), both DNA damage and cytotoxicity could be suppressed. A relationship between the sensitivity of cells from different species to cytotoxicity and the yield of DNA single strand breakage has also been demonstrated [6]. Importantly, these cell lines had similar capacities to consume hydrogen peroxide from the culture medium [6]. Other studies have also attempted to find a relationship between DNA damage induction and cell inactivation and, although exposure to the oxidant was performed at ice temperature, no cause–effect relationship could be found [7, 8]. This exposure protocol may be responsible for the different results. Indeed, data obtained at 4° should only reflect the exclusive action of chemical/free radical processes since biochemical/enzymatic processes are not operative at ice temperature. Nevertheless, the available information does not allow any straightforward interpretation of the mechanism by which micromolar concentrations of hydrogen peroxide impair the reproductive capacity of the cells, and whether or not DNA single strand breakage is responsible for this effect remains unclear at present. We have tested this hypothesis by investigating the sensitivity to the induction of DNA damage in cell variants characterized by increasing levels of resistance to the growth inhibitory effect of hydrogen peroxide, and found that resistant cells also showed a moderate decrease in their sensitivity to DNA single strand break induction by hydrogen peroxide; this effect, however, did not correlate with the level of resistance to cytotoxicity.

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† Abbreviations: CHO, Chinese hamster ovary; SSB, single strand break; PFGE, pulsed field gel electrophoresis; DSB, double strand break.

MATERIALS AND METHODS

Materials. Hydrogen peroxide was purchased as a

30% solution from J. T. Baker Chemicals B. W. (Deventer, Holland). Metal compounds 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.). Methyl-[^{14}C]-thymidine was from NEN (Boston, MA, U.S.A.).

Cells and cell culture conditions. Cell lines were grown in McCoy's 5a medium supplemented with 10% foetal bovine serum and 1% penicillin–streptomycin in a humidified atmosphere containing 5% CO_2 in air at 37°. The procedure utilized for isolating hydrogen peroxide resistant variants has been described elsewhere [18]. Briefly, cells (2×10^6 cells/75 cm^2 flask) were adapted to 850 μM H_2O_2 (treatment was for 1 hr followed by 3 days of growth) by stepwise increases from 150 to 850 μM H_2O_2 over a period of 4.5 months. These cells will hereafter be called V 150, V 250 and V 850 (depending on the oxidant concentration to which the cells had been adapted). Cell variants were cultivated for a further 127 or 209 days and never exposed to the oxidant and are referred to as R 127 and R 209, respectively.

Cell growth inhibition studies. Cells (4.5×10^5) were inoculated into 60 mm tissue culture dishes and after 6 hr the monolayers were exposed for 30 min to increasing concentrations of hydrogen peroxide (added from a freshly prepared solution in distilled water). At the end of the treatment, the original medium was removed and replaced with fresh culture medium. Cells were allowed to grow for 48 hr and then counted with a Coulter counter particle-size analyser. Cell numbers present in the oxidant-treated dishes were expressed as a function of the number of cells in untreated dishes to assess the influence of hydrogen peroxide on cell reproduction.

Alkaline elution assay. Experimental cultures for alkaline elution were labelled overnight with methyl-[^{14}C]thymidine (0.05 μCi/mL) plated in 35 mm tissue culture dishes, and then chased for 6 hr in label-free medium. Cells containing [^{14}C]DNA were exposed to H_2O_2 for 30 min and then analysed for DNA damage. Cells were removed from the dishes by trypsinization (1% trypsin for 5 min at ice temperature). The filter elution assay was carried out by a procedure virtually identical to that described by Kohn *et al.* [19] with minor modifications [9]. Briefly, 5×10^5 cells were gently loaded onto 25 mm, 2 μm pore polycarbonate filters and then rinsed twice with 10 mL of ice cold saline A (0.14 M NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM glucose) containing 5 mM EDTA (disodium salt). Cells were then lysed with 5 mL of 2% sodium dodecyl sulphate, 0.025 M EDTA (tetrasodium salt), pH 10.1. Lysates were rinsed with 7 mL of 0.02 M EDTA (tetrasodium salt) and the DNA was eluted overnight in the dark with 1.5% tetraethyl ammonium hydroxide/0.02 M EDTA (free acid)/0.1% sodium dodecyl sulphate (pH 12.1), at a flow rate of ca. 30 μL/min. Fractions of approximately 3 mL were collected and counted in 7 mL of Lumagel containing 0.7% glacial acetic acid. The DNA remaining on the filters was recovered by heating for 1 hr at 60° in 0.4 mL of 1 N HCl followed by the addition of 0.4 N NaOH (2.5 mL) and was again determined by scintillation counting. DNA was also recovered from the interior of the membrane holders after vigorous flushing with 3 mL of 0.4 N NaOH. This solution was processed for scintillation counting as described above.

Strand Scission Factors (SSF) were calculated from the resulting elution profiles by determining the absolute log of the ratio of the percentage of DNA retained in the filters of the drug-treated sample to that retained from the untreated control sample (both after 8 hr of elution).

PFGE assay. Cells were labelled overnight with [^{14}C]thymidine (0.05 μCi/mL) and incubated for a further 6 hr in a medium containing unlabelled thymidine (1 μg/mL).

PFGE was performed essentially as described by Blocher and Kuhni [20]. To prepare agarose plugs, cells were sedimented at 1000 rpm for 5 min at 4° and the pellet was resuspended in PBS containing 5 mM EDTA, pH 8.3. This procedure was repeated three times, and the final suspension in PBS was adjusted to give a density of 3.64×10^6 cells/mL. Suspension (300 μL) was mixed with 300 μL of melted agarose (low melt agarose, Biorad, Richmond, CA, U.S.A.; 1% solution in PBS) in a 15 mL conical tube maintained at 45°. The cell–agarose mixture was vortexed, transferred to a gel plug former on ice, and refrigerated for 15 min. The plugs (four per experimental point) were removed and incubated in 1 mL of ESP (0.5 M EDTA, 1% sarkosyl, 1 mg/mL proteinase K, pH 9) for 1–2 hr at 4° then at 45° for 20 hr. The plugs were washed five times for 1 hr in sterile 0.5 × TE buffer (10 mM Tris–HCl, 1 mM Na_2EDTA; pH 7.5) and stored at 4° in 0.5 M EDTA (pH 8).

The DNA in the plugs was separated by PFGE in a 0.5% agarose gel (chromosomal grade, Biorad, Richmond, CA, U.S.A.) in 0.5 × TAE buffer (44.5 mM Tris base, 34.5 mM sodium borate, 1 mM Na_2EDTA, pH 7.2). The gel was run for 20 hr on a CHEF-DR II system (BioRad) operating at 1.21 V/cm with a switch time of 75 min. The buffer (0.5 × TAE) was maintained at 25°. The gel was stained with ethidium bromide, viewed under a UV transilluminator and photographed. The distribution of radioactivity in the gel was determined by cutting portions of the gel containing the DNA (well and lane). These portions were then melted in glass scintillation vials in the presence of 50 μL of 1 N HCl and processed for liquid scintillation counting.

RESULTS

In this study we sought to determine whether the level of resistance of hydrogen peroxide-resistant CHO cell variants to challenge with the oxidant correlated with resistance to DNA SSB-induction. We therefore incubated wild type cells and variants (characterized by increasing levels of resistance) with 50 μM H_2O_2 for 30 min and estimated the number of DNA SSBs using the filter elution assay. Results shown in Fig. 1 indicate that cells resistant to killing by H_2O_2 also showed a decreased susceptibility to the DNA damaging action elicited by the oxidant, although V 250 cells seemed somewhat more resistant than V 850 cells. Figure 1B shows the dose–response curves for DNA SSB-induction by H_2O_2 in wild type and V850 cells and

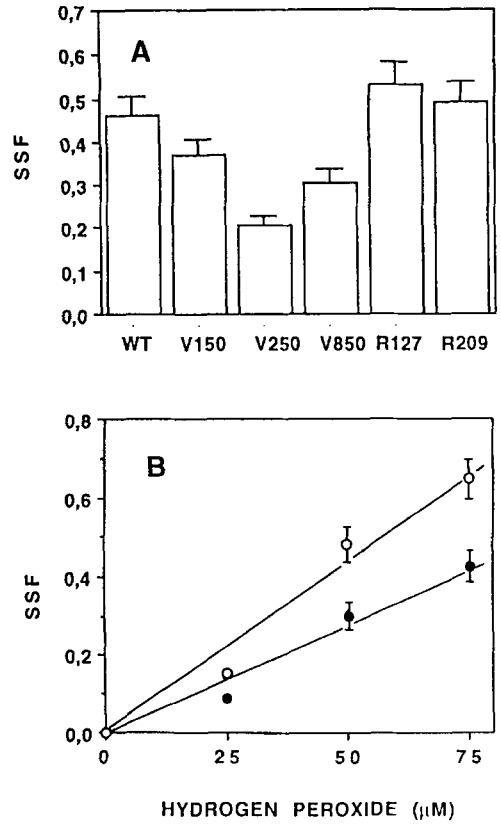

Fig. 1. H_2O_2-induced DNA single strand breakage in sensitive and resistant cells. (A) Cells were treated for 30 min at 37° in saline A with 50 μM H_2O_2 and assayed for DNA SSBs. (B) Wild type and V 850 cells were exposed to increasing concentrations of H_2O_2 for 30 min at 37° and processed as described above. SSFs were calculated as described in Materials and Methods section. Experimental values shown in panels A and B represent the means ± SEM calculated from three separate experiments each performed in duplicate.

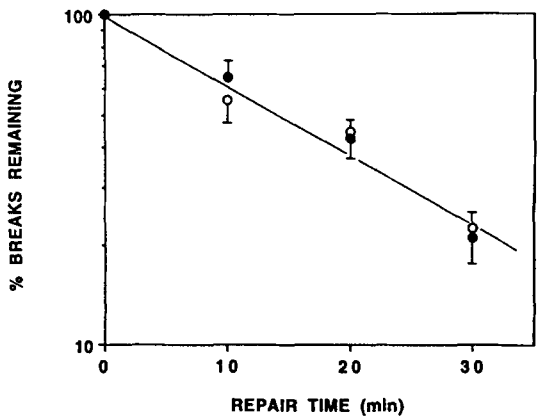

Fig. 2. Kinetics of removal of DNA SSBs produced by H_2O_2 in sensitive and resistant cells. Wild type cells (open circles) were exposed to 50 μM H_2O_2 whereas V 850 cells (closed circles) received a bolus of 75 μM H_2O_2. After treatment (10 min at 37°), cells were rinsed and allowed to repair in a complete culture medium and, at specific time intervals, analysed for DNA SSBs. Values represent the means ± SEM of three separate experiments, each performed in duplicate.

Table 1. IC_{50} values for growth inhibition by H_2O_2 in sensitive and resistant cells

Cell line*	IC_{50} (μM)
wild type	75
V 150	232
V 250	397
V 850	712
R 127	150
R 209	78

* Cells were incubated for 30 min at 37° with increasing concentrations of the oxidant and, following 48 hr of growth, assayed for cell number, as detailed in Materials and Methods. The IC_{50} values were calculated from the corresponding growth inhibition curves and indicate the oxidant concentration inhibiting cell proliferation by 50%.

indicates that resistance was maintained over the entire concentration range investigated. Similar results were obtained with V 150 and V 250 cells. In order to assess whether a relationship exists between the level of DNA damage and cytotoxicity, the data shown in Fig. 1A should be compared with the ID_{50} values for cytotoxicity reported in Table I. Clearly, the results obtained in DNA damage studies correlate poorly with those obtained in cytotoxicity studies. For example, V 250 and V 850 cells were more resistant to DNA damage induction by a factor of 2.2 and 1.45 with a dose-modifying factor (DMF) for cytotoxicity of 5.23 and 9.5, respectively. DNA strand scission was also investigated in variants adapted to the highest oxidant concentration (850 μM) and then grown in peroxide-free medium for 127 and 209 days. The sensitivity of the variants to DNA damage returned to that of the parental cell line after 127 days of growth in peroxide-free medium and remained unchanged upon additional growth for a further 82 days (Fig. 1A). Importantly, these cells maintained some resistance to the growth inhibitory action elicited by hydrogen peroxide (Table I). These results once again emphasize the lack of relationship between the sensitivity of the cells to DNA single strand breakage and cytotoxicity.

We have previously shown that the level of DNA single strand breakage found after exposure of CHO cells to H_2O_2 for a fixed period of time is not only a function of the total level of breaks that are produced, but also depends on the proportion of these breaks that have been rejoined during the treatment [9, 10]. Thus, although the assessment of the steady state level of DNA single strand breakage at a fixed time of exposure may give an indication of the responsiveness, at the molecular level, to the insult elicited by the oxidant in sensitive and resistant cells, it is also important to assess whether

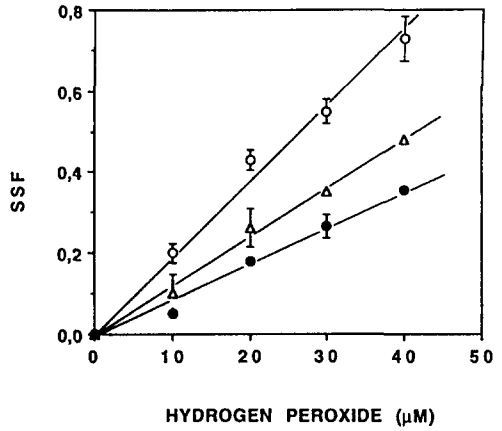

Fig. 3. Effect of hydrogen peroxide at ice temperature on DNA SSB-induction in sensitive and resistant cells. Wild type (○), V 250 cells (△) and V 850 cells (●) were exposed to increasing concentrations of H_2O_2 for 30 min at 4° and assayed for DNA SSBs. Values represent the means ± SEM calculated from three separate experiments, each performed in duplicate.

the observed differences in sensitivity were a consequence of different rates of removal of DNA strand breaks. In order to do so, we investigated the kinetics of repair of DNA SSBs generated by a short term exposure of wild type and V 850 cells to the oxidant. In these experiments, treatments were performed at 37° for 10 min and H_2O_2 was used at 50 μM in the parental cell line and at 75 μM in V 850 cells. Under these experimental conditions, similar levels of initial damage to the DNA, as measured by the filter elution assay, were produced. Following treatment, cells were allowed to repair in fresh, pre-warmed medium for various time intervals, and assayed for residual DNA damage. The rate of removal of DNA lesions generated by H_2O_2 was similar in wild type and V 850 cells, and was characterized by a $t_{1/2}$ of approx. 15 min (Fig. 2). Similar results were obtained in V 250 and R 127 cells (not shown). We therefore conclude that the lower level of DNA single strand breakage observed in the variants, compared to wild type cells, was due to the production of a decreased number of strand breaks, and not dependent on different velocities in DNA repair processes.

Different rates of depletion of the oxidant from the culture medium might be responsible for the observed resistance of the variants to DNA damage induced by hydrogen peroxide. Indeed, an increased activity of the enzymes inactivating the oxidant, i.e. catalase and glutathione peroxidase, could markedly affect the level of DNA SSB-induction by regulating the intracellular concentration of the oxidant. In a previous paper [18] we investigated catalase levels in sensitive and resistant cells and found that the parental cell line and the variants displayed moderate variations in catalase content. Due to the marked differences in total cell proteins, we expressed these results both on a per million cell basis and on a per milligram protein basis. V 150, V 250 and V 850 cells had a greater catalase activity (expressed on a per million cell basis) than wild type cells (1.1-, 1.44- and 1.29-fold, respectively). Catalase activity was also higher in R 127 cells (1.22-fold) and returned to control levels in R 209 cells. By using a different normalization procedure (per milligram protein basis), variants were found to display a lower content of this detoxifying enzyme as compared to wild type cells (0.75-fold for V 150 cells; 0.92-fold for V 250 cells; 0.51-fold for V 850 cells; 0.77-fold for R 127; 0.83-fold for R 209). Obviously, the decrease is only apparent and is due to fluctuations in total cell proteins. As previously noted [18], the moderate variations in catalase activity do not fully explain the molecular basis for the resistance of the variants to growth inhibition by hydrogen peroxide. A correlation, however, can be found between resistance to DNA damage induction and catalase activity, at least when the data of enzyme activity are expressed on a per million cell basis. Indeed, V 250 cells, which had the highest catalase activity, also displayed the lowest level of DNA single strand breakage following treatment with the oxidant. If such a relationship exists, then sensitivity to DNA damage induction should be similar in sensitive and resistant cells exposed to the oxidant at 4°, a temperature at which biochemical and enzymatic processes are not operative. It was somewhat surprising to find that V 250 and, to a greater extent, V 850 cells retained resistance to DNA SSB-induction by hydrogen peroxide at ice temperature (Fig. 3); therefore, one must conclude that additional mechanisms, and not scavenging enzymes alone, protect cells against this type of insult. Although it is important to emphasize that the underlying molecular mechanisms involved in the production of DNA single strand breakage are different at physiologic and ice temperature [9–11] it can be that even at 37° these factors might be of importance. In a previous study [9] we performed a detailed characterization of DNA SSBs produced by hydrogen peroxide at ice temperature and noted that the degree of strand breakage was not a linear function of exposure time. Indeed, the rate of production of DNA SSBs decreased continuously with time, reaching a plateau after approximately 60 min. This effect was due to time-dependent depletion of the oxidant from the culture medium. In this study treatments were for 30 min and, consistent with our previous observation, we found evidence of a slightly reduced rate of accumulation of DNA strand breaks over the time of exposure (not shown). In addition, this effect was cell density dependent (not shown). Again, these findings may be interpreted as an indication of a progressive depletion of the oxidant from the culture medium. Since scavenging enzymes are not operative at ice temperature, it is likely that hydrogen peroxide is "consumed" via its reaction with divalent iron to form highly reactive oxygen species (hydroxyl radicals). It was therefore important to determine whether the resistance of the variants to the induction of DNA single strand breakage induction by H_2O_2 was dependent on the different cellular masses. Indeed, we had previously reported that V 250 and V 850 cells had approx. 2.5 and 3.5 times more proteins than wild type cells,

respectively [18]. To find experimental support for the above hypothesis, we estimated the level of DNA damage produced by H_2O_2 in wild type cells at different cell densities. As expected, the sensitivity to DNA damage was an inverse function of cellular density (not shown).

Finally, we investigated whether DNA DSBs were produced at growth-inhibitory-range concentrations of hydrogen peroxide in sensitive and resistant cells. Experimental results (not shown) have demonstrated that, under these experimental conditions no DNA DSBs can be detected using the highly sensitive PFGE assay [20].

Conclusions

The purpose of isolating cells resistant to hydrogen peroxide is to understand the underlying molecular mechanisms governing cellular resistance to the oxidant and, possibly, the mechanisms involved in cell injury. As far as the mechanism of cytotoxicity of micromolar levels of hydrogen peroxide is concerned, a number of studies have suggested an association between the production of DNA single strand breakage and the loss of reproductive capacity [5, 6]. The data presented in this paper do not support this hypothesis. Indeed, we find no correlation between the level of resistance to the growth inhibitory effect of the oxidant and the susceptibility to DNA damage in cell variants characterized by different degrees of resistance to killing by H_2O_2. The obvious conclusion is that DNA single strand breakage has hardly anything to do with cell death in oxidatively injured cells.

Ward and co-workers [7, 8] had calculated that 0.4 million DNA SSBs are required to kill a cell following challenge with hydrogen peroxide and that DNA DSBs cannot be detected under these conditions. Likewise, we report that no DNA DSBs are produced in sensitive and resistant cells, at least at oxidant growth–inhibitory-range concentrations. We have also shown that a very high number of DNA SSBs are generated by hydrogen peroxide, as compared to X-rays, under equitoxic conditions [21] and that the susceptibility of CHO cells to killing by H_2O_2 was only slightly augmented when the repair of DNA SSBs was slowed down by incubating the cells in the presence of 3-aminobenzamide, an inhibitor of the enzyme poly(ADP-ribose) polymerase [10]. Finally, we recently demonstrated that although L-histidine increases both DNA SSBs and cytotoxicity generated by hydrogen peroxide, the L-histidine-mediated enhancement of hydrogen peroxide-induced cytotoxicity is not necessarily associated with an increased formation of DNA single strand breakage. Three lines of evidence support this inference: (1) addition of an excess of L-glutamine, virtually inhibiting L-histidine uptake, reduced cytotoxicity but did not prevent the increased formation of DNA SSBs [22]; (2) treatment with the oxidant following pre-loading with the amino acid leads to an enhanced cytotoxic response with no increased formation of DNA SSBs [23]; and (3) DNA single strand breakage is increased by treatment with L-histidine and hydrogen peroxide at ice temperature, but lethality is not [12]. These observations are in keeping with recently published data from Coleman *et al.* [24] which indicate a dissociation of the accumulation of DNA single strand breakage from the killing of hepatocytes treated with various agents producing an oxidative stress and therefore suggest that DNA single strand breaks caused by oxygen derived radicals do not represent a lethal lesion.

In conclusion, the results presented in this paper demonstrate that in cell variants resistant to hydrogen peroxide the level of resistance to killing elicited by the oxidant does not correlate with sensitivity to DNA SSB-induction, and therefore suggest that, consistent with previous results obtained in our and other laboratories, this type of lesion is not the cause of cytotoxicity in oxidatively-injured cells. These negative findings, however, do not provide any evidence for the actual mechanism involved in cell killing elicited by hydrogen peroxide levels in the micromolar range. Recent reports have demonstrated that U 937 human myeloid leukemia [25] and LLC-PK_1 renal tubular epithelial cells [26] challenged with 1 mM hydrogen peroxide will eventually die by apoptosis. On the other hand, experiments in progress in our laboratory demonstrate that wild type CHO cells, under the conditions utilized in this study, do not die by apoptosis: another negative finding which leaves the mechanism whereby low levels of hydrogen peroxide kill CHO cells still unsolved.

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