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Growth Inhibition and Differentiation Induction in Murine Erythroleukemia Cells by 4-Hydroxynonenal

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4-Hydroxynonenal (HNE) is one of the major end products of lipid peroxidation. Here we show that the exposure of murine erythroleukemia (MEL) cells to 1 μM HNE, for 10.5 h over 2 days, induces a differentiation comparable with that observed in cells exposed to DMSO for the whole experiment (7 days). The exposure of MEL cells for the same length of time demonstrates a higher degree of differentiation in HNE-treated than in DMSO-treated MEL cells. The protooncogene c-myc is down-modulated early, in HNE-induced MEL cells as well as in DMSO-treated cells. However, ornithine decarboxylase gene expression first increases and then decreases, during the lowering of the proliferation rate. These findings indicate that HNE, at a concentration physiologically found in many normal tissues and in the plasma, induces MEL cell differentiation by modulation of specific gene expression.

Keywords: Lipid peroxidation, HNE, MEL cells, differentiation, gene expression

INTRODUCTION

Lipid peroxidation (LPO) leads to the production of several aldehydes of relatively high half-life.[1] A negative correlation exists between both the proliferative activity and/or anaplastic grade, and the ability of neoplastic cells to peroxidize.[2] In fact, highly undifferentiated anaplastic cells show undetectable levels of both basal and inducible LPO and a low or undetectable level of LPO products.[3] The most important class of aldehydes derived from LPO is the 4-hydroxy-2,3-trans alkenal group. 4-Hydroxynonenal (HNE) is quantitatively the major alkenal formed.[3,4] Its concentration in normal tissues ranges from 0.2 to 2.8 μM.[4] Within this range of concentration,

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HNE can affect signaling pathways through modulation of enzymatic activities or gene expression (see review[5,6,7]). Biological and toxic effects of HNE largely depend on its ability to react with sulphydryl and amino groups of low molecular weight compounds and proteins.[8] When added to cell suspension, HNE shortly disappears since it rapidly reacts with proteins and is also metabolized by the cells.[9] HPLC experiments demonstrated that HNE is detectable in the culture medium for no more than 45 minutes after its addition.[9] To prolong the exposure of the cells to the low aldehyde concentrations, repeated treatments were performed in K562 and HL-60 human leukemic cells.[9,10] When HNE was maintained in the cell suspension for 7.5 h, through repeated treatments at intervals of 45 min, the aldehyde induced a terminal, granulocytic-like differentiation of HL-60 cells[10] and a time-dependent inhibition of K562 cell growth.[9] In these cell lines, in which endogenous HNE production is not detectable, a single addition of 1 μM HNE inhibited ornithine decarboxylase (ODC) activity[11] as well as c-myc expression;[12,13] while it induced, in K562 cells, a transient increase of γ-globin expression.[12]

Murine erythroleukemia (MEL) cells represent a good model to study the inducer-mediated differentiation of transformed cells. They can be induced to terminally differentiate and to express the erythroid phenotype by a variety of chemical agents, such as DMSO and hexamethylenebisacetamide (HMBA).[14,15] As observed in K562[12] and HL-60 cells[10] the malonyldialdehyde (MDA) and HNE content in MEL cells was undetectable even if exposed to prooxidant stimuli (data not shown). Thus, they represent a good model for the study of exogenous HNE effect on erythroid differentiation. However, the inducer must be maintained in the culture medium for some hours to obtain the onset of differentiation. Thus our first aim was to define the procedure of repeated HNE treatments able to induce the highest level of MEL cell differentiation. Moreover, to elucidate the mechanism of HNE action, we examined the expression patterns of an early response gene, c-myc, and of a delayed-early response gene, ornithine decarboxylase (ODC), which were modulated differently in MEL cells during the differentiation induced by HMBA and DMSO.[16,17]

MATERIALS AND METHODS

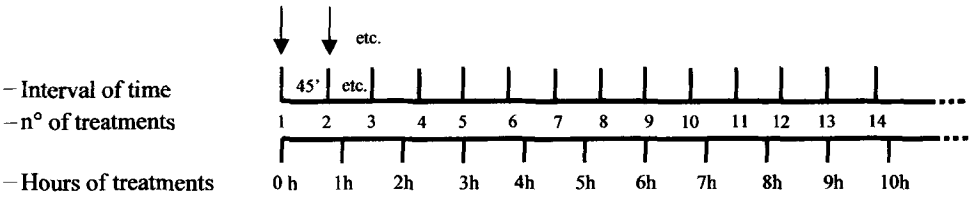
Cells and Culture Conditions

Mouse erythroleukemic (MEL) cells (clone 86, kindly provided by Prof. Pontremoli, University of Genova, Italy) were cultured at 37 °C in a humidified atmosphere of 5% CO-air in RPMI 1640 medium supplemented with 2 mM glutamine, antibiotics and 10% fetal calf serum (Gibco BRL). Cells were maintained at a density of $1–2 \times 10^5$/ml. Cell viability was assessed by the trypan blue exclusion test.

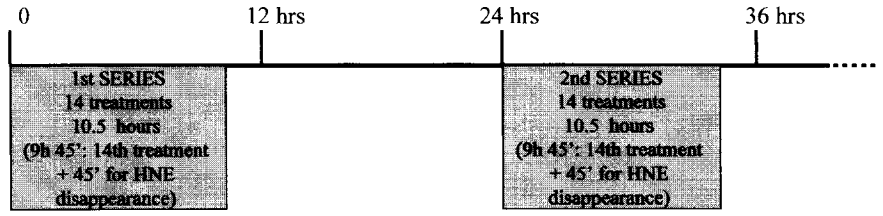
Induction of Differentiation

4-Hydroxynonenal (HNE, gift from Dr. J. Schaur, University of Graz, Austria) was prepared as previously reported.[10] Exponentially growing cells were resuspended at a concentration of 2×10^5/ml in fresh complete medium. HNE treatments (final concentration 1 μM) were performed at regular intervals of time (45 min) as illustrated in Figure 1:

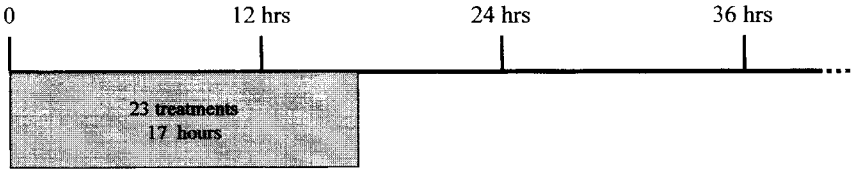
(A) repeated treatments over 2 days: 14 treatments at day 1 (first series) followed by 14 treatments at day 2 (second series). The overall time of exposure was evaluated by counting 45 min following the last treatment. In this case, it was 10.5 h for each series (45 min after the 14th treatment performed at 9 h and 45 min);
(B) 23 treatments over 1 day (17 h of exposure to HNE);



A) REPEATED TREATMENTS OVER 2 DAYS



B) 23 TREATMENTS OVER 1 DAY



C) 14 TREATMENTS OVER 1 DAY

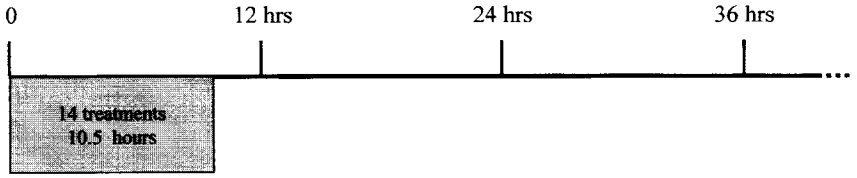


FIGURE 1 Schedules of treatments with HNE.

(C) 14 treatments over 1 day (10.5 h of exposure to HNE).

The HNE concentration, at the end of each treatment, was measured by HPLC as previously reported.[9] No accumulation of HNE was detected: its concentration in the culture medium did not exceed 1 μM even soon afterwards the start of each treatment and, 45 min after the last addition, HNE was undetectable (data not shown). DMSO (Sigma Chemical Co) was added to the culture medium at the beginning of experiments, at a final concentration of 1.5%. When needed, the culture was washed to remove DMSO and resuspended in fresh complete medium without inducer.

Hemoglobin

Hemoglobin (Hb) concentration in MEL cells was measured as follows: 10×10^6 cells were lysed in 1 ml of lysing buffer (10 mM Tris pH 7.5, 137 mM NaCl, 1.4 mM Mg-acetate and 0.5% Nonidet P-40), and incubated on ice for 15 min. The lysate was then centrifuged at 1,500 rpm for 15 min (ALC, 4236 centrifuge). Hb concentration was quantified by optical density (OD) at 414 nm (O.D. 1,000 corresponds to 0.13 mg/ml Hb).

RNA Isolation

Total cytoplasmic RNA was isolated from MELC as described by Sambrook *et al.*[18] with minor modification. Each sample was examined for purity and degradation on a nondenaturating agarose gel prior to Northern blot analysis. Total RNA was isolated at different time points after addition of inducers (HNE or DMSO). Uninduced cells were used as time 0.

Northern Blot Analysis

Equal amounts of RNA (15–30 μg), as determined by UV-absorption at 260 nm, were separated on 0.9% agarose –2.2 M formaldehyde gel and transferred to nitrocellulose filters (Hybond-C, Amersham). Hybridizations were carried out as previously described[11] with probes labelled by the random priming method (Megaprime DNA labelling system RPN 1607, Amersham; 1.5×10^6 cpm/ml hybridization buffer). Each filter was hybridized with mouse β-globin, human c-myc exon III, mouse ODC cDNA probes and with rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to control the differences in the amount of RNA transferred.

RESULTS

Effect of HNE on Cell Growth and Differentiation

Figure 1 (panels A, B, C) shows the procedures of different HNE treatments. In cells repeatedly treated with HNE over 2 days (as shown in Figure 1, panel A), both the proliferation rate and the hemoglobin production of MEL cells were analysed and compared with those induced by DMSO (Figure 2). DMSO is a potent and well-known inducer of MEL differentiation and it was used as positive control. In this set of experiments, DMSO was maintained in the culture medium until the end of experiment (7 days). Cell proliferation was strongly inhibited by HNE treatments and this effect was more pronounced

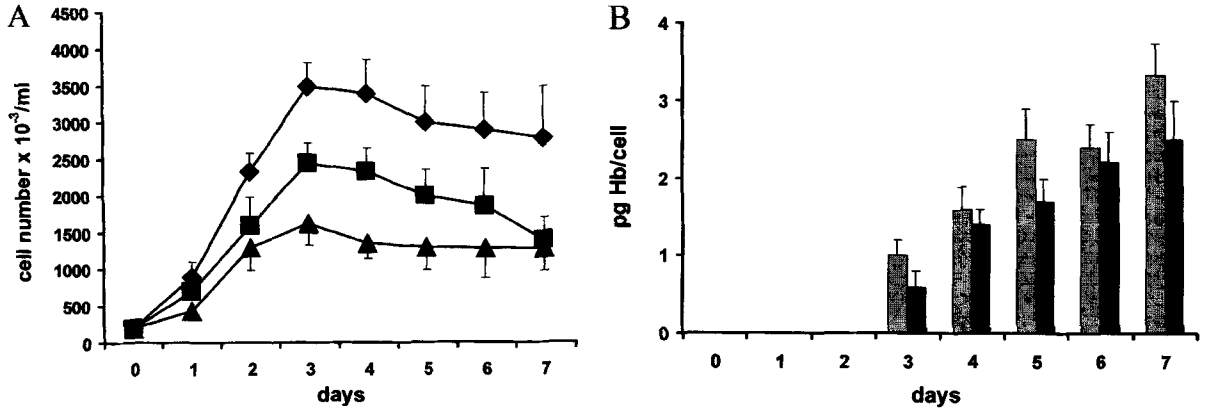


FIGURE 2 Panel A: Growth of MEL cells treated with HNE (repeated treatments over 2 days, see Figure 1) or with 1.5% DMSO (maintained in cell culture for the whole experiment). (◆) MEL cell control, (■) DMSO, (▲) HNE. Data are the mean ± S.D. of four separate experiments; Panel B: Hemoglobin content in MEL cells treated with HNE or with 1.5% DMSO as described in panel A. (▩) DMSO, (■) HNE. Data are the mean ± S.D. of four separate experiments.

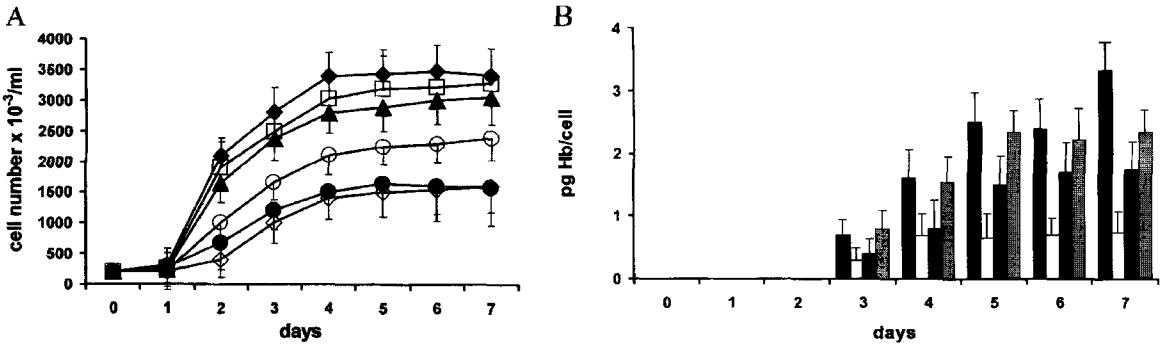


FIGURE 3 Panel A: Growth of MEL cells exposed for 17 or 10.5 h to HNE (see Figure 1 panel B and C, respectively) or exposed to 1.5% DMSO for 10.5 and 17 h (same times of HNE exposition) or for the whole experiment. (◆) MEL cell control, (□) DMSO 10.5 h, (▲) DMSO 17 h, (○) HNE 10.5 h, (◇) HNE 17 h, (●) DMSO for the whole experiment. Data are the mean ± S.D. of four separate experiments; Panel B: Hemoglobin content in MEL cells exposed for 17 or 10.5 h to HNE (see Figure 1 panel B and C, respectively) or exposed to 1.5% DMSO for the whole experiment or for 17 h. (■) DMSO for the whole experiment, (□) DMSO 17 h, (■) HNE 10.5 h, (■) HNE 17 h. Data are the mean ± S.D. of four separate experiments.

in HNE-treated cells than in DMSO-treated cells (Figure 2, panel A). On the other hand, the antiproliferative effect of DMSO was comparable with that obtained by other authors in this cell line.[14]

The values of hemoglobin were determined throughout the experiments (7 days) by subtracting the control values from the values obtained after HNE or DMSO treatments at the corresponding time. Hemoglobin was detected by day 3 and increased during the following days (Figure 2, panel B). The values were similar in both HNE- and DMSO-treated cells.

Cell viability, at the end of treatments and during the following days, was not affected in HNE- and DMSO-treated cultures (data not shown).

To demonstrate a correlation between the length of HNE exposure and the effectiveness to induce terminal differentiation, MEL cells were continuously treated with 1 μM HNE for 10.5 h or for 17 h, respectively (as shown in Figure 1, panel B and C, respectively). In these experiments, cells were also exposed to DMSO for 10.5 h or for 17 h, then washed, and resuspended in the medium without inducer. Results obtained showed that both schedules of HNE treatment inhibited cell growth in a time-dependent way (17 h treatments is more effective than 10.5 h) and this inhibition is stronger than that observed in cells treated with DMSO for the same length of time (Figure 3, panel A).

The hemoglobin content (Figure 3, panel B), in cells treated with HNE, increased in relation to the time of exposure to the aldehyde. In fact, the treatments with HNE for 17 h produced a higher level of hemoglobin accumulation than that seen in cells exposed to the HNE for 10.5 h. Moreover, the hemoglobin content in cells treated with HNE for 17 h was comparable to that detected in cells continuously exposed to DMSO (7 days), whereas the treatments with DMSO for a limited time (17 h) induced a very low level of hemoglobin accumulation and 10.5 h of exposure to DMSO did not induce any hemoglobin accumulation (data not shown).

Effect of HNE Treatment on Gene Expression

Since the repeated treatments over 2 days with 1 μM HNE induced a high degree of MEL cell differentiation, we studied the expression of β-globin, c-myc and ODC genes, in cells subjected to this experimental procedure. The analysis was performed during the early period of HNE treatments (2–6 h) and during the following days, when a stable terminal differentiation occurs (Figure 4). DMSO was maintained in cell culture for the whole experiment (4 days).

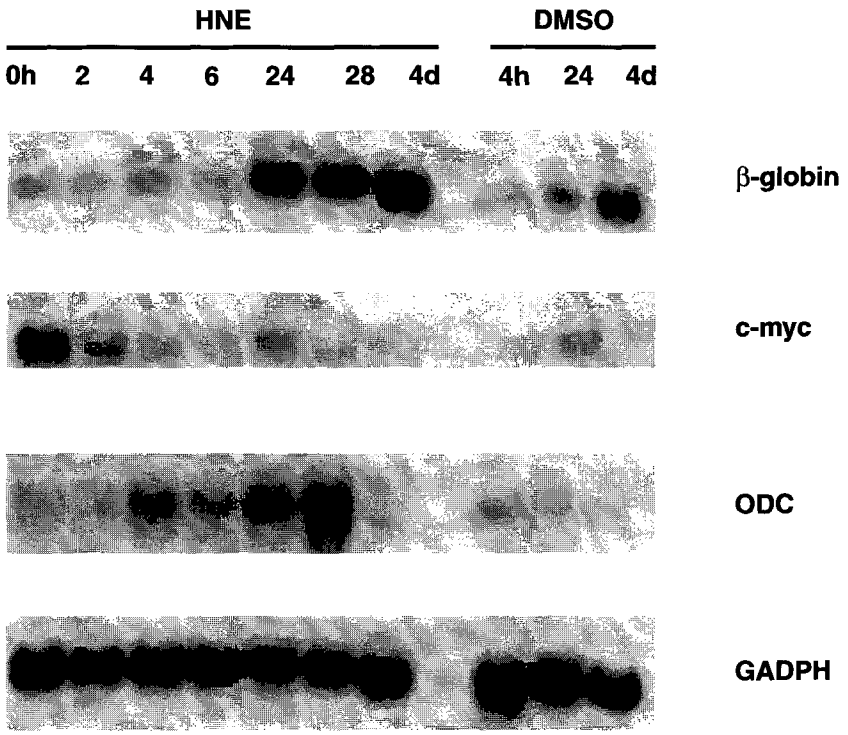


FIGURE 4 Northern blot analysis of total RNA of MEL cells treated with HNE (repeated treatments over 2 days, see Figure 1) or exposed for the whole experiment with 1.5% DMSO. Panel A: β-globin expression; Panel B: c-myc expression; Panel C: ornithine decarboxylase (ODC) expression.

The basal level of β-globin mRNA remains relatively constant during the first 6 h of HNE treatment, whereas a greater increase appears after 24 h. It is noteworthy that the β-globin expression in HNE-treated cells, after 24 h of treatment, is higher than that seen, at this time, in DMSO-treated cells (Figure 4, panel A).

The level of c-myc mRNA, 2–6 h after the beginning of treatments with HNE, strongly decreased, whereas it began to reaccumulate at 24 h (Figure 4, panel B). Thereafter c-myc mRNA declined gradually, when the cells underwent terminal differentiation. A similar biphasic change in c-myc mRNA levels was also observed in cells treated with DMSO. However, the inhibition of c-myc expression induced by DMSO at 4 h, was higher than that observed after HNE treatment.

In contrast to the decline of c-myc mRNA levels, the ODC gene expression increased during the first 30 h of HNE treatments (Figure 4, panel C). Thereafter, the mRNA levels largely declined, appearing loosely correlated with the proliferation rate. A similar pattern was observed in DMSO-treated cells, in which ODC expression first slightly increased and then decreased at day 4, according to the decline of the proliferation rate. Expression of the GAPDH gene was evaluated to verify that no differences existed between RNA amounts of different samples (Figure 4, panel D).

DISCUSSION

Our results point out that HNE is able to induce terminal differentiation in the erythroleukemic cell line. The differentiative ability of HNE was previously demonstrated in HL-60 promyelocytic

leukemic cells in which the aldehyde induced a granulocytic-like differentiation when maintained in cell cultures for 7.5 h.[10] Moreover, a recent work confirms that HNE can induce differentiation and apoptosis in K562 cells,[19] according with our previous observations.[12]

With the procedure standardized in this work (14 repeated treatments over 2 days) HNE causes an accumulation of hemoglobin comparable with that obtained with the continuous exposure to DMSO. Moreover, the inhibition of cell proliferation induced by HNE, at days 3, 4 and 5, was stronger than that observed after DMSO treatment. This may depend, at least in part, by a different pathway of cell proliferation control. In fact, our previous observation in HL-60 cells, indicated that the accumulation in G0/G1 phase of the cell cycle occurs 24–48 h after HNE treatment, whereas, it is observed after 5 days from DMSO treatment.[20]

The greater effectiveness of HNE with respect to that displayed by DMSO has been demonstrated by maintaining the cells for the same amount of time in the presence of two inducers. In fact, 17 h of exposure to HNE induced a strong increase in hemoglobin accumulation, whereas the hemoglobin accumulation in cells, treated with DMSO for 17 h, is very low. Moreover, 10.5 h of exposure to HNE, only at day 1, induced a low but detectable increase of hemoglobin, while the exposure to DMSO for the same period was completely ineffective. These results show that DMSO, but not HNE, needs at least 17 h of contact with the cells to produce a detectable effect on differentiation. This time, named "latent period", is observed also for other inducers.[21]

Our results demonstrated that an increase of hemoglobin accumulation occurs even after 10.5 h of exposure to the HNE (14 repeated treatments), but this increase is greater and more stable when 14 treatments with 1 μM HNE are repeated at day 2.

Changes in gene expression are associated with the induction of MEL cell differentiation.[22] In particular, a common observation is the increase of β-globin gene expression, which we found to be increased in both HNE- and DMSO-treated cells. However, 24 h from the beginning of treatments, the increase in β-globin gene expression is stronger in HNE- than in DMSO-treated cells. This may suggest that the differentiation induced by HNE appears earlier than that induced by DMSO.

It has long been demonstrated that the c-myc protooncogene is involved in the regulation of cell differentiation[22,23] and that it is down-regulated in a biphasic manner during the differentiation of MEL cells.[17] We have previously demonstrated that c-myc is down-regulated by HNE in K562 and HL-60 cells.[12,13] In MEL cells, HNE induces a biphasic modulation of c-myc expression. It behaves, therefore, as other differentiation inducer for this cell line. The reduction of c-myc transcript at 4 h was stronger in DMSO- than in HNE-treated cells. However, HNE treatments was not finished at 4 h, thus the effect displayed by the aldehydes might be partial. In fact, if we compared the c-myc expression after 4 days, the level of inhibition was similar in both HNE and DMSO-treated cells.

The ODC gene is the key regulatory enzyme of polyamine synthesis which is essential for cell proliferation,[24] whereas the ODC involvement in the differentiation process has not been elucidated as yet. Some authors suggested that the increase in ODC activity is a necessary event in the differentiation process,[25] whereas others got opposite results.[16] It has been suggested that the chemical inducers can be broadly divided into two types: those which act via stimulation of ODC expression, and those which do not increase the activity of this enzyme.[25] From our results it arises that HNE, like DMSO, belongs in the first group of inducers. In fact, HNE induces an early increase in ODC expression, followed by a reduction of mRNA transcripts at day 3 and 4. This decrease may be linked to the decline of proliferation, whereas, the increase of ODC expression in HNE treated cells may be related

to the cycle of replication necessary before the cells became terminally committed. The slight increase in ODC expression was observed also in DMSO treated cells, in which the decrease of ODC expression after 4 days was similar to that observed in HNE-treated cells.

In conclusion, data accumulating in past years indicated that HNE is an important molecule involved in intracellular signalling pathway.[26,27,28] Results obtained by other laboratories[19] and our present findings demonstrated that HNE can be definitely included in the class of differentiation inducers. Its action in MEL cells involves a persistent modulation of differentiation-related gene expressions, that precedes and accompanies the cessation of proliferation and the hemoglobin accumulation. Moreover, it is noteworthy that HNE acts at concentration near to those found in several types of normal cells and fluids, thus it might exert in such cells a control in proliferation and differentiation under physiological conditions.

Acknowledgements

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