Effect of Dexamethasone on Conditioned Enhancement of Natural Killer Cell Activity

Key Words
Hypothalamic-pituitary-adrenocortical axis
Dexamethasone
Methionine-enkephalin
Conditioned response
Natural killer cell activity

Abstract
It is believed that the expression of the conditioned natural killer cell activity is regulated through the hypothalamus-pituitary axis. Since the conditioned expression of both the plasma level of adrenocorticotropic hormone and natural killer (NK) cell activity show a sequential rise after exposure to the conditioned stimulus, it was of interest to determine if treatment with dexamethasone could inhibit this response. These studies suggest that treatment with dexamethasone was able to block the expression of the conditioned NK cell activity but was unable to interfere with the pairing of the conditioned stimulus with the unconditioned stimulus. We conclude that the hypothalamic pituitary axis is an integral part of the pathway for stimulation of NK cell activity in the spleen.

Introduction
There is strong evidence that the immune system and the central nervous system communicate through the hypothalamus-pituitary-adrenocortical (HPA) axis. We have used conditioning as a tool to investigate the interaction between the central nervous system (CNS) and the immune system (IS). Natural killer (NK) cell activity can be generated by the injection of poly I:C into BALB/c mice. This activity can be readily conditioned by associating the odor of camphor (conditioned stimulus, CS) with the injection of poly I:C (unconditioned stimulus, US). In animals that have been conditioned, a subsequent exposure to the conditioned stimulus alone will trigger a rise in NK cell activity in the spleen. This rise in NK cell activity is prompted presumably by the CS signal activating central neuronal pathways that activate the HP axis to release neuroendocrine hormones which stimulate NK cell activity in the spleen. We have characterized this response with respect to all of the necessary controls including camphor odor and other odorant controls and have shown repeatedly that the comparison between conditioned and nonconditioned groups reliably reflects a conditioned response when it is present [1-3]. This includes using animals exposed to the same number of exposures to camphor odor by random' trials or backward conditioning. Control groups in which the CS or US are not given in proper sequence or conditioned animals that are not exposed to the CS are unable to mount a conditioned response [1,2]. There is specificity for the odor used in the CS/US association. The conditioned groups receiving different odor cues under identical conditions showed a conditioned response only when
the same odor cue was given at the association and recall step. The conditioned response was expressed only in relation to the specific odor of the conditioned stimulus [3]. Exposure of normal mice to camphor odor on different schedules showed no effect on NK cell activity. We have found that the noncondi-

Materials and Methods

**Animals**

BALB/c female mice used in this study were 6 weeks old and were obtained from Charles River Breeding Company (Wilmington, Del., USA). All mice were kept in standard animal facilities, on a 12-hour light/dark cycle with food and water ad libitum. Mice were separated into groups of 8 or 9 per cage at least 1 week before the experiments were started. All of the experimental procedures were performed between 07.30 and 08.30.

**Drugs**

Dexamethasone phosphate disodium salt and Met-Enk were purchased from Sigma (St. Louis, Mo., USA) and dissolved in sterile saline (0.9\% NaCl) prior to their use in the experiments. Poly I:C was obtained from Pharmacia, and dissolved in sterile saline at 200 ug/ml and stored at 4°C.

**Conditioning Procedure**
A 3-day conditioning paradigm was employed in the present study. Camphor odor was used as the CS and 20 or 36 ug of poly I:C as the US. For exposure to the camphor odor, mice were placed inside a cabinet, a hot bottle containing camphor which was dissolved partially in mineral oil was placed on the cage top, and a second cage was inverted over the cage holding the mice. Animals were left undisturbed for 1 h. On the day of acquisition (day 0), animals in the conditioned group (CND) were exposed to the odor of camphor, followed by an injection of poly I:C intraperitoneally (i.p.). The animals in the nonconditioned (NC) group were injected i.p. with poly I:C only. On day 2, both groups were exposed to the odor of camphor for 1 h and injected with a suboptimal dose of 1 ug poly I:C. The suboptimal dose has been shown to increase the sensitivity of NK cells to neuroendocrine signals that trigger the conditioned response.

**Cisterna magna Injection of Met-Enk**

A 3-day protocol was used in these studies in order to relate the information to the conditioning data. Mice were injected i.p. on day 0 with 36 ug poly I:C to initiate the NK cell response. On day 2, the poly-I:C-treated animals were anesthetized by injection i.p. with 0.1 ml ketamine/rompun mixture (K/R, 85 mg/kg and 13 mg/kg, respectively). Five minutes later when the animals were unresponsive to toe pressing, injections were made into the cisterna magna with a 250-ul capacity Hamilton syringe using a 27-gauge needle. Met-Enk was diluted in sterile saline to 4 ug/ml so that a 5-ul injection would contain 0.02 ug of the drug. Control mice were injected with saline. Following the injection into the cisterna magna, a suboptimal dose of 1 ug poly I:C was given to all of the animals. NK cell assay was performed on day 3.

**Effect of Dexamethasone in Poly I:C-Pretreated Mice**

To determine whether dexamethasone had any direct effect on NK cell activity, we treated mice with dexamethasone using the 3-day protocol. Two groups of mice received 36 ug poly I:C i.p. on day O. On day 2, one group was injected with 100 ug/kg of dexamethasone i.p. and the other group was injected with saline. Animals in both groups were then injected with 1 ug poly I:C i.p. An NK cell assay was performed 24 h later. In a separate experiment, two groups of mice received 36 ug poly I:C i.p. on day 0. On day 2, the animals were anesthetized and one group of animals was injected intravenously (i.v.) with dexamethasone. The other group was injected with saline. Both groups of animals were then injected with 1 ug poly I:C.

**Dexamethasone Treatment of Conditioned Mice**

To determine whether dexamethasone can block the conditioned increase of NK cell activity, dexamethasone was injected into the animals at one of two places: either prior to pairing of the CS with the US, or just prior to recall of the conditioned response with the CS. Dexamethasone was given immediately before the exposure to CS on day 0 or prior to recall with the CS on day 2. The nonconditioned groups were also treated with dexamethasone in the same manner as the conditioned animals. Control groups injected with saline were monitored along with the experimental groups.

**Dexamethasone Treatment of Met-Enk-Injected Mice**

To determine the effect of dexamethasone on the central stimulatory effect of Met-Enk on NK cell activity, the animals were divided into 4 groups of 8 mice each. All of the animals were injected with 36 ug poly I:C on day 0. On day 2, animals in groups 1 and 2 were injected with saline i.p. and groups 3 and 4 with 100 ug/kg dexamethasone. The animals in groups 1 and 3 were then anesthetized and injected into the cisterna magna with Met-Enk. Group 2 and 4 mice were injected into the cisterna magna with saline. NK cell assay was performed on day 3.

**Preparation of Spleen Cells**
Animals were sacrificed by CO2 asphyxiation. Spleens were removed immediately and placed into individual petri dishes containing sterile saline on ice. Single-cell suspensions of spleens were obtained with a forceps and a 3-ml syringe with a 23-gauge needle. The spleen cell suspension was collected with the same syringe into a sterile 15-ml tube. The tubes were filled with saline and centrifuged at 2,000 rpm (700 g) for 5 min at 4°C in a Beckman centrifuge. The supernatant was discarded and the washing was repeated. The pellet was suspended in 1 ml of sterile saline with a sterile Pasteur pipette. Spleen cell counts were made in a Coulter counter following lysis of red blood cells with saponin. The whole spleen cells (with red blood cells) were used in the NK cell assay.

**NK Cell Assay**

YAC-1 target cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 U penicillin, 100 ug streptomycin, and 5 x 10⁻⁵ M 2-mercaptoethanol. The YAC-1 cells were cultured into fresh tissue culture flasks with fresh medium 24 h before harvesting for the assay. With this procedure, the viability is >95% and the spontaneous release in the chromium release assay is <10%. YAC-1 cells were labeled with sodium chromate (Amersham, Chicago, IL, USA) at a ratio of 100 μCi/10⁶ cells in a total volume of 0.2 ml at 37°C in a CO2 incubator for 30 min. The cells were washed twice with a large excess of medium and suspended at a final density of 1 x 10⁶ cells/ml in RPMI 1640 supplemented with 5% FCS. 0.1 ml of spleen effector cells were mixed with target cells at ratios of 200:1, 100:1, and 50:1 (E:T ratio) in triplicate wells containing 0.1 ml of 1 x 10⁵ ⁵¹Cr-labeled YAC-1 target cells in 96-well, flat-bottomed microtiter plates (Linbro, Hamden, Conn., USA). Plates were incubated for 4 h in a humidified, 37°C, CO2 incubator. 0.1 ml of supernatant from each well was collected after centrifugation of plates at 1,400 rpm (345 g) for 5 min at 25°C in a Beckman centrifuge. The radioactivity of the samples was counted in a Beckman y counter. Maximum ⁵¹Cr released (MR) from the target cells was measured after incubation in the presence of 0.17VHC1 and spontaneous release (SR) in the presence of medium. Percent specific ⁵¹Cr released was calculated as: [(test release-SR)/(MR-SR)] x 100.

**Statistical Analysis**

Statistical analysis of the data was calculated using analysis of variance (ANOVA) and Duncan's multiple-range tests with an a value of 0.05.

**Table 1.** Effect of dexamethasone on NK cell activity on day 3

| Groups | Percent specific ⁵¹Cr released at E:T ratios | p  
|--------|------------------------------------------|-----|   |
|        | 200:1 | 100:1 | 50:1 |
| Experiment 1  
Dexamethasone | 7.0±0.6 | 6.0±0.6 | 4.1±0.3 | 0.99 |
| Saline | 7.1±0.6 | 6.3±0.5 | 3.7±0.4 |
| Experiment 2  
Dexamethasone | 11.8±0.6 | 10.3±0.6 | 6.9±0.5 | 0.44 |
| Saline | 10.7±0.7 | 6.8±0.5 |

Values are means ± SE for each group. Each group in both of the experiments had 8 mice and all of the animals were injected with 36 ug poly I:C i.p. on day 0. On day 2, animals in the dexamethasone and saline groups were anesthetized with K/R and injected i.v. with dexamethasone (100 ug/kg) in 0.1 ml or with 0.1 ml saline, respectively. The animals were then injected with 1 ug poly I:C/mouse. The NK cell assay was performed on day 3.
On day 2, animals were injected i.p. with 100 ug/kg dexamethasone in 0.1 ml or with 0.1 ml saline. All animals were then injected i.p. with 1 ug poly I:C/mouse. The NK cell assay was performed on day 3.

Results

Effect of Dexamethasone on Poly I: C-Induced NK Cell Activity

In order to ascertain that treatment with dexamethasone did not directly affect the poly-I:C-induced NK cell activity, a dose of 100 ug/kg of dexamethasone was administered to the animals 10 min before poly I:C injection. The activities measured at 24 h were found to be comparable in the dexamethasone- and saline-treated groups (for dexamethasone, 21.5 ± 1.2, 18.8 ± 1.0, and 12.5 ± 0.6; for saline, 23.3 ± 3.4, 18.9 ± 2.7, and 12.6 ± 1.8 at ratios of 200:1, 100:1, and 50:1, respectively, with p = 0.8055). These results suggest that NK cell activity peaked at the same time in both the dexamethasone- and saline-treated groups and the level of their activities was not significantly different.

Dexamethasone Injected into Animals i.v. or i.p. Had No Effect on the Poly-I:C-Induced NK Cell Activity Measured 3 Days Later

The injection of poly I:C induces a rise in NK cell activity that peaks at 24 h and declines by 3-5 days. Experiment 1 (table 1) showed that i.v. injection of dexamethasone on day 2 at the time NK cell activity was in

Hsueh Rogers Hiramoto/Ghanta

Dexamethasone and Conditioning

Table 2. Dexamethasone blocked the conditioned expression of NK cell activity

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Percent specific 51Cr released at E:T ratios</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>200:1</td>
</tr>
<tr>
<td>CND</td>
<td>7</td>
<td>14.7±1.0*</td>
</tr>
<tr>
<td>NC</td>
<td>7</td>
<td>13.0±0.5</td>
</tr>
<tr>
<td>CNDex</td>
<td>7</td>
<td>10.5±0.7</td>
</tr>
<tr>
<td>NEx</td>
<td>7</td>
<td>9.0±1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE for each group. * = Significant difference from the other 3 groups.

Animals in the CND and CNDex groups were conditioned by exposure to camphor odor for 1 h, followed immediately by an injection i.p. with 20 ug poly I:C. The NC and NEx groups received only poly I:C at this time. On day 2, the animals in the CNDex and NCex groups were injected i.p. with 100 ug/kg dexamethasone and the positive control groups CND and NC were injected with saline. Statistical analysis was performed by repeated measures of ANOVA and Duncan’s multiple range tests. There is a significant difference between groups overall (p = 0.0001, F 1 10.78). Significant differences were found between the CND and NC groups (p = 0.01). However, there was no significant difference between the CNDex and NEx groups (p = 0.31).

Dexamethasone at acquisition

(1)CND  8 | 16.9±1.8 | 14.1±1.7 | 9.3±1.1            |
(2)NC   8 | 11.0±1.3 | 8.5±0.8  | 5.3±0.5            |

Dexamethasone at expression

(3)CND  9 | 11.2±1.3 | 8.8±1.1  | 6.3±0.7            |
The conditioning was carried out as follows: on day 0, groups 1 and 2 were first injected with dexamethasone 100 ug/kg i.p. The CND groups 1 and 3 were exposed to the CS for 1 h followed by an injection of poly I:C. This constituted the CS-US association. The NC groups 2 and 4 were injected with poly I:C only on this day. On day 2, groups 3 and 4 were injected with 100 ug/kg dexamethasone i.p. All groups (1-4) were exposed to the CS for 1 h, and NK cell activity was measured on day 3. Values are means ± SE or each group.

Groups 1 and 2 were used to evaluate the effect of dexamethasone on acquisition of the conditioned response. Statistical analysis was performed with repeated measures of ANOVA (p = 0.0226, F = 6.684).

Groups 3 and 4 were used to evaluate the effect of dexamethasone on expression of the conditioned response (p = 0.3786, F = 0.823).


decline had no effect on the NK cell activity when compared to the saline-treated control group (p = 0.99). Similarly the same dose of dexamethasone delivered via the i.p. route showed no effect on NK cell activity (table 1, experiment 2, p = 0.44). Routes of administration of a drug often play an important role in the effect produced. Since no effect on NK cell activity was noted when dexamethasone was injected via the i.v. or i.p. route, we selected the i.p. route for dexamethasone administration for all the subsequent experiments.

**Dexamethasone Blocked the Conditioned Enhancement of NK Cell Activity at the Expression Step**

To evaluate the effect of dexamethasone on the conditioned response, animals were conditioned by exposure to the CS followed by an injection of poly I:C. Prior to recall of the conditioned response, the positive control groups were injected with saline and the experimental groups were injected with dexamethasone. The NK cell activity was elevated in the positive control, CND group when compared to its NC group (table 2). Significant differences were found between the CND versus the NC group. The NK cell activity between CNDdex and NCdex groups showed no significant difference (p = 0.31). The expression of the conditioned response as measured by the NK cell activity was blocked by the administration of dexamethasone prior to the reexposure to the CS. Interestingly, the overall NK cell activities in the dexamethasone-treated groups were significantly decreased when compared to the saline-treated groups. In the light of the observations that dexamethasone at the dose used did not appear to have a direct suppressive effect on NK cell activity, the reason for this difference is not clear.

**Dexamethasone Did Not Prevent the Acquisition of the Conditioned Response**

The effect of dexamethasone on conditioning was next evaluated at both the acquisition phase, i.e. at the time of pairing of the CS with the US and at the recall step of the conditioned response (table 3). The results indicate that dexamethasone had no effect when administered at the

<table>
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<tbody>
<tr>
<td></td>
<td></td>
<td>200:1</td>
</tr>
<tr>
<td>(1)Met-Enk</td>
<td>10.0±1.0*</td>
<td>7.8±0.6*</td>
</tr>
<tr>
<td>(2)Saline</td>
<td>7.0±0.5</td>
<td>6.5±0.5</td>
</tr>
<tr>
<td>(3)Met-Enkdex</td>
<td>9.1±0.7*</td>
<td>7.6±0.6*</td>
</tr>
<tr>
<td>(4)Salinedex</td>
<td>5.4±0.4</td>
<td>5.2±0.4</td>
</tr>
<tr>
<td>(4)NC</td>
<td>8</td>
<td>10.2±1.2</td>
</tr>
</tbody>
</table>

Table 4. Dexamethasone did not block the enhancement of NK cell activity induced by injection into the cisterna magna of Met-Enk
Values are means ± SE for each group. Statistical analysis indicated a significant overall difference between the groups (p = 0.0015, F = 6.74). Significant differences were also observed between Met-Enk vs. saline (p = 0.02), and Met-Enkdex vs. salinejex (p a 0.003).

* = Significant difference from control groups.

time of pairing the CS with the US. The NK cell activity in the CND was significantly elevated over the NC group (p = 0.0226), indicating that conditioning had taken place in the animals that were treated with dexamethasone. However, when dexamethasone was given prior to recall of the conditioned response, the expression of the conditioned response was blunted (p = 0.3786). The latter observation is consistent with the earlier (table 2) results. Taken together, the data suggest that treatment with dexamethasone inhibited the expression of the conditioned response. The drug appears not to interfere with the pairing of the CS with the US.

Discussion

Earlier work from our laboratory [5] suggested that a central opioid-receptor-mediated efferent pathway was involved in regulating the conditioned increase in NK cell activity at the expression step. Based on this initial observation, conceptually, we believed that p-End or Met-Enk plays a stimulatory role on NK cell activity and could be one of the opioids involved in the CNS for the expression of the conditioned response [4]. The injection of Met-Enk into the cisterna magna prompted a rise in NK cell activity suggesting that an opioid-receptor-mediated efferent pathway may be utilized during the recall of the conditioned response [4, 5]. We have also demonstrated that the level of ACTH in the plasma and interferon (IFN)-a gene expression in the spleen were higher in the conditioned animals than in the controls [18]. These observations suggest the possibility that the neuroendocrine system, and in particular the HPA axis, might play an important role in mediating the conditioned expression of NK cell activity. Substantial evidence indicates that opioid peptides are important modulators of the HPA axis which direct the release of ACTH and p-End from the pituitary, the latter being capable of stimulating NK cell activity in the spleen [6-9]. Consequently, it seems reasonable that in conditioned animals, the HPA axis is involved in triggering the rise in NK cell activity in the spleen in response to exposure of the animal to the CS. Dexamethasone has been shown to block the HPA activity at both central sites (hypothalamus and other brain regions) [13-15] and at the pituitary level [16,17]. In order to use dexamethasone in our studies, the effect of dexamethasone on NK cells was examined to eliminate the possibility that dexamethasone might block the conditioned response by directly acting at the level of splenic NK cells rather than through the HPA axis. Others have shown that dexamethasone can suppress the NK cell activity in vitro and in vivo [19-23]. In our hands, treatment of the normal animals with dexamethasone appears not to influence the level of NK cell activity either at 24 h or at 3 days after

Dexamethasone Did Not Block the Enhancement of NK Cell Activity Induced by the Injection of Met-Enk into the Cisterna Magna

We have previously reported that an opioid-receptor-mediated efferent pathway can be stimulated by the injection of Met-Enk into the cisterna magna to raise NK cell activity. To test whether dexamethasone can block the enhancement of NK cell activity induced by Met-Enk, the drug was injected into the animals prior to treatment oral Met-Enk or saline. The stimulatory effect of Met-Enk on NK cell activity was not altered in the dexamethasone-treated group (Met-Enkdex vs. salinejex) table 4). Significant differences were observed between Met-Enk vs. saline, and Met-Enkdex vs. salinedex groups. These results suggest that pretreatment of the animals with dexamethasone did not block the opioid receptor mediated efferent pathway of NK cell activation.
the injection of poly I:C. These results suggest that treatment with dexamethasone at the dose level used did not interfere with the IFN production or trafficking of NK cells. Dexamethasone used at 100 |ig/kg was equivalent to administering 2 p.g of dexamethasone to a 20 g mouse. This dose appeared to be far below that required to alter NK cell activity in vivo. Nevertheless, Kusnecov et al. [24] showed that the dose was sufficient to reduce the plasma corticosteroid level and indicated that the HPA axis was down-regulated (or blocked) by dexamethasone.

### Dexamethasone and Conditioning

At the dose used by Kusnecov et al. [24], we observed that dexamethasone blocked the conditioned enhancement of NK cell activity at the expression stage but it did not inhibit the pairing of the CS with the US at the acquisition step. These results infer that activation of the HPA axis may be required for the recall of the conditioned response but not for linking the CS with the US. The glucocorticoid analogue is capable of modulating the CRF-induced ACTH/α-End secretion from the pituitary [25]; it also negatively regulates the proopiomelanocortin (POMC) gene expression in the pituitary [10, 26]. Whether the mechanism by which dexamethasone exerts its inhibitory activity on conditioned NK cell activity during recall is by blocking CRF-induced production of ACTH/α-End from the pituitary or by blocking the expression of POMC remains to be determined. Although dexamethasone is known to bind to multiple sites within the brain and the pituitary [12, 13, 15], it is not known which site(s) is important in mediating the observed blocking of the conditioned response.

Our previous findings [5] suggested that an opioid-receptor-mediated pathway was involved only in the recall step and not the association step. Therefore it is conceivable that during the recall of the conditioned response, the odor (CS) signal initiated by camphor induced the release of endogenous opioids which in turn acted at the HPA axis and produced an enhancement of NK cell activity via release of ACTH into the plasma and elevation of IFN-α in the spleen. Met-Enk has been reported to have the ability to enhance NK cell activity when injected into the cisterna magna [4]. Met-Enk appears to activate the effector pathway and in some ways mimic the conditioned recall of the NK cell activity. Although Met-Enk may be potentially involved in the regulation of the conditioned NK cell activity [4], its central stimulatory effect was not blocked by dexamethasone. There are at least two ways that Met-Enk might bypass the blocking effect of dexamethasone on the HPA axis: (a) it may be possible that the dose of Met-Enk (1 ug/kg) given into the cisterna magna can override the block by dexamethasone, and counteract the inhibition and restore the activity of the pathway; (b) on the other hand, the site of action of Met-Enk could be independent of the site of the dexamethasone block, in which case the Met-Enk pathway would be uninterrupted. The activation of central opioid receptors (β and 8) by Met-Enk can stimulate the release of hormones from the HPA axis. Nikolarakis et al. [27] have shown that these actions might be mediated through alternative pathways that are not dependent on CRF (i.e. through CRF-independent pathways). If this should prove to be the case, then dexamethasone would not be able to block the Met-Enk-activated pathway for stimulation of the NK cell activity.

Although we have repeatedly used the cisterna magna route to inject drugs and mediators into the CNS, the use of the cisterna magna route appears to be controversial. Nowaczyn et al. [28] reported that substances injected into the cisterna magna will not reach interior brain regions. However, Cross et al. [29] have shown that injection of 6-hydroxydopamine (6-OHDA) into the cisterna magna depleted catecholamine levels in the hypothalamus, mid brain, and pons-medulla and this was in agreement with the depletion of catecholamines produced by injection of 6-OHDA intraventricularly. It is unclear how signals are transmitted into the central nervous system via the cisterna magna; however, the fact that function at the level of NK cell activity is observed suggests that the brain is receiving such signals.

This study was done to ascertain whether the HPA axis was used in conditioned animals to relay signals to the NK cells of the spleen. Treatment with dexamethasone was able to inhibit the expression of the
conditioned response. Despite the fact that dexamethasone did not block the central stimulatory opioid pathway which could be activated by Met-Enk, dexamethasone, in like manner as naltrexone (an opioid antagonist), also blocked the expression of the conditioned response. The acquisition (CS-US association) was not inhibited by either naltrexone or dexamethasone. We conclude that learning of the conditioned NK cell activity in BALB/c mice appears not to involve pathways blocked by dexamethasone or naltrexone. These results, however, do not eliminate the possibility of other signaling pathways of the HPA axis that might be utilized to allow pairing of the CS with the US. Further studies between the HPA axis and NK cell activity are needed to clarify the intricate role of the hypothalamus on the conditioned response.

Acknowledgments

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References


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