Induction of mammary cancer and lymphoma by multiple, low oral doses of 7,12-dimethylbenz[a]anthracene in SENCAR mice

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Existing models of mouse mammary carcinogenesis induced by the model polycyclic aromatic hydrocarbon 7,12-dimethylbenz[a]anthracene (DMBA) typically use a small number of bolus doses applied intragastrically. In contrast to this, typical human exposures to carcinogens are thought to be at lower doses and to occur with chronic or sporadic timing. When the classical dosage (1 mg DMBA given once a week for 6 weeks) was split into five daily doses of 20 µg given intragastrically to female SENCAR mice each week for 6 weeks, toxicity was high and the major tumor type seen was lymphoma. Lowering the dose to 60 µg/day gave less toxicity, a 75% incidence of lymphoma and a 30% incidence of mammary carcinoma. However, 20 µg DMBA given five times per week for 6 weeks resulted in a 65–70% incidence of mammary carcinoma within ~50 weeks. This represents a 50-fold lower daily dosage of DMBA than that used in the classical model. DNA was prepared from 10 mammary adenocarcinomas and 10 lymphomas and exons 1 and 2 of the H-ras1, K-ras and N-ras genes were sequenced using PCR techniques. Mutations altering codons 12 or 61 of one of the ras family genes were found in 4/10 mammary carcinomas and 5/10 lymphomas. Three mammary tumors exhibited codon 61 mutations, one in each of the genes studied, and a fourth tumor contained a codon 12 mutation in the K-ras gene. Among the lymphomas, two mutations in codon 12 of K-ras, one mutation in codon 61 of K-ras and two mutations in codon 61 of N-ras were also found. Each of the mutations could be interpreted as a G→T or A→T transversion. It is suggested that the high incidence of lymphoma at the higher, repetitive doses may be related to immunotoxicity. These low dose models of lymphomagenesis and mammary carcinogenesis should prove useful for tests of chemopreventive agents that target the initiation phase of carcinogenesis.

Introduction
Polycyclic aromatic hydrocarbons (PAH*) are a well-studied class of environmental contaminants that are implicated as human carcinogens (1,2). Based primarily on experimental studies in rodents and in vitro, it is thought that PAH carcino-

*Abbreviations: PAH, polycyclic aromatic hydrocarbons; DMBA, 7,12-dimethylbenz[a]anthracene; MNU, N-methylN-nitrosourea.

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Table I. Amplification of ras gene exons

<table>
<thead>
<tr>
<th>Exon amplified</th>
<th>Primersa</th>
<th>Annealing temperature (°C)</th>
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<tbody>
<tr>
<td>H-ras1, exon1</td>
<td>5'-CTTGCGTCTGCTGCTGCTGCTGCTCATTGG-3'</td>
<td>68b</td>
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<tr>
<td>H-ras1, exon2</td>
<td>5'-ACAGCCCCCTTGCCAGTTAGG-3'</td>
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<tr>
<td>K-ras, exon1</td>
<td>5'-GAAGGCGCCTGCTGAAAAATGG-3'</td>
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<tr>
<td>K-ras, exon2</td>
<td>5'-GGGTCTGACTCATCCACAA-3'</td>
<td>51c</td>
</tr>
<tr>
<td>N-ras, exon1</td>
<td>5'-GCTCTTACAGAAGAACAGT-3'</td>
<td>58b</td>
</tr>
<tr>
<td>N-ras, exon2</td>
<td>5'-ACCAAGTTCTACCGAAGCAGT-3'</td>
<td>55b</td>
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</table>

aPrimer pairs are listed with the sense strand first. All reactions were performed in the presence of 1.5 mM MgCl₂.
bAnnealing temperature was 68°C for the first eight cycles, 60°C for the next 32 cycles.
cAmplification reactions were 40 cycles at the indicated annealing temperature.

Table II. Incidence of tumors induced by different doses of DMBAa

<table>
<thead>
<tr>
<th>Category</th>
<th>Control</th>
<th>20 µg</th>
<th>60 µg</th>
<th>200 µg</th>
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<tbody>
<tr>
<td>Total</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Dead without tumor</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Normal at termination</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>Mice with any tumor</td>
<td>1</td>
<td>18</td>
<td>20</td>
<td>12</td>
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<td>Mice with lymphoma</td>
<td>1</td>
<td>5</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Thymic lymphoma</td>
<td>0</td>
<td>3</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Reticular cell sarcoma</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Lymphoblastic lymphoma</td>
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<td>4</td>
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<td>Undifferentiated lymphoma</td>
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<td>2</td>
<td>11</td>
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<tr>
<td>Single lymphoma</td>
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<td>1</td>
<td>2</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Mice with mammary carcinoma</td>
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<td>13</td>
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<tr>
<td>Adenocarcinoma type B</td>
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<tr>
<td>Mice with other tumors²</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
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aFour groups of mice fed AIN-76A diet were treated by gavage with different doses of DMBA, 0, 20, 60 or 200 mg/mice/day, 5 days/week for 6 weeks. The cumulative number of mice in each category at termination is given in the table.
bComprised of carcinomas that could not clearly be assigned to Dunn’s classification groups, including two tumors with atypical tubular structures and three tumors with a predominant spindle cell-type component.
²Other tumors included ovarian tumor, myoma and endometrial tumor.

Any visible tumors or other abnormal tissues were removed and fixed in 10% buffered formalin for routine histopathological examination. The classification of all tumors is based on examination of hematoxylin/eosin stained paraffin sections. The experiment was terminated when all mice in the experimental groups had been killed due to morbidity, 53 weeks after beginning DMBA treatment (47 weeks from last treatment). At this time, the remaining control mice were necropsied to check for macroscopic tumors, but none were found. A second experiment with a single dose group (60 µg/day) was performed in the same way but with a larger number of mice.

Statistics

Tumor incidence was recorded and survival time analysis performed using the Statview 4.5 software package. The Breslow–Gehan–Wilcoxon test was used to compare survival curves; data are presented using P < 0.05 as the criterion for significance.

Molecular correlates of tumorigenesis

Ten representative mammary adenocarcinomas and 10 lymphomas that were frozen at the time of necropsy were chosen for molecular analysis. Total DNA was prepared from tumor slices by digestion with proteinase K and organic solvent extraction. Pairs of primers specific for exons 1 and 2 of the cellular H-ras, K-ras and N-ras genes (Table I) were used to directly amplify these regions from the genomic DNA of the tumors using Taq polymerase. The amplified products were purified by agarose gel electrophoresis and sequenced with Taq polymerase using end-labeled primers.

Results

To determine the effects of administration of multiple low doses of DMBA on SENCAR mice, four groups of mice were treated 5 days/week for 6 weeks with 0, 20, 60 or 200 µg/day DMBA by gavage. By the end of the treatment period, three mice were dead in the high dose group and one mouse was dead in the intermediate dose group due to non-neoplastic disease. As shown in Figure 1, weight gain was decreased significantly in the 200 µg group (triangles) compared with the control group (open circles) during treatment with DMBA (0–6 weeks in Figure 1). A slight decrease in weight gain in the 60 µg (squares) group was also observed at week 5, but it recovered to normal quickly after cessation of treatment. The decrease in body weight gain at the 200 µg/day dose level suggested that this split-dose approach produces enhanced toxicity, even though the total dose (6 mg) is the same as in the previous model (10). This enhanced toxicity was borne out by the mortality data (Table II). In the highest dose group (200 µg), 40% of the mice were dead due to acute toxicity, infection or other non-neoplastic diseases within 11 weeks.

Tumors began to appear at week 4 after the last treatment with DMBA in mice in the highest dose group (Figure 2, triangles). The incidence of lymphomas increased rapidly from week 4 through week 11, at which time all mice in this group had died or been killed. Lymphoma and mammary tumor incidence reached 50 and 10% respectively 11 weeks after the last treatment. This distribution of tumors was unexpected, since in a previous study (10) lymphomas were five times less frequent than mammary carcinomas.

In the 60 µg group, lymphomas also began to appear in week 4, similar to the onset of lymphomas in the high dose group (Figure 2A, squares). The onset of mammary tumor appearance was delayed to ~16 weeks (Figure 2B). At this time, ~40% of the mice had already developed lymphoma. All mice in this group were killed by 32 weeks after the last treatment, 75% of the mice with lymphomas and 30% with mammary tumors (two mice had both mammary tumors and lymphomas simultaneously). None of the mice died due to acute toxicity (Table II).

In the 20 µg group, both lymphomas and mammary carci-
Mammary tumorigenesis by low-dose DMBA

Fig. 1. Weight gain of mice treated with different doses of DMBA. Four groups of 20 female SENCAR mice were fed AIN-76A diet ad libitum and treated intragastrically with DMBA at a dose of 20 (closed circles), 60 (squares) or 200 (triangles) µg/mouse/day, 5 days/week for 6 weeks. Control animals (open circles) received vehicle only. The average weights of the mice in each group are plotted for each week.

Fig. 2. Effect of different doses of DMBA on incidence of lymphomas and mammary tumors. The mice described in the legend to Figure 1 were observed for palpable tumors and were killed when tumors exceeded 1 cm in diameter or when the mice became moribund. The cumulative number of mice killed with either (A) lymphomas or (B) mammary carcinomas is plotted. Symbols as in Figure 1.

In summary, two opposite dose-dependent relationships were seen: (i) the higher the dose, the higher the lymphoma incidence; (ii) the lower the dose, the higher the mammary tumor incidence. The time course of DMBA-induced tumorigenesis, for either lymphoma or mammary tumors, also showed a dose-dependent relationship: the higher the dose, the earlier the time of onset (Figure 2). However, analysis of the tumor incidence curves was complicated by the relatively high level of toxicity in the high dose group and by the appearance of two distinct tumor types in all groups. Since the time of onset of fatal tumors was dose dependent and the dose dependence appeared to be different for the two tumor types, the final analysis suggests that at the 60 µg dose rate the relative risk of mammary cancer is greater than that at the 20 µg dose rate, even though the final incidence (Figure 2) is lower. Statistical comparisons of the survival curves by the Breslow–Gehan–Wilcoxon test indicated a significant difference between the 20 and 60 µg dose rates for both mammary cancer and lymphoma (P < 0.05).
Reproducibility of lymphoma risk measurements

<table>
<thead>
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<th>$t_{25}$</th>
<th>$t_{50}$</th>
<th>$t_{75}$</th>
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<tbody>
<tr>
<td>Experiment 1</td>
<td>15.3</td>
<td>23.6</td>
<td>29.0</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>16.6</td>
<td>22.0</td>
<td>33.6</td>
</tr>
</tbody>
</table>

*The risk of death from lymphoma in mice treated with 60 $\mu$g/day DMBA was calculated from the censored incidence data. The estimated mean time to 25, 50 and 75% mortality is tabulated.

Data from Experiment 1 ($n = 20$ mice) are presented in full in Figure 4. Experiment 2 was a repetition using a larger number of animals ($n = 38$).

Several different anatomical locations for lymphoma occurrence were seen. Among mice with a single macroscopic tumor (local lymphoma), the site of occurrence was either a mammary gland-associated lymph node or the thymus. In the 60 $\mu$g group, thymic lymphoma, often well advanced, was found in 40% of mice (Table II). In mice with multiple tumors (systemic lymphoma), Peyer’s patches, mesenteric lymph nodes and spleen were also involved. Systemic lymphomas were more common than local lymphoma (10/15 in the 60 $\mu$g group, 7/10 in the 200 $\mu$g group) and were sometimes found to infiltrate other organs, including liver, kidney, ovary and muscle. Lymphoblastic lymphoma and reticulum cell sarcoma were identified among the tumors, but most of the lymphomas could not be further classified from the histopathology slides.

Mammary tumors also appeared of different types. Adenocarcinoma resembling Dunn’s type B was the most common tumor seen, but acanthomas, undifferentiated and unclassified carcinomas were also found. Histopathology of tumors characteristic of this model have been presented previously (10). Most mammary tumors were single and localized in the anterior thoracic glands. In addition to mammary tumors and lymphomas, other tumors including ovarian tumors, myomas and endometrial tumors were occasionally found.

To verify the high lymphoma incidence found in the intermediate dose group, a second trial with the 60 $\mu$g/day protocol was initiated with a larger number of mice ($n = 38$). Incidence curves for both lymphomas and mammary carcinomas were similar to those in the first experiment (data not shown). The final incidences of lymphomas and mammary carcinomas were 71 and 26% respectively. Three points (25, 50 and 75% survival) from the Kaplan–Meyer survival curves in the two experiments are compared in Table III, indicating that the survival times were very similar for both tumor types.

Molecular correlates of tumorigenesis

Mutation of the H-ras1 gene is a common feature of DMBA-induced mouse skin carcinogenesis (11,12) and codon 61 mutations have been observed in mammary tumors induced in mice by treating preneoplastic mammary cells with DMBA (13,14). Very recently (15) H-ras1 mutations have been observed in primary DMBA-induced mouse mammary carcinomas. In addition, point mutation of the K-ras gene has been observed in N-methylN-nitrosourea (MNU)-induced mammary tumors (16) and transgenic mice that overexpress the N-ras gene exhibit a high spontaneous incidence of mammary carcinomas (17). K-ras mutations have been described in MNU-induced T cell lymphomas in mice (18,19).

To begin to determine whether mutations of ras family genes are involved in mammary tumorigenesis in the low dose model, we isolated genomic DNA from 10 representative adenocarcinomas from mice in the 20 and 60 $\mu$g/day DMBA groups and amplified known mutational hotspots in the H-ras1, K-ras and N-ras genes by PCR using primers specific for exons 1 and 2 of these genes. The amplification products were then sequenced by cycle sequencing with Taq polymerase; examples of the wild-type and mutant sequences obtained from each of these genes are shown in Figure 4. Using these
techniques we identified 4/10 mammary tumors with apparent mutations (Table IV), two in the K-ras, one in the N-ras and one in the H-ras1 gene. All detected mutations were in codons 12 and 61, previously noted hotspots for DMBA-induced mutations in several tumorigenesis models, and all mutations were A→T or G→T transversions, consistent with previously noted DMBA mutational spectra.

Mutations in ras family genes were also detected in genomic DNA purified from 10 representative lymphomas produced in the repetitive, low dose DMBA model (Table IV). Half of the lymphomas tested exhibited a mutation in either codon 12 or 61 of either the N-ras or K-ras genes, similar to the findings among the mammary tumors. Again, all the mutations were apparently A→T or G→T transversions and all altered the predicted amino acid sequence of the protein.

Discussion

The three doses of DMBA used in these studies produced three very different effects. The highest dose of DMBA (200 µg) caused acute toxicity, affecting ~40% of the mice within 10 weeks, and produced lymphomas in most of the remaining mice. Reducing the daily DMBA dose to 60 µg prevented the toxicity and induced lymphomas in 75% of the mice. The remaining mice developed mammary tumors. The lowest dose of DMBA resulted in a high incidence of mammary tumors and a correspondingly lower incidence of lymphomas. From these results, it appears that the intermediate dose (60 µg) may represent an advantageous new lymphomagenesis model, because only a short observation time (~25 weeks) is needed. The low dose DMBA (20 µg) model may be advantageous to study mammary tumorigenesis, with 1/10 of the total dose used in the previous model (10) and only 1/50 of the daily dose. The drawbacks are the increased manipulation of the animals during dosing and the longer observation time.

DMBA-induced lymphoma was reported after percutaneous treatment (100 µg twice weekly for 7 weeks) in DBA/2 mice; tumor incidence can reach 100% with a short latency period. This strain is homozygous for the recessive Ahb allele and is not inducible for aryl hydrocarbon hydroxylase activity (20). If mice are homozygous or heterozygous for the dominant Ahp allele, like C57BL/6 mice, aryl hydrocarbon hydroxylase is inducible and they are susceptible to skin tumors after treatment with DMBA (20). SENCAR mice are also sensitive for DMBA-induced skin cancer after topical application (21). However, in SENCAR mice, Fischer and colleagues reported only 13% lymphoma incidence after weekly oral administration of DMBA (total dose 6 mg; 10). The obvious question is why such a high incidence of lymphoma (up to 50–70%) was seen when we changed to multiple split dose administration (5 doses/week for 6 weeks) with the same or a lower total dose (6 and 1.8 mg). There are many studies that show that DMBA has immunosuppressive effects (22–24). For instance, a daily oral exposure of B6C3F1 mice to DMBA at dose rates of 30, 90 and 300 µg/day/mouse for 14 days (total exposure 0.42, 1.26 and 4.2 µg/mouse) produced a dose-dependent decrease in the number of viable cells recovered from the spleen, thymus and Peyer’s patches and also inhibited phytohemagglutinin and lipopolysaccharide mitogen responses in these lymphoid cells (25,26). Our high frequency approach (20, 60 and 200 µg/day/mouse for 6 weeks, totally 0.6, 1.8 and 6 mg) is similar to their dose schedule, suggesting that immunosuppression may also occur in the DMBA-treated SENCAR mice.

It is possible that immunosuppression, particularly cytotoxicity in the lymphocyte populations, becomes a key factor in the development of lymphoid neoplasia when the lymphoid system is repeatedly exposed to a high enough dose of carcinogenic. A pharmacokinetics study in B6C3F1 mice indicated that DMBA was nearly completely eliminated within 24 h when a single dose was administered. The highest tissue concentrations of DMBA were detected in gut-associated lymphoid tissues and the peak level was reached in this lymphoid tissue 2 h earlier than that in serum, suggesting DMBA first appeared in and had a high affinity for lymphoid tissue. The amount of covalent binding of [3H]DMBA in spleen was 2-fold higher than that in the other organs (27). In our high frequency approach, at the two higher dose rates the immunological system was repeatedly exposed to necrogenic doses of DMBA. The resulting, almost continuous cytotoxicity and mutagenic activity would have assured that proliferating cells were exposed to the mutagenic activity of DMBA and may have resulted in a very high potential to produce lymphoma. Based on the data from B6C3F1 mice (25), the lowest dose used here (20 µg/day) may not have been necrogenic for lymphoid tissue and therefore did not lead to high levels of lymphomagenesis. With a longer interval between doses, even the much higher dose (1 mg once a week) may not be lymphomagenic. Because the lymphoid system is a very proliferative tissue, it may recover a normal population within 1 week, before the next toxic and mutagenic exposure to DMBA. So we may hypothesize that strong and continuing DMBA-induced immunosuppression (hormone and other factors may be involved, see below) may be a key factor in causing lymphoid neoplasia.

The molecular mechanism of immunosuppression by DMBA is still not clear. An important question is whether the parent compound, DMBA, or metabolites of DMBA are responsible for the immunosuppressive effect. Some studies have shown that unmetabolized DMBA has immunosuppressive effects and metabolism is not necessary (24,28). However, other studies indicated that metabolism by cytochrome P450 IA1 is required for DMBA to produce immunosuppression. When purified metabolites were studied, one metabolite had 65-fold more potent immunosuppressive effects than the parent compound DMBA (29). Thus, it seems likely that both DMBA and some metabolites of DMBA can produce immunosuppressive effects. However, metabolites of DMBA that can cause immunosuppressive effects may not be produced in large enough amounts in vivo and therefore DMBA itself may play a dominant role in immunosuppression. In other words, DMBA itself may indirectly enhance tumorigenesis through damage...
of immune surveillance against tumor cells, in addition to the expected role of electrophilic metabolites of DMBA as mutagens.

Another factor that may contribute to the target selectivity of tumorigenesis due to different dose schedules is differences in the metabolic processing of DMBA in mammary and lymphoid tissues. As indicated above, DMBA is fairly rapidly cleared from lymphoid tissues. However, the mammary gland accumulates DMBA and retains significant levels for at least 3 days after a single administration (30 mg) (30), suggesting that the fat tissue in the mammary gland may act as a reservoir for DMBA. Thus, with daily exposure to DMBA, levels of the carcinogen in mammary tissue may tend to increase with time of treatment. Since the estrous cycle in mice is relatively short (4–6 days; 31), hormonally induced proliferation of mammary epithelial cells must occur at a time when potentially mutagenic DNA damage is present in proliferating cells. When a longer interval between doses or sub-nectrogenic doses of DMBA is used, accumulation of DMBA in the mammary gland could become a key factor in provoking mammary tumorigenesis.

Previous studies of mouse mammary tumors (13–17) implicated the involvement of ras family genes in chemical carcinogenesis in this tissue. However, only one of these studies (15) demonstrated mutation in a ras gene (H-ras1) in primary, DMBA-induced tumors. It is therefore interesting that our preliminary results indicate that ras gene mutations are fairly common (4/10 tumors) in the multiple, low dose model. In addition, K-ras, N-ras and H-ras1 mutations were detected. K-ras mutations as a result of MNU treatment were reported in an in vitro model system (16), but to our knowledge N-ras mutations have not been seen in mouse mammary tumors. T cell lymphomas induced by MNU have been found to exhibit K-ras exon 1 mutations (18,19), but ras family mutations have not previously been described in DMBA-induced lymphomas. The incidence in the current model appears to be high and both K-ras and N-ras mutations were seen. Interestingly, in addition to the CAA→CTA mutation in codon 61 that is commonly seen in DMBA-induced tumors of several types (11–15,32–34), a CAA→CAT transversion in codon 61 of the K-ras gene was found in a single lymphoma (Table IV). The analogous mutation in the H-ras gene, producing a Gln→His amino acid substitution, is known to produce a transforming protein (35). The CAA→CAT mutation has also been described in DMBA-initiated melanocytic lesions of mice (36) and in several non-Hodgkins’ lymphomas in AIDS patients (37).

Although these data suggest that all three ras family genes can contribute to mammary tumorigenesis and K-ras and N-ras mutations to lymphomagenesis, clearly ras mutations are not necessary in these models. In addition, the present data do not distinguish whether in those tumors that bear a ras gene mutation, that mutation is an early or a late event in carcinogenesis. The lengthy treatment time of the current model, during which DMBA may contribute mechanistically to both initiation and promotion, is designed to mimic expected human exposures, not to allow discrimination between initiation and promotion mechanisms.

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References


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