

Effect of Diets Containing Different Levels of Linoleic Acid on Human Breast Cancer Growth and Lung Metastasis in Nude Mice¹

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ABSTRACT

The purpose of the study was to determine the effect of three different levels of dietary linoleic acid (LA) intake on the growth of MDA-MB-435 human breast cancer cells in the mammary fat pads of nude mice, and their metastasis to the lungs. These diets were isocaloric, and contained different mixtures of safflower (LA-rich) and coconut (saturated fatty acid-rich) oils to provide 23% (w/w) total fat, with 2, 8, and 12% (w/w) LA. A fourth group was fed a low-fat, 5% (w/w) corn oil diet. There were 25 mice in each dietary group. At necropsy, 12 weeks after the tumor cell injections, the primary tumor weights in the 12% LA (4.1 ± 2.7 g)- and 8% LA (3.5 ± 1.7 g)-fed groups were significantly greater ($P < 0.05$) than those fed the 2% LA diet (2.5 ± 1.5 g); they did not differ significantly from the weights of mammary fat pad tumors in the 5% corn oil-fed mice. The incidence of grossly visible pulmonary metastatic nodules was not significantly different between the 8 and 12% LA-fed mice, but was higher for both groups compared with the 2% LA-fed group ($P < 0.05$), with a similar trend in comparison with the 5% corn oil group. The mean total calculated volumes of the macroscopic metastases per tumor-bearing mouse were significantly greater in the 8 and 12% LA (157 ± 250.7 and 99.1 ± 140.0 mm³, respectively), compared with the 2% LA (23.3 ± 51.8 mm³)- and 5% corn oil (24.5 ± 35.1 mm³)-fed mice; all $P < 0.05$. Micro-metastases were observed most frequently in the 5% corn oil and 2% LA dietary groups, but none of the differences were statistically significant. No differences were detected in the concentrations of prostaglandin E, leukotriene B₄, or 5-hydroxyeicosatetraenoic acid in tumors from mice fed the four different diets.

INTRODUCTION

While it is well established that the level and type of fat in the diet influences experimental mammary carcinogenesis in rodents (1-4), and the growth and metastasis of transplantable mammary carcinomas (5-8), there continues to be uncertainty regarding the existence of a corresponding effect in human breast cancer (9-11). Nevertheless, some clinical studies have shown that obesity is associated with a reduced disease-free interval after mastectomy (12, 13), that dietary fat may impact upon disease stage at the time of diagnosis (14), and that a high fat consumption decreases survival time in patients with metastatic breast cancer (15).

The issue is complicated further by a lack of consistency in those data that do support such a relationship; some studies have indicated that the association between breast cancer risk (16) or progression (17, 18) applies specifically to saturated fats, whereas others support a role for polyunsaturated fats (14, 19).

In previous studies (20-22), we found that a high-fat diet stimulates growth of the MDA-MB-435 human breast cancer cell line in athymic nude mice, and enhances its capacity to metastasize to the regional lymph nodes and lungs. The comparisons were between diets containing 2 levels of corn oil, a lipid rich in the ω -6 polyunsaturated fatty acid LA.³ The high-fat (23% CO) diet contained approximately 12%,

and the low-fat (5% CO) diet 2.6% LA, and so left open the question of whether the absolute level of fat in the diet or its LA content was responsible for the observed effects on breast cancer growth and metastasis.

We now report that both growth rate of this human breast cancer cell line at the primary site, and the occurrence and extent of pulmonary metastasis, are influenced by the level of LA present in diets which are isocaloric and contain the same high (23%) level of total fat. These results provide the first experimental support for the view that dietary polyunsaturated fatty acids may exert an adverse influence on the prognosis of breast cancer patients.

As an initial approach to mechanistic studies, the levels of 3 eicosanoids, PGE, arising from cyclooxygenase activity, and LTB₄ and 5-HETE, two products of the 5-lipoxygenase-mediated metabolic pathway, were determined in primary tumors from each of the dietary groups.

MATERIALS AND METHODS

Animals. Female athymic nude mice (NCr-nu/nu) aged 3 weeks were obtained from Simonsen Laboratories (Gilroy, CA) and were maintained in microisolator cages with housing in a pathogen-free isolation facility. They were assigned randomly to 1 of the 4 experimental diets, with 25 mice included in each group.

Diets. The diets, prepared by BioServ Inc. (Frenchtown, NJ), were based on the semipurified AIN-76A diet (23, 24), and were analyzed for their actual macronutrient content and screened for potential contaminants by the supplier. Three of the diets had the same high-fat content (23%, w/w), but this was provided by LA-rich safflower oil and saturated FA-containing coconut oil in differing ratios to give 2, 8, or 12% (w/w) LA (7). They were isocaloric, with 4.45 kcal/g of diet. A fourth group of mice was fed the same low-fat 5% CO diet, with an energy content of 3.53 kcal/g, as we used in our earlier study (20), in order to provide a comparison with the safflower oil/coconut oil-based diets. The diets were sterilized by irradiation with ⁶⁰Co, and stored in heat-sealed plastic bags filled with nitrogen at 4°C (25). Fresh diet was provided, and that not consumed discarded, twice a week. Feeding the experimental diets was commenced 7 days before injecting the breast cancer cells so that the initial phase of tumor proliferation occurred in the desired lipid environment.

Cell Line. The estrogen receptor-negative MDA-MB-435 human breast cancer cell line was originally isolated from a pleural effusion (26). Its growth in serum-free, estrogen-free culture medium is stimulated by LA.⁴ For the study described here, the cells were maintained in Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY) with 5% fetal bovine serum and grown to 70-90% confluence in T-150 flasks. The medium was changed 24 h before cell harvesting by trypsinization. For injection, the cells were resuspended in medium at a concentration of 20×10^6 cells/ml and kept on ice.

Experimental Procedure. The mice were anesthetized with pentobarbital, and the tumor cells were injected into a right-sided thoracic mammary fat pad which had been exposed by a small incision (20). A 50- μ l volume of inoculum containing 1×10^6 cells was injected, and the incision was closed with a skin clip. The mice were weighed and the inoculation site was palpated at weekly intervals. When tumors became palpable their maximum diameters (L) and the perpendicular diameter (W) of each were measured with a vernier caliper weekly until completion of the study. The formula

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³ The abbreviations used are: LA, linoleic acid (18:2, n -6); CO, corn oil; FA, fatty acid;

PGE, prostaglandin E; LTB₄, leukotriene B₄; 5-HETE, 5-hydroxyeicosatetraenoic acid.

⁴ Unpublished observations.

$$\left(\frac{L}{2}\right) \times \left(\frac{W}{2}\right) \times \pi$$

was used to calculate the surface areas (20).

The experiment was terminated 12 weeks after injection of the tumor cells, when the mice were killed by CO₂ euthanasia. At necropsy, body and primary tumor weights were determined, and the extent of lung metastases was assessed as previously described (20), using the method of Welch *et al.* (27). The volumes of lung surface metastatic deposits were calculated as for a sphere, and summed to obtain an estimate of the total volume of lung metastases per mouse. The lungs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined by light microscopy.

Tumor Eicosanoid Assays. Portions of the primary tumors were trimmed to remove necrotic tissue and immediately frozen in liquid nitrogen until the eicosanoid extractions were carried out. These were performed as described by Ip *et al.* (28). Briefly, 250 mg of tissue were finely minced, sonicated in 2.5 ml of 0.1 M Tris-HCl buffer (pH 7.4), and the homogenate was centrifuged at 800 × *g* for 15 min to remove the fat layer and cell debris; the resulting supernatant was then re-centrifuged at 100,000 × *g* for 1 h. Protein determinations were performed on 5-μl aliquots with the use of the Bio-Rad protein assay (Bio-Rad, Richmond, CA) with bovine serum albumin as standard. The high-speed supernatant was acidified to pH 3.5 with glacial acetic acid and extracted 3 times with 3 volumes of ethyl acetate. The 3 extracts were pooled and the solvent was evaporated under nitrogen. The residue was resuspended in 750 μl of the radioimmunoassay buffer and stored at -70°C until analyzed. Radioimmunoassays for PGE₂, LTB₄, and 5-HETE were performed by using ³H-label kits obtained from Advanced Magnetics (Cambridge, MA). The sensitivities per assay tube are: PGE₂, 2.6 pg; LTB₄, 3.2 pg; 5-HETE, 4.9 pg. The PGE₂ antibody exhibits cross-reactivities of 50, 6, and 3% with PGE₁, PGA₂, and PGA₁, respectively. In view of the 50% cross-reactivity between the antibody and PGE₁, the radioimmunoassay results were designated PGE. The 5-HETE antibody cross-reacts 100% with 5-HETE-lactone, and 7% with 8-HETE. When aliquots of the labeled eicosanoids were added separately to several tumor homogenates to determine extraction efficiencies, the recoveries were found to be 62.0 ± 0.4%, 57.2 ± 1.0%, and 49.9 ± 2.8% (SE) for PGE₂, LTB₄, and 5-HETE, respectively. Results are expressed as pg of eicosanoid/mg protein.

Statistics. The differences in the incidence with which primary tumors and metastases occurred were evaluated by the χ² test, and the lung metastasis total volumes by the nonparametric Mann-Whitney *U* test. Other statistical comparisons were made by Student's unpaired *t* test; *P* < 0.05 was considered statistically significant.

RESULTS

Body Weight Changes and Growth of Mammary Fat Pad Tumors. Body weights of the mice in all 4 dietary groups were similar at the time of tumor cell injection (12% LA, 20.9 ± 1.6 g; 8% LA, 20.8 ± 1.4 g; 2% LA, 20.7 ± 1.8 g; low-fat 5% CO, 21.0 ± 1.5 g), and remained so over the first 8 weeks of the study (at week 8: 24.4 ± 2.0 g; 24.6 ± 2.0 g; 24.4 ± 1.8 g; 24.3 ± 1.9 g, respectively). Subsequently, the larger size of the mammary fat pad tumors in the 8 and 12% LA groups began to affect total body weight, so that at necropsy there were no significant differences between the 3 high-fat

groups when the tumor weight was subtracted from the body weight for each animal (2% LA, 25.7 ± 1.9 g; 8% LA, 25.5 ± 3.0 g; 12% LA, 25.4 ± 2.3 g). At necropsy, the mice fed the 5% fat (w/w) CO diet weighed, on average, 1 g less (24.6 ± 3.0 g) than those fed the 23% fat diets after correction for the tumor weights, a difference which was not statistically significant.

The final incidence of mammary fat pad tumors in each dietary group was 22 of 25 (88%) for the 2% LA, 17 of 25 (68%) for the 8% LA, 21 of 25 (84%) for the 12% LA high-fat diets, and 22 of 25 (88%) for the 5% CO low-fat diet. The difference between the 8% and the 12% LA-fed mice in tumor development was not statistically significant, and the growth rates of the established primary tumors were similar, and more rapid, than that of those in mice fed the 2% LA-containing diet. Thus, in Fig. 1, the mean surface areas of the tumors from the groups of mice fed the two highest levels of LA were significantly greater (*P* < 0.01) than that of tumors in mice fed the 2% LA diet at all time points beyond week 4. The low-fat (5% CO) diet providing approximately 2.6% (w/w) LA, was associated with a tumor growth rate which did not differ significantly from that of the high-fat 2% LA group. At necropsy, the weights of the primary tumors from the 8 and 12% LA-fed groups, 3.5 ± 1.7 and 4.1 ± 2.7 g, respectively, were not significantly different, but in both cases were higher than those from the 2% LA-fed mice: 2.5 ± 1.5 g (*P* < 0.05). The 5% CO diet was associated with primary tumors which were intermediate in final weight (3.2 ± 1.7 g).

Tumor Metastases. The occurrence of macroscopically detectable lung metastases and micrometastases in mice bearing mammary fat pad primary tumors is summarized in Table 1. Seven (32%) of the 22 in the 2% LA, 11 (65%) of 17 in the 8% LA, and 13 (62%) of 21 in the 12% LA dietary groups had visible pulmonary metastatic nodules,

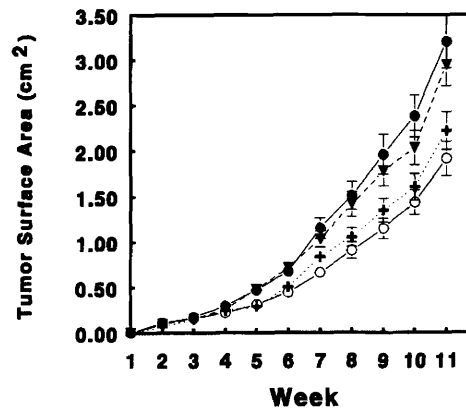


Fig. 1. Growth of MDA-MB-435 cell solid tumors in the mammary fat pads of mice fed high-fat diets containing 2% (○), 8% (▼), or 12% (●) LA (w/w), or a low-fat, 5% CO (+) diet. Points, means ± SEM of the tumor surface area for each mouse with a palpable tumor. There were no statistically significant differences in the size of tumors in mice fed 8 or 12% LA at any time point. The tumors in mice fed the two highest LA levels were significantly larger than those in mice fed 2% LA (all *P* < 0.01) or 5% CO (all *P* < 0.05) diets after week 4.

Table 1 Effects of dietary fatty acid intake on incidence of grossly visible pulmonary metastatic nodules and micrometastases from MDA-MB-435 human breast cancer solid tumors in mammary fat pads of nude mice

Dietary group	Grossly visible nodules	Micrometastases	Total metastatic incidence
2% linoleic acid, 23% fat (w/w)	7/22 (32) ^a	5/22 (23)	12/22 (55)
8% linoleic acid, 23% fat (w/w)	11/17 (65) ^b	2/17 (12)	13/17 (77)
12% linoleic acid, 23% fat (w/w)	13/21 (62) ^b	4/21 (19)	17/21 (81)
5% corn oil, 5% fat (w/w)	10/22 (45)	7/22 (32)	17/22 (77)

^a Numbers in parentheses, percentage.

^b Significantly different from 2% LA-fed group, *P* < 0.05.

Table 2 Effects of dietary fatty acid intake on extent of lung metastasis from MDA-MB-435 human breast cancer solid tumors

Metastases	High (23%) fat diet			Low (5%) fat diet
	2% LA	8% LA	12% LA	5% CO
No./mouse				
Total	2.5±5.5 ^a	12.1±18.7	10.1±14.1	3.1±4.4
<1 mm	0.82	4.94	3.76	1.41
1–3 mm	1.23	5.59	5.48	1.50
>3 mm	0.45	1.53	0.86	0.18
Total volume of metastatic nodules/mouse (mm ³)	23.3±51.8	157.4±250.7 ^{b,d}	99.1±140.0 ^c	24.5±35.1

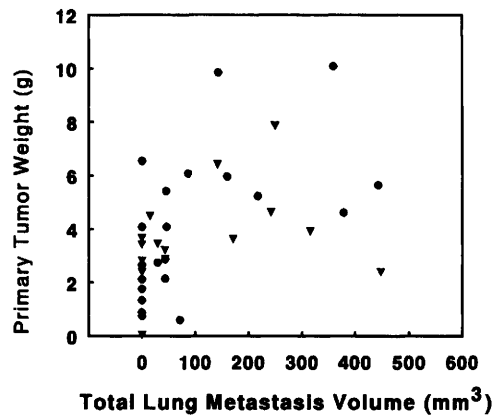
^a Mean ± SD.^b Significantly different from 2% LA-fed group, *P* < 0.01.^c Significantly different from 2% LA-fed group, *P* < 0.02.^d Significantly different from 5% CO-fed group, *P* < 0.05.

Fig. 2. Total volume of lung metastasis for individual tumor-bearing mice fed 8% (▼) or 12% (●) LA high-fat diets plotted with the corresponding primary tumor weight at necropsy. Metastasis was frequently extensive and independent of the size of the primary tumor.

as did 10 (45%) of 22 primary tumor-bearing mice in the low-fat, 5% CO, group. While the frequencies with which macroscopic lung metastases were observed were not statistically significant between the 8 and 12% LA-fed groups and the 5% CO group, both the 8 and 12% LA-fed animals showed a higher incidence of metastasis compared with the 2% LA-fed group (*P* < 0.05) despite identical total fat and energy intakes. Micrometastases were seen most frequently in the 5% CO- and 2% LA-fed mice, but none of the differences between dietary groups were statistically significant (Table 1).

The mean total calculated volumes (± SD) of the grossly visible pulmonary metastatic nodules per tumor-bearing mouse were significantly less in the 23% total fat, 2% LA group, and the 5% total fat, CO group (23.3 ± 51.8 and 24.5 ± 35.1 mm³, respectively) compared with the 23% total fat, 8 and 12% LA-fed animals (157.4 ± 250.7 and 99.1 ± 140.0 mm³, respectively); *P* < 0.05 for each comparison (Table 2).

Each primary tumor weight determined at necropsy and the corresponding estimate of total lung metastatic volume were plotted together for the mice fed the 8 and 12% LA-containing, high-fat diets (Fig. 2), and for those fed the 2% LA, high-fat and the 5% CO, low-fat diets (Fig. 3). While the primary tumors were often larger in mice fed the two LA-rich diets, the extent of lung metastasis was not governed by the size of the primary tumor ($\tau = 0.312$; *P* = 0.107). However, in the case of the two diets containing low levels of LA, when present the relatively limited extent of pulmonary metastatic involvement occurred in association with the larger primary tumors, and there was a significant correlation between metastatic burden and primary tumor weight ($\tau = 0.460$; *P* = 0.002).

Tumor Eicosanoids. Radioimmunoassays for PGE, LTB₄, and 5-HETE were performed on 12 tumors taken at random from the 2 and 12% LA, high-fat and the 5% CO, low-fat groups, and 11 tumors from

the 8% LA, high-fat group. No significant differences were evident in the eicosanoid content of these tumors taken at necropsy (Table 3).

DISCUSSION

A frequently expressed concern regarding experimental studies of the influence of dietary fat on mammary tumorigenesis is whether the observed promotional effects are due specifically to the lipid, or to increased energy intake (29–32), and the same question might be raised in relation to tumor progression and metastasis. In an earlier study (20) we found that the growth and metastasis of MDA-MB-435 breast cancer cells in nude mice were stimulated by feeding a 23% CO high-fat diet compared with the progression of tumors in mice fed 5% CO low-fat diets. Now we have demonstrated unequivocally that diets with the same energy and total fat content differ in their capacity to stimulate the growth and metastasis of a human breast cancer cell line depending upon the FA composition. These results are consistent with experiments *in vitro* on the effects of FAs on the growth of cultured human breast cancer cells; LA stimulated cell proliferation, whereas

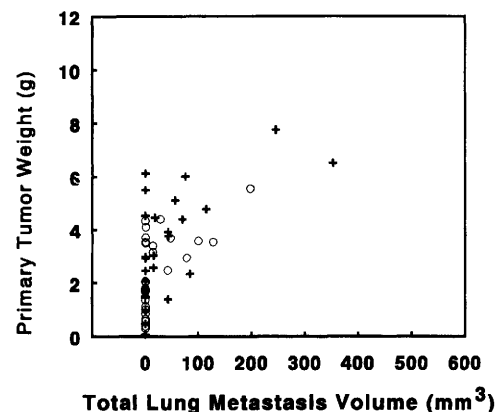


Fig. 3. Total volume of lung metastasis for individual tumor-bearing mice fed a 2% LA high-fat diet (○) or a 5% corn oil low-fat diet (+) plotted with the corresponding primary tumor weight at necropsy. When present, metastasis was of limited extent, and generally occurred in association with the larger primary tumors.

Table 3 Eicosanoid levels in mammary fat pad tumors (mean ± SD)
None of the differences between groups were statistically significant.

Dietary group	No. of tumors	Eicosanoid concentration (pg/mg protein)		
		PGE	LTB ₄	5-HETE
2% linoleic acid, 23% fat (w/w)	12	628.3±141.8	81.1±20.4	730.7±186.5
8% linoleic acid, 23% fat (w/w)	11	644.4±223.8	90.1±33.2	821.1±229.9
12% linoleic acid, 23% fat (w/w)	12	669.5±207.9	93.2±28.5	727.0±200.5
5% corn oil, 5% fat (w/w)	12	724.1±250.4	91.8±19.6	650.1±202.5

stearic and palmitic acids, two saturated FAs, were mildly inhibitory at the same concentrations (33). We also found that a high-fat (23% w/w) diet providing 4.45 kcal/g, but containing only 2% LA, was no more effective in influencing MDA-MB-435 cell growth and metastasis than the 5% CO diet with 3.53 kcal/g and approximately an equal amount of LA which we used in our previous study (20). Thus, there appears to be a specific function for LA in producing the observed effects of a high-fat diet on the biological behavior of this breast cancer cell line in nude mice.

A number of studies have demonstrated similar specific effects of LA in experimental mammary tumorigenesis (2, 34, 35), the growth of transplantable mammary carcinomas (5, 36, 37), and metastasis in rodent models (7, 38). Abraham *et al.* reported an enhancing effect of dietary LA on the growth of transplanted tumors (5, 36), which appeared to be related to the availability of arachidonic acid for cell membrane phospholipid synthesis, but not its conversion to prostaglandins (37). Katz and Boylan (38) used a retired breeder rat model (6) to compare different diets in which CO, olive oil, or beef tallow provided the high-fat intake, and found that CO was associated with the greatest mean lung metastatic tumor volume; a low-fat CO group had the lowest pulmonary tumor burden.

The 23% (w/w) total fat isocaloric diets used in the present study were modeled on those used by Hubbard and Erickson (7) to study the mouse metastatic mammary tumor 4526 cell subline. They found that when the cells were growing as solid tumors in mammary fat pads, the number of metastatic nodules on the lung surfaces was 3- or 4-fold fewer in mice fed 8% or less LA compared with mice fed 12% LA. Thus, in this model also, LA exerted a specific effect on metastasis beyond that which might be ascribable to fat intake *per se*, although in the case of the MDA-MB-435 human breast cancer cell line 8 and 12% LA-containing diets exerted similar effects on metastasis, the dose-dependent response or response threshold being within the 2–8% LA range.

Most likely, several independent or related mechanisms are involved in the effects of dietary FAs on breast cancer growth and metastasis, and, as in the case of experimental mammary tumorigenesis (2, 39, 40), there is some evidence in support of a role for the eicosanoids. A comparison of PGE₂ levels in mouse mammary lesions ranging from preneoplastic benign masses to highly metastatic carcinomas, including those formed from the 4526 subline, showed a close relationship between tumorigenicity and metastatic potential, and tissue PGE levels (41). Hubbard *et al.* (42) found that the stimulatory effect of a high-fat, LA-rich, diet on lung metastasis in their 4526 mouse mammary carcinoma model was suppressed by indomethacin; this inhibitor of eicosanoid synthesis also reduced the tumor PGE content, as reported previously by Fulton (43). Yet, despite these indications that tumor eicosanoid synthesis is involved in cell growth and metastasis, we found no effect of dietary LA intake on MDA-MB-435 tumor PGE, LTB₄, or 5-HETE content, and neither did Hubbard *et al.* (7, 42) when tumor PGE₂ levels were assayed in their study of dietary LA and metastasis of the 4526 mouse mammary tumor cell subline. These results do not, however, exclude the possibility that eicosanoid production rates were influenced by FA intakes at earlier, and critical, stages in tumor progression.

Dietary FAs may also exert effects on the host tissues, including eicosanoid levels (44), which are favorable to the establishment of metastases. In their mouse mammary tumor cell model, Hubbard and Erickson (8) found that LA appeared to enhance metastasis development by influencing the host rather than tumor cell proliferation, and the recipient target tissue is undoubtedly an important component of the metastatic cascade. However, in contrast to these earlier studies, we included micrometastases when assessing the effects of dietary FAs on the metastatic process. These often consisted of a few small

nests of tumor cells, a pathological feature which we described in detail elsewhere (21), and which were present most frequently in the 5% CO dietary group. Thus, the size and multiplicity of the metastases, rather than simply the overall incidence, were increased by a high intake of LA, suggesting that the *n-6* FA enhanced their growth once colonization of the lungs had taken place, a mechanism consistent with stimulation of MDA-MB-435 cell growth *in vitro* (33), and of the mammary fat pad tumor progression by LA.

In conclusion, while caution is appropriate in attempting to translate our experimental results to the clinical situation, it is worth noting that in White American breast cancer patients, a relatively high consumption of polyunsaturated fat has been associated with the presence of regional or distant metastasis at the time of diagnosis (14).

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