

Activity of human contact allergens in the murine local lymph node assay

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The murine local lymph node assay (LLNA) is a predictive test for the identification of chemicals that have the potential to cause skin sensitization. Since its original development, the assay has been the subject of national and international evaluation studies and extensive comparisons with guinea pig tests and human data. On the basis of these investigations, the LLNA has recently been endorsed by ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods) as a stand-alone method for skin sensitization hazard identification. At the same time, ICCVAM confirmed that, although the LLNA is not an *in vitro* method, it does represent a refinement in the way animals are used and can provide a means for reducing the number of animals used in sensitization hazard assessment. The investigations described here were designed to explore further the ability of the LLNA to identify accurately those chemicals that cause allergic contact dermatitis in humans. To that end we have measured, amongst 3 independent laboratories, LLNA responses induced by a total of 18 test chemicals, 11 of which are known to cause skin sensitization and 7 of which are believed not to be associated with any significant evidence of allergic contact dermatitis in humans. The LLNA correctly classified 16 of the 18 materials. The 11 chemicals tested which are associated with allergic contact dermatitis in humans were found to be positive in the LLNA. Of the 7 materials believed to be non-sensitizers, 5 were negative in the LLNA and 2 produced positive results. Collectively, these data provide additional evidence that the LLNA is able to discriminate skin sensitizers from those chemicals which do not possess a significant skin sensitization potential and thus provides a method for hazard identification that offers important animal welfare benefits.

Key words: local lymph node assay; contact allergy; predictive testing; mouse sensitization; isopropyl myristate; linalool; methyl 4-hydroxybenzoate; parabens; fragrance. © Munksgaard, 2000.

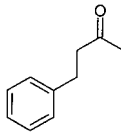
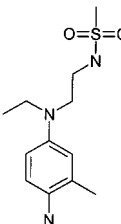
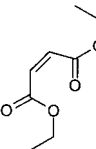
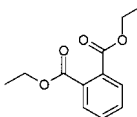
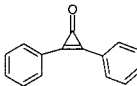
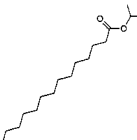
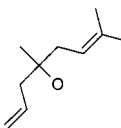
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The prospective identification of skin sensitizing chemicals is an important step in the safety assessment of new ingredients or products. Traditionally, sensitization potential has been determined largely by the conduct of guinea pig assays such as the guinea pig maximization test (1, 2) and the occluded patch test of Buehler (3, 4). These guinea pig test methods employ a biphasic protocol and are based on the visual assessment of erythema produced upon the elicitation of a contact allergic response in previously sensitized animals. In contrast, the murine local lymph node assay (LLNA) seeks to identify potential skin sensitizing chemicals as a function of events that are associated with the induction phase of contact sensitization. The LLNA is a mechanistically based, alternative test method which identifies contact allergens as a function of lymphocyte proliferative responses

provoked in draining lymph nodes following epicutaneous application of the test material (5, 6). In addition, the magnitude of the proliferative response correlates closely with the skin sensitizing capacity of the chemical (7). Compared with the traditional guinea pig test methods, the LLNA offers the advantages of: (i) having an objective endpoint; (ii) being a significant refinement in the way in which animals are used for contact sensitization testing (i.e., no use of adjuvants, shaving or depilation or occlusive patches and bandages); (iii) providing a means for reducing the number of animals used and, iv) requiring less time for completion than most currently used guinea pig test methods.

Recently, the LLNA was reviewed thoroughly by the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM). The Committee concluded that the method was

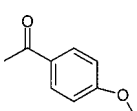
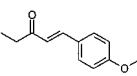
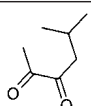
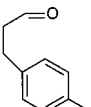
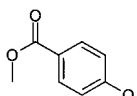
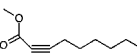
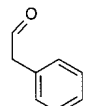
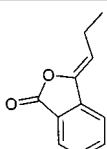
Table 1. Structures, chemical class and references to human sensitization

Chemical name [CAS number]	Chemical structure	Classification and reference for human sensitization
benzylidene acetone (4-phenyl-3-buten-2-one) [122-57-6]		sensitizer 36, 37
1-butanol [71-36-3]		non-sensitizer
CD-3 (4-(N-ethyl-N-2-methan-sulfonamido-ethyl)-2-methyl-1,4-phenylenediamine [25646-71-3])		sensitizer 38, 39
diethyl maleate [141-05-9]		sensitizer 36, 40, 41
diethylphthalate [84-66-2]		non-sensitizer 42
diphenylcyclopropenone [886-38-4]		sensitizer 43, 44
glycerol [56-81-5]		non-sensitizer 45
isopropyl myristate [110-27-0]		non-sensitizer 19
linalool [78-70-6]		non-sensitizer 15 sensitizer 17, 46

sufficiently validated to be used as a stand-alone test for the identification of skin sensitizing chemicals. In addition, the ICCVAM peer review panel confirmed that the LLNA offered important animal welfare benefits by both refining the way in which animals are used for skin sensitization testing and potentially reducing the number of animals required for this purpose (8).

Since its original development, the LLNA has been the subject of national and international

Table 1 (cont'd)

Chemical name [CAS number]	Chemical structure	Classification and reference for human sensitization
4' methoxyaceto-phenone [100-06-1]		non-sensitizer 47
1-(p-methoxyphenyl)-1-penten-3-one [104-27-8]		sensitizer 48
5-methyl-2,3-hexanedione [13706-86-0]		sensitizer 49
p-methylhydrocinnamic aldehyde [5406-12-2]		sensitizer 14
methyl 4-hydroxybenzoate (methylparaben) [99-76-3]		non-sensitizer
methyl 2-nonynoate [111-80-8]		sensitizer 50, 51
phenylacetaldehyde [122-78-1]		sensitizer 52, 53
3-propylidenephthalide [17369-59-4]		sensitizer 54
squaric acid [2892-51-5]		sensitizer 55, 56, 57

evaluation studies (9, 10) and extensive comparisons with guinea pig tests and human data (11, 12). The LLNA was found to be at least as sensitive as, and more reliable than, current guinea pig tests for predictive identification of important skin sensitizers. While comparisons of LLNA data with results of guinea pig tests are of value, the ultimate purpose of any animal assay is to identify chemicals which are capable of inducing allergic contact sensitization in humans. The investigations described here were designed to explore further the ability of the LLNA to identify accurately those chemicals that cause allergic contact dermatitis in humans. To that end, we have measured LLNA responses induced by a total of 18 test chemicals,

11 of which are known to cause skin sensitization and 7 of which are believed not to be associated with allergic contact dermatitis in humans.

Materials and Methods

Participating laboratories

Participating laboratories were the Procter & Gamble Company, Miami Valley Laboratories, Cincinnati, OH (Laboratory A), AstraZeneca Central Toxicology Laboratory, Macclesfield, Cheshire, UK (Laboratory B), and Unilever Safety and Environmental Assurance Centre Toxicology Group, Unilever, Sharnbrook, Bedfordshire, UK (Laboratory C).

Animals

Female CBA/J strain mice (Laboratory A; Harlan Sprague Dawley, Inc., Indianapolis, IN or Jackson Laboratories, Bar Harbor, ME) or female CBA/Ca strain mice (Laboratories B and C; Harlan Olac, Bicester, Oxfordshire, UK), age 6–12 weeks old, were used in these studies. Animals used in studies conducted by Laboratory A were housed, fed, and handled in compliance with standards set forth by the U.S. Animal Welfare Act and recommendations in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" and all procedures performed on animals were reviewed and approved by a veterinarian and the Institutional Animal Care and Use Committee. Animals used in studies conducted by Laboratories B and C were housed under standard conditions and all procedures were approved by the UK Home Office and carried out in compliance with the Animals (Scientific Procedures) Act 1986 under a Home Office granted Project License.

Chemicals

Test chemicals were chosen for this study based on two criteria: (i) the potential of the chemical to induce contact allergy in humans was well established; and (ii) the chemical had not been previously tested in the LLNA. The following test materials were obtained from Sigma-Aldrich Co. Ltd., Dorset, UK: diethylphthalate, 99%; diphenylcyclopropanone, 98%; glycerol, 99%; methyl 2-nonyanoate, 99%; 3-propylenephthalide, 96+%; phenylacetaldehyde, 100%; 4-methoxyacetophenone, 100%; diethyl maleate, 97%; linalool, 97% and 4-methyl hydroxybenzoate, 99%. Benzylidene acetone, 99+%; 1-(p-methoxyphenyl)-1-penten-3-one, 99% and 3,4-dihydroxy-3-cyclobutene-1,2-dione (squaric acid), 99% were obtained from Aldrich, Milwaukee, WI. Isopropyl myristate, 98% was purchased from Sigma,

St. Louis, MO. The test materials also included 1-butanol, 100%, (J.T. Baker, Phillipsburg, PA), p-methyl hydrocinnamic aldehyde, 100% (Firmenich, Middlesex, UK), 4-(N-ethyl-N-2-methanesulfonamidoethyl)-2-methyl-1,4-phenylenediamine, technical quality (CD-3; Eastman Kodak Company, Rochester, NY) and 5-methyl-2,3-hexanedione, 99% (Frutarom (UK) Ltd., Berkhamsted, Herts, UK). The concentrations of each chemical and the vehicle used, as well as the testing laboratory are detailed in Table 2. Dose levels of each material were chosen to provide the highest possible test concentration based on either solubility in the selected vehicle or lack of systemic toxicity of the chemical. For those chemicals which were deemed to be non-sensitizing in man, the highest possible dose, up to 100% in some cases, was used.

Local lymph node assay

A standard local lymph node assay (LLNA) protocol, as described previously, was used by laboratories B and C (13). Laboratory A employed the standard protocol except that lymph nodes were pooled for individual mice rather than from experimental groups. The robustness of the LLNA has been demonstrated previously that such minor procedural modifications, i.e., using lymph nodes pooled for experimental groups versus nodes from individual animals, are without consequence to the test's performance (9, 10). Briefly, groups of mice ($n=5$, Laboratory A; $n=4$, Laboratories B and C) were treated 1× a day for 3 consecutive days on the dorsum of both ears with 25 μ l of 1 of 3 concentrations of test material or with vehicle alone. 5 days after the initial treatment, all mice were injected intravenously, via the tail vein, with 250 μ l of phosphate buffered saline (PBS) containing 20 μ Ci of ^{3} H-methylthymidine (^{3} H-TdR; specific activity 5.0 Ci/mmol for Laboratory A, 2.0 Ci/mmol for Laboratories B and C; Amersham Corporation, Arlington Heights, IL and Amersham International, Amersham, Buckinghamshire, UK, respectively). 5 h later, the mice were euthanised and the draining auricular lymph nodes were removed and pooled for each individual mouse (Laboratory A) or for each experimental group (Laboratories B and C). Single cell suspensions were prepared by gentle mechanical disaggregation through either nylon mesh (100 μ m pore size; Laboratory A) or 200-mesh stainless steel gauze (Laboratories B and C). Cell suspensions were washed 2× with an excess of PBS and precipitated with 5% trichloroacetic acid (TCA) and left at 4°C overnight. The samples were then centrifuged, resuspended in 1 ml 5% TCA and transferred to 10 ml of scintillation cocktail (Laboratory A, Ecolume, ICN Radio-

Table 2. Local lymph node assay results

Chemical and testing lab	Vehicle ^a and concentrations	dpm/node or *mean dpm ($\pm$ SEM)	Stimulation index	Sensitization classification by LLNA	Human sensitization classification
benzylidene acetone	AOO	*595 $\pm$ 185	1		
	10% w/v	5059 $\pm$ 773	8.5		
Lab A	25% w/v	8070 $\pm$ 352	13.6	+	+
	50% w/v	7619 $\pm$ 892	12.8		
1-butanol	dH ₂ O	*366 $\pm$ 44	1		
	5% v/v	598 $\pm$ 157	1.6		
Lab A	10% v/v	451 $\pm$ 17	1.2	—	—
	20% v/v	530 $\pm$ 91	1.4		
CD-3	DMSO	*370 $\pm$ 36	1		
	1% w/v	1675 $\pm$ 155	4.5		
Lab A	5% w/v	2199 $\pm$ 119	5.9	+	+
	10% w/v	2312 $\pm$ 657	6.3		
diethyl maleate	AOO	361	1		
	25% w/v	5902	16.3		
Lab C	50% w/v	8153	22.6	+	+
	100% w/v	4725	13.1		
diethylphthalate	AOO	380	1		
	25% v/v	384	1.0		
Lab B	50% v/v	506	1.3	—	—
	100% v/v	557	1.5		
diphenylcyclopropenone	acetone	353	1		
	0.3% w/v	8631	24.5		
Lab B	1% w/v	8710	24.7	+	+
	3% w/v	11170	31.7		
glycerol	DMF	363	1		
	25% v/v	406	1.1		
Lab B	50% v/v	240	0.7	—	—
	100% v/v	187	0.5		
isopropyl myristate	AOO	*595 $\pm$ 185	1		
	25% v/v	1220 $\pm$ 188	2.1		
Lab A	50% v/v	1946 $\pm$ 502	3.3	+	—
	100% v/v	1999 $\pm$ 297	3.4		
linalool	AOO	361	1		
	25% w/v	919	2.5		
Lab C	50% w/v	1718	4.8	+	—
	100% w/v	2988	8.3		
4' methoxyacetophenone	AOO	392	1		
	10% w/v	490	1.3		
Lab C	25% w/v	404	1.0	—	—
	50% w/v	395	1.0		
1-(p-methoxyphenyl)-1-penten-3-one	AOO	*493 $\pm$ 102	1		
	10% v/v	1743 $\pm$ 415	3.5		
Lab A	25% v/v	4946 $\pm$ 1344	10.0	+	+
	50% v/v	12854 $\pm$ 2113	26.1		
5-methyl-2,3-hexanedione	AOO	353	1		
Lab B	25% v/v	1017	2.9		
	50% v/v	2103	6.0	+	+
	100% v/v	5058	14.3		
p-methylhydrocinnamic aldehyde	AOO	361	1		
	25% w/v	1302	3.6		
Lab C	50% w/v	3254	9.0	+	+
	100% w/v	5908	16.4		
methyl 4-hydroxybenzoate	DMF	356	1		
	10% w/v	267	0.8		
Lab C	25% w/v	321	0.9	—	—
	50% w/v	287	0.8		

Table 2 (cont'd)

Chemical and testing lab	Vehicle ^a and concentrations	dpm/node or *mean dpm (±SEM)	Stimulation index	Sensitization classification by LLNA	Human sensitization classification
methyl 2-nonynoate Lab B	80% EtOH	314	1	+	+
	5% v/v	3258	10.4		
	10% v/v	5550	17.7		
	20% v/v	7660	24.4		
phenylacetaldehyde Lab C	AOO	392	1	+	+
	25% w/v	6094	15.5		
	50% w/v	9315	23.8		
	100% w/v	9436	24.1		
3-propylidenephthalide Lab B	AOO	526	1	+	+
	5% v/v	2554	4.9		
	10% v/v	4803	9.1		
	20% v/v	7954	15.1		
squaric acid Lab A	DMSO	*426±53	1	+	+
	2.5% w/v	1692±254	4.0		
	5.0% w/v	1163±226	2.7		
	10% w/v	1439±114	3.4		

^a AOO=acetone: olive oil, 4:1; dH₂O=distilled water; EtOH=ethanol; DMSO=dimethyl sulfoxide; DMF=dimethylformamide

chemicals, Irvine, CA; Laboratories B and C, OpiPhase MP, LKB Scintillation Products, FSA Laboratory Supplies, UK). ³H-TdR incorporation was measured by β -scintillation counting and expressed as disintegrations per min (dpm) per individual mouse (Laboratory A) or as dpm per node for each experimental group (Laboratories B and C). Stimulation indices (SI) for each experimental group were determined as the increase in ³H-TdR incorporation relative to the concurrent vehicle treated control group. A material is considered to be positive in the LLNA if a 3-fold or greater increase in proliferation, compared with the concurrent vehicle control, is obtained at 1 or more test concentrations.

Results

The skin sensitization potential of 18 chemicals representing a variety of chemical classes was tested in the LLNA amongst the 3 participating laboratories. While the majority of chemicals tested are fragrance materials, they represent a number of chemical classes including ketones, aromatic amines, aliphatic esters, and alcohols. References to the evidence for the skin sensitizing activity of these materials in humans is shown in Table 1 together with the corresponding chemical structure.

The results obtained for these materials are summarized in Table 2. Laboratory A measured lymph node isotope incorporation for each individual mouse. The results in Table 2 for this laboratory are reported as the group mean dpm and standard error of the mean (SEM) and SI value. Results from Laboratories B and C are from lymph nodes pooled for each experimental group and are pre-

sented as dpm per node, together with an SI value.

Vehicle control values obtained were very consistent across the 3 laboratories and ranged from 366–595 (Laboratory A), 314–526 (Laboratory B) and 359–392 (Laboratory C). The LLNA identified correctly 16 out of the 18 materials. All of the 11 chemicals tested which were judged in man to be significant contact allergens elicited positive reactions in the LLNA, provoking in each case a greater than 3-fold increase in proliferation compared with concurrent vehicle treated controls at 1 or more test concentrations. Indeed, 9 out of 11 materials stimulated an SI of greater than 3 at all 3 concentrations tested. Furthermore, such responses were generally vigorous, with the majority (9 out of 11) of chemicals eliciting maximal SIs of 13.6 to 31.7, which were in many cases achieved at relatively low concentrations, for example, 0.3% diphenylcyclopropanone stimulating an SI of 24.5. The remaining 2 chemicals, squaric acid and CD3, did not induce such high levels of thymidine incorporation, peaking at SIs of 4.2 and 6.3, respectively. However, positive (SI of 3 or greater) responses were recorded at relatively low test concentrations of 1% CD3 and 2.5% squaric acid. Of the 7 chemicals believed to be non-sensitizers, 5 failed to provoke an SI of 3 or more at any test concentration examined, despite employing relatively high doses of material in each case (maximum concentrations 20–100%). For the remaining 2 chemicals, linalool and isopropyl myristate, in both cases positive responses were recorded in the LLNA. In keeping with the strategy of testing putative negative compounds at the highest achievable concentration, these materials were tested at a maximum of 50% and 100%, respectively, with the former inducing SIs of 4.8 and 8.3 at 25% and 50%

Table 3. Comparison of LLNA and human classifications

LLNA classification	Human classification		unclear	total
	Human positive	Human negative		
LLNA positive	11	2	0	13
LLNA negative	0	5	0	5
total	11	7	0	18
table statistics for the shadowed 2×2 table				
sensitivity:	100%	prevalence:	1.57	
specificity:	71%			
positive predictivity:	85%			
negative predictivity:	100%			
accuracy:	89%			
χ^2	7.61	(≥ 3.8)		

and the latter provoking SIs of 3.3 and 3.4 at 50% and 100%, respectively.

A 2×2 contingency table was used to compare the human sensitization classification with the predictions obtained in the LLNA for the 18 chemicals tested in this study (Table 3). The accuracy, or closeness of agreement between the LLNA test result and an accepted reference value, was found to be 89%. Sensitivity and specificity values of 100% and 71% were calculated, and reflect the proportion of all correctly identified positive and negative materials, respectively. The percentage of chemicals positive in the LLNA, that are positive in humans (positive predictivity), was 85% and the % of LLNA negatives, that are human negatives (negative predictivity), was 100%.

Discussion

The objective of the work presented here was to investigate further the ability of the LLNA to predict accurately the potential of chemicals to cause allergic contact dermatitis in humans. Chemicals were chosen for these investigations based on both human maximization test data and clinical case reports of allergic contact dermatitis.

The LLNA predicted successfully the human sensitization potential for 16 of the 18 test materials used in this study. However, 2 of 7 of the chemicals tested which are believed not to be associated with allergic contact dermatitis, isopropyl myristate and linalool, were misclassified in so far as they were found to be positive in the LLNA. Linalool is one of the most commonly found fragrances utilized in toiletries and cosmetics (14), therefore, the incidence of exposure is relatively high. It is generally considered to be a non-sensitizer, producing negative results in the human maximization test (15). There are, however, a few isolated reports which indicate that linalool is a contact allergen (16, 17). It is possible, but unproven, that positive findings with this material are the re-

sult of impurities (e.g., oxidation products) in the sample tested.

Isopropyl myristate, tested according to the recommended dose selection criteria, selecting the highest test concentrations achievable based on solubility of the material in the chosen vehicle and lack of local/systemic toxicity (8), was found to be weakly positive in the LLNA. Isopropyl myristate has been reported to be a very rare human contact allergen (18). However, it is generally considered to be devoid of sensitizing properties as the human maximization test with isopropyl myristate at 20% in petrolatum did not produce any positive responses (19). This material has been shown to be an irritant when tested in mice (20), rabbits and humans (21), and it has been reported that some irritants can produce a positive response in the LLNA (10, 22, 23). The results obtained with isopropyl myristate are suggestive of a false-positive response according to criteria published by Basketter et al. (24): (i) there were no structural alerts for skin sensitization (data not shown) as determined by DEREK, an expert chemical rule-based system (25, 26); (ii) the test material is known to be a skin irritant at high concentrations; and (iii) the test substance was only weakly positive at the highest concentrations tested (stimulation index of 3.4 when tested at 100%). Strategies for evaluating false-positive results in the LLNA have been proposed and are discussed in detail elsewhere (24).

Methyl 4-hydroxybenzoate (methyl paraben), along with other paraben esters, is widely used as a preservative in "leave on" products, such as cosmetics and topical medicaments, and is generally considered to have little sensitization potential (except in relation to chronic use on skin ulcers) and, for the purposes of this study, was therefore classified as a non-sensitizer. While contact sensitivity to parabens has been reported (27), their sensitization potential in humans is considered low (28, 29). Patch test studies confirm this view. Menné and Hjorth (30) report a frequency of response to the pa-

rabens mixture of only 1% in 8020 patients patch tested over a 15-year period. Jacobs et al. (31) reported a frequency of <0.5% in 5167 patients tested over a 12-year period. Hannuksela et al. (32) found an incidence of positive responses to parabens in 0.3% of 4097 patients. Methyl 4-hydroxybenzoate has been reported to be negative in predictive guinea pig tests (33, 34). Therefore, it is not surprising that it was also found to be negative in the LLNA. It is important to bear in mind that while predictive tests such as the LLNA may not always detect weak or infrequently sensitizing materials, they are, however, capable of identifying chemicals that are significant human allergens (11, 35).

Key elements of method performance used for evaluating new tests include: (i) *sensitivity* – the proportion of all positive chemicals that are identified correctly as positive; (ii) *specificity* – the proportion of all negative chemicals that are identified correctly as negative; (iii) *positive predictivity* – the proportion of correct positive responses among materials testing positive; (iv) *negative predictivity* – the proportion of correct negative responses among materials testing negative; (v) *accuracy* – the closeness of agreement between a test result and an accepted reference value. Since the goal of the LLNA is the identification of materials that are capable of inducing allergic contact dermatitis in humans, the use of human data as the reference for comparison of LLNA results would seem the most appropriate method for evaluating its performance. As part of the ICCVAM review of the LLNA, the National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods (NICEATM) conducted an independent analysis of the performance of the LLNA, using a list of 74 chemicals for which both LLNA and human test data exist (8). In comparison with human data, which comprised 39 chemicals tested using the human maximization test and 35 compounds included in human patch test allergen panels, they found the LLNA to have a sensitivity of 72% (49/68), a specificity of 67% (4/6), a positive predictivity of 96% (49/51), a negative predictivity of 17% (4/23) and an accuracy of 72% (53/74); broadly similar values to those obtained in the current investigation. The ICCVAM Peer Review Panel concluded that "... the LLNA can reasonably predict human responses to sensitizers when compared with currently accepted methods for regulatory decision making." (8).

In the present study, the LLNA classified correctly the human sensitization potential of 16 of the 18 chemicals tested. Both human maximization test data and clinical case reports were used to evaluate the performance of the LLNA. It is important to consider that human clinical data are

quite complex, being influenced by many factors including the nature and extent of exposure which induced the positive response as well as the individual sensitivities of each patch test subject. Despite these caveats, the accuracy of the prediction of the LLNA was found to be 89% (Table 3). The data obtained from this current study provide further evidence that the LLNA is a reliable and accurate method for identifying chemicals that are classified as significant human skin sensitizers.

Acknowledgement

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