Perspectives on the Current State of Evaluation of Skin Sensitization

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Skin sensitization caused by chemicals is one of the high social interest diseases in terms of maintenance and control of working environments and general consumer safety. In recent years, there have been strong demands to replace animal testing with non-animal testing to detect the skin sensitizing potential of chemicals. Several non-animal tests have already been developed and are being used for regulation of each country. In this review, we provide an overview of skin sensitization, and introduce international regulatory trends, new non-animal tests, and our approach and future perspectives to the evaluation of skin sensitizing potential.

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Introduction

With growing global concerns regarding environmental protection as well as the need to maintain and promote human health, evaluating the adverse effects of chemical substances such as agrochemicals on human health and the environment has become increasingly important. With these concerns, skin sensitization in humans by industrial chemicals found in daily household products as well as agrochemicals used for protecting crops from pests, weeds, and pathogens has become an essential endpoint that is required to be evaluated for registration worldwide. Skin sensitization is an allergic reaction,[1] that occurs in response to skin contact with a single or a mixture of substances, and repeated contact with a sensitizing substance can cause localized inflammation in the form of allergic contact dermatitis. Skin sensitization has increasingly become a disease of great social interest, since it was accompanied by "allergic contact dermatitis" caused by products used by general consumers as well as those used in workplaces. Thus, detailed evaluation of skin sensitization by chemicals is of utmost importance.

Traditionally, skin sensitization is evaluated in guinea pigs and mice. However, recently, alternative methods have been developed with no experimental animals due to animal welfare concerns and are being utilized for safety control of agrochemicals, pest control chemicals, and industrial chemicals.

In our company, to develop chemical substances that can be handled safely without concerns for human health, we always update the technical knowledge in response to the global regulatory trends and evaluate the product safety for skin sensitization using the latest study methods. We herein provide an overview of the evaluation of skin sensitization to introduce global regulatory trends and new evaluation methods, present our company's approach in response to these trends and new methods, and offer our perspectives for the future.

Evaluation of skin sensitization

1. Principles of evaluation of skin sensitization

(1) Allergy and skin sensitization

The immune system is designed to protect the human body from pathogens and viruses as well as harmful substances. However, the immune system may become hypersensitive or respond inappropriately against harmless substances such as pollen and food, which is referred to as allergy. The 2011 report by the Ministry of Health, Labour, and Welfare describes allergy as "a national affliction, affecting approximately half of the population", and in 2014, Basic Act on Allergic Diseases Measure was established. These reflect the recent dramatic rise in allergic diseases, which has become a major social issue.[2]

Table 1 Classification of allergic reactions

Reaction type	Immune mechanism	Example
I (immediate)	IgE-basophils and/or mast cells	Acute asthma Acute allergic rhinitis
II (cytotoxic)	IgG and/or IgM antigens in the cells membrane	Hemolytic anemia
III (immune complexes)	IgM and/or IgG complexes-soluble antigens	Serum sickness
IV (late)	T cells	Tuberculin reaction Contact dermatitis

Allergies can be broadly classified into four types (**Table 1**).[3] In contrast to types I–III, which involve antibodies, skin sensitization is a type IV allergy (delayed type allergy) which involves immune cells such as T lymphocytes; the inflammatory response occurs following contact with the causative chemical substance, i.e., the allergen, with the reaction peaking at 24 to 48 hours after exposure. Type IV allergy is clinically referred to as allergic contact dermatitis, which accounts for approximately 60% of all occupational skin diseases.[4] Substances most frequently reported as causes of occupational allergic contact dermatitis include metals, such as nickel, cobalt, and chrome; epoxy and acrylic resins; rubber products, and *p*-phenylenediamine used in hair dyes.

Skin sensitization is not a life-threatening disease. However, in occupational allergies, the affected individual may have to change to a different work role or leave the workplace to avoid aggravation, pain, or discomfort. Meanwhile, with consumer products, a large number of individuals may be affected simultaneously. Thus, skin sensitization is not only a workplace issue but also may have a massive impact on social life.

(2) Mechanism of skin sensitization

It is known that two phases are involved in onset of allergy; the "induction" phase to acquire sensitivity to the allergen, and the "challenge" phase where allergic symptoms occur. In the case of skin sensitization, T lymphocytes which target a specific chemical substance proliferate in a body as the chemical substance repeatedly penetrates through the skin into the body. Once T lymphocytes are disseminated throughout the body and acquire "induction," they begin to release various cytokines, eliciting an inflammatory response, i.e., allergic contact dermatitis, at the area of contact with the chemical substance ("challenge").

In consideration of the mechanism of skin sensitization, it is critical to understand the adverse outcome pathway (AOP) (**Fig. 1**). AOP describes a pathway of adverse events elicited by a chemical substance by linking key events (KEs) at each different levels; at molecular, cellular, tissue, and individual levels, that lead the adverse events. The AOP of skin sensitization was published in the Organization for Economic Co-operation and Development (OECD) guidance document in 2012.[5]

The "induction" phase in skin sensitization is acquired through four KEs based on the AOP (**Fig. 2**). In KE1, the chemical substance penetrates into the skin and binds to proteins in the epidermis. Normal skin performs a barrier function by preventing the entry of chemical substances with a molecular weight of 1000 or greater. Thus, majority of the allergens that exhibit skin sensitization are low-molecular-weight chemicals. By binding to skin proteins, the low-molecular-weight chemical becomes a complete allergen that can exert antigenicity. In KE2, keratinocytes become activated.

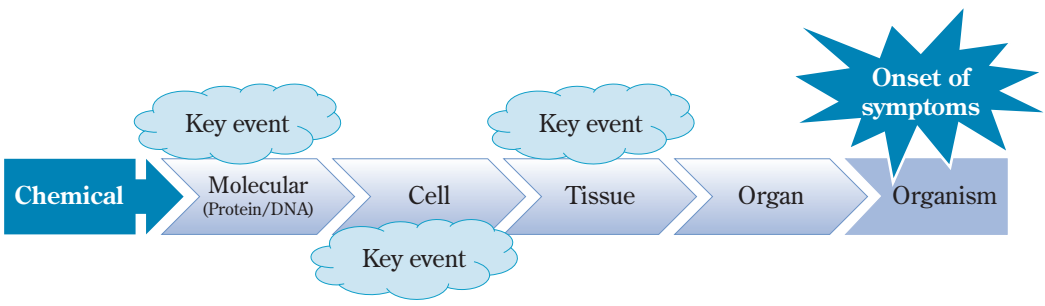


Fig. 1 A concept of Adverse Outcome Pathway (AOP)

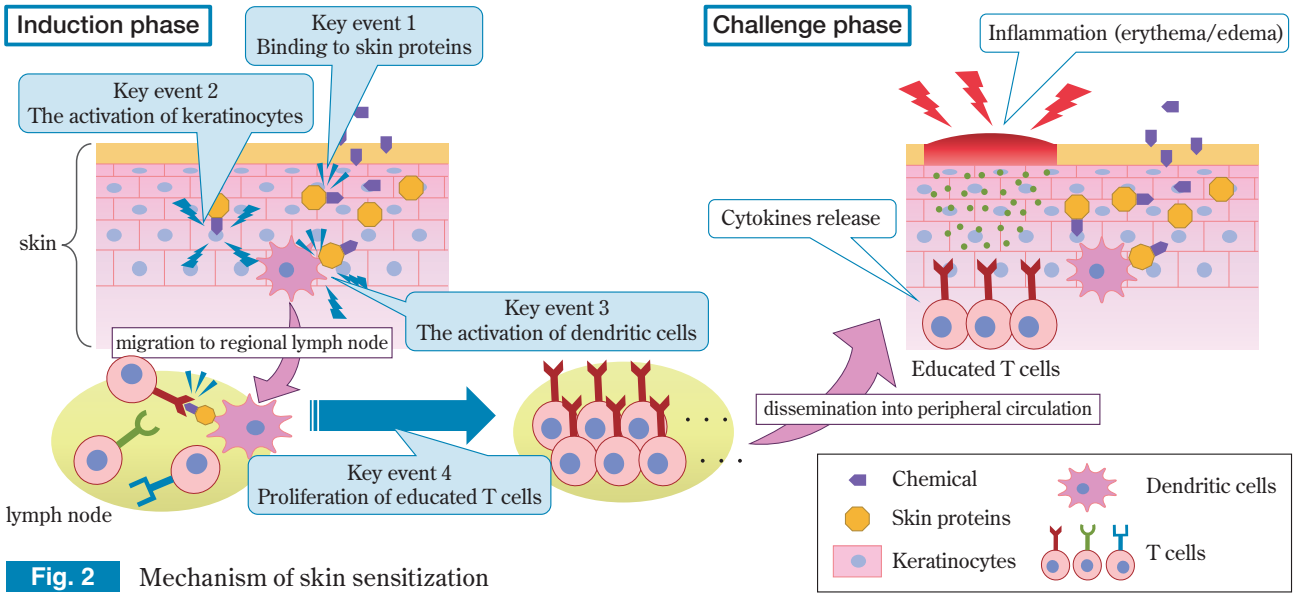


Fig. 2 Mechanism of skin sensitization

When a chemical substance bound to protein penetrates through the skin, keratinocytes, which make up approximately 90% of the epidermal layer, become activated, elicit an inflammatory response to protect cells from the chemical substance, and induce dendritic cells (DC) to respond to the inflammatory reaction. In KE3, DCs become activated. The complete allergens are captured by DCs in the epidermal or the dermal layer and are migrated to regional lymph nodes along with the activated DCs. In KE4, T lymphocytes become activated and proliferate. The immature T lymphocytes in the lymph node become activated by recognizing the allergen presented by the DCs and start to differentiate, mature, and proliferate into educated T cells. When the T lymphocytes become educated T cells and start disseminating into peripheral circulation throughout the body, "induction" is acquired.

(3) Representative skin sensitization tests using animals

There is a long history of test development to evaluate skin sensitization to chemical substances, and a large number of methods using humans and animals have been reported since the 1940s.

Well known skin sensitization tests using animals included in the OECD test guideline (TG) are the guinea pig maximization test (GPMT) and the Buehler test in guinea pigs, which utilize skin reaction as the endpoint (TG406)[6], and the local lymph node assay (LLNA), which utilizes lymph node proliferation in mice as the endpoint (TG429).[7] Below is the summary of these three tests.

[1] Guinea pig maximization test (GPMT)

The GPMT has been widely used since its development by Magnusson and Kligman in 1969.[8] In this test in a highly sensitive method, the test substance is injected intradermally with Freund's complete adjuvant (FCA) as an immune enhancer. If the test substances are non-irritating, an irritant reaction is provoked at the test site by pretreatment with sodium lauryl sulfate.

For the first induction, FCA and the test substance are intradermally injected into the shoulder region of the guinea pig. One week later, the test substance is topically applied by occlusive dressing for 48 hours for the second induction. Two weeks after the second induction, the test substance is applied and occluded on the flank of the animal for 24 hours for challenge. If the positive skin reactions such as erythema and/or swelling at the application site are observed at 24 and 48 hours after the challenge, it is concluded that the test substance is a skin sensitizer.

[2] Guinea pig/Buehler test

The Buehler test was developed by Buehler in 1965.[9] The test substance is applied topically from induction through challenge. This test does not use FCA, and it is considered to more closely reflect the realistic potential for human exposure. Thus, depending on the result of the GPMT using FCA, this test may be particularly valuable to assess the risk of skin sensitization with practical use of chemicals.

For induction, the test substance is applied and occluded on the flank of the guinea pig for six hours once a week for three weeks. Two weeks after the final

induction, the test substance is applied and occluded on the opposite flank of the induction side for six hours for challenge. The methods for observation and assessment are the same as those used for the GPMT.

[3] Mouse/Local Lymph Node Assay (LLNA)

The LLNA, developed by Kimber *et al*. in 1986,[10] evaluates the proliferative response of T lymphocytes during induction (KE4) via the uptake of radioactively-labeled tritiated thymidine into the DNA of T lymphocytes.

Test substance is applied topically to the dorsum of each ear of the mouse once a day for three consecutive days, and tritiated thymidine is injected via the tail vein three days after the final application; the lymph nodes from each ear are then excised. The ratio of incorporated radioactivity between the treatment group that receives the test substance and the control group that receives vehicle alone is calculated to determine the stimulation index (SI). A dose-response increase in radioactivity, with an SI of 3 or higher, is considered positive. Moreover, concentration of the test substance that elicits an SI value of 3 (i.e., EC3) can be used as a quantitative endpoint for prediction of skin sensitization potency (**Fig. 3**).

The LLNA allows quantitative evaluation in contrast to the tests using guinea pigs. Moreover, given that the LLNA uses fewer animals and reduces pain in animals as FCA is not used, this test provides advantages with regard to animal welfare. Furthermore, as it enables evaluation of coloring substances that hinder the evaluation of the skin reaction, the LLNA is recommended globally. However, the LLNA can show false negative for certain metals (skin sensitizers in humans) and false positive for irritating substances (non-sensitizers in humans). Thus, the LLNA is also known to have a limitation.

In the LLNA, radioactive substances are used to measure lymphocyte proliferation and therefore the assay has limited use in regions where the acquisition, use, or disposal of radioactivity is problematic. Thus, improved methods that do not use radioactive substances but utilize adenosine triphosphate (ATP) and 5-bromo-2'-deoxyuridine (BrdU) have been included in the 2010 OECD TG as TG442A (LLNA-DA)[11] and TG442B (LLNA-BrdU-ELISA or FCM).[12] We participated in the validation study for LLNA-DA and contributed to the creation of the guideline.

2. Recent trends in evaluation for skin sensitization

(1) Registration requirements of each region (Japan, The United States of America, The European Union)

Since there are slight differences between regions regarding skin sensitization tests included in the registration requirements for agrochemicals, pest control chemicals, and industrial chemical products, we provide a summary of the registration requirements for Japan,

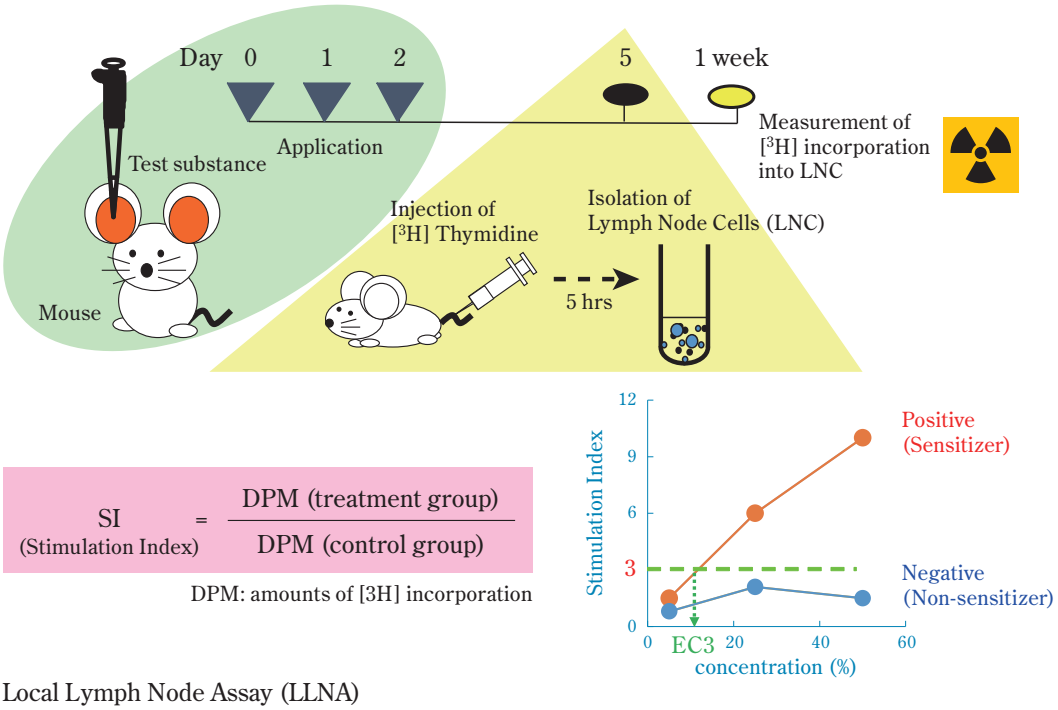


Fig. 3 Local Lymph Node Assay (LLNA)

Table 2 Skin sensitization test acceptability for registration of chemicals in Japan, US and EU

Region	Chemical class	Regulating authority	Regulation Act	Acceptability		
				GPMT/Buehler	LLNA	Non-animal test method
Japan	Agricultural chemicals	MAFF	Agricultural Chemicals Control Act	○	○	○
	Insecticides for infectious disease control	MHLW	PMD Act	○	○ (quasi drug only)	○ (quasi drug only)
	Industrial chemicals	MHLW METI MOE	JCSCA ISHA	← Not necessary →		
US	Pesticides	US EPA (OPP)	FIFRA	○	◎	○
	Industrial chemicals	US EPA (OPPT)	TSCA	← Not necessary →		
EU	Plant protection products	EFSA	PPPR (1107/2009)	○	◎	—
	Biocidal products	ECHA	BPR (528/2012)	○	◎	—
	Industrial chemicals	ECHA	REACH (1907/2006)	○	○	◎

○: acceptable; ◎: high priority method; —: not listed; MAFF: Ministry of Agriculture, Forestry and Fisheries; MHLW: Ministry of Health, Labour and Welfare; PMD Act: Pharmaceutical and Medical Device Act; METI: Ministry of Economy, Trade and Industry; MOE: Ministry of the Environment; JCSCA: Japanese Chemical Substances Control Act; ISHA: Industrial Safety and Health Act; OPP: Office of Pesticide Programs; OPPT: Office of Pollution Prevention and Toxics; PPPR: Plant Protection Products Regulation; BPR: Biocidal Products Regulation; REACH: Registration, Evaluation, Authorization and Restriction of Chemicals

the United States of America (USA), and the European Union (EU) in **Table 2**.

In all regions, there is a trend of moving away from animals models as primary test approaches toward alternative methods that do not use animals.

(i) Japan

The authorities responsible for the regulations differ depending on the purpose of use. Agrochemicals used for crops are governed by the Agricultural Chemicals Control Act (the Ministry of Agriculture, Forestry and Fisheries), pest control chemicals other than agrochemicals such as insecticides are governed by the Pharmaceutical and Medical Device Act*1 (Ministry of Health, Labour and Welfare), and industrial chemicals are governed by Japanese Chemical Substances Control Act (JCSCA)*2 (Ministry of Economy, Trade and Industry/Ministry of Health, Labour and Welfare/Ministry of the Environment) and Industrial Safety and Health Act (ISHA) (Ministry of Health, Labour and Welfare).

Agrochemicals comprise the active substance, which is the active ingredient, and the plant protection product, which is the actual product. Previously, for evaluating skin sensitization, GPMT was recommended for the active substance, and the Buehler test was recommended for the plant protection product.[13] However, with the revision of "On documents to be submitted at application for registration of agrochemicals," the use of alternative methods to animal testing has become acceptable for the active substance in agrochemicals, as outlined in the OECD TG.[14]

For pest control chemicals that are designated as pharmaceuticals, tests using FCA such as the GPMT are recommended in the "Guideline for Toxicity Studies of Drugs."[15] Depending on the dosage form and the active ingredient, some pest control chemicals are designated as quasi drugs. Traditionally, quasi drugs have been evaluated by the methods using guinea pigs or by the LLNA, similar to pharmaceuticals.[16] However, in 2018, the "Guidance on the testing strategy combining multiple alternative methods to animal testing for skin sensitization in the safety assessment of quasi-drug and cosmetic products" was created, which allowed alternative methods to animal testing for the

*1 Pharmaceutical and Medical Device Act: Act on Securing Quality, Efficacy and Safety of Pharmaceuticals, Medical Devices, Regenerative and Cellular Therapy Products, Gene Therapy Products, and Cosmetics

*2 Japanese Chemical Substances Control Act: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances

assessment of these substances (for details, see **2.** (2) (v)).[17]

For industrial chemicals, neither JCSCA nor the ISHA requires skin sensitization as an essential test for the notification of new substances and does not specify the test method.

(ii) USA

In the USA, regardless of whether the product is to be used on crops or pests, pesticides such as fungicides, herbicides, insecticides, and rodenticides are under the jurisdiction of the United States Environmental Protection Agency (US EPA) and regulated in accordance with the Federal Insecticide, Fungicide, and Rodenticide Act. For registration of pesticides, the evaluation of skin sensitization is normally required for both the active substance and the plant protection product. Although the US EPA guideline lists the LLNA, GPMT, and Buehler test as options, it recommends the LLNA as the first choice for testing.[18] Meanwhile, alternative methods to animal testing are considered, and an interim policy that permits submission of data combining alternative test methods for single substances such as the active substance or the inactive substance was published in April, 2018.[19] Albeit an interim policy, the US EPA Office of Pesticide Programs, which oversees registrations, has stated that if the tests are performed in line with this policy, they will accept the data promptly, indicating that they are perceptive to the promotion of alternative methods to animal testing.

Industrial chemicals are also under the jurisdiction of the US EPA and regulated in accordance with the Toxic Substances Control Act (TSCA). For the registration of industrial chemicals, data submission is not essential at application. However, as a result of review, if the agency determines that information is required to perform risk assessment, data submission will be requested.

(iii) EU

Agrochemicals are designated as plant protection products (PPP), which are under the jurisdiction of the Europe Food Safety Agency (EFSA), and regulated in accordance with The Plant Protection Products Regulation (1107/2009). While application for the active substance is submitted collectively to the European Commission (EC), application for the plant protection product must be submitted to the authority of one of three regions (north, central, or south) of the EU where the product is to be marketed. The data requirements are specified in the Commission Regulation 283/2013 (active substance) and 284/2013 (plant protection product), which are under the regulation 1107/2009. In both commission regulations, the LLNA is the first choice; the GPMT is permitted only if the LLNA cannot be performed, after presenting justification.[20),21] Meanwhile, pest control chemicals are designated as biocidal products, which are under the jurisdiction of the European Chemicals Agency (ECHA), and regulated in accordance with the Biocidal Products Regulation (528/2012).[22] The regulation stipulates to first evaluate based on any usable data, and if this evaluation is not possible, tests using animals (*in vivo* tests) are conducted. However, as with PPP, the LLNA is the first choice, and other test methods must be justified.

Industrial chemicals are under the jurisdiction of the ECHA and regulated in accordance with REACH regulations. At REACH, chemical substances that are manufactured or imported in quantities over a ton annually require registration, and the required toxicity tests differ depending on the quantity manufactured/imported: 1–10, 10–100, 100–1000, and over 1000 tons. Skin sensitization must be evaluated for all categories of tonnage, and *in vivo* tests can be performed only when evaluation by alternative methods to animal testing is not available. For *in vivo* testing, as with PPP and Biocidal Products, the LLNA is the first choice, and any other test method must be justified.[23]

(2) Development status of alternatives methods to animal testing

The "Seventh Amendment of EU Cosmetics Directive (2003/15/EC)," which was distributed across Europe in 2003, gradually abolished animal tests in cosmetics development and, since 2013, prohibited the sales of cosmetics including the ingredients that were tested on animals within the EU. The amendment prompted acceleration of the development of alternative methods to animal testing not only in the cosmetic industry but various industries worldwide.[24] This trend, as shown above, had a massive impact on regulations in various countries.

Among the various toxicities, skin sensitization in particular has a fairly well-studied mechanism of action, and a number of test methods not using animals are currently in development for the evaluation of KE1, KE2, and KE3 of the AOP, summarized above. Below,

we introduce the main evaluation methods for each of the KEs that are already included in the OECD TG and summarize recent trends, such as *in silico* techniques used for toxicity prediction and evaluation methods combining these techniques.

(i) KE1: covalent protein binding

Low-molecular-weight compounds become complete antigens by covalently binding with amino acid residues (particularly cysteine and lysine) of proteins. The direct peptide reactivity assay (DPRA), which was developed to evaluate the bond between chemical substances and proteins, was included in the OECD TG442C as a test method for KE1 in 2015. Recently, FUJIFILM Corporation has devised an improved version of DPRA, the amino acid derivative reactivity assay (ADRA), and we participated in the validation study for the ADRA. The ADRA was added to the OECD TG442C in June 2019.[25]

The DPRA utilizes two synthetic peptides containing cysteine or lysine instead of endogenous proteins. After two synthetic peptides are mixed with the test substance and incubated for 24 hours, the unbound peptide is measured by high-performance liquid chromatography (HPLC) to evaluate the test substance binding potency with proteins, which allows qualitative as well as quantitative evaluation. As assessment criteria, mean rates of ≤6.38% and >6.38% in the reduction of cysteine or lysine peptides are considered as negative and positive, respectively. Moreover, if the rate of reduction is >6.38% but <22.52%, the test substance is considered to have low reactivity. If the reduction rate is >22.52% but <42.47%, the test substance is considered to be moderately reactive. Finally, a reduction rate of >42.47% is considered to indicate a highly reactive test substance. Thus, the DPRA can be classified into three levels.[26),27)]

The ADRA utilizes the DPRA principles but includes N-acetyl cysteine (NAC) or N-acetyl lysine (NAL) instead of synthetic peptides as the supplement forms of cysteine or lysine with naphthalene; these compounds exhibit strong ultraviolet absorption and increase detection sensitivity. In the ADRA, the test substance is mixed with NAC or NAL at 25 °C for 24 hours, and the unreacted NAC or NAL is measured by HPLC. If the mean rate of reduction in NAC and NAL is 4.9% or greater, the result is considered positive. Since the detection sensitivity is higher than that of the DPRA, the reaction can be performed at lower concentrations, which provides advantages such as a reduction in the amount of test substance and an expansion of applicability domain to less soluble substances (**Fig. 4**).[28)]

(ii) KE2: activation of keratinocytes

When penetrating the skin, chemical substances stimulate keratinocytes and activate a specific signal transduction pathway. Before "induction" is acquired, many of the skin-sensitizers induce genes that are controlled by the antioxidant response element (ARE) in keratinocytes; particularly involving to activating Nrf2-Keap1-ARE pathway. Nrf2 is bound to its repressor, Keap1, and in an inactive state normally. However, when an electrophilic substance, such as a skin-sensitizer, binds with the cysteine residue of Keap1, Nrf2 dissociates from Keap1 and translocates to the nucleus to bind with the ARE in the DNA. Consequently, gene group downstream is induced to express, and initiate the cellular protection program against damage from

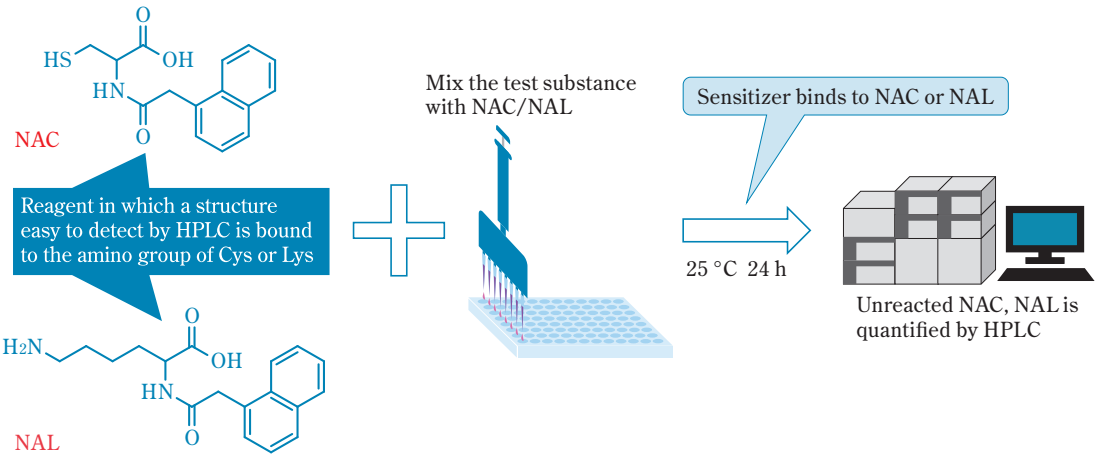


Fig. 4 Method of ADRA
Key event 1 can be addressed using ADRA. Key event 1 is the covalent binding of electrophilic substances to nucleophilic centers in skin proteins.

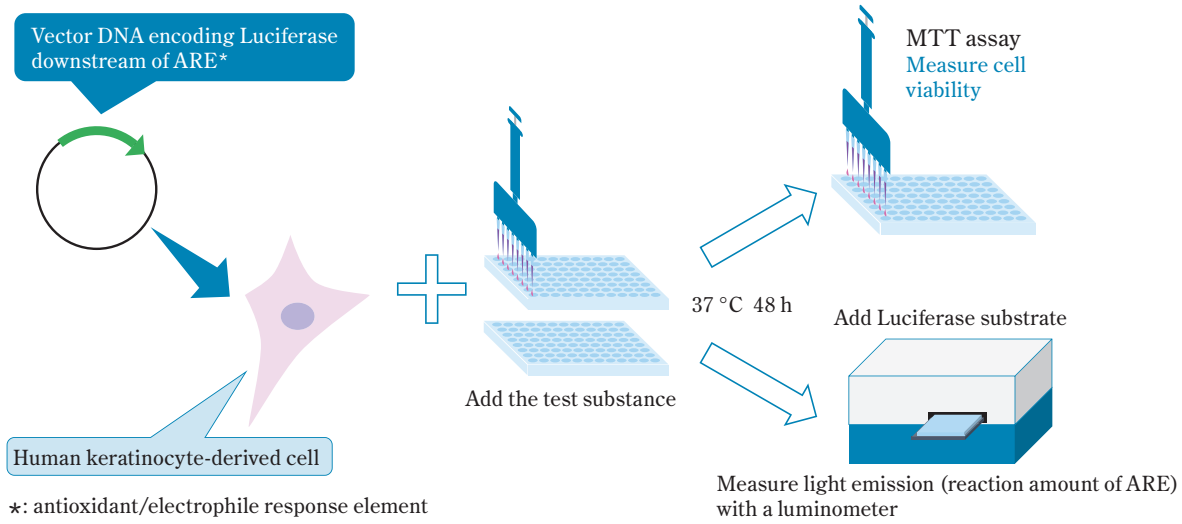


Fig. 5 Method of KeratinoSens
Key event 2 can be addressed using KeratinoSens. Key event 2 is gene expression changes or inflammatory responses associated with specific cell signaling pathways such as ARE-dependent pathways in the keratinocytes.

the chemical substance. KeratinoSens and LuSens assays were listed in the OECD TG442D in 2015 and 2018, respectively.[29] Both assays use cell lines derived from human keratinocytes, and evaluation is based on the amount of fluorescence generated by luciferase located downstream of ARE. When cell viability is at least 70% and luciferase expression relative to control is 1.5-fold or more, the result is considered as positive (**Fig. 5**).

(iii) KE3: DC activation

DCs become activated directly through stimulation by the skin-sensitizing substance or indirectly via cytokines and interleukins (ILs) released from keratinocytes. The activated DCs produce various signaling molecules including cytokines and chemokines; they also engulf the antigen, recognize the structure specific to the antigen, and present the antigen on the major histocompatibility complex (MHC) on the cell surface to activate antigen-specific T lymphocytes. As just described, the activated DCs undergo various structural and functional changes, such as increases in the expression of the cell surface molecules CD86 and CD54, which are costimulatory molecules necessary for antigen presentation, and production of signaling molecules including IL-8 to communicate with the surrounding cells.[30),31)] As representative methods for detecting these changes, the human cell line activation test (h-CLAT), U937 cell line activation test (U-SENS), and IL-8 reporter gene assay (IL-8 Luc Assay) were successively added to the OECD TG442E between 2016 and 2018.[32)] We took part in the validation studies for the IL-8 Luc Assay and contributed to the creation of its guideline.

The h-CLAT utilizes THP-1 cells, cultured human monocytic cells, instead of DCs, and measures the expression levels of CD86 and CD54 as indicators. The levels of fluorescently labeled CD86 and CD54 are measured by flow cytometry at 24 hours after the test substance has been added, and the relative fluorescence intensity (RFI) of the exposed cells relative to those that were not treated with the test substance is determined. If the cell viability is at least 50% and the RFIs of CD86 and CD54 are at least 150% and 200%, respectively, in two replicates, the test substance is considered as positive. This method is also considered for quantitative evaluation by using minimum induction threshold (MIT). Substances with an MIT of less than 10 μg/mL are considered as strong sensitizers and those with an MIT between 10 and 5000 μg/mL are considered as weak sensitizers.[33),34)]

The U-SENS, as with h-CLAT, utilizes U937 cells, cultured human histiocytic lymphoma cells, instead of DCs and measures the expression level of CD86 as the indicator.[35)] The rate of CD86-positive cells is compared between the treated and the untreated cells at 45 hours after the addition of the test substance using the stimulation index (SI). If the cell viability is at least 70% and the SI is at least 150% in two replicates, the test substance is considered positive.

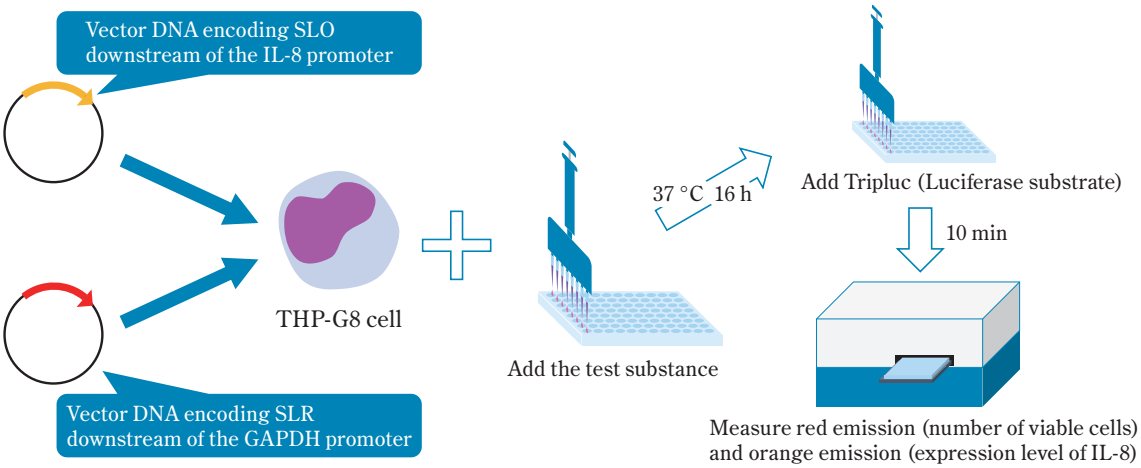


Fig. 6 Method of IL-8 Luc Assay
Key event 3 can be addressed using IL-8 Luc Assay. Key event 3 is the activation of dendritic cells, typically assessed by expression of specific cell surface markers, chemokines and cytokines.

The IL-8 Luc assay, unlike the two methods summarized above, uses the expression levels of IL-8 as the indicator.[36] The assay utilizes THP-G8 cells, which are THP-1 cells transfected with DNA vectors harboring two luciferase genes, stable luciferase orange (SLO) and stable luciferase red (SLR), downstream of the IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promotor sequences. GAPDH is a housekeeping gene that is constantly expressed and used as an indicator to calculate changes in relative expression levels of target genes. The SLO and SLR substrates are added 16 hours after the application of the test substance, and fluorescence intensity is measured using a luminometer. The result is considered as positive if the IL-8/GAPDH ratio is 1.4-fold or higher in the test substance group compared with the solvent control group. The final result is considered positive in two replicates; a negative result replicated three times indicates a negative final result (**Fig. 6**).

(iv) *In silico* method for skin sensitization prediction

Predicting toxicity based on the physicochemical properties of a chemical substance or available information on similar compounds has been utilized mainly for determining genotoxicity. However, recent advances in computer technology and demands for minimizing animal tests led to the widening of the scope of toxicity prediction techniques to include prediction of local effects such as irritation and skin sensitization. Physicochemical properties that are utilized for this purpose are not limited to basic information such as molecular weight and octanol/water partition coefficient and include various descriptors such as the state of electrons based on the substance structure; the toxicity prediction of novel substances is performed by analyzing large volume of information in databases.

The tools for toxicity prediction can be categorized into statistical- and expert rule-based tools (**Table 3**). The statistical-based tools utilize mathematical models derived from the dataset of the reference compound and are based on the quantitative structure activity relationship (QSAR), which analyses the relationship between the chemical structure and its activity, in this case its toxicity, based on the partial structure, physical

Table 3 Summary of *in silico* tools

In silico Model	Builders	Characterization		Freely available software
		Statistical-based	Expert rule-based	
OECD QSAR Toolbox	OECD	✓	✓	✓
Toxtree	JRC		✓	✓
VEGA	Caesar project	✓		✓
CASE Ultra	MultiCASE	✓		
Derek Nexus	Lhasa		✓	
TIMES-SS	LMC	✓	✓	
TOPKAT	Accelrys	✓		

properties, and polarity of electrons. The main statistical-based models are VEGA, CASE Ultra, and TOPKAT. Conversely, the expert rule-based tools use structural rules and alerts and are derived from known information such as literatures and expert data analysis. Structural alert is defined as a partial structure involved in the toxicity effect or mechanism. The representative expert rule-based models are Toxtree and Derek Nexus.

Some models are free, such as OECD QSAR Toolbox, Toxtree, and VEGA, whereas others are for profit, such as CASE Ultra, Derek Nexus, TIMES-SS, and TOPKAT. The for-profit models have a greater variety of functions compared with the free models, such as the ability to predict skin sensitization by taking metabolites in skin into consideration.

(v) Combining multiple alternative methods to animal testing

Skin sensitization is a complex immune response; therefore, alternative methods that evaluate a single KE is considered inadequate for evaluating the capability of a test substance for skin sensitization. Therefore, focus has been increasingly placed on comprehensive assessment approaches that combine not only *in chemico* or *in vitro* methods, but also *in silico* methods.

In recent years, the defined approach (DA) has been drawing increasing attention as a method that combines *in chemico* and *in vitro* test results with *in silico* prediction data. The DA combines data from studies included in OECD TGs and evaluates these data in accordance with clearly defined rules via the data interpretation procedure, which takes into account predictability and limitations in scope. As the evaluation does not involve any judgment by experts, it is superior in objectivity. In 2016, the OECD has created a guidance document, in which it introduced 12 case studies[37] (**Table 4**).

In 2018, the US EPA became the first regulatory authority to present the following two DA models for qualitative evaluation of skin sensitization (positive/negative assessment)[19] (**Fig. 7** and **8**).

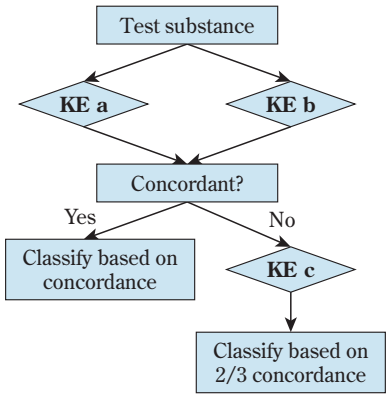


Fig. 7 Defined Approach: "2 out of 3 approach" (US EPA)

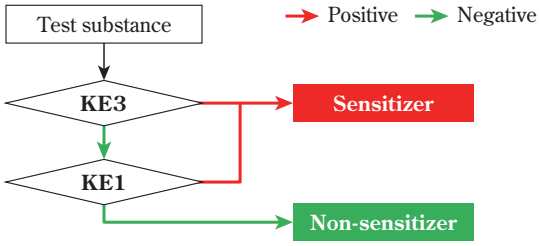


Fig. 8 Defined Approach: "Sequential Testing Strategy" (US EPA)

Table 4 Case study of defined approach

	Case study	Proposer	Purpose
1	An AOP-based "2 out of 3" integrated testing strategy approach to skin hazard identification	BASF	Hazard identification
2	Sequential Testing Strategy for hazard identification of skin sensitisers	RIVM	
3	A non-testing Pipeline approach for skin sensitisation	G. Patlewicz	
4	Stacking meta-model for skin sensitization hazard identification	L'Oreal	
5	Integrated decision strategy for skin sensitization hazard	ICCVAM	
6	Consensus of classification trees for skin sensitization hazard prediction	EC-JRC	
7	Sensitizer potency prediction based on Key event 1+2: Combination of kinetic peptide reactivity data and KeratinoSens data	Givaudan	Potency prediction
8	The artificial neural network model for predicting LLNA EC3	Shiseido	
9	Baysian Network DIP for hazard and potency identification of skin sensitizers	P&G	
10	STS for sensitizing potency classification based on *in chemico* and *in vitro* data	Kao	
11	ITS for sensitizing potency classification based on *in silico*, *in chemico* and *in vitro* data	Kao	
12	DIP for skin allergy risk assessment (SARA)	Unilever	

(a) Based on the "2 out of 3 approach" proposed by BASF, in which two arbitrary tests are performed for two KEs among KE1, KE2, and KE3, the approach concludes when the results of the two tests are concordant. However, if the results of the two tests are different, a test is performed for the untested KE. When the results of two tests are concordant, the result is adopted as the final assessment (**Fig. 7**).

(b) Based on the "sequential testing strategy (STS)," proposed by Kao Corporation, the test for KE3 is performed first, and the final result is considered as positive if the result of the test is positive. If the result of the test is negative, the test for KE1 is performed. If the result of the test for KE1 is positive, the final result is considered as positive; however, if the result of the test is negative, the final result is considered as negative (**Fig. 8**). As with (a), the model does not specify the type of test, provided it corresponds to the respective KE.

In Japan, the "bottom-up 3 out of 3" method was presented as a means of positive/negative assessment for quasi drugs and cosmetic products in 2018 by the Ministry of Health, Labour and Welfare. If the results of the DPRA, KeratinoSens assay, and h-CLAT for assessing KE1, KE2, and KE3, respectively, are negative for all, the test substance is negative[17] (**Fig. 9**).

OECD is creating a guideline for the DA, and three evaluation methods have been proposed in the draft. The "2 out of 3 approach" is considered for qualitative evaluation, The STS and the integrated testing strategy (ITS) proposed by Kao Corporation are considered for quantitative evaluation of the potency of skin sensitization. Test substances are classified into strong sensitizers, weak sensitizers, and non-sensitizers based on the evaluation results.[38] In the STS, the designated tests that can be performed are the h-CLAT for KE3 and the DPRA for KE1. If the result is positive for h-CLAT and the MIT is less than 10 μg/mL, the substance is considered to be a strong sensitizer. If the MIT is more than 10 μg/mL, the substance is assessed as a weak sensitizer. If the h-CLAT result is negative, the DPRA is performed; if the result is positive, the substance is assessed as a weak sensitizer, whereas a negative result indicates that the substance is a non-sensitizer (**Fig. 10**). In the ITS, in addition to the DPRA for KE1 and the h-CLAT for KE3, the OECD QSAR Toolbox is recommended as the *in silico* tool. The results of the respective tests are scored between 0 and 3, and a total score of 7 indicates that the substance is a strong sensitizer. A score between 2 and 6 is deemed to indicate a weak sensitizer, whereas a score of 0 or 1 is considered to define a non-sensitizer (**Fig. 11**). Moreover, the consistency of the integrated testing strategy with the GHS classification[1] is currently being examined.

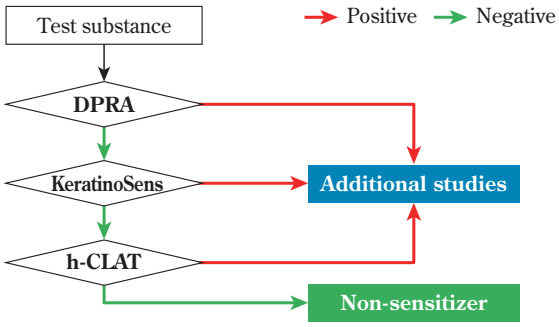
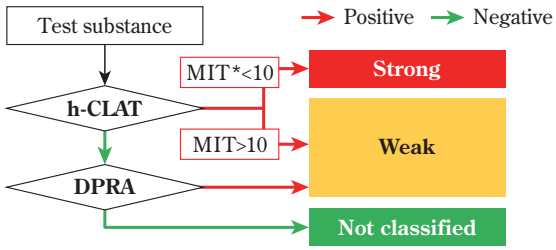


Fig. 9 Defined Approach: "Bottom up 3 out of 3 approach" (JP MHLW)



*: Minimum induction threshold (μg/mL)

Fig. 10 Defined Approach: "KE 3/1 Sequential Testing Strategy" (OECD)

Score	h-CLAT MIT (μg/mL)	DPRA depletion (%)	OECD QSAR toolbox
3	≤10	≥42.47	—
2	>10, ≤150	≥22.62, <42.47	—
1	>150, ≤5000	≥6.376, <22.62	Sensitiser
0	Not calculated	<6.376	Non sensitiser

Total battery score

7: Strong	2 ~ 6: Weak	0, 1: Not classified

Fig. 11 Defined Approach: "KE 3/1 Integrated Testing Strategy" (OECD)

Our company's action for using alternative methods to animal testing for skin sensitization

In our company, a "study on binding ability of glutathione, a peptide composed of glutamate, cysteine, and glycine" has been conducted to detect skin sensitization as a preliminary evaluation target at mainly the substances handled by our company in the early phase of development since 2005.[39),40)] Glutathione binding study is in principle same category as KE1; however, unlike the DPRA and ADRA in which the rate of substrate reduction is assessed, the compound formed through binding with glutathione is detected by liquid chromatography/mass spectrometry. On the other hand, there are some difficulties (i.e. detection power when using a single method and the judgement of potency of skin sensitization) as with other methods for KE1.

From the perspective of worker safety, in early stages of development during research-level synthesis, workers handle many chemicals, including ingredients and intermediates, that have not yet been evaluated for skin sensitization. Thus, there is an urgent need for the development of simple and rapid techniques that can evaluate skin sensitization of chemicals without the need for animal testing. Moreover, techniques which can not only assess whether a substance is a skin sensitizer but also distinguish strongly sensitizing substances that can cause allergic contact dermatitis in humans at low concentrations can be used for other purposes. For example, such techniques can aid in consideration of alternative substances or preventive measures such as the use of protective gear for the prevention of allergic contact dermatitis before they occur.

Currently, in our company, we utilize test methods that we introduced through participation in validation studies, and we are investigating simple and accurate evaluation methods by the appropriate combination of Derek Nexus and the OECD QSAR Toolbox as *in silico* tools with the DPRA and ADRA for KE1, the LuSens assay for KE2, and the IL-8 Luc assay for KE3.

Our future vision

Skin sensitization is one of the few toxicities for which the AOP is well elucidated. With increasing desire to reduce animal testing in global society, new alternative methods to animal testing are being developed successively. Recently, the genomic allergen rapid detection (GARD) assay, which analyses changes in 200 genes through machine learning, has been attracting attention. In addition, validation studies of a number of tests are under way, with the aim to be included in the OECD.[41)]

As mentioned above, although new alternative methods and the testing strategy combining multiple alternative methods are being actively developed, there are some tasks for the development of alternative methods to animal testing, such as the accuracy of prediction and the applicability domain of chemical substances that can be tested; further improvement is currently ongoing to tackle these challenges.

1. Prediction of the potency of skin sensitization

In evaluation of skin sensitization, in addition to the positive/negative categorization, the potency of skin sensitization is also an important information from the viewpoint of worker safety. As mentioned above, testing strategy that combines multiple tests, such as the STS and ITS proposed by the OECD, is capable of classifying the potency of sensitization, albeit only in two categorizations. There is a need for testing strategy that allow substance discrimination in multiple categorizations depending on their skin sensitization ability, which will enable a more accurate prediction of sensitization potency.

2. Solubility

In test systems using cultured cells in *in vitro* methods, one common issue is that the chemicals which are highly hydrophobic and poorly water-soluble are not applicable. For the evaluation of skin irritation, a test system using three-dimensional reconstructed human epidermis model that is suitable for the evaluation of poorly water-soluble substances is already included in the OECD TG (TG439).[42)] In this test, the compound is applied directly to the epidermis without dissolving in culture medium and evaluation is based on the cell viability. Evaluation methods, which apply this model to skin sensitization and monitor changes in the expression of target genes within the keratinocytes of the epidermal layer, are currently assessed with the intent to be included in OECD test guideline (EpiSensA, SENS-IS assay).[43),44)]

3. Pro- and pre-haptens

There are no suitable tests for evaluating "pro-haptens," chemicals that show reactivity only after skin penetration and being metabolized, or "pre-haptens," chemicals that show reactivity after autoxidation. Thus,

development of appropriate pretreatment methods or utilization of *in silico* techniques that include a predictive function for metabolites are needed.

Currently, there are still several tasks for completely replacing animal testing with non-animal testing for the evaluation of skin sensitization. Therefore, when we evaluate skin sensitization using alternative methods, there is a need to understand the characteristics and the applicability domain of each method to perform evaluations by combining multiple methods. In the future, we will continue to actively introduce new alternatives to animal testing; we also aim to develop alternative methods utilizing the characteristics of each of the *in silico*, *in chemico*, and *in vitro* tests to be able to evaluate skin sensitization potency. We believe these approaches will contribute to improvement in the safety of our products and workers as well as a reduction in animal testing.

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