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David Basketter & Gavin Maxwell

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IN VITRO APPROACHES TO THE IDENTIFICATION AND CHARACTERIZATION OF SKIN SENSITIZERS

David Basketter

St John's Institute of Dermatology, St Thomas' Hospital, London, UK

Gavin Maxwell

Safety and Environmental Assurance Centre, Unilever, Sharnbrook, Bedford, UK

Allergic contact dermatitis (ACD) is to a considerable extent a preventable disease. Limitation of ACD can be achieved by correct detection of skin sensitizers, characterization of potency, understanding of human skin exposure, and the application of adequate risk assessment and management strategies. A range of methods now exist that have been proven to be very accurate in terms of the predictive identification of chemicals that possess skin sensitizing properties. In addition, certain methods, notably the local lymph node assay (LLNA), also deliver valuable information of the relative potency of identified sensitizers. Great use can be made of this potency information in the application of quantitative risk assessments (although of course such assessments depend also on the availability of accurate data on human skin exposure). However, the challenge now to be faced is how to obtain the same quality of information on the potency of skin sensitizing chemicals using solely in vitro and in silico methods. With the forthcoming elimination of in vivo tests, the opportunities being exploited for in vitro test development focus on key elements of the sensitization process, such as peptide binding and dendritic cell activation. What has to then be addressed is how information from such in vitro assays is integrated, together with data on epidermal bioavailability, to deliver an assessment of the allergen potency.

Keywords: Hazard identification; *In vitro* alternatives; Risk assessment; Skin allergy; Skin sensitization

BACKGROUND

Allergic contact dermatitis (ACD) is a relatively common skin disease. Predictive tests that have the capacity to identify sensitizers before they are placed on the consumer/occupational market exist; these methods are highly successful. So, what is the problem? Firstly they use animals and, secondly, they provide an integrated response that permits the potency of potential contact allergens to be measured. Both of these aspects present a real challenge to *in vitro* toxicology. The reality is that the identification of hazards *in vitro* (the intrinsic properties of chemicals, in this case skin sensitization) is just the starting point of a much greater challenge, understanding relative skin sensitizing potency and then assessing the real risk of the induction

Address correspondence to Dr. David Basketter, St John's Institute of Dermatology, St Thomas' Hospital, London SE1 7EH, UK. Tel.: 01234782944; Fax: 01234782944; E-mail: david.basketter@ukonline.co.uk

of skin sensitization in the context of either a specific pattern of, or multiple patterns of, skin exposure.

MECHANISM OF THE INDUCTION OF SKIN SENSITIZATION

A skin sensitizer/contact allergen is a chemical substance that possesses the intrinsic ability to cause skin sensitization/contact allergy. Where there has been sufficient exposure to a skin sensitizer, an individual will become sensitized and thus have a contact allergy, which is not associated with clinical symptoms, but which can be detected by a 48 h diagnostic patch test with the sensitizing chemical. For an individual who has contact allergy, further skin exposure to a sufficient dose of the sensitizing chemical will lead to the elicitation of an eczematous reaction called ACD. Additionally, a sensitized individual may also experience ACD in response to structurally similar chemicals, a phenomenon called cross-reactivity. Skin sensitization is an intrinsic property of a chemical substance and is referred to as a hazard; the likelihood that this hazard will be expressed in humans is referred to as risk and is a function of hazard potency and exposure. The likelihood that a particular individual will become sensitized is also a function of individual susceptibility.

The immunobiology of skin sensitization has been investigated for many years and as a consequence is relatively well understood. A comprehensive review has just been published in the standard textbook of contact dermatitis (1). So, what follows here is a presentation of the mechanism in a manner that is most appropriate for a discussion of the identification and characterization of potentially skin sensitizing chemicals. However, first, the properties of chemicals that can behave as skin sensitizers must be mentioned.

Why is a chemical a skin sensitizer? The most important property that a chemical must possess in order to cause skin sensitization is the ability to modify skin protein, normally by covalently binding to it, such that the modified protein, or rather the part to which the chemical is bound, then stimulates the immune system. The sensitizing chemical, or at least the part of it that binds to skin protein, is usually referred to as the hapten. Consequently, the process is often referred to as hapten-protein binding. The reaction mechanisms associated with sensitization chemistry have been well reviewed (2,3). More complex is the issue of chemicals that are not themselves reactive, but require air oxidation (4,5) or metabolic processes in skin (6,7) to produce a reactive species: such chemicals are referred to as prohaptens. The likely metabolic processes involved have been intensively considered (8), but remain largely theoretical. Most recently, there has been consideration of not only how chemicals bind to protein, but to which protein and at what site(s) on the protein. Again, there is relatively limited knowledge, but what exists has been reviewed in a recent publication (9). Nevertheless, the extent of knowledge on the chemistry of skin sensitization has led to the development of tools to help in the predictive identification of new sensitizers, material that is discussed later in this article.

To induce contact allergy, however, a skin sensitizing chemical must do much more than simply react with skin proteins. Firstly, it must gain access to the viable epidermis, a factor likely to be facilitated by an existing eczema (10). The skin is composed of three layers (the epidermis, the dermis, and the deeper localized subcutis)

with the outermost layer of the epidermis being formed by a cornified layer of keratinocytes known as the stratum corneum. Within these layers several cell types are known to be involved in cutaneous immune responses and we will now review what role they are thought to play in chemical-induced skin sensitization.

The epidermal layer of skin is predominantly composed of keratinocytes that in addition to forming an effective barrier via the stratum corneum, are known to play a key role in the initiation of skin inflammatory and immune reactions (11). When exposed to physical or chemical stresses keratinocytes release a cocktail of soluble proinflammatory mediators [such as interleukin (IL) -1 α , tumor necrosis factor α (TNF- α), IL-8, and prostaglandin (PG) E₂], anti-inflammatory mediators [such as IL-10 and IL-1 receptor antagonist (Ra)] and growth factors [such as IL-6, IL-7, IL-15, granulocyte/macrophage colony-stimulating factor (GM-CSF)] (12– 19). Keratinocyte activation induces further mediator release by innate immune cells resident in the skin, such as Langerhans' cells (LC) in the epidermis and mast cells, dendritic cells (DC), and fibroblasts in the dermis (14).

LC are immature DCs that reside in the epidermis and display a unique dendritic morphology, which allows them to sample their environment and thereby act as "sentinels" of the adaptive immune response (20). LC activation induced by IL-1 α and/or TNF- α has been shown to result in the upregulation of IL-1 β and IL-18 expression (21,22) followed by antigen uptake and migration from the epidermis, via the afferent lymphatics to the draining lymph node (dLN) (23). During migration LCs undergo functional maturation in response to epidermal mediators and assume mature DC characteristics, such as upregulation of HLA-DR, CD40, CD54, CD80, (B7.1) and CD86 (B7.2) (24). Upon arrival at the dLN the LC, now a mature DC, is guided via its expression of the chemokine receptor CCR7 to T cells in the paracortex (25,26). Although DC: T cell interactions have been frequently described it is worth reviewing that early T cell activation is controlled by two signals that need to be successfully delivered by the DC to fully activate a naïve T cell. Signal 1 is the engagement of the T cell receptor (TCR) with an appropriate peptide-MHC complex, signal 2 is the co-stimulatory signal most simply characterized as the engagement of CD28 on the T cell with either CD80 or CD86 on DC (27). If both of these signals are delivered successfully the T cell will become activated and enter into a proliferative cycle. Of the effector T cell populations that have the potential to differentiate following this proliferative event, it is CD8⁺ cytotoxic T cells that have been characterized as having a dominant role in the elicitation of ACD (1). However, the cellular regulation of these CD8⁺ T cell responses has yet to be fully resolved, although a recent publication by Vocanson and colleagues strongly suggests a role for CD4⁺ T cells (28).

Cell proliferation that is stimulated in the lymph node is the pivotal step for the induction of skin sensitization and it is this endpoint that is measured by the mouse local lymph node assay (LLNA). The LLNA was originally developed as a method for the identification of chemicals that have the ability to cause skin sensitization and allergic contact dermatitis, but may also give an indication of the potency of an allergen (29). Briefly, the standard LLNA protocol measures the level of cellular proliferation in the auricular lymph nodes, via the incorporation of ³H-labelled thymidine, five days after the first of three daily applications of chemical to the dorsum of each ear. The extent of dLN proliferation observed for the novel chemical, relative to

vehicle control, is then used as the starting point of an evaluation whose ultimate aim is to determine the level of human exposure that is acceptably safe.

CHEMISTRY

A number of *in silico* methods currently exist that aim to predict a novel chemical's reactivity based on the known *in vivo* reactivity of existing chemicals. This software is referred to as Structural Activity Relationship (SAR) or Quantitative Structural Activity Relationship (QSAR) models (differentiated by their ability to quantify the level of reactivity, in addition to simply identifying a hazard) and use the identification of motifs within a novel compound's structure to draw parallels or read-across to well-characterized chemicals containing those same motifs. In this way, the likelihood of a sensitization hazard can be predicted from a chemical structure alone and this information can then be used to guide any subsequent *in vitro* or *in vivo* testing strategy.

At a basic level, all of this work is predicated on the hypothesis that all organic chemical contact allergens must react covalently with (haptenate) protein, that is, they must be electrophiles. The chemistry likely to underlie this has been extensively discussed in various reviews (30,31). Very recently, leading experts in this area have categorized a number of mechanistic domains into which essentially all organic contact allergens can be placed (32,33). Despite this theoretical understanding there is actually very little knowledge of the true *in vivo* haptentation reactions that have been proposed to take place in the skin (34). Nevertheless, based on the theoretical considerations from classic organic chemistry, a system that identified structural alerts for skin sensitizers, DEREK, has been implemented (35). As knowledge has been acquired, the DEREK system has evolved to take it into account (36). Using this Windows-based software, a user can input a chemical structure and obtain a rapid readout of any chemical structures therein that may cause sensitisation together with an explanation of the background with references. As with any other predictive tool, there is an incidence of false positives and false negatives. For all computer-based methods, current information indicates scope for improvement, with none of them having undergone a formal independent validation (37).

As well as basic SAR, quantitation has long been added to the evaluation of closely related groups of chemicals. From the pioneering publication by Roberts in 1982 (38), has flowed a steady stream of publications (39–41). Although these QSARs can give useful predictions not only of whether a chemical is a skin sensitizer, but also of just how strong it might be, they are always restricted to the narrow chemistry on which they are based. The possibility of developing a global QSAR for skin sensitization still seems some distance away, not least because the challenge of combining the chemical reactivity parameters used to represent reaction rates proves substantial (42). Some hope in this respect may be derived from the recent adoption of quantitative mechanistic modeling allied to the developing appreciation that the majority of skin sensitizers actually fall into a relatively limited number of chemical classes (33,34).

Adding complexity to the aforementioned is the challenge presented by chemicals that are not reactive in their own right but may become so as a result of air oxidation or metabolic activation; these are referred to commonly as prohaptens,

or perhaps better as pro-electrophiles (43). Dermatologists are very familiar with examples of these: colophony requires air oxidation to sensitize (44); the hair dye p-phenylenediamine sensitizes via both chemical and metabolic oxidation (45,46). It seems likely that a significant minority of chemicals are pro-electrophiles (47), and it is certainly the case that up to a third of the standard patch test series are in this category. Efforts focusing on the incorporation of metabolic considerations into computer-based systems are underway (48). However, much work on this topic remains to be done, not least in terms of substantiating the quality of these predictions via enhancement of our knowledge of the relevant skin metabolic steps that are associated with ACD.

PROTEIN BINDING

As discussed in the Chemistry section, the current understanding of the mechanism of skin sensitization is that chemicals react with many skin proteins, perhaps at many amino acid sites, to produce altered (haptenated) self protein(s) (35). In an attempt to investigate whether the intrinsic sensitizing potential of a chemical can be predicted from its peptide reactivity kinetics, the haptenation of model peptides or proteins has been studied *in vitro* (49,50). *In vitro* peptide binding assays based around the detection of hapten modification of model peptides, although still in development, show promise and support the theory that the covalent modification of proteins or peptides is a property shared by the majority of skin sensitizing chemicals. At present, with testing of >100 chemicals, the accuracy of predictions compared to existing *in vivo* methods is in the region of 85% (51). It seems probable that this type of approach will improve further as our understanding of the protein-chemical interactions involved in skin sensitization continues to increase (52,53). However, one of the most useful advances would be to gain an appreciation of how the type or extent of protein modifications in the skin affect the induction of sensitization, but this remains a difficult challenge (54,55).

It should be noted that elegant studies have been done, notably on nickel and trinitrophenol, which provide some insights (56,57). However, it is not clear at present how the information can be more generally applied in predictive toxicology.

SKIN PENETRATION/METABOLISM

In vitro skin penetration studies are currently performed to gauge to what extent a chemical is likely to penetrate the skin and enter the circulation, resulting in systemic exposure. These assays are generally performed using *ex vivo* human or pig skin, with the extent of skin penetration being assessed by measurement of the amount of radioactively labelled compound that is present in the supernatant in contact with the underside of the skin section. This relatively crude measurement of the extent of chemical penetration through the skin is useful at present as a gauge for the extent of systemic exposure; however, future risk assessments will require a more accurate measurement of the extent of chemical penetration into, rather than through, the skin (58). Without this information we will have no way of relating the predicted extent of human exposure to the applied dose applied to *in vitro* assays. *In silico* models are currently being developed to predict the extent of

penetration into the skin based on the identification of motifs within the compound's structure (59).

At present the majority of *in vitro* assays for the prediction of skin sensitization potential require a positive test chemical to be active, and thereby induce an effect, in the test system without prior metabolic conversion. *In vivo* chemical metabolism in the skin, either to a more or less active species, is believed to be an important mechanism in the induction of skin sensitization for a particular sub-class of compounds known as pro- or pre-haptens (43,60). Therefore, in order to be confident that a test or a battery of tests has excluded all potential for test chemical reactivity at least one of these assays must be metabolically competent to a similar or greater extent than human skin. Ongoing research aims to address this problem via two approaches; firstly, through the characterization of the metabolic capacity of existing cell-based assay systems to establish how they compare to human skin and secondly, through the development of metabolic enzyme "cocktails" that could be used to supplement metabolically incompetent model systems (61).

ACTIVATION OF KERATINOCYTES

Keratinocytes are the predominant cell type in the skin and under-acknowledged participants in cutaneous immune responses. They are known to produce large quantities of IL-1 α and TNF- α that serve to alert LC to the presence of a pathogenic infection or physical/chemical stressor (12–19). Although no keratinocyte-based predictive assays are currently being widely promulgated for the prediction of skin sensitization potential *per se*, reconstituted skin equivalents composed of human epidermal keratinocytes seeded on synthetic matrices are currently being evaluated by the European Centre for the Validation of Alternative Methods (ECVAM) for the prediction of skin irritation [i.e., EpiDermTM (MatTek, USA) and EpiskinTM (Episkin, France) models] (62,63).

These models are generated through sophisticated protocols that allow the keratinocytes to develop a stratum corneum at the air–medium interface, which acts as a functional barrier. However, it has to be stressed that skin equivalents do differ from normal human skin, for example, the penetration rate of substances through the stratum corneum has been found to be 10–30-fold higher than normal skin *in vivo* (64), suggesting these models may be prone to over prediction. In contrast to ACD, skin irritation is initiated through a direct inflammatory effect on the skin and in the absence of the adaptive immune system mechanisms that characterize skin sensitization (65). However, observed tissue pathology with either condition [e.g., erythema, oedema, dryness, desquamation, pain and itching (66)] makes irritant contact dermatitis and ACD difficult to distinguish, suggesting some molecular mechanisms may be common to both inflammatory reactions and *in vitro* models developed for skin irritation predictive testing could potentially be used to generate data on the relative irritancy of different skin sensitizers.

Due to the absence of any visual symptoms of irritancy in skin equivalent models (e.g., erythema or oedema) other biomarkers have been selected for the prediction of irritant effects. Of these, cell viability (measured by MTT assay) remains the most commonly used parameter for the prediction of *in vitro* skin irritation. However, despite the broad correlation between irritant potential and reduced cell viability

the results of the ECVAM pre-validation exercise have shown that measuring cytotoxicity alone does not always allow irritants and non-irritants to be distinguished (67). Consequently, IL-1 α release was investigated during the ECVAM skin irritation validation study, in addition to cell viability measurements, as a potential supplementary endpoint. We await the official output of this trial; however, preliminary results suggest there is promise in this approach and encourage suggestions that skin equivalent models could be used to generate data for use in future skin sensitization risk assessment approaches.

DENDRITIC CELL ACTIVATION

It is widely recognized that DC play a pivotal role in the induction and maintenance of adaptive immune responses through their ability to process and present antigen to T cells and in particular their unique ability to prime and activate naïve T cells in the lymph node (27). With respect to the induction of cutaneous immune responses, LC residing in the epidermis are believed to be the key DC cell type and consequently the phenotypic changes that LC undergo upon sensitizer exposure have been extensively studied over the last twenty years and form the basis for a raft of DC-based predictive assays (68,69). The hypothesis that underlies the development of DC-based assays for skin sensitization is that sensitizer-induced changes in DC phenotype can be differentiated from irritant- or non-sensitizer-induced changes. This hypothesis derives from the belief that in addition to presenting the processed antigen in the context of the MHC receptor the DC's role is to convert the range of environmental and antigenic signals encountered at the skin into a receptor expression and soluble mediator release profile that a T cell can understand and that some part of this profile is key to predicting a sensitizer's potential or potency.

As mentioned previously, alterations in LC phenotype that accompany changes in the epidermal environment and/or antigen/sensitizer exposure can be broadly separated into two main categories; receptor changes, for example, changes in adhesion molecule and chemokine receptor expression facilitate migration out of the skin (70,71) and increases in MHC Class II and co-stimulatory molecule expression facilitate lymph node interactions with responsive T cells, and soluble mediator release, for example, changes in cytokine release potentiate LC maturation and the epidermal inflammatory response (72). Morphological changes are also observed upon LC activation (73); however, these are less easily quantified as *in vitro* assay endpoints and therefore have remained underexploited. In terms of changes in cytokine expression, interleukin (IL) 1 β , IL-18 and tumor necrosis factor alpha (TNF- α) have all been shown to promote LC maturation and migration to the lymph node whereas IL-10 has been implicated in down-regulating these processes (74–76). However, translation into predictive assays has proven difficult (77,78).

LC are a relatively minor population of the epidermis (2–5%) and as a result their direct isolation for experimental analysis is a difficult task, which tends to result in the activation of the target population (79). Consequently, several cell culture protocols have been developed to derive LC-like dendritic cells from bone marrow, cord

blood, or peripheral blood precursors (68,80). DC can be generated from mouse bone marrow CD34 + hematopoietic cell precursors (81); however, this method is time-consuming and requires a significant number of animals which, in combination with the recent implementation of the 7th Amendment to the EU Cosmetics Directive (82), has favored the adoption of methods that utilize human tissues. Two main protocols have been developed, using either peripheral blood monocytic cells (PBMC) or CD34 + hematopoietic cell precursors (HPC) isolated from cord blood as the starting cell type. The PBMC or CD34 + HPC are then cultured *in vitro* for up to fourteen days in a cocktail of growth factors and cytokines until a immature or mature DC phenotype is achieved (83). Several studies have then gone on to investigate the effect of chemical sensitizers on these cultured DC, noting the upregulation of co-stimulatory molecule (e.g., CD40, CD80, CD83, and CD86) and cytokine expression (e.g., IL-12 p40, TNF- α and IL-1 β) (84–87). These observations support the hypothesis that sensitizers can directly induce DC maturation and this has led to the development of *in vitro* predictive assay protocols that use DC-like cell lines as surrogates for DC.

These DC-like cell line assay protocols have the benefit of reducing the amount of the time spent on cell culture (i.e., rapid cell expansion and relatively simple culture protocols) and eliminating the donor-to-donor variability that is intrinsic to primary cell culture (78,88). Various cell lines have been investigated as DC-like model systems for the purpose of predicting skin sensitization potential, including THP1 (89,90), U937 (91), KG-1 (89,92), and MUTZ-3 (93) lines. Of these model systems, two are currently undergoing inter-laboratory assessment to evaluate their reproducibility and predictive capacity within the European Cosmetic, Toiletry and Perfumery Association, COLIPA. The first of these *in vitro* tests, the human Cell Line Activation Test (h-CLAT) is based on the human monocytic leukaemia cell line, THP1, and was developed as part of a collaboration between two Japanese companies (89,90). The second is the U937/CD86 test which is based on the human histiocytic lymphoma cell line, U937 (90). Both test systems are based on the measurement of relative changes in receptor expression (CD54 and CD86 in h-CLAT test and CD86 in U937/CD86 test), as measured by flow cytometry, with sensitizer potential being identified when receptor expression thresholds are crossed in relation to vehicle control values. Further evaluation of one of the methods has been published very recently (94,95).

Despite the substantial effort to date, these approaches, although promising, have not yielded an assay that is suitable for formal validation. Identified issues relate to the sensitivity of the *in vitro* test, with chemical-induced cellular cytotoxicity (although controlled) playing a pivotal role in the *in vitro* assay result and consequently placing a high degree of importance on chemical dose setting. It may be possible that the sensitivity of these assays could be enhanced by introducing “danger signals” provided by the activation of toll-like receptors (TLRs). Work on this topic is currently underway, but has yet to yield insights that could lead to immediate practical application (96). In addition to these problems, issues surrounding phenotypic-drift within cell line cultures have made standardization of these approaches challenging, plus there also exists the need to ensure that the metabolic capacity of the cells (or perhaps of the test system) can properly reflect that which exists in the skin to activate prohaptens (8,59).

T CELL PROLIFERATION

Naïve T cell proliferation in response to chemical treatment is a robust indicator that a substance is immunogenic and is the endpoint measured in the mouse LLNA. Consequently any *in vitro* approach that could accurately predict naïve T cell proliferation could potentially yield information on sensitizer potency, something that few other *in vitro* approaches seem capable of generating at present.

Several publications exist within the literature demonstrating the experimental feasibility of inducing an antigen-specific proliferative response by naïve T lymphocytes *in vitro* following co-incubation with chemical sensitizer-treated DC or LC. Among these published studies is the work of Conrad Hauser (97) who has demonstrated the induction of primary mouse T cell proliferation and a similar approach has also been employed to measure the proliferative expansion of human T cells *in vitro* to a range of known skin sensitizing chemicals (98,99). In these experiments, naïve T lymphocytes mounted a proliferative response following stimulation by DC exposed to chemical sensitizers, but not following treatment with the irritant sodium dodecyl sulphate (SDS). However, the sensitivity of this approach has always been questionable as significant proliferative responses have generally only been detectable following stimulation with strong sensitizers [such as trinitrophenyl (TNP) and fluorescein isothiocyanate (FITC)], with weak or moderate skin sensitizers [such as citronellal, hydroxycitronellal, and coumarin] rarely generating a significant response and the strong allergen p-phenylenediamine only doing so in a limited proportion of the experiments. Furthermore, due to the complexity of DC: T cell co-culture protocols, these approaches have been found to be both labor intensive as well as difficult to standardize between different laboratories. However, several programs of research are ongoing in this area and as such their still remains hope that advances in cell culture or analytical technology may yield a robust predictive assay based on this approach.

CONCLUSIONS

It seems quite possible that in just a few years time toxicologists will have both existing approaches to the identification of skin sensitizers available as well as more novel strategies. As is the case in 2007, which approach is adopted will depend on various considerations—the need for accurate identification, the need to limit/avoid animal testing, the need to have data that can be taken into a risk assessment. In our view (but recognizing that this is only a best guess) it is probable that, by 2012, we could see a substantial reduction in the use of animals in the predictive identification of skin sensitizing chemicals, but that total replacement of *in vivo* tests presents a substantial challenge. There will be a subset of chemicals for which predictions are difficult, perhaps because they represent novel chemistry, or simply as a result of the situation where hierarchical tiered strategies have been implemented, but at the end of such strategies, where the answer is uncertain, the gold standard remains an *in vivo* test. For reduction (and refinement) in animal testing, the regulatory acceptance of the reduced LLNA, which uses just 4 mice, will be critical; however, this will only identify hazard, a yes/no decision about the presence of sensitizing properties (100). To eliminate animal testing requires the ability to integrate data

from a variety of sources (chemical structure, peptide binding, disturbance of keratinocytes, activation of Langerhans cells, etc.) to provide a perspective on relative potency compared to benchmark allergens. The challenge of how to combine and use data from *in vitro* endpoints for skin sensitization has only been addressed so far in theory (101). Only if toxicologists can develop such methods and have them accepted by other experts, and regulatory authorities, will progress be made both in the complete replacement of animal tests while maintaining, or enhancing, human safety.

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