ISJ 11: 240-246, 2014 ISSN 1824-307X

RESEARCH REPORT

Immune response of *Phyllophaga polyphylla* larvae is not an effective barrier against *Metarhizium pingshaense*

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Accepted September 3, 2014

Abstract

Previous research has uncovered that the cuticle of *P. polyphylla* larvae acts as a good non-immunological barrier against *M. pingshaense*. In the present study we investigated whether *P. polyphylla* larvae also show a similarly robust immunological response against *M. pingshaense*. Firstly, we estimated a median lethal dose (LD50) of blastospores to be injected into the hemocoel. Secondly, we injected the estimated LD50 of blastospores into the hemocoel of larvae to quantify phenoloxidase (PO), nitric oxide (NO) and antimicrobial activity as a response against fungal invasion. In contrast to a previous report that showed that *M. pingshaense* is unable to kill *P. polyphylla* after topical applications, here we demonstrate that: (a) 100 % of *P. polyphylla* larvae died when blastospores were injected into the hemocoel and (b) when injecting the LD50 into the hemocoel of the larvae, immune response did not differ with control. Our results imply that immunological responses do not protect *P. polyphylla* larvae against *M. pingshaense* infections. Thus, the cuticle seems a better defense mechanism compared to PO, NO and antimicrobial activity. One proximate explanation for our results is that blastospores are not detected by the host's immune machinery. An ultimate explanation is that there may be a resource-based tradeoff between non-immunological and immunological barriers, in which white grubs may be investing more in cuticle at the cost of PO, NO and antimicrobial activity.

Key Words: non-immunological barriers; ecoimmunology; white grubs; *Metarhizium pingshaense*

Introduction

Understanding the basis of host resistance is an intriguing biological phenomenon given that pathogens are ubiquitous and impose a strong selective pressure on their host (Schmid-Hempel, 2011). In both vertebrates and invertebrates, resistance consists of both non-immunological and immunological barriers (Hart, 2011; Parker *et al*., 2011). The former could be a behavioral, mechanical and/or hostile environment against invaders (Smilanich *et al*., 2009). On the other hand, the immunological defence prevents foreign agents to cause infection by humoral and cellular components.

It is assumed that non-immunological and immunological barriers can complement each other's defensive action against parasites and/or pathogens (e.g. Dubovsky *et al*., 2013; Fedorka *et al*., 2013; reviewed by Moreno-García *et al.*, 2013). However, just how complementary both barrier types are has not been studied in detail (Parker *et al*., 2011). One pathogen type towards which a host can use both type of barriers is the

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entomopathogenic fungi (Lundgren and Jurat-Fuentes, 2012; reviewed by Arsavanitis *et al*., 2013). These fungi use mechanical pressure and enzymatic degradation to damage the insect cuticle and penetrate the host (Hajek and St. Leger, 1994). Hence, the cuticle represents a first non-immunological barrier against entomopathogens (Wilson *et al*., 2001). However, after injury (*i.e.*, by the fungus penetration), the insect host responds to fungal infection by producing antimicrobial peptides and a cellular response to protect the hemolymph from invasion (Lemaitre and Hoffmann, 2007). Once the pathogen penetrates the cuticle, the insect immune response (humoral and/or cellular) in the hemolymph attacks the fungi by phagocytosis, lytic activity and the activation of phenoloxidase (PO), the latter producing nodule formation, encapsulation and melanization (Lavine and Strand, 2005; Bogus *et al*., 2007).

In the present study we have investigated the immune response of the white grub, *Phyllophaga polyphylla*, when infected with the entomopathogenic fungi *Metarhizium pingshaense*. White grubs are soil dwelling herbivore insects that continuously interact with a large variety of pathogens (Jackson and Klein, 2006), including entomopathogenic fungi. The wide use of these microorganisms to regulate white grub species (Shah and Pell, 2003) has demonstrated a differential susceptibility of these insects to fungal infection (Rodríguez del Bosque *et al*., 2005; Morales-Rodríguez *et al*., 2010; Nong *et al*., 2011; Guzmán-Franco *et al*., 2012). Previous studies showed that *P. polyphylla* larvae are fairly resistant to infection by *M. pingshaense* when immersed in a conidial suspension of this fungus, with mortality never exceeding 20 % after 36 days of incubation (Enríquez-Vara *et al.,* 2012; Guzmán-Franco *et al*., 2012). Thus, these studies concluded that cuticle acts as a fairly good non-immunological barrier. However, whether the same defensive capacity applies when the insect's immune system is challenged, is unclear. Given that 20 % of infected animals is high enough, one would expect that, if the fungus penetrates the insect, immune response should complement the defensive action of the cuticle. One approach for testing this is via artificially by-passing the mechanical barrier imposed by the cuticle by injecting blastospores (the fungal form that multiplies inside the insect) into the host hemocoel. Using this approach, we had two aims in the present study: a) finding a median lethal dose (LD50) of *M. pingshaense* blastospores; and, b) measuring immune response of *P. polyphylla* larvae after injection with different doses of *M. pingshaense*. For immune response, we assessed PO, antimicrobial activity and nitric oxide (NO), three key actors in the defense against parasites and pathogens in insects (reviewed in Beckage, 2008).

Materials and methods

Insects

Third-instar *Phyllophaga polyphylla* larvae were collected from corn fields in Guanajuato, Mexico (20° 02´30.12" N, 100 ° 28´36.4"). Once collected, the larvae maintained individually in plastic cups (100 mL) at 20 °C with damp peat moss (Growing Mix®, Canada) for 4 weeks before they were used in the experiment.

Production of blastospores

The fungus *Metarhizium pingshaense* isolate GC01 was used. Enríquez-Vara *et al*. (2012) and Gúzman-Franco *et al*. (2012) used this isolate against *P. polyphylla*. In both works the isolate GC01 was referred as *M. anisopliae* (morphospecie) but Carrillo-Benítez *et al*. (2013) used molecular methods to demonstrate that the isolate GC01 is indeed *M. pingshaense*. Hence we will refer to the isolate GC01 as *M. pinshaense*. First, conidia were produced in petri dishes containing Sabouraud Dextrose Agar medium (SDA). After 20 days of incubation at 25 ºC in complete darkness, conidia from the medium were harvested with a sterile scalpel. Conidia and mycelium were deposited into a sterile 50 mL volume centrifuge tube containing 30 mL of 0.03 % Tween 80. The mixture of conidia and mycelium was stirred for 15 min. Conidia were separated from mycelium by filtration trough sterile cloth and deposited into a new sterile 50 mL volume centrifuge tube. Conidia concentration was estimated using a haemocytometer. Conidial suspension was then inoculated and grown in 50 mL of sterile liquid medium containing yeast extract, sucrose and Tween 80 (2:2:0.4 p/v). Liquid medium contained in a 250 mL Erlenmeyer flask and with a concentration of $1x10^6$ con mL was incubated on a shaker at 120 rpm at 28 °C for three days. Blastospores were harvested by filtration through sterile cloth and, to remove any remaining liquid medium, the suspension was centrifuged three times at 10,000 rpm for 10 min and suspended in phosphate buffered saline solution pH 7.4 (PBS) (Sigma). The concentration of blastospores was determined using a haemocytometer. The percentage of viable blastospores was estimated prior to experiments using the plate count technique on SDA (Goettel and Inglis 1997). In all cases more than 95 % were viable.

Survival of P. polyphylla *larvae injected with* M. pingshaense *blastospores*

Different groups of 30 third-instar *P. polyphylla* larvae were injected with different doses of blastospores of *M. pingshaense* (10^3, 10^4, 10^5 and 10^6 blastospores, in a total volume of 5 µL per larva) suspended in PBS. Before injection, white grubs were anesthetized on ice and immobilized. The blastospore suspension was injected into the larvae hemocel through the dorsal surface at the junction between the second and third abdominal segments. Injections were carried out using a 30-gauge needle fitted to a 1 mL syringe mounted on a calibrated micro-applicator. As control group, larvae were only injected with PBS. The larvae were transferred individually to 12-well cell culture plates (COSTAR ®, Corning Inc. NY, USA) (1 larva per well), which contained a 2 cm diameter filter paper which had been moistened with 80 µl of sterile distilled water. The 12-well culture plates were incubated at 25 °C

in complete darkness and mortality was assessed every 24 h for 10 days. Dead larvae were incubated at 25 ºC and 100 % RH for 7 - 10 days, to encourage sporulation thereby allowing fungal infection to be confirmed. Data were analyzed using Kaplan-Meier survival curves, and the log-rank test was used to evaluate statistical differences between white grubs injected with PBS only or with different doses of blastospores of *M. pingshaense*. Kaplan-Meier survival curves were constructed for each treatment. The log-rank test was used to compare survival amongst curves constructed for each treatment.

Immune response of P. polyphylla *against* M. pingshaense *infection*

The immune response of *P. polyphylla* against *M. pingshaense* infection was estimated by quantifying the production of PO, NO and antimicrobial activity in the insect´s hemolymph as a response to infection (see below). To achieve this, a lethal dose (LD50) concentration of blastospores was injected into the hemolymph. Injecting a LD50 increased the survival time before death thereby allowing immune parameters to be quantified. The LD50 was estimated by dose-response assays.

Estimation of LD50

The estimation of LD50 was obtained using the same methodology described above with some modifications. Twelve third-instar *P. polyphylla* larvae were exposed to four doses. Based on the results of the previous experiment, a different set of doses was selected 5.10^3, 1.10^4, 5.10^4 and 1.10^5 blastospores of *M. pingshaense* in PBS. The complete experiment was repeated on two different occasions. Larval mortality was recorded every 24 h for five days. Mortality was corrected using Abbott´s formula (Abbott, 1925). Data from the bioassays were analysed using a generalized linear model with binomial error and probit link in the statistical package GenStat v. 8.0 (Payne *et al*., 2005). The numbers of infected larvae were assumed to follow a binomial distribution with sample sizes equal to the number of larvae tested. Before combining two replicates, a parallel model analysis was done for each replicate. First, a single line was fitted to data from replicates. Second, intercepts were allowed to vary amongst the replicates and third, slopes were also allowed to vary amongst the replicates. If the single line model was the best for each replicate, then data from the two replicates could be combined. Concentration causing 50 % infection (LD50) of larvae was estimated from best fit model and confidence interval for LD50 was calculated according to Fieller's theorem (Fieller, 1944).

Quantification of immune parameters

Five groups of 12 larvae each were injected with the LD50 estimated previously (5.10^3 blastospores) to quantify PO, NO and antimicrobial production. These parameters were estimated in the hemolymph of larvae at five different times after injection (0, 2, 6, 12 and 24 h). The immune response of each of the five different times after injection was estimated in a different group of 12 larvae. A different set of five groups of 12 larvae were injected with only PBS and treated as described before. A total of 120 larvae were used for both treatments and times of quantification. All treated larvae were maintained as described before until hemolymph was collected.

Hemolymph collection

For the hemolymph collection, the integument of each larva was surface sterilized with 70 % ethanol and then rinsed twice using sterile distilled water. Hemolymph samples were obtained by cutting the third thoracic leg of each larva and four drops (approximate 30 µL) of hemolymph were collected into sterile and precooled Eppendorf tubes (1.5 mL) containing 100 µL of PBS, and vortexed for 10 s. The mixture was centrifuged for 10 min at 10, 000 rpm and 4 ºC to remove hemocytes and cell debris. The supernatant was divided into three aliquots, two of 50 µL and one of 30 µL. The two 50 µL aliquots were mixed separately with 50 µL of PBS, while the third was placed in a 0.5 mL Eppendorf tube and kept at -80 °C until required. The first 50 µL subsample was used to measure protein hemolymph content and PO activity. The second subsample was used to estimate NO production, and the 30 µL sample was used to estimate antimicrobial activity. All measurements were carried out immediately after hemolymph collection.

Protein content

Proteins were measured using the BCA (Pierce Biotechnology, Rockford, IL) protein assay kit with BSA as the protein standard. Two replicates of 10 µl of hemolymph/PBS mixture were used to measure the protein in each sample (see Enríquez-Vara *et al*., 2012). The absorbance was measured on a Varioskan Flash microplate reader (Thermo Fhiser Scientific, Waltham, MA) at 562 nm.

PO activity

Hemolymph PO activity was measured using the method described by Enríquez-Vara *et al*. (2012). Briefly, an aliquot that contained 40 µg of protein was placed in a 96 well microplate (Corning Inc, Corning NY), and then dose-tittered to a volume of 50 µL of sample and PBS. To this mixture, 50 µL of L-DOPA (4 mg/mL) was added to obtain a final volume of 100 µL. PO activity was assayed spectrophotometrically with dopamine as a substrate. The slope of the curve was calculated by using the optical density at 490 nm. Optical density readings were taken every minute for one hour at 30 °C (Enríquez-Vara *et al*., 2012).

NO production

A colorimetric nitrate/nitrite assay kit (SIGMA) was used to prepare the standard curve and to estimate NO in each sample following the manufacturer's instructions. The basis of this technique is that nitric oxide is a highly unstable radical that rapidly reacts with other oxygen-reactive species to form stable products, such as nitrites, nitrates and toxic radicals (*i.e.*, peroxynitrite). Hence, the total nitrate and nitrite content is used to indirectly estimate the amount of nitric oxide in each sample. The amount of NO (µM) in samples was

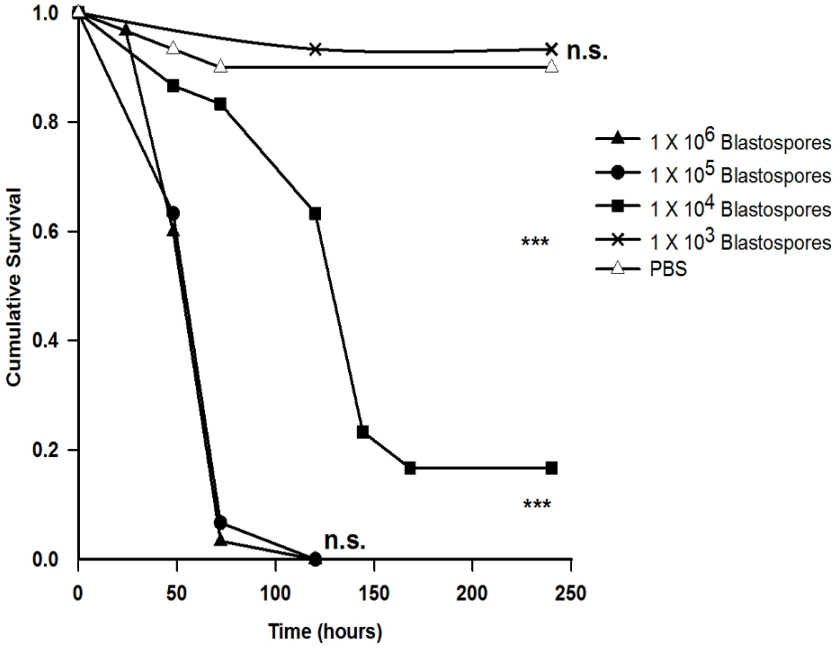

Fig. 1 Survival of third-instar larvae of *Phyllophaga polyphylla* injected with different concentrations of blastospores of *Metarhizium pingshaense*. **p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001, denote differences in survival between concentrations of blastospores and PBS lines by Log-rank statistics. The n.s. indicates that the survival were not significantly different between lines.

estimated by extrapolation with a standard curve of known concentrations. Readings were performed at 540 nm.

Antimicrobial activity

Antimicrobial activity in the haemolymph samples was measured using the cup agar-diffusion assay technique (Mohring and Messner, 1968). The microbial activity was measured according to the methods described by Bogus *et al*. (2007), with some modifications. Briefly, the assays were performed in 90-mm Petri dishes containing 66 mM Sörensen buffer, pH 6.4 (10 mL), using *Micrococcus lysodeiticus* (7 mg) as substrate, agar (100 mg) and streptomycin sulfate (0.7 mg). Hemolymph samples (4 µL) free of insect cells were added to the Petri dishes, samples formed a circle of three mm in diameter. The diameters of the lytic zones around the three mm diameter samples were measured after incubation of the Petri dishes for 24 h at 37 °C. The antimicrobial activity of the insect hemolymph samples was expressed in equivalents of chicken egg white lysozyme. Increasing concentrations of lysozyme (10 - 1000 ug/mL) were used as a standard for comparisons. The quantities of NO and antimicrobial activity found were so small that we were unable to record them. Data from PO activity were analysed using analysis of variance (ANOVA) with the statistical package SAS v. 9.0 (SAS, North Carolina,USA). We compared PO measurements between PBS (control) and injected with blastospores treatments, and their interaction with the time after inoculation. PO data were ln-transformed to meet assumption of normal distribution and equality of variances.

Results

Survival of P. polyphylla *larvae injected with* M. pingshaense *blastospores*

Significant differences were found in the survival rates of *P. polyphylla* larvae amongst all treatments compared (X^2 = 145.86; $p < 0.05$; Fig. 1). When the larvae were injected with 1.10^5 and 1.10^6 blastospores of *M. pingshaense*, some died within the first 48 hours post-injection and 100 % mortality was recorded at 120 hours. An intermediate effect in survival was obtained when the larvae were injected with 1.10^4 blastospores (Fig. 1). The survival rate in larvae injected with 1.10^3 blastospores and larvae in the PBS control were similar and mortality was never greater than 10 % (Fig. 1).

Immune response of P. polyphylla *against* M. pingshaense *infection*

Estimation of LD50

No evidence of non-parallelism (χ_I^2 = 0.22, $p > 0.05$) or differences in intercepts (χ_I^2= 2.93, $p > 0.05$) amongst replicates were found, justifying the pooling of data from separate replicates for further analyses. The LD50 value estimated for M. pingshaense was $5.2x10^3$ (CI=$3.3x10^3$-$7.5x10^3$) blastospores. Therefore, larvae were injected with

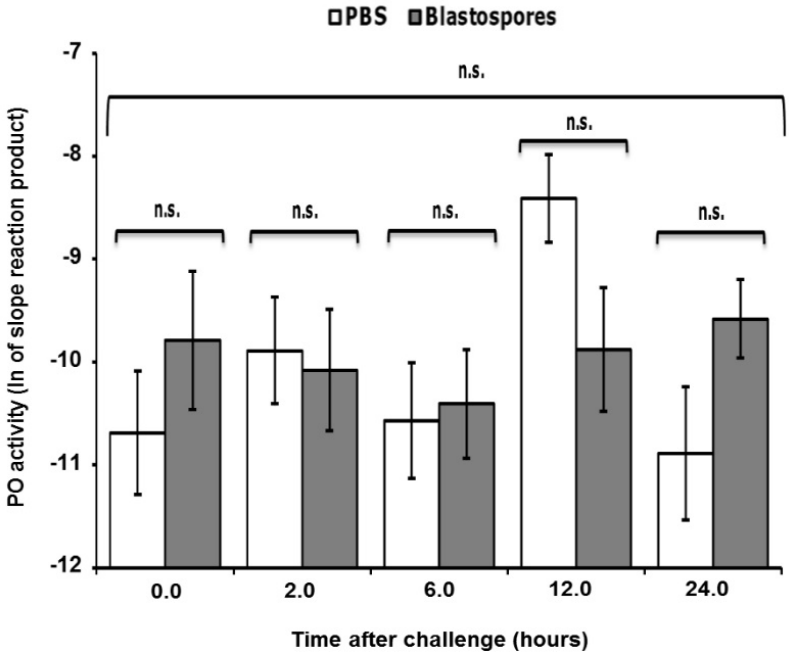

Fig. 2 Slope PO activity expression according to PBS or blastospores and time. Sample size was 12 larvae per time point and treatment. Each bar indicates mean ± SE. The n.s. indicates that the PO activity were not significantly different between PBS and blastospores.

the LD50 estimated to assess immune response parameters.

Quantification of immune parameters

After injection of blastospores, only PO production was activated. Neither NO nor antimicrobial activity was recorded. PO production was similar in larvae injected with blastospores and PBS ($F_{1,150}$ = 0.16, p > 0.05, Fig. 2), and this result was consistent throughout all measurement times ($F_{4,150}$= 1.75, p > 0.05, Fig. 2).

Discussion

By injecting blastospores directly into the hemocel of white grub larvae, we found that nearly 100 % of individuals were infected after 120 h of incubation at the greatest doses. In perspective with previous studies using the same host-pathogen interaction (Enríquez-Vara *et al.,* 2012; Guzmán-Franco *et al*., 2012), it seems that the *P. polyphylla* cuticle is a more effective barrier against *M. pingshaense* than PO, NO and antimicrobial activity. Related to this, Bogus *et al*. (2007) found that mortality associated with the topical application of conidia was explained by the cuticle thickness in three insect larvae. The cuticle may be important as a barrier in soil systems because it prevents the negative impact of abiotic factors (*i.e.*, the damage to the cuticle due to friction with the soil), and therefore may favour resistance against a wide variety of pathogens and parasites (Villani *et al*., 1999). As the epicuticle is more variable in its components than the procuticle, this could be implicated in the differential insect resistance to invaders (see Golebiowski *et al*., 2008).

It is unclear why the PO quantities did not differ between the PBS and blastospore treated larvae. We propose that blastospores of *M. pingshaense* were not detected by the cellular or humoral innate immune response of *P. polyphylla*. Related to this, it is known that the entomopathogenic fungi must be discreet to avoid being recognized as a foreign agent by the host's immune response (Wang and St. Leger 2005; Vilcinskas, 2010). Poprawski and Yule (1991) injected 3.10^6 spores of *Metarhizium anisopliae* in *Phyllophaga anxia* larvae, obtaining 42 % mortality. Interestingly, we injected only 1.10^6 blastospores, which produced 100 % mortality. It is likely that spores are better detected by the insect immune response than blastospores (Wang and St. Leger, 2006), as well as by the fact that blastospores are the stage of replication of the fungus leading to a faster invasion of the host´s hemocel, which is not the case for spores (Gillespie *et al*., 2000). Notice, however, that the immune response caused by a fungal infection may vary according to the distinct host and fungal pathogen species. For example, when the fungus *Conidiobolus coronatus* were inoculated in different insect species, the immune response based on the PO levels determined varied: PO levels decreased when *G. mellonella* was inoculated whereas no modification was found in *Diprion pini*, but an increase was observed in *Calliphora vicina* (Bogus *et al*., 2007). In relation to *Metarhizium*, even the

same host species can produce different immune responses. For example, *G. mellonella* larvae infected with *M. anisopliae* reduced PO activity (Slepneva *et al*., 2003), a result that could not be corroborated when the same host and fungus species were used (Dubovskiy *et al*., 2013). Again, one mechanism is that blastospores may become undetected.

An explanation is needed as for why immune response was so reduced (PO) or non-existent (NO and antimicrobial activity) in *P. polyphylla* larvae in the face of a fungal infection. This may be related to the evolutionary ecology of immune response. It is known that non-immunological and immunological barriers are costly to produce and so their costs can limit the expression of other traits (Schmid-Hempel, 2005; McKean and Lazzaro 2011; Parker *et al*., 2011). For example, in *Acheta domesticus* the investment in cuticle thickness (a non-immunological barrier) decreased egg production and adult body size (Bascuñan *et al*., 2010). As for immunological barriers, Ardia *et al*. (2012) found that in insects, encapsulation response led to increased levels of PO and CO_2 production but decreased levels of lysozyme. On the other hand, both barriers can conflict each other's expression. In support of this, immunological barriers are inefficient in species whose non-immunological barriers are effective at combating parasites or pathogens (Parker *et al*., 2011). For example, Dubovskiy *et al*. (2013) found that PO and lytic activity were lower in hemolymph that in cuticle in *G. mellonella* against *M. anisopliae*. Thus, one explanation for our results is that the efficiency of the cuticle against *M. pingshaense* is traded-off with PO, NO and antimicrobial activity.

In practical terms, the studies by Enríquez-Vara *et al.* (2012), Guzmán-Franco *et al*. (2012) and those we have shown here suggest that entomopathogenic fungi could be used as a strategy to control white grubs. However, since fungi need to break the cuticle to penetrate the insect, one way to facilitate fungal infection is to use nematodes. To this aim, we suggest the use of *Heterorhabditis* nematodes (Bedding and Molyneux, 1982). Future experiments should see whether this strategy is viable.

Acknowledgements

JN Enriquez-Vara had a scholarship grant from the Consejo Nacional de Ciencia y Tecnología (CONACyT). Fundación Produce Guanajuato A.C. (Project FGP251/09-SIFP-11-2007-0271) financed this study. J Contreras-Garduño was supported by a grant from CONACyT (152666) and Universidad de Guanajuato-2013. Finally, thanks to an anonymous reviewer for key comments to an original version.

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