Association of antibiotic resistance and higher internalization activity in resistant *Helicobacter pylori* isolates

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Received 24 October 2005; returned 1 December 2005; revised 9 December 2005; accepted 12 December 2005

Objectives: *Helicobacter pylori* resistance to antibiotics is the main factor for therapy failure, while other features remain largely unknown. The aims of this study are to investigate the relationship of antibiotic resistance and *in vitro* internalization activity between cure and failure isolates and to determine whether failures are associated with persistence of the same predominant strain.

Methods: Fifty-three isolates from forty-seven patients (cure group, n = 31; failure group, n = 16) receiving one of two lansoprazole-based therapies before and/or after therapy were investigated. Antibiotic susceptibility was determined by Etest. Genotyping was determined by cagA, babA, vacA and RAPD analyses. Target cells of internalization assay were AGS cells.

Results: Five of six paired pre- and post-treatment isolates had the same predominant genetic profiles and exhibited similarly high internalization activities. The A2143G point mutation of the 23S rRNA gene conferred clarithromycin resistance. Moreover, increased antibiotic resistance after therapy was found for these five cases. Pre-treatment isolates from the failure group (n = 11) had higher level of internalization activity than those from the cure group (n = 31) (P = 0.00005). Antibiotic-resistant strains were significantly associated with higher internalization activity than were susceptible strains (metronidazole, P < 0.005; clarithromycin, P < 0.005).

Conclusions: Our results suggest that resistant *H. pylori* strains are associated with antibiotic resistance and superior internalization activity, protecting them against antibiotic treatment.

Keywords: *H. pylori*, *in vitro* internalization, triple therapy, 23S rRNA

Introduction

*Helicobacter pylori*, a gastric pathogen, infects approximately half of the human population, in which it may persist for a lifetime, making it one of the most successful pathogens of mankind.¹–³ Enduring infection of this peculiar microbe is linked to chronic inflammation in gastric epithelial cells, which may further develop into peptic ulcers, gastric atrophy and is believed to be a risk factor for gastric adenocarcinoma and low-grade B-cell lymphoma.²

Two complete genomic sequences and other results demonstrate unexpectedly high genetic heterogeneity of this microbe, and shed light on its extraordinary ability to adapt into different ecological niches created by the diversity of humans, their ancestors, their environments and their diets.⁴,⁵ Several virulence factors including urease, flagella, mucous-damaging enzymes and cell-surface adhesins such as blood group antigen-binding adhesin (BabA) are crucial for persistent inhabitation of *H. pylori*.⁶ Furthermore, strains with two non-conserved exoproteins, vacuolating toxin (VacA) and cytotoxin-associated antigen (CagA) (type I strains), result in more severe clinical outcomes than type II strains, which secrete only little VacA and are devoid of CagA.⁷ The differential effects of bacterial virulence determinants along with host factors contribute to the development of distinct clinical sequelae during persistent infection of *H. pylori*.⁶

Despite the general notion of *H. pylori* as a predominant extracellular pathogen localized in the mucus overlaying the gastric mucosa, a minor subset of *H. pylori* have been observed near...
the intercellular junctions or the lamina propria, or even inside gastric mucosal cells both in vitro and in vivo. By use of time-lapse video microscopy and gentamicin assays in cultured epithelial cells, viable and motile H. pylori residing in multivesicular bodies for at least 24 h were found to repopulate the extracellular space, indicating release from the intravacuolar niche inside cells. CagA"VacA" rather than CagA"VacA"− isolates are also found to have higher entry into cultured epithelial cells. Moreover, some strains, particularly those associated with peptic ulcer are more aggressively invasive than *Shigella flexneria*. It is not surprising, given the high use of antibiotic therapy for *H. pylori* infection, that there is a 10–40% failure rate of treatment, demonstrating a rise of resistant strains. Consistent with this trend, we have previously found ~15% failure rates even with two high-dosage antibiotics in 1 week triple therapy regimens. In this study, we sought to investigate whether failures were associated with persistence of the same predominant strain. Since the relationship of in vitro internalization activity and antibiotic resistance in the *H. pylori* resistant infection is thus far not known, we also investigated whether there was any association between antibiotic resistance and the level of internalization activity of clinical isolates.

Materials and methods

**H. pylori strains, bacterial culture and antibiotic resistance**

Fifty-three available clinical isolates from forty-seven patients enrolled in a previous study were investigated. These patients have received one of two lansoprazole-based triple therapies (LAC, lansoprazole 30 mg twice daily, clarithromycin 500 mg twice daily and amoxicillin 1 g twice daily; LMC, lansoprazole 30 mg twice daily, clarithromycin 500 mg twice daily and metronidazole 500 mg twice daily) for 1 week in Taichung Veterans General Hospital, Taiwan. All these patients were *H. pylori*-positive on the basis of *^{13}C* urea breath test and bacterial culture performed on biopsies before therapy. Successful cure was defined as a negative *^{13}C* urea breath test in the follow-up visit (at least 6 weeks) after therapy.

Isolates were recovered from frozen stocks on Wilkins–Chalgren agar with 10% sheep blood, 0.2% β-cyclodextrin, 1% IsoVitaleX, 10 mg/L nalidixic acid and 8 U/mL polymyxin B in a microaerophilic atmosphere for 3–7 days at 37°C until colonies were visible. After passages onto fresh plates, the bacteria were routinely cultured on Brucella agar plates (Becton Dickinson, NY, USA) with 6 mg/L vancomycin and 2 mg/L amphotericin B. For liquid culture, the bacteria harvested from plates were cultured in Brucella broth containing 2% fetal bovine serum, 0.2% β-cyclodextrin, 1% IsoVitaleX, 6 mg/L vancomycin and 2 mg/L amphotericin B for 2 days.

The *H. pylori* strains were tested for metronidazole, clarithromycin and amoxicillin susceptibility using the Etest (AB Biodisk, Solna, Sweden). The MIC was defined as the concentration on the Etest strip closest to the point of intersection with growth on the plate. Metronidazole resistance was defined as an MIC of >8 mg/L, amoxicillin resistance and clarithromycin resistance were defined as MIC of >2 mg/L.

**PCR and RAPD typing**

Chromosomal DNA was prepared from *H. pylori* strains as described elsewhere. The presence of the *cagA* gene was detected by a PCR method described elsewhere. Genotyping of *vacA* signal sequence region (*vacA s*: s1a, s1b and s2) and the middle region (*vacA m*: m1 and m2) was determined as described elsewhere. The *babA2* status was determined by a PCR method with primers *babA2*-F and *babA2*-R or with primers *nhu_babA2*-F and *babA2*-R. RAPD typing was done essentially as described elsewhere, using primer 1281 (5′-AAGCGGCAAC-3′) or primer 1283 (5′-GCGATCCCCCA-3′). The PCR cycling reaction of the 23S rRNA is as follows: an initial denaturation for 5 min at 95°C, 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min; and a final extension at 72°C for 10 min. The PCR products were purified and sequenced directly on both strands by the deoxy chain termination procedure with an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, MA, USA) in an automated DNA sequencer (model 377-96; Perkin-Elmer).

**Sequencing of the 23S rRNA gene**

The primers used for PCR amplification of a 1.4 kb fragment of the 23S rRNA gene are 23S rRNA-F (5′-AGTCGGGACCTAAGGC-GAG-3′) and 23S rRNA-R (5′-TTCCCCGTTAGTGCTTTTCAG-3′). The PCR products were purified and sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) in an automated DNA sequencer (model 377-96; Perkin-Elmer). The primers used were: primer 1281 (5′-GCGATCCCCCA-3′) or primer 1283 (5′-AAGCGGCAAC-3′), and primer 1315 (5′-BAAACAGGCCACG-3′)

**Bacterial internalization assay**

*H. pylori* invasion into cultured AGS cells (ATCC CRL 1739) was assayed using a standard gentamicin method described by Lozniewski et al. In brief, mid-logarithmic phase grown bacteria in suspension were quantified by optical density measurements. Approximately 2 × 10⁵ cells were cultured in 24-well plates and then infected with *H. pylori* suspension (~10⁸ cfu) for 6 h at 37°C. To determine the number of viable intracellular bacteria, infected cells were washed three times in PBS and incubated with 100 mg/L of the membrane-impermeable antibiotic gentamicin (Sigma-Aldrich) for 1.5 h at 37°C to remove extracellular bacteria. The infected monolayers were washed three times with PBS and then lysed with distilled water for 10 min. Lysates were serially diluted in PBS, plated onto Brucella blood agar plates and cultured for 4–5 days, after which the cfu were counted. The internalization activity was defined as the proportion of bacteria surviving to the total number of bacteria infected to the AGS monolayer in each cell. We counted viable epithelial cells for normalization of the samples. The internalization activity was determined as the mean of at least six experiments performed in duplicate.

**Statistical analysis**

Data of each comparison (between cure and failure groups or between antibiotic-susceptible and antibiotic-resistant groups) in relation to internalization activity were analysed by Kolmogorov–Smirnov test. All comparisons of this study were determined as normal distribution. Student’s t-test was used to compare the data. The box plots were used to show summary statistics for the distribution of bacterial internalization activities. Statistical analyses were carried out using the SPSS program (version 10.1, SPSS Inc., IL, USA). A P value of less than 0.05 was considered statistically significant.

**Results**

**Patient and bacterial properties**

There were 31 pre-treatment isolates randomly selected from 31 different patients in the cure group and 20 available isolates from all 15 therapy-failure patients (5 paired pre- and post-treatment isolates, 6 pre-treatment isolates and 4 post-treatment isolates). In addition, there was one additional set of paired pre- and post-treatment isolates (from patient no. 47, who was considered to
be cured by LAC therapy, but had positive bacterial culture 9 months after the enrolled date.

**Change of susceptibility to antibiotics after treatment in the failure group**

Antibiotic resistance was determined for all isolates. No resistance to amoxicillin was found. Resistance to metronidazole was detected in 7 of 31 pre-treatment isolates (23%) in the cure group, as compared to 6 of 12 pre-treatment isolates (50%) and 7 of 10 post-treatment isolates (70%) in the therapy failure group. Resistance to clarithromycin was detected in 5 of 12 pre-treatment isolates (42%) and 8 of 10 post-treatment isolates (80%) in the therapy failure group, as opposed to none of the 31 isolates (0%) in the cure group.

Analysis of the MIC values between the pre- and post-treatment isolates indicated that the post-treatment isolate of three cases (no. 42, no. 45 and no. 46) had an increased MIC value for clarithromycin, turning from the susceptible into the resistant phenotype after therapy (Table 1). In patient no. 43 who received the LMC therapy, the post-treatment isolate displayed a significantly increased MIC value for metronidazole (from 0.75 to 64 mg/L), showing a susceptible-to-resistant change after therapy. The paired isolates from patient no. 44 were resistant to both metronidazole and clarithromycin, in which the post-treatment isolate had even higher MIC values. On the other hand, isolates from patient no. 47 were susceptible to both clarithromycin and metronidazole. These results together suggested that there was an increased antibiotic resistance after receiving antibiotic treatment for all paired isolates except one (no. 47).

To evaluate whether there were A2142G and/or A2143G mutations in the 23S rRNA gene that conferred clarithromycin resistance, we have characterized the 23S rRNA gene of the paired isolates. As shown in Table 1, three pairs (no. 42, no. 45 and no. 46) had a wild-type nucleotide (A) at the position 2143 of the 23S rRNA gene in the pre-treatment isolate while an A2143G point transition in the post-treatment isolate, consistent with the susceptible-to-resistant phenotype after therapy. Two cases (no. 43 and no. 44) that carried clarithromycin resistance before and after therapy indeed had the specific point mutation in the paired isolates, suggesting that the clarithromycin-resistant strains had inhabited the stomach before treatment. On the other hand, both pre- and post-treatment isolates of patient no. 47 had the wild-type nucleotide at position 2143, in line with their susceptibility to clarithromycin.

**Genetic characterization of H. pylori paired isolates from patients who failed in therapy**

To investigate whether failures were associated with persistence of the same predominant strain, we have characterized genotypes of the paired isolates in the failure group. As shown in Table 1, all strains were cagA-positive and babA2-positive. For vacA genotype, two pairs (no. 42 and no. 46) were typed as s1a/m2 and three (no. 43, no. 44, and no. 45) were s1a/m1. The paired isolates from patient no. 47 were noted to have different vacA genotype; the pre-treatment isolate was s1a/m1 while the post-treatment isolate was s1a/m2.

By use of RAPD analysis, five pairs (42–46) demonstrated indistinguishable RAPD patterns (data not shown). In contrast, patient no. 47 harboured a strain that had a distinct RAPD pattern after therapy (data not shown). Six single colonies of pre- and post-treatment isolates respectively were further characterized, in which there were identical RAPD profiles within each population (data not shown). It was also noted that the unique pairs (no. 47) have different levels of bacterial internalization, in

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**Table 1. Analysis of pre- and post-treatment paired isolates from therapy-failure patients**

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Therapy</th>
<th>Pre/Post</th>
<th>Genotype</th>
<th>MIC (mg/L)</th>
<th>Viable cfu/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>cagA</td>
<td>babA2</td>
<td>vacA s</td>
</tr>
<tr>
<td>42</td>
<td>LMC</td>
<td>Pre</td>
<td>+</td>
<td>+</td>
<td>s1a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post</td>
<td>+</td>
<td>+</td>
<td>s1a</td>
</tr>
<tr>
<td>43</td>
<td>LMC</td>
<td>Pre</td>
<td>+</td>
<td>+</td>
<td>s1a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post</td>
<td>+</td>
<td>+</td>
<td>s1a</td>
</tr>
<tr>
<td>44</td>
<td>LMC</td>
<td>Pre</td>
<td>+</td>
<td>+</td>
<td>s1a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post</td>
<td>+</td>
<td>+</td>
<td>s1a</td>
</tr>
<tr>
<td>45</td>
<td>LAC</td>
<td>Pre</td>
<td>+</td>
<td>+</td>
<td>s1a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post</td>
<td>+</td>
<td>+</td>
<td>s1a</td>
</tr>
<tr>
<td>46</td>
<td>LMC</td>
<td>Pre</td>
<td>+</td>
<td>+</td>
<td>s1a</td>
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<td></td>
<td></td>
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<tr>
<td>47</td>
<td>LAC</td>
<td>Pre</td>
<td>+</td>
<td>+</td>
<td>s1a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post</td>
<td>+</td>
<td>+</td>
<td>s1a</td>
</tr>
</tbody>
</table>

*The presence of cagA and babA2 in each isolate was determined by PCR. vacA s is the genotype of vacA signal sequence region (s1a, s1b or s2), while vacA m is the genotype of the vacA middle region (m1 or m2).

MIC, minimum inhibitory concentration; MTZ, metronidazole; CLR, clarithromycin; AMX, amoxicillin. Resistant phenotype is indicated by a bold number.

Each subject no. identifies a unique patient.

Table 1. Analysis of pre- and post-treatment paired isolates from therapy-failure patients

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contrast with the similar levels seen for the other pairs (Table 1). Moreover, patient no. 47 was initially considered to be cured, but had positive bacterial culture 9 months after the enrolled date. Taken together, these results suggested that patient no. 47 was re-infected by a distinct strain. This case was thus excluded in the subsequent statistical analyses.

**Therapy-failure isolates were associated with greater intracellular invading activity**

Bacterial isolates from the therapy-failure group were found to have a greater level of invading activity than those in the cure group. By use of the gentamicin protection assay, *H. pylori* 60190 was found to invade AGS cells to a relatively high extent; the invading population corresponded to 0.10 ± 0.02% of the total number of added bacteria or 1.07 ± 0.13 viable cfu per cell for strain 60190. We then compared bacterial invading activity in all isolates from the therapy-failure group (*n* = 20) and cure group (*n* = 31). The level of bacterial survival was 0.02–1.44 cfu per cell in therapy-failure isolates, as compared with 0.03–0.85 in cure group isolates, revealing a greater overall level of intracellular survival in the therapy-failure group.

Analysis of the level of internalization activity of pre-treatment isolates between the cure group (*n* = 31) and failure group (*n* = 11) revealed that therapy-failure isolates were associated with higher level of internalization activity (*P* = 0.00005, Student’s *t*-test). No differences in the levels of internalization were found depending upon whether the patient received LAC or LMC therapies or between m1 and m2 strains (data not shown).

**Paired isolates had similarly high internalization activity**

To evaluate whether therapy failure affected the internalization activity, we found that the five paired isolates (42–46) had relatively high levels of internalization activity (at least 0.55 cfu/cell). Moreover, the paired isolates that had the same predominant genetic profiles displayed similar levels of internalization activity. The pre- and post-treatment isolates from patient no. 47, in contrast, had distinct internalization activities (0.19 versus 0.44 cfu/cell), in accordance with that this subject was likely to re-infected by a different strain.

**Diverse distribution of internalization activity was found for antibiotic-resistant isolates in the failure group**

To investigate the level of internalization activity within a given antibiotic group, data of 46 isolates were analysed: (i) pre-treatment isolates in the cure group (*n* = 31); (ii) pre-treatment isolates in the failure group except no. 47 (*n* = 11); and (iii) unpaired post-treatment isolates in the failure group (*n* = 4).

As shown in Figure 1(a), there was a higher median value of metronidazole-susceptible strains in the failure group (*n* = 6) than that in the cure group (*n* = 24), showing statistical significance (*P* < 0.01). Similarly, there was a significantly higher level of internalization activity for clarithromycin-susceptible isolates in the failure group (*n* = 7) than in the cure group (*n* = 31) (*P* < 0.05).

For metronidazole-resistant isolates, there was a much broader range of internalization activity distributed in the failure group (Figure 1a). Analysis of data between the cure (*n* = 7) and failure (*n* = 9) groups revealed a higher median value of internalization activity in the failure group. However, no statistical significance was found possibly because of limited sample size (*P* = 0.074). There was also a high median value of internalization activity for clarithromycin-resistant strains in the failure group. On the other hand, no clarithromycin-resistant strains were found in the cure group.

**Antibiotic-resistant strains were associated with greater internalization activity**

As primary antimicrobial resistance is the major factor influencing the success of triple therapy, data were analysed for 42 pre-treatment isolates from both the cure and therapy-failure groups. As shown in Figure 1(b), resistant strains were significantly associated with higher internalization activity than were susceptible strains (metronidazole, *P* < 0.005; clarithromycin, *P* < 0.005). There was even higher statistical significance when data from all 53 isolates were analysed (metronidazole, *P* = 0.00012; clarithromycin, *P* < 0.0001). These results suggest a close link between antibiotic resistance and higher level of internalization activity.

**Discussion**

In this investigation, we have characterized all available isolates from 16 patients that failed in triple therapies. Of six patients with isolates before and after therapy, we found that one was re-infected by a new strain while the other five had paired isolates with indistinguishable RFLP and RAPD profiles. Thus, treatment failure did not generally lead to a change in the predominant strain. Not surprisingly, the post-treatment isolates had higher antibiotic resistance than did their corresponding pre-treatment isolates, in accord with other results.

By use of *in vitro* internalization assay, the five paired isolates were noted to have relatively high level of internalization activities. Of interest, these paired isolates had similar internalization activities, unlike the apparently higher antibiotic resistance after therapy. One possible explanation is that there may be complex sequences involved in *H. pylori* internalization that requires several determinants, as opposed to simple point mutations of a specific gene that confer antibiotic resistance. Indeed, an A2143G point mutation in the 23S rRNA gene was found for three cases after therapy (no. 42, no. 45 and no. 46), resulting in clarithromycin resistance after therapy.

We found that isolates from the therapy-failure group were associated with significantly elevated internalization activity (*P* = 0.00005). Metronidazole-susceptible and clarithromycin-susceptible isolates respectively were also associated with a significantly higher level of internalization activity in the failure group. For metronidazole-resistant strains, there was a higher median value in the failure group. A highly diverse distribution of internalization activity among failure isolates was noted, reflecting that a certain fraction of resistant strains had relatively high internalization activity. This observation however lacks statistical significance (*P* = 0.074) possibly due to the small sample size.

This elevated internalization activity was also significantly linked to primary antibiotic resistance (metronidazole, *P* < 0.005; clarithromycin, *P* < 0.005) when all pre-therapy isolates (*n* = 42) from the cure group and therapy-failure group are considered. The close link between the phenotype of higher invading activity and that of antibiotic resistance is interesting, notably, for

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clarithromycin that is able to enter eukaryotic cells. Most significantly, the post-treatment isolates that acquired clarithromycin resistance displayed relatively high internalization activity, as opposed to no clarithromycin-resistant isolates in the cure group.

As the administered antibiotic transports across the membrane or travels into deeper sites of the stomach, it would diffuse down their concentration gradient. It is thus likely that the emergence of resistance is predisposed in internalized or penetrated bacteria.

Figure 1. (a) Comparison between the cure and failure groups in relation to the internalization activity. Data of the cure group are from 31 randomly selected pre-treatment isolates, while those of the therapy-failure group are from pre-treatment paired (n = 5) or unpaired (n = 6) isolates, and post-treatment isolates (n = 4). (b) Comparison between the susceptible and resistant groups in relation to the internalization activity. Data are pre-treatment isolates (n = 42) from both cure and therapy-failure groups. A box plot is utilized to show the summary statistics for the distribution of the data. The ends of the boxes define the 25th and the 75th percentiles. Lines drawn from the ends of the box represent the largest and the smallest values that are not outliers. Each extreme value (more than three box lengths) is indicated by an asterisk. Each outlier value (between 1.5 and 3.0 box lengths) is represented by an open circle. P-values were determined by Student’s t-test. MTZ-S, metronidazole-susceptible; MTZ-R, metronidazole-resistant; CLR-S, clarithromycin-susceptible; CLR-R, clarithromycin-resistant; NS, non significant. P < 0.05 was considered statistically significant.
due to exposure to lower concentrations of antibiotics. Efficient adaptive point mutations by \textit{H. pylori} would presumably take place for the rapid development of high-level resistance to the applied antibiotics since most \textit{H. pylori} strains have a hypermutator phenotype.\textsuperscript{24–26} In support of this view, A2143G point mutation in the 23S rRNA gene and/or higher degree of antibiotic resistance is found after therapy. The 1 week treatment thereby selectively enriched the stomach with strains of both resistant phenotypes. These results strongly suggest that bacteria with high internalization activity jeopardize treatment success, apart from other factors including primary antibiotic resistance of some strains, high bacterial load and high gastric acid production in some hosts.\textsuperscript{27,28} The combined phenotypes thus allow bacteria to persist long-term, yielding an even more resistant strain as seen in the rise with the global use of antibiotic therapy.\textsuperscript{12}

In conclusion, we provide evidence that bacteria with high antibiotic resistance and/or elevated invading activity escape better during antibiotic treatment of \textit{H. pylori} infections. Therapy failure thus resulted in selection and enrichment of strains with both refractory phenotypes, leading to even more resistant strains. These results support the hypothesis that the stomach can serve as a temporary reservoir, with some bacteria capable of invading the gastric epithelium and persisting intracellularly in a quiescent state, later reemerging to initiate another round of infection.\textsuperscript{8,9} Such mechanisms may also underlie the success of \textit{H. pylori} in surviving in its unique niche during human history.\textsuperscript{29}

Acknowledgements

This work was supported by National Science Council (NSC93-3112-B-007-013 and NSC94-3112-B-007-005), Taiwan and partially by Veterans General Hospitals University System of Taiwan Joint Research Program, Chi-Shuen Tsou’s Foundation (VGHUST93-P6-29).

Transparency declarations

No declarations were made by the authors of this paper.

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