Brief Definitive Report

Expression of Variants of the Major Surface Glycoprotein of *Pneumocystis carinii*

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Summary

Previously, we have shown that a multicopy family of related but unique genes encodes the major surface glycoprotein (MSG) of *Pneumocystis carinii*. To examine whether different members of this gene family are expressed by *P. carinii*, antisera were prepared against peptides whose sequences were determined from the deduced amino acid sequences of variants of rat-derived MSG. Immunohistochemical staining of serial sections of rat lungs of infected animals showed that at least three variants of MSG were expressed in an individual lobe, that there was a focal expression of these variants within the lung, and that the relative numbers of these foci were different. Indirect immunofluorescent staining of purified *P. carinii* organisms using these antisera revealed that at least three variants of MSG were present in organisms isolated from an individual rat and that both cysts and trophozoites reacted with each antiserum. A substantial difference in the fraction of organisms reacting with a specific antipeptide antiserum was seen when comparing organisms isolated from rats raised in a single colony over a period of two years as well as organisms isolated at one time point from rats raised in different colonies. This demonstration of antigenic variation in *P. carinii* supports the hypothesis that *P. carinii* utilizes such variation for evading host defense mechanisms.

Pneumocystis carinii is a major opportunistic pathogen of patients with HIV infection (1, 2). Although *P. carinii* cannot be reliably cultured in vitro, studies using organisms obtained from immunosuppressed animals have led to important advances in our understanding of the basic biology of this pathogen, including strong evidence to support its reclassification from a protozoan to a member of the fungi (3).

While important differences exist in *P. carinii* organisms isolated from different hosts, all possess an abundant, highly immunogenic, major surface glycoprotein (MSG), also called gpA (4–6). MSG shows a size on reducing SDS-PAGE of between 95 and 120 kD (7). cDNA or genomic clones encoding MSG derived from rats, humans, and ferrets have been isolated and sequenced (8–10). These studies indicate that MSGs from each host are encoded by a unique family of related but distinct genes, which, for rat-derived *P. carinii*, are present as tandem arrays within the genome (11). Recently, we and others have shown that there appears to be a single or very limited number of expression sites for the MSG genes (12, 13).

MSG appears to be strongly involved in *P. carinii* attachment mechanisms (14–17) and to be a target for host immune response (18, 19) and thus is a good candidate for vaccine development. Recently it has been shown that cDNAs produced from mRNA obtained from the lungs of a single immunosuppressed infected rat code for multiple variants of MSG (20), supporting the concept that a single isolate of *P. carinii* can express different MSG variants. The potential ability of *P. carinii* to express different MSG variants suggests that the organism is utilizing antigenic variation as a means of evading host immune defenses, which may interfere with the efficacy of a vaccine. To examine this question more directly, we used epitope-specific antibodies to examine the expression of MSG variants in a single host. In this report, we demonstrate that multiple variants of MSG are expressed within one rat and that the expression pattern may change with time and with location. In addition, we present evidence that within a single lobe this variation is focal, suggesting a clonal expansion of *P. carinii* organisms within the lung.

Materials and Methods

Preparation of Organisms. *P. carinii* organisms were obtained from the lungs of male Sprague–Dawley rats as previously described (21). National Institutes of Health (NIH) rats were purchased from the National Cancer Institute Frederick Animal Production Unit and housed at one facility at the NIH. Biocon rats

were purchased from Sasco (Omaha, NE) and housed at a facility in Rockville, MD. Cages were not isolated from each other. Animals were killed when they appeared ill, as evidenced by ruffled hair, weight loss, and rapid respiration. Lungs were aseptically removed and used only if they were found to be heavily infected with *P. carinii* and free of significant bacterial or other fungal infection. For histopathologic studies, lungs were preserved in Histochoice and embedded in paraffin. For studies using intact *P. carinii*, organisms were partially purified by Ficoll–Hypaque density gradient centrifugation, washed in PBS, pelleted, and stored at −70°C.

Indirect Immunofluorescence. Partially purified *P. carinii* organisms were thawed on ice, resuspended twice in 1 ml of 0.1 M glycine, pH 2.5, to remove bound immunoglobulins, resuspended in 3 ml of PBS, filtered through a nylon 10-μm filter (Micron Separations Inc., Westborough, MA), and adjusted with PBS to OD_{600} = 1.0. 20 μl was smeared on a plain glass slide, allowed to air dry, fixed in ice-cold acetone for 5 min, and stored at 4°C until staining.

For immunofluorescent staining, slides were washed once in water, layered with 0.25 ml of 10% lamb serum (Life Technologies, Grand Island, NY) for 1 h, and then washed twice with pH 7.2 Tris-buffered saline (TBS). Anti-peptide antisera were diluted 1:50 in 10% lamb serum, and 0.25 ml was layered onto the slides for 1 h and then washed twice in TBS. The slides were next layered for 30 min with 0.25 ml of fluorescein-conjugated goat anti–rabbit antibodies (Organon Teknika, West Chester, PA) diluted 1:50 in 10% lamb serum, then washed twice in TBS, after which they were coated with 0.25 ml of rhodamine-conjugated concanavalin A (Sigma Chemical Co., St. Louis, MO), 0.25 μg/ml for 30 min, and washed twice in TBS. Concanavalin A has been shown to bind to both trophozoites and cyst forms of *P. carinii* (22). Finally, slides were mounted under VectaShield (Vector Laboratories, Inc., Burlingame, CA).

Preparation of Anti-peptide Antisera. Antisera GP3/PC3 and PC5 were produced by Research Genetics (Huntsville, AL) in rabbits against keyhole limpet hemocyanin–conjugated peptides as described (8). The peptide used for the production of GP3/PC3 corresponds to amino acid residues 446–460 of GP3 and 453–467 of PC3 (ELRGNLGLVRFYSDP); PC5 corresponds to residues 365–379 of the homologous region of PC5 (ELKGKLGHVRFYSDP), based on the numbering as reported in reference 8. Antiserum MGP3 was raised by Chiron Mimotopes (Clayton, Australia) in 15 BALB/c mice immunized with peptide covalently coupled to diphtheria toxoid via a cysteine residue added to the NH_2 terminus of the peptide TPGGETGASGGTPGT (residues 848–853 of GP3 [8]). Mice were injected with 20 μg of peptide per injection, with Freund's complete adjuvant on day 1 and Freund's incomplete adjuvant on day 14. Animals were bled on day 58, and sera from all animals were pooled.

Anti–P. carinii mAb. Mouse mAb 4D7 was prepared against rat-derived *P. carinii* and characterized as described (23).

Immunohistochemistry. Lungs were fixed via tracheal infusion and immersion in Histochoice fixative (Amresco, Inc., Solon, OH) and embedded in paraffin. Tissue sections were processed with a streptavidin–alkaline phosphatase (SAAP) detection system and evaluated for immunoreactivity with rabbit antisera GP3/PC3 or PC5 diluted 1:50 in PBS with Tween 20 (PBST), or a 1:50 dilution in PBST of tissue culture supernatant of mouse anti–pneumocystis mAb 4D7. In brief, serial 5-μm tissue sections were collected on positively charged glass slides (SuperfrostPlus; Fisher Scientific, Pittsburgh, PA), deparaffinized, rehydrated, and then blocked in 2% normal goat or horse serum in PBST for 20 min at room temperature in a humidified chamber. After the blocking serum was drained, sections were incubated with primary antibodies diluted in PBST for 1 h under the same conditions. Sections were washed with PBST and then incubated for 30 min with biotinylated goat anti–rabbit or horse anti–mouse IgG antibody (Vector Laboratories), diluted 1:200 in PBST. After another PBST wash, sections were incubated for 30 min with streptavidin–alkaline phosphatase (GIBCO BRL, Gaithersburg, MD), diluted 1:50 in PBST. The chromogenic substrate for alkaline phosphatase was HistoMark Red (Kirkegaard and Perry, Gaithersburg, MD). Color development progressed for 50 min in the dark, and then sections were washed in distilled water and counterstained with Mayer's hematoxylin (Poly Scientific, Bay Shore, NY). Negative controls included replacing the primary antisera with appropriate preimmune or nonimmune sera and applying the primary antisera to uninfected rat lung tissue.

Results and Discussion

To determine whether different MSG variants were expressed in a single lung, and whether there was a discrete pattern to the expression of MSG variants, we performed immunohistochemistry on serial sections of a *P. carinii*–infected rat lung, using mouse mAb 4D7 to visualize all *P. carinii* (Fig. 1). When sequential serial sections were probed with the polyclonal anti-peptide antisera anti-GP3/PC3 or anti-PC5, distinct differences in the immunostaining pattern were observed (Fig. 2). There are clusters of organisms that stain preferentially with either anti-GP3/PC3 or with anti-PC5. Since most organisms that react with one of these anti-peptide antisera appear as tightly clustered foci, they seem likely to have arisen through the clonal proliferation of *P. carinii* that express either the GP3/PC3 variant or the PC5 variant. In addition, a comparison between the relative numbers of foci stained with the anti-peptide antisera and the total number of organisms as visualized by 4D7 shows that whereas both GP3/PC3 and PC5 epitopes are found frequently in this population, they by no means account for all the MSG variants that are found in the lung of a single infected rat. These findings are consistent with a recent report (20) in which the simultaneous presence of at least seven distinct mRNA species encoding MSG was demonstrated in a single rat lung.

Whereas the majority of clusters stain with only one of the anti-peptide antisera, there are clearly groups of organisms that stain with both. These dual-staining clusters are found mostly in bronchioles or in alveoli continuous with bronchioles and may represent organisms being cleared from deeper alveoli. Alternatively, they may represent variants that contain epitopes common to both peptides, since there is an overlap in the sequences used to generate the anti-peptide antisera.

To determine whether any variation in MSG expression occurred with respect to either time or location, we performed indirect immunofluorescence assays (IFAs) on purified *P. carinii* organisms. Fig. 3 shows the results obtained when probing these organisms with the anti-peptide antisera. Since *P. carinii* binds concanavalin A (22), organisms are seen as red when visualized with concanavalin A–rhodamine

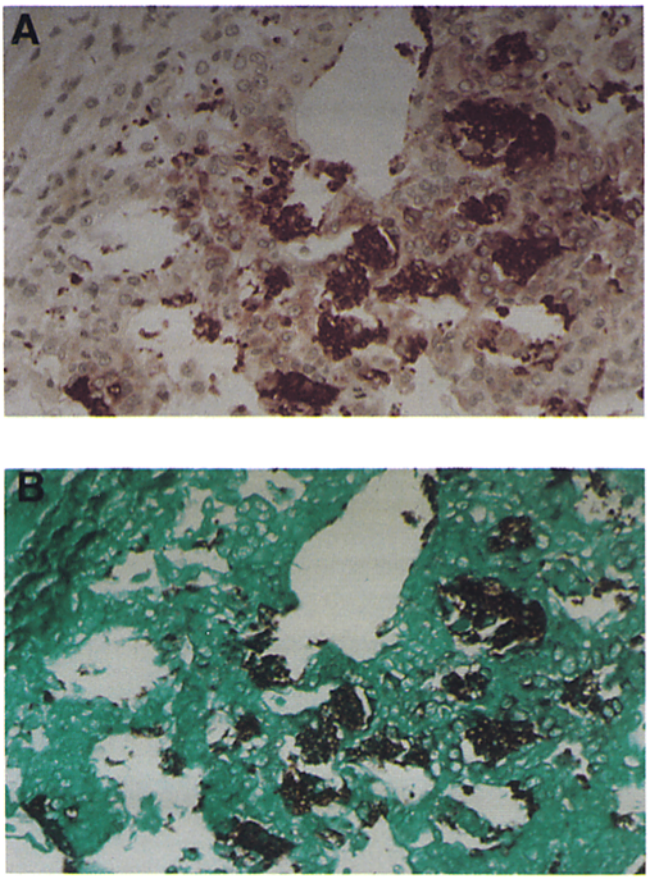

Figure 1. Demonstration that mouse mAb 4D7 detects all *P. carinii* detected by methenamine silver. (*A*) Methenamine-silver staining of a 5-μm section of Histochoice-fixed, paraffin-embedded tissue. *P. carinii* cysts appear brown. (*B*) Immunohistochemical staining of the same section of rat lung. *P. carinii* appear as deep-red clusters. Magnification: 400.

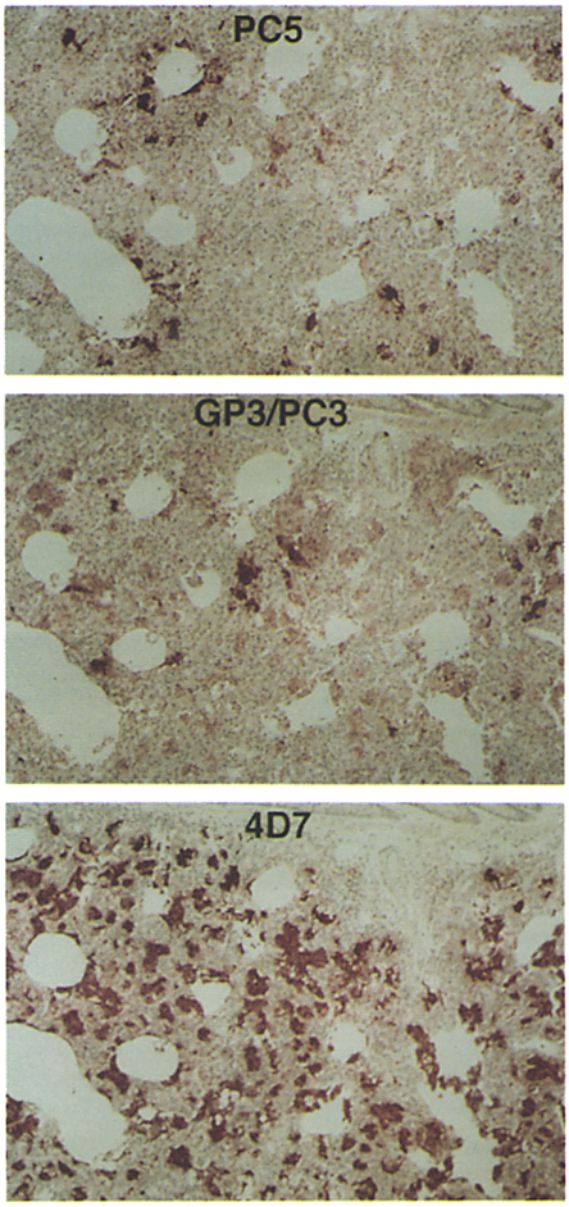

Figure 2. Focal expression of MSG variants in 5-μm serial sections of *P. carinii*–infected rat lung. Immunohistochemistry was performed using as primary antibodies either anti-peptide antisera PC5 (*top*) or GP3/PC3 (*middle*), or mouse mAb 4D7 (*bottom*). The staining pattern is different for the two epitope-specific antibodies; all areas of reactivity correspond, however, to *P. carinii* organisms as identified by antibody 4D7. Magnification: 400.

alone. Yellow fluorescence is due to a combination of the red from concanavalin A–rhodamine, which reacts with all organisms, and the green fluorescence from the binding of specific rabbit anti-peptide antisera as visualized by goat anti–rabbit FITC. IFA of organisms obtained from the lungs of a rat housed in the NIH colony through January 1990 revealed that the majority of organisms expressed epitopes that were recognized by either anti-PC5 or anti-GP3/PC3 (Fig. 3, *bottom left*). Both cyst and trophozoite forms were recognized by each antibody. No specific immunofluorescence was seen when applying preimmune sera (not shown). By January 1993, there was a substantial decrease in the fraction of organisms that reacted with these two antisera (Fig. 3, *bottom row, left and center*). Most dramatic was a decline between 1990 and 1993 in the fraction of organisms isolated from NIH-housed rats that were reactive with anti-PC5 (*top row, left versus center*). Fig. 3 also shows that organisms obtained at the same time from rats housed at a commercial facility (Biocon) contained a much higher fraction that were IFA-positive with anti-PC5 plus anti-GP3/PC3 (*bottom row, center and right*). Much of this is apparently due to the higher levels of immunofluorescence obtained with anti-PC5 against the Biocon organisms (*top row, center and right*). The results shown in Fig. 3 were obtained for individual infected rats killed at the indicated times. Similar results were obtained at each time for four other rats killed at the same stage of infection within a 3-wk period (not shown). Problems of clumping of the *P. carinii* organisms (especially trophozoites) (24) prevented quantification of these findings.

Since anti-GP3/PC3 recognizes an epitope common to both GP3 and PC3, we utilized a second anti-peptide antiserum, raised in mice and designated as anti-MGP3 to de-

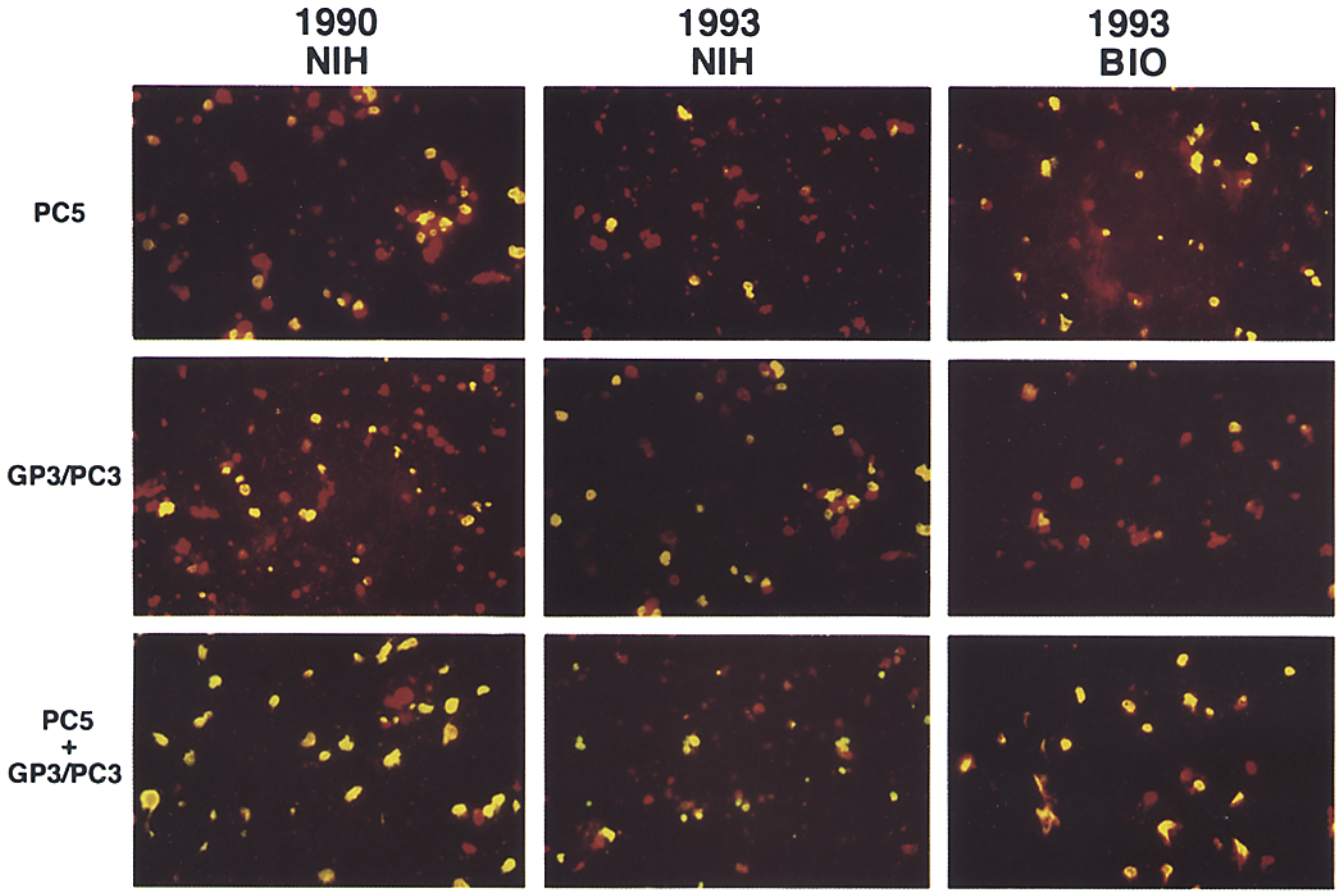

Figure 3. Binding of specific anti-peptide antisera raised in rabbits to three preparations of rat-derived *P. carinii* organisms. Double-exposure photography yields a red fluorescence for organisms that react with concanavalin A–rhodamine only and yellow fluorescence for organisms that react with both the concanavalin A–rhodamine and the green goat anti–rabbit FITC. The year in which the specimens were obtained and the source of the rats are shown across the top, while the anti-peptide antiserum used is shown on the left. Magnification: 630.

termine which variant is expressed. Anti-MGP3 is directed to a region of GP3 (residues 843–857) that is more COOH-terminal than that used to produce anti-GP3/PC3. Fig. 4 shows the immunofluorescent pattern obtained with this antiserum. The red fluorescence is from organisms that react with only anti-GP3/PC3, whereas a green fluorescence would be seen for those that react with only anti-MGP3, and a yellow fluorescence for those that react with both. Although many organisms react with anti-GP3/PC3, only a small fraction of the organisms are immunoreactive to both, indicating that only a minority of expressed MSGs possess GP3-like epitopes at both positions. No *P. carinii* reacted with only anti-MGP3. Since the deduced amino acid sequence in this region of PC3 is unknown, the corresponding epitope in PC3 cannot be determined, but is presumably different from that of GP3, since not all organisms reactive with anti-GP3/PC3 are reactive with anti-MGP3. There appear then to be at least four variants of MSG expressed in these preparations: PC5-like, PC3-like, GP3-like, and at least one other variant that reacted with no epitope-specific antibody.

On the basis of these data, it is not possible to definitively conclude whether there is a variation in the levels of ex-

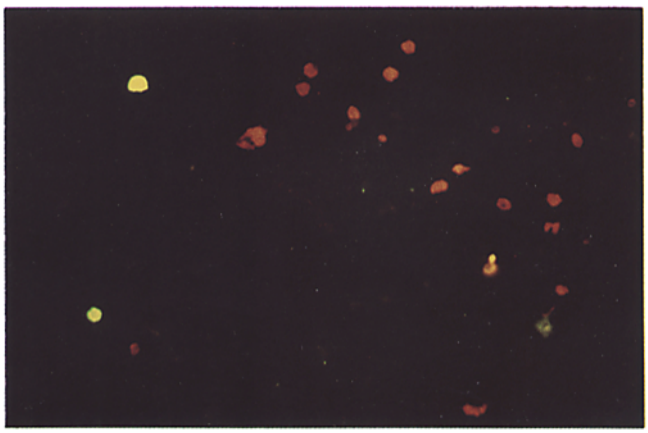

Figure 4. Expression of GP3-specific epitopes in rat-derived *P. carinii*. Double-exposure photography yields a red fluorescence for organisms that react with anti-GP3/PC3 only and yellow fluorescence for organisms that react both with anti-MGP3 detected by goat anti–mouse FITC and with anti-GP3/PC3 detected by goat anti–rabbit rhodamine. No organisms reacted only with anti-MGP3. Magnification: 630.

pression of these specific epitopes among individual organisms, although it appears that the preponderance of organisms are either negative by IFA or show fluorescence of similar strong intensity. It is also unclear whether both of these epitopes can be expressed simultaneously in an individual cyst or trophozoite. It is clear, however, that a shift in the pattern of epitope expression can occur among animals in a given colony. Since it has been established that different strains of *P. carinii* can infect rats in a single colony (25, 26), one explanation for this observed variation is that the colony harbored different strains, each expressing different epitopes that have intrinsically different growth rates, and that this led to a shift in the expression pattern over time. Thus by 1993, the dominant strain in the NIH colony was one that expressed neither GP3/PC3 nor GP3, but a third variant. Another, more interesting, possibility is that *P. carinii* is varying the expression of MSG to evade host defense mechanisms. The best-characterized example of this type of antigenic variation is that found in the variable surface glycoprotein of African trypanosomes, where hundreds of variants can be expressed as an evasion strategy (27).

Since these studies were performed on rats that were immunosuppressed by steroid administration, there are inherent limitations. These include time of onset of the *P. carinii* pneumonia, the severity and duration of the infection, source of latent or transmitted *P. carinii*, and the presence of other pathogens. Further, if *P. carinii* has developed a strategy of antigenic variation, it presumably was developed to evade immune responses in an immunologically normal host, not an immunosuppressed host. In the former, the expression of MSG variants may be more ordered, while in the latter, the need for antigenic variation may be lost, allowing multiple MSGs to be expressed at one time. Nevertheless, this work has clearly shown that there is temporal and geographic variation in the expression of specific epitopes found in MSG, and that in an individual animal, the expression of these epitopes is presented in a focal fashion.

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