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*J Immunol* 1998; 160:4254-4261; ; http://www.jimmunol.org/content/160/9/4254

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Extensive Junctional Diversity of Ig Heavy Chain Rearrangements Generated in the Progeny of Single Fetal Multipotent Hematopoietic Cells in the Absence of Selection

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We analyzed the progeny of individual multipotent hemopoietic cells, derived from the para-aortic splanchnopleura, the earliest identified source of lymphocyte precursors in pre-liver mouse embryos. Single precursors were expanded in an in vitro culture system that permits both commitment and differentiation of B cell precursors. We show that from one single multipotent progenitor we could obtain large numbers of B cell precursors that rearrange the Ig heavy chain genes and generate a repertoire as diverse as that observed in adult populations. N region additions are present at V(D)J junctions, showing that terminal deoxynucleotidyl transferase expression has been switched on and is not, consequently, an intrinsic property of adult stem cells. Throughout the culture period, cells show a majority of DJ vs V(D)J rearrangements and a ratio of 2:1 of nonproductive to productive V(D)J rearrangements, which is close to the expected frequency in the absence of selection. In addition, counterselection for D-J rearrangements in reading frame 2 is observed in V(D)J joints, and allelic exclusion was consistently observed. We conclude that of the three events associated with heavy chain rearrangement, two of them, namely allelic exclusion and counterselection of cells in which the D segment is in reading frame 2, are intrinsic to the cell, while selection of productive heavy chain rearrangements is induced in the bone marrow environment.

Received for publication October 15, 1997. Accepted for publication January 5, 1998.

allelic exclusion and selection for cells with productive rearrangements are dependent on the expression of an Ig heavy chain, the first is intrinsic to the cell, while the second seems to be environmentally determined.

Materials and Methods

**Cell culture**

Fetal livers and para-aortic splanchnopleuras were obtained from timed mating of C57BL/6 or C57BL/6×BALB/c F1, mice (day of plug = day 0) as previously described (24). B220-positive cells (B220<sup>+</sup>) were isolated from fetal liver by a panning procedure using anti-B220 Abs (14:8) as described previously (23). The positive population was seeded at 2 to 3×10<sup>5</sup> cells/well in 24-well plates and grown over adherent layers of irradiated (2000 rads) S17 fibroblasts (20,000–50,000 per well) in 2 ml of Opti-MEM (Life Technologies, Cergy Pontoise, France) supplemented with 10% FCS, 5×10<sup>-5</sup> 2-ME, and 100 U/ml recombinant IL-7. For the single-cell experiment, cells were isolated and seeded by micromanipulation onto 96-well plates, then cultured as previously described (24).

**Flow cytometry analysis**

Single-cell suspensions were stained with phycoerythrin-labeled anti-B220 (PharMingen, CA), FITC-labeled anti-m<sub>μ</sub> (Jackson ImmunoResearch, West Grove, PA), and biotin-labeled anti-CD43 (PharMingen, San Diego, CA), followed by a second step of streptavidin-TriColor (Tebu, Le Perray-en-Yvelines, France). Dead cells were eliminated from the analysis by propidium iodide exclusion. Fluorescence was measured with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA) using the CellQuest software.

**RT-PCR for semiquantitative analysis of TdT**

At different time points of culture, flow cytometry analysis was performed with anti-B220-phycoerythrin and anti-IgM-FITC Abs, and 10<sup>5</sup> cells were recovered for RNA preparation. Total RNAs were extracted from the cells as described (26). First-strand cDNAs synthesis were performed with 2.5 μg of RNA primed with 0.5 μg oligo(dT) using 200 U of MmULV reverse transcriptase. cDNAs were amplified by PCR using, for TdT amplification, primers SLS<sup>5</sup> (GAAGGCCATCCGTGTAGATC) and SLS<sup>3</sup> (GTT TCAATGTAGTCCAGTCC); or for hypoxanthine phosphoribosyltransferase (HPRT) amplification, HPRT<sup>5</sup> (GTAATGATCGTCAACGG ACCG GGGAC) and HPRT<sup>3</sup> (CCAGCAAGCTTGCAACCTTAACCA). HPRT dosage is used to normalize differences in reverse reaction efficacy or in cDNA input between the samples. PCR reactions were performed in 50-μl volume containing 5 μl of cDNA sample, 1× PCR buffer, 100 μM of each of four deoxynucleotides triphosphates, 0.5 μM of each sense and antisense primers, and 1.25 U of Taq polymerase. Amplifications consisted of a denaturing step, 2 min at 94°C, followed by cycles (26–30) each consisting of 15 s at 94°C, 30 s at 55°C (TdT) or 59°C (HPRT), and 30 s at 72°C. Five microliters of the PCR product was separated on 1.5% agarose gel electrophoresis and detected by Southern blot analysis using internal oligonucleotides as probes: HPRT int., 5′-TGTTATGATGACTACACGG CCC-3′, and TdT int., 5′-ATGATGATGATGACACCGAATCAACACCG-3′; which reveals both of the TdT isoforms (27) amplified in this PCR assay. The signals obtained were then quantified by scanning the filters on a phosphorimager.

**CDR3 length analysis with immunoscope**

The repertoire analysis of Ig heavy chain has been described in detail elsewhere (28, 29). In brief, PCR amplifications were performed with a sense primer specific for each of the variable gene families; and an anti-sense primer specific for either the IgM constant region or the intron region of J<sub>μ</sub>4 was used for cDNA or DNA amplification, respectively. A run-off elongation on the amplification product was then performed with a fluorescent primer specific for J<sub>μ</sub>4 and the product analyzed in an automated DNA sequencer (Applied Biosystems, Foster City, CA). A software (immunoscope; Ref. 28) converts the bands detected on the gel into peaks for which the length and intensity are known. Equivalent amounts of DNA corresponding to B220<sup>+</sup> cells were used in all experiments. For the semiquantitative analysis of rearrangements, a variant of this technique was used. Briefly, equivalent amounts of DNA were amplified for 22, 25, and 28 cycles with different pairs of primers. The intensities of peaks detected were added and ratios calculated under nonsaturating conditions of amplification.

**Cloning and sequencing**

J558-J<sub>μ</sub>4 amplification products were purified by electroelution and cloned into pCRII using a TA cloning kit (Invitrogen, San Diego, CA), and recombinant clones were sequenced using the T7 sequencing kit (Pharmacia, Uppsala, Sweden). All of the sequences obtained derived from independently rearranged clones.

**Results**

**Progression analysis of differentiating B cell precursors in vitro**

We isolated multipotent hemopoietic precursors from day 9 embryos. These precursors, located in the para-aortic splanchnopleura, were micromanipulated as individual cells and cultured as previously described (24).

We followed by flow cytometry analysis the B lineage cells obtained from these precursors. Figure 1 shows the patterns of B220/IgM staining of the progeny of one of six representative multipotent hemopoietic precursors. The first B220<sup>+</sup> cells are detected around day 12 of culture. By day 19, >80% of the cells express the B220 marker and the first B cells, able to respond to LPS stimulation and secrete IgM (30), are seen. Between day 19 and day 35 to 40 of culture, IgM<sup>+</sup> cells can be detected, representing 2 to 3% of total cells, with a maximum of 20 to 30% between days 20 and 25. The low level of IgM<sup>+</sup> cells observed at most time points is probably due to the nonresponsiveness of mature B cells to IL-7, so that IgM<sup>+</sup> cells readily die. We assume that this level remains constant, as differentiation is continuously occurring. Indeed, throughout the whole experiment, cells proliferated with a mean division time of 16 h, and the majority expressed B220 and CD43 (data not shown) indicating that most cells in culture are from the pro-B cell type (31). The highest proportion of IgM<sup>+</sup> cells, detected around days 20 to 25 in all cultures surveyed, indicates a synchronization of the differentiating precursors, with numbers decreasing thereafter.

**Individual multipotent precursors that rearrange the Ig heavy chain in vitro can generate a diverse repertoire**

We followed the pattern of heavy chain variable gene rearrangement from the progeny of six independent multipotent cells growing in the presence of IL-7 and stromal cells. Due to the multipotent nature of the precursor that was isolated, commitment to the B lineage and all D-J<sub>H</sub> and V-D-J<sub>H</sub> rearrangements necessarily occurred in vitro.

To analyze the Ig heavy chain rearrangement, the first step was a PCR amplification using a 5′ V gene-specific primer together with a 3′ primer specific for the μ constant region for cDNA amplification (28) or a 3′ primer specific for the intron downstream of the J<sub>H</sub>4 gene for DNA amplification (29). The fluorescent run-off products covering the CDR3 region were then separated on a sequencing gel. Figure 2 shows the CDR3 size spectrum of the V(D)J rearrangement obtained with cDNA isolated from single bone marrow cells amplified with a V region primer specific for the J558 family. The pattern seen with any diverse population of cells is that of a normal size distribution represented by the gaussian-shaped curve. cDNA and DNA were isolated from the progeny of the six individual precursors at three different time points. The profiles of V<sub>H</sub>4-D-J<sub>H</sub> rearrangement in the cDNA from one of these precursors, isolated at day 15, 22, and 32 of culture, are shown in Figure 2. A complex pattern of diversity was observed for all samples analyzed, showing that a wide variety of heavy chain rearrangements is achieved in vitro by the progeny of a single precursor. At day 15 and 22, the diversity obtained with cDNA was comparable with that resulting from equivalent numbers of bone marrow cells. Nevertheless, the mean of CDR3 size distribution is evolving throughout the culture period. A diverse pattern...
was also obtained with other V gene primers used (VQ52 and V7183, data not shown).

By day 32 of culture, the same precursor population was tested again. The pattern that was previously diverse had changed drastically. We observed fewer major peaks having an irregular distribution. The expression pattern was now consistent with an oligoclonal population and was observed in both cDNA and DNA samples. After 40 to 50 days in culture, cells could no longer respond to LPS, although they continued to grow in the presence of IL-7 (data not shown), indicating that no more B cells were being generated.

We conclude that in the progeny of single multipotent cells in vitro, B cell precursors undergo Ig gene rearrangements, and a fraction of them differentiate, in a time-regulated manner, into IgM⁺ cells. Longer time periods in culture induce increasing oligoclonality in their pattern of V(D)J rearrangements, and after 4 wk the cell population is no longer representative of the initial rearrangements observed.

**TdT expression in fetus derived B cell precursors**

It has been shown that TdT “switch-on” can occur in fetal T cell precursors, depending on environmental cues. Here, we observe that TdT expression can be equally switched on in B cell precursors generated in vitro, starting with multipotent precursors from pre-liver embryos, as well as with committed B cell precursors from fetal liver.

The multipotent nature of the splanchnopleura precursors implies that they do not express TdT ex vivo. As shown in Figure 3A, TdT was also not detectable in fetal liver at day 12 or day 15 of gestation, even in an enriched population of B cell precursors, whereas it was readily detected in adult bone marrow containing an identical percentage of B cell precursors. However, TdT mRNA was detected in B cell precursors of fetal liver origin from day 12 and day 15 after 4 days of culture and in the progeny of cells isolated from splanchnopleura analyzed at day 22 of culture (Fig. 3A).

By a semiquantitative PCR assay, the level of TdT present, after several days of culture, in B precursors from fetal liver at day 14 of development and from para-aortic splanchnopleura was compared with that from adult bone marrow (Fig. 3B). We observed a fourfold increase of TdT mRNA level in fetal liver precursors between 4 and 8 days of culture (Fig. 3B). The amount of TdT mRNA in precursors developed in vitro is lower than the level detected in adult bone marrow when normalized for the representation of B220⁺ cells. This weak expression is nevertheless sufficient to add N regions at the rearranged V(D)J junctions in B cell precursors originating from the para-aortic splanchnopleura. The profiles obtained in cDNA of cultured cells show a shift in the mean of CDR3 size distribution from day 15 to day 22 of about three amino acids (Fig. 2), suggesting that TdT expression also...

**FIGURE 1.** Flow cytometry profiles of the progeny of clone 15. Cells isolated at day 12, 19, 22, and 32 of culture were stained with anti-B220 PE (FL2-H) and anti-IgM FITC (FL1-H). The percentages of B220⁻/IgM⁻ and B220⁺/IgM⁺ cells in the lymphocyte population defined by a FSC/SSC gate are shown.

**FIGURE 2.** Shown are the profiles generated by the immunoscope software of the PCRs performed in samples of DNA (left panel) and cDNA (right panel). Samples of sIg⁺ bone marrow cells were analyzed along with cells isolated at three different time points during the development of the progeny of one multipotent para-aortic splanchnopleura-derived precursor (designated clone 15). The first time point analyzed was at day 15 of culture, and subsequently samples were isolated at day 22 and 32. The PCR reaction was performed with the variable gene family specific J558 primer and either a constant region-specific primer or a primer hybridizing with the JH 43⁻9 intron region in cDNA or DNA analysis, respectively. y-axis: intensity of the detected band on the gel shown in arbitrary units. In both panels featuring BM the relative intensities corresponding to the size of the different CDR3 rearrangements are figured.
increases upon culture of the splanchnopleural precursors. Nevertheless, as expected, at day 22, the mean of the gaussian distribution is still one to two amino acids shorter than the mean obtained for adult bone marrow.

The presence of TdT in cultured cells is confirmed by sequence analysis (Fig. 4), since for two individual clones from splanchnopleural origin we were able to find 36 and 57% of sequences containing N additions, with a mean of four additions per junction. It is interesting to note that a substantially higher number of N sequences are observed in the V-D than in the D-J joints (Fig. 4). These results are consistent with a gradual increase in TdT levels during culture of splanchnopleura-derived B cell precursors. An up-regulation of TdT following culture of fetal B cell precursors is also suggested by the presence of N regions in V(D)J junctions from day 12 fetal liver cells after 10 days of culture (32).

Altogether, these results indicate that the differential expression of TdT in fetal vs adult life is not an intrinsic property of the hemopoietic precursors, but seems to depend on environmental cues.

Cells undergoing nonproductive heavy chain rearrangements are maintained in vitro

The immunoscope images obtained for DNA and cDNA PCR analysis isolated from bone marrow depleted of sIg" were indistinguishable (Fig. 2). As previously reported, we found a minority of the peaks of intermediate length corresponding to nonproductive rearrangements. This result is consistent with the notion that most sIg" bone marrow cells have undergone selection for the expression of a productive rearrangement (33).

The DNA profiles seen in DNA isolated from the progeny of multipotent cells at day 15 and 22 of culture (Fig. 2), although diverse, are drastically different from those seen for bone marrow, and peaks at all lengths of CDR3 are observed indicating the presence of a majority of out-of-frame rearrangements. We could never detect any nonproductive rearrangement at the cDNA level, which shows that these transcripts are probably unstable. These results show that cells with productive rearrangements are not favored under our culture conditions.

It is interesting that once in vitro-developed B cells are mature and respond to LPS, they can express only the heavy chain product of one of the chromosomes; in other words, they can accomplish allelic exclusion (30).

A semiquantitative PCR analysis performed in DNA samples after 10 and 21 days of culture indicates that the ratio of productive to nonproductive rearrangements, when the J558 VH gene primer is used, is kept constant during this period of time and is about 10-fold lower than in adult spleen (Table I). Using the same semiquantitative PCR approach, we calculated the ratio of DJ/V(D)J rearrangements in cells that develop in culture: as shown in Table I, the representation of DJ rearrangements is 5-fold higher than in splenic B cells, which indicates an enhanced proportion of pro-B cells in the cultured population.

To ensure that the intermediate-size peaks correspond to nonproductive rearrangements, we cloned and sequenced the DNA PCR products of two populations of cells that rearranged in vitro and compared them to sIg" bone marrow-derived sequences amplified and cloned under the same conditions. Figure 4 shows the CDR3 region of these sequences. Among all sequenced clones, we never found any repeated sequences. While in bone marrow we could detect ~70% of productive heavy chain rearrangements, in the cell populations developed in culture we found a minority (26–37%) of productive rearrangements. As predicted, counterselection for D regions in reading frame 2 (RF2) is observed, suggesting that cells expressing the Dμ protein are arrested in the D-J configuration. Actually, sequence analysis of D-Jμ rearrangements shows an identical representation of all reading frames, namely, 32% RF1, 36% RF2, and 32% RF3 for clone 15 and 23% RF1, 32% RF2, and 36% RF3 for clone 16 (data not shown). A similar analysis (33) done at the single-cell level in pro-B cells isolated from the bone marrow gave the same results.

These results confirmed the global PCR analysis and indicate that, in contrast to what is observed in vivo, B cell precursors in vitro express a ratio of productive to nonproductive rearrangements close to the expected frequency in the absence of selection (1:2).

Discussion

The location of the embryonic origin of hemopoietic cells has long been a matter of debate. Recently, we and others (25, 34, 35) have identified a site in the mouse embryo proper that harbors the first detectable multipotent hemopoietic precursors. This site is called the para-aortic splanchnopleura. We have previously shown that single micromanipulated cells, isolated from the para-aortic

![Figure 3](http://www.jimmunol.org/)
splanchnopleura at day 9 of gestation, that can generate lymphocytes in vitro can also generate multiple myeloid cells and are therefore multipotent (24). As a consequence, lymphocyte commitment and differentiation occur in vitro. S17 stromal cells supplemented with IL-7 and c-kit ligand fulfill the requirements for B lymphocyte precursors expansion and differentiation into

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Clone & RF 1-5% & RF 2-10% & RF 3-33% & In frame sequences 70% \\
\hline
\hline
GTT GCA GAG & TA GCT TAT GAT TAC GAC TAC TTA & C TAT & ATG & AAA AAG ACC TAC C TAA CTA GCA GAG TAC TTA \\
\hline
\hline
\end{tabular}
\end{table}

**FIGURE 4.** Sequences of the CDR3 region isolated from the progeny of two multipotent precursors. The PCR products obtained with the J558/J4 primers, shown in Figure 2, were cloned and sequenced. Clone 15 and 16 correspond to the progeny of two independent multipotent precursors. Bone marrow depleted of sIg \( ^{1} \) cells was processed the same way, as a control. No repeated sequences were found.
B cells that can respond to LPS and differentiate into plasma cells.

We have observed that from one single multipotent progenitor we could generate in vitro large numbers of B cell precursors that rearrange heavy chain genes with a large pattern of diversity, evolving during the time course of culture. After 15 days of culture, the mean of CDR3 size distribution was shorter than that of normal bone marrow, reflecting the reduced junctional diversity observed in fetal V(D)J joints in which N regions are lacking. Surprisingly, at day 22 of culture, the mean of the CDR3 size distribution is increased due to the appearance of N nucleotides additions, yet in a reduced proportion compared with bone marrow. It was noticeable that the number of N regions per junction was about sixfold higher in V-D than in D-J joints. As no such discrepancy was recorded in the same V(D)J joints from bone marrow, this difference could be explained by a lower TdT expression level during D-J rearrangement in cultured cells, rather than by differential accessibility between gene segments.

It has been proposed that external signals from the microenvironment of the developing thymus might provoke a switching on of TdT gene expression (36–38), or alternatively, that several waves of precursors harboring different potentials in the capacity to add N nucleotides sequentially colonize the lymphopoietic organs (39, 40). Induction of TdT is known to occur upon culture of neonatal thymocytes (41, 42), and N region diversity has also been shown in rearranged TCR genes in fetal thymus organ culture (36). Moreover, in adult SCID mice reconstituted with fetal liver precursors, N nucleotides are present in TCR rearrangements. In this article, we show that TdT expression occurs upon culture of fetal B precursors corresponding to different stages of the ontogeny of the immune system: from the earliest detected source of hemopoietic stem cells (day 9) to the committed B cell precursor in fetal liver (day 15). Altogether, these data suggest that, as their T cell counterparts, fetal B cell precursors have the potentiality to express TdT and are not consistent with the existence of different waves of precursors colonizing fetal liver vs bone marrow. The data support the idea that TdT expression is dependent on environmental cues, which could either induce up-regulation of TdT gene expression or gradually change the property of the stem cell lineage.

As time in culture increased, the polyclonal pattern of rearrangements became oligoclonal irrespective of whether DNA or cDNA was analyzed. Consequently, long periods of in vitro culture bias the rearrangement pattern of these samples. Our interpretation of the data is based on the fact that cells that can make a productive rearrangement at the heavy chain locus can probably progress into B cells, which are short lived under these culture conditions. The flow cytometry analysis supports this hypothesis, since we consistently detected in all clones a maximum rate of differentiation into B cells at around day 20 that quickly decreased, showing that cells cannot survive for long and that most cells differentiate synchronously. This apparent synchronization has been previously noticed in fetal liver cells (43).

Assuming a limited degree of self-renewability of the multipotent precursor, cells with V_{H}D-I_{H} rearrangements will become rare and will consequently generate an oligoclonal pattern. Finally, a majority of cells that make D-I_{H} rearrangements only will dominate the cultures, as previously reported (44) (A.C., unpublished observations). Abelson-transformed pre-B cell lines have been shown to loose expression of the RAG genes with time. Whether or not the same phenomenon is present in primary cells, it is clear that the predominance of cells at D-J_{H} stage can only be explained by a reduction in the capacity to further rearrange the Ig loci (44). In addition, cells with the D region in RF2 will stop further rearrangement due to the production of D_{μ} protein. These phenomena could also explain why LPS responsiveness is lost with time in culture.

Surprisingly, DNA isolated from the culture samples generated a pattern of rearrangement different from bone marrow, and out-of-frame rearrangements outnumbered in-frame rearrangements. Sequence analysis of cloned DNA rearrangements confirmed, at all time points tested, that a minority of rearrangements (30%) are in-frame as compared with 70% in the bone marrow sample that we used as a control. This result shows that, in vitro, cells that make nonproductive rearrangements are maintained and constitute a large proportion of the population.

We could explain these data as a result of limited survival in vitro of cells that make a productive rearrangement, leading to the accumulation of CD43^{+} cells. If accumulation of CD43^{+} cells is likely to occur with time, it can hardly be the only explanation for our observations. The predominance of out-of-frame rearrangements is seen early in the culture (day 15) before the first IgM^{+} B cells are detected. In vitro precursors can advance into later stages of differentiation and still respond to IL-7 (a part of fraction D in the Hardy nomenclature (31) or pre-B I in the Melchers nomenclature (45). If selection of cells undergoing productive rearrangements would be as efficient in culture as in vivo, we were expecting to find ratios of PNPs (productive/nonproductive) rearrangements close to those in bone marrow. The constant predominance of out-of-frame rearrangements argues for the absence of positive selection in culture.

Sequence analysis of D-J_{H} rearrangements shows equivalent proportions of all reading frames in the dominant population of precursor cells at the D-J_{H} stage, consistent with previous observations (33). In contrast, in V(D)J rearrangements, a counterselection for D-J_{H} in RF2 is seen both in our test DNA as well as in bone marrow. According to the current view, cells with the D region in RF2 will stop further rearrangement in the heavy chain locus, due to the production of D_{μ} protein. Assuming that the mechanism leading to counterselection of cells that express D-J in RF2 is the same that mediates allelic exclusion, we infer that allelic exclusion operates normally in vitro. Although in the present report allelic exclusion has not been directly tested, our previously published data demonstrate that B cells are allelically excluded in vitro. We have previously shown that cells generated in culture from Igh^{a}xlgh^{b} precursors from pre-liver embryos in response to LPS secrete the product of only one chromosome (30).

We conclude from the three major functions of the expression of a pre-B cell receptor, namely allelic exclusion, counterselection for cells expressing the D_{μ} protein, and selection for cells that made productive V(D)J rearrangements, that the first two, but not the third, are efficiently achieved in vitro. Counterselection for nonproductive rearrangement is therefore not an intrinsic phenomenon but rather is environmentally induced. We could envisage several possibilities as to the nature of this environmental influence. A ligand(s) external to the pre-B cell receptor and absent in our cultures could be the required signal inducing the expansion of pre-B cells after successful heavy chain rearrangement. Alternatively,
differential survival thresholds in B-lineage cells could account for the expansion and survival of pre-B receptor-expressing cells. Different levels of bcl-2 expression have indeed been reported at different stages of B cell development, which in situations of limiting growth factor availability might also account for differential cell survival. The experiments reported here could provide an assay system to identify these environmental stimuli required for progression of B-lineage cells.

Acknowledgments

We acknowledge the help of Isabelle Godin in the isolation of splanchnopleura regions of day 9 embryos, Sylvie Darche, and Sylvie Delassus for help in the PCR experiments, and Michèle Goodhart for critical reading of the manuscript.

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