Aggregates Are the Biologically Active Units of Endotoxin*

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For the elucidation of the very early steps of immune cell activation by endotoxins (lipopolysaccharide, LPS) leading to the production and release of proinflammatory cytokines the question concerning the biologically active unit of endotoxins has to be addressed: are monomeric endotoxin molecules able to activate cells or is the active unit represented by larger endotoxin aggregates? This question has been answered controversially in the past. Inspired by the observation that natural isolates of lipid A, the lipid moiety of LPS harboring its endotoxic principle, from Escherichia coli express a higher endotoxic activity than the same amounts of the synthetic E. coli-like hexacylated lipid A (compound 506), we looked closer at the chemical composition of natural isolates. We found in these isolates that the largest fraction was hexacylated, but also significant amounts of penta- and tetraacylated molecules were present that, when administered to human mononuclear cells, may antagonize the induction of cytokines by biologically active hexacylated endotoxins. We prepared separate aggregates of either compound 506 or 406 (tetraacylated precursor IVa), mixed at different molar ratios, and mixed aggregates containing both compounds in the same ratios. Surprisingly, the latter mixtures showed higher endotoxic activity than that of the pure compound 506 up to an admixture of 20% of compound 406. Similar results were obtained when using various phospholipids instead of compound 406. These observations can only be understood by assuming that the active unit of endotoxins is the aggregate. We further confirmed this result by preparing monomeric lipid A and LPS by a dialysis procedure and found that, at the same concentrations, only the aggregates were biologically active, whereas the monomers showed no activity.

Bacterial lipopolysaccharide (LPS)† is one of the most potent activators of the immune system in mammals. During cell growth or as a result of the action of antibacterial host factors or antimicrobial peptides, LPS is released from the outer leaflet of the cell wall of Gram-negative bacteria. Manifold interactions of LPS with host factors have been described, such as the activation of the complement system, activation of immune cells, and interaction with a variety of serum proteins, to name only a few. The most prominent activity of LPS is its immunostimulatory potency leading to the complex clinical syndrome of Gram-negative sepsis when the initial host response to an infection becomes dysregulated. The clinical manifestation of sepsis is characterized by fever, hypotension, respiratory and renal failure, and intravascular disseminated coagulation (1). These effects are not the result of LPS toxicity but are rather a consequence of cell activation by LPS and a subsequent dysregulation of the inflammatory host response. The biological activity of LPS is harbored in the lipid anchor of the molecule, termed lipid A or “the endotoxic principle” of LPS (2).

A variety of investigations in the structural prerequisites for biological activity of lipid A have revealed that the immune response is critically dependent on a certain chemical structure. Based upon detailed investigations showing that variations of the chemical structure of Escherichia coli-type lipid A, such as the reduction of the number of acyl chains or the cleavage of phosphate groups, lead to a strong decrease in biological activity (3, 4), it is generally accepted that the most potent lipid A is the hexacylated lipid A from E. coli with a bisphosphorylated diglucosamine backbone. In contrast, the nature of the physical state of biologically active LPS has long been under debate. In an aqueous environment and at concentrations above a lipid-specific concentration, amphiphilic molecules like lipid A form supramolecular aggregate structures. The type of aggregate strongly depends on the chemical structure of the aggregate-forming molecules. Combined biophysical and immunological investigations into the role of the aggregate structure for biological activity have shown that only conical molecules, which form cubic inverted aggregate structures, exhibit high endotoxic activity, whereas cylindrical molecules, forming lamellar aggregate structures, exhibit low or no endotoxic activity (5). Thus, the shape of the aggregate-forming molecules, including the aggregate structure, correlate with the ability to activate host cells. This dependence has been shown for a variety of endotoxin and non-endotoxin structures (6). The information on the nature of the biologically active unit of LPS is of utmost importance and can be very valuable for an understanding of the molecular mechanisms of cell activation. Thus, there is only limited information on the specific interaction of LPS with cellular receptors such as CD14 or Toll-like receptor 4 (TLR4) (7).

Synthetic lipid A analogues and partial structures are invaluable tools for systematic elucidation of the physicochemical requirements for biological activity. The available preparations

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have a broad range of biological activities, ranging from high endotoxicity for compound 506, corresponding to the hexacylated lipid A from *E. coli*, to compounds without endotoxic activity in humans such as compound 406, corresponding to the tetracylated lipid A precursor IVa. However, we describe here that, in contrast to what one might expect and what was found earlier (8), the biological activity of pure synthetic preparations of hexacylated lipid A from *E. coli* (compound 506) is much lower than that of preparations of natural lipid A. Based on this observation, we analyzed the chemical composition of several natural lipid A preparations. The data presented reveal high heterogeneity of natural lipid A preparations, an observation that has been described earlier by others as well (9). The consequences of mixing chemically different lipid A for biological activity are, however, not well understood. We asked the question whether the higher biological activity of these heterogeneous lipid A preparations is due to the influence of heterogeneity on the physical state of such preparations. Using chemically well characterized lipid A and LPS preparations, we performed a panel of experiments to investigate the importance of the chemical composition for the biological activity of lipid A preparations. Thus, we prepared mixtures of compounds 506 and 406 in varying molar ratios and observed an increase in endotoxic activity as compared with the same total amount of pure 506 at low admixtures of 406 (<20%) when the compounds were mixed in chloroform prior to their transference into the aqueous phase to form mixed aggregates (aggr.(506 + 406)) and their administration to the immune cells. However, when pure aggregates of compound 506 and of compound 406 were mixed in water (aggr.(506) plus aggr.(406)), the expected antagonistic action of 406 was observed. We then performed experiments using various phospholipids instead of compound 406 and arrived at very similar results and concluded that underacylated endotoxin or even phospholipid molecules introduce heterogeneities in endotoxin aggregates and, thus, reduce the binding energy. These results seemed, therefore, to support the hypothesis that the biologically active state of endotoxin is the aggregate and not the monomer. To further prove this hypothesis, we applied a dialysis procedure to separate endotoxin monomers from mixed suspensions of aggregates and monomers and compared the biological activities of monomers and aggregates at same concentrations. These experiments provided unequivocal evidence that only endotoxin in the aggregated form is biologically active.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**

Lipids and Other Chemicals—Deep rough mutant Re LPS from *E. coli* strain F515 and rough mutant Ra LPS from *Salmonella enterica* sv. Minnesota strain R60 were extracted by the phenol/chloroform/petroleum ether method (10), purified, lyophilized, and transformed into the triethylamine salt form. The synthetic hexacylated lipid A compound 506 of the synthetic tetracylated lipid A compound 406 were synthesized as described earlier (11–13). Diposphatidylglycerol (cardiolipin, CL) was obtained from Avanti Polar Lipids (Alabaster, AL).

For the preparation of LPS, lipid A, or CL suspensions, the compounds were first suspended in chloroform, and the solvent was evaporated under N₂. The dry lipids were then resuspended in distilled water by thorough vortexing and the suspension was temperature cycled at least twice between 4 and 56 °C, each cycle followed by intense vortexing for a few minutes, and then stored at 4 °C for at least 12 h prior to measurement. We prepared mixtures of compounds 506 and 406 in two different ways: 1) The two compounds were mixed in chloroform at different molar ratios, the solvent was evaporated, and the dry mixtures were resuspended in distilled water to produce aggregate suspensions that were mixed on the molecular level, consisting of both types of molecules, 406 and 506. These mixed aggregates are termed “aggr.(506 + 406)” 2) Aggregates containing only compound 506 or compound 406 were prepared as described above (evaporation of solvent, and resuspension in distilled water), and the aggregates were mixed in the aqueous phase in different molar ratios. These aggregate mixtures are termed “aggr.(506) plus aggr.(406).” Preparations with CL instead of compound 406 were done analogously.

**Preparation of Monomer Suspensions**—Monomer solutions were obtained in a dialysis setup consisting of a donor and an acceptor chamber, separated by a dialysis diaphragm with a cut-off of 5000 Da, thus allowing only monomers to pass. The donor chamber was filled with endotoxin suspensions at a concentration (10⁻⁶ μl) well above the assumed critical micellar concentration of about 10⁻⁹ M (14). The acceptor chamber was filled with distilled water. After 48 h, the concentrations of endotoxin aggregated from monomers and endotoxin molecules at the acceptor side, respectively, were determined mass spectrometrically via the measurement of the hydroxy-fatty acid concentration. For this, a deuterium-labeled standard (30H-D5-14:0, Amersham Biosciences) was added to each sample. The samples were then lyophilized, resuspended in 1 ml of 4 N HCl, and heated for 4 h at 100 °C. After centrifugation, the samples were resuspended in 1 ml of 5 N NaOH and incubated for 30 min at 100 °C. The fatty acids were extracted with chloroform, and the solvent was evaporated under N₂. For analysis in the GC-MS (GC model 5890, MSD model 5970, both Hewlett Packard) the fatty acids were methylated with diazomethane. The endotoxin concentration was determined by calculating the area ratio of the standard peak and hydroxy fatty acid mass peak.

**Stimulation of Human Mononuclear Cells**—In experiments for determining the cytokine-inducing capacity of LPS, human mononuclear cells (MNCs) were stimulated with LPS, and tumor necrosis factor (TNF) α production of the cells was determined in the supernatant. Blood for human mononuclear cell preparation was obtained from healthy volunteers. MNCs were isolated by separation from whole blood, using Ficoll-Hypaque as previously described (15).

Stimulation experiments were done either under serum-free conditions or in the presence of serum or LPS-binding protein (LBP). For stimulation experiments under serum-free conditions, cells were washed several times in RPMI 1640 (containing 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin), and 900 μl MNCs (1 × 10⁶ cells/well) were transferred to 96-well culture plates. For the measurement of the presence of serum or LBP either 4% AB serum or 100 ng/ml LBP (kind gift of R. Dedrick, XOMA Corp.) was added to the cell culture medium. LPS was serially diluted in RPMI 1640 and added to the cultures at 20 μl per well. The cultures were incubated for 4 h at 37 °C and 5% CO₂. Supernatants were collected after centrifugation of the culture plates for 10 min at 400 × g and stored at −20 °C until determination of cytokine content.

The immunological determination of TNFs in the cell supernatant was determined in a sandwich–enzyme-linked immunosorbent assay as described elsewhere (16). Microtiter plates (Greiner, Solingen, Germany) were coated overnight at room temperature with a monoclonal mouse antibody against human TNFs (Intex AG, Switzerland) followed by three washings with water. One-hundred-microliter samples of culture supernatants were dispensed into the wells and incubated with horseradish peroxidase-conjugated rabbit anti-human TNFα antibody (Intex) for 16–24 h at 4 °C. After washing, the color reaction was started by addition of tetramethylbenzidine/H₂O₂ and stopped by the addition of 1 M sulfuric acid. Serial dilutions of human recombinant TNFα (Intex) provided a standard curve. Plates were read at 450 nm with an enzyme-linked immunosorbent assay photometer. Quantification of TNFα was determined in duplicate with detection ranges of 0–500 pg/ml. Due to donor-specific variations in the TNFα production by the different serums, the data set out of three is shown, and data given are mean of triplicates ± S.D.

**Lymphoid Amebocyte Lysate Assay**—Endotoxin activity of aggregates and monomers was determined by a quantitative kinetic assay based on the reactivity of Gram-negative endotoxin with *Limbulus* amebocyte lysate (LAL) at 37 °C using test kits of LAL Coasym Chromo-LAL K (Sturm & Schindler GmbH, Kempten, Germany). The hemolymph concentrate used in this test was from *E. coli* (O55:B5), and 1 ng/ml of this LPS corresponds to ∼10 endotoxin units/ml according to the manufacturer. In this assay, saturation occurs at 125 endotoxin units/ml and the resolution limit is >0.1 endotoxin unit/ml (maximum value for ultrapure water for embryo transfer, Sigma). Endotoxin-free water (Aqua B. Braun) was used for all dilution. Data shown are mean of triplicates ± S.D. of two individual experiments.

**Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry**—The chemical structure and the biological heterogeneity of the lipid A used in the different experiments were controlled by...
MALDI-TOF MS applying a Bruker-Reflex II instrument (Bruker-Daltonik, Bremen, Germany) in linear configuration at an acceleration voltage of 28 kV. Lipid A samples were dissolved in chloroform/methanol (50:50, v/v) at a concentration of 2 μg/µl and treated with a small amount of an Amberlite IR-120 (H⁺) cation exchange resin to remove disturbing sodium and potassium ions as described elsewhere (18). A 1-µl sample was mixed with a 1-µl matrix solution (0.5 M 2,4,6-trihydroxyacetophenone, Aldrich) in methanol. Aliquots of 0.5 µl were deposited on a metallic sample holder and analyzed immediately after drying in a stream of air. Mass spectra were recorded in negative and positive ion modes. Mass scale calibration was performed externally with similar compounds of known chemical structure.

RESULTS

Variations in the Chemical Structure and Biological Activity of Synthetic Lipid A Analogue 506 and Natural Lipid A Preparations—The chemical structure of the lipid A part of LPS critically determines the biological activity of lipid A and LPS preparations. When stimulating MNCs with synthetic and natural lipid A preparations ex vivo to produce TNFα, the biological activity of pure preparations of synthetic hexaacylated compound 506 showed reduced TNFα production in comparison to equal amounts of preparations of natural lipid A, such as lipid A derived from E. coli strain F515 (Fig. 1). Based upon this observation, we analyzed the chemical composition of the synthetic compound 506 and several natural lipid A preparations by MALDI-TOF mass spectrometry. As expected the negative ion mass spectrum of compound 506 (Fig. 2A) comprises only one abundant molecular ion at m/z 1796 representing the pure hexaacylated bisphosphorylated lipid A. In contrast, the analysis of two different batches of natural lipid A isolated from LPS of E. coli A and B. Data represent one out of three independent experiments, and data given are mean of triplicates ± S.D.

Considering the significant quantities of antagonistic structures in the natural preparations of lipid A, their biological activity is particularly surprising. We put up the hypothesis that the presence of endotoxically inactive structures in those preparations have an impact on the physical state of the biologically active structures and by this means increase their endotoxic activity.

Molecular Composition of Aggregates Critically Determines Their Biological Activity—Even though there is a broad collection of data showing a strong relationship between aggregate structure and biological activity, the role of the physical state of endotoxin for the display of endotoxic activity is not very well understood. We sought to investigate whether the observed differences in biological activity of homo- versus heterogeneous endotoxin preparations is the result of differences in the physical state of such preparations. We used two chemically pure and highly defined synthetic lipid A preparations to address this question, the endotoxically active compound 506 and the endotoxically inactive and even antagonistic compound 406, and prepared mixtures of these in two different ways to obtain either mixed aggregates (aggr. 506 + 406) or aggregate mixtures (see “Experimental Procedures”). The two differently prepared mixed suspensions were used to stimulate human mononuclear cells to produce TNFα. In Fig. 3A the result of a representative stimulation experiment is shown. It is obvious that there is a clear difference in the biological effects in dependence on the type of aggregates added to the cells. When compounds 406 and 506 were added as aggregate mixtures (aggr. 506 + plus aggr. 406) to stimulate the cells, the production of TNFα induced by compound 506 was reduced in the presence of compound 406, representing the known antagonizing effect of compound 406. This antagonistic effect of compound 406 was already evident at a ratio of 406 to 506 of 0.1:1.

However, when mixed aggregates of compounds 406 and 506 (aggr. 506 + 406) were used to stimulate the cells, the results were different depending on the molar ratios of compound 406 and compound 506. In contrast to stimulation with aggregate mixtures (aggr. 506 plus aggr. 406), stimulation with mixed aggregates (aggr. 506 + 406), at molar ratios of 406 to 506 between 0.1:1 and 0.2:1, resulted in clearly higher biological activity than stimulation with compound 506 alone. This observation clearly shows that the antagonistic action of 406 depends on the physical state of the molecule, which is obviously different in mixed aggregates than in pure aggregate suspensions. However, at molar ratios of 406 to 506 equal or above 0.5:1, the biological activity was reduced, showing an antagonistic effect of compound 406. Analogous results can be observed, when, instead of compound 406, suspensions of phospholipids (PLs) such as diphosphatidylglycerol CL (Fig. 3B), phosphatidylcholine, or lysophosphatidylcholine, respectively, were used (data not shown). Thus, the presence of these phospholipids at low molar ratios in preparations of compound 506 (aggr. 506 + 1PL) led to an increase of TNFα production of MNCs, whereas aggregate mixtures of phospholipids and compound 506 (aggr. 506 plus aggr. 1PL) led to a decrease of TNFα production. The observed differences in the biological activities of the differently prepared aggregate suspensions allow us to conclude that the activation of cells, as well as the inhibition of cell activation by antagonistic structures, are most likely mediated by aggregates instead of monomeric molecular endotoxin structures.

Lipid A and LPS Display Their Biological Activity Only in the Form of Aggregates but Not as Monomers—To get further evidence for the nature of the biologically active unit of endotoxin, we constructed a dialysis system that allows the separation of monomers from aggregate suspensions and that always

![Graph](image-url)
contains a certain number of monomers. The dialysis system consists of two Teflon chambers, separated by a dialysis membrane with a molecular mass cut-off of 5000 Da. Because the molecular mass of hexaacylated *E. coli*-type lipid A is 1797 Da, only monomers or dimers are supposed to pass the membrane. Dimers are, however, not stable in aqueous environments and decompose readily to monomers. Aggregate suspensions at a concentration of $10^{-6}$ M, which is still well above the assumed critical micellar concentration of lipid A of $-10^{-8}$ M (14, 22), were filled into one side of the dialysis chamber, and samples were taken from both sides after 48 h and analyzed to determine the endotoxin concentration by GC-MS via the determination of the concentration of the hydroxy fatty acids. With this method concentrations of endotoxin as low as $5 \times 10^{-10}$ M could be determined.

Using aggregated and monomeric samples of lipid A or of Re LPS (Fig. 4) to stimulate human mononuclear cells showed, that aggregate suspensions have significantly higher biological activity than monomer solutions of identical concentration. In independent stimulation experiments using cells from various

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**Fig. 2. Variations in the chemical structures of synthetic lipid A analogue 506 and of natural lipid A.** Negative ion MALDI-TOF mass spectra of synthetic hexaacyl lipid A analogue 506 (A) and natural lipid A isolated from different batches of LPS obtained from *E. coli* strain F515 (B and C).
down to concentrations of 10

The elucidation of the biologically active chemical structure of LPS has been addressed by a number of scientists in the past. The definition of lipid A as the “endotoxic principle” was a benchmark, initiating a variety of investigations to pinpoint the chemical groups that harbor the activating principles within the lipid A structure. The data available assemble into a detailed picture of the submolecular structures that are important for biological activity. However, bacteria display and release a variety of different LPS species from their outer membranes. As we describe here and has been discussed by others in the past (23, 24), natural preparations of LPS or lipid A do not contain a single molecular species with defined chemical structure but are complex mixtures of molecules differing mainly in the degree of acylation (Fig. 2). This heterogeneity leads to surprising consequences for biological activity. In contrast to what one might expect, the presence of low concentrations of structures, which per se express low agonistic or even antagonistic activity found in natural LPS preparations such as pentaacetylated or tetraacetylated lipid A, respectively, does not lead to a decrease but to an increase in the inflammatory activity of the respective LPS or lipid A preparation. In line with this observation is the finding, that the synthetic compound 506 is less active than the according natural lipid A preparation derived from E. coli (Fig. 1). The difference in the biological activity between the different preparations becomes even more obvious when considering that in the case of sample A only ~80% and in the case of sample B ~45% of the total mass correspond to hexaacylated Lipid A. These observations could be confirmed and can be explained by the results from mixing experiments, in which we compared the biological activity of defined mixtures with those of the respective pure components of the mixture. The presence of chemical structures such as biologically non-active compound 406 at low molecular ratios in preparations of hexaacylated lipid A leads to an increase in biological activity (Fig. 3A). The same effect could be observed when, instead of compound 406, tetraacylated diphosphatidylglycerol CL (Fig. 3B), diacylated phospholipids such as PC, or even lysophospholipids were used. Because these non-lipid A structures give rise to the same biological effect as antagonist lipid A structures, the underlying mechanism is unlikely to be based on specific chemical structures of these compounds. The enhancing effect on the biological activity of homogenous hexaacyl lipid A by the admixture of low amounts of amphiphilic compounds with less than six fatty acid residues was observed, however, only when the two compounds were mixed in an organic phase and the mixture was then transferred to the aqueous phase. The mixtures thus represented mixed aggregates. By mixing the preformed aggregates of the two compounds in the aqueous phase, the aggregates stay separated, and under these conditions the presence of aggregates made from per se antagonist molecules leads to the expected antagonistic effect already at low concentrations (Fig. 3, A and B). Thus, it seems more likely that a mechanism responsible for the enhancing effect of compound 406 or phospholipids in mixed aggregates could be the reduction of the binding energy in aggregates by the presence of molecules of different chemical structure. In crystallography such heterogeneities would be defined as imperfections. These results definitely support the idea that aggregates play an important role in cell activation by endotoxin.

The question, whether LPS activates host cells as monomeric molecules or as multimeric aggregates, has been under debate for a long time. Several publications have addressed this question with contrasting results. Shnyra et al. (25) showed that aggregated LPS activated the LAL cascade to a higher degree than monomerized LPS and was lethal in galactosamine-sensitized mice in lower doses, whereas Takayama et al. (26) found that LPS is an order of 2.5 more active in stimulating a pro-B-cell line in a disaggregated state (26) and was more active in the LAL assay than aggregated LPS (27). Several years ago,
the term pathogen-associated molecular pattern was introduced as a superordinate term for a variety of molecules displayed by pathogens such as LPS, peptidoglycan, and lipopeptides. These molecules are recognized by pattern recognition receptors (PRRs) on the host cell, leading to an inflammatory response. The term pattern implies that PRRs recognize recurrent molecular motives such as those displayed in supramolecular structures represented by aggregates. With the aim of supporting the results from the mixing experiments (Fig. 3), which give evidence that the biological activity of LPS, whether agonistic or antagonistic, is displayed by aggregates rather than by single molecules, we employed a dialysis system to separate monomers from aggregate suspensions. With these experiments we could confirm these results and show that monomers, separated from aggregate suspensions, are not able to stimulate cells. Thus, the presentation of molecules, monomers or aggregates, by CD14 and LBP seems to be as important as the transport activity of these proteins. For example, very recent publications show that CD14 delivers

**Fig. 4.** Endotoxin aggregates are more potent stimulators of immune cells than monomers. TNFα production from MNCs after stimulation with lipid A and Re LPS aggregates (light boxes) and monomers (dark boxes) in the same concentrations. A, serum-free conditions; B, addition of 100 ng/ml LBP; C, addition of 4% AB serum. Data represent one out of three independent experiments, and data given are mean of triplicates ± S.D.; N.D., not detectable.

**Table I**

| Culture conditions | Lipid A monomers (\(10^{-9}\) M) | Re LPS monomers (\(10^{-9}\) M) | Ra LPS monomers (\(10^{-9}\) M) |
|-------------------|-------------------------------|-------------------------------|-------------------------------|
|                   | Control                       | 2.0 ± 0.9                     | 2.1 ± 1.8                     | 6.6 ± 4.3                     |
|                   | Serum-free                    | 1.4 ± 1.3                     | 0.7 ± 1.2                     | 0.6 ± 1.0                     |
|                   | + LBP (100 ng/ml)             | 2.0 ± 0.9                     | 5.2 ± 5.2                     | 2.0 ± 1.9                     | 2.2 ± 2.4                     |
|                   | + AB-serum (4%)              | 5.4 ± 7.2                     | 7.2 Not done                  | 2.4 Not done                  |
LPS Activates Cells Only in the Aggregated State

Fig. 5. Endotoxin aggregates are more active in the Limulus amoebocyte lysate assay than monomers. Endotoxic activity of lipid A and Re LPS aggregates (light boxes) and monomers (dark boxes) in same concentrations (10⁻⁹ m) in the Limulus amoebocyte lysate assay. Data shown are mean of duplicates.

LPS to a complex of TLR4 and MD2 (31). However, the nature of LPS in these complexes has not been defined. Our data unequivocally show that aggregates are the biologically active unit of LPS under serum-free conditions as well as in the presence of LBP or of human serum. The fact that LPS monomers show no endotoxic activity even when LBP or Re serum is present indicates that these molecules can obviously not be presented to cells in a manner that is sufficient for cell activation.

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