Synergism in the activation of human CD8 T cells by cross-linking the T-cell receptor complex with the CD8 differentiation antigen

(T-cell activation/T-cell differentiation antigens/T-cell receptor)

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ABSTRACT Resting human T cells can be activated and induced to proliferate by cross-linking the T-cell receptor complex (Ti/CD3) with anti-CD3 (T3) antibodies, such as OKT3, together with interleukin 2. Here we describe functional properties of another monoclonal anti-CD3 antibody (BMA 030) that, cross-linked in various ways, only weakly stimulates accessory-cell-depleted T-cell cultures. However, when cross-linked to anti-CD4 or anti-CD8 antibodies a markedly enhanced proliferation (mAb) to the corresponding subpopulation is observed. We have concentrated on the analysis of CD8 cells and have found that BMA 030, when cross-linked together with anti-CD8 (T811), induced proliferation more than 100-fold greater than BMA 030 alone, whereas cross-linking with antibodies to other T-cell membrane antigens (HLA-A, B, or CD5) provided no or marginal synergistic signals. There was no synergistic effect when only one of the two antibodies, BMA 030 or T811, was cross-linked and the other was applied in soluble form. In contrast, each of the two antibodies alone, when applied in soluble form, inhibited activation induced by the cross-linked antibodies. The T-cell differentiation antigen CD8 has been implicated in the major histocompatibility complex (MHC) class I restricted specificity of CD8 T cells. In previous work from other laboratories only the negative influences of soluble anti-CD8 antibodies have been noted. In contrast, our results suggest that cross-linking between Ti/CD3 and CD8 may be a critical event in the activation of mature CD8 cells. We hypothesize that, in antigen-induced T-cell activation, CD8 and Ti/CD3 become cross-linked by their simultaneous binding to class I-associated structures. Such a mechanism, if required for proliferation in early T-cell ontogeny, could generate a selective pressure for CD8 cells to recognize class I-associated antigens.

Most human T lymphocytes bearing the CD4 (T4) differentiation antigen display helper activity and are restricted to major histocompatibility complex (MHC) class II antigens, whereas most T cells bearing the CD8 (T8) antigen are restricted to MHC class I antigens and exhibit cytotoxic or suppressor function (1, 2). The correlation of phenotype with MHC restriction appears more stringent than that with function (1, 2), and there is indirect evidence that CD4 and CD8 might be implicated in T-cell recognition: Monoclonal antibodies (mAbs) to CD4 or CD8 inhibit activation and effector functions of cells with the corresponding phenotype (3–5). Moreover, results show abrogation of the development of the T4 lineage by treating mice from birth with anti-Id antibodies (6).

Functional roles of several T-cell membrane antigens in T-cell activation have been identified. In particular, anti-CD3 (T3) mAbs, when coupled to Sepharose beads, induce T-cell proliferation without accessory cells (7). This effect requires addition of exogenous interleukin 2 (IL-2) for resting but not for activated T cells (7, 8). The CD3 antigen is noncovalently associated with the antigen receptor of T cells (9, 10), and triggering of the T-cell receptor complex (Ti/CD3) via anti-CD3 is thought to exert signals on intracellular second messengers similar to triggering by antigen (11). In contrast, anti-CD4 and anti-CD8 antibodies, even when coupled to Sepharose beads, did not activate T cells, and soluble anti-CD4 and anti-CD8 antibodies were shown to inhibit T-cell activation (5, 7).

Here we show a synergistic effect in the induction of T-cell proliferation by cross-linking Ti/CD3 and CD8 antigens on the T-cell membrane. For these experiments an anti-CD3 mAb (BMA 030) was used that on its own is less efficient in T-cell activation than the prototype anti-CD3 antibody OKT3. We think that interaction of the Ti/CD3 complex with BMA 030 may more closely reflect its interaction with antigen. Cross-linking of Ti/CD3 with CD8 could be achieved in antigen activation by simultaneous binding of both to MHC class I-associated structures on the stimulator cells. Moreover, a similar cross-linking might induce growth during T-cell ontogeny, thus providing a selective pressure for the generation of CD8 cells whose receptors have affinity for MHC class I products.

MATERIALS AND METHODS

mAb to T-Cell Antigens. OKT3 and BMA 030 are anti-CD3 mAb of the mouse IgG2a isotype obtained from Ortho Pharmaceutical (Raritan, NJ) and from Behringwerke, respectively. The anti-HLA-A,B was obtained from Hybritech (San Diego, CA); the anti-β2-macroglobulin antibody was from Becton Dickinson. T321 (IgG1, anti-CD4), T811 (IgG1, anti-CD8), and T61 (IgG1, anti-CD5) were kindly provided by P. Rieber, Munich. All antibodies were classified by the International Workshop on Human Leucocyte Differentiation Antigens (12). The antibodies were calibrated for mouse immunoglobulin concentration.

T-Cell Isolation. Human peripheral blood mononuclear cells were isolated from healthy volunteer donors by Ficoll/metrizoate (Pharmacia) density-gradient centrifugation. They were further purified by depletion of adherent cells on plastic surface (Nunc, or Greiner, Nürtingen, F.R.G.) at 37°C for 2 hr followed by gentle pipetting of nonadherent cells. Subsequently, T cells were separated from B cells and residual monocytes by rosetting with 2-aminoethylisothiouronium bromide hydrobromide (Sigma)-treated sheep erythrocytes and centrifuged on Ficoll/metrizoate gradients (13). The erythrocytes were lysed by 0.87% Tris-buffered NH4Cl, pH 7.2, for 10 min. For the experiments shown in Table 2 the T-cell populations were in addition incubated with an antimonocyte mAb (Mono BRL, Bethesda Research Laboratories) and an anti-natural killer cell mAb (NKH-1). After

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Abbreviations: mAb, monoclonal antibody; MHC, major histocompatibility complex; PHA, phytohemagglutinin; IL-2, interleukin 2; r, recombinant; Ti/CD3, T-cell receptor complex.
indirect staining with fluoresceinated anti-mouse immunoglobulin, the fluorescence-negative population was collected by cell sorting (see next section for details). For the experiments depicted in Figs. 2–4 negative selection was performed with the mAb Mono-BRL in conjunction with an anti-monomorphic Ig mAb (MAX.21) developed in our laboratory (14). Each preparation was controlled by staining with α-naphthylacetate esterase. No monocyte could be found by microscopic analysis that included regularly 5–10 × 10^6 cells.

Flow Cytometry and Cell Sorting. Phenotypic analysis with anti-CD3 mAb was performed by indirect immunofluorescence with fluorescein isothiocyanate-conjugated F(ab')2 goat anti-mouse immunoglobulin (Tago, Burlingame, CA) as described (15). Human T cells (10⁴ cells) were analyzed in each sample on an Ortho Cytofluorograf 50H (Ortho Instruments). For fluorescence-activated cell sorting 10⁴ cells were incubated under agitation with anti-T-cell mAb at 5 μg/ml for 30 min at 4°C. Unbound mAb was removed by washing (twice with 2 ml) the cells in medium, and fluoresceinated F(ab')2 goat anti-mouse immunoglobulin was added for another incubation period followed by two washing steps.

Cell Culture and Proliferation Assay. Cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 2-mercaptoethanol (10⁻⁵ M, final concentration)/l-glutamine (2 mM)/penicillin (100 units/ml)/streptomycin (100 μg/ml)/Hepes (25 mM), pH 7.2/10% (vol/vol) preselected human serum (blood group AB) for 4 or 5 days at 37°C in a humidified atmosphere of 5% CO₂/95% air. Reconstituant (r) IL-2 was calibrated (16) and added in a final concentration of 100 units/ml (=80 ng/ml). Proliferative responses were determined by adding 1 μCi of [³H]thymidine per microculture well for 16 hr and harvesting the cultures onto glass fiber filters. [³H]Thymidine incorporation was measured in a liquid scintillation spectrometer.

Surface Labeling and Immunoprecipitation Procedures. Viability, proliferating Jurkat cells (1 × 10⁶ cells) were surface labeled with ¹²⁵I (Amersham) by the Enzymobead radiolabelling system (Bio-Rad). Labeled cells were washed four times in cold phosphate-buffered saline (PBS, pH 7.2) and lysed by Nonidet P-40 (final concentration, 0.5%/1 mM phenylmethylsulfonyl fluoride, pH 8.0, for 25 min at 4°C). Aggregated proteins were removed by centrifugation for 20 min at 100,000 × g. Cell lysates were then preclarified by incubation with an equal volume of protein A-Sepharose (Pharmacia) and 20 μg of a nonreactive mAb. Preclarified lysates were incubated with 15 μg anti-T-cell mAb for 2 hr at 4°C followed by protein A-Sepharose for another 1 hr. Precipitates were washed three times in 1% Nonidet P-40, once in 0.5 M NaCl, once in distilled H₂O, and dissolved in sample buffer for NaDodSO₄/PAGE. After addition of 10%/vol/2-mercaptoethanol and heating (3 min, 95°C), aliquots of each sample were applied to 10% acrylamide gels (17). Radioactively labeled precipitates were visualized by autoradiography.

Cytotoxicity Assay. Cells were assayed for cytotoxicity in a 4-h ⁵¹Cr release assay. Effector cells were added to 2 × 10³ ⁵¹Cr-labeled P815 mouse mastocytoma cells or K-562 human erythroleukemia cells. For lectin-facilitated lysis phytohemagglutinin (PHA, GIBCO 670/0576, 1% of stock solution) was added. After incubation, supernatant was removed, and radioactivity was measured. Percent specific lysis was calculated from cpm as 100 × (experimental − lower control)/acid lysis − lower control). Each effector/target cell ratio was assayed in triplicate.

RESULTS

Responses of Fluorescence-Activated Cell Sorter-Purified CD8 and CD4 T Cells to Subsequent Stimulation with Anti-CD3 and IL-2. Sorting of human T-cell subpopulations with anti-CD8 or anti-CD4 was done with fluoresceinated anti-mouse immunoglobulin as second antibody. Positively and negatively selected T cells (Fig. 1) were cultured in the presence of the anti-CD3 mAb BMA 030 in soluble form, together with exogenous rIL-2. A considerable proliferative response was observed in subpopulations positively selected either for CD4 or for CD8 (Table 1), whereas the corresponding negatively selected populations did not respond. The response of CD8 cells was higher than that of CD4 cells possibly reflecting the different accessory cell requirements that were demonstrated for mitogen-activated LYT-2* cells and LYT4* cells in mice (18). Alternatively, a lower density of CD4 molecules on resting T cells might be critical (see Fig. 1). The marginal response of CD8 cells with IL-2 alone could be explained by in vivo-activated cells that may be present in small amounts in peripheral blood. They were excluded in further experiments by adding an anti-IA antibody during negative selection.

The increased responses of positively selected T cells suggested that the previous exposure of the cells to the subpop-specific antibodies sensitized the cells to a subsequent stimulation with anti-CD3. The critical step in this reaction may have been either the binding of anti-CD8 (anti-CD4) to its antigen or the cross-linking of several CD8 (CD4) molecules by microprecipitation of the antibodies by the fluoresceinated anti-mouse immunoglobulin antibody. As a third possibility, free valences of the anti-mouse immunoglobulin antibody may bind the subsequently added anti-CD3 antibody and may lead to CD3–CD8 (CD4) cross-linking as the critical event.

Anti-CD8 and Anti-CD3 (BMA 030) Synergistically Induce Proliferation of CD8 Cells When Cross-Linked on the T-Cell

Table 1. Proliferative response of human T-cell subpopulations in the presence of BMA 030 (anti-CD3) and rIL-2

<table>
<thead>
<tr>
<th>Cells</th>
<th>BMA 030</th>
<th>IL-2</th>
<th>BMA 030/IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4/anti-CD4⁺</td>
<td>55 ± 5</td>
<td>124 ± 92</td>
<td>1.959 ± 205</td>
</tr>
<tr>
<td>CD4/anti-CD8⁻</td>
<td>38 ± 13</td>
<td>46 ± 8</td>
<td>107 ± 39</td>
</tr>
<tr>
<td>CD8/anti-CD4⁻</td>
<td>27 ± 2</td>
<td>546 ± 16</td>
<td>2.141 ± 1.096</td>
</tr>
<tr>
<td>CD8/anti-CD8⁺</td>
<td>85 ± 33</td>
<td>484 ± 75</td>
<td>31.639 ± 4.626</td>
</tr>
</tbody>
</table>

T cells were stained with anti-CD4 or anti-CD8 followed by fluoresceinated anti-mouse immunoglobulin. They were positively or negatively selected in a fluorescence-activated cell sorter as shown in Fig. 1. Cells (5 × 10⁶ cells per well) were cultured in round-bottomed wells for 5 days. BMA 030 was at 20 ng/ml, and rIL-2 was at 100 units/ml. Results are expressed as mean cpm ± SD (n = 3).
Membrane by F(ab')2 Anti-Mouse Immunoglobulin. In the following set of experiments we tested whether cross-linking by anti-mouse immunoglobulin antibody was necessary for the sensitization of T cells by anti-CD8 to subsequent exposure to anti-CD3 antibody. Negatively selected CD8 cells were first exposed to a variety of anti-T-cell mAb, followed after washing by anti-mouse immunoglobulin antibodies. Cells treated in this way and appropriate control cells were then exposed to anti-CD3 and tested for their proliferative response. It should be noted that the CD8 cells used in this experiment had been depleted of natural killer cells, some of which have been reported to express CD8 and/or CD3 (19, 20). The two experiments in Table 2 show that the anti-mouse immunoglobulin antibody was necessary for sensitization to occur. Moreover, antibodies to other surface antigens (CD5, β2-microglobulin) had no or only marginal effects. Most importantly, application of anti-CD3 together with anti-CD8 in the first step, in the presence of anti-mouse immunoglobulin, had a similar stimulating effect as the pretreatment with anti-CD8 and anti-mouse immunoglobulin followed by stimulation by anti-CD3. This latter finding suggested that the critical event may be a simultaneous cross-linking of CD3 with CD8, rather than the presensitization due to binding or cross-linking of the CD8 antigen.

Solid-Phase Attached Mixtures of Anti-CD3/Anti-CD8 Cause Activation of Individual Resting T Cells. The experiments in Tables 1 and 2 had been performed in round-bottom microc wells, albeit at low cell numbers (<10⁶ cells). It was nevertheless possible that one of the requirements for T-cell stimulation was agglutination of cells, which is expected to take place under these conditions. To minimize this possibility we employed flat-bottom microc wells coated with antibodies, which leads to individual attachment of cells with little contact between them. Anti-mouse immunoglobulin antibodies were coated to the plastic surface, and anti-T-cell mAb were added, alone or mixed at 1:1 molar ratios. Prior to addition of cells excess antibodies were removed by washing.

The T-cell population was depleted extensively of Ia⁺ positive cells and residual monocytes by negative selection with anti-Ia and anti-monocyte antibodies such that no esterase-positive monocyte could be found upon inspection of 10⁵ T cells. As can be seen in Fig. 2 over a broad range of cell numbers cross-linked BMA 030 alone was far less effective than BMA 030 cross-linked with an anti-CD8 antibody. Anti-CD8 alone was ineffective. Cross-linking of BMA 030 with HLA class I antigens also increased the proliferative response to a certain extent. This effect was more pronounced as in the experiments described in Table 2, perhaps because of the more effective cross-linking achieved by solid-phase attachment of antibodies. In comparison to BMA 030, the standard anti-CD3 mAb (OKT3) gives the maximal response also when used alone.

These cultures also allow repeated microscopic observation of individual cells. After 4 days blast transformation and cell division could be seen in 5–15% of the T cells. This represents a good proportion of all CD8 cells (20–40% in unseparated T cells), corresponding to frequencies of activation reported for other polyclonal activation protocols (21).

T-Cell Activation by Cross-Linking of CD3 (BMA 030) and CD8 Is Inhibited by Soluble Antibodies to Either Membrane Antigen. We tried to inhibit the activation caused by BMA 030 cross-linked with T811 by soluble antibodies. For these experiments (Fig. 3) solid-phase antibodies had to be coupled.

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**Table 2.** Proliferative response of CD8 cells in the presence of BMA 030 and IL-2 after incubation with anti-T-cell mAbs with or without cross-linking by F(ab')2 anti-mouse immunoglobulin

<table>
<thead>
<tr>
<th>Pretreatment of CD8 cells</th>
<th>[H]Thymidine incorporation</th>
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<tbody>
<tr>
<td></td>
<td>mAb</td>
<td>amlg</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>T811 -</td>
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<tr>
<td></td>
<td>T811 +</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BMA 030 +</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>T61 +</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2 + -</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>T811 +</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>T61 +</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>αβ μm +</td>
<td>-</td>
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</tbody>
</table>

CD8 cells of two different donors were negatively selected after incubation with anti-CD4 (T321) and anti-natural killer (NKH-1) mAb. Subsequently, they were incubated again with antibodies to CD8 (T811), CD3 (BMA 030), CD5 (T61), β2-microglobulin (+) or without (−) cross-linking by F(ab')2 anti-mouse immunoglobulin (amlg). Cells (2 × 10⁶ cells per well) were cultured in round-bottomed wells for 5 days. Values with stimulation indices > 5 compared to the controls with BMA 030 plus IL-2 are enclosed in boxes. BMA 030 was at 20 ng/ml, and IL-2 was at 100 units/ml. Results are mean cpm ± SD (n = 4).
directly to the plastic surface without a first layer of anti-mouse immunoglobulin, because small amounts of soluble mAb could become incorporated into the solid phase by binding to the anti-mouse immunoglobulin. Direct coupling provided a somewhat less efficient activation signal (data not shown) that could be overcome by slightly increased cell numbers and/or culture period.

The experiment in Fig. 3 shows that cross-linking either of CD8 molecules by T811 or of CD3 molecules by BMA 030 does not sensitize resting T cells to a subsequent addition of the other mAb in soluble form. There is a marginal activation by cross-linked BMA 030 alone as already described in the former experiments. The synergistic activation signal provided by cross-linked BMA 030 and T811 together could be inhibited dose dependently by each of the two antibodies in soluble form. These results suggest that separate fixation of CD8 and T1/CD3 provides a negative signal to the cells.

T-Cell Activation by Cross-Linking CD3 (BMA 030) and CD8 Gives Rise to Functionally Competent T-Killer Cells. We addressed the question whether CD8 T cells activated by cross-linking the T-cell receptor complex with the CD8 antigen become functionally competent (Fig. 4). Resting T cells were cultured on flat-bottomed microculture wells coupled with BMA 030 and T811. After 7 days T-cell blasts were harvested that consisted exclusively of CD8 positive cells (data not shown). These cells were tested in a cytotoxicity assay on two different target cells (P815 and K562) with and without PHA. As demonstrated in Fig. 4, both targets are killed in the presence of PHA whereas direct, presumably natural killer-mediated, cytotoxicity is low. These results show that by the employed cross-linking protocol cytotoxic T-cell precursors differentiate into functionally competent cytotoxic T lymphocytes.

**Comparison of Immunochemical Properties of OKT3 and BMA 030.** mAbs OKT3 and BMA 030 are of the same IgG subclass (IgG2a) and appear to bind in a similar way to the 24-kDa and the 28-kDa species of the CD3 molecule complex from the 125I-surface-labeled human T-cell leukemia line Jurkat (Fig. 5). Under reducing conditions we found in precipitates with OKT3 two additional faint bands of 42 kDa and 52 kDa as described (22). These molecules have been identified as the α and β chains of the T-cell antigen receptor (22, 23). They appear to be somewhat less represented in the immunoprecipitate of BMA 030. Binding curves with graded amounts of BMA 030 and OKT3 are nearly identical (data not shown).

**DISCUSSION**

The experiments described in this paper suggest a mechanism for T-cell recognition (24) and provide the first positive evidence that this mechanism may have a decisive role in T-cell activation and generation of the repertoire.

We studied the effect of an anti-CD3 antibody (BMA 030), which by itself poorly stimulates T cells, even after cross-
linking in various ways. The antibody precipitates the classical components of the Ti/CD3 complex although the receptor heterodimer may be somewhat less represented than in precipitates of the prototype OKT3 antibody. The antibody is of the same immunoglobulin class (IgG2a) as OKT3 and does not belong to the already described nonstimulatory IgG2b antibodies. It binds to T cells quantitatively similar to OKT3. In our opinion, this antibody leads to a less-effective cross-linking of the Ti/CD3 complex, insufficient for maximal T-cell stimulation.

In contrast, cross-linked OKT3 antibody provides a maximal stimulatory signal for T cells. We consider that this may be due to an unintended inclusion of CD8/CD4 molecules into the precipitate of the Ti/CD3 complex. In addition, by physiological antigen-activation cross-linking of the Ti/CD3 complex with itself may be difficult to achieve. The T-cell receptor is an asymmetrical, monovalent structure presumably binding to individual monoclonal epitopes on stimulator cells. T-cell stimulation by cross-linked antibodies to Ti/CD3 may, therefore, in any case be quite artificial. We think that OKT3 in some way overrules the physiological requirements. For example, it may bind to a different CD3 epitope than does BMA 030, thus inducing signal transmission on its own.

Our experiments show that the functional deficiency of BMA 030 in the absence of accessory cells can be fully compensated by its cross-linking to anti-CD8. Technically, effective cross-linking could be achieved by soluble F(ab')2 anti-mouse immunoglobulin, by anti-mouse immunoglobulin coupled to plastic surfaces, or by directly coating both antibodies to plastic surfaces. In either case, anti-CD8 and anti-CD3 can be assumed to be in close proximity and to be immobilized relative to one another. It thus appears that CD8 and the Ti/CD3 complex must be brought together physically for the triggering signal to occur. Activation of resting T cells in this way required the presence of exogenous IL-2, and their proliferative response was, therefore, presumably a consequence of IL-2 receptor expression.

When soluble anti-CD3 or anti-CD8 antibodies were applied together with the mixture of both mAbs in solid-phase condition, activation is prevented. Other groups have made similar observations (25, 26) for anti-CD4 antibodies and have interpreted their data to indicate that CD4 molecules may be receptors for negative (suppressive) signals. Our data, particularly the observation that even soluble anti-CD3 antibody can block activation by the cross-linked mixture of anti-CD3 with anti-CD8, argue against the conclusion that any of these surface molecules may be receptors for negative (suppressive) signals per se. The mechanism for inhibition by soluble antibody may—more simply—be the prevention of the cross-linking of its antigen together with the other and may thus resemble the action of soluble suppressor molecules. In other words, we conclude from our data that the separate fixation of CD8 and of the Ti/CD3 complex may counteract activation and may, therefore, be a critical mechanism for suppression.

Cross-linking of Ti/CD3 with other surface molecules such as HLA-A class I molecules also led in some experiments to synergistic T-cell activation, particularly when both antibodies were coated via anti-immunoglobulin to plastic surfaces. In all of these experiments, synergy was much weaker than with CD8. We, therefore, at present, attach no particular significance to these results. Future experiments may, however, reveal other antigen combinations whose cross-linking leads to strong T-cell activation.

We think that these results offer an attractive mechanism for the generation of the MHC class I and class II restricted repertoires of CD8 and CD4 cells, respectively. We suggest that physiological activation of T cells by antigen and antigen-presenting cells occurs by simultaneous binding of CD8 (CD4) and Ti/CD3 to MHC class I (class II)-associated epitopes, thus cross-linking CD8 (CD4) and Ti/CD3. In mature T cells, this favors the activation of CD8 (CD4) cells by class I (class II)-associated epitopes. We further suggest that induction of proliferation in early T-cell ontogeny has similar requirements, which would provide the selective pressure for the generation of two distinct repertoires in mature T cells, associated with two distinct differentiation antigens on their membrane.

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