Dissection of the Role of Macrophages in Triggering T Lymphocytes for Interleukin 2 Production by Monoclonal Antibody OKT3

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Abstract Unfractionated human peripheral blood mononuclear cells produce a small amount of interleukin 2 (IL 2) by stimulation with a monoclonal anti-T3 antibody (OKT3) in vitro. The IL 2 production could be greatly augmented by the addition of a phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA).

In the presence of TPA, the T cell enriched fraction deprived of macrophages did not produce IL 2, but the T cells pulse-incubated with OKT3 and reconstituted with macrophages efficiently produced IL 2 in subsequent culture in the presence of TPA as did T cells reconstituted with OKT3-pulse-incubated macrophages. The stimulating effect of OKT3 in the presence of macrophages was inhibited dose-dependently by the addition of an immunoglobulin, particularly by mouse IgG2a which is the same isotype as that of the OKT3 antibody, showing that it inhibits by blocking the binding of OKT3 to Fc receptors on macrophages. The same extent of IL 2 production was induced in T cells when paraformaldehyde-fixed macrophages were substituted for intact macrophages. Remarkable IL 2 production was also induced by OKT3 when latex beads coated with rabbit anti-mouse IgG2a antibody and TPA were added to the culture. It was confirmed that the production induced by these stimulations was due to an increase of IL 2 mRNA. These results show that effective signals for IL 2 production are generated by efficient crosslinking of T3 molecules which results from multi-interaction of T3 molecules on the T cell membrane and anti-T3 antibody molecules on macrophage membrane or on the surface of the latex particle.

Recent studies have demonstrated that the T cell antigen receptor consists of the clonotypic disulfide-linked heterodimer (Ti) containing $\alpha$ and $\beta$ chains, and is noncovalently associated with a monomorphic T3 complex consisting of $\gamma$, $\delta$, and $\varepsilon$ chains (1–4, 12, 14, 16–18, 23). The function of the T3 complex is not clear, but it appears to play a critical role in the activation of T cells by antigenic stimulation. Several monoclonal antibodies (mAb) directed against the T3 molecules bind to its reactive molecules on T cells and stimulate polyclonal T cell proliferation by a mechanism presumably analogous to specific antigen stimulation (6, 22, 24, 25).
Therefore, T cell stimulation by anti-T3 mAb is considered as an appropriate model for T cell activation and growth stimulation.

The mitogenic effect of anti-T3 mAb is known to be macrophage-dependent, but the precise mechanism by which macrophages facilitate T cell activation is not clearly defined (5, 10, 15, 24). The available data suggest that binding of anti-T3 mAb to macrophages is necessary to activate T cells, probably to mediate the cross-linking of the T3 complex on the T cell surface. In addition, macrophages perhaps provide still other signals (e.g., by secreting interleukin 1) to potentiate the cell activation.

Meuer et al (13) reported that Sepharose-bound anti-T3 mAb alone induced interleukin 2 (IL 2) production in human alloreactive T cell clones without any other supplement. On the other hand, Weiss et al (26, 27) demonstrated that human T cell leukemia Jurkat cells required two stimuli, anti-T3 mAb (soluble form) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA).

Induction of IL 2 production in stimulated lymphocytes can be greatly enhanced, in general, by addition of TPA as we previously reported (9, 29). The levels of IL 2 mRNA and IL 2 produced are unusually high compared with those induced by phytohemagglutinin (PHA) stimulation. This seems to be due to a remarkable effect of TPA-activated regulatory component, which is highly sensitive to a potent protein kinase C (C kinase) inhibitor, H-7, on the magnitude of the IL 2 mRNA induction (unpublished data). In the presence of a sufficient concentration of TPA, the magnitude of IL 2 production is then dependent on the other signals generated by receptor stimulation with, for example, anti-T3 mAb and PHA because TPA by itself is not sufficient to induce IL 2 production (7, 9, 27, 29, 30). Accordingly, we considered that it will be advantageous to analyze the conditions required for receptor mediated signal generation for IL 2 production in the presence of TPA.

The present study was performed to clarify the mechanisms required for signal generation to induce IL 2 production in normal peripheral T cells. Our results show that macrophage dependence of anti-T3 stimulated IL 2 production is most probably due to a necessity for receptor aggregation which can be reproduced by the use of anti-T3 mAb fixed on inert particles in the presence of TPA. Using an IL 2 cDNA as a probe, it was demonstrated that a particle-bound anti-T3 and TPA are necessary and sufficient to trigger IL 2 mRNA synthesis.

MATERIALS AND METHODS

Reagents and culture medium. OKT3 mAb was purchased from Ortho Pharmaceutical Corp. (Raritan, N.J., U.S.A.). Purified mouse IgG1, IgG2a, IgA, and rabbit anti-mouse IgG2a Ab were prepared in our laboratory. RPMI 1640 medium was obtained from Gibco (Grand Island, N.Y., U.S.A.), fetal calf serum (FCS) from Kyoto Biken Co. (Kyoto, Japan), PHA-M from Difco laboratories (Detroit, MI, U.S.A.), Ficoll-Hypaque from Pharmacia, Uppsala, Sweden, TPA from Sigma Chemical Co. (St. Louis, MO, U.S.A.), [methyl-3H]thymidine from New England
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Nuclear (Boston, MA, U.S.A.), paraformaldehyde from Wako Pure Chemical Industries Ltd. (Osaka, Japan), formaldehyde from Nakarai Chemical Ltd. (Kyoto, Japan), and Nonidet P-40 from Bethesda Research Laboratories (Rockville, MD, U.S.A.).

Preparation of cells. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Hypaque density gradients as described previously (9). The cells were incubated in plastic dishes for 2 hr at 37 C and the non-adherent cells were passed through a nylon wool column. The nylon wool passed fraction referred to as purified T cells was more than 90% positive for OKT3 and contained less than 2% macrophages. The adherent cell fraction obtained by plastic dish adherence contained 80–95% macrophages as judged by non-specific esterase staining.

Culture for IL 2 production and IL 2 assay. PBMC (5 x 10^6/ml) or purified T cells (2 x 10^6/ml) were cultured in flat-bottom microtiter plates (Costar, Cambridge, MA, U.S.A.) in a volume of 0.2 ml in RPMI 1640 medium containing 10% FCS, 2-mercaptoethanol (5 x 10^-5 M), streptomycin (100 μg/ml), gentamycin (100 μg/ml), and penicillin G (100 U/ml) with or without OKT3 (0.5–100 ng/ml) and TPA (5 or 10 ng/ml) for 48 hr at 37 C. IL 2 activity of the culture supernatant was assayed using an IL 2-dependent murine T cell line (MTH.41.16) originating from a cytotoxic T cell line, a gift of Dr. J. Hamuro of the Central Research Laboratory of Ajinomoto Co., Ltd., Yokohama, Japan, as described (9). In brief, the cells (5 x 10^3/0.2 ml) were added to the serial dilutions of the culture supernatants in flat-bottom microtiter plates in RPMI 1640 containing 10% FCS. After 24 hr of culture, the cells were pulsed with 0.2 μCi/well of [3H]thymidine during the last 4 hr and then harvested by a multiple cell harvester (Labo Science, Tokyo). [3H]Thymidine uptake was measured by a liquid scintillation counter. From the regression line, the concentration of IL 2 that gave 33% of the maximum response was defined as 0.1 U/ml.

Fixation of macrophages with paraformaldehyde. Macrophages were washed three times, fixed with 2% paraformaldehyde in PBS for 20 min at room temperature and washed three times with RPMI 1640.

Antibody coated latex. It was prepared by incubating 50–200 μl of the purified antibody (OKT3, 2.5 μg/ml; rabbit anti-mouse IgG2α antibody (RAMG), 6 mg/ml; mouse IgG2α, 6 mg/ml) with 5–20 μl of pelleted Bacto Latex 0.81 (Difco, Detroit, MI, U.S.A.) at 50 C for 30 min, washed three times, and then suspended in RPMI 1640 supplemented with 10% FCS to block any remaining latex protein-binding sites.

Measurement of relative levels of IL 2 mRNA. Relative levels of IL 2 mRNA were determined by the cytoplasmic dot hybridization method (28) as described in the previous report (29). In brief, 2 x 10^6 cells stimulated under various conditions were lysed in 45 μl of 10 mM Tris (pH 7.4), 1 mM EDTA and 5 μl of 5% Nonidet P-40. After pelleting the nuclei, the supernatants were mixed with 75 μl of 20 x SSC (0.15 M NaCl/0.015 M trisodium citrate, pH 7) and 75 μl of 37% formaldehyde, and denatured by heating at 60 C for 15 min. A portion (100 μl) of the denatured cytoplasmic extract was serially 2-fold diluted with 15 x SSC and spotted on a nitrocellulose sheet on a Minifold apparatus (Schleicher and Schuell, Dassel, FRG). The hybridization was performed as described by Thomas (21) using a probe of
32P-labeled IL 2 cDNA (PstI-StuI fragment) that did not contain vector DNA and a poly (A) region. Results were evaluated by autoradiography. Relative levels of IL 2 mRNA were expressed as the final dilution which gave a definite positive autoradiographic spot comparing with the spots of control samples.

RESULTS

IL 2 Production by PBMC by Synergistic Action of OKT3 and TPA

PBMC were cultured with various concentrations of monoclonal anti-T3 antibody OKT3 in the presence or absence of TPA as shown in Table 1. In the absence of TPA, a significant but small amount of IL 2 was produced by stimulation with 10–100 ng/ml of OKT3. The OKT3 stimulation (10–100 ng/ml) induced almost the same level of [3H]thymidine incorporation into PBMC as did PHA (3–4 x 10^4 cpm/2 x 10^5 cells). In the presence of 10 ng/ml of TPA, IL 2 secretion by PBMC reached more than 80 U/ml by stimulation with 10–100 ng/ml of OKT3. Since OKT3 induced submaximal IL 2 production at 10 or 12.5 ng/ml in the presence of 10 ng/ml of TPA, these concentrations of OKT3 were mainly used in the following experiments.

Kinetics of IL 2 mRNA and IL 2 Production

PBMC were cultured with OKT3 plus TPA and the time course of the induction of IL 2 mRNA and IL 2 production were examined. As shown in Fig. 1, a significant amount of IL 2 mRNA was detected after 1 hr of culture. Then the mRNA rapidly increased reaching a peak after 9–12 hr, maintained a relatively high level until 48 hr and declined by 72 hr. A significant increase of IL 2 activity in the culture supernatant was first observed after 3 hr and the activity increased rapidly up to 12 hr and rose gradually until 72 hr. The kinetics of IL 2 mRNA and IL 2 production induced by OKT3 plus TPA was essentially identical to that observed by stimulation with PHA plus TPA in our previous study (29).

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<th>OKT3 (ng/ml)</th>
<th>IL 2 activity (U/ml)</th>
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<td></td>
<td>without TPA</td>
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<tr>
<td>0.5</td>
<td>n.d.</td>
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<tr>
<td>1.0</td>
<td>n.d.</td>
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<tr>
<td>5.0</td>
<td>0.03</td>
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<tr>
<td>10.0</td>
<td>0.17</td>
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<tr>
<td>12.5</td>
<td>0.27</td>
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<tr>
<td>50.0</td>
<td>0.14</td>
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<td>100.0</td>
<td>0.15</td>
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PBMC (5 x 10^6/ml) were stimulated with the indicated concentrations of OKT3 in the presence or absence of TPA (10 ng/ml) and cultured at 37 C for 48 hr. The IL 2 activities of the supernatants were assayed using an IL2-dependent mouse T cell line and expressed as U/ml as described in “MATERIALS AND METHODS.” n.d.: not detectable.
Macrophage Dependency of IL 2 Production by T Cells Stimulated with OKT3 Plus TPA

As shown in Fig. 2, IL 2 production by macrophage-depleted T cells was greatly reduced. When macrophages were returned to the T cell culture, IL 2 production recovered. Addition of 10–20% macrophages to T cells was needed to return IL 2 production to the same level as that induced by PBMC.

To examine the mode of interaction among OKT3, macrophages and T cells, macrophages or T cells were pulse-incubated with OKT3 for 2 hr and the cells washed were cultured with T cells or macrophages for IL 2 production. As shown in Fig. 3, both the T cells cultured with OKT3-pulsed macrophages and the OKT3-pulsed T cells cultured with macrophages could efficiently produce IL 2. OKT3-pulsed T cells or OKT3-pulsed macrophages could not by themselves produce IL 2. These results indicate that macrophages are required to induce IL 2 production in T cells by OKT3 even in the presence of TPA and that the macrophage-T cell interaction is efficiently achieved by pulse-treatment of either T cells or macrophages with OKT3.
Because the Fc portion of OKT3 has been shown to interact with the Fc receptor on macrophages (5, 10, 11), OKT3 probably mediates direct association of T cells with macrophages by binding to T3 molecules on T cells and Fc receptors on macrophages, and this interaction seems to generate a signal for IL 2 production by T cells in the presence of TPA. In fact, IL 2 production by PBMC induced by OKT3 plus TPA was dose-dependently inhibited by the addition of immunoglobulins, especially mouse IgG2a, which competitively inhibited the binding of OKT3 to Fc receptors on macrophages (Fig. 4).
Effect of Metabolically Inactive Macrophages on the Induction of IL 2 Production by Anti-T3 Antibody

To determine whether the metabolic processes of macrophages were required for OKT3-induced IL 2 production in T cells, paraformaldehyde-fixed macrophages were added to the T cell culture with OKT3 and TPA. Figure 5 shows that the accessory cell function of the macrophages was maintained after treatment with 2% paraformaldehyde, indicating that certain mechanical roles of metabolically inactive macrophages are involved in the stimulation of T cells by OKT3.
Fig. 6. Effect of OKT3-coated latex on IL 2 production by T cells. Purified T cells \((2 \times 10^6/ml)\) were cultured in U-bottom microtiter plates with varying concentrations of OKT3-coated latex (○), irrelevant mouse IgG2a Ab-coated latex (△) or FCS-treated control latex (●) in the presence of TPA (5 ng/ml) at 37°C for 48 hr. The IL 2 activities were measured using an IL 2-dependent mouse T cell line and expressed by [³H]thymidine uptake (cpm).

Fig. 7. Effect of RAMG-latex on IL 2 production by T cells in the presence of OKT3 and TPA. Purified T cells \((2 \times 10^6/ml)\) were cultured with varying concentrations of RAMG-latex in the presence of TPA (5 ng/ml) with (○) or without (●) addition of 10 ng/ml of OKT3 at 37°C for 48 hr. T cells were also cultured with varying concentrations of FCS-treated control latex in the presence of TPA (5 ng/ml) and OKT3 (10 ng/ml) (△). The IL 2 activities of the supernatants were assayed as described in “MATERIALS AND METHODS.”
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*IL 2 Production by T Cells in the Presence of Anti-T3-Coated Particles and TPA*

Whether artificial particles such as OKT3-coated latex could replace the macrophage function was investigated. As shown in Fig. 6, significant IL 2 production by purified T cells was observed by adding OKT3-coated latex at a concentration of more than 0.001% in the presence of TPA, but FCS-treated or irrelevant mouse IgG2a-coated latex had no effect on the induction of IL 2 production. When the purified T cells were cultured with OKT3 and TPA in the presence of RAMG-coated beads which bind OKT3 to the beads, large amounts of IL 2 were produced in the supernatants at concentrations of 0.01-0.05% of RAMG-latex. The level of IL 2 production was almost equivalent to that produced by PBMC stimulated with OKT3 and TPA. However, T cells stimulated with OKT3 in the presence of RAMG-coated latex without TPA did not secrete any IL 2 as shown in Fig. 7. These results indicate that OKT3-coated latex beads could be substituted for macrophages in this system and that the presence of TPA in the culture is essential for efficient induction of IL 2 production by T cells.

*Synergistic Effect of the Cross-Linking of T3 Complex and TPA on IL 2 mRNA Induction*

The relative levels of IL 2 mRNA induced by various combinations of stimulants were determined by the cytoplasmic dot hybridization technique. As shown in Fig. 8, no significant level of IL 2 mRNA was detected in the T cells stimulated with TPA or OKT3 alone and in the T cells stimulated with TPA or OKT3 in the presence of RAMG-latex. The combination of soluble OKT3 and TPA induced a minimal amount of IL 2 mRNA. Stimulation of T cells with OKT3 and TPA in the presence of RAMG-latex caused an extreme increase in IL 2 mRNA which was comparable to that found in T cells stimulated with OKT3 and TPA in the presence of macrophages. These results show that multi-interaction between T3 molecules on T cells and OKT3 molecules on latex beads as well as the effect of TPA were necessary for

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<tr>
<th>Addition</th>
<th>IL2 mRNA</th>
<th>IL2 (U/ml)</th>
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<tr>
<td>Medium</td>
<td>n.d.</td>
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<tr>
<td>TPA</td>
<td>n.d.</td>
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</tr>
<tr>
<td>OKT3</td>
<td></td>
<td>1.1</td>
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<tr>
<td>OKT3+TPA</td>
<td></td>
<td>681</td>
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<tr>
<td>RAMG-latex+TPA</td>
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<td>1,484</td>
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<td>RAMG-latex+OKT3</td>
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<td>RAMG-latex+OKT3+TPA</td>
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<td>Mφ+OKT3+TPA</td>
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<td>1,484</td>
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Fig. 8. Effect of RAMG-latex on IL 2 mRNA induction by OKT3 plus TPA. Purified T cells (2 × 10⁶/ml) were cultured with or without macrophages (2 × 10⁶/ml) in the presence or absence of indicated agents (TPA, 5 ng/ml; OKT3, 10 ng/ml; RAMG-latex, 0.025% w/v) at 37°C for 48 hr. The IL 2 activities and relative IL 2 mRNA levels were determined as described in “MATERIALS AND METHODS.” n.d.: not detectable.
efficient triggering of IL 2 mRNA induction and IL 2 production by normal peripheral blood T cells.

DISCUSSION

The present study showed that IL 2 production by unfractionated PBMC stimulated with soluble anti-T3 antibody was greatly enhanced by the addition of TPA but such response to soluble anti-T3 disappeared even in the presence of TPA when macrophage-depleted T cell fraction was stimulated. Thus, the macrophage function could not be replaced by TPA alone. The macrophage function seems to be primarily to fix anti-T3 antibody molecules on the cell surface through binding to Fc receptors as reported by other investigators (5, 10, 11, 20). Indeed, our experiments showed the dose-dependent blocking of this function by immunoglobulins, particularly by mouse IgG2a which competes with anti-T3 antibody of the IgG2a subclass for binding to Fc receptors. It was also indicated that the anti-T3 bound to metabolically inert macrophages or bound to rabbit anti-mouse IgG2a which had been adsorbed on latex particles had a similar ability to stimulate IL 2 production of T cells in the presence of TPA. These results showed the importance of an efficient crosslinking of T3 molecules by multiple interaction with anti-T3 antibody molecules fixed on a particle. It was noted that treatment of OKT3 pulse-incubated T cells with soluble rabbit anti-mouse IgG2a antibody did not induce IL 2 production in the presence of TPA (data not shown). This could be due to a low efficiency of crosslinking by anti-T3-anti-mouse IgG2a antibody complexes not fixed on particles and/or the requirement for multi-interaction of T3 molecules and anti-T3 antibody molecules on a membrane or solid surface.

In contrast to our study, Hara and Fu (8) have recently reported that human macrophage-depleted peripheral T cells could produce IL 2 by stimulation with soluble anti-T3 antibodies (IgG1, IgG2a, and IgM) in the presence of TPA. This discrepancy may be due in part to the fact that they used a high concentration of anti-T3 antibodies (1 μg/ml), which might induce sufficient crosslinking of T3 molecules in the presence of possibly contaminated residual macrophages to produce IL 2 to some extent in the presence of TPA.

Our results are consistent with the report by Schwab et al (19) that Sepharose-bound anti-T3 antibody did not induce any IL 2 secretion or DNA synthesis in human T cells in the absence of macrophages, but it should be noted that some human T cell clones primed with alloantigens were activated in producing IL 2 by the stimulation of Sepharose-bound anti-T3 antibody alone (13).

Apart from the above discrepancy, it is concluded from the present study that optimal activation of T cells for IL 2 production require multi-interaction of T3 molecules and anti-T3 molecules which can be visualized in a very exaggerated way by comparing the effect of particle-bound antibody and soluble antibody in the presence of TPA. The present study further confirmed that the induction of IL 2 production by such stimulation is due to the induction of an increase of IL 2 mRNA in the stimulated cells. On this basis it is reasonable to suggest that the role of
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Macrophages or other antigen-presenting cells is not limited to processing and presenting antigen to T cells for associative recognition of antigen and 1a but also allows multiple interaction of T cell receptor molecules with antigen-1a molecules on the cell membrane which facilitates efficient receptor crosslinking. Under physiological conditions, the macrophage should be alive to process antigen and also to secrete molecules such as interleukin 1 to potentiate T cell activation in the absence of TPA.

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REFERENCES


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