HUMAN T CELL ACTIVATION

I. Monocyte-independent Activation and Proliferation Induced by Anti-T3 Monoclonal Antibodies in the Presence of Tumor Promoter 12-0-Tetradecanoyl Phorbol-13-acetate

BY TOSHIRO HARA AND SHU MAN FU

From the Cancer Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

T3 antigen is a 20–25 kD complex on human T cells (1–3). The recently described T cell receptors specific for antigen (5–9) are noncovalently linked to this antigen (6, 10). The role of T3 antigen in T cell activation has been probed using monoclonal antibodies (mAb) directed against the T3 antigen. They induced proliferation of T cells (11, 12), secretion of lymphokines (13–17), expression of interleukin 2 (IL-2) receptors (18, 19), and mobilization of Ca++ (20, 21). The majority of these effects appear to be dependent on monocyte interaction with the Fc portions of the antibodies (22–29). Recently, Jurkat, a T3+ human T cell leukemia line, has been used as a model for T cell activation. Anti-T3 antibodies were shown to deliver one of the two signals required for IL-2 gene activation, its synthesis, and secretion (30). Phorbol ester (TPA) rendered the other signal.

Herein, three anti-T3 mAb of different isotypes (IgG1, IgG2a, and IgM) were generated and used to probe activation and proliferation of normal human peripheral blood T cells. One of these (IgM) was not mitogenic for T cells in the presence of monocytes. All three anti-T3 mAb, in the absence of monocytes, and in collaboration with TPA, were able to induce isolated T cells to express IL-2 receptors, to secrete IL-2, and to proliferate.

Materials and Methods

Monoclonal Antibodies. Three mAb against T3 were generated and named T3-I (IgG2a), T3-II (IgG1), and 235 (IgM). TE, an mAb inhibiting T cell rosette formation and reacting with a 55 kD glycoprotein, was an IgG2a. T3-I and T3-II mAb were produced in C57 mice immunized with activated T cells. TE and 235 were generated in B6F1 mice, using thymocytes as the immunogen. Mice were immunized intraperitoneally...
with $2 \times 10^7$ cells, with alum as an adjuvant. 3 wk later, $2 \times 10^7$ cells in phosphate buffered saline (PBS) were given intraperitoneally. Spleens were taken 3 d later. Fusion with tumor SP2/0 was carried out as described (31). Hybridoma supernatants were screened against the immunizing cells for their binding activities and their abilities to induce T cell proliferation or block T cell proliferation induced by allogeneic cells. The desired hybridomas were cloned twice on soft agar. Antibody subclass determination was made by enzyme-linked immunosorbent assay with subclass-specific rabbit anti–mouse Ig antiserum (Meloy Laboratories, Inc., Springfield VA) and alkaline phosphatase–linked goat anti–mouse Ig. mAb were purified from culture supernatants using goat anti–mouse Ig–Sepharose 4B column. Bound antibodies were eluted with glycine-HCl buffer, pH 2.9, dialyzed against PBS and sterilized by filtration.

OKT3 and fluorescein isothiocyanate (FITC)–conjugated OKT3 were purchased from Ortho Pharmaceuticals, Raritan NJ. Anti-Leu 1, Leu 4, and FITC-conjugated Leu 4 mAb, and FITC-conjugated avidin were from Becton Dickinson and Co., Mountain View CA. FITC-labeled, affinity-purified goat anti–mouse IgM (μ heavy chain–specific) was purchased from Southern Biotechnology Associates, Birmingham, AL.

G3.5 (T12 equivalent) and 10.2 (Leu 1 equivalent) mAb (32) were generously provided by Dr. J. A. Hansen (Fred Hutchinson Cancer Research Center, Seattle WA). Anti–putative T cell receptor antibodies (anti-Ti) S160 (10) and C37 were generous gifts from Dr. R. Bigler (Rockefeller University, NY) and Dr. C. Y. Wang (The Sloan-Kettering Cancer Center, NY), respectively.

Biotinylation of Purified AT-1 mAb. AT-1, an mAb against IL-2 receptor, was produced as described previously (33). Biotinylation of purified AT-1 was carried out according to a standard method. 0.5 ml of AT-1 (1 mg/ml) in 0.2 M Tris HCl, pH 8.1, was mixed quickly with 0.1 ml of 3.4 mg/ml biotin N-hydroxysuccinimide ester (Calbiochem-Behring, San Diego CA) solution and stirred for 30 min at room temperature. The biotinylated AT-1 was extensively dialyzed against PBS.

Cell Preparation. Peripheral mononuclear cells (PMC) were isolated from buffy coats from normal donors or peripheral blood, using Ficoll-Hypaque density gradient centrifugation. To isolate monocyte-free T cells, PMC were further separated by Percoll continuous gradient centrifugation, followed by carbonyl-iron treatment, nylon wool column, and plastic adherence (34–36). Monocyte-free T cells were also obtained by sheep red blood cell (SRBC) rosetting (37), carbonyl-iron treatment, nylon wool column, and plastic adherence. These T cell preparations contained <0.1% monocyte, by nonspecific esterase staining. SRBC rosetting separation had no effect on the results, although T11 antigen was found to be an alternative pathway of T cell activation (38).

Cells were suspended in complete media consisting of RPMI 1640 (Gibco, Grand Island NY) supplemented with 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan UT), 2 mM L-glutamine, and 50 μg/ml gentamicin (Gibco).

Immunofluorescence Studies. Cells were stained with mAb at 4°C for 30 min. After extensive washing, the cells were further incubated with FITC-conjugated goat anti–mouse F(ab)2 at 4°C for 30 min. For the study on IL-2 receptor expression, biotinylated AT-1 was used as the first ligand, and FITC-conjugated avidin was used as the second ligand. After wash, the cells were analyzed with a Coulter Epics V flow cytometer (Coulter Electronics, Hialeah FL). Integrated fluorescence of the gated population was measured, and 10,000 cells were analyzed.

Proliferation Assays. PMC or monocyte-depleted T cells (2 × 10^5 cells) in 200 μl of complete medium were incubated in triplicate in flat-bottomed 96-well plates at 37°C for 3 d in a 5% CO₂ atmosphere. During the last 8 h, cells were pulsed with 0.4 μCi of [³H]thymidine (specific activity 75 Ci/mmol; Amersham Corp., Arlington Heights IL) and incorporated radioactivity was determined as described (39).

For proliferation assays, the following reagents were used: Phytohemagglutinin-P (PHA-P) (Difco Laboratories, Inc., Detroit MI), Concanavalin A (Con A) (Pharmacia Fine Chemicals, Piscataway NJ), Pokeweed mitogen (PWM) (Gibco), tetanus toxoid (Commonwealth Scientific Corp. Alexandria VA), 12-o-tetradecanoyl phorbol-13-acetate (TPA)
Modulation Studies. PMC (10^6 cells/ml) or cells of the HPB-ALL leukemic line (2.5 × 10^4 cells/ml) in complete medium were incubated with a saturating dose of mAb for 18 h at 37°C. Cells were washed three times, then stained as described above.

IL-2 Activity Assay. 4 × 10^6 murine IL-2-dependent HT-2 cells (a gift from Dr. K. Himeno from Oklahoma Medical Research Foundation, Oklahoma City OK) in 100 µl of complete media containing 2.5 × 10^-5 M 2-mercaptoethanol (2-ME) were incubated with 100 µl of culture supernatants of various dilutions, in triplicate, at 37°C for 24 h. Then, 0.4 µCi of [3H]thymidine was added, and incubation was continued for another 4 h. The incorporated radioactivity was assayed as described above.

IL-2 Production. T cells (10^6 cells/ml) in complete medium were cultured for 12 h in the presence of various activators. Supernatants were collected by centrifugation, filtered, and assayed for IL-2 activity as described above.

IL-1 Activity Assay. Single-cell suspensions of mouse thymocytes (1.5 × 10^7 cells/ml) in complete medium containing 2.5 × 10^-5 M 2-ME were cultured for 3 d with or without 1 µg/ml PHA in the presence of various dilutions of IL-1 (40). [3H]thymidine incorporation was determined after an 8 h pulse, as described above.

Immunoprecipitation and Autoradiography. T lymphocytes or HPB-ALL cells (2 × 10^7) were labeled with ^125I (specific activity 15.8 mCi/µg of iodine; Amersham Corp.) by the lactoperoxidase technique, or with ^[35S]methionine (specific activity 1,265 Ci/mmol; Amersham Corp.) in methionine-free RPMI 1640 with dextral fetal calf serum. The labeled cells were solubilized in PBS containing 0.5% Nonidet P-40 (NP-40) and 1 mM phenylmethylsulfonyl fluoride. After centrifugation, supernatants were added to 30 µl of Sepharose 4B conjugated with goat anti-mouse Ig (5 mg/ml) that had been incubated with mAb. The mixture was incubated for 60 min at 4°C with constant mixing, and then washed three times with a buffer containing 10 mM Tris HCl, pH 7.8, and 0.6 M NaCl. Two additional washings with a buffer containing 10 mM Tris HCl, pH 8.8, 0.6 M NaCl, 0.1% sodium dodecyl sulfate (SDS), and 0.05% NP-40 were carried out. The absorbed proteins were released from the Sepharose by boiling for 3 min in a sample buffer containing 0.125 M Tris HCl, pH 6.8, 2% SDS, 5% 2-ME, and 10% glycerol, and were subjected to electrophoresis on 15% polyacrylamide gels in the presence of 0.1% SDS. Gels were stained, destained, and dried. Autoradiography was carried out against X-OMAT AR films at -70°C (41).

Results

Characterization of Anti-T3 mAb. We found two mAb to be mitogenic for PMC. They were T3-I (IgG2a) and T3-II (IgG1). They precipitated a 22-24 kD molecule (Fig. 1, lanes 1 and 2) that had a similar electrophoretic mobility as the T3 antigen precipitated by OKT3 and Leu 4 in our gel system. mAb 235, an IgM, was not mitogenic. It blocked T cell proliferation. Despite failure to precipitate the T3 complex from either ^125I surface-labeled or ^[35S]methionine metabolically-labeled T cells, we obtained data to indicate that it was reactive with T3 antigen.

In immunofluorescence assays, all three antibodies stained 85-95% of peripheral blood T cells, and ~60% thymocytes. They failed to stain B cells, granulocytes, monocytes, erythrocytes, and platelets.

The relationship between our anti-T3 antibodies and those commercially available was investigated. Unlabeled mAb Leu 4, T3-II, and 235 significantly blocked the staining of T cells by OKT3-FITC (Fig. 2 A). T3-I was less effective. OKT3 and T3-II significantly blocked the staining of T cells by Leu 4-FITC, while T3-I and 235 were less effective (Fig. 2 B). When FITC-labeled goat anti-
mouse IgM and mAb 235 (IgM) were used, OKT3, Leu 4, and T3-II markedly blocked the staining by 235. T3-I blocked it very weakly (Fig. 2 C). These data indicate that OKT3, Leu 4, T3-II, and 235 react either with a similar antigenic determinant or determinants closely located on the T3 complex. T3-I appears to react with a distinct antigenic determinant.

The reactivity of 235 against the T3 complex was further documented by comodulation experiments. In the first experiment, PMC were incubated with a saturating dose of anti-T3-II or 235 mAb at 37°C for 18 h. Cells were washed, stained, and analyzed. Modulation of the T3 complex by T3-II rendered T cells nonreactive to 235 (Table I). Conversely, cells modulated by 235 were not stained by T3-II. Other T cell antigens, such as T11 (55 kD) and Leu 1 (67 kD), were not affected by the modulation of the T3 complex. In a second experiment, we used two mAb (C37 and S160) against putative T cell receptors (Ti). The first one (C37) was against a 80–90 kD heterodimer complex on the leukemia cell line HPB-ALL (Dr. C. Y. Wang, personal communication), and S160 was against a similar structure on leukemic cell SU (9). These two Ti structures
FIGURE 2. Flow-cytometer analysis of the interaction between mAb 235 and anti-T3. (A) Inhibition of FITC-labeled OKT3 staining of PMC. PMC were incubated for 30 min at 4°C with an unlabeled mAb (OKT3, Leu 4, T3-I, T3-II, or 235) and then washed three times. Second incubation was carried out by adding a FITC-labeled OKT3 followed by analysis on a Coulter Epics V flow cytometer. Control indicates the staining with FITC-labeled OKT3 alone. (B) Inhibition of FITC-labeled Leu 4 staining of PMC. Staining was performed as described above, except that FITC-labeled Leu 4 was used instead of FITC-labeled OKT3. Control indicates the staining with FITC-labeled Leu 4 alone. (C) Inhibition of the staining with 235 IgM and FITC-labeled goat anti-mouse IgM of PMC. First incubation was done as described above. Second incubation was performed by adding 235, followed by washing. Then, FITC-labeled goat anti-mouse IgM was added and analyzed on a Coulter Epics V flow cytometer. Control indicates the staining with OKT3, Leu 4, T3-I, or T3-II and FITC-labeled goat anti-mouse IgM.

TABLE I
Comodulation of 235 and T3 Antigens On Peripheral Mononuclear Cells

| Modulating antibody | Stained cells |
|---------------------|---------------|
|                     | 235 | T3-II (T3) | TE (T3) | Leu 1 |
| %                   | 83.1 | 81.2 | 92.7 | 82.4 |
| 235                 | 1.7  | 4.0   | 93.4 | 84.2 |
| T3-II               | 0.9  | 1.7   | 92.0 | 81.3 |

Peripheral mononuclear cells (10⁶ cells/ml) were incubated with a saturating dose of anti-T3-II or 235 mAb at 37°C for 18 h. Cells were washed, stained, and analyzed with a Coulter Epics V flow cytometer as described in Materials and Methods.

comodulated with T3 antigens. Table II details the experiment involving C37. Modulation of HPB-ALL cells by T3-II or 235 rendered the cells unreactive to C37. Modulation of the Ti antigen with C37 also modulated the T3 complex, as detected by T3-I, T3-II, or 235. Similar results were obtained by using S160 mAb and leukemic cell SU.

Inhibition of T Cell Proliferation by Nonmitogenic Anti-T3 mAb 235. When the
TABLE II
Comodulation of Ti and T3 Antigens on HPB-ALL. Evidence That mAb 235 Is Directed Against the T3 Complex

| Modulating antibody | Stained cells | 235 | T3-I | T3-II | Ti (C37) | Leu 1 |
|---------------------|---------------|-----|------|-------|----------|-------|
| None                | %             | 93.8| 94.3 | 95.2  | 93.3     | 91.8  |
| 235                 | %             | 12.0| 13.1 | 13.9  | 16.3     | 92.2  |
| T3-II               | %             | 11.0| 11.5 | 12.1  | 14.5     | 90.7  |
| Ti (C37)            | %             | 19.7| 18.7 | 21.6  | 9.7      | 89.6  |

Modulation was carried out as described in Table I.

TABLE III
Inhibition of T Cell Proliferation by mAb 235

| IgM antibody | [\(^{3}H\)]Thymidine incorporation* |
|--------------|-----------------------------------|
|              | Con A | PHA | PWM | MLC | Tetanus toxoid |
|              | cpm   | cpm | cpm | cpm | cpm            |
| A-235 µg/ml  |       |     |     |     |                |
| 0.05         | 41,842| 107,537| 29,211| 35,323| 9,271         |
| 0.1          | 35,664| (14.2)| 105,427| 26,971| 34,253| 10,124       |
| 0.5          | 24,637| (40.7)| 101,154| 28,350| 32,825| 10,705       |
| 1.0          | 8,192 (80.3)| 100,908| 20,018 (25.4)| 9,615 (72.2)| 3,813 (59.4) |
| 5.0          | 2,731 (93.4)| 16,548 (84.4)| 13,907 (48.2)| 4,851 (86.0)| 2,727 (71.0) |
| 10.0         | 1,517 (96.3)| 9,437 (91.1)| 12,661 (52.8)| 2,344 (93.2)| 1,983 (79.0) |
| B-control µg/ml|       |     |     |     |                |
| None         | 41,556| 106,417| 26,858| 34,575| 9,403         |
| 0.05         | 42,714| 102,968| 26,631| 33,441| 9,421         |
| 0.1          | 40,932| 104,883| 25,338| 32,680| 10,001        |
| 0.5          | 39,688| 104,892| 28,539| 33,707| 9,563         |
| 1.0          | 40,824| 103,653| 23,897| 35,200| 8,380         |
| 5.0          | 40,032| 103,407| 26,033| 34,901| 10,195        |
| 10.0         | 39,778| 102,702| 27,197| 32,990| 9,929         |

Without mitogen

* Peripheral mononuclear cells (2 × 10⁵) were stimulated with Con A (5 µg/ml), PHA (10 µg/ml), PWM (0.1%), irradiated allogeneic cells (MLC, 2 × 10⁵), or tetanus toxoid (20 µg/ml) in the presence of various concentrations of mAb. [\(^{3}H\)]Thymidine incorporation was measured after 3 d (for Con A, PHA, and PWM) or 6 d (for MLC and tetanus toxoid) of incubation. Standard deviations of each value were within 10%.

* Values in parentheses indicate percent inhibition.

purified preparation of IgM 235 was added at the start of cultures, it blocked T cell proliferation induced by mitogens (Con A, PHA, and PWM), allogeneic cells, and soluble antigen (tetanus toxoid) in a dose-dependent manner (Table III).
the three mitogens employed in this investigation, mAb 235 was less effective in blocking the proliferation induced by PHA or PWM. This was observed in several experiments involving different individuals. To clarify which phase of T cell activation was affected, 235 was added at different time points after the initiation of mixed lymphocyte cultures (MLC). When 235 was added at the start of cultures, it blocked the proliferation of allogeneic T cells by 85%. This inhibition decreased to 65% and 25% when the antibody was added 24 and 48 h, respectively, after the cultures were begun. In contrast, the addition of 235 (0.05–50 μg/ml) to cultures of PHA-stimulated T blasts did not interfere with proliferation induced by IL-2.

**T Cell Proliferation Induced by Anti-T3 mAb and TPA.** Although antibody 235 was not mitogenic, and, in fact, inhibited T cell proliferation induced by antigens and mitogens, it became mitogenic for PMC in the presence of TPA (Table IV, Exp. A). For PMC, TPA was found to be quite mitogenic, in a dose-dependent manner. At all doses, antibody 235, as well as the other two mitogenic anti-T3 mAb showed a synergistic effect with TPA in the induction of T cell proliferation.

Antibody T3-II is an IgG1. We confirmed reports (23, 28) that there were nonresponders in the control population. In three nonresponders, T3-II became mitogenic in the presence of TPA, and the degree of proliferation was similar to that induced by T3-I (IgG2a).

We looked at the role of monocytes in this system. Two methods were used to deplete monocytes from T cell preparations. In the absence of monocytes, T3-I

| TABLE IV | Effect of Various Concentrations of TPA on the Mitogenic Activity of mAb |
|-----------|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| mAb (1 μg/ml) against: | TPA concentration (ng/ml) | 0  | 0.3  | 1.0  | 3.0  |
|-----------|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| A. Peripheral mononuclear cells | | | | | |
| Medium | 625 | 1,496 | 17,572 | 47,776 |
| Control IgM | 632 | 1,537 | 17,842 | 45,005 |
| 235 IgM | 452 | 12,866 | 91,611 | 84,577 |
| T3-I | 58,033 | 71,986 | 96,711 | 91,908 |
| T3-II | 55,381 | 66,734 | 90,891 | 92,973 |
| B. Monocyte-depleted T cells | | | | | |
| Medium | 194 | 540 | 2,132 | 6,952 |
| 235 IgM | 188 | 16,090 | 66,340 | 52,983 |
| T3-I | 230 | 22,151 | 53,671 | 47,912 |
| T3-II | 226 | 12,356 | 56,525 | 43,606 |
| 10.2 (Leu 1) | 235 | 916 | 3,044 | 6,676 |
| TF (T11) | 255 | 535 | 1,189 | 4,919 |
| G3.5 (T12) | 150 | 988 | 1,945 | 6,606 |

*2 X 10⁵ cells were incubated with 1 μg/ml mAb in the presence of various concentrations of TPA. [³H]Thymidine uptake was measured after 3 d. Standard deviations of each mean value were within 15%.*
and T3-II were no longer mitogenic (Table IV, Exp. B). TPA was also much less mitogenic. In this system, all three anti-T3 mAb (1 μg/ml) showed a similar potency in the induction of T cell proliferation in the presence of TPA. Other pan-T mAb, such as 10.2 (Leu 1), TE (TII), and G3.5 (T12) showed no mitogenic effect in the presence of TPA, with or without monocytes.

The magnitude of the responses of isolated T cells to anti-T3 in the presence of TPA was similar to that induced by T3-I and T3-II in the presence of monocytes. It appears that more vigorous proliferation was observed when anti-T3 and TPA were added to unseparated mononuclear cells (Table IV). This was observed in three separate experiments. Although T cell preparations isolated by rosetting with SRBC were utilized in these experiments, comparable results were obtained with monocyte-depleted T cell preparations isolated by Percoll gradient centrifugation and nylon wool column purification.

With a nonmitogenic dose of TPA (0.3 ng/ml), increasing doses of anti-T3 induced more proliferation (Table V). mAb 235 was less potent than T3-I and T3-II. The pentameric nature of IgM may account for this discrepancy in potency.

IL-2 Receptor Expression and IL-2 Production. T cell activation with anti-T3 mAb in the presence of TPA was associated with a marked increase in IL-2 receptor expression as detectable by our mAb AT-1. (Table VI). Because the cells were cultured in the presence of mouse mAb, biotinylated AT-1 was used with avidin-FITC. With anti-T3 alone, 10% of the T cells weakly expressed IL-2 receptors at all time points studied. Although TPA did not induce a high level of proliferation, a significant number of T cells were positive for IL-2 receptors. However, the intensity of fluorescence was quite weak (Fig. 3). In the presence of anti-T3 mAb and TPA, 80% of the T cells were positive by 12 h, and ~90% became strongly positive by 60 h (Table VI and Fig. 3). This was observed with all three anti-T3 mAb.

IL-2 production by T cells stimulated by anti-T3 in the presence of TPA was detected 12 h after the initiation of cultures (Table VII). Significant proliferation by HT-2 cells was detected even when the supernatants were diluted to 1:20 and 1:60. Supernatants from cultures with either anti-T3 or TPA alone did not show IL-2 activities. As an additional control, addition of anti-T3 to collected TPA supernatant did not support the growth of HT-2 cells.

IL-1 and IL-2 Did Not Restore T Cell Responsiveness to Anti-T3 in the Absence of

| TABLE V |

Lymphocyte Proliferation by Various Concentrations of mAb in Presence of TPA

| mAb   | Antibody concentration (ng/ml) |
|-------|-------------------------------|
|       | 0    | 1    | 10   | 100  | 100  |
|       | cpm  |      |      |      |      |
| 235 (IgM) | 289  | 471  | 603  | 12,478 | 25,269 |
| Tα-1 (IgG2a) | 177  | 7,330| 25,188 | 31,472 | 29,540 |
| Tα-II (IgG1) | 227  | 200  | 10,191 | 32,743 | 31,508 |

Monocyte-depleted T cells (2 × 10^5 cells), prepared as described in Materials and Methods, were cultured in the presence of various concentrations of mAb and 0.3 ng/ml TPA for 3 d. Standard deviations of each mean value were within 15%.
TABLE VI

Time Course of Expression of IL-2 Receptors on Monocyte-depleted T Cells

| Culture condition | IL-2 receptor-positive cells |  |  |  |
|-------------------|-----------------------------|---|---|---|
|                   | 12 h | 36 h | 60 h | [\(^{3}H\)Thymidine incorporation] |
|                   | %    | cpm  | cpm  | cpm  |
| 235 + TPA         | 80.6 | 89.2 | 89.2 | 73,539 |
| T3-I + TPA        | 80.2 | 89.4 | 88.4 | 78,194 |
| T3-II + TPA       | 79.6 | 89.1 | 90.9 | 72,548 |
| 235 (1 \(\mu\)g/ml) | 2.6  | 3.5  | 3.9  | 147   |
| T3-I (1 \(\mu\)g/ml) | 8.2  | 8.9  | 9.7  | 116   |
| T3-II (1 \(\mu\)g/ml) | 8.7  | 9.3  | 9.9  | 116   |
| TPA (0.6 ng/ml)   | 42.7 | 66.6 | 68.6 | 1,306  |
| Medium            | 2.8  | 3.6  | 4.0  | 173   |

Monocyte-depleted T cells (10^6 cells/ml) were stimulated as described above. The percentages of IL-2 receptor-positive cells were determined by biotinylated AT-1 (anti-IL-2 receptor) and avidin-FITC at the indicated times. 10^4 cells were analyzed with a Coulter Epics V flow cytometer as described in Materials and Methods. [\(^{3}H\)Thymidine incorporation was determined after 3 d in culture. These data are representative of three independent experiments.

**Figure 3.** Flow-cytometer analysis of the expression of IL-2 receptors on T cells. Monocyte-depleted T cells were incubated with medium or 235 (1 \(\mu\)g/ml) alone (2), TPA (0.6 ng/ml) alone (3), or TPA and 235 (4). IL-2 receptor-positive cells were determined by biotinylated AT-1 and avidin-FITC, and analyzed with a flow cytometer as described in Table VI. (1) indicates the staining with nonbiotinylated AT-1 and avidin-FITC.

**Monocytes.** IL-1 and IL-2 were added to monocyte-depleted T cell cultures to determine if they could restore their responsiveness to anti-T3 mAb. As shown in Table VIII, IL-1 did not restore T cell responsiveness to anti-T3 in the absence of monocytes. The IL-1 preparation was shown to be active by its augmentation of PHA stimulation of mouse thymocytes. In this assay, mouse...
### TABLE VII
**IL-2 Production by Monocyte-depleted T Cells Stimulated by Anti-T3 mAb and TPA**

| Culture condition | [3H]Thymidine incorporation at final dilutions of: |  |
|-------------------|-------------------------------------------------|---|
|                   | 1:2 | 1:6 | 1:20 | 1:60 |
| 235 + TPA         | 9,714 | 5,639 | 2,815 | 1,477 |
| T3-I + TPA        | 10,306 | 6,945 | 4,579 | 2,445 |
| T3-II + TPA       | 10,796 | 6,952 | 4,210 | 2,165 |
| 235 (1 µg/ml)     | 1,479 | 1,203 | 1,092 | 883 |
| T3-I (1 µg/ml)    | 1,683 | 1,359 | 1,329 | 819 |
| T3-II (1 µg/ml)   | 1,654 | 1,433 | 1,444 | 858 |
| TPA (0.6 ng/ml)   | 1,564 | 1,405 | 1,219 | 857 |
| Medium            | 1,635 | 1,450 | 1,446 | 1,107 |

Table VII shows the IL-2 production by monocyte-depleted T cells (10⁶ cells/ml) cultured in the presence of stimulator(s). After 12 h, supernatants were harvested, and their ability to stimulate [3H]thymidine incorporation into murine IL-2-dependent HT-2 cells was determined. The standard deviation of each value was within 15%. Three independent experiments showed similar results.

### TABLE VIII
**Effects of IL-1 and IL-2 on T Cells in Presence of Anti-T3**

| Interleukin | Medium | mAb (1 µg/ml) | 235 | T3-I | T3-II |
|------------|--------|---------------|-----|------|-------|
| U/ml       |        |               |     |      |       |
| IL-1       |        |               |     |      |       |
| 0          | 173    | 331           | 538 | 544  |       |
| 2.5        | 279    | 173           | 778 | 803  |       |
| 5          | 262    | 263           | 665 | 699  |       |
| 10         | 165    | 310           | 907 | 703  |       |
| IL-2       |        |               |     |      |       |
| 0          | 294    | 174           | 370 | 230  |       |
| 125        | 254    | 1,329         | 1,905| 1,527|       |
| 250        | 291    | 2,715         | 3,272| 2,852|       |
| 500        | 428    | 3,673         | 5,739| 4,455|       |
| IL-2 (recombinant) | |               |     |      |       |
| 125        | 440    | 3,176         | 4,716| 3,276|       |
| 250        | 385    | 4,376         | 7,531| 5,469|       |
| 500        | 917    | 5,065         | 8,237| 6,374|       |

Table VIII shows the effects of IL-1 and IL-2 on T cells cultured for 3 d in the presence of various concentrations of IL-1 or IL-2 with or without anti-T3 mAb (1 µg/ml). Standard deviations of each mean value were within 15%.
thymocytes incorporated 796 cpm of [3H]thymidine in the presence of PHA (1 μg/ml). With 2.5 U/ml of IL-1 added, 15,630 cpm was taken up.

Both purified and recombinant IL-2 augmented anti-T3 mAb in the induction of T cell proliferation. However, the effect was quite small in comparison to that of TPA.

**Discussion**

We raised three mAb (T3-I, T3-II, and 235) and used them to probe the role of the T3 complex in human T cell activation. T3-I (IgG2a) and T3-II (IgG1) precipitated a T3 complex and behaved similarly to OKT3 (IgG2a) and Leu 4 (IgG1), respectively, in terms of their mitogenicity for T cells in the presence of monocytes. 235, an IgM mAb stained T cells, blocked the staining of T cells by FITC-OKT3 or FITC-Leu 4, and induced the modulation of the T3/Ti complex. Modulation of the T3/Ti complex by anti-T cell idiotypic antibodies also rendered the T cells unreactive to 235. Although repeated attempts to precipitate the T3 complex with 235 were unsuccessful, the accumulated evidence led to the conclusion that 235 was reactive with the T3 complex.

235 by itself was not mitogenic for T cells in the presence of monocytes. This is similar to other anti-T3 antibodies of IgM isotype (29, 42). In addition, 235 IgM was an effective inhibitor of T cell proliferation (Table III). Recently, Leu 4 (IgG1) has been shown to inhibit T cell mitogenesis in individuals whose T cells were nonresponsive to Leu 4 in the presence of autologous monocytes (43). That nonmitogenic anti-T3 mAb can block antigen- and mitogen-induced T cell proliferation underscores the central role of the T3/Ti complex in T cell activation.

Despite its inhibitory effect, 235 in the presence of TPA was mitogenic for T cells. T3-I and T3-II were also mitogenic to monocyte-depleted T cells in the presence of TPA. This indicates that 235, as well as T3-I and T3-II, was capable of delivering an inductive signal in the absence of monocytes. This is consistent with the finding that anti-T3 antibodies induce Ca++ influx in the absence of monocytes (20, 21). Preliminary experiments using 3,3'-dipentyloxacarbocyanine iodide as a probe (44) showed that all three anti-T3 mAb used in this investigation induced membrane depolarization of T cells in the absence of monocytes. Our T cell preparations contained <0.1% monocytes, as measured by nonspecific esterase staining, and were no longer responsive to T3-I and T3-II. Furthermore, nonmitogenic 235 IgM, which appeared to have little interaction with monocytes, behaved similarly to T3-I and T3-II in T cell activation and proliferation in the presence of TPA. Thus, the T cell mitogenic effect of anti-T3 plus TPA is independent of monocytes. The effect was preceded by the induction of IL-2 receptors and by IL-2 secretion. (Tables VI and VII). These data suggest that two signals are necessary for normal T cell activation and proliferation. This is analogous to the two-signal model (30) of IL-2 induction and secretion in Jurkat cells, a T leukemia line.

In a recent study by Clement (42), a direct anti-T3-mediated interaction of the T3 molecules with monocyte membrane appeared to be necessary for
mitogenesis. In his system, anti-T3 (IgG1) was used to deliver an inductive signal to T cells from nonresponsive individuals. An attempt to generate a second signal was carried out by incubating anti-T3 (IgG1)-coated T cells with monocytes in the presence of an anti-p67 (a Leu 1-equivalent IgG2a). This interaction did not induce T cell proliferation. It is likely that close interaction between T cells and monocytes, mediated by anti-T3, activates a structure on or near the T3 complex. TPA may act on this structure, and thus it can substitute for the required monocyte function. At any rate, these results further support the use of anti-T3-induced T cell proliferation as a good model for antigen-induced T cell proliferation that also requires close contact between T cells and antigen-presenting cells (45).

We, as well as others (46, 47) clearly establish that TPA can deliver a second signal in normal T cell activation; the first signal being rendered by anti-T3, or T cell mitogens. TPA has been shown to have diverse cellular effects (48, 49). We do not yet know which of these effects is responsible for the results observed in our system. Note that TPA, by itself, could induce a low level of IL-2 receptor expression without inducing IL-2 production in monocyte-depleted T cells. These results indicate the complexity in the events leading to T cell proliferation.

Our attempts to substitute IL-1 or IL-2 for TPA were not successful. IL-1 had no effect on T cell proliferation when it was added, together with anti-T3, to monocyte-depleted T cells. IL-2 did collaborate with anti-T3 to induce T cell proliferation. However, this effect was quite small. Under this condition, the expression of IL-2 receptors was only slightly enhanced. Furthermore, the addition of 1% of monocytes to these cultures markedly enhanced both T cell proliferation and IL-2 receptor expression (T. Hara and S. M. Fu, manuscript in preparation). These results are in agreement with those reported recently by Welte et al. (50). While our results did not support the assignment of a primary role of IL-1 and IL-2 in anti-T3-induced mitogenesis, the possibility that a higher concentration of these interleukins, achievable in the case of cell-cell contact, might play such a role, cannot be discarded. Our results do not rule out the possibility that other monokines yet to be identified may also be involved.

Our experimental approach has also been used to probe action by other anti-T cell antibodies. Anti-p55 (T11, SRBC receptor), anti-p67 (Leu 1), and anti-p100 (T12) antibodies were found not to be mitogenic for T cells in the presence of TPA. Certain anti-T11 antibodies in combination have been shown to be mitogenic, and T11 has been postulated to be an alternative T cell activation pathway (38). The lack of responsiveness of T cells to anti-T11 plus TPA further suggests that the T11 activation pathway differs from that involving the T3/Ti complex. Of four anti-T cell-subset antibodies (anti-p55 [T4], anti-p32-45 [T8], anti-p40 [3A1], and anti-p44 [9.3]), only 9.3 induced T cells to proliferate in collaboration with TPA (T. Hara, S. M. Fu, and J. A. Hansen; submitted for publication.) It is of interest to note that the 9.3 antigen is a disulfide-linked dimer of a 44 kD polypeptide. The activation of T cells by 9.3 and TPA is accompanied by IL-2 secretion and IL-2 receptor expression in a manner very
similar to that seen with anti-T3 and TPA. Thus, our approach has proven useful for probing T cell-antigen function.

**Summary**

Three monoclonal antibodies (mAb), of IgG1, IgG2a, and IgM isotypes, raised against the T3 complex, were used to probe the activation of human T cells. The IgM antibody 235 was not mitogenic for peripheral blood mononuclear cells (PMC). It efficiently blocked the proliferation of PMC induced by T cell mitogens, alloantigens, and soluble antigens. The other two antibodies were mitogenic, and behaved similarly to Leu 4 and OKT3, respectively. In T cell preparations with <0.1% monocytes (as assayed by nonspecific esterase staining), all three mAb were not mitogenic. They failed to induce either interleukin 2 (IL-2) receptor expression or IL-2 secretion. Addition of IL-1 failed to collaborate with anti-T3 mAb to induce these T cells to proliferate, but IL-2 enhanced T cell proliferation slightly. Monocyte-depleted T cells, however, proliferated in response to all three anti-T3 mAb, when TPA was added, in a dose-dependent manner. TPA induced a low level of IL-2 receptor expression in monocyte-depleted T cells, without inducing IL-2 secretion. Anti-T3 plus TPA induced a marked enhancement in both quantity and intensity of IL-2 receptor expression. IL-2 secretion was also detected. These results indicate that anti-T3 IgM can deliver an inductive signal despite its blockage of T cell proliferation, and that two signals are necessary and perhaps sufficient to induce human T cell activation and proliferation.

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