

INVOLVEMENT OF T44 MOLECULES IN AN ANTIGEN-
INDEPENDENT PATHWAY OF T CELL ACTIVATION
Analysis of the Correlations to the T Cell Antigen-Receptor Complex

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Recently, several laboratories have derived monoclonal antibodies (mAb)¹ that react with clonotypic determinants of the heterodimeric antigen receptor molecules expressed on human (1–6) and murine T cells (7–12). In some instances these antibodies (also referred to as anti-Ti) mimic the effect of specific antigens by inducing proliferation of and lymphokine release by T lymphocytes expressing the corresponding clonotype (1, 4, 13). On the other hand, antibodies to the monomorphic T3 molecules are also able to induce T cell proliferation and lymphokine release, but this effect is not clonotypically restricted (13–14). In addition, soluble anti-T3 antibody blocks antigen-induced specific T cell stimulation (13) as well as specific target cell lysis by cytolytic T lymphocytes (15–19). The effects of anti-T3 antibody have been explained by the fact that T3 molecules are physically and functionally linked to the Ti-bearing receptor molecules (20). Recently (21, 22), however, some antibodies directed against the T11 molecule, which is physically unrelated to the Ti-T3 complex, have also been shown to activate T cells leading to cell proliferation and lymphokine release. In the present study we have analyzed a third nonclonotypic molecule (designated T44) that appears to be involved in human T cell activation. T44 has been recently defined by the 9.3 mAb as a T-specific (23) surface glycoprotein of approximately M_r 44,000 (44 K) (24). The 9.3 antibody has been reported (25) to enhance the proliferative responses to phytohemagglutinin or to allogeneic cells, but to have no effect on resting T lymphocytes. Here we show that the 9.3 (anti-T44) mAb, in the presence of adherent cells, can directly induce human peripheral blood T lymphocytes to proliferate and release interleukin 2 (IL-2). The availability of a variant of the IL-2-producing Jurkat leukemia cell line (termed JA3), and of four corresponding anticlonotypic antibodies, allowed a better analysis of the molecular and functional characteristics of this molecule and its relationship with Ti, T3, and T11 structures. Thus, upon interaction with the anti-T44 mAb, T44 molecules underwent rapid modulation from the

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¹ *Abbreviations used in this paper:* CTL, cytotoxic T lymphocyte; IL-2, interleukin 2; mAb, monoclonal antibody; MLC, mixed leukocyte culture; NP-40, Nonidet P-40; PBMNC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PMA, phorbol myristate acetate; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TdR, thymidine.

JA3 cell surface; moreover, neither T3, T11, nor clonotypic molecules co-modulated with T44. Modulation of T3 or Ti structures by the corresponding antibodies, although not affecting T44 antigen expression, did abrogate IL-2 production in response to anti-T44 antibody. In contrast, modulation of T44 antigen had no effect on IL-2 production elicited by anti-T3 or anti-Ti antibodies. Thus, T44 may be part of an antigen-independent pathway of T cell activation that is not physically linked to the T3-Ti receptor complex or the T11 molecule.

Materials and Methods

Cells. Mononuclear cells (MNC) were isolated from human peripheral blood (PB) and adherent cells removed by incubation on plastic petri dishes. Purified T cells were isolated from PBMNC by rosetting with sheep erythrocytes as previously described (26). Mixed leukocyte culture (MLC)-activated T cell populations were obtained by stimulating PBMNC (10^6 per well) with irradiated (5,000 rad) human allogeneic spleen cells (10^6 per well) as described (27). On day 7, cells were harvested and centrifuged on Ficoll-Hypaque gradients, to remove dead cells. Human thymuses (age, 1–8 yr) were removed during corrective cardiac surgery and used as a source of thymocytes, as previously described (28). The JA3 cloned cell line was derived by mutagenesis from the IL-2-producing Jurkat T-ALL by exposing these cells to 200 rad gamma radiation (cesium source). Cells were then selected on the basis of their resistancy to 6-thioguanine (hypoxanthine guanine-phosphoribosyltransferase-deficient mutants) containing medium. This selection was performed six times over a period of 40 d. Selected microcultures containing cells resistant to 6-thioguanine were cloned and tested for both IL-2 production and expression of T3 antigen. JA3 was selected because it was the clone that displayed the highest levels of IL-2 production and also expressed large amounts of T3 antigen as assessed by indirect immunofluorescence.

Monoclonal Antibodies. The UCHT1 mAb (29) (directed to surface T3 antigen) was kindly provided by Dr. M. J. Crumpton and Dr. P. C. L. Beverley (Imperial Cancer Research Fund, London). The 4F2 and 3A1 mAb (30) were a generous gift of Dr. A. S. Fauci (National Institutes of Health, Bethesda, MD). Leu-4 mAb was purchased from Becton, Dickinson & Co., Basel, Switzerland, and the 9.3 (NEI-o12) mAb from New England Nuclear, Dreieich, Federal Republic of Germany. mAb with specificity for the antigen receptor-like molecule expressed on JA3 cells were derived from a fusion between the P3-U1 mouse myeloma cell line and spleen cells from BALB/c mice that had been immunized intravenously with 10^7 JA3 cells in phosphate-buffered saline (PBS). A booster injection with 1.5×10^7 JA3 cells was performed 15 d later, and immune splenocytes were fused with P3-U1 myeloma cells 3 d after the injection, as described (31). Screening of the hybridomas was based on the capacity of the various hybridoma supernatants to induce IL-2 production by JA3 in the presence of phorbol myristate acetate (PMA). Four different hybrids were isolated for further characterization, as described in more detail elsewhere (Moretta, A., G. Pantaleo, M. Lopez-Botet, and L. Moretta, submitted for publication). These four hybrids, proved to be specifically reactive with the immunizing JA3 cells and to immunoprecipitate from ^{125}I -labeled JA3 cells a protein with a diffuse band of 80–85 kD (nonreduced). Under reducing conditions, this protein could be resolved into two major peptides of 40 and 45 kD. The four mAb were termed JTi₁, JTi₂, JTi₃, and JTi₄, respectively.

Characterization of Radioiodinated Cell Surface Proteins. JA3 cells were surface labeled with ^{125}I using lactoperoxidase-glucose-oxidase-catalyzed iodination (32). $\sim 30 \times 10^6$ cells were washed and resuspended in 1 ml PBS containing glucose (5.5 mM) and the following reagents: 20 μl KI (5×10^{-5} M), Na^{125}I (1 mCi), 40 μl lactoperoxidase (1 mg/ml solution in PBS), 10 μl glucose oxidase (stock solution diluted 1:100 in PBS). Cells were incubated at 4°C. After 10 and 20 min, 10 μl glucose oxidase was added and the incubation was carried out for a total period of 30 min. Cells were then washed five times in cold RPMI 1640, twice in PBS, and lysed in 10 mM Tris-buffered saline (pH 7.5) containing 1%

Nonidet P-40 (NP-40), 1 mg/ml bovine serum albumin, and 0.1 mM phenylmethylsulphonyl fluoride (PMSF), for 20 min at 4°C. After spinning at 100,000 g, the supernatant was then immunoprecipitated as follows. Supernatants were dialyzed with PBS containing 0.05% NP-40 and 0.1 mM PMSF and precleared three times with 100 μ l of packed protein A–Sepharose beads for 2 h under rotation. Aliquots (200 μ l) were then incubated for 2 h with 20 μ l of a 1:10 dilution of anti-T44 (9.3) mAb or 10 μ l of anti-Ti (JT_{i4}) ascite diluted 1:50. 20 μ l of protein A–Sepharose beads were then added and samples incubated for 4 h at 4°C. The immunoprecipitate was eluted from protein A–Sepharose by boiling for 2 min in 5% sodium dodecyl sulfate (SDS) in the presence or absence of 5% 2-mercaptoethanol and analyzed on 11% discontinuous SDS-polyacrylamide gels (33).

For absorption studies, after incubation with the packed anti-T44 (or anti-JT_{i4}) mAb-specific immunoabsorbent, the cell lysate was reincubated twice with the same immunoabsorbent, following the procedure described above. Using this protocol, virtually complete depletion of the corresponding T44 (or JT_i) molecules was achieved. The resulting “cleared” cell lysate was then incubated with the anti-JT_i (or anti-T44) mAb-specific immunoabsorbent for an additional 3 h at 4°C. After washing, the specific bound material was eluted and separated by SDS-PAGE as described above.

Flow Cytofluorometric Analysis. The techniques used have been described in detail elsewhere (34). Briefly, aliquots of 10⁵ cells (peripheral lymphocytes, MLC-induced blast cells, thymocytes, or JA3 cells) were stained with the appropriate mAb followed by fluoresceinated goat anti-mouse Ig. Control aliquots were stained with the fluorescent reagent alone. All samples were then analyzed on a flow cytometer (FACS II; Becton Dickinson Immunocytometry Systems, Mountain View, CA) gated to exclude nonviable cells. Results are expressed as arbitrarily normalized fluorescence histograms, i.e., number of cells vs. fluorescence intensity.

Proliferation of PB T Lymphocytes in Response to mAb or Phytohemagglutinin (PHA). Proliferative responses of PB T lymphocytes to mAb or PHA were analyzed as follows. T lymphocytes (or unfractionated PBMNC) were cultured as described elsewhere (31) in triplicate microwells (10⁵ cells/well) (Greiner Labor Technik, Nurtinger, FRG) either alone or with 1–2 \times 10⁴ adherent cells in 0.2 ml with RPMI 1640 supplemented with 10% fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY), L-glutamine, penicillin-streptomycin, and one or another stimulus, for varying culture intervals. PHA was used at a final dilution of 1:100. mAb were used at the following dilutions: Leu-4 mAb, 1:100 to 1:1000 of the stock solution; 9.3 mAb, 1:40 to 1:500 of the stock solution; 3A1, 4F2, and UCHT1 mAb, 1:1000 dilution of ascitis. After incubation at 37°C in a 6% CO₂, humidified atmosphere for different culture intervals, individual wells were pulsed with 1 μ Ci of [³H]thymidine ([³H]TdR) (Amersham U.K.) 16–18 h before harvesting. Each value represents the mean of triplicate cultures. Standard deviations were <10%.

Determination of IL-2 Production by Peripheral T Lymphocytes. To evaluate the amount of IL-2 produced by lymphocytes after stimulation with lectin or mAb, we used a murine T cell proliferation assay as described (35). Briefly, peripheral blood lymphocytes (10⁵) were cultured for varying periods of time in the presence of one or another stimulus (see above) under the same culture conditions mentioned above. In experiments performed to study the effect of modulation of T3 or T44 antigens on the subsequent antibody-induced IL-2 production, “modulated” or “unmodulated” purified T lymphocytes were cultured (10⁵ per well) in the presence of mAb and 10⁴ adherent cells for 48 h before collection of culture supernatants. 100 μ l of culture supernatants were removed from each microculture and added at a final concentration of 50% to 4 \times 10³ murine indicator cells (CTLL line, kindly provided by Dr. K. Smith [36]). IL-2 activity was assessed by [³H]TdR uptake by CTLL after a 6 h pulse with 1 μ Ci of [³H]TdR at the end of a 24 h culture period.

Determination of IL-2 Production by JA3. To quantitate the amount of IL-2 secreted by JA3 cells after stimulation with mitogen or mAb, JA3 cells (1.5 \times 10⁵ per well) were cultured in the presence of phorbol myristate acetate (PMA) (10 ng/ml) or 10⁴ adherent cells and one or another stimulus for a 24 h period. Thereafter, 100 μ l of supernatant were removed and assessed for IL-2 activity on the murine CTL line, as previously

described (35). PHA was used at a final dilution of 1:100; Leu-4 and 9.3 mAb, 1:200 to 1:500; 3A1 and 4F2 mAb, 1:1000 dilution of ascitis; UCHT-1 mAb, 1:5000 dilution of ascitis; JTi₁ and JTi₄ mAb, 1:10 dilution of the hybridoma culture supernatant.

Modulation Experiments. Antigenic modulation of JA3 cells or purified T cells was performed by addition of one or another mAb and incubation for 24 h at 37°C in 6% CO₂. To ensure that modulation was performed in the presence of a saturating amount of mAb, a large antibody excess was used. Cells were cultured in flat-bottomed wells (Costar, Data Packaging, Cambridge, MA) at a concentration of 10⁶/ml in RPMI 1640 (Seromed, Biochrom, FRG), 10% FCS, with the following final dilutions of mAb: Leu-4 and 9.3, 1:100 of the stock solution; 3A1, MAR 206 (anti-T11), 4F2, UCHT1 (anti-T3), JTi₁, and JTi₄ mAb, 1:100 of the ascitic fluid. After modulation at 37°C for 24 h, cells were harvested, washed twice, and either stained directly with fluorescent anti-mouse antibodies or incubated with one or another mAb followed by the fluorescent anti-mouse reagent (17). The percentage of modulation as well as the percentage of reexpression of the various surface molecules was calculated according to Reinherz et al. (37). The same experimental procedure was used in experiments in which cells were stimulated with mAb and PMA (or adherent cells) after antigen modulation (see above).

Results

Functional Consequences of T44 Triggering of Peripheral Blood T Lymphocytes: Induction of Cell Proliferation and IL-2 Release. Prior studies (25) indicated that anti-T44 antibody specifically reacted with human T lymphocytes and enhanced the proliferative responses to PHA or to allogeneic cells, whereas no effect was observed on unstimulated lymphocytes. In view of the potential importance of T44 molecules in T cell activation, we investigated the ability of anti-T44 mAb to induce lymphocyte proliferation, in comparison with anti-T3 antibody or PHA. First, the distribution of T44 antigen in different T cell populations was investigated. Fig. 1 shows the reactivity pattern of anti-T44 or anti-T3 antibody with peripheral blood lymphocytes, thymocytes, and T cells activated in 7-d MLC. In adherent cell-depleted peripheral blood lymphocytes, a large proportion of cells (65–75%) was T44 positive. In addition, the fluorescence distribution was clearly bimodal (Fig. 1A), similar to that observed with anti-T3 antibody (Fig. 1D). In addition, sequential staining with anti-T44 and anti-T3 antibody

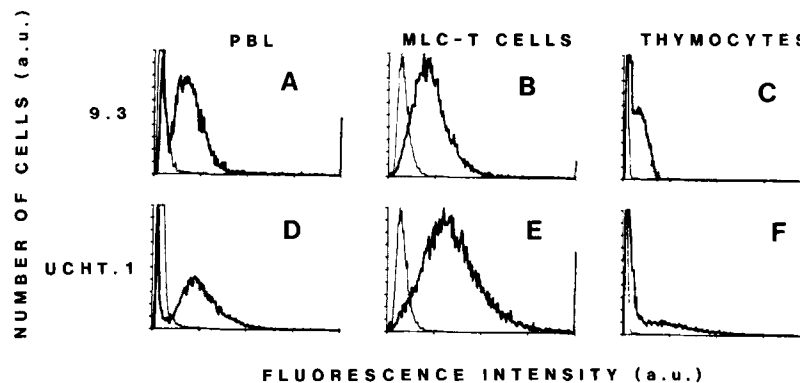


FIGURE 1. Fluorescence distribution of T44 (A–C) and T3 (D–F) antigen in peripheral blood unfractionated lymphocytes, MLC-activated T cells, and thymocytes. The various cell populations were stained with either 9.3 or UCHT1 mAb, followed by fluoresceinated anti-mouse Ig. Samples were run on a FACS II, gated to exclude nonviable cells.

(and vice versa) did not lead to any increase in the percentages of stained cells, indicating that T44 and T3 antigen were largely, if not totally, expressed by the same cells. Virtually 100% of T cells activated in 7-d mixed lymphocyte culture expressed both T44 and T3 antigen (Fig. 1, *B* and *E*). In contrast, only minor subsets of thymocytes expressed T44 or T3 antigen (*C* and *F*). In several experiments, the percentages of T44⁺ thymocytes were 10–20%.

mAb directed to T3 molecules have been shown (21) to promote polyclonal proliferation of resting T lymphocytes provided that monocytes are present in the culture assay. The mitogenic potential of anti-T44 mAb under culture conditions giving optimal responses to anti-T3 was then examined. The anti-T44 mAb was compared with PHA and anti-T3 and 3-A1 mAb for the capacity to induce proliferation of peripheral blood T cells in the presence of 10–20% adherent cells. As shown in Fig. 2A, anti-T44 mAb had a strong mitogenic effect, comparable in magnitude to that obtained with anti-T3 antibody or PHA. However, unlike T cell proliferation with PHA or anti-T3 mAb, that induced by 9.3 mAb was maximal at day 5 of culture instead of day 3. Like anti-T3, anti-T44 antibody required macrophages to mediate this mitogenic effect. Comparable results were obtained with 10 other donors (data not shown). The efficient T cell proliferation obtained with anti-T44 antibody could be mediated via IL-2 production by the responding T cell populations. To determine whether anti-T44 antibody was indeed capable of inducing IL-2 release, the same cell culture combinations analyzed in proliferation assays were tested for IL-2 release. In these experiments, culture supernatants were collected at different intervals and analyzed for IL-2 activity by a proliferation assay, using the CTLL cells as an indicator system (36). Anti-T44 antibody did induce IL-2 production (Fig. 2B), as did PHA or anti-T3. Note, however, that while IL-2 activity with PHA or

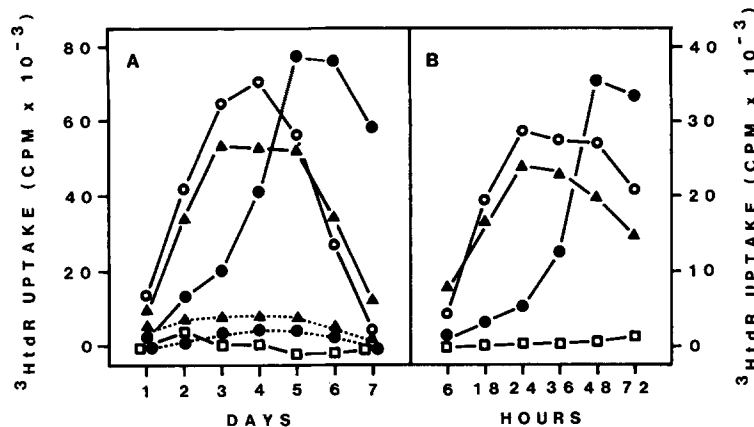


FIGURE 2. Anti-T44 antibody induces human peripheral blood T lymphocytes to proliferate (A) and release IL-2 (B). Purified T lymphocytes to which 10–20% adherent cells had been added (or simply PBMNC) were cultured (10^5 cells per well) for different intervals with one of the following reagents: (●) 9.3 (anti-T44), final concentration 1:100 of the stock solution; (▲) UCHT1 (anti-T3), 1:1000 of ascitic fluid; (□) 3A1, 1:1000 of ascitic fluid; (○) mAb or PHA. Cell proliferation was evaluated by [³H]TdR uptake (16 h pulse), whereas IL-2 production was assessed by adding culture supernatant to a standard 24 h proliferation assay using the IL-2-responsive CTL indicator cells. In A, the proliferative responses of T cells alone (i.e., in the absence of adherent cells) to UCHT1 (▲···▲) or 9.3 (●···●) mAb are also shown.

anti-T3 was maximal at 24 h, it peaked at 48 h in cultures induced with anti-T44.

Derivation of Clonotypically Restricted Antibodies Stimulating IL-2 Production from the T44-positive JA3 Cell Line. Analysis of the physical and functional relationship between T44 molecule and other surface molecules involved in T cell activation, including the clonotypically restricted Ti and the monomorphic T3 or T11 molecules, required the availability of an appropriate IL-2-producing T cell clone or tumor T cell line and anticolonotypic antibodies selectively reactive with such cells. We recently derived by mutagenesis a variant of the Jurkat leukemic T cell line, designated JA3 (surface phenotype: T11⁺, T3⁺, 3A1⁺, T4⁻, T8⁻, DR⁻, TAC⁻, 4F2⁺, T44⁺), which produces large amounts of IL-2 upon stimulation with PHA or with anti-T3 mAb in conjunction with PMA or adherent cells. To produce mAb against clonotypic structures of JA3 cells, we used these cells to immunize BALB/c mice. Subsequently, immune splenocytes were fused with P3-U1 mouse myeloma line by standard hybridization techniques. Supernatants of the 372 individual hybridomas were screened according to their ability to induce JA3 to produce IL-2 in the presence of PMA. By this screening procedure, we selected four stimulatory mAb that reacted with JA3 but lacked reactivity with any other T tumor cell line tested, with resting or activated T cell populations and several T cell clones, as assessed by indirect immunofluorescence on a FACS. These antibodies were termed anti-JTi₁, -JTi₂, -JTi₃, and -JTi₄ and were of the IgG2a (JTi₁₋₃) and IgG2b (JTi₄) murine isotype, respectively. JTi₁₋₄ antibodies were shown to immunoprecipitate in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) a single diffuse band of 85 K *M_r* under nonreducing conditions and two major peptides of *M_r* 40 and 45 K under reducing conditions (see below). In some experiments, however, a third band could be visualized under reducing conditions. In this respect it should be noted that anti-Ti mAb directed against T cell clones (1), T cell lines such as REX (38) or HPB-ALL (2, 5), or T cell leukemias (3, 4) usually precipitate only two bands under reducing conditions. However, our results are in line with the data reported by Weiss and Stobo (6), who used an anti-Ti mAb directed against a nonmutagenized Jurkat clone. In addition, cross-inhibition and sequential immunoprecipitation experiments provided clear evidence that anti-JTi₁₋₄ reacted with the same surface molecule on JA3 cells. The reactivity pattern of the antibodies, their functional properties, and the characteristics of the immunoprecipitated molecules clearly indicate that JTi₁₋₄ recognize clonotypic determinants of the T cell receptor molecule. While further characteristics of JTi₁₋₄ antibodies have been described elsewhere (Moretta, Pantaleo, Lopez-Botet, and Moretta, submitted for publication), here such anticolonotypic antibodies were used as a tool for analysis of the physical and functional relationship between T44 molecules and the T3-Ti molecular complex.

*Anti-T44 Antibody Immunoprecipitates a Prevalent *M_r* 80 K Band Under Nonreducing Conditions.* The molecule immunoprecipitated by the 9.3 antibody was originally termed T44 on the basis of the apparent *M_r* under reducing conditions (24). The molecule precipitated by the same anti-T44 antibody on JA3 cells appeared (Fig. 3B) as a diffuse band of *M_r* 40–45 K under reducing conditions. In addition, a second band of 80–85 K could be seen. Moreover, under nonre-

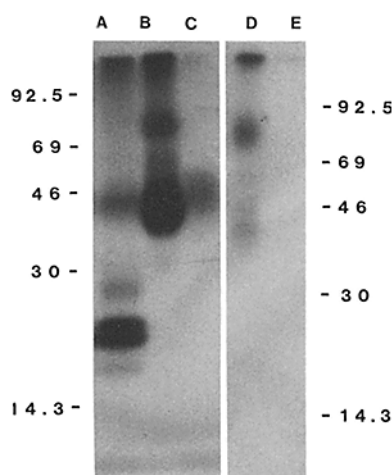


FIGURE 3. Analysis of T44 molecule by SDS-PAGE under reducing or nonreducing conditions. JA3 cells were surface labeled with ^{125}I and lysed as described in Materials and Methods. Cell lysates were immunoprecipitated with mAb and protein A-Sepharose. SDS-PAGE was performed under reducing (A-C) and nonreducing (D-E) conditions in an 11% polyacrylamide gel followed by autoradiography. The internal molecular weight markers were lysozyme (14.3 K), carbonic anhydrase (30 K), ovalbumin (46 K), bovine serum albumine (69 K), and phosphorylase *b* (92.5 K). (B and D) T44 molecules under reducing and nonreducing conditions, respectively. T3 (A) and T11 (C) molecules are shown for comparison (reduced). Anti-Tac, which does not precipitate any detectable material (E), was used as a negative control.

ducing conditions (Fig. 3D), the majority of the immunoprecipitated material displayed an M_r of 80–85 K, and the 40–45 K band was greatly decreased in intensity. Thus, the apparent M_r under both reducing and nonreducing conditions appeared similar to those of the receptor molecules precipitated by anti-clonotypic antibody from JA3 cells (see Fig. 5). Similar results have been recently obtained on 9.3 immunoprecipitates from the HPB-ALL cell line (39).

Comparison Between Activation Induced by Anti-T44 Antibody and that Elicited by PHA or Antibodies to T3 or JTi in JA3 Cells. The availability of the T44⁺ JA3 cells and of anti-JTi clonotypic antibodies capable of inducing IL-2 production from these cells made it possible to analyze the response of JA3 cells to a variety of stimuli, such as PHA and antibodies directed against T44, T3, or Ti molecules. As shown in Table I, PHA and antibodies to T3, JTi, or T44 molecules induced production of large amounts of IL-2 in the presence of PMA. Antibodies directed against other antigens expressed by JA3 such as 3A1 and 4F2 (30), used as controls, had no stimulatory effect. Although not shown, similar results were obtained in the presence of adherent cells without the addition of PMA. It is noteworthy that lower but significant IL-2 production from JA3 was also detected with anti-T44 mAb in the absence of both PMA and adherent cells, whereas no proliferative response by resting T cells was observed under the same conditions.

Independent Expression of T44 Molecules and T3-Ti Receptor Complex at the T Cell Surface. T44 molecules display an M_r similar to that of T cell receptor molecules under both reducing and nonreducing conditions. This, together with the common ability of the two structures to trigger T cell proliferation and IL-2 release, suggested a possible molecular relationship between the two molecules.

TABLE I
Anti-T44 mAb Induces IL-2 Production from JA3 Cells

Stimulus	Specificity	Ig subclass	IL-2 activity	
			+PMA*	-PMA
<i>U/ml</i>				
PHA	—	—	100	4
Leu 4	T3	IgG1	124	2
UCHT1	T3	IgG1	117	2
9.3	T44	IgG2a	108	48
4F2	4F2	IgG2a	5	4
JTi1	Ti	IgG2a	113	10
JTi4	Ti	IgG2b	131	15
3A1	3A1	IgG1	3	1

JA3 cells (1.5×10^5 /well) were cultured in the presence of the indicated stimulus for 24 h. The IL-2 activity in culture supernatants was assessed as described in Materials and Methods. Similar results were obtained in eight additional experiments.

* PMA was added at 10 ng/ml final dilution.

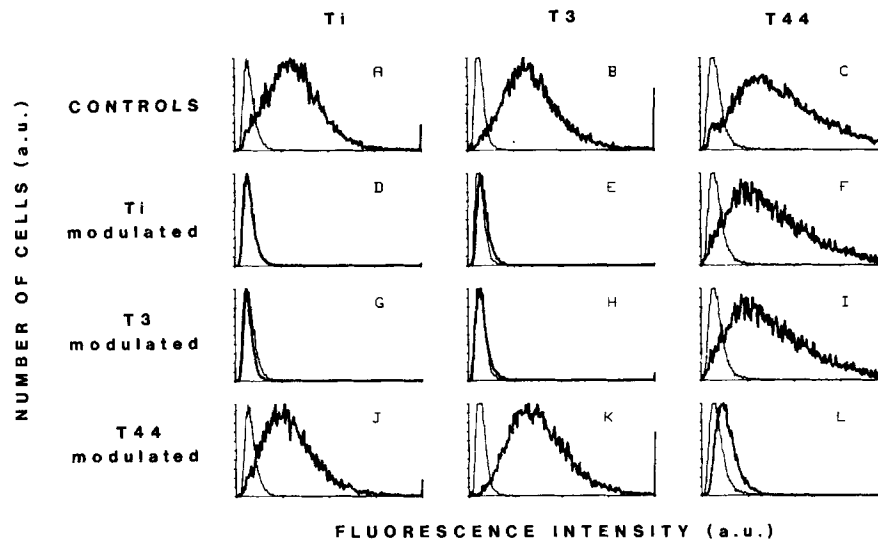


FIGURE 4. Modulation (or lack thereof) of T44, T3, and Ti molecules on JA3 cell surface after preincubation with mAb. JA3 cells were incubated with anti-T3, anti-T44, or anti-JTi mAb for 24 h at 37°C, and subsequently washed several times. Cells incubated with one or another antibody were either stained directly with the anti-mouse Ig fluorescent reagent or incubated (30 min) with the same or one of the other antibodies before staining with the second reagent and FACS analysis. Background fluorescence is given for cells stained with the fluoresceinated anti-mouse Ig reagent only.

Antibodies directed to the T3 or Ti structures have been shown (13) to induce modulation of the corresponding molecules. In addition, modulation of either one of the two molecules resulted in comodulation of the other. In agreement with these data, anti-T3 or anti-JTi mAb induced complete modulation of T3-Ti molecular complex in JA3 cells (Fig. 4). Given the ability of anti-T44 antibody to induce IL-2 production in a similar manner as anti-T3 or anti-JTi antibody,

we wanted to determine whether anti-T44 mAb could induce modulation of T44 molecules and whether a physical link existed between T44 molecules and the T3-Ti molecular complex. To this end, JA3 cells were incubated for 24 h with antibodies to T3, JTi, or to T44 (to induce antigen modulation) and subsequently stained with each other antibody. Although anti-T44 did not induce a complete modulation of the corresponding molecules in JA3 cells, a strong reduction (~80%) of the T44 expression was observed (Fig. 4). T3-Ti expression was unchanged after modulation of the T44 molecules. Conversely, as previously shown (40), the expression of T44 was unaffected by modulation of the T3-Ti complex. In these experiments we also investigated the physical relationship between T44 and T11 molecules, in view of the involvement of both these structures in T cell proliferation and lymphokine release. In agreement with a previous report (21), expression of T11 molecules was not affected by modulation of the T3-Ti complex; moreover, it remained unchanged after modulation of T44 molecules, indicating that T11 and T44 molecules are not physically linked on the T cell surface (data not shown).

Given the susceptibility of T44 molecules to antibody-induced modulation and given the functional consequences of T44 triggering, it was important to determine whether T44 molecules were reexpressed at the cell surface soon after modulation or whether, similarly to T3-Ti complex, reexpression occurred after a 48–72 h interval. To this end, JA3 cells were incubated for 24 h with antibodies to T44, T3, or JTi molecules, washed extensively, and cultured in the absence of antibody, before analysis of antigen reexpression at different culture intervals. Neither T3-Ti complex nor T44 were reexpressed during the first 48 h, (i. e., T3 and Ti were undetectable whereas T44 was still reduced by ~80%) as assessed by indirect immunofluorescence and FACS analysis. T44 started to be reexpressed at 72 h and the reexpression was complete at 96 h. Interestingly, reexpression of T44 at the JA3 cell surface paralleled that of T3 and JTi molecules.

To further document that T44 and JTi molecules were neither identical nor physically related, sequential immunoprecipitation experiments were performed. JA3 cells were surface labeled using lactoperoxidase-catalyzed iodination. After detergent lysis, cell extracts were immunoprecipitated and analyzed by SDS-PAGE. Under reducing conditions (Fig. 5), the anti-T44-specific immunoprecipitate consisted of M_r 44 K molecules migrating as a broad band (Fig. 5*a*). Under the same conditions, the anti-JTi-specific immunoprecipitate showed three distinct bands of M_r 49, 45, and 40 K, respectively (Fig. 5*b*). Under nonreducing conditions, the molecules recognized by anti-T44 and anti-JTi₄ mAb consisted of a similar 85 K band (data not shown). Preclearing of anti-T44-specific molecules virtually eliminated the reactivity of such antibody for ¹²⁵I-labeled JA3 cell lysates (Fig. 5*d*) but did not affect at all the reactivity of anti-JTi₄ mAb (Fig. 5*c*). It must be stressed that absorption experiments using the anti-T44 antibody were performed using twice as much cell lysate with respect to the immunoprecipitations shown in lanes *a* and *b*. In addition, preclearing of anti-JTi-specific molecules abrogated the reactivity of anti-JTi antibodies for ¹²⁵I-labeled JA3 cell lysates but had no effect on the immunoprecipitation of anti-T44 mAb-reactive molecules (not shown).

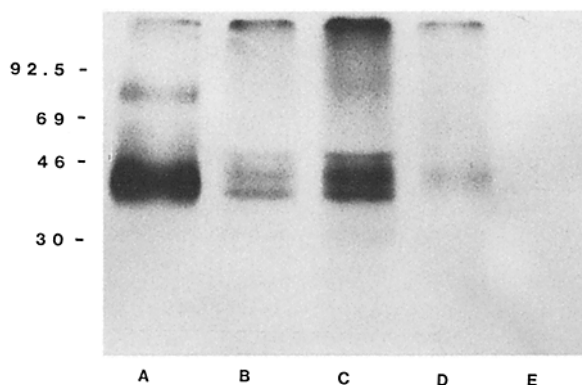


FIGURE 5. Sequential precipitation of JA3 surface antigens identified by JT₄ and 9.3 mAb. JA3 cells were surface-labeled with ¹²⁵I and lysed as described in Materials and Methods. The cell lysates were immunoprecipitated with mAb and protein A-Sepharose. SDS-PAGE was performed under reducing conditions in an 11% polyacrylamide slab gel system. The following molecular weight markers were used: lysozyme (14.3 K), carbonic anhydrase (30 K), ovalbumin (46 K), bovine serum albumin (69 K), and phosphorylase *b* (92.5 K). Specific immunoprecipitates were obtained by using: (a) anti-T44 mAb, (b) anti-Ti mAb, (c) anti-Ti mAb after preclearing of the anti-T44-reactive population, (d) anti-T44 mAb after preclearing with the same mAb, (e) negative control consisting of cell lysate reacted with the anti-TAC mAb. In *c* and *d*, twice as much cell lysate was used compared with *a* and *b*.

T44 Triggering of IL-2 Production Is Regulated by the T3-Ti Receptor Complex. Since both T44 and T3-Ti receptor complex triggered IL-2 production and, moreover, both structures were susceptible to antibody-induced modulation, it was important to determine whether the capacity to trigger via one structure was dependent on the other. To determine whether anti-T3 or anti-JTi antibodies affected subsequent activation by anti-T44 antibody, and, conversely, whether anti-T44 modulation had any effect on subsequent triggering with anti-T3 or anti-Ti antibodies, JA3 cells were incubated for 24 h at 37°C with one or another mAb, washed to remove excess antibody, and stimulated with appropriate concentrations of the various antibodies and PMA. As shown in Table II, preincubation of JA3 cells with anti-T44 antibody virtually abrogated IL-2 production induced by the same antibody, but did not affect the anti-T3- or anti-Ti-triggered IL-2 production. In contrast, preincubation with anti-T3 or anti-JTi mAb abrogated IL-2 production triggered by the same antibodies as well as by anti-T44. Similar results were obtained in experiments in which adherent cells (and not PMA) were added to the cultures. Similar experiments designed to analyze the functional consequences of antibody-induced modulation on IL-2 production were performed with purified (resting) peripheral blood T cells. Cells were preincubated for 48 h with either anti-T44, anti-T3, or anti-T11 antibody. After extensive washings and FACS analysis, cells were cultured in the presence of one of the antibodies and adherent cells (10⁴/well). The 48-h supernatants were assessed for IL-2 activity in the CTL indicator system. Preincubation of T cells with anti-T44 antibody abrogated the IL-2 production induced by the same antibody but had no effect on anti-T3-mediated IL-2 production. In contrast, preincubation with anti-T3 antibody abrogated the IL-2 production triggered by anti-T3 or anti-T44 antibody. The anti-T11 antibody

TABLE II
*Influence of mAb-induced Modulation on JA3 Activation by
 Anti-T44 mAb*

JA3 cell	Stimulus*				
	Anti-T3	Anti-Ti [‡]	Anti-T44	Anti-T11	—
Unmodulated	113	138	119	1	2
T3 modulated	6 [§]	11	9	5	2
Ti modulated	19	3	5	3	3
T44 modulated	118	127	2	2	3
T11 modulated	124	133	128	8	2

JA3 cells were incubated for 24 h with one or another mAb as described in Materials and Methods, washed, and subsequently seeded at 1.5×10^5 /well in the presence of the various mAb and PMA (10 ng/ml). Supernatants were collected 24 h later and tested for IL-2 activity as described in Materials and Methods.

* PMA was added to all cultures.

[‡] Anti-JTi₄ antibody was added.

[§] Values indicate the arbitrary units of IL-2 per ml.

used as control neither induced modulation of the corresponding molecules nor promoted any detectable IL-2 production by T cells (not shown). Thus, it appears that modulation of the T3-Ti receptor complex, although having no effect on T44 expression at the cell surface, regulated the ability of the T44 structure to induce IL-2 release.

Discussion

In the present report we describe a new pathway of human T lymphocyte activation triggered by the T cell lineage-specific T44 surface molecule after binding to a specific mAb. Some physical and functional characteristics of the T44 molecules appeared similar to those of the T cell receptor molecules, including comparable M_r under both reducing and nonreducing conditions, susceptibility to antibody-induced modulation, and the capability of triggering T lymphocyte proliferation and IL-2 release. A possibility was that anti-T44 antibody recognized a monomorphic determinant of the T cell receptor. However, our present data provide clear evidence that the two surface structures are physically independent. Moreover, we show that T44 molecules are not physically related to the T11 structure, which is also capable of mediating a T cell activation alternative to that initiated by the T3-Ti receptor complex (21).

The T44 molecule was expressed in both resting and activated T lymphocytes and in some T cell leukemias (23), while it was weakly expressed on a subpopulation of thymocytes. In this respect, distribution of T44 molecules differs from that of the M_r 50 K T11 or the 40 K 3A1 molecules, which are present on 98% of thymocytes and thus represent early T cell differentiation antigens. In addition, according to Hansen et al. (23), distribution of the T44 molecules could be distinguished from that of T3 antigen on the basis of differential reactivity with some T cell leukemias.

Similar to anti-T3 or anti-T11, anti-T44 antibody induced both T cell proliferation and IL-2 release. However, whereas T11 triggering in resting T lympho-

cytes could be achieved with antibody in soluble form (21), triggering of T44 molecule, like that of T3, required the presence of adherent cells. On the other hand, triggering of JA3 cells could be achieved with anti-T44 in absence of PMA or adherent cells, while triggering of JA3 with either anti-T3 or anti-Ti antibody required PMA or adherent cells. In a previous report (25) the same anti-T44 antibody did not induce proliferation of resting T cells. A likely explanation for this discrepancy may be that, in contrast to lymphocyte suspensions used in our study, lymphocytes were nylon wool-passed and therefore depleted of most adherent cells.

We also studied the relationships between T44 molecules and the other monomorphic or clonotypic structures involved in T cell activation, on the JA3 tumor T cell line. JA3 appeared to be particularly suitable for these studies in view of its ability to produce large amounts of IL-2 after appropriate stimuli and because mAb to the JA3 clonotypic structures were available. Antibody-induced antigen modulation experiments showed that T44 molecules, like T3 or Ti, were susceptible to modulation. However, while modulation of T3 or Ti molecules resulted in reciprocal comodulation, it had no effect on T44 surface antigen expression. Moreover, modulation of T44 molecules had no effect on T3, Ti, or T11 antigen expression. These experiments, together with the finding that preclearing ¹²⁵I-labeled JA3 lysates with anti-T44 did not remove molecules immunoprecipitated by anti-Ti antibodies, and that preclearing with anti-JTi did not remove molecules reactive with anti-T44 mAb, indicate that the T44 molecule is not identical and probably not physically related to the α or β chains of the T cell receptor. The T cell receptor complex, however, appears to regulate the capacity of the pathway of T cell activation initiated by T44 molecule to induce T cell responses. This conclusion is based on the observation that modulation of T3 or Ti molecules leads to inhibition of T cell response to anti-T44 antibody. A similar dependency upon the T3-Ti antigen receptor complex has been described by Meuer et al. (21) for the T11-dependent pathway of T cell activation. Study of the functional correlation between these two antigen-independent pathways of T cell activation could not be carried out because anti-T11 mAb with stimulatory capacity were not available in our lab; however, modulation experiments indicated that T44 and T11 molecules were not physically linked at the cell surface.

The T44 pathway of T cell activation may be triggered by a still undefined natural ligand. It cannot be excluded that the T44 molecule itself may be important for cell-to-cell contacts, as suggested by preliminary experiments in which anti-T44 antibody had a strong inhibitory activity on specific target cell lysis mediated by alloreactive cytolytic cells. Whatever the natural ligand of T44 molecule may be, the late reexpression of this molecule after antibody-induced modulation is strikingly similar to that of the T3-Ti antigen receptor complex. This latency will likely be important for the regulation of the T44-triggered pathway of T cell activation. Future studies characterizing the relationship of the T3-Ti antigen receptor complex and the T44 molecule, by the study of T44⁻ mutants of JA3 similar to the Ti⁻ mutants described by Weiss and Stobo (6), should better define the relative function and behavior of these surface molecules.

Summary

Prior studies indicate that the 9.3 monoclonal antibody (mAb) which defines a 44 kD T lineage-specific glycoprotein (T44) enhances the proliferative response of peripheral blood T lymphocytes to phytohemagglutinin (PHA) or allogeneic cells. The T44 molecule was expressed in both resting and activated T lymphocytes and in a subset of thymocytes, as assessed by indirect immunofluorescence and flow cytometry. In view of the potential importance of T44 in T cell activation, we investigated the ability of the 9.3 (anti-T44) antibody to stimulate peripheral blood T lymphocytes under culture conditions giving optimal proliferative responses to anti-T3 mAb. Like UCHT1 (anti-T3) mAb, the 9.3 (anti-T44) mAb promoted strong proliferative responses of purified T cells, provided that adherent cells were added to the culture. Maximal proliferation in response to 9.3 antibody was consistently detected at day 5 (at day 3 with anti-T3 or PHA). Moreover, triggering of T lymphocytes with 9.3 antibody (in the presence of adherent cells) resulted in strong IL-2 production that peaked at 48 h. Analysis of the physical and functional relationship between the T44 molecule and other molecules involved in T cell activation, including the clonotypically restricted Ti and the monomorphic T3 or T11 molecules, was carried out on a mutagenized Jurkat T leukemia cell line. This mutant, termed JA3 (surface phenotype: T11⁺, T3⁺, 3A1⁺, T4⁻, T8⁻, DR⁻, Tac⁻, 4F2⁺, T44⁺) produced large amounts of IL-2 upon stimulation with PHA, anti-T3, or anticolonotypic mAb in conjunction with phorbol myristate acetate (or adherent cells). The molecules precipitated by anti-T44 mAb from ¹²⁵I-labeled JA3 cells appeared as a diffuse band of *M_r* 40–45,000 under reducing conditions; under nonreducing conditions, a prominent band of *M_r* 80–85,000 was observed, while the *M_r* 40–45,000 band was greatly reduced. Thus, T44 molecules in both reducing and nonreducing conditions had relative molecular weights similar to that of molecules carrying clonotypic (Ti) determinants. In addition, like anti-Ti or anti-T3 mAb, anti-T44 antibody induced JA3 cells to produce large amounts of IL-2 in the presence of phorbol myristate acetate. Other similarities between T44 and molecules carrying clonotypic structures included the susceptibility to antibody-induced modulation and the late reexpression (72 h) at the cell surface after modulation. Taken together, these experiments suggest that anti-T44 mAb might recognize a monomorphic determinant of the T cell receptor molecule or be physically or functionally linked to the T3-Ti complex. However, antibody-induced modulation of T44 molecules did not lead to the loss of T3, Ti, or T11 molecules, nor did modulation of T3 or Ti affect the expression of T44 antigen at the JA3 cell surface. In addition, sequential immunoprecipitation experiments showed that preclearing of JA3 cell lysates with anti-T44 antibody did not remove molecules carrying clonotypic structures, and that preclearing with anticolonotypic mAb did not affect subsequent precipitation with anti-T44 mAb. Thus, the antigen-independent, T44-mediated mode of T cell triggering is distinct from that initiated by the T3-Ti antigen receptor complex or by T11 molecules. However, modulation of the T3-Ti receptor complex, although it had no effect on T44 expression, did regulate the ability of T44 molecules to induce IL-2 release. The nature of the natural ligand of T44 molecule and the

role in vivo of T44-triggered pathway of human T cell activation remains to be determined.

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