PERTURBATION OF THE T4 MOLECULE TRANSMITS A NEGATIVE SIGNAL TO T CELLS

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Thymocytes differentiate into two mutually exclusive subsets of T lymphocytes that can be distinguished by the expression of the T4 and T8 surface proteins (1). T4+ and T8+ subsets can be further distinguished with respect to recognition of distinct classes of major histocompatibility complex (MHC) molecules, as well as expression of different immunological function. T4+ lymphocytes recognize foreign antigen predominantly in conjunction with class II MHC molecules, while T8+ cells recognize antigen predominantly in association with class I MHC molecules (2–4). Functionally, the T4+ subset contains the majority of helper T cells, while the T8+ subset is devoid of helper cells but contains the majority of cytotoxic and suppressor T cells (5). The relationship of the T4+ subset to MHC recognition of class II molecules is probably more stringent than the relationship of T4 expression to helper function, since T4+ lymphocytes are functionally heterogeneous, and can be induced to differentiate into cytotoxic and suppressor cells (6–8).

Recently, data from many laboratories (9–16) have suggested that the T4 molecule is not solely a marker of a unique T cell subset, but plays a crucial role in both the inductive and effector phases of T cell functions. For example, monoclonal antibodies (mAb) directed against certain epitopes of the T4 glycoprotein inhibit antigen-induced in vitro proliferation (9), lymphokine release (10, 11), helper cell function (12), as well as autologous reactions induced by class II-bearing stimulator cells (13). In addition, antibodies against T4 inhibit cytotoxic activity of T4+ lymphocytes specific for target cells bearing class II MHC determinants (14–16). Taken together, these data have been interpreted to be consistent with the hypothesis that the T4 molecules are receptor structures for nonpolymorphic regions of class II molecules (10, 14, 15). Alternative interpretations of the antibody inhibition studies are that the interaction of anti-T4 antibodies with the T4 molecule delivers a negative signal to T4+ cells, or interferes with the recognition of important non-MHC structures on stimulating and target cells.

To study these possibilities in greater detail, we have triggered T4+ cells in vitro with antibodies against the T3 surface molecule, in the presence or absence of antibodies to the T4 molecule. T3 is the 20/25 kilodalton (kD) molecular
complex known to be physically associated with the polymorphic T cell receptor for antigen (17–19). Anti-T3 triggering mimics antigen activation even with respect to the requirement for accessory cells (20). We have found that antibodies to certain epitopes of T4 inhibit anti-T3 activation of T4+ cells. This inhibition by anti-T4 was observed even under experimental conditions in which the accessory cell was devoid of MHC class II molecules. In addition, we have found that T4 cells depleted of accessory cells can be triggered by anti-T3 bound to Sepharose beads, and this activation is also inhibited by anti-T4 antibodies. Thus, perturbation of the T4 molecule can deliver inhibitory signals to T cells, independent of MHC class II recognition.

Materials and Methods

Lymphocyte Preparation and Isolation of T Cells. Fresh peripheral blood mononuclear cells were isolated from consenting human volunteers by Ficoll hypaque density gradient centrifugation. The methods for isolation of lymphocyte subpopulations and distinct T cell subsets have been previously described (21). On the basis of erythrocyte (E) rosette formation with sheep red blood cells (SRBC), cells were further fractionated into E+ (T cells) and E- (non-T cells). E+ cells were further treated by 1-h adherence to plastic petri dishes at 37°C in a humidified atmosphere with 5% CO2. Nonadherent E+ cells (T cells) were then negatively selected as follows: T4+ cells were negatively selected from the E+ population by treatment with OKT8 and OKM1 antibody plus complement (C). T8+ cells were selected by the use of OKT4 and OKM1 antibody plus complement (22). Phenotypic analysis of the resulting populations by cytofluorography showed that the OKT8-treated population contained >90% OKT3+ cells, >90% OKT4+ cells, <2.0% OKT8+ cells, and <1% OKM1+ cells. The OKT4-treated population contained >90% OKT3+ cells, >85% OKT8+ cells, <10% OKT4+ cells, and <1% OKM1+ cells.

Monoclonal Antibodies. mAb were used in the ascites form or as purified antibodies prepared from ascites as described (23). The mAb used in these studies were OKT4 (IgG2), OKT4C (IgG1), OKT1 (IgG1), OKT3 (IgG2), OKJ2 (IgG1), and OKT8A (IgG2). OKT4 and OKT4C react with noncompeting epitopes of the T4 molecule (24). OKT1 binds to >90% of peripheral T cells (25). OKJ2 recognizes nonpolymorphic antigenic determinants of class II MHC products (26). All ascites antibodies were used at a final dilution of 1:100–1:200. OKT3 was used in purified form at a final dilution of 500 ng/ml. Coupling of purified OKT3 to CNBr-activated Sepharose 4 B beads was performed as described by the manufacturer (Pharmacia Fine Chemicals, Uppsala, Sweden).

Cultures. The final medium for all cultures consisted of Iscoves modified Dulbecco’s medium (IMDM) supplemented with 1% penicillin-streptomycin (Gibco Laboratories, Grand Island, NY) and 10% fetal calf serum (HyClone Laboratories, Logan, UT). T cells were placed in culture at a final concentration of 10⁶ cells/ml in a volume of 200 μl, in 96-well, flat-bottom microtiter plates (Costar, Cambridge, MA). E+ accessory cells were irradiated with 2,000 rad before addition to culture. The cell line U937 (27) was maintained in bulk culture at a concentration of 0.5 × 10⁶ cells/ml. For proliferative experiments, U937 cells were irradiated (7,000 rad) and added to the cultures at the indicated concentrations. The phenotype of U937 was Ia−, OKM1+, OKM5−, OKT3−, OKT4+, and OKT8−. Proliferation was assessed in triplicate after 72 h of cell culture by [³H]thymidine (1 μCi/well; New England Nuclear, Boston, MA) incorporation as previously described (21).

In experiments in which anti-T3 bound to Sepharose beads were used, cultures were performed in round-bottom plates. To achieve maximum proliferation, ~1,000 beads were added per culture.

Results

Antibodies to Certain Epitopes of the T4 Molecule Inhibit Anti-T3 Activation of T4 Cells. In an initial series of experiments, isolated T4+ cells were triggered with
OKT3 in the presence of varying concentrations of accessory E\(^{-}\) cells. At the initiation of cell culture, antibodies raised against distinct epitopes of the T4 molecule (OKT4 and OKT4C), as well as a control antibody, OKT1, were added, and T cell activation was assessed 72 h later by \[^3\text{H}\]thymidine incorporation. A representative experiment is shown in Fig. 1. The activation of T4\(^{+}\) cells by OKT3 was dependent on E\(^{-}\) accessory cells, and this activation was markedly inhibited by OKT4C. In contrast, OKT4 was only minimally inhibitory, and the control antibody, OKT1, had no inhibitory effects. Since OKT4C and OKT1 are both IgG1 antibodies, the inhibition by OKT4C was not a function of isotype alone. The inhibition by OKT4C but not OKT1 is dose dependent, with significant inhibition observed at concentrations of OKT4C as low as 2.5 \(\mu\text{g/ml}\) (Fig. 2).

To rule out nonspecific inhibitory properties of OKT4C antibodies, as well as to assess the tissue specificity of the inhibition, OKT4C was added to OKT3-activated T4\(^{+}\) and T8\(^{+}\) subsets in the presence of E\(^{-}\) accessory cells. As shown in Fig. 3, OKT4C markedly inhibited activation of T4\(^{+}\) cells, but had no significant effect on the OKT3-activated T8\(^{+}\) cells. Thus, the inhibition observed was not nonspecific, but was dependent on the interaction of OKT4C with T4\(^{+}\) cells.

**Figure 1.** T4\(^{+}\) cells, \(10^6\) cells/ml, were incubated with OKT3 (500 ng/ml) in the presence of increasing numbers of irradiated E\(^{-}\) cells (●). At the beginning of the culture, mAb OKT4C (■), OKT1 (△) or OKT4 (□) were added. \[^3\text{H}\]Thymidine incorporation was measured after 72 h. Antibodies were used at concentrations as described.

**Figure 2.** T4\(^{+}\) cells (10\(^6\) cells/ml) were incubated in the presence of OKT3 (500 ng/ml) and irradiated E\(^{-}\) cells (0.2 × 10\(^6\) cells/ml). Proliferation was measured by \[^3\text{H}\]thymidine incorporation after 72 h (---). OKT4C (■) or OKT1 (□) were added at increasing concentrations.
Figure 3. T cells were fractionated by antibody plus C treatment into T8⁺T4⁻ (A) and T4⁺T8⁻ (B) populations. The respective populations were triggered with OKT3 and irradiated autologous E⁺ cells, and proliferation was measured after 72 h.

Figure 4. T4⁺ cells (10⁶ cells/ml) were cultured in the presence of OKT3 (500 ng/ml) and E⁻ cells (0.2 x 10⁶ cells/ml). The antibodies OKT4C (II), OKT1 (A) or OKT4 (F-I) were added at time 0, and cultures were harvested after 1, 2, 3, and 5 d of culture; [³H]thymidine incorporation was measured. The control cultures (no antibody) are labeled (●).

Conversely, OKT8 inhibited the proliferation of T8⁺ cells, as previously described (28).

One interpretation of the inhibition by OKT4C observed above is that the antibodies altered the kinetics of OKT3-induced proliferation of T4⁺ cells. To address this point, we assessed the proliferative response of T4⁺ cells triggered by OKT3 in the presence or absence of OKT4C, and terminated the cultures on various days. In the representative experiment shown in Fig. 4, OKT4C, when added at the initiation of culture, inhibited OKT3 activation throughout the culture periods (days 1–5). Therefore, OKT4C was not inducing a shift in the kinetics of T3 activation. In a related experiment, OKT4C was added to OKT3-activated T4⁺ cells at various times after the initiation of cell culture. Inhibition was marked only when OKT4C was added during the first 24 h of cell culture. No inhibition was seen when OKT4C was added at 48 h of culture (Fig. 5). These studies show that OKT4C is not nonspecifically cytotoxic or interfering with [³H]thymidine incorporation.

Inhibition of OKT3-activated T4⁺ Cells by OKT4C Is Not Dependent on Ia⁺ Accessory Cells. In the next series of experiments, we asked whether the inhibition of OKT3-activated T4⁺ cells by OKT4C depends on the presence of Ia⁺ accessory cells. Since OKT3 activation is dependent on E⁻ cells, which contain Ia⁺ monocytes and B cells (Fig. 1), we devised alternative experimental approaches to activating T4⁺ cells by OKT3 in the absence of Ia⁺ cells. First, we asked whether the accessory cell function of E⁻ cells could be replaced by the human myelomonocytic cell line, U937, which does not express Ia on the cell surface.

We found that, despite the lack of Ia, the U937 cells were an excellent source of accessory cells for T3 activation. We then added OKT4C, OKT1, or OKI2
to T4⁺ cells cultured with graded numbers of irradiated U937 cells in the presence of OKT3, and assessed proliferation after 3 d. The results of a representative experiment are shown in Fig. 6. The OKT3-induced activation of T4⁺ cells in the presence of the Ia⁻ U937 accessory cell was markedly inhibited by OKT4C. The control OKT1 antibody was not inhibitory. In addition, OKI2, an antibody that binds to nonpolymorphic regions of class II MHC molecules, had no inhibitory effect on activation induced by U937 cells. Taken together, these results show that the mechanism of inhibition by OKT4C was independent of the interaction of T4⁺ cells with Ia⁺ accessory cells.

To further address this point we found that T4⁺ cells could be activated in the absence of E⁻ accessory cells if OKT3 was presented on Sepharose beads. In a representative experiment (Fig. 7), we found that T4⁺ cells, depleted of any residual monocytes by OKM2 and C, could be activated by OKT3 covalently linked to beads. As expected, in the absence of accessory cells, the T4⁺ cells did not respond to soluble OKT3. However, these T4⁺ cells did proliferate in the presence of OKT3 bound to Sepharose beads. Importantly, OKT4C, but not OKT4 or OKT1, significantly inhibited this activation. These experiments show that, even in the absence of accessory cells, OKT4C could inhibit T4⁺ cell activation.
Figure 7. Monocyte-depleted T4+ cells (10^6 cells/ml) were cultured in the presence of medium, soluble OKT3, or Sepharose-bound OKT3. Antibodies OKT4C, OKT4, or OKT1 were added to cultures containing Sepharose-bound OKT3. After overnight pulse, [3H]-thymidine incorporation was measured.

Discussion

These studies were undertaken to further explore the functional role of the T4 molecule in T cell activation. Specifically, we asked whether the T4 molecule participates in T cell activation solely by interacting with class II MHC molecules. To address this question, we devised experimental approaches to activating T4+ cells by OKT3 in the absence of Ia-bearing accessory cells, and asked whether anti-T4 antibodies could still inhibit function. This experimental system was chosen because T4+ cell activation by anti-T3 bears analogies to antigen-mediated triggering. For example, triggering T4+ cells with either anti-T3 or antigen in the presence of Ia+ accessory cells results in comodulation of the T cell receptor–T3 molecular complex, with ensuing interleukin 2 (IL-2) production, IL-2 receptor expression, and proliferation (29, 30).

Our experiments show that certain antibodies against T4 specifically inhibit the proliferative response to anti-T3 (Fig. 1). Since our initial experiments included autologous Ia+ accessory cells, one interpretation of these results was that OKT4C inhibits by abrogating putative T4-Ia interactions. This interpretation has been offered as an explanation for experiments in which anti-T4 antibodies inhibit T4+ cell-mediated help, cytotoxicity, or proliferation (9–14).

In further experiments, however, we ascertained that this could not be the only explanation for anti-T4–induced inhibition. In these experiments, we took advantage of the fact that in some experimental systems, Ia− cells are capable of functioning as accessory cells. For example, in the murine system, concanavalin A (Con A)-induced T cell proliferation can be supported by Ia− fibroblasts. We therefore rigorously depleted our T4+ population of Ia-bearing monocytes, and replaced them with an Ia− macrophage-derived cell line, U937. Evidence that the depletion of monocytes was in fact complete was demonstrated functionally by the lack of response of T4+ cells to soluble OKT3, and also by complete lack of OKM1-bearing cells by cytofluorographic analysis. The U937 cell line lacks Ia surface molecules, but bears the OKM1 cell surface marker, and is capable of producing IL-1 (31). Our experiments show that U937 could substitute for Ia+ macrophages in supporting anti-T3 triggering of T4+ cells. More importantly,
anti-T4 potently inhibited this response (Fig. 6). These results are analogous to the findings of Beckoff et al. (32), who showed that anti-L3T4, the mouse analogue of T4, inhibited Con A–driven T cell responses in the presence of Ia– fibroblasts. Taken together, these data show that Ia+ accessory cells are not required for the inhibition of T4+ cell activation by antibodies against T4.

In our last set of experiments, we asked whether anti-T4 inhibition could be observed in the virtual absence of accessory cells. It has recently been demonstrated that it is possible to activate T cells with anti-T3 in the absence of accessory cells. For example, Weiss et al. (33) activated the Jurkat T cell leukemia by anti-T3 in the presence of phorbol-12-myristate-13-acetate (PMA). In addition, in the apparent absence of accessory cells, Meuer et al. (30) were able to activate long term–cultured antigen-specific T cell clones with Sepharose-bound anti-T3. In our experiments, fresh peripheral T4+ cells were activated with Sepharose-bound anti-T3. Under these conditions, we showed that anti-T4 antibodies markedly inhibit T4+ cell activation (Fig. 7). These results suggested that the T4 molecule is capable of regulating T cell function independently of interactions with accessory cells, and ruled out the formal possibility that anti-T4 inhibits by abrogating the putative interaction of T4 with non-MHC molecules on the surface of accessory cells. We cannot rule out the possibility that the T4 molecule may interact with other surface components of the T4+ cell itself.

Taken together, our data is consistent with the hypothesis that the T4 molecule may function as an independent transducer of negative signals to the T cell surface. This hypothesis is not incompatible with the idea that the T4 molecule serves as a receptor for class II MHC antigens. If the T4 molecule were in fact the receptor for Ia molecules, then negative signals transmitted by T4-Ia interactions could be advantageous in certain situations. Since T4+ cells usually interact with Ia-bearing accessory cells in lymphoid tissue in the absence of antigen (34, 35), these cells would usually be receiving negative signals. Only in the presence of a strong positive signal, delivered by the interaction of the polymorphic T cell antigen receptor with antigen and Ia, could the cell be turned on. This view requires the postulate that either the negative signal delivered by the T4-Ia interaction is easily overcome by higher-avidity antigen interactions, or that the T4 molecule, in the presence of antigen, could deliver a positive signal. Thus, T4 might function as a switch-like molecule on the T cell surface, being turned off when T4 interacts with Ia alone, and turned on when antigen is present. Recently, a cDNA encoding the T4 molecule has been isolated and sequenced (36). The deduced amino acid sequence demonstrates an N-terminal domain with amino acid and structural homology to immunoglobulin light chains. In addition, the molecule has a cytoplasmic domain of 41 amino acids. This structural data is compatible with the idea that T4 can both function as a receptor for Ia and deliver regulatory signals to the T cell.

**Summary**

We investigated the functional role of the T4 molecule in the activation of T cells by OKT3. T4+ cells were induced to proliferate by OKT3 and erythrocyte rosette–negative accessory cells in the presence or absence of OKT4C, OKT4, and OKT1. OKT4C (IgG1), and not OKT4 (IgG2) or OKT1 (IgG1) inhibited
proliferation when OKT4C was added during the first 24 h of cell culture. The inhibition of OKT3 activation by OKT4C did not require Ia+ accessory cells, since T4+ cells could be activated by OKT3 in the presence of Ia- U937 cells, and this activation was markedly inhibited by OKT4C. Furthermore, T4+ cells could be induced to proliferate by OKT3 covalently linked to Sepharose beads, in the absence of any accessory cells. Under these conditions, OKT4C, but not OKT4 or OKT1 significantly inhibited proliferation. These data demonstrate that at least one mechanism by which anti-T4 antibodies inhibit T cell activation is independent of any putative role of T4 molecules in the recognition of Ia on target cells. The data are compatible with the idea that perturbation of the T4 molecules can transmit a negative signal to T4+ cells.

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