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MONOCLONAL ANTIBODIES TO CD2 AND LYMPHOCYTE FUNCTION-ASSOCIATED ANTIGEN 3 INHIBIT HUMAN THYMIC EPITHELIAL CELL-DEPENDENT MATURE THYMOCYTE ACTIVATION[1]

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Recent study of human thymocyte-thymic epithelial (TE) cell interactions has demonstrated that thymocytes bind to TE cells, and a consequence of this binding is the provision of accessory cell signals by TE cells for phytohemagglutinin (PHA)-induced mature thymocyte activation. In this paper we report on studies of the molecules involved in TE cell-dependent mature thymocyte activation. TE-thymocyte interactions necessary for PHA-induced thymocyte activation were inhibited by monoclonal antibodies against the cluster of differentiation (CD)2 antigen on thymocytes and lymphocyte function-associated (LFA)-3 antigen on TE cells. Inhibition of TE accessory cell signals by antibodies against CD2 (αCD2) and LFA-3 (αLFA-3) antigens occurred early on during thymocyte activation and prevented thymocyte interleukin 2 receptor expression. Further, αCD2 and αLFA-3 inhibited PHA-induced thymocyte activation in whole thymic explant cultures suggesting a significant role of the CD2 and LFA-3 antigens in thymocyte activation when accessory cell signals for PHA-induced thymocyte triggering were delivered by cells within an intact thymic microenvironment.

The human thymic microenvironment is known to be critical for generation of immunocompetent T lymphocytes (1-5). However, the roles that individual thymic microenvironment components play in activation of T cells are unknown. Recently, the development of a long term in vitro culture system for human thymic epithelial (TE)[3] cells (6) has allowed for the investigation of human TE-thymocyte interactions in vitro. We have previously shown that both autologous and allogeneic mature and immature thymocytes bind to cultured human TE cells (7). Contrary to results expected based on current theories of intrathymic T cell maturation, binding of thymocytes to TE cells was not inhibited by monoclonal antibodies against major histocompatibility complex (MHC) class I or class II antigens (7), but rather was inhibited by antibodies against the cluster of differentiation (CD) 2 antigen (lymphocyte function-associated antigen (LFA) 2, T11, E-rosette receptor), and LFA-3 (8). To study functional sequelae of TE-thymocyte binding, we have established thymocyte proliferative assays that are strictly TE cell-dependent, and shown that TE cells can function as potent accessory cells for phytohemagglutinin (PHA)-induced mature ($CD1^-$, $CD3^+$, $p80^+$) thymocyte activation (9). We have postulated that TE cells may provide accessory cell activation signals necessary for completion of the terminal stages of T cell maturation or necessary for intrathymic response of mature thymocytes to antigens (10, 11).

In this report, we show that monoclonal antibodies against the CD2 and LFA-3 antigens inhibit TE cell-dependent PHA-induced mature thymocyte activation.

MATERIALS AND METHODS

Monoclonal antibodies. Antibodies against the LFA-1 (TS1/22), LFA-2 (TS1/8), and LFA-3 (TS2/9) antigens were used as previously described (12, 13). Antibodies 3F10 (14) and L243 (15), which identify nonpolymorphic determinants on MHC class I and II structures respectively, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The following monoclonal antibodies were obtained through the Second International Workshop and Conference on Leukocyte Differentiation Antigens (reviewed in Haynes (16)): Sk9/Leu 6 (CD1), T11/3Pt 2H9 (CD2), T11/ROLD2-1H8 (CD2), T11/3T4-8B5 (CD2), T11/7T4/7A9 (CD2), T11/7T4-7E10 (CD2), T3/RW2-4B6 (CD3), T3/RW2-8C8 (CD3), T4/19Thy 5D7 (CD4), L17F12/Leu1 (CD5), T12/3Pt12B8 (CD6), T8/21Thy 2D3 (CD8), TAC (IL-2 Receptor, CD25). Antibodies 3A1 (CD7) and A1G3 (anti-p80) were used as previously described (17, 18).

Acquisition and preparation of tissue. Thymic tissue was obtained from normal children (age 31 mo to 16 yr) undergoing median sternotomy and corrective cardiac surgery. Human TE cell cultures were established as previously described (6). Thymocyte single cell suspensions were prepared by gently teasing thymic tissue with forceps followed by passing the suspension through gauze to remove large tissue chunks and purification by Ficoll-Hypaque density centrifugation (19). Purified thymocytes were used immediately or were slow frozen at 1 to 3×10^8 cells/ml in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 20% fetal calf serum (GIBCO), gentamicin 10 μg/ml (Schering, Kenilworth, NJ), and 7.5% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO), and stored in liquid nitrogen until use. Thymocytes were thawed by incubation at 37°C for 1 hr in thawing medium consisting of RPMI 1640 supplemented with 30% fetal calf serum, gentamicin 10 μg/ml, heparin 20 U/ml (The Upjohn Co., Kalamazoo, MI), and deoxyribonuclease I (Sigma) 0.01 mg/ml.

Cytocentrifuge preparations of cultured TE cells used in TE cell thymocyte co-cultures were evaluated in indirect immunofluores-

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[3] Abbreviations used in this paper: TE, thymic epithelial; TE_M, mitomycin C-treated TE cells; LFA, lymphocyte function-associated antigen; CD, cluster of differentiation; PHA, phytohemagglutinin; ACD, accessory cell-depleted; IL-2, interleukin 2; IL-2R, interleukin 2 receptor.

cence assays using a panel of monoclonal antibodies including AE-3 (antikeratin) (20) Mol (antimonocyte, macrophage) (21), TE-7 (antifibroblast) (22), L243 (anti-MHC class II) (15), TE-3 (anticortical TE cell) (23), and A2B5 (antimedullary and subcapsular cortical TE cell) (24). TE cells were 92.0 ± 1.9% AE-3-positive, less than 0.5% Mol-positive, 8.0 ± 1.9% TE-7-positive, 59.0 ± 3.0% TE-3-positive, 19.1 ± 1.9% A2B5-positive, and 2.8 ± 1.1% L243-positive.

Accessory cell-depleted (ACD) thymocytes were obtained by passage of thymocytes over nylon wool column (25). Thymocytes and ACD thymocytes were characterized by indirect immunofluorescence (26) with monoclonal antibodies 35.1 (anti-CD2) (27), Mol, L243, and NA1/34 (anti-CD1, cortical thymocyte) (28). Thymocytes utilized in this study were 94.1 ± 0.8% 35.1-positive, 2.5 ± 0.9% Mol-positive, 8.4 ± 1.4% L243-positive, and 50.0 ± 1.6% NA1/34-positive. ACD thymocytes were 96.1 ± 0.8% 35.1-positive, 48.8 ± 1.9% NA1/34-positive, and less than 0.5% Mol-positive. Thymocytes which were NA1/34-negative were mature thymocytes and were positive for the A1G3 (p80) antigen.

Thymocyte proliferation assays. Proliferative assays were performed as previously described (29). Briefly 1×10^5 thymocytes, 1×10^5 ACD thymocytes, or 1×10^5 ACD thymocytes and 1×10^4 mitomycin C-treated (40 μg/ml for 1 hr) TE cells (TE_M) in 0.2 ml of RPMI 1640 + 15% human A serum (Plasma Alliance Inc., Knoxville, TN) either in the presence or absence of PHA, 0.5 μg/ml (Burroughs Wellcome Co., Research Triangle Park, NC) were placed into 96-well round-bottomed plates (Costar, Cambridge, MA). After a 3-day incubation at 37°C in humidified 5% CO_2 environment, 0.4 μCi of [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) was added to each well and incubation continued for an additional 4 hr. At the end of culture, wells were harvested onto glass fiber filters using a Mash II harvester (Whittaker M.A. Bioproducts, Walkersville, MD). Isotope incorporation was determined by scintillation counting in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). Results are expressed as Δ cpm = cpm of PHA-stimulated culture − cpm of unstimulated culture. As previously reported, PHA-induced thymocyte activation in this culture system was found to be completely TE cell-dependent by rigorous depletion of macrophages and MHC class II-positive cells from both thymocyte suspensions and TE cell suspension (9).

Antibody inhibition assays. Monoclonal antibodies used for inhibition of thymocyte proliferation were sterile-filtered through 0.2 μm filter (Millipore Products, Bedford, MA) and added to thymocyte assays or TE-thymocyte co-cultures at saturating concentrations. All antibodies were used at a 1/200 dilution of ascites except αCD2 and αLFA-3 which were used at 1/2000 and 1/1000 dilutions, respectively. P3X63/Ag8 was used as control ascites for all experiments. Results are expressed as percent of control [^{3}H]thymidine incorporation = [Δ cpm in presence of antibody/ Δ cpm in presence of control (P3X63/Ag8) antibody] X100. Data were compared by Student's *t* test.

Thymic explant cultures. Fresh thymic tissue was cut into 4 × 4 × 2 mm specimens and placed into culture in 3 ml of RPMI 1640 + 15% human A serum in the presence or absence of PHA 10 μg/ml in 12-well tissue culture plates (Costar). Monoclonal antibodies were added at the same concentrations as for thymocyte proliferation assays with P3X63/Ag8 serving as control.

After a 4-day incubation at 37°C in humidified 5% CO_2 environment, each specimen was teased apart with sterile forceps and thymocytes were purified by Ficoll-Hypaque density centrifugation. Thymocytes were placed into 96-well round-bottomed plates at 1×10^5 cells in 0.2 ml RPMI + 15% human A serum. [^{3}H]Thymidine, 0.4 μCi, was added to each well and plates were incubated for 4 hr. Harvesting and isotope incorporation were determined as for thymocyte proliferation assays.

Interleukin 2 receptor (IL-2R) assays. ACD-thymocytes, ACD-thymocytes + 10% TE_M, or ACD-thymocytes + 10% TE_M with saturating concentrations of αCD2 or αLFA-3 were cultured in 1 ml of RPMI 1640 + 15% human A serum in round-bottomed tubes for 3 days in the presence of PHA, 0.5 μg/ml. Cells were washed once, stained with directly fluoresceinated anti-IL-2R antibody (Coulter Immunology) and analyzed (10^4 cells counted) on an Ortho Diagnostic Systems Cytofluorograf (Westwood, MA), model 50H. Thymocytes which had not been cultured or were cultured for 3 days without PHA were less than 5% IL-2R-positive.

RESULTS

TE accessory cell function. As previously reported (9), TE cells function as potent accessory cells for PHA-induced thymocyte activation. Thymocytes cultured with PHA for 3 days incorporated little [^{3}H]thymidine (Fig. 1). Thymocytes depleted of accessory cells (ACD) by nylon wool filtration incorporated even less [^{3}H]thymidine, and TE_M incorporated essentially no [^{3}H]thymidine. However, co-cultivation of 1×10^5 ACD thymocytes with 1×10^4 TE cells resulted in marked [^{3}H]thymidine incorporation (Fig. 1). This TE cell-dependent ACD thymocyte [^{3}H]thymidine incorporation was most marked at suboptimal PHA concentrations of 0.25 μg/ml to 1.0 μg/ml. Furthermore, as previously shown, the TE cell-dependent ACD thymocyte activation was dependent upon the number of added TE cells, was directed primarily toward the $CD1^-$, $CD3^+$ (mature) thymocyte subpopulation, and was IL-2-dependent (9). Previous kinetic studies have shown that maximal thymocyte activation in this system occurred at day 3 to day 4 of culture (9).

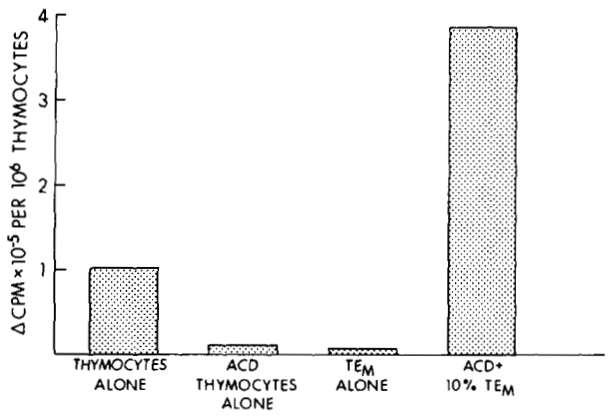

Figure 1. TE cell-dependent assay of PHA-induced ACD thymocyte activation. Aliquots of 10^5 thymocytes, 10^5 ACD thymocytes, 10^4 TE_M cells, or 10^5 ACD thymocytes + 10^4 TE_M were cultured as described in *Materials and Methods*. Data are expressed as Δcpm/10^6 thymocytes = cpm of PHA-stimulated culture − cpm of unstimulated culture. Data shown are from one experiment that is representative of nine separate experiments performed.

Inhibition of TE accessory cell function. Using this TE-thymocyte co-culture system and a PHA concentration of 0.5 μg/ml, we examined the effect of a large group of monoclonal antibodies added at the initiation of culture at saturating concentrations. Representative antibodies against the CD1, CD4, CD5, CD7, and CD8 groups had no inhibitory effect upon TE accessory cell function for PHA-induced thymocyte activation (data not shown). Next, monoclonal antibodies against the CD2 (αCD2), LFA-3 (αLFA-3), and MHC class I and II antigens were added to PHA-stimulated autologous (Fig. 2*A*) and allogeneic (Fig. 2*B*) TE-ACD thymocyte co-cultures. αCD2 ($p < 0.001$) and αLFA-3 ($p < 0.001$) inhibited TE cell-dependent PHA-induced ACD thymocyte activation in both autologous and allogeneic cultures. In contrast, antibody L243 against MHC class II antigen did not inhibit thymocyte [^{3}H]thymidine incorporation in either co-culture type (Fig. 2, *A* and *B*). Antibody 3F10 against MHC class I antigen did not significantly inhibit ACD thymocyte PHA responses in autologous TE-thymocyte co-cultures (Fig. 2*A*), but did inhibit by 38% ($p < 0.02$) the TE cell-dependent PHA response of ACD thymocytes in allogeneic TE-thymocyte suspensions (Fig. 2*B*).

Antibody inhibition in thymic explant cultures. The relevance of the in vitro antibody inhibition of TE-thymocyte proliferation to in vivo interactions of thymocytes

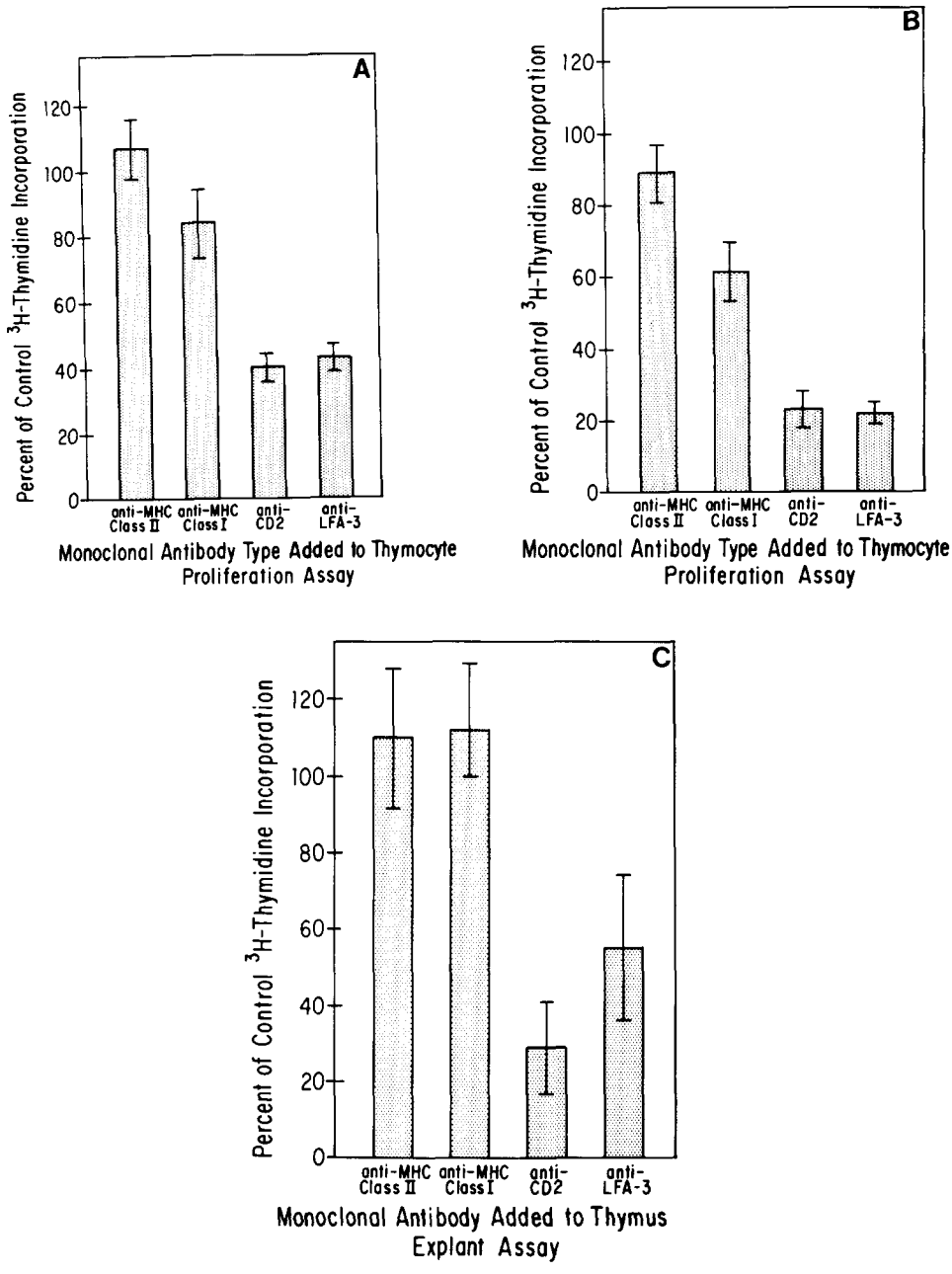

Figure 2. Effect of addition of monoclonal antibodies on TE cell-dependent PHA-induced thymocyte activation. Either 10^5 ACD thymocytes + 10^4 autologous TE_M (*A*), or 10^5 ACD thymocytes + 10^4 allogeneic TE_M (*B*) were placed into culture as described in *Materials and Methods*. Saturating concentrations of monoclonal antibodies were added and cultures incubated 3 days. Cultures were harvested and [^{3}H]thymidine incorporation determined as described. Fresh thymic explants 32 mm^3 (*C*) were cultured, harvested, and [^{3}H]thymidine incorporation determined as described in *Materials and Methods*. *A*, *B*, and *C* represent the mean ± SEM of seven, eight, and four experiments, respectively. Mean [^{3}H]thymidine incorporation of control cultures in *A*, *B*, and *C* was 230,000, 116,000, and 31,000 cpm per 10^6 thymocytes, respectively.

with an intact thymic microenvironment was investigated by assaying the effect of monoclonal antibody on PHA-induced activation of thymocytes in situ in 32 mm^3 thymic explants. As shown in Figure 2*C*, αCD2 and αLFA-3 inhibited PHA-induced thymocyte activation when mitogen stimulation and accessory cell signals were provided in the context of an intact thymic microenvironment. In contrast, antibodies against MHC class I and class II antigens had no inhibitory effect on PHA-induced thymocyte activation in thymic explants (Fig. 2*C*).

Antibody inhibition of IL-2R expression. Since IL-2R expression and interaction of IL-2 with its receptor are necessary for mature T cell proliferation (30), we investigated the effect of addition of αCD2 and αLFA-3 on ACD thymocyte IL-2R expression. As shown in the representative experiment in Figure 3*A*, in the presence of PHA (0.5 μg/ml) only 23% of ACD thymocytes expressed IL-2R after a 3-day culture without TE cells. Addition of 10% autologous TE cells to ACD thymocytes + PHA resulted in an increase after 3 days (to 78%) in the number of IL-2R$^+$ thymocytes (Fig. 3*B*). Addition of αCD2 (Fig. 3*C*) or αLFA-3 (Fig. 3*D*) to the PHA-stimulated TE-ACD thymocyte co-culture markedly inhibited thymocyte IL-2R expression after 3 days (to 5 and 6% of thymocytes, respectively). Similar results were obtained in three separate experiments.

Kinetics of inhibition of TE accessory cell function. Previous studies of the kinetics of TE accessory cell function for PHA-stimulated ACD thymocytes demonstrated that PHA-induced thymocyte [^{3}H]thymidine incorporation in the presence of TE cells peaked on days 3 and 4 of culture with a reduction of [^{3}H]thymidine incorporation occurring at later times (9). Addition of αCD2 and αLFA-3 inhibited thymocyte [^{3}H]thymidine incorporation maximally on day 2 to day 4 of co-culture with less inhibition at later times (Fig. 4*A*). As shown in Figure 4*B*, addition of αCD2 and αLFA-3 at time 0 or 4 hr after

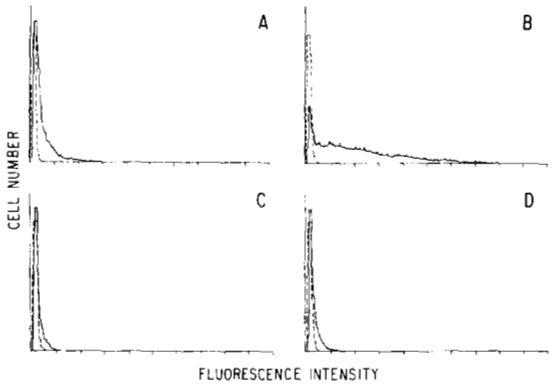

Figure 3. Antibodies against CD2 and LFA-3 antigens inhibit thymocyte IL-2R expression in TE-thymocyte co-cultures. ACD thymocytes (*A*), ACD thymocytes + 10% TE_M (*B*), ACD thymocytes + 10% TE_M + αCD2 (*C*), or ACD thymocytes + 10% TE_M + αLFA-3 (*D*) were cultured and IL-2 R determined as described in *Materials and Methods*. Control cells stained with directly fluoresceinated P3X63/Ag8 control ascites are represented by *dotted lines* in each panel. Data shown are from one experiment representative of three separate experiments performed.

PHA addition inhibited TE cell-dependent ACD thymocyte [^{3}H]thymidine incorporation. However, when αCD2 and αLFA-3 were added 16 or 24 hr after PHA, no inhibition of [^{3}H]thymidine incorporation was observed. αCD2 antibody treatment of thymocytes for 1 hr at room temperature or 37°C, followed by initiation of co-culture did not inhibit PHA-induced thymocyte activation (Δcpm = 266,000 for αCD2-treated thymocytes and Δcpm = 253,000 for control, $p > 0.5$). Similarly, αLFA-3 treatment of TE cells under the same conditions did not inhibit PHA-induced thymocyte activation (Δcpm = 247,000 for αLFA-3-treated TE cells while Δcpm = 253,000 for control, $p > 0.5$).

DISCUSSION

In this study, we have used an assay that is strictly TE cell-dependent to determine functional molecules involved in TE-mature thymocyte interactions. Moreover, we have shown that the same molecules that appear to be involved in TE accessory cell function for mature thymocytes in suspension cultures are also involved in provision of accessory cell signals to thymocytes in cultured thymic explants.

αCD2 and αLFA-3 both bind to peripheral T cells and have been previously reported to inhibit peripheral T cell activation in mitogen-induced [^{3}H]thymidine incorporation assays (13). Since αCD2 (but not αLFA-3) binds to thymocytes (8, 31), αCD2 inhibition of TE cell-dependent PHA-induced thymocyte activation could occur by delivery of a negative signal for activation to thymocytes (32–34) rather than by antibody blocking of TE-thymocyte binding. Against the hypothesis that αCD2 delivers a negative activation signal is the observation that preincubation of αCD2 with thymocytes followed by washing away excess antibody before co-culture did not inhibit TE cell-dependent PHA-induced thymocyte [^{3}H]thymidine incorporation at 3 days. Rather, we found αCD2 and αLFA-3 had to be present in saturating amounts continuously over 3 days in TE-thymocyte co-cultures to maximally inhibit thymocyte activation. That αCD2 and αLFA-3 antibody inhibited TE accessory cell function maximally during the first 24 hr after addition of PHA (Fig. 4*A*) suggested that later in culture on days 3 to 4,

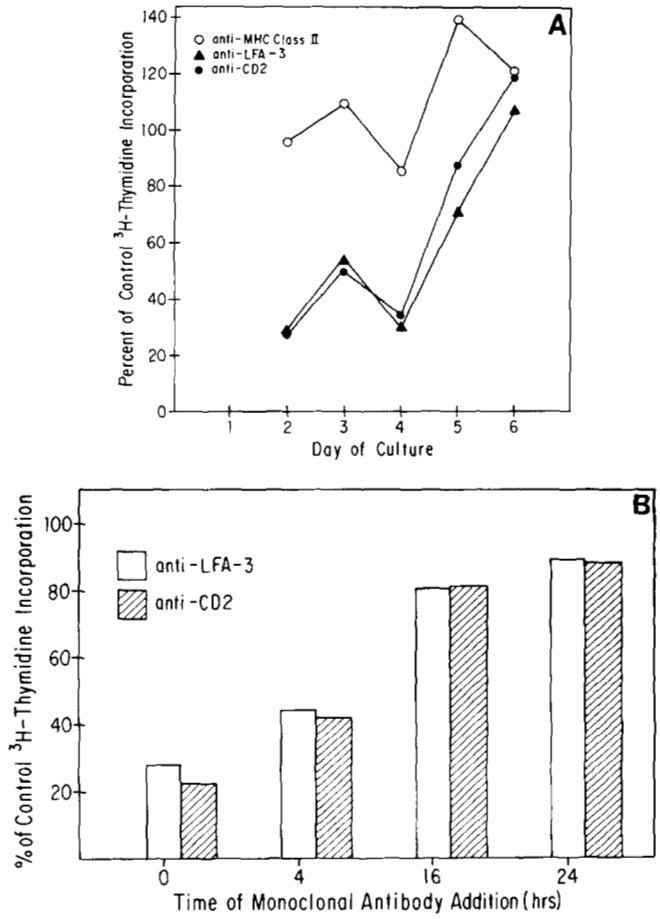

Figure 4. Kinetics of αCD2 and αLFA-3 inhibition of TE cell-dependent PHA-induced ACD thymocyte activation. In *A*, replicate cultures of 10^5 ACD thymocytes + 10^4 TE_M in the presence of PHA 0.5 μg/ml were established. Saturating concentrations of anti-MHC class II antibody, αCD2, αLFA-3 or control P3X68/Ag8 ascites were added. Cultures were incubated for the times indicated, harvested, and [^{3}H]thymidine incorporation determined as described in *Materials and Methods*. Data are expressed as percent of control [^{3}H]thymidine incorporation with control antibody added. Control [^{3}H]thymidine incorporation was 293,000, 360,000, 83,000, 64,000, and 75,000 cpm per 10^6 thymocytes on day 3 to 7, respectively. In *B*, replicate cultures containing 10^5 ACD thymocytes + 10^4 TE_M were placed into co-culture in round-bottomed plates in the presence of PHA 0.5 μg/ml. Saturating concentrations of αCD2 or αLFA-3 antibodies were added at 0, 4, 16, or 24 hr. [^{3}H]Thymidine was added during the final 4 hr of a 3-day culture. Cultures were harvested and [^{3}H]thymidine incorporation was determined. Data are expressed as percent of control [^{3}H]thymidine incorporation with control antibody added. Control [^{3}H]thymidine incorporation was 137,000 cpm/10^6 thymocytes.

TE cells in co-cultures eventually overcame the blocking effect of αCD2 and αLFA-3 for TE-thymocyte interactions. Thus, αCD2 most likely inhibits thymocyte proliferation in this assay by binding to thymocytes and blocking TE-thymocyte binding. Similarly, αLFA-3, by binding to TE cells, likely inhibits thymocyte proliferation in a similar manner, i.e., via inhibition of TE-thymocyte binding. This hypothesis is especially attractive since it implies a single set of cell-surface molecules may mediate both the binding necessary for cytotoxic T cell killing (35, 36) and the binding of thymocytes to TE cells necessary for mature thymocyte activation.

In previous studies, using a TE-thymocyte binding assay, we found that αCD2 antibodies inhibited TE-thymocyte interactions by binding to thymocytes, whereas αLFA-3 inhibited TE-thymocyte interactions by binding to TE cells (8). Moreover, fluorescence-activated cell sorter analysis of thymocytes and TE cells in suspension

confirmed that αCD2 bound to thymocytes and not to TE cells whereas αLFA-3 bound TE cells but not thymocytes (8). Thus, in this assay of TE cell-dependent PHA-induced thymocyte activation, αCD2 inhibited by binding to thymocytes whereas αLFA-3 inhibited by binding to TE cells.

Anti-MHC class I antibodies have been recently reported to inhibit human peripheral T cell triggering by anti-CD3 antibodies (37). It was of interest that anti-MHC class I antibody inhibited thymocyte activation in allogeneic but not in autologous TE-thymocyte co-cultures or in thymic explant cultures, implying a selective role for class I MHC antigens in allogeneic TE-thymocyte interactions. The absence of an inhibitory effect on thymocyte activation of anti-MHC class II antibodies despite the induction of MHC class II antigen expression on TE cells during TE-thymocyte co-cultures (9) suggests that this TE cell-dependent thymocyte activation is not MHC-restricted.

The simplest interpretation of the involvement of the CD2 antigen on thymocytes and the LFA-3 antigen on TE cells in TE-thymocyte cell binding is a receptor ligand interaction between the CD2 and LFA-3 molecules. The CD2 antigen has been recently shown to bind directly to the LFA-3 antigen on human erythrocytes (38), and to induce rosetting of human erythrocytes (39). Similarly, purified LFA-3 antigen has been shown to bind to CD2 antigen on Jurkat T cells and to induce rosetting of Jurkat T cells, peripheral blood T cells (40), and thymocytes (41). The ability of TE cells to act as a functional CD2 ligand to trigger immature ($T4^-$, $T8^-$, $CD7^+$) thymocyte proliferation in the absence of PHA (42) also argues for direct involvement of the CD2 antigen in TE-thymocyte binding. Furthermore, Hunig has shown that the sheep red blood cell analogue of LFA-3 antigen can activate human peripheral blood T cells in concert with an anti-CD2 antibody (43). Since the CD2 antigen has been implicated in T cell activation via PHA (44–46) and via triggering with combinations of anti-CD2 antibodies (47–51), these data suggest that a natural ligand for the CD2 molecule is present in thymus on human thymic epithelial cells. Our studies of αCD2- and αLFA-3-induced inhibition of TE-thymocyte binding (8) and inhibition of TE cell-dependent thymocyte activation (present study) suggest that one ligand for the CD2 molecule in thymus may be LFA-3 molecules on TE cells.

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