

A NOVEL MODEL FOR ANTIGEN-DEPENDENT ACTIVATION
OF NORMAL HUMAN T CELLS

Transmembrane Signaling by Crosslinkage of the CD3/T Cell
Receptor- α/β Complex with the Cluster Determinant 2 Antigen

By MANIKKAM SUTHANTHIRAN

*From The Rogosin Institute and Departments of Medicine and Biochemistry,
Cornell University Medical College, New York, New York 10021*

Cell surface molecules that regulate antigen-dependent signaling of T cells have been the focus of much investigation. Data have emerged indicating that the clonotypic α/β heterodimer, expressed on the surface of T cells in association with the lineage-specific CD3-protein complex, recognizes antigen in the context of MHC gene products expressed on the surface of antigen-presenting (accessory) cells (1-5). Additional T cell surface molecules, the CD4¹ and the CD8 antigens, appear to contribute to the T cell activation process by functioning as associative recognition/restriction elements for the monomorphic component of MHC antigens. Although the CD2 antigen has also been implicated in many aspects of T cell functions, its role in T cell activation initiated via the CD3/TCR- α/β is controversial (6-12).

The studies reported here were prompted by our original demonstration of differential effects of mAb directed at the SRBC-binding epitope of the CD2 antigen. We found that the anti-CD2 mAb prevents clustering between T cells and a B-lymphoblastoid cell line (B-LCL)² endowed with accessory cell activity, and inhibits T cell proliferation dependent on co-stimulatory signals provided by the B-LCL but not with 12-*o*-tetradecanoyl phorbol-13-acetate. Interestingly, the same anti-CD2 mAb, when crosslinked, was an effective substitute for accessory cell signals in the activation of normal human quiescent T cells (13, 14). Because these observations raised the possibility that the CD2 antigen might participate in physiological T cell-accessory cell interactions and because activation via the α/β heterodimer of the TCR is dependent upon accessory cell signals (15-17), we reasoned, despite previous suggestions by others of mutually antagonistic signaling via the TCR complex and CD2 antigen (9-11), that signaling via the TCR and the CD2 antigen would be synergistic in promoting T cell activation. Here we have tested this hypothesis using highly purified normal human T cells and T cell subsets (CD4⁺ or CD8⁺ T cells), and mAbs directed at the CD3/TCR- α/β and at the CD2 antigen, as probes.

Address correspondence to Dr. M. Suthanthiran, Immunogenetics and Transplantation Center, The Rogosin Institute, 430 East 71st Street, New York, NY 10021.

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² *Abbreviations used in this paper:* B-LCL, B-lymphoblastoid cell line; CD, cluster determinant; PCR, polymerase chain reaction; PKC, protein kinase C.

Results from our investigation demonstrate explicitly that signals initiated by cross-linking the TCR complex with the CD2 antigen activates normal human T cells (CD4⁺ or CD8⁺ T cells). Furthermore, this novel model of activation is homologous to physiologic antigenic signaling of T cells with respect to signal transduction via Ca²⁺ mobilization and protein kinase C activation, transcriptional activation, and translation of the gene for IL-2, and the utilization of IL-2-IL-2 receptor pathway for T cell proliferation.

Materials and Methods

Materials. mAbs directed at a framework determinant of CD3/TCR- α/β , anti-TCR-1 (15-17), anti-Leu-5b (CD2), anti-Leu-3a + 3b (CD4), anti-Leu-1 (CD5), anti-Leu-M3 (CD14), anti-Leu-M1 (CD15), anti-IL-2 receptor α (CD25), and anti-HLA-DR were purchased from Becton Dickinson & Co., Mountain View, CA. OKT11 (CD2), OKT4 (CD4), and OKT4A (CD4) mAbs were purchased from Ortho Pharmaceuticals, Raritan, NJ. MO2 (CD14) and NKH-1A (CD56) mAbs were purchased from Coulter Corp., Hialeah, FL. Affinity-purified goat antibodies specific for mouse IgG and affinity-purified goat antibodies specific for γ -1 or γ -2a heavy chains were purchased from Southern Biotechnology Associates, Inc., Birmingham, AL. Staurosporine and K-252a were purchased from Kyowa Hakko USA Inc., New York, NY; and 1-5 (isoquinolinylsulfonyl)-2-methyl-piperazine (compound H-7) was from Sigma Chemical Co., St. Louis, MO.

Generation and Purification of F(ab')₂ Fragments of mAbs. An immunopure F(ab')₂ preparation kit (Pierce Chemical Co., Rockford, IL) was used for the generation and purification of F(ab')₂ fragment of anti-TCR-1 or OKT11. In brief, the mAbs were dialyzed against 20 mM sodium acetate (pH 4.5) and concentrated. F(ab')₂ fragments were generated using immobilized pepsin and a digestion buffer (0.1 M sodium citrate buffer, pH 4.1 and 60-min incubation for IgG2a OKT11 and pH 3.5 and 8-h incubation for IgG1 anti-TCR-1). The F(ab')₂ fragments were purified with an immobilized protein A column using immunopure binding and elution buffers (Pierce Chemical Co.). The eluted fragments were dialyzed against PBS (pH 7.4) for 24 h using dialysis tubing of 50,000 molecular weight cut off, and concentrated. SDS-PAGE of eluted fragments under nonreducing conditions demonstrated the presence of F(ab')₂ fragments and the absence of intact IgG mAbs.

Affinity-purified F(ab')₂ fragments of rabbit antibodies specific for mouse IgG, purchased from Southern Biotechnology Associates, were used to crosslink the F(ab')₂ fragments of anti-TCR-1, OKT11, or anti-TCR-1 and OKT11.

Isolation of T cells and Subsets of T Cells. Human PBMC were isolated by Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation of venous blood obtained from healthy normal individuals. T cells were isolated from PBMC with a previously described sequential multi-step procedure (18) that includes: (a) depletion of adherent cells by Sephadex-G10 (Pharmacia) gel filtration; (b) depletion of residual monocytes by treatment with 5 mM L-leucine methyl ester (Sigma Chemical Co., St. Louis, MO); (c) depletion of HLA-DR⁺ cells and NK cells by treatment with anti-HLA-DR mAb, anti-NKH-1A mAb, and prescreened rabbit complement; and (d) utilization of the SRBC-rosetting technique as the final preparative step for the isolation of highly purified T cells. FACS analysis of cells isolated revealed >98% of cells to be positive for CD3 and CD2 antigens and <1% of cells to be positive for NKH-1, HLA-DR, CD14, CD19, or CD25 antigen.

The CD4⁺ and the CD8⁺ T cell subsets were isolated with a modified panning procedure of Wysocki and Sato (19). In brief, highly purified T cells were pretreated with saturating concentrations of anti-CD8 mAb (OKT8 and anti-Leu-2A) or anti-CD4 mAbs (OKT4A and OKT4) and then placed into petri dishes coated with affinity-purified goat anti-mouse IgG and incubated for 70 min at 4°C. Nonadherent cells (negative selection) were removed following the incubation period. FACS analyses of negatively selected CD4⁺ or CD8⁺ T cell subset revealed >95% purity for the corresponding phenotype, and <1% contamination with the reciprocal subset or with NKH-1, HLA-DR, CD14, CD19, or CD25 antigen positive cells.

Measurement of T Cell Activation. IL-2 receptor α (CD25) expression was measured by FACS analysis using Epics C cell sorter (Coulter Corp.), as described (18). The cells were labeled with PE-conjugated anti-CD25 mAb and the percentage of cells expressing IL-2 receptor α was determined by analysis of immunofluorescence histograms, with Epics C multidata acquisition and display systems, software version 3.1. PE-conjugated isotypic control mAb was used to determine nonspecific fluorescence signal.

T cell proliferation was quantitated by determining [3 H]thymidine incorporation. In brief, T cells, CD4 $^+$ or CD8 $^+$ T cells (10^6 cells/ml) were cultured in a total volume of 200 μ l in round-bottomed microwell plates at 37°C in a 5% CO $_2$, 95% air humidified atmosphere and [3 H]thymidine incorporation into DNA during 48–64 h of culture was determined in a liquid scintillation counter. T cells were cultured in RPMI medium 1640 (catalogue no. 380-2400, Gibco Laboratories, Grand Island, NY) supplemented with 5% FCS (catalogue no. 230-6140, Gibco Laboratories) and 0.1% gentamycin.

Measurement of Intracellular free Ca $^{2+}$ Concentration. Intracellular free Ca $^{2+}$ concentration of T cells was determined using indo-1 (Molecular Probes, Junction City, OR), as described (18). In brief, T cells were incubated with acetoxy-methyl ester of indo-1 (10 μ M, 90 min, 37°C), washed thrice, and resuspended at 10^6 cells/ml in the buffer medium. The ratio of 398 (violet)/482 (blue) fluorescence of indo-1 loaded T cells was measured at designated time intervals (R) and after lysis of cells with 1% Triton X-100 in the absence (R_{\max}) or presence of 10 mM EGTA (R_{\min}), using a LS-5 Perkin-Elmer spectrofluorometer (Perkin-Elmer, Long Island, NY). Ca $^{2+}$ concentration was calculated using the formula: $[Ca^{2+}] = K_d \times (R - R_{\min}) / (R_{\max} + R) \times sf_2/sb_2$, where K_d is the dissociation constant (250 nM) and sf_2/sb_2 is the ratio of the blue fluorescence intensity of the Ca $^{2+}$ -free and bound dye, respectively (18).

Detection of IL-2 Gene Activation by Polymerase Chain Reaction (PCR). A new methodology, reverse transcription followed by PCR (20), was utilized for the detection of mRNA encoding IL-2. In brief, total cellular RNA, isolated from T cells by guanidinium isothiocyanate/cesium chloride method, was reverse transcribed into cDNA with 0.1 μ g/ml oligo(dT) primers and 200 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Bethesda Research Corp., Bethesda, MD). The first strand cDNA copies were then amplified (30 cycles) with either an IL-2 sequence specific primer pair (5'-CCTCTGGAGGAAGTGCTAAA-3' and 5'-ATGGTTGCTGTCTCATCAGC-3') or a β -actin sequence specific primer pair (5'-GTGG-GCCGCTCTAGGCACCA-3' and 5'-TGGCCTTAGGGTGCAGGGGG-3') and Thermo aquatic DNA polymerase (Taq polymerase, Perkin-Elmer/Cetus Corp.), using a DNA thermal cycler (Gene Machine; USA/Scientific Plastics, Ocala, FL). The amplification profile consisted of denaturation at 95°C for 45 s, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR-generated products were fractionated by agarose gel electrophoresis, and validated by the predicted size of the amplified cDNA fragments and by restriction enzyme analysis.

Results

Synergism between mAbs Directed at the TCR and CD2 Antigen. We examined whether anti-TCR-1 and anti-CD2 mAbs are synergistic in promoting T cell proliferation. This experimental approach was prompted by: (a) the observation that the mitogenic activity of anti-TCR-1, a mAb that reacts with a framework determinant of CD3/TCR- α/β for antigen, is fully dependent upon the presence of monocytes (15–17), and (b) our earlier demonstration that the crosslinked anti-CD2 can function as an effective substitute for monocytes in oxidative mitogenesis (14).

Fig. 1 illustrates that OKT11, a prototypic mAb directed at the SRBC-binding epitope of CD2 antigen (21), and anti-TCR-1 are indeed synergistic in promoting T cell proliferation: [3 H]thymidine incorporation was only $1,395 \pm 501$ cpm/culture with T cells incubated alone, and it increased significantly to $102,070 \pm 7,733$ cpm/culture when T cells were simultaneously signaled with anti-TCR-1 and OKT11

(mean \pm SEM, $n = 8$, $p < 0.0001$, ANOVA). It is important to note that the marked proliferative response is absolutely dependent upon crosslinking of anti-TCR-1 with OKT11 and that neither the combined presence of soluble phase anti-TCR-1 and OKT11 nor the crosslinking of anti-TCR-1 alone or crosslinking of OKT11 alone promotes T cell proliferation (Fig. 1 A).

Several additional features of the synergism between anti-TCR-1 and OKT11 are also illustrated in Fig. 1: (a) specificity of synergism: whereas anti-TCR-1 and OKT11 are synergistic, neither mAb is synergistic with soluble phase or crosslinked anti-Leu-1 (anti-CD5) mAb (Fig. 1 B), (b) the need for crosslinking anti-TCR-1 with OKT11: whereas crosslinking anti-TCR-1 with OKT11 induces marked proliferation, selective crosslinking of anti-TCR-1 (with affinity-purified antibodies specific for mouse IgG1) fails to induce significant proliferation of T cells treated with soluble phase OKT11, and selective crosslinking of OKT11 (with affinity-purified antibodies specific for mouse IgG2a) fails to elicit marked proliferation of T cells treated with soluble phase anti-TCR-1. Furthermore, selective but independent crosslinking of anti-TCR-1 and OKT11 does not induce T cell proliferation (Fig. 1 C), and (c) dose-response characteristics: the magnitude of T cell proliferation elicited with crosslinked anti-TCR-1 and OKT11 is dependent upon the concentrations of anti-TCR-1 as well as OKT11 (Fig. 1, D and E).

Synergism between F(ab')₂ Fragments of mAbs Directed at the TCR and CD2 Antigen. In a fashion analogous to the signaling activity of intact anti-TCR-1 and OKT11, F(ab')₂ fragments of anti-TCR-1 and OKT11 were clearly synergistic in stimulating T cells. Fig. 1 F illustrates the marked proliferation resulting from stimulating T cells with F(ab')₂ anti-TCR-1, F(ab')₂ OKT11, and F(ab')₂ rabbit anti-mouse IgG. Fig. 1 F shows also that neither the combined presence of soluble phase F(ab')₂ anti-TCR-1 and F(ab')₂ OKT11 nor crosslinking of F(ab')₂ anti-TCR-1 alone or F(ab')₂ OKT11 alone induces marked proliferation of T cells. The stimulation indices (³H]thymidine incorporation of treated T cells/[³H]thymidine incorporation of untreated T cells) were 137 with T cells treated with F(ab')₂ anti-TCR-1 (0.25 μ g/ml), F(ab')₂ OKT11 (0.5 μ g/ml), and F(ab')₂ rabbit anti-mouse IgG (R α M IgG 2.5 μ g/ml); 1.3 with T cells treated with crosslinked F(ab')₂ OKT11 alone; 1.2 with T cells treated with crosslinked F(ab')₂ anti-TCR-1 alone; and 1.6 with T cells treated with soluble phase F(ab')₂ OKT11 and F(ab')₂ anti-TCR-1.

The magnitude of T cell proliferation was influenced not only by the relative concentrations of F(ab')₂ fragments of R α M IgG (Fig. 1 F) but also by the concentrations of F(ab')₂ fragments of mAbs. For example, treatment of T cells with F(ab')₂ OKT11 (1.0 μ g/ml), F(ab')₂ R α M IgG (2.5 μ g/ml), and 0.06, 0.125, 0.25, or 0.50 μ g/ml of F(ab')₂ anti-TCR-1 resulted in stimulation indices of 8, 31, 48, and 90, respectively.

Anti-TCR-1 and Anti-CD2 Are Synergistic in Inducing Proliferation of CD4⁺ T Cells and CD8⁺ T Cells. Data summarized in Fig. 1 demonstrate that the crosslinkage of TCR with CD2 antigen promotes T cell proliferation independent of co-stimulatory signals (e.g., accessory cells, exogenous cytokines or phorbol esters). It was of interest to determine whether this novel method of activation is effective in each of the two major subsets of T cells, the CD4⁺ and CD8⁺ T cells.

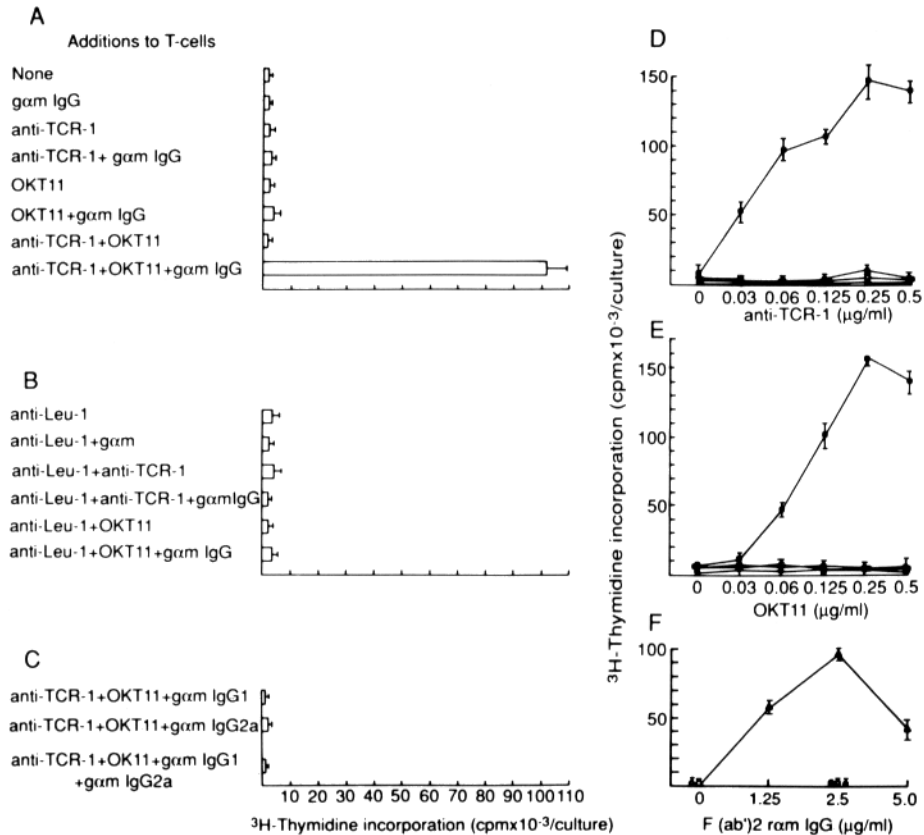


FIGURE 1. Synergism and unique aspects of signaling T cells with anti-TCR-1 and anti-CD2 mAbs. Highly purified human T cells (200,000 cells/200 μ l) were incubated with antibodies shown and the resultant proliferation was quantified (cpm, mean \pm SEM, $n = 3-8$) by determining [³H]thymidine incorporation during 48-64 h of culture. Concentrations of reagents (panels A-C): anti-TCR-1, 0.125 μ g/ml; OKT11, 0.5 μ g/ml; anti-Leu-1, 0.5 μ g/ml; and γ M IgG specific for mouse IgG, IgG1, or IgG2a, 5.0 μ g/ml. (D) Treatment of T cells: (O) anti-TCR-1 alone; (●) anti-TCR-1 + γ M IgG (5.0 μ g/ml); (□) anti-TCR-1 + OKT11 (0.5 μ g/ml); (■) anti-TCR-1 + OKT11 (0.5 μ g/ml) + γ M IgG (5.0 μ g/ml). (E) Treatment of T-cells: (O) OKT11 alone; (●) OKT11 + γ M IgG (5.0 μ g/ml); (□) OKT11 + anti-TCR-1 (0.125 μ g/ml); (■) OKT11 + anti-TCR-1 (0.125 μ g/ml) + γ M IgG (5.0 μ g/ml). (F) Treatment of T-cells: (O) none; (□) F(ab)₂ RαM IgG; (■) F(ab)₂ OKT11 + RαM IgG; (●) F(ab)₂ anti-TCR-1 + RαM IgG; (Δ) F(ab)₂ anti-TCR-1 + F(ab)₂ OKT11; (▲) F(ab)₂ anti-TCR-1 + F(ab)₂ OKT11 + F(ab)₂ RαM IgG [F(ab)₂ OKT11: 0.5 μ g/ml, F(ab)₂ anti-TCR-1: 0.25 μ g/ml].

Crosslinked anti-TCR-1 and anti-CD2 did induce significant proliferation of highly purified CD4⁺ subset and the CD8⁺ subset of T cells. Anti-TCR-1 or anti-CD2, however, did not synergize with anti-CD4 or anti-CD8 mAbs and induce proliferation, under similar experimental conditions. The stimulation indices ([³H]thymidine incorporation of CD4⁺ or CD8⁺ T cells treated with crosslinked mAbs/[³H]thymidine incorporation of untreated CD4⁺ or CD8⁺ T cells) were 264, 4, 1.4, and 0.7 when CD4⁺ T cells were treated with crosslinked anti-TCR-1 (0.125 μ g/ml) and

anti-CD2 (OKT11, 0.5 $\mu\text{g}/\text{ml}$), crosslinked anti-TCR-1 and anti-CD4 (OKT4A, 0.5 $\mu\text{g}/\text{ml}$), crosslinked anti-CD2 and anti-CD4 (OKT4A), or crosslinked anti-CD2 and anti-CD4 (OKT4, 0.5 $\mu\text{g}/\text{ml}$), respectively. The stimulation indices were 225, 2, and 1 when CD8⁺ T cells were treated with crosslinked anti-TCR-1 and anti-CD2, crosslinked anti-TCR-1 and anti-CD8 (OKT8, 0.5 $\mu\text{g}/\text{ml}$), or crosslinked anti-CD2 and anti-CD8 mAbs, respectively. Also, simultaneous but independent crosslinking of TCR and the CD2 antigen on the surface of CD4⁺ T cells or CD8⁺ T cells did not result in marked proliferation of either subset: the stimulation indices were only 0.8 and 1.3 when CD4⁺ T cells and CD8⁺ T cells, respectively, were treated with anti-TCR-1 (0.125 $\mu\text{g}/\text{ml}$), OKT11 (0.5 $\mu\text{g}/\text{ml}$), $\text{g}\alpha\text{M}$ IgG1 and $\text{g}\alpha\text{M}$ IgG2a (2.5 $\mu\text{g}/\text{ml}$, each). Thus, crosslinkage of TCR with CD2 antigen, and not simultaneous but independent crosslinking of TCR and CD2 antigen or crosslinking of TCR or CD2 antigen with the CD4 or the CD8 antigen, is a sufficient stimulus for the induction of proliferation of normal human CD4⁺ or CD8⁺ T cells.

Effects of Crosslinkage of TCR and/or CD2 Antigen on Ca²⁺ Mobilization. An increase in the concentration of intracellular free Ca²⁺ is considered to be an early obligatory event for T cell proliferation (22, 23). Given that crosslinkage of TCR with the CD2 antigen promotes T cell proliferation and selective crosslinkage of TCR or CD2 antigen does not, effects of crosslinking of anti-TCR-1 with OKT11 as well as effects of selective crosslinking of anti-TCR-1 and of OKT11 on intracellular Ca²⁺ homeostasis were determined.

Fig. 2 shows a rapid and marked increase in the concentration of intracellular free Ca²⁺ by crosslinking anti-TCR-1 with OKT11: intracellular free Ca²⁺ concentration increased from a precrosslinked value of 201 \pm 8 nM to a peak value of 1,235 \pm 63 nM following the addition of goat anti-mouse IgG to T cells pretreated with anti-TCR-1 and OKT11 (mean \pm SEM, $n = 6$, $p < 0.0001$, ANOVA).

Selective crosslinking of anti-TCR-1 or OKT11, in the presence of reciprocal mAb in soluble phase, also resulted in an increase in the concentration of intracellular free Ca²⁺. However, the magnitude of increase and the kinetics of increase found with selective crosslinking were distinctly different from that found with crosslinking of OKT11 with anti-TCR-1. As illustrated in Fig. 2, the peak intracellular Ca²⁺ con-

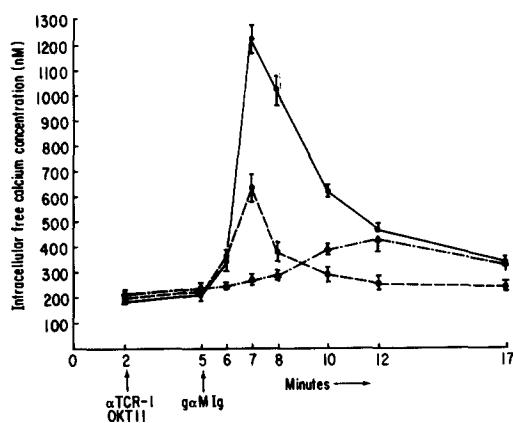


FIGURE 2. Calcium mobilization from crosslinked anti-TCR-1 and/or OKT11. Intracellular free calcium concentration was determined with indo-1 loaded T-cells (10^6 cells) as described in Materials and Methods. Anti-TCR-1 (0.125 $\mu\text{g}/\text{ml}$) and OKT11 (0.5 $\mu\text{g}/\text{ml}$) were added to T-cell after determining the resting T-cell calcium concentration; the mAbs were crosslinked with $\text{g}\alpha\text{M}$ IgG (●), $\text{g}\alpha\text{M}$ specific for IgG1 (■) or IgG2A (▲—▲). Results (mean \pm SEM) from six consecutive experiments (concentration of crosslinking reagent: 5.0 $\mu\text{g}/\text{ml}$).

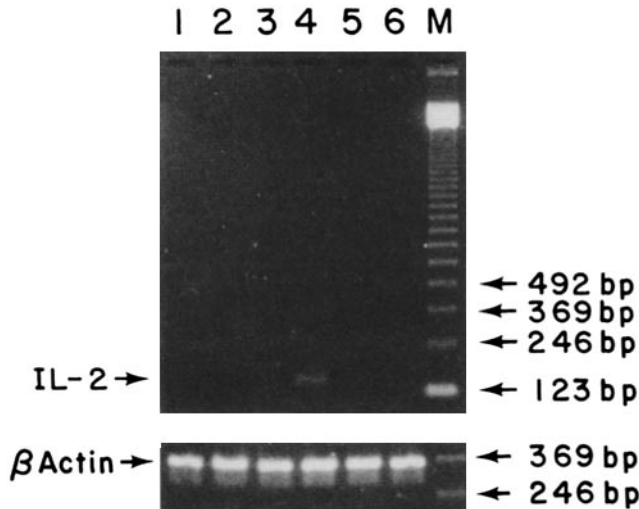
centrations were 421 ± 46 nM and 635 ± 58 nM with selective crosslinking of OKT11 or anti-TCR-1, respectively, in the presence of reciprocal mAb in soluble phase as compared with $1,235 \pm 63$ nM following crosslinkage of anti-TCR-1 with OKT11 ($p < 0.0001$, ANOVA). The peak concentration was reached rapidly by crosslinking anti-TCR-1 with OKT11 or by selective crosslinking of anti-TCR-1 alone, and was reached slowly by selective crosslinking of OKT11. A sustained increase in the concentration of Ca^{2+} above the precrosslinked Ca^{2+} value was evident by crosslinking anti-TCR-1 and OKT11 or by selective crosslinking of OKT11, and not from selective crosslinking of anti-TCR-1. Thus, crosslinking of anti-TCR-1 and OKT11 resulted in a Ca^{2+} mobilization profile that included the rapid increase in the Ca^{2+} levels found with selective crosslinking of anti-TCR-1 alone and the sustained increase resulting from selective crosslinking of OKT11 alone.

Effects of Regulators of Protein Kinase C (PKC) on the Signaling of T Cells by Crosslinkage of TCR with the CD2 Antigen. Ca^{2+} and PKC activation participate in signal transduction in a variety of eukaryotic cells (24, 25). Having documented Ca^{2+} mobilization by crosslinking the TCR with the CD2 antigen, we investigated the role of Ca^{2+} mobilization and PKC activation in the transduction of signals initiated by crosslinking the TCR with the CD2 antigen. The contribution of Ca^{2+} was examined with EGTA, and that of PKC activation with direct or competitive inhibitors of PKC.

T cell proliferation induced by crosslinking anti-TCR-1 (0.125 $\mu\text{g}/\text{ml}$) with OKT11 (0.5 $\mu\text{g}/\text{ml}$) was inhibited by EGTA: dose-response experiments revealed that the half-maximal inhibitory concentration (IC_{50}) was 0.28 mM. The compound H-7, a derivative of isoquinolinesulfonamide and a competitive inhibitor of PKC (26), inhibited T cell proliferation and the IC_{50} was 0.019 mM. Staurosporine, a microbial alkaloid and a direct inhibitor of PKC (27), also mediated marked inhibition and the IC_{50} was 0.003 mM. An additional compound, K-252a, a competitive inhibitor of PKC whose mechanism of action is similar to that of compound H-7 but with a greater potency (28), also inhibited T cell proliferation induced by crosslinkage of anti-TCR-1 with OKT11 and the IC_{50} for K-252a was 0.05 μM .

T Cell Proliferation Induced by Crosslinkage of TCR with the CD2 Antigen Is IL-2 Dependent. IL-2-dependent as well as IL-2-independent pathways of T cell activation have been reported (29–32). We therefore determined whether crosslinking the TCR with the CD2 antigen results in transcriptional activation and translation of the gene for IL-2. We also examined whether IL-2 receptor α (a subunit of IL-2 receptor that facilitates high-affinity binding of IL-2) is induced and whether successful interaction between IL-2 and its receptor is required for T cell proliferation elicited by crosslinking the TCR with the CD2 antigen.

Agarose gel electrophoretic analysis of PCR products generated with IL-2 sequence-specific primers or with β -actin sequence-specific primers and Taq polymerase are illustrated in Fig. 3. It is evident that: (a) T cells signaled with crosslinked anti-TCR-1 and OKT11 express the IL-2 gene (lane 4); (b) crosslinking of anti-TCR-1 alone, OKT11 alone or simultaneous but independent crosslinking of anti-TCR-1 and of OKT11 does not result in IL-2 mRNA accumulation (lanes 2, 3, 5 and 6), and (c) β -actin mRNA (control mRNA) expression is similar in mAb treated or untreated T cells (lanes 1–6). In accordance with the data shown in Fig. 3, cell-free supernatants, obtained from T cells treated with crosslinked anti-TCR-1



IL-2 cDNA generated the expected 61 and 88 bp fragments. (Lane 1) T + 0, (lane 2) T + anti-TCR-1 + α M IgG, (lane 3) T + OKT11 + α M IgG, (lane 4) T + anti-TCR-1 + OKT11 + α M IgG, (lane 5) T + anti-TCR-1 + OKT11 α M IgG2a and (lane 6) T + anti-TCR-1 + OKT11 + α M IgG1 (anti-TCR-1: 0.25 μ g/ml, OKT11: 0.5 μ g/ml, α M IgG, IgG2a or IgG1: 5.0 μ g/ml). Arrows on the right indicate 4 repeats of 123 bp ladder in the marker (M) lane.

FIGURE 3. Detection of IL-2 gene activation with the PCR. Total cellular RNA, isolated from treated or untreated T cells, was reverse transcribed into cDNA with oligo (dT) primers and M-MLV reverse transcriptase. The first strand cDNA was amplified (30 cycles) with an IL-2 sequence-specific primer pair or with a β -actin-specific primer pair and Taq polymerase. The amplified PCR products were fractionated by 1.5% agarose gel electrophoresis using Tris-acetate EDTA buffer (pH 8.0), stained with ethidium bromide, and validated by the predicted size of IL-2 cDNA fragment (149 bp for IL-2) and β -actin cDNA fragments (350 bp). Mse I restriction enzyme digestion of

and OKT11, induced marked proliferation of an IL-2-dependent cytotoxic T cell line (33).

Signaling of T cells with crosslinked anti-TCR-1 and OKT11 resulted also in the induction of IL-2 receptor α expression. FACS analyses illustrating the synergism between anti-TCR-1 and OKT11 in the elicitation of IL-2 receptor α expression are summarized in Fig. 4.

In a fashion homologous to physiologic signaling of T cells, the proliferative response elicited by crosslinking TCR with the CD2 antigen was dependent upon successful interactions between IL-2 and its receptor. As shown in Fig. 5, mAb directed at IL-2 receptor α (anti-CD25 mAb) inhibited T cell proliferation induced with crosslinked anti-TCR-1 and OKT11 ($p < 0.001$, ANOVA). The inhibition resulting from anti-CD25 mAb was reversed, in a concentration-dependent manner, by recombinant IL-2 (Fig. 5, B-E).

Discussion

Results from this study demonstrate for the first time that crosslinkage of CD3/TCR- α/β complex with the CD2 antigen on the surface of CD4⁺ or CD8⁺ T cells results in their activation independent of costimulatory signals (e.g., accessory cells, exogenous lymphokines, or phorbol esters). Furthermore, crosslinkage of TCR with the CD2 antigen elicits an activation cascade that is homologous to physiological antigenic signaling of T cells with respect to: (a) Ca²⁺ mobilization and PKC activation for cell-surface signal transduction, (b) transcriptional activation and translation of the gene for IL-2, and (c) utilization of IL-2 and IL-2 receptor pathway for the proliferative response.

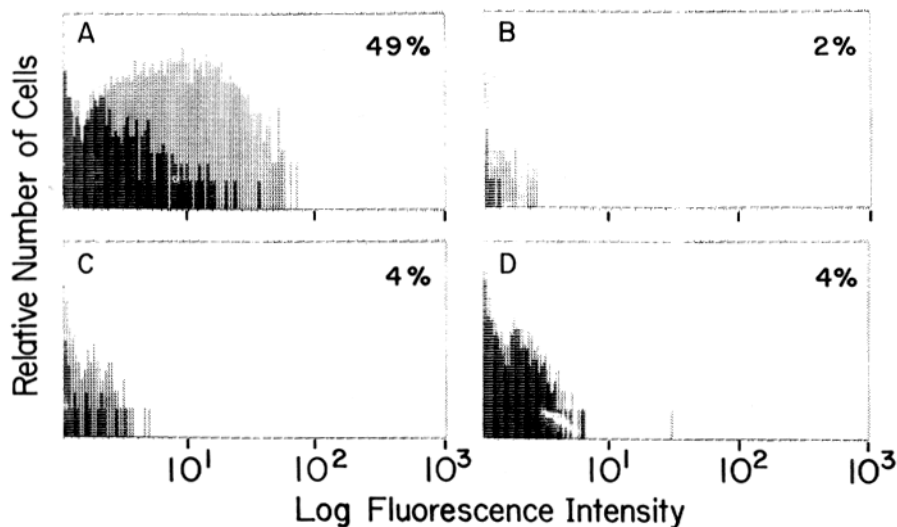


FIGURE 4. FACS analysis of T cells for IL-2 receptor α expression. T cells (10^6 cells) were incubated with various agents for 16 h at 37°C in a 5% $\text{CO}_2/95\%$ air humidified atmosphere; the cells were then labeled with PE-conjugated anti-CD25 mAb or with PE-conjugated isotypic IgG1 control mAb. The percentage of T cells positive for IL-2 receptor α (shown in each panel) was determined by analysis of immunofluorescence histograms using Epic C multidata acquisition and display systems, software version 3.1. Nonspecific fluorescence (*dark lines*) found with the isotypic control mAb is also shown. (A) T cells + anti-TCR-1 ($0.125\ \mu\text{g/ml}$) + OKT11 ($0.5\ \mu\text{g/ml}$) + $\text{g}\alpha\text{M}$ IgG ($5.0\ \mu\text{g/ml}$); (B) T-cells + none; (C) T cells + anti-TCR-1 ($0.125\ \mu\text{g/ml}$) + $\text{g}\alpha\text{M}$ IgG ($5.0\ \mu\text{g/ml}$); (D) T cells + $0.5\ \mu\text{g/ml}$ OKT11 + $\text{g}\alpha\text{M}$ IgG ($5.0\ \mu\text{g/ml}$).

Several features of our experimental design are worthy of emphasis: First, normal human resting T cells and T cell subsets and not abnormal or preactivated T cells were used in our study. This approach represents a significant physiological advantage in light of the differential signaling requirements of T cell lines, clones, or hybridomas compared with resting T cells (34–37). Second, mAbs directed at a framework determinant of the CD3/TCR- α/β complex rather than mitogenic lectins were used. This strategy permits the study of the signaling activity of a cell-surface molecule involved in physiological antigen signal transduction. It is important to note that anti-TCR-1, at concentrations used in our study, reacts only with the T cells expressing the α/β heterodimer of the TCR and not with T cells that do not express this heterodimer (38). Third, mAbs directed at the CD2 antigen were used to generate the obligatory accessory signal in our investigation. Given that the CD2 antigen is expressed on all peripheral blood T cells, our approach has the potential to uncover signaling pathways operative in the CD4 as well as the CD8 subset of normal human T cells. Fourth, mAbs directed at the SRBC-binding epitope (CD2.1 epitope) of the CD2 antigen were used herein to generate the stimulatory signal. This is significant in view of earlier suggestions that the CD2.1 epitope generates inhibitory signals and that the CD2.2 and CD2.3 epitopes need to be engaged in order to generate stimulatory signals (6–9). Moreover, the CD2.1 epitope, unlike some of the other epitopes of the CD2 antigen (CD2.3 or 9-1), is constitutively expressed on resting T cells and thus has the potential to initiate and/or participate in the very first event obligatory for T cell activation. Fifth, activation was accomplished in our

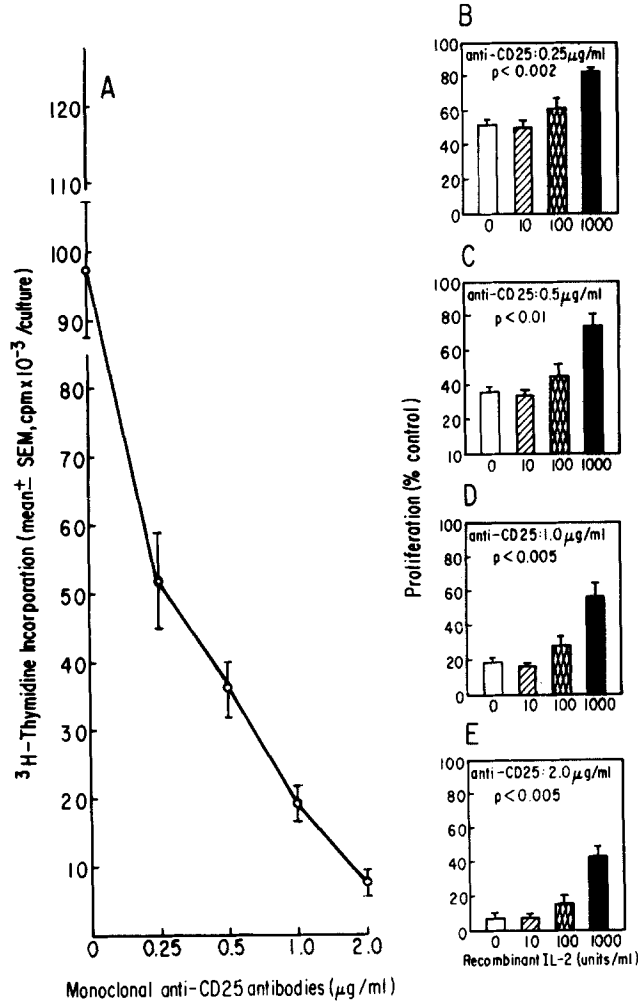


FIGURE 5. Effects of anti-CD25 mAbs and/or recombinant IL-2 on T cell proliferation. T cells (200,000 cells/200 μl) were activated by crosslinking anti-TCR-1 (0.125 $\mu\text{g/ml}$) with OKT11 (0.5 $\mu\text{g/ml}$), and proliferation was measured by determining [^3H]thymidine incorporation during 48-64 h of culture. Concentration-dependent inhibition found with anti-CD25 mAb is shown in A. The ability of recombinant IL-2 to reverse anti-CD25 mAb-associated inhibition in B-E. Results (mean \pm SEM) found in three consecutive experiments.

study in the absence of co-stimulatory agents (e.g., feeder cells, exogenous IL-2 or tumor promoters). These additional stimuli are mandatory for T cell proliferation induced with mAb combinations such as CD2 - D66 + CD2 9.6/T11₁ (39), CD3-BMA030 + mAb directed at T cell surface antigens (40, 41), or CD3-RW24B6 + CD4-19 Thy 5D7 or CD8-21 Thy 2D3 (42).

Signaling by Crosslinkage of TCR with the CD2 Antigen. An important requirement for the induction of proliferation of normal human T cells demonstrated herein is that the TCR should be crosslinked with the CD2 antigen: selective or simultaneous but independent crosslinking of these cell surface molecules is insufficient to elicit the proliferative response. Furthermore, crosslinkage of TCR with CD2 but not of either antigen with CD4 or CD8 antigen elicits marked proliferation (of the corresponding subset) independent of co-stimulatory signals. This differential signaling potential of T cell surface molecules, viewed especially in the light of observations

of others that crosslinking CD4 or CD8 with CD3 antigen induces T cell proliferation only in the presence of exogenous IL-2 or phorbol esters (40, 42), suggests that: (a) crosslinking the TCR with the CD4 or CD8 antigen generates signals that are sufficient only for the induction of T cell competence to growth factors such as IL-2 but not for IL-2 production and/or T cell's progression through the entire cell-cycle, and (b) crosslinkage of TCR with the CD2 antigen generates instructive signals that are adequate not only for the acquisition of competence but also for the production of IL-2 and the progression of T cells through the entire cell cycle.

Participation of Ca^{2+} and PKC in Signal Transduction. A unifying theme for the transmembrane regulation of cellular functions is that Ca^{2+} mobilization and PKC activation function as synergistic intracellular signals, (22-25, 43). Indeed, crosslinkage of the TCR with the CD2 antigen resulted in a marked and sustained increase in intracellular free Ca^{2+} levels. Our finding that a sustained increase in Ca^{2+} is required for the full activation of normal human T cells is reminiscent of the observation that a sustained but not a transient increase in intracellular free Ca^{2+} is associated with the expression of the IL-2 gene in Jurkat T cell line (44). Our additional observations that EGTA and regulators of PKC are inhibitory are consistent with Ca^{2+} mobilization and PKC activation participating in the transduction of signals generated by crosslinkage of TCR with the CD2 antigen.

Utilization of an Operational IL-2 System. Antigen-induced T cell proliferation is dependent upon the expression of receptors for IL-2, production of IL-2, and successful interaction between IL-2 and its receptors (29, 30). Evidence for an interleukin-independent pathway for human lymphocyte proliferation however, has also been presented (31, 32). Our data that crosslinkage of TCR with the CD2 antigen induces IL-2 gene activation and IL-2 receptor expression, and that mAb directed at the IL-2 receptor inhibits T cell proliferation, support the contention that T cell proliferation induced by this novel approach is dependent upon an operational IL-2 system, and is homologous to physiological antigen-dependent T cell proliferation.

A Novel Model for Antigen-dependent T Cell Signaling. A unifying theme has emerged in which T cells view antigens in the context of MHC gene products expressed on antigen-presenting (accessory) cells. T cell surface proteins, in addition to the TCR, that contribute to the informative T cell-accessory cell interactions, however, have remained an enigma despite the paradigm that the CD4 and CD8 molecules function as associative recognition/restriction elements for the MHC gene products expressed on the surface of accessory cells.

Based on our current as well as earlier investigations, it is possible to conceptualize a novel model for antigen-dependent activation of T cells. As schematized in Fig. 6, instructive T cell-accessory cell interactions might proceed not only via molecular contacts between the TCR and the MHC-peptide but also via the CD2 antigen and the LFA-3 and/or other sites on accessory cells (step 1). These cell-surface molecular interactions then, lead to the coclustering of the TCR with the CD2 antigen (step 2). The antigen- and accessory cell-induced association between the TCR and the CD2 antigen is necessary for the optimum generation of second messenger molecules (Ca^{2+} , DAG, etc.) and transcriptional activation and translation of genes (IL-2, IL-2 receptor, etc.) responsible for the antigen-dependent clonal expansion of normal human T cells (step 3). A critical observation that supports the concept formulated herein is our earlier demonstration that the anti-CD2 mAb (the same

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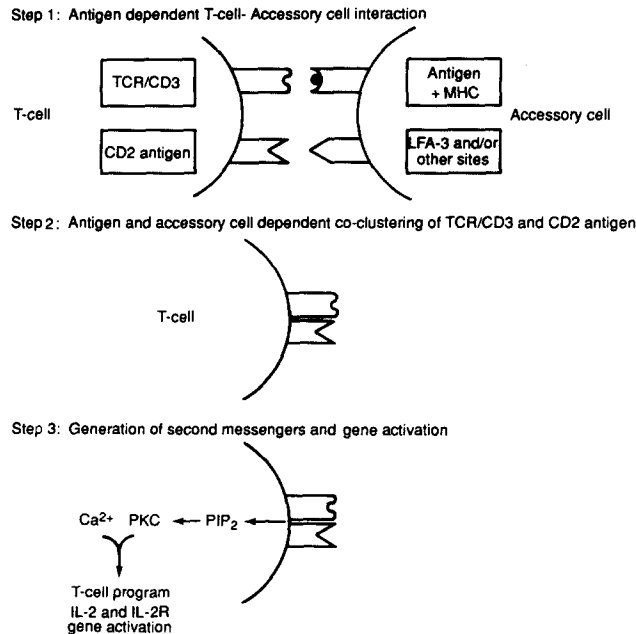


FIGURE 6. Schema for antigen-dependent T cell signaling. Antigen-dependent T cell-accessory cell interactions involve not only molecular contacts between CD3/TCR- α/β complex and the antigenic peptide (●) viewed in the context of MHC antigens on accessory cells but also physical associations between the CD2 antigen with LFA-3 and/or other sites (step 1). These molecular contacts lead to the redistribution and coclustering of the TCR and the CD2 antigen on the surface of T cells (step 2). This coclustering is required for the optimum generation of second messenger molecules and the activation of genes responsible for antigen-dependent clonal expansion of T cells (step 3).

mAb used in this investigation) prevents clustering between T cells and accessory cells; an important additional supportive observation from our laboratory is that anti-CD2 (soluble phase) inhibits T cell proliferation only when the obligatory co-stimulatory signal is provided with accessory cells but not when TPA (accessory cell substitute) provides the accessory signal (13, 14). Elegant data showing that antigen-dependent IL-2 production is enhanced by the expression of CD2 antigen in a murine T cell hybridoma (45) and that LFA-3, a natural ligand for CD2 antigen (46), is mitogenic (47), are in accord with some of the features of the minimal model formulated here for the activation of normal human quiescent T cells.

Summary

Transmembrane signaling of normal human T cells was explored with mAbs directed at TCR, CD2, CD4, CD5, or CD8 antigens and highly purified CD4⁺ T cells and CD8⁺ T cells. Our experiments explicitly show that: (a) crosslinkage of TCR with the CD2 antigen, and not independent crosslinking of TCR and of CD2 antigen or crosslinking of either protein with the CD4 or CD8 antigen induces significant proliferation independent of co-stimulatory signals (e.g., accessory cells, recombinant lymphokines, or tumor promoter), (b) F(ab')₂ fragments of mAb directed at the TCR and F(ab')₂ anti-CD2, crosslinked with F(ab')₂ fragments of rabbit anti-mouse IgG, promote the proliferation of highly purified T cells, (c) a prompt and sustained increase in intracellular free Ca²⁺ concentration results from crosslinkage of TCR with the CD2 antigen, (d) T cell proliferation induced by this novel approach is curtailed by EGTA and by direct or competitive inhibitors of PKC, (e) crosslinkage of TCR with the CD2 antigen results in the transcriptional activation and translation of the gene for IL-2 and in the expression of IL-2 receptor α (CD25), (f) anti-CD25

mAbs inhibit T cell proliferation initiated by crosslinkage of TCR with the CD2 antigen, and recombinant IL-2 restores the proliferative response. Our first demonstration that crosslinkage of TCR with the CD2 antigen induces proliferation of normal human CD4⁺ T cells and CD8⁺ T cells, in addition to revealing a novel activation mechanism utilizable by the two major subsets of T cells, suggest that the CD2 antigen might be targeted for the regulation of antigen-specific T cell immunity (e.g., organ transplantation).

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