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Activation of human peripheral blood T lymphocytes by pharmacological induction of protein-tyrosine phosphorylation

(calcium/interleukin 2/CD28)

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ABSTRACT **Protein-tyrosine kinase and protein-tyrosine phosphatase (PTPase) activities are essential for T-cell antigen receptor-mediated signaling. To assess the functional consequences of alteration of the levels of tyrosine phosphorylation in normal human T cells, the effects of vanadate and hydrogen peroxide were studied. In combination, these agents induced tyrosine phosphorylation of cellular substrates, elevated cytosolic free calcium, and induced interleukin 2 receptor (IL-2R) α chain expression but not IL-2 secretion. However, anti-CD28 antibody in combination with vanadate and hydrogen peroxide induced IL-2 secretion, consistent with the requirement for a costimulatory signal in the induction of this gene. The effects of vanadate and hydrogen peroxide were enhanced in the absence of the T-cell PTPase, CD45. Thus, acute pharmacologic manipulation of the level of tyrosine phosphorylation in normal T cells correlates with partial, but not full, activation of these cells; in concert with a costimulatory signal provided by perturbation of the CD28 molecule, the complete program of activation is initiated. These agents should prove useful in dissecting signaling pathways involved in the regulation of genes critical to the immune response.**

In contrast to the growth factor class of receptors that display intrinsic protein-tyrosine kinase (PTK) activity, many immunologically relevant, oligomeric receptors including the T-cell antigen receptor (TCR) and Fc receptors lack intrinsic kinase activity. Nonetheless, stimulation of these receptors results in protein-tyrosine phosphorylation of cellular substrates (reviewed in refs. 1–10), and pharmacologic inhibition of tyrosine kinases prevents both the early biochemical and late functional events that occur after receptor stimulation (11–14). Thus, PTK activity appears to be necessary for T-cell activation, but is PTK function alone sufficient to initiate any or all of the events involved in this process? Expression of PTKs in T-cell lines can enhance TCR-mediated interleukin 2 (IL-2) induction or in the case of v-*src* can induce constitutive but suboptimal IL-2 secretion (15–17). However, the limitation of transfection studies is that the consequences of acute alteration of the levels of tyrosine phosphorylation in normal T cells cannot be addressed.

Vanadate is a well-documented inhibitor of protein-tyrosine phosphatases (PTPases), but its effects on intact cells are variable (18–20). By contrast, the combination of vanadate and H_2O_2 generates the compound pervanadate, the efficacy of which has been demonstrated to be far greater than that of vanadate (21–23). Pervanadate treatment of hepatoma cells and adipocytes results in protein-tyrosine phosphorylation, stimulation of lipogenesis, protein synthesis, and inhibition of lipolysis. Therefore, we examined the utility of vanadate and H_2O_2 to activate normal human T cells.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells from normal donors were separated on Ficoll/Hypaque. Adherent cells (monocytes and B cells) were removed by incubation on plastic dishes and nylon wool. Highly enriched populations of $CD3^+$ T cells (>95%) were obtained by centrifugation of nylon wool-passed cells on discontinuous density gradients of Percoll (24). Two-color fluorescence measurements were performed on a FACScan IV flow cytometer (Becton Dickinson) to assess purity. The murine thymoma cell line, BW5147.3, and its $CD45^-$ counterpart were grown in RPMI 1640 medium supplemented with glutamine, antibiotics, and 10% fetal bovine serum.

Anti-phosphotyrosine [anti-Tyr(*P*)] Immunoblotting. Stock solutions of H_2O_2 and sodium orthovanadate, Na_3VO_4, were freshly prepared, premixed where indicated for 15 min at room temperature to generate pervanadate (21), added to T cells suspended in medium, and incubated for 20 min. Lysates were prepared from cells as described (25), electrophoresed on a SDS/10.5% polyacrylamide gel, transferred to nitrocellulose paper, and immunoblotted with anti-Tyr(*P*) monoclonal antibody (mAb), 4G10 (Upstate Biotechnology, Lake Placid, NY). The filters were then incubated with horseradish peroxidase-conjugated monoclonal anti-mouse immunoglobulin (Amersham) and developed by the enhanced chemiluminescence method according to the manufacturer's directions. The filters were subsequently stained with Ponceau S (Sigma) to ensure that equivalent amounts of protein were added to each lane.

Measurement of Intracellular Cytosolic-Free Calcium Concentration ($[Ca^{2+}]_i$). Fresh peripheral blood human T cells were preincubated with various concentrations of herbimycin A or vehicle (0.18% dimethyl sulfoxide) overnight, then incubated for 30 min in 5 μM Indo-1 AM, washed, and suspended in Dulbecco's phosphate-buffered saline containing 5 mM glucose at 37°C. Stock solutions of Na_3VO_4 and H_2O_2 were premixed, allowed to react at room temperature for 10 min, and then added to the cell suspension, yielding final concentrations of 0.1 mM and 1.0 mM, respectively. Changes in $[Ca^{2+}]_i$ were monitored continuously in a fluo-

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Abbreviations: PAO, phenylarsene oxide; PTK, protein-tyrosine kinase; PTPase, protein-tyrosine phosphatase; TCR, T-cell antigen receptor; IL-2, interleukin 2; IL-2Rα, IL-2 receptor α chain; mAb, monoclonal antibody; $[Ca^{2+}]_i$, intracellular cytosolic-free calcium concentration; PMA, phorbol 12-myristate, 13-acetate; Tyr(*P*), phosphotyrosine.

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rimeter (Photon Technology, Princeton, NJ) by exciting Indo-1 at 358 nm and monitoring the ratio of fluorescence of the Ca^{2+}-bound dye at 402 nm (violet) to that of unbound dye measured at 486 nm (blue).

RNA Blot-Hybridization (Northern) Analysis. Isolated T cells ($1–2 \times 10^6$ per ml) were stimulated with H_2O_2, Na_3VO_4, both, or with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 6 or 24 hr; then the cells were washed and subjected to RNA extraction. Total cellular RNA was purified from T cells by using RNAzol (Cinna/Biotecx Laboratories, Friendswood, TX). Twenty micrograms of RNA was subjected to electrophoresis on a 0.8% agarose/formaldehyde gel, transferred to Nytran (Schleicher & Schuell), and hybridized with ^{32}P-labeled cDNA probes. The blots were then exposed to Kodak X-OMAT AR film for 1–3 days at −70°C. The human IL-2 cDNA was obtained from N. Holbrook (National Institute of Aging, Baltimore); the human p55 IL-2R cDNA, from W. Leonard (National Institute of Child Health and Human Development, Bethesda, MD); and the chicken β-actin cDNA, from D. W. Cleveland (Johns Hopkins School of Medicine, Baltimore).

RESULTS AND DISCUSSION

Consistent with previous reports, we first observed that the combination of sodium orthovanadate and H_2O_2 potently inhibited T-cell PTPase activity (data not shown). Therefore, we treated isolated normal human T cells with vanadate or H_2O_2 or both to determine if these agents would induce substrate-tyrosine phosphorylation. Vanadate alone at every dose tested had an undetectable effect on tyrosine phosphorylation (Fig. 1, lanes 5, 9, and 13), and H_2O_2 alone (Fig. 1, lanes 1–4) only induced phosphorylation at the highest concentrations (10 mM). However, the combination of vanadate and H_2O_2 was markedly synergistic in stimulating protein-tyrosine phosphorylation. At lower doses of vanadate and H_2O_2, selective phosphorylation of substrates was observed (lane 8), whereas at higher concentrations of these agents, intense phosphorylation of a large number of substrates was seen. We next compared the substrates seen after TCR crosslinking with those seen with pervanadate stimulation. We observed that at early time points similar substrates did appear to become phosphorylated after addition of both stimuli (Fig. 2, compares lanes 2 and 8). However, whereas protein-tyrosine phosphorylation was very transient after

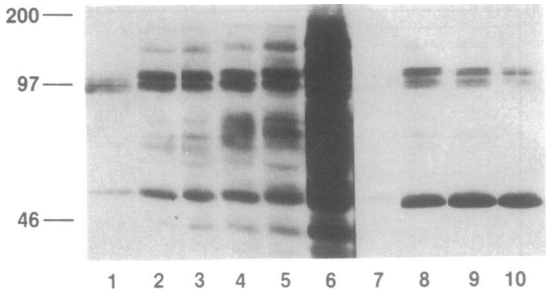

FIG. 2. Time course of protein-tyrosine phosphorylation induced by 0.1 mM Na_3VO_4 and 0.1 mM H_2O_2 versus anti-CD3 stimulation. Lanes: 1 and 7, unstimulated cells; 2–6, cells stimulated with Na_3VO_4 and H_2O_2 for 5, 10, 15, 20, and 30 min, respectively; 8–10 cells stimulated with anti-CD3 (mAb G19.4) for 2, 10, and 15 min, respectively. Molecular weight is shown $\times 10^{-3}$; the protein of $M_r \approx$ 50,000 seen in lanes 8–10 represents immunoglobulin heavy chain.

TCR occupancy, pervanadate stimulation of protein phosphorylation did not reach its peak until 60 min (not shown), at which time a large number of additional substrates were phosphorylated. These effects of vanadate and H_2O_2 in T cells were consistent with previous studies in adipocytes and hepatoma cells (21–23) and were similar to the effects of the PTPase inhibitor phenylarsenine oxide (PAO) on T cells (26).

TCR-mediated inositol phospholipid hydrolysis and Ca^{2+} mobilization have previously been shown to be PTK dependent (11–14). Additionally, we have recently demonstrated that expression of the v-*src* PTK in T-cell hybridomas induces spontaneous Ca^{2+} oscillations (27). Therefore, we hypothesized that stimulation of T cells with the combination of H_2O_2 and vanadate might be sufficient to increase $[Ca^{2+}]_i$ in normal T cells. Indeed, while neither H_2O_2 nor vanadate alone induced Ca^{2+} mobilization, the combination induced inositol phospholipid hydrolysis (data not shown) and substantially raised $[Ca^{2+}]_i$ (Fig. 3). The magnitude of this response can be appreciated by the fact that stimulation of T cells with anti-CD3 antibody under similar conditions resulted in roughly a doubling of the $[Ca^{2+}]_i$ (data not shown). The tracings reflecting $[Ca^{2+}]_i$ in cells stimulated with either sodium orthovanadate or H_2O_2 separately were superimposible on that of the unstimulated cells (not shown). Treatment of T cells with catalase-treated pervanadate (21) also induced Ca^{2+} mobilization (data not shown), suggesting that

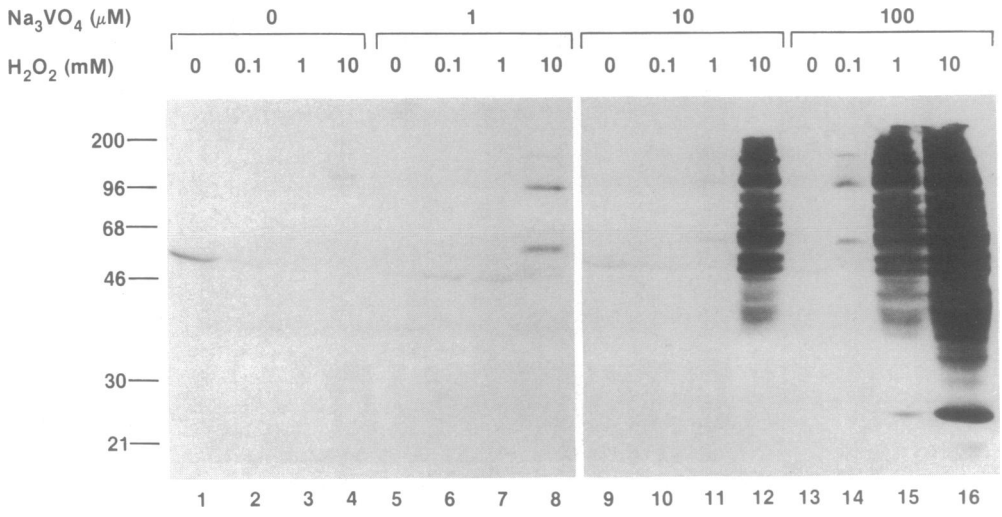

FIG. 1. Anti-Tyr(*P*) immunoblot analysis of isolated human peripheral blood T lymphocytes treated with various doses of H_2O_2 and sodium orthovanadate separately and in combination. Stock solutions of Na_3VO_4 and H_2O_2 were prepared, premixed for 15 min at room temperature (refs. 21–23) where indicated, and added to lymphocytes that were then stimulated for 20 min at 37°C. The lymphocytes were washed and lysed. Postnuclear supernatants were electrophoresed and immunoblotted with anti-Tyr(*P*) mAb (4G10).

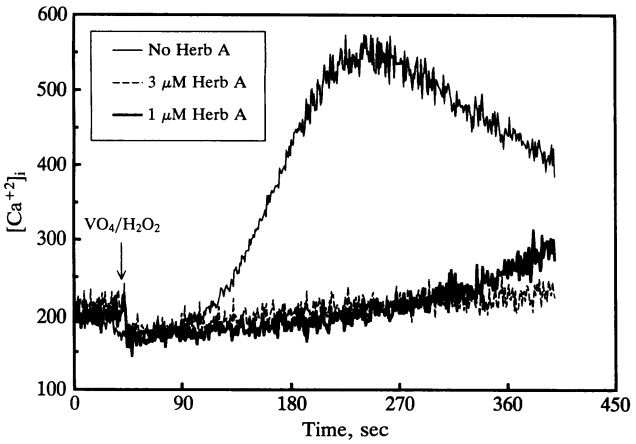

FIG. 3. $[Ca^{2+}]_i$ in H_2O_2- and vanadate-stimulated cells. Isolated human peripheral blood lymphocytes were preincubated for 16 hr at 37°C with or without herbimycin (Herb) A as indicated. Na_3VO_4 (0.1 mM) and H_2O_2 (1 mM) were premixed and added to Indo-1 AM-loaded cells, and $[Ca^{2+}]_i$ was measured by monitoring in a fluorometer the emission fluorescence ratio at 402/486 nm. Strong increases in $[Ca^{2+}]_i$ was induced by 1 μm ionomycin in each of the cell populations tested, confirming the potential responsiveness of herbimycin A-treated cells and the adequacy of loading with Indo-1.

pervanadate, not free H_2O_2, was the moiety responsible for the effect.

Since tyrosine phosphorylation is an early and requisite event in TCR-mediated signaling, we next asked whether vanadate and H_2O_2 stimulation induced the distal events typically observed in activated T cells. Some of the best characterized events in T-cell activation include the secretion of IL-2 and expression of IL-2 receptor α chain (IL-2Rα) (CD25) (28). We observed that T cells treated with both vanadate and H_2O_2 expressed IL-2Rα mRNA (Fig. 4) and protein (Fig. 5 *Upper*) but failed to express IL-2 or other T-cell cytokine mRNA (e.g., γ interferon, tumor necrosis factors α and β, and granulocyte/macrophage colony stimulating factor) (data not shown). In contrast, PMA and ionomycin stimulated the expression of both IL-2Rα and IL-2 (Fig. 3). However, vanadate and H_2O_2, like PMA and ionomycin, activated T lymphocytes to become larger and more granular as reflected by an increase in both forward and side scatter; the increase in mean channel fluorescence seen with H_2O_2 and vanadate stimulation was about 50% of that seen with PMA and ionomycin. Because of the toxicity of PAO, we could not compare the functional effects of this compound with those induced by pervanadate stimulation.

TCR occupancy and its associated biochemical consequences are usually not sufficient to induce IL-2 secretion in normal peripheral blood T cells. Therefore, it has been proposed that complete T-cell activation requires a costimulatory signal (reviewed in ref. 29) such as that delivered by perturbation of CD28 (30–34). We sought to determine if such a costimulatory signal would complement activation of T cells with vanadate and H_2O_2. We observed that, whereas vanadate and H_2O_2 alone did not induce IL-2 production, when they were used in combination with anti-CD28 mAb, IL-2 secretion was observed to be greater than that seen with anti-CD3 mAb and PMA stimulation (Fig. 5 *Lower*). Anti-CD28 alone or vanadate and H_2O_2 in combination with anti-CD3 mAb failed to induce substantial IL-2 secretion.

How are vanadate and H_2O_2 activating T cells? Naturally, vanadate and particularly H_2O_2 have other effects unrelated to PTPase and PTK activity, and this caveat must be considered. To address the specificity of these compounds with respect to the parameters of cell activation investigated, we studied the ability of a PTK inhibitor to block the effects of these agents. We observed that the vanadate/H_2O_2-mediated Ca^{2+} mobilization was PTK dependent in that the PTK inhibitor, herbimycin A, which also blocked pervanadate-induced protein-tyrosine phosphorylation (35), completely abrogated the vanadate/H_2O_2-induced Ca^{2+} flux (Fig. 3). Additionally, the induction of IL-2Rα and IL-2 by vanadate/H_2O_2 ± CD28 was also inhibitable by herbimycin A (data not shown). Therefore, we concluded that, while vanadate and H_2O_2 have numerous actions unrelated to the induction of

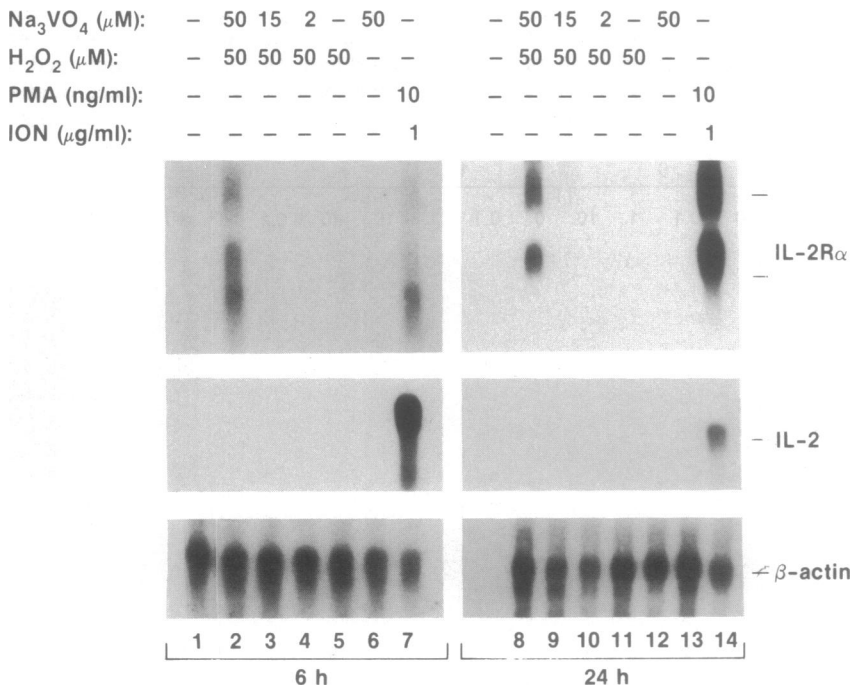

FIG. 4. Expression of activation genes in H_2O_2- and vanadate-treated human T cells. Isolated peripheral blood lymphocytes were stimulated as indicated for 6 or 24 hr (h), following which total cellular RNA was extracted, electrophoresed, transferred to filters, and hybridized with the indicated labeled cDNA probes. ION, ionomycin.

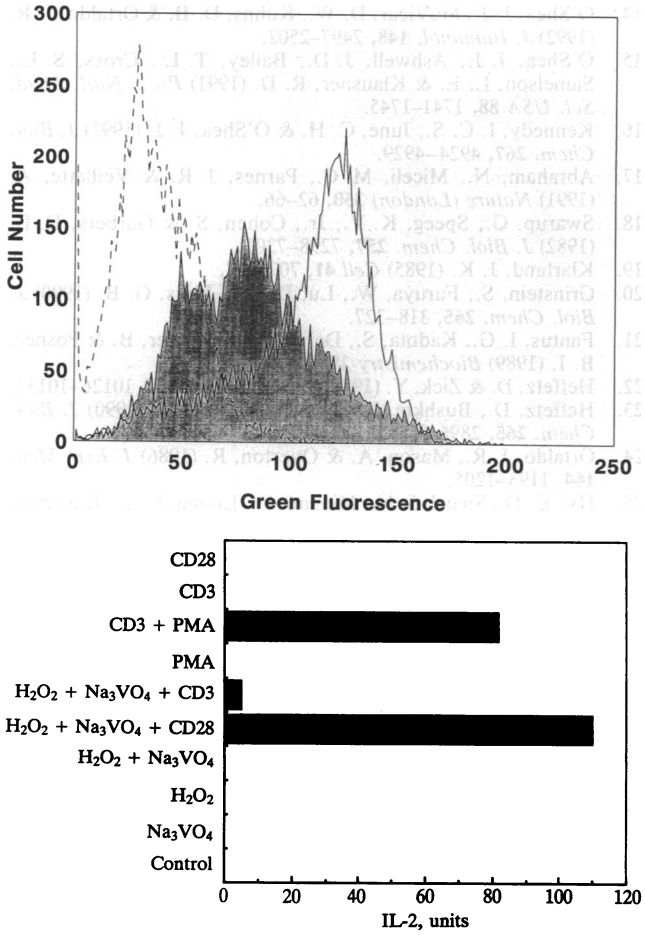

FIG. 5. (*Upper*) Flow cytometric analysis of IL-2Rα induction. Isolated human T cells were stimulated or not (- - -) with 0.1 mM H_2O_2 and 0.1 mM Na_3VO_4 (shaded profile) or PMA (10 ng/ml) (——) for 48 hr. T cells were stained with anti-CD25 mAb and analyzed by flow cytometry. The histogram representing unstained cells was superimposible on that of unstimulated T cells. (*Lower*) IL-2 secretion. Isolated human T cells (5×10^6 cells per ml; >95% purity) were stimulated with Na_3VO_4 (0.1 mM), H_2O_2 (0.1 mM), PMA (10 ng/ml), ionomycin (1 μg/ml), anti-CD3 mAb (OKT3, 10 μg/ml), and anti-CD28 (10 μg/ml) separately or in combination as indicated. Twenty-four hours later, supernatants were harvested and IL-2 was assayed by ELISA.

protein-tyrosine phosphorylation, the effect of these compounds on Ca^{2+} mobilization and other parameters of T-cell activation was PTK dependent.

Having determined that vanadate and H_2O_2 potently inhibited PTPase activity in normal T cells as reported in other cell types, we sought to determine whether vanadate and H_2O_2 functioned by inhibiting CD45, a prominent T-cell PTPase that is requisite for TCR-mediated signaling (36–38). We therefore studied the effect of these agents in cell lines that lack this enzyme and observed that, relative to the parental murine lymphocyte cell line BW5147, cells lacking CD45 (39) had a reproducibly greater sensitivity to vanadate and H_2O_2 (Fig. 6, compare lanes 2 and 6). These data indicate that, although the effect of these agents was influenced by CD45 expression, it was not required. This finding suggests that other PTPases are likely to be involved in T-cell activation induced by pervanadate and perhaps by TCR-mediated activation.

We next asked if TCR expression was required for the effects of vanadate and H_2O_2. We observed these agents to be equally effective in inducing phosphorylation and inositol phosphate production in cells lacking surface TCR

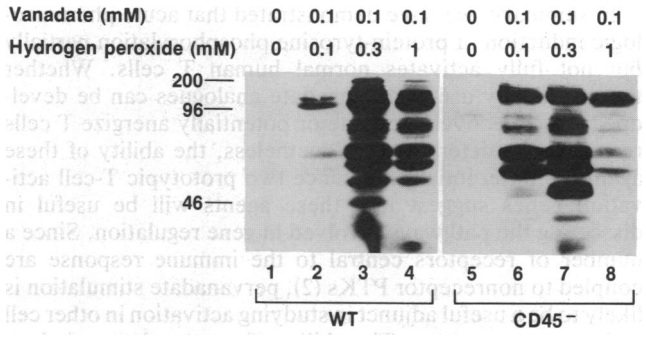

FIG. 6. Stimulation and anti-Tyr(*P*) mAb immunoblot analysis of a murine T-cell line BW5147 and a CD45⁻ variant derived from it. The cells were stimulated for 20 min 37°C as indicated. Cell lysates were electrophoresed and immunoblotted with anti-Tyr(*P*) mAb. WT, wild type.

(BW5147), as compared with a TCR^+ T-cell hybridoma produced by using BW5147 as a fusion partner (2B4.11; data not shown). Since the TCR is required for signaling via several other lymphocyte surface molecules (40, 41), this finding suggested that H_2O_2 and vanadate were not simply inducing cross-linking of surface molecules; rather it is likely that these agents pharmacologically bypass the need for receptor-coupled phosphorylation.

Tyrosine phosphorylation of cellular substrates has emerged as a critical component in the signal-transduction mechanism of a variety of cells and is the earliest demonstrable event observed with TCR signaling (4). While PTK function has been demonstrated to be necessary for T-cell activation, determination of whether PTK activity is sufficient to explain the constellation of events comprising T-cell activation has been hampered in part by the lack of pharmacologic inducers of protein-tyrosine phosphorylation. The combination of phorbol ester and ionomycin, which activate protein kinase C and increase $[Ca^{2+}]_i$, respectively, is typically used to fully activate T cells, bypassing the need for receptor-coupled kinase activation, inositol phospholipid hydrolysis, and Ca^{2+} mobilization. Herein, we have demonstrated that pharmacologically inducing the most proximal demonstrable event in physiologic receptor-mediated signaling—i.e., protein-tyrosine phosphorylation—is sufficient to induce many of the biochemical and functional events seen in typical T-cell activation. However, this was not sufficient to induce IL-2 gene expression; the additional signal provided by the costimulatory molecule CD28 was required. The data suggest that protein-tyrosine phosphorylation *per se* appears not to be a substitute for CD28 occupancy in the regulation of the IL-2 gene. This may be interpreted to indicate that tyrosine phosphorylation of proteins is not the critical signaling role of CD28. Alternatively, it is conceivable that CD28 ligation induces the selective phosphorylation of a specific substrate that is poorly phosphorylated through vanadate and peroxide stimulation.

PAO inhibition of PTPase activity previously has been shown to uncover constitutive PTK activity (17); however, the toxicity of this compound precluded the study of downstream events associated with PAO activation (unpublished observations). The present results with vanadate and H_2O_2 are consistent with this mechanism of activating the T cells. The effects of these agents appear to have been mediated partially by the CD45 PTPase, but the data also suggest that phosphatases other than CD45 are involved in the process of T-cell activation. However, alteration of redox states can also activate PTK (42); therefore, the induction of tyrosine phosphorylation may result from either or both of these mechanisms.

In summary, we have demonstrated that acute pharmacologic induction of protein-tyrosine phosphorylation partially but not fully activates normal human T cells. Whether therapeutically useful pervanadate analogues can be developed that selectively activate or potentially anergize T cells remain to be determined. Nonetheless, the ability of these agents to differentially influence two prototypic T-cell activation genes suggest that these agents will be useful in dissecting the pathways involved in gene regulation. Since a number of receptors central to the immune response are coupled to nonreceptor PTKs (2), pervanadate stimulation is likely to be a useful adjunct in studying activation in other cell and receptor systems. The ability of pervanadate to induce Ca^{2+} mobilization should also be useful in dissecting the mechanisms by which PTKs regulate Ca^{2+} homeostasis.

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