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SYNERGY BETWEEN THE T3/ANTIGEN RECEPTOR COMPLEX AND Tp44 IN THE ACTIVATION OF HUMAN T CELLS[1]

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In addition to the T3/antigen receptor complex (T3/Ti), other T cell surface molecules participate in early events involved in human T cell activation. In this report we document that monoclonal antibody 9.3, which recognizes a 90,000 dalton homodimer expressed on human T cells, synergizes with ligands reacting with T3/Ti to activate purified T cells and Jurkat, a human T cell leukemic line. Unlike phorbol myristate acetate (PMA), 9.3 was able to synergize only with anti-T3 or anti-Ti if these antibodies were immobilized. Moreover, 9.3 failed to synergize with the calcium ionophore ionomycin. At high concentrations only, 9.3 could synergize with PMA in the activation of Jurkat and a T3/Ti negative mutant of Jurkat. At such high concentrations of 9.3, small transient increases in cytoplasmic free calcium ($(Ca^{++})_i$) were detected in quin 2-loaded Jurkat cells. This increase in $(Ca^{++})_i$ was the result of release of internal stores of calcium. 9.3 induced the hydrolysis of polyphosphoinositides, albeit the magnitude of inositol phosphates generated in response to 9.3 was substantially less than that observed with anti-Ti. No effect on pkC translocation was observed in Jurkat cells stimulated with 9.3. Although the small increase in $(Ca^{++})_i$ induced by 9.3 may account for its synergy with PMA, this effect is unlikely to account for the more potent synergistic effect observed with 9.3 and phytohemmaglutinin or immobilized anti-T3 and anti-Ti antibodies.

Human T lymphocytes recognize antigen in association with major histocompatibility antigens on the surface of antigen-presenting cells, and as a consequence of this interaction, can be activated to perform their effector functions. Central to the recognition of specific antigen is the T cell antigen receptor, a multimeric structure composed of an 80,000 to 90,000 dalton polymorphic disulfide-linked heterodimer and three associated 20,000 to 28,000 dalton chains termed T3 (T3/Ti) (1–8). This receptor not only recognizes specific antigen but also can transmit at least one of the signals that can initiate activation (9–12). Because antigen recognition requires a physical interaction between T cells and antigen-presenting cells, it is possible that T cell surface molecules other than the T3/Ti complex also contribute to either antigen recognition or to the initiation of activation. Monoclonal antibodies (MAb)[4] reactive with several distinct non-T3/Ti molecules can modulate T cell function. For example, antibodies directed against T4, T8, and lymphocyte function-associated antigen 1 can block the ability of cytolytic T lymphocytes to kill appropriate target cells (13–15). Alternatively, under appropriate conditions antibodies reactive with T11 and Tp44 can induce T cell proliferation (16–18). Despite these observations, the functions of these molecules and their ligands have not been defined.

We have used the human T cell leukemic line Jurkat, which can be activated to produce interleukin 2 (IL 2), as a model to study the cell surface molecules and molecular events involved in T cell activation. In the presence of phorbol myristate acetate (PMA), ligands that interact with the T3/Ti complex (such as soluble anti-T3 or anti-Ti MAb, as well as the lectin phytohemmaglutinin; PHA) activate Jurkat to produce IL 2 (19). Perturbation of T3/Ti by these agents results in an increase in the concentration of cytoplasmic free calcium ($(Ca^{++})_i$) in Jurkat (9, 10). Because interactions involving the T3/Ti complex can be bypassed with calcium ionophores, it is probable that this T3/Ti-mediated increase in $(Ca^{++})_i$ is an intracellular signal for activation. However, this increase in $(Ca^{++})_i$ alone is not sufficient to trigger activation (as measured by IL 2 production), but requires the additional signal(s) supplied by PMA (9, 10, 19). PMA binds to and activates protein kinase C (pkC) (20, 21). This synergism between increases in $(Ca^{++})_i$ and the activation of pkC is not unique to Jurkat, but has been confirmed with normal T cells (22, 23) and appears to be a general pathway by which many non-lymphoid cells can be activated as well (24).

Although PMA provides a potent synergizing stimulus, it is not clear which physiologic signal it substitutes for in the activation of T cells. Because antigen recognition occurs at the T cell-macrophage interface, it is reasonable to speculate that some macrophage cell surface molecule or secreted product interacts with a T cell surface molecule and provides this stimulus. The T cell surface molecule that functions as a receptor for such ligands and transduces this second stimulus has not been identified.

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[4] Abbreviations used in this paper: pkC, protein kinase C; MAb, monoclonal antibody; IP_3, inositol trisphosphate; IP_2, inositol bisphosphate; and IP, inositol phosphate.

In a recent study, antibodies reactive with either T1 or Tp44 could synergize with anti-T3 antibody in the induction of proliferative responses of peripheral blood mononuclear leukocytes (PBL) (25). In this study, we confirm and extend these observations demonstrating that MAb 9.3 (reactive with Tp44), unlike PMA, can only synergize with immobilized but not soluble anti-T3 or anti-Ti antibodies in the activation of purified T cells or Jurkat. In additional contrast with PMA, 9.3 fails to synergize with ionomycin, a calcium ionophore. The mechanism of transmembrane signalling by Tp44 was studied in Jurkat. Although 9.3 induced the hydrolysis of polyphosphoinositol, the low levels induced are unlikely to account for its potent synergistic effects with anti-T3 or anti-Ti antibodies.

MATERIALS AND METHODS

Cells. Jurkat, clone E6-1, and mutants of Jurkat that fail to express T3 and Ti have been characterized and were maintained in RPMI 1640 supplemented with fetal calf serum (FCS) and antibiotics (8). CTLL-20 is a murine IL 2-dependent cell line that has been described (19). Heparinized peripheral blood was collected from normal volunteers, and the mononuclear cell fraction was isolated by Ficoll-Hypaque density gradient centrifugation. After removal of adherent cells by adherence to plastic, T cells were enriched by sorting for cells that failed to stain by indirect immunofluorescence with MAb CA 206 and Leu-M3 as analyzed on a fluorescence-activated cell sorter (FACS IV; Becton Dickinson, Mountainview, Ca.) as described (8).

Monoclonal antibodies (MAb). Hybridomas w6/32 (anti-class I HLA), OKT3 (anti-T3), OKT4 (anti-T4), and OKT11 (anti-T11) were obtained from the American Type Culture Collection (Rockville, MD), and ascitic fluids were generated after pristane priming BALB/c mice. C305 (anti-Ti on Jurkat), C373 (anti-T11), and J64 (anti-T9) were derived in this laboratory and have been described (8, 26). CA 206 is reactive with HLA class II molecules (27). Leu-M3 (reacts with monocyte antigens) was obtained from Becton Dickinson (Mountainview, CA). 10.2 (anti-T1) and 9.3 (anti-Tp44) ascitic fluids were obtained from Dr. Jeffrey Ledbetter, and purified IgG fractions of 9.3 were purchased from New England Nuclear (25). Antibodies were coupled to Sepharose beads at 8 to 10 mg/ml.

Cell cultures. Peripheral blood T cells, 5×10^4 cells/culture, or PBL, 1×10^5 cells/culture, were stimulated in duplicate or triplicate flat-bottomed microcultures containing 200 μl of RPMI medium supplemented with 10% FCS. Unless otherwise indicated, cells were stimulated with OKT3 or W6/32 (1/1000 final dilution of ascitic fluid) in soluble fluid phase or were immobilized to the culture dish with affinity-purified goat anti-mouse Ig (Cappel, Cochranville, PA) that had been adhered to the plastic dish in a carbonate buffer (pH 9.5). Cells were also stimulated with ionomycin (0.5 μg/ml; Calbiochem, La Jolla, CA), PHA (1 μg/ml; Burroughs-Wellcome, Research Triangle Park, NC), or PMA (0.5 ng/ml; Sigma Chemical Co., St. Louis, MO). After 72 hr of culture at 37°C, cultures were pulsed with 1 μCi of [^{3}H]thymidine (6.4 Ci/mmol) for 4 hr. Cells were collected on glass fiber filters by using an automated harvester (Scatron, Norway), and [^{3}H]thymidine uptake was determined by liquid scintillation counting. Jurkat cells or mutants were stimulated in microcultures for 24 hr and IL 2 production was determined as described by using CTLL-20 as an indicator cell (19).

Immunofluorescence. Cell surface antigens of Jurkat, T3/Ti negative mutants of Jurkat, and PBL were analyzed by indirect immunofluorescence on a FACS IV according to described methods (8). Saturating amounts of the indicated MAb and a fluoresceinated $(Fab')_2$ goat anti-mouse Ig preparation (Cappel) were used.

Determination of cytoplasmic free calcium. Jurkat cells were loaded with the 5 μM acetoxy-methyl ester of the calcium-sensitive fluorescent dye quin 2, and changes in fluorescence were monitored at 37°C as described (9). Either purified IgG preparations (6 μg/ml final) or dilutions of ascitic fluid (1/300) were added as stimuli to quin 2-loaded Jurkat cells suspended in HEPES-buffered saline containing 1.0 mM Ca^{++}. In some experiments, extracellular calcium was depleted by the addition of EGTA, pH 7.40, final concentration of 10 mM. $(Ca^{++})_i$ was calculated by the method of Tsien (28).

Determination of inositol phosphates. Inositol phosphates were quantitated as described (29). Briefly, incorporation of [^{3}H]inositol into phospholipid was achieved by incubating Jurkat cells (10^7 cells/ml) in a HEPES-buffered saline solution with 40 μCi/ml of [^{3}H]inositol (37 MBq/ml; Amersham Corp., Arlington Heights, IL) for 3 hr followed by dilution to 10^6/ml with RPMI 1640 supplemented with 10% FCS. After an overnight incubation at 37°C, cells were washed and were resuspended in HEPES-buffered saline at 5×10^6 cells/ml. Antibodies were added to a final dilution of 1/1000. The [^{3}H]inositol phosphates were extracted from Jurkat cells, were separated by anion exchange chromatography, and were quantified as described by us in detail (29) by using published methods (30).

pkC translocation assays. Jurkat cells (10^7 cells/ml) were incubated at 37°C for the indicated time intervals with the indicated stimuli: 9.3 or w6/32 antibodies (1/1000 final dilution); PHA (1.0 μg/ml); or PMA (50 ng/ml). Thereafter, cells were washed twice in Ca^{++} and Mg^{++} containing phosphate-buffered saline and were lysed in 100 μl of distilled water. The lysate was reconstituted with 2 ml of buffer (20 mM Tris, pH 7.5, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 0.5 mM EGTA, and 0.33 M sucrose) and was centrifuged at 100,000 × G for 30 min. While the cytosolic fraction was stored, the particulate membrane pellets were homogenized in 1% Nonidet P-40 in buffer, and after 30 min, were centrifuged at 12,000 rpm for 10 min. Cytosol and solubilized membrane fractions were passed over a 1 ml DE52 column and were eluted with 0.1 M NaCl. pkC activity was assayed as described in detail with 100 μM γ-^{32}ATP (ICN Radiochemicals, Irvine, CA), 50 μg III-S histone (Sigma), 0.75 mM Ca^{++}, and 10 mM Mg^{++}, in the presence and absence of 24 μg phosphatidylserine and 1.6 μg diolein (Sigma) (31). Specific pkC activity, in the presence of phospholipids, is expressed as pmol ^{32}P incorporated into histone per 3 min/total vol of cytosol or membrane fraction.

RESULTS

MAb 9.3 can substitute for PMA in the mitogenic response of purified T cells to immobilized but not soluble anti-T3 antibody. Recent studies demonstrated that MAb 9.3 could augment the anti-T3 proliferative response of partially purified T cells (25). Therefore, it was of interest to confirm whether 9.3 could substitute for accessory cell function and to determine under what conditions 9.3 could substitute for adherent cells in the activation of PBL by anti-T3. As expected, adherent cell-depleted PBL failed to respond to soluble OKT3 (Table I). Although 9.3 had a small augmenting effect upon the OKT3 response of unfractionated cultures, it failed to substitute for adherent cell function in the response of nonadherent PBL to soluble anti-T3. At least one adherent cell function in the anti-T3 response can be attributed to the anchoring of the anti-T3 antibody via the Fc receptor (32, 33). This function can be restored with Sepharose bead-coupled anti-T3 antibody (34). Similarly, by using micro-culture plates coated with goat anti-mouse Ig to immobilize the OKT3, the anti-T3 response was restored,

TABLE I

9.3 Fails to reconstitute adherent cell function in cultures stimulated with soluble OKT3[a]

Responding Cells	Stimulus	cpm	
		−9.3	+9.3
Unfractionated PBL	0	613[b] ± 55	571 ± 18
	OKT3	66,503 ± 1,389	79,336 ± 218
	w6/32	459 ± 31	396 ± 54
Nonadherent PBL	0	233 ± 35	269 ± 23
	OKT3	297 ± 70	452 ± 111
	G αMIg	109 ± 22	241 ± 25
	G αMIg + OKT3	167,481 ± 4,445	174,515 ± 13,322
	w6/32	222 ± 18	356 ± 74
	G αMIg + w6/32	289 ± 41	512 ± 22

[a] Cultures of unfractionated PBL or PBL depleted of adherent cells by two cycles of plastic adherence, 10^5/well, were stimulated in microcultures that were or were not coated with goat anti-mouse Ig and were stimulated with medium, OKT3, or w6/32 in the presence or absence of 9.3 (1/2,000 dilution of ascitic fluid) for 72 hr. [^{3}H]Thymidine uptake of triplicate cultures was determined during the last 4 hr of culture. Representative of six separate experiments.

[b] Mean ± SEM.

and this effect was specific because no mitogenic response was noted with soluble or immobilized anti-HLA antibody (w6/32) (Table I). No additional effect of 9.3 was noted in the cultures reconstituted by immobilizing anti-T3.

In a recent study, highly purified T cells failed to respond to anti-T3 antibodies even if they were immobilized, suggesting that additional stimuli might be provided by some contaminating adherent cells or other non-T cells in the above cultures (35, 36). Therefore, it was of interest to determine whether 9.3 antibody would synergize with soluble or immobilized antibody in cultures of highly purified T cells. For these experiments, we isolated nonadherent cells that failed to stain with Ca 206 (anti-class II HLA) or Leu-M3 (anti-monocyte) MAb on a FACS. In agreement with the previously published data, purified T cells failed to respond not only to soluble OKT3 antibody but also to immobilized OKT3 (35, 36). These responses were reconstituted if PMA was included in the cultures (Table II) (23). Although 9.3 failed to reconstitute the response of purified T cells stimulated with soluble OKT3 antibody, responses comparable with those seen in cultures stimulated with PMA plus OKT3 were observed when 9.3 was added to cultures of purified T cells stimulated with immobilized OKT3 (Table II).

In previous studies, we and others have demonstrated that anti-T3 antibodies increase $(Ca^{++})_i$ in T cell lines and peripheral T cells, and calcium ionophores could substitute for anti-T3 antibodies in synergizing with PMA (8–12, 22). Therefore we also examined whether 9.3 would synergize with a calcium ionophore, ionomycin, in the mitogenic response of purified T cells. Although PMA synergized with ionomycin in inducing a proliferative response by purified T cells, 9.3 failed to synergize with ionomycin (Table II).

9.3 synergizes with immobilized anti-T3, anti-Ti, or PHA in the activation of Jurkat. Because 9.3 is not expressed on all peripheral T cells (all T4-positive cells, but only approximately 50% of T8-positive cells (37), and the synergistic effects observed in the experiments described above might be the result of interactions of different subpopulations of T cells, the effects of 9.3 upon the activation of the homogeneous T cell line Jurkat were studied. Jurkat cells could be activated to produce substantial levels of IL 2 in response to immobilized anti-T3, immobilized anti-Ti (C305), or PHA in the presence of 9.3, even at a final dilution of $1/10^5$ (Table III). Immobilization of anti-T3 or anti-Ti could be accomplished either by using antibody coupled to Sepharose beads (as in Table III), or as in the case of purified T cells, bound to the plastic culture dish via goat anti-mouse Ig (data not shown). As in the case of purified T cells, no synergy was observed between 9.3 and soluble antibody (OKT3 or C305) or ionomycin. Unlike 9.3, none of the other MAb tested that reacted with other cell surface determinants expressed on Jurkat (OKT4, OKT11, C373, w6/32, 10.2, or J64), synergized with PHA, C305-coupled beads, ionomycin, or PMA in the production of IL 2 by Jurkat.

9.3 synergizes with PMA in Jurkat and a mutant of Jurkat that fails to express the T3/Ti complex. Of interest was the synergistic effect of 9.3 observed in the presence of PMA. At high concentrations, (1:1000 of ascitic fluid, a saturating concentration), 9.3 plus PMA consistently induced Jurkat to produce some, albeit lower levels of IL 2 (Table III). A similar synergistic effect of 9.3 in the presence of PMA has been reported by others for both the activation of Jurkat and PBL (17, 18). However, a point of contention arises in these other studies regarding the role of the T3/Ti complex in this response. Modulation of T3 from Jurkat eliminated the response of these cells to 9.3 plus PMA (18), whereas responsiveness was maintained in T3-modulated peripheral T cells (17). Because modulation of T3 involves exposing the cell to activating antibodies, the resultant response may be difficult to interpret due to the influence of the modulating antibody.

Because well characterized mutants of Jurkat that failed to express the T3/Ti complex were generated in our laboratory (8, 38), we made use of these cells to examine the requirement for T3 in the activating effects of 9.3 upon Tp44. Of six mutants examined, five expressed abundant levels of antigens detected by 9.3 on the cell surface as assessed by indirect immunofluorescence and flow cytometry (data not shown). One mutant, J.RT3-T3.5, failed to react with 9.3, but this did not correlate with the defect in this cell (other cells also lacking β-chain transcripts expressed antigens detected by 9.3). These results suggest that no close structural dependence exists between Tp44 and the T3/Ti complex. Moreover, because the T3/Ti mutant J.EMS-T3.3 produced IL 2 levels of comparable magnitude with that observed in Jurkat (Table IV) in response to 9.3 plus PMA, no functional dependence seems to be evident between these cell surface structures.

Signal transduction by Tp44. The mechanisms by which cell surface molecules regulate intracellular events during T cell activation are of considerable interest. At high concentrations, 9.3 can partially substitute for

TABLE II

Synergy between MAb 9.3 and immobilized OKT3 in the activation of purified T cells

	Stimulus	cpm			
		0	OKT3		Ionomycin
			Soluble	Immobilized	
Experiment 1	0	166 ± 67	497 ± 35	1,658 ± 220	719 ± 52
	PMA	556 ± 3	37,691 ± 6,511	120,768 ± 8,872	37,691 ± 6,511
	9.3	219 ± 108	577 ± 75	80,289 ± 7,567	770 ± 308
Experiment 2	0	173 ± 27	411 ± 468	2,947 ± 503	261 ± 81
	PMA	126 ± 8	34,947 ± 3,568	65,496 ± 3,540	24,347 ± 803
	9.3	215 ± 72	533 ± 130	102,954 ± 15,949	533 ± 120

[a] Purified T cells, obtained by cell sorting as described in the *Materials and Methods*, were stimulated in replicate cultures for 72 hr in the presence of the indicated stimuli. [^{3}H]Thymidine uptake was determined during the last 4 hr of culture. Two separate representative experiments are shown.

[b] The means ± SEM are shown.

TABLE III

Production of IL 2 by Jurkat stimulated with MAb 9.3[a]

Stimulus	Stimulus (IL 2 u/ml)							
	0	PMA	PHA	C305	C305-beads	OKT3	OKT3 beads	Ionomycin
0	<2	<2	3.5	<2	3.5	<2	<2	<2
PMA	<2	<2	48	15	38	32	44	27
9.3								
1/1,000	<2	12	32	<2	24	<2	48	<2
1/10,000	<2	<2	38	<2	30	<2	32	<2
1/100,000	<2	<2	24	<2	18	<2	32	<2
w6/32								
1/1,000	<2	<2	3.5	<2	3.5	<2	<2	<2

[a] Jurkat cells, 10^6/ml, were cultured in the presence of the indicated stimuli as described (5). Stimuli were used as follows: PMA, 50 ng/ml; PHA, μg/ml; C305 or OKT3 ascitic fluid, 1/1,000 final dilution; C305-coupled or OKT3-coupled Sepharose beads, 1% final dilution; ionomycin, 1 μM; 9.3 ascitic fluid, as indicated; and w6/32 ascitic fluid. After 24 hr of culture, cell-free supernatants were collected and were assessed for IL 2 activity. The results shown are representative of six separate experiments.

TABLE IV

IL 2 production by Jurkat and J.EMS-T3.3, a T3/Ti negative mutant, in response to 9.3 plus PMA[a]

Stimulus	Responding Cell (IL 2 u/ml)	
	Jurkat	J.EMS-T3.3
None	<2	<2
PMA	<2	<2
9.3	<2	<2
9.3 + PMA	30.6	12.0
Ionomycin	<2	<2
Ionomycin + PMA	96.2	162.0

[a] Jurkat and J.EMS-T3.3, a mutant of Jurkat that fails to express T3 or Ti antigens were stimulated as indicated and IL 2 production was determined after 24 hr. The results are representative of three separate experiments.

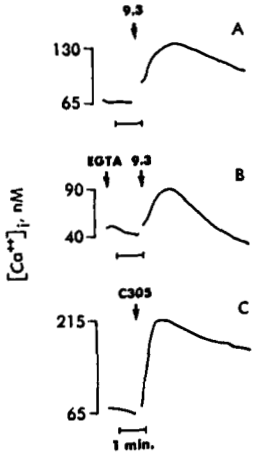

Figure 1. Perturbation of Tp44 by MAb 9.3 increased $(Ca^+)_i$ in Jurkat. Jurkat cells, 10^7/ml, were loaded with the calcium-sensitive fluorescent dye quin 2, 5 μM, and changes in fluorescence were monitored at 37°C as described (18). MAb 9.3 (1/300 dilution of ascitic fluid) was added to quin 2-loaded Jurkat cells in medium containing 10 mM extracellular calcium (*Graph A*) or to cells in which extracellular calcium had been depleted to less than 40 nM by the addition of EGTA (*Graph B*). MAb C305 (anti-Ti) (1/300 dilution of ascitic fluid) was added in the presence of 10 mM extracellular calcium (*Graph C*). $(Ca^+)_i$ was determined as described (18).

either anti-Ti or ionomycin, an observation suggesting that 9.3 might increase $(Ca^{++})_i$. On the other hand, 9.3 can also mimick some of the effects of PMA, an activator of pkC. Accordingly, we examined the ability of 9.3 to affect $(Ca^{++})_i$ and to activate pkC.

When added to Jurkat cells loaded with the Ca^{++}-sensitive fluor, quin 2, 9.3 caused an increase in $(Ca^{++})_i$ from a basal level of 72 ± 8 nM to a peak of 130 ± 11 (n = 7, representative tracing in Fig. 1*A*). Moreover, a substantial portion of this increase could be attributed to the release of internal stores of calcium, because substantial increases were observed when extracellular Ca^{++} was depleted by the addition of EGTA (n = 4, Fig. 1*B*). It should be noted that the changes in $(Ca^{++})_i$ were detected only at relatively high concentrations of 9.3, 1/300 final dilution of ascites or 6 μg/ml of purified IgG. Additionally, the level of $[Ca^{++})_i$ increase and its duration were substantially less than that seen with anti-Ti (compare Fig. 1*A* and *C*). As previously reported, no change in $(Ca^{++})_i$ was noted in response to comparable amounts of several other antibodies including w6/32 (9, 10).

Because a substantial portion of the 9.3-induced increase in $(Ca^{++})_i$ was the result of release of internal stores of Ca^{++}, this suggested that 9.3 might induce the generation of inositol trisphosphate (IP_3), a product of the hydrolysis of phosphatidylinositol 4,5-bisphosphate, and the putative second messenger responsible for the release of internal stores of Ca^{++} (24). The phospholipid pool of Jurkat was labeled with [^{3}H]inositol, and the production of inositol phospholipids was measured after the addition of 9.3, or as a control, after the addition of w6/32 (a MAb reactive with class I HLA determinants). Figure 2 demonstrates that addition of 9.3 antibody to Jurkat results in a prompt increase in the levels of ^{3}H-IP_3 and [^{3}H]inositol biphosphate (^{3}H-IP_2). Neither ^{3}H-IP_3 nor ^{3}H-IP_2 appeared subsequent to the addition of the anti-HLA antibody (w6/32). Although the increase of ^{3}H-IP_3 in response to 9.3 was at least two-fold or greater than the level observed with w6/32-stimulated or unstimulated cells (seven separate determinations), this increase was substantially less than that observed with C305 antibody (Table V).

Activation of pkC by both phorbol esters or its physiologic activator, diacylglycerol, is associated with translocation of pkC activity from the cytosol to the membrane fraction (21). Inasmuch as PMA exerts a potent effect upon the activation of pkC as determined by a translocation of enzyme activity from the cytosol to the membrane fraction of Jurkat cells, we were unable to detect pkC activation by 9.3 (Table VI). In contrast, PHA exerted a modest effect upon pkC translocation.

DISCUSSION

T cell antigen recognition involves complex cell-cell interactions between the T cell and the antigen-presenting cell. Abundant evidence suggests that cell surface molecules other than those involved in antigen recognition are important in accessory events leading to T cell

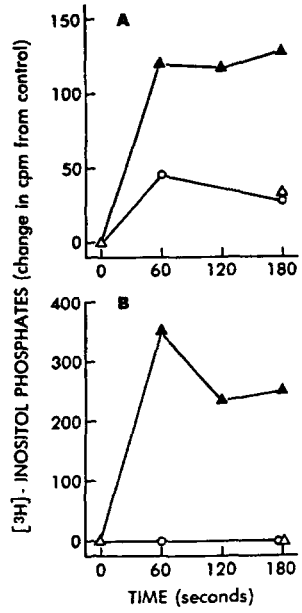

Figure 2. Perturbation of Tp44 by MAb 9.3 generates increases in [^{3}H]-IP$_3$ (*Panel A*) and [^{3}H]-IP$_2$ (*Panel B*). The results are expressed as the change in cpm from unstimulated Jurkat cells at time 0 after the addition of 9.3 (▲), w6/32 (○), or medium (△). Basal values of ^{3}H-IP$_3$ and ^{3}H-IP$_2$ were 90 cpm and 645 cpm, respectively. These data are representative of three separate experiments. MAb were added in a final dilution of 1/300. The [^{3}H]inositol phosphates (from 5 × 10^6 cells) were extracted, were separated by anion exchange chromatography, and were quantified as described.

TABLE V

Appearance of [^{3}H]Inositol phosphates after stimulation with 9.3 or C305[a]

Stimulating Antibody	[^{3}H]Inositol Phosphates		
	IP$_1$	IP$_2$	IP$_3$
w6/32 (α HLA)	147 ± 1[b]	422 ± 11	73 ± 5
9.3 (α Tp44)	204 ± 5	667 ± 24	165 ± 2
C305 (α Ti)	329 ± 6	2263 ± 2	991 ± 10

[a] The phospholipid pool of Jurkat was equilibrated with [^{3}H]inositol. After stimulation with the indicated MAb for 2 min at 37°C, the concentrations of [^{3}H]inositol phosphates were determined.

[b] The results are expressed as mean ± SD of a single experiment performed in triplicate.

TABLE VI

MAb 9.3 does not induce pkC translocation[a]

Stimulus	pkC Activity (pmol ^{32}P incorporated)[b]	
	Expt. 1	Expt. 2
0	2637/547	6706/302
9.3 (2 min)	1773/559	ND
9.3 (10 min)	3201/365	6312/307
9.3 (30 min)	2999/661	ND
w6/32 (10 min)	1881/341	ND
PHA (10 min)	ND	4863/1092
PMA (10 min)	549/5754	698/2696

[a] Jurkat cells (1 × 10^7) were incubated at 37°C with the indicated stimuli (1/1000) dilution of ascites for 9.3 and w6/32, 1 μg/ml PHA, and 50 ng/ml PMA for the indicated time intervals. pkC activity was assayed as described in *Materials and Methods*.

[b] Specific pkC activity in the presence of phospholipids is expressed as pmol ^{32}P incorporated into histone per 3 min per total volume of cytosol fraction/membrane fraction. Figures are mean of duplicates.

activation. The studies reported here suggest that 9.3 may interact with a cell surface molecule involved in such accessory events. MAb 9.3 has recently been reported to be capable of activating human T cells or Jurkat in the presence of PMA (17, 18). We also demonstrated that at high concentrations, 9.3 can synergize with PMA and activate Jurkat. However, our studies demonstrate that 9.3 is much more potent as an agonist in providing a second signal (i.e., non-T3/Ti complex mediated) required for the activation of T cells and Jurkat. This finding is in agreement with the studies of Ledbetter et al. (25).

The results of this study support those made by others in the dual role of non-T accessory cells in the activation of T cells by anti-T3 MAb (35, 36). One function of such accessory cells is the immobilization of anti-T3 antibody via an interaction of the antibody with the Fc receptor on adherent cells. This is supported by numerous studies involving adherent cell depletion experiments and the demonstration that anti-T3 MAb of the IgG1 subclass fail to stimulate certain individuals that lack Fc receptors capable of binding mouse IgG1 (32, 34). This function of adherent cells can be mimicked by immobilizing anti-T3 MAb to Sepharose or plastic surfaces (34). However, a second function of accessory cells is revealed only in cultures of highly purified T cells, and thus suggests the involvement of a soluble mediator. Immobilized anti-T3 antibody fails to activate highly purified T cells. Although PMA apparently can substitute for both functions of accessory cells (see below), 9.3 appears to be able to substitute only for this latter function. IL 1 may also be able to substitute for this second function (35, 39).

MAb 9.3 appears to be capable of triggering Tp44 independent of the expression of T3/Ti, because it synergized with PMA in the production of IL 2 by a mutant of Jurkat that fails to express T3/Ti. These results are in agreement with the observations of Hara et al. (17) but appear to contradict those of Moretta et al. (18). This discrepancy probably stems from the methods used. Antigenic modulation of T3/Ti, which involved exposure of cells to agonist anti-T3 antibody, was used in both studies (17, 18). Because such modulation may have physiologic effects upon signalling mechanisms, the results of such studies may be misleading. The use of T3/Ti mutants provides a preferable model for studying the consequences of ligand-receptor interactions independent of T3/Ti. Thus Tp44 appears to be able to signal via events that do not appear to involve T3/Ti surface molecules.

Increases in $(Ca^{++})_i$ and activation of pkC can regulate intracellular events that culminate in T cell activation. Perturbation of the T3/Ti complex is linked to polyphosphoinositide turnover, indicating that the T3/Ti complex probably can activate pkC by generating diacylglycerol, as well as increases in $(Ca^{++})_i$ by IP$_3$ (29). Perturbation of Tp44 also leads to polyphosphoinositide turnover as evidenced by the ability of high concentrations of 9.3 to generate IP$_3$ and mobilize intracellular Ca^{++}. The resulting increase in $(Ca^{++})_i$ may explain the ability of 9.3 in high concentrations to synergize with PMA in the activation of Jurkat and in the activation of PBL (17, 18). However, the 9.3-induced increases in $(Ca^{++})_i$ and inositol phosphates are substantially less than those induced by anti-Ti, and therefore are an unlikely explanation for the synergism observed between 9.3 and either PHA or immobilized anti-Ti (or anti-T3). It is also unlikely that this synergism can be fully explained by a direct effect of 9.3 on pkC activity because we were unable to detect any translocation of pkC activity to the membrane after the addition of even high concentrations of 9.3 to Jurkat cells. Although the failure to detect any pkC translocation

may reflect a relative insensitivity of the translocation assay, our results do establish that any effect of 9.3 on pkC activation is substantially less than that of PMA or even PHA (which also induces polyphosphoinositide turnover) (40). It is possible that the synergism between 9.3 and perturbation of the T3/Ti complex is due, not to a direct effect of 9.3 on $(Ca^{++})_i$ or pkC, but to modulation by 9.3 of signal generation by T3/Ti. Of interest in this regard is our observation that 9.3 activates T cells or Jurkat in the presence of immobilized MAb but fails to synergize with soluble MAb. It is not known what the effects of immobilization of anti-T3 or anti-Ti are, but immobilized antibody may more effectively activate pkC, or may prolong the activation signal by preventing receptor internalization. Preliminary results indicate that soluble anti-T3 has a modest, transient effect on pkC translocation, and this effect is prolonged when these MAb are immobilized (B. Manger, unpublished results). One may speculate that immobilized anti-T3 or anti-Ti as opposed to the soluble form may more closely mimick antigen as it is presented on an accessory cell. Alternatively, instead of an effect upon polyphosphoinositide turnover, 9.3 may influence an as yet unidentified intracellular messenger that is distinct from either Ca^{++} or pkC. A role for such a signal in T cell activation has been recently suggested by the observations of others (41, 42). Moreover, other than pkC activation, additional roles for PMA are suggested by recent experiments with the use of more physiologic activators of pkC that do not reconstitute all of the functions of PMA (43).

The physiologic ligand for Tp44 is not known. Although IL 1 and perturbation of Tp44 have similar effects on activation of Jurkat, it is unlikely that Tp44 is the IL 1 receptor based on differences in tissue distribution and molecular size (44). Additional studies will focus upon defining the ligand that reacts with the molecule identified by 9.3, as well as clarifying the events involved in signal transduction in the T cell.

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