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Mitogen-Induced Modulation of CD3, CD4, and CD8

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ABSTRACT: It is not clear whether CD3 contacts CD4 or CD8 directly, nor have the regulation and interregulation of expression of these three receptor molecules been determined. We explored these issues by first stimulating human peripheral blood lymphocytes *in vitro* with three well-characterized T-cell receptor–directed mitogens (phytohemagglutinin [*PHA*], concanavalin A [*ConA*], and anti-CD3 monoclonal antibody [αCD3]) and then using multiparameter flow cytometric techniques to investigate modulation of surface (*sur*) and cytoplasmic (*c*) CD3, CD4, and CD8. Cultures with αCD3 had a rapid, large, and persistent decline in surCD3; the cCD3 median fluorescent intensity (*MFI*) declined gradually, over the entire culture period. With αCD3, surCD4 MFI and cCD4 MFI declined by days 4 to 8 (31% of *ex vivo* value, $p < 0.001$ and 47%, $p = 0.033$), as did surCD8 MFI (58%, $p = 0.010$). PHA was associated with an increase in surCD8%, surCD8 MFI, and cCD8% at days 4 to 8 (178% of *ex vivo*, $p = 0.003$; 168%, $p = 0.025$; and 331%, $p = 0.001$). For PHA at days 4 to 8, cCD8 MFI was highly variable but always higher than in unstimulated cultures (5 of 5 experiments). With ConA, at 3 to 5 hours *ex vivo*, there was a decrease in surCD3 MFI relative to *ex vivo* (64%), surCD4% (83%), cCD4% (87%), surCD4 MFI (50%) and cCD4 MFI (48%), surCD8% (85%) and an increase in cCD8% (260%). As with PHA, at days 4 to 8, surCD8% was high relative to *ex vivo* (169%). Thus, we found that αCD3 had delayed effects on CD4 and CD8; PHA had delayed effects on CD8 only; and ConA had very rapid effects on CD3, CD4, and CD8, as well as a delayed effect on surface CD8. These effects involve both surface and cytoplasmic antigen expression and are more consistent with degradation or retention, rather than with shedding or increased production. They may reflect direct interactions between CD4 or CD8 and CD3 and/or interregulation of CD3 expression with expression of these coreceptor molecules. *Human Immunology 61, 202–211 (2000).* Published by Elsevier Science Inc.

KEYWORDS: PHA; ConA; T lymphocytes; T-cell antigens; CD3; CD4; CD8

INTRODUCTION

Monoclonal anti-CD3 antibodies (αCD3), phytohemagglutinin (PHA), and concanavalin A (ConA) all bind to various parts of the T-cell antigen receptor (TCR). Investigations to determine the nature and effects of this binding provided crucial insights into the structure of the TCR [1, 2]. Extensive work by many researchers has lead to the conclusion that PHA binds to the idiotypic portion of the TCR (Ti), whereas ConA binds to CD3 and to Ti [3].

Mitogen- and αCD3-related studies have been invaluable in defining the steps in receptor-mediated signal transduction, cellular activation, and effector function, as well as in determining the interactions among coreceptor molecules. Low concentrations of αCD3 act in many ways like ConA; αCD3 is frequently referred to as a mitogen [3, 4]. αCD3 decreases lytic activity by CD8+ lymphocytes but may or may not affect target binding; this effect can be long lasting [5, 6]. Following αCD3 treatment, decreases in antigen-specific proliferation and cytotoxic function occur in association with the decreased expression of CD3 and reappear with the reexpression of this molecule [1].

The interaction between the TCR and the coreceptor molecule CD4 has been examined in relation to the human immunodeficiency virus (HIV), but the interactions between CD3 and CD8 have been examined less

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Received July 16, 1999; accepted August 18, 1999.

0198-8859/00/$–see front matter
PII S0198-8859(99)00128-7

fully [7–12]. CD3, CD4, and CD8 have similar phosphorylation signal systems; however, they differ in terms of (a) whether or not endocytosis occurs subsequent to phosphorylation and (b) the endocytic pathways followed if endocytosis does occur [9, 13–18]. These pathways are further affected by the nature of the stimulation [15]. It is debatable whether any direct physical association actually occurs between the TCR and CD4 and/or CD8; certainly, the TCR and these coreceptor molecules come in very close proximity during antibody-induced T-cell activation [17, 19, 20].

We used cell permeabilization methods, surface/cytoplasmic fluorescent staining, and multiparameter flow cytometric techniques to investigate the effects of mitogens and αCD3 on the modulation of surface and cytoplasmic CD3, CD4, and CD8. We cultured human peripheral blood mononuclear cells (PBMCs) with PHA, ConA, or αCD3 and assessed the effects of these mitogens on the expression and intensity of expression of these key receptor molecules. We then examined the effects of secondary protein kinase C stimulation with the phorbol ester 12-myristate 13-acetate (PMA) and/or pre-Golgi blockade with brefeldin A (BFA) on CD3, CD4, and CD8 expression.

MATERIALS AND METHODS

Cells

PBMCs were obtained from normal human donors seronegative for HIV and hepatitis viruses. These were gradient separated and cultured in RPMI 1640 containing antibiotics and 10% heat-inactivated (56°C for 45 min) donor serum (culture condition referred to as Resting) or 10% heat-inactivated (56°C for 45 min) fetal calf serum (all other cultures) at 37°C in 5% CO_2 in a humidified incubator at a concentration of 2×10^6 cells/ml. Cells were retained in the above media without stimulation or were stimulated with 5 μg/ml PHA (culture condition referred to as PHA; Difco Laboratories, Detroit, MI), 4 μg/ml Con A (ConA) (Pharmacia Applied Biosystems, Uppsala, Sweden), or 0.05 μg/ml monoclonal antibody to the CD3 ϵ chain (αCD3) (clone SK7, Becton Dickinson Immunochemistry Systems [BD], San Jose, CA). For PHA only, 10% interleukin-2 was added after the first 1.5 days of culture (volume percentage; 640 half-maximal units/ml; Advanced Biotechnologies, Columbia, MD). Cells were harvested from the cultures at between 3 and 5 hours *ex vivo* (ConA and αCD3, 1 experiment), between 24 and 32 h *ex vivo* (Resting, 1–2 experiments; other culture conditions, 5 experiments), and between 4 and 8 days *ex vivo* (Resting and PHA, 6 experiments; ConA and αCD3, 5 experiments). At days 4 to 8, cell survival did not differ significantly among mitogen groups (data not shown).

Reagents

These included phycoerythrin-conjugated (PE) monoclonal antibodies (MAb) to CD3 (clone SK7, BD), peridinin chlorophyll protein (PerCP)-conjugated MAb to CD8 (clone SK1, BD), and allophycocyanin (APC)-conjugated MAbs to CD4 (clone SK3, BD). Isotype controls included mouse IgG2b PE (clones 27–35, PMG), mouse IgG1 PerCP (clone X40, BD), and mouse IgG1 APC (clone X40, BD). Unconjugated MAbs to CD4 (clone SK3, BD), CD8 (clone SK1, BD), and CD3 (clone SK7, BD) were used to block surface staining.

Other reagents

Permeabilization was done with ORTHO PermeaFix™ (Ortho Diagnostics, Raritan, NJ). Other reagents included PMA (Sigma Chemical Co., St. Louis, MO) (50 ng/ml for 4–5.5 h) and BFA (Sigma; 10 μg/ml for 4–5.5 h), added on the day of harvest (2 experiments for Resting and PHA at days 6 or 7; 1 experiment for ConA and αCD3 at day 1.5).

Blocking of Surface Antigens

For experiments in which cytoplasmic antigen expression and intensity were assessed in parallel with surface assessments, surface staining was blocked as follows. Cells were incubated with unconjugated MAbs for 15 min at room temperature (RT) in the dark, centrifuged, and washed with a buffered saline solution. They were then permeabilized and fixed with Ortho Permeafix and stained with identical, conjugated MAbs. With this protocol, $<1\%$ of cells were positive when incubated with the corresponding MAbs after blocking but before permeabilization (Fig. 1).

Flow Cytofluorometry

Multiparameter cytofluorometry was done with a FACSort (BD) and Lysis II or CellQuest software. As negative controls, cell aliquots were stained with the isotype controls above. Prior to permeabilization, surface antigens were stained for 15 min at RT in the dark. Staining for cytoplasmic antigens was done after blocking (above), after permeabilization for 30 min at RT in the dark.

At least 25,000 ungated events were collected for each tube; lymphocytes were defined by their forward- and side-scatter properties. Duplicate or triplicate tubes were run for each sample. Cell collection was done on a single Facsort (BD) by a single operator. The flow cytometer was standardized daily using chicken erythrocytes. Voltage was adjusted to maintain the standard in the same target channel throughout any given experiment. The same lots of MAbs were used throughout any given experiment; saturating amounts of antibodies were used. All analyses for a given experiment were done by a single

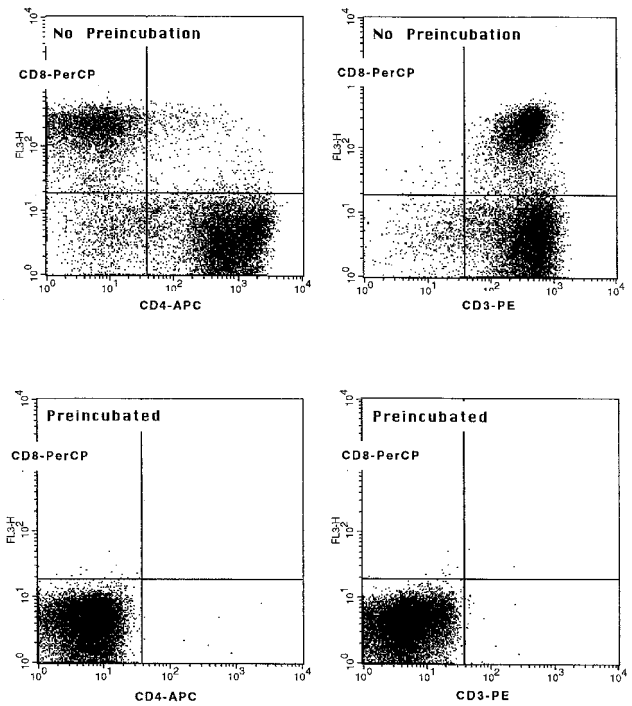


FIGURE 1 Dot plots illustrating blocking of surface antigen staining. Cultured peripheral blood mononuclear cells (PBMCs) were incubated for 15 min at room temperature in the dark with saturating amounts of directly conjugated monoclonal antibodies to CD3 (F12, PE), CD8 (F13, PerCP), and CD4 (F14, APC) prior to permeabilization. Lower panels represent PBMCs preincubated with identical, nonconjugated monoclonal antibodies for 15 min at room temperature in the dark prior to above incubation with conjugated antibodies.

individual; gates and margins were consistent throughout any given experiment.

Statistical Analyses

Data were collected in terms of both percentage positive and median fluorescence intensity of the positive cells (MFI); mean values of duplicate or triplicate tubes were used in all analyses. Percent positivity and fluorescence intensity varied from donor to donor, fluorochrome to fluorochrome, and surface antigen to surface antigen. Therefore, for statistical analyses and graphing of results, summary results for each culture condition or antigen were standardized in 2 ways. First, results were expressed as ratios of the pertinent value to the value for that donor at the first assessment, prior to inoculation or culture. For example, if for a given donor, 60% of PBMCs expressed CD4 *ex vivo*, and 30% expressed CD4 at the time of harvest from a certain culture condition, the harvest value was recorded as being 50% of the *ex vivo* value. Second, for experiments in which PMA and/or BFA were used, the effects of these were expressed as the percentage change from nontreatment, for example, (post-treatment value − nontreatment value) ÷ (nontreatment value) × 100. This standardization approach has been used successfully by others to compare relative fluorescence intensities, despite the inherently qualitative nature of these measurements [9]. Statistical analyses were done using paired Student *t*-tests, the latter providing comparisons between culture conditions and time points that take into account the parallel cultures and shared donor nature of the study design. Results of significance testing will be provided if the *p* value associated with the two-sided paired *t*-test was <0.10; these are provided in Tables 2 through 4.

TABLE 1 Percentage and median fluorescence intensity (MFI) of lymphocytes expressing surface and cytoplasmic CD3, CD4, and CD8 at initiation of cultures

		Location[a]	
Cluster of differentiation	Statistic	Surface	Cytoplasmic
Percentage of lymphocytes expressing antigen			
CD3	Mean	72.8 (8.4)	62.8 (11.1)
	Median	72.0	62.8
CD4	Mean	50.0 (9.2)	47.5 (10.5)
	Median	50.1	46.9
CD8	Mean	20.6 (5.6)	11.5 (6.0)
	Median	20.6	12.0
MFI of lymphocytes expressing antigen			
CD3	Mean	579.6 (296.5)	193.2 (84.8)
	Median	489.2	174.8
CD4	Mean	607.5 (201.6)	271.0 (79.0)
	Median	521.0	276.3
CD8	Mean	63.5 (18.8)	28.8 (5.7)
	Median	56.5	26.6

[a] Numbers in parentheses are standard deviations.

RESULTS

The mean and median proportions of lymphocytes expressing surface CD3, CD4, and CD8 (surCD3%, surCD4%, surCD8%) *ex vivo* are provided in Table 1, as are the mean and median proportions expressing cytoplasmic CD3, CD4, and CD8 post–surface blocking (cCD3%, cCD4%, and cCD8%). Also provided in Table 1 are the MFIs for lymphocytes expressing surface CD3, CD4, or CD8 (surCD3 MFI, surCD4 MFI, and surCD8 MFI) or cytoplasmic CD3, CD4, or CD8 (cCD4 MFI, cCD4 MFI, and cCD8 MFI).

αCD3

As shown in Table 2 and by the triangles in the figures, cultures with αCD3 had a rapid, large, and persistent decline in surCD3% and MFI. The cCD3% did not decline significantly; however, the cCD3 MFI declined

TABLE 2 Modulatory effects associated with monoclonal antibody to CD3[a]

Parameter	Time point[b]	Mean value[c] (%)	*p* Value[d]	Comparison group[e]	*p* value	Comparison group
CD3						
Sur %	1	0.4	NA			
	2	7.0	<0.001	a		
	3	2.1	<0.001	a	0.004	b
Sur MFI	1	42.3	NA			
	2	11.9	<0.001	a		
	3	7.9	<0.001	a, b		
c MFI	1	70.1	NA			
	2	68.9	0.026	a		
	3	28.6	0.010	a		
CD4						
Sur MFI	1	76.0	NA			
	2	75.1	0.006	a		
	3	31.0	<0.001	a, c	0.027	b
c MFI	1	65.6	NA			
	2	75.4	0.053	a		
	3	47.3	0.033	a		
CD8						
c %	3	215.6	0.024	c[f]		
Sur MFI	1	98.0	NA			
	2	86.1	0.016	a		
	3	58.1	0.010	a	0.008	c

[a] Percentage of lymphocytes expressing surface CD3, 4, or 8 (sur %); percentage expressing cytoplasmic CD3, 4, or 8 (c %), median fluorescence intensity (MFI) of cells expressing surface CD3, 4, or 8 (sur MFI), and MFI of cells expressing cCD3, 4, or 8 (c MFI).

[b] 1 = 3–5 h *ex vivo* (n = 1 experiment); 2 = 24–32 h *ex vivo* and 3 = 4–8 days *ex vivo* (n = 5 experiments for surface parameters; 4 for cytoplasmic parameters)

[c] Mean proportionate values are provided in relation to the *ex vivo* values

[d] p value determined by a paired Student t-test; nonapplicable (NA) when n = 1.

[e] Comparisons include to *ex vivo* (a), to unstimulated cells at time point 3 (b), time point 3 value compared to time point 2 value (c).

[f] At time 2, = 119.9%.

gradually over the entire culture period. At 24 to 32 h, for CD4+ lymphocytes compared to CD8+ lymphocytes, the surCD3 MFI was lower (4 of 5 experiments), and the cCD3 MFI was higher (4 of 4 experiments; differences not statistically significant, NS). SurCD4 MFI and cCD4 MFI had declined with αCD3, PHA, and ConA at 24 to 32 h, but additional declines at days 4 to 8 were found only with αCD3 (5 of 5 and 3 of 4 experiments, respectively; Figs. 2 and 3). At 4 to 8 days, cCD8% had increased but not significantly more than in the Resting condition (diamonds; Fig. 4). As found for surCD4 MFI, surCD8 MFI continued to decline through days 4 to 8 in αCD3 cultures (5 of 5 experiments; Fig. 5); this was not found with PHA or ConA. Unlike cCD4, cCD8 MFI did not decline (increased in 3 of 4 experiments, NS). When PMA or BFA was added prior to culture harvest, the surCD3% declined by >90%, from a mean of 6% to means of 0.3–0.6% of cells remaining surCD3 positive. PMA was associated with a 14% increase in the cCD3%. When BFA was added prior to culture harvest, the surCD3 MFI and cCD3 MFI both increased—the former markedly so (Fig. 6A); cCD3 MFI may have also been affected by PMA (Fig. 6B). The BFA effect on surCD3 MFI may have been greater in CD4+ lymphocytes (CD4+: +181% change, CD8+: +78% change); the PMA effect on cCD3 MFI may have been greater in CD8+ lymphocytes (CD4+: +7% change, CD8+: +23% change). Changes in CD4 and CD8 parameters with PMA and/or BFA treatment did not differ in any clear way between αCD3 and Resting (Figs. 7A,B, 8A,B and data not shown).

PHA

As shown in Table 3 and by circles in figures, PHA was associated with declines in surCD3 MFI, surCD4 MFI (Fig. 2), and cCD4 MFI (Fig. 3) at 24 to 32 h *ex vivo* and with an increased surCD3% and cCD3% at days 4 to 8; however, these changes were similar to those seen with Resting. CD3 findings did not differ between CD4+ and CD8+ lymphocytes. The percentage of lymphocytes expressing surCD8 and cCD8 (Fig. 4) had increased by days 4 to 8, as had surCD8 MFI (Fig. 5). At days 4 to 8, cCD8 MFI was highly variable but was always higher than that of Resting (5 of 5 experiments). Generally unlike Resting, PHA in association with PMA and/or BFA had little effect on CD3 and CD4 parameters (Figs. 6A, B, 7A, B) but may have been associated with increases in cCD8 MFI (PMA/BFA: +13.8% change).

TABLE 3 Modulatory effects associated with phytohemagglutinin[a]

Parameter	Time point[b]	Mean value[c] (%)	*p* Value[d]	Comparison group[e]	*p* Value	Comparison group
CD3						
Sur %	3	132.2	0.083	a	0.041	c[f]
c %	2	99.2	0.031	c		
	3	145.2	0.008	a		
Sur MFI	2	54.1	<0.001	a		
	3	68.3	0.023	a		
CD4						
Sur MFI	2	63.6	0.006	a		
c MFI	2	63.6	0.026	a		
CD8						
Sur %	2	102.2	0.032	c		
	3	178.1	0.003	a	<0.001	b
c %	2	142.5	0.042	c		
	3	330.8	0.001	a	0.009	b
Sur MFI	3	167.8	0.025	b		

[a] Percentage of lymphocytes expressing surface CD3, 4, or 8 (sur %); percentage expressing cytoplasmic CD3, 4, or 8 (c %), median fluorescence intensity (MFI) of cells expressing surface CD3, 4, or 8 (sur MFI), and MFI of cells expressing cCD3, 4, or 8 (c MFI).

[b] 2 = 24–32 h *ex vivo* and 3 = 4–8 days *ex vivo* (n = 6 experiments for surface parameters; 5 for cytoplasmic parameters). This mitogen was not done at time point 1.

[c] Mean proportionate values are provided in relation to the *ex vivo* values

[d] *p* value determined using a paired Student *t*-test.

[e] Comparisons include to *ex vivo* (a), to unstimulated cells at time point 3 (b), time point 3 value compared to time point 2 value (c).

[f] At time 2, = 99.7.

Con A

As shown in Table 4 and by squares in figures, with ConA, at 3 to 5 h *ex vivo*, there were declines in surCD3 MFI (64.1% of *ex vivo*), surCD4% (83.3% of *ex vivo*), cCD4% (86.6% of *ex vivo*), surCD4 MFI (49.8% of *ex vivo*; Fig. 2), and cCD4 MFI (48.4% of *ex vivo*; Fig. 3), surCD8% (85.2% of *ex vivo*), and surCD8 MFI (56.0% of *ex vivo*; Fig. 5); cCD8% had increased (259.8% of *ex vivo*; Fig. 4). At days 4 to 8, similar to PHA, surCD8% was high relative to *ex vivo* and to Resting, and values for cCD8% were widely variable but were always greater than those for *ex vivo* and Resting. Unlike Resting, ConA

TABLE 4 Modulatory effects associated with concanavalin A[a]

Parameter	Time point[b]	Mean value[c] (%)	*p* Value[d]	Comparison group[c]	*p* value	Comparison group
CD3: Sur %	3	132.2	0.041	c[f]		
Sur MFI	1	64.1	NA			
	2	66.4	0.034	a		
	3	56.8	0.029	a	0.041	b
CD4: Sur MFI	1	49.8	NA	a		
	2	69.7	0.046	a		
c MFI	1	48.4	NA	a		
	2	56.0	0.032	a		
CD8: Sur %	1	85.2	NA			
	3	169.0	0.041	a	0.096	b
Sur MFI	1	56.0	NA			
	2	71.4	0.014	a		
c %	3	397.6	0.063	c[g]		

[a] Percentage of lymphocytes expressing surface CD3, 4, or 8 (sur %); percentage expressing cytoplasmic CD3, 4, or 8 (c %), median fluorescence intensity (MFI) of cells expressing surface CD3, 4, or 8 (sur MFI), and MFI of cells expressing cCD3, 4, or 8 (c MFI).

[b] 1 = 3–5 h *ex vivo* (n = 1 experiment); 2 = 24–32 h *ex vivo* and 3 = 4–8 days *ex vivo* (n = 5 experiments for surface parameters; 4 for cytoplasmic parameters)

[c] Mean proportionate values are provided in relation to the *ex vivo* values

[d] *p* value determined using a paired Student *t*-test; nonapplicable (NA) when n = 1.

[e] Comparisons include to *ex vivo* (a), to unstimulated cells at time point 3 (b), time point 3 value compared with time point 2 value (c).

[f] At time 2, = 103.1%.

[g] At time 2, = 120.9%.

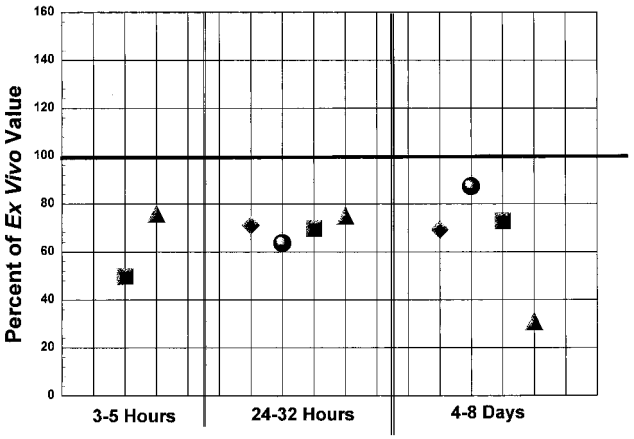


FIGURE 2 Surface CD4 median fluorescence intensity, by culture condition and time, relative to the first value for Resting lymphocytes, expressed as a percentage of the *ex vivo* value (see *Materials and Methods*). Nonstimulated human peripheral blood mononuclear cells (diamonds; $n = 1$ at 24–32 h and $n = 6$ at 4–8 days), PHA-stimulated cells (circles; $n = 5$ at 24–32 hours and $n = 6$ at 4–8 days), Con A–stimulated cells (squares) and αCD3-treated cells (triangles) (n's = 1 at 3–5 h; n's = 5 at 24–32 h and at 4–8 days).

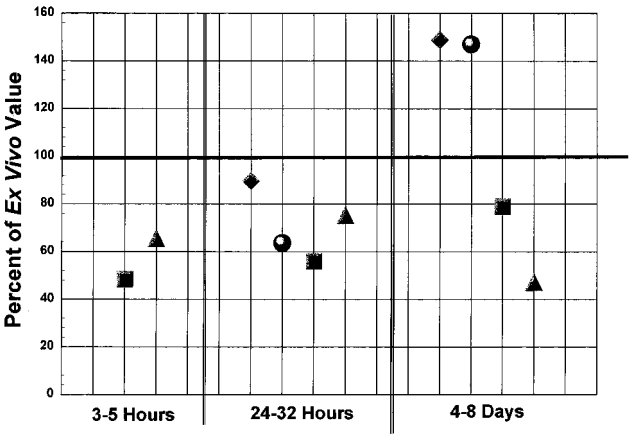


FIGURE 3 Cytoplasmic CD4 median fluorescence intensity, by culture condition and time, relative to the first value for Resting lymphocytes, expressed as a percentage of the *ex vivo* value (see *Materials and Methods*). Nonstimulated human peripheral blood mononuclear cells (diamonds; $n = 1$ at 24–32 hours and $n = 5$ at 4–8 days), PHA-stimulated cells (circles; $n = 4$ at 24–32 hours and $n = 5$ at 4–8 days), Con A–stimulated cells (squares) and αCD3-treated cells (triangles) (n's = 1 at 3–5 h; n's = 4 at 24–32 h and at 4–8 days).

in association with PMA and/or BFA treatments was associated with increases in cCD3% (PMA/BFA: +26%) and cCD3 MFI (Fig. 6B) and decreases in surCD8% (Fig. 8A) and surCD8 MFI (Fig. 8B).

DISCUSSION

The TCR, CD4, and CD8 are all associated with $p56^{lck}$, a cytoplasmic, membrane-associated 56 kDa protein tyrosine kinase [17]. In normal human PBMCs, CD4 undergoes a low rate of constitutive endocytosis, which is inversely proportional to $p56^{lck}$ expression [21]. This endocytosis requires at least either a pair of leucine residues and/or a neighboring phosphorylated serine residue [22], which is present on both CD4 and CD3 [12]. It has been suggested that with phosphorylation, unlike CD3 and CD4, CD8 does not undergo dissociation from $p56^{lck}$ and/or endocytosis [9, 14, 23]. However, others suggest that although CD8 is normally present on the outer cell membrane, when cross linking occurs, it can become protoplasmic, like CD4 [16].

We assessed three mitogens known to interact with the human TCR in regard to their effects on surface and cytoplasmic CD3, CD4, and CD8 expression. We examined the CD3-related effects more fully and to determine if there were any additional effects on coreceptor molecules. We also examined the effects of secondary PMA stimulation [9, 14, 17] and/or pre-Golgi blocking with BFA [24] to assess whether phosphorylation/activation was complete with the primary stimulation and to assist in determining the location of molecules lost from the cell surface.

We found that with αCD3 almost all CD3 was immediately blocked or lost from the cell surface. This effect was not complete, however. Long after initial stimulation, the addition of PMA led to additional surface declines and cytoplasmic increases. Further, the effect appeared to be greater in CD4+ lymphocytes, supporting the possibility of an association between CD3 and CD4 in activated cells [17, 20] and being perhaps at variance with a report that CD4+ and CD8+ lymphocytes respond similarly to αCD3 [7]. The addition of BFA led to increases in both surface and cytoplasmic CD3 expression, suggesting that with αCD3, the primary mechanism of CD3 depletion, other than masking, was endocytosis and not shedding, as had been suggested in a very early murine study and in a recent clinical study [1, 25]. An additional effect—not, to our knowledge, previously reported—was the very gradual loss of cytoplasmic CD3. This effect may be due to some form of intrinsic regulation of CD3 production, perhaps related to the inability of endoplasmic reticulum–located CD3 to be recirculated to the surface if the αCD3 had affected the expression of the ϵ chain [26]. Culture with αCD3 also led to a decreased expression of both surface and cytoplasmic CD4 and CD8, with little additional effect associated with BFA. This suggests that long-term decreased expression of CD3 may influence the expression of CD4 [9] and CD8. This effect on coreceptors may serve a self-regulatory function [11] and cannot be solely

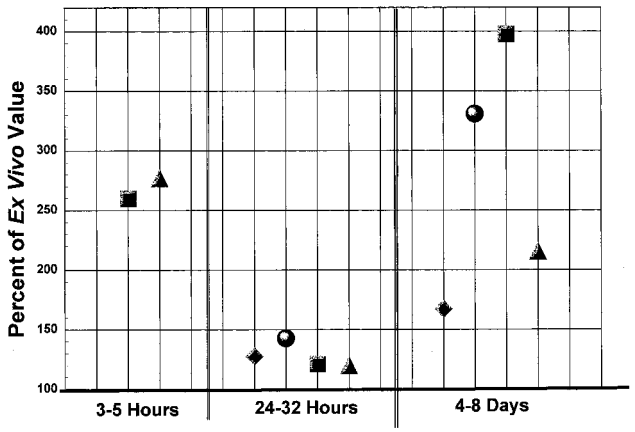
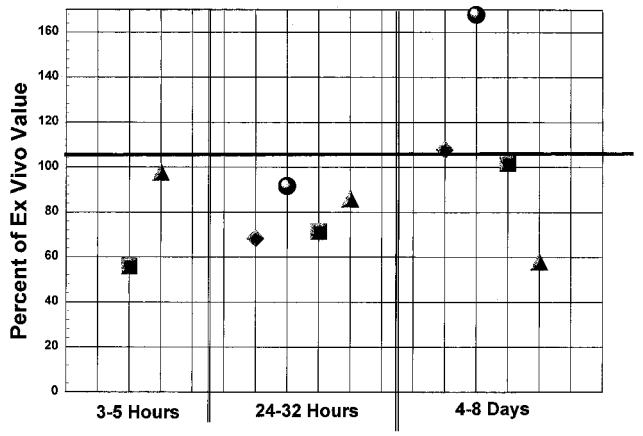


FIGURE 4 Proportion of lymphocytes expressing cytoplasmic CD8 by culture condition and time, relative to the first value for Resting lymphocytes, expressed as a percentage of the *ex vivo* value (see *Materials and Methods*). Nonstimulated human peripheral blood mononuclear cells (diamonds; $n = 1$ at 24–32 h and $n = 5$ at 4–8 days), PHA-stimulated cells (circles; $n = 4$ at 24–32 h and $n = 5$ at 4–8 days), Con A–stimulated cells (squares) and αCD3-treated cells (triangles) (n's = 1 at 3–5 h; n's = 4 at 24–32 hours and at 4–8 days).

due to the shared presence of paired leucine motifs, since these are present in CD3 and CD4 [12] but are not in CD8 (personal communication, M. Marsh, 1999).

As with αCD3, with PHA, a latent effect was found on CD8 expression; however, in this case there was an increased—not decreased—surface and cytoplasmic expression after 4 to 8 days of culture, enhanced by the presence of PMA with BFA. Also unlike αCD3, PHA had minimal effect on CD4 or CD3, findings consistent with 1 previous study [27]. These differences between PHA and αCD3 may be related to 1 or both of 2 characteristics of PHA stimulation that differ from that of αCD3: (a) its microtubule-associated protein 2 kinase activation is Ca^{2+} and protein kinase C-independent [15]

FIGURE 5 Surface CD8 median fluorescence intensity, by culture condition and time, relative to the first value for Resting lymphocytes, expressed as a percentage of the *ex vivo* value (see *Methods*). Nonstimulated human peripheral blood mononuclear cells (diamonds; $n = 1$ at 24–32 h and $n = 6$ at 4–8 days), PHA-stimulated cells (circles; $n = 5$ at 24–32 h and $n = 6$ at 4–8 days), Con A–stimulated cells (squares) and αCD3-treated cells (triangles) (n's = 1 at 3–5 h; n's = 5 at 24–32 h and at 4–8 days).



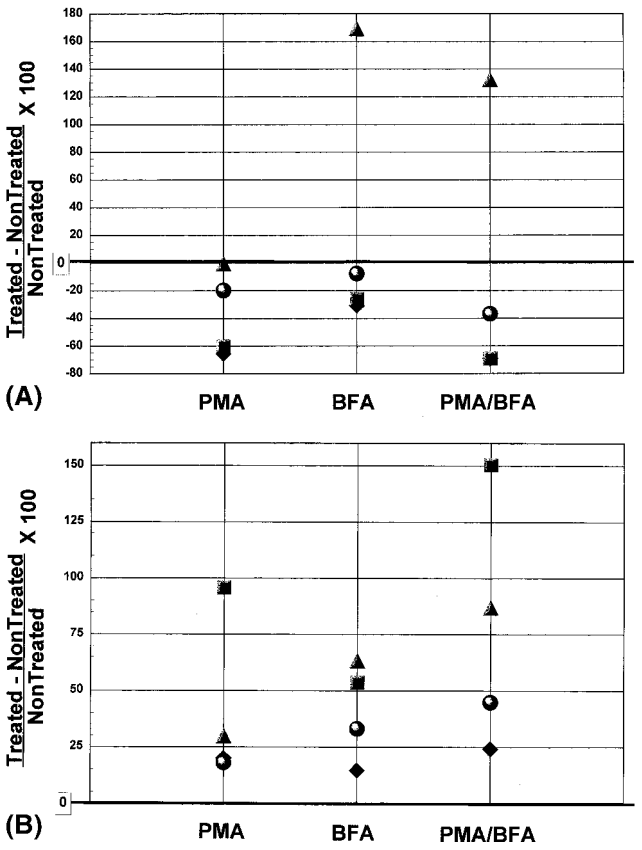


FIGURE 6 (A) Mean proportionate difference in surface CD3 median fluoresence intensity, compared to cultures receiving no additional treatment, by culture group and treatment received. Phorbol ester 12-myristate 13-acetate (PMA), Brefeldin A (BFA), both, or neither added to cultures for the last 4 h of culture period. Values expressed as the percentage change from no treatment, for instance, (posttreatment value − nontreatment value) ÷ (nontreatment value) × 100 (see *Materials and Methods*). Nonstimulated human peripheral blood mononuclear cells (diamonds; $n = 2$), PHA-stimulated cells (circles; $n = 2$), Con A–stimulated cells (squares; $n = 1$), and αCD3-treated cells (triangles; $n = 1$). (B) Mean proportionate difference in cytoplasmic CD3 median fluorescence intensity, compared with cultures receiving no additional treatment, by culture group and treatment received. Phorbol ester 12-myristate 13-acetate (PMA), Brefeldin A (BFA), both, or neither added to cultures for the last 4 h of culture period. Values expressed as the percentage change from no treatment, for instance, (posttreatment value − nontreatment value) ÷ (nontreatment value) × 100 (see *Materials and Methods*). Nonstimulated human peripheral blood mononuclear cells (diamonds; $n = 2$), PHA-stimulated cells (circles; $n = 2$), Con A–stimulated cells (squares; $n = 1$), and αCD3-treated cells (triangles; $n = 1$).

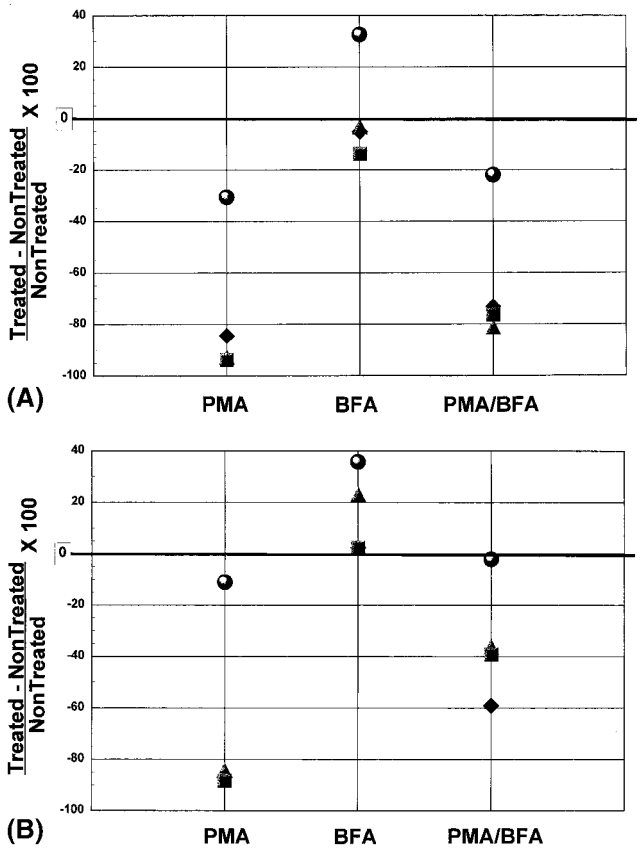


FIGURE 7 (A) Mean proportionate difference in surface CD4 median fluorescence intensity, compared with cultures receiving no additional treatment, by culture group and treatment received. Phorbol ester 12-myristate 13-acetate (PMA), Brefeldin A (BFA), both, or neither added to cultures for the last 4 h of culture period. Values expressed as the percentage change from no treatment, for instance, (posttreatment value − nontreatment value) ÷ (nontreatment value) × 100 (see *Materials and Methods*). Nonstimulated human peripheral blood mononuclear cells (diamonds; $n = 2$), PHA-stimulated cells (circles; $n = 2$), Con A–stimulated cells (squares; $n = 1$), and αCD3-treated cells (triangles; $n = 1$). (B) Mean proportionate difference in cytoplasmic CD4 median fluorescence intensity, compared with cultures receiving no additional treatment, by culture group and treatment received. Phorbol ester 12-myristate 13-acetate (PMA), Brefeldin A (BFA), both, or neither added to cultures for the last 4 h of culture period. Values expressed as the percentage change from no treatment, for instance, (posttreatment value − nontreatment value) ÷ (nontreatment value) × 100 (see *Materials and Methods*). Nonstimulated human peripheral blood mononuclear cells (diamonds; $n = 2$), PHA-stimulated cells (circles; $n = 2$), Con A–stimulated cells (squares; $n = 1$), and αCD3-treated cells (triangles; $n = 1$).

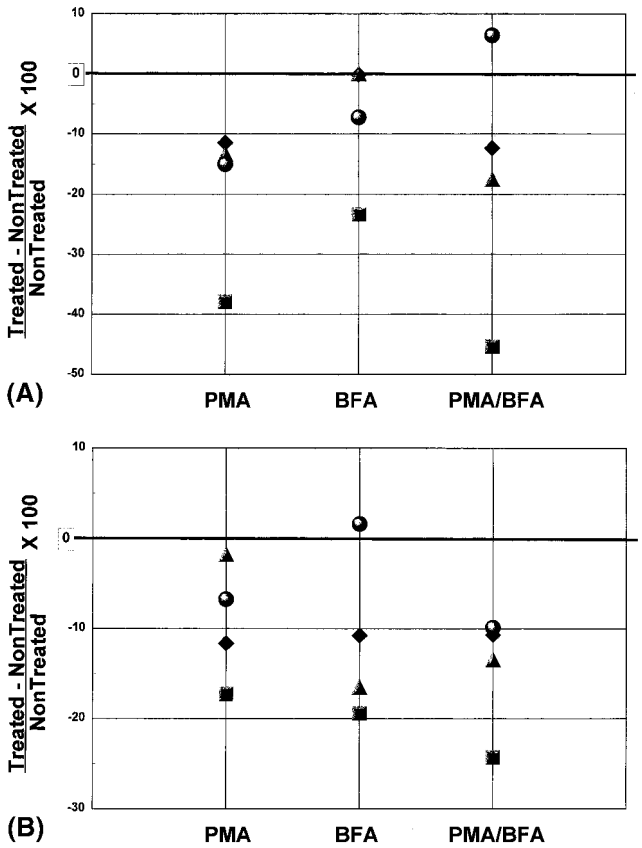


FIGURE 8 (A) Mean proportionate difference in percentage of lymphocytes expressing surface CD8 compared with cultures receiving no additional treatment, by culture group and treatment received. Phorbol ester 12-myristate 13-acetate (PMA), Brefeldin A (BFA), both, or neither added to cultures for the last 4 h of culture period. Values expressed as the percentage change from no treatment, for example, (posttreatment value − nontreatment value) ÷ (nontreatment value) × 100 (see *Materials and Methods*). Nonstimulated human peripheral blood mononuclear cells (diamonds; $n = 2$), PHA-stimulated cells (circles; $n = 2$), Con A–stimulated cells (squares; $n = 1$), and αCD3-treated cells (triangles; $n = 1$). (B) Mean proportionate difference in surface CD8 MFI compared with cultures receiving no additional treatment, by culture group and treatment received. Median fluorescence intensity of cells expressing CD4 (MFI). Phorbol ester 12-myristate 13-acetate (PMA), Brefeldin A (BFA), both, or neither added to cultures for the last 4 h of culture period. Values expressed as the percentage change from no treatment, for example, (posttreatment value − nontreatment value) ÷ (nontreatment value) × 100 (see *Materials and Methods*). Nonstimulated human peripheral blood mononuclear cells (diamonds; $n = 2$), PHA-stimulated cells (circles; $n = 2$), Con A–stimulated cells (squares; $n = 1$), and αCD3-treated cells (triangles; $n = 1$).

and (b) PHA interacts with the α/β or γ chains of the TCR, not with CD3 [3].

Unlike the more latent effects of PHA and αCD3 on CD4 and/or CD8, most of the effects of ConA were found at 3 to 5 h. As with αCD3, these effects included declines in both CD4 and CD8 as well as in CD3. There was a decrease in the expression of all three molecules, with the only concurrent cytoplasmic increase being of cCD8%. PMA and/or BFA were associated with an increase in cytoplasmic CD3 and a decrease in surface CD8,

suggesting that phosphorylation/activation was not complete for these 2 molecules and that overall declines in cytoplasmic CD3 and CD4 were due to degradation of molecules endocytosed, not to lack of production. As with αCD3 and PHA, these ConA results are at variance with Hoxie et al., who reported that these mitogens were not associated with CD4 endocytosis or declines in CD4 [27], but the early time point findings are consistent with those of other authors' reports of similarities between and blocking of αCD3 effects and ConA [4, 5]. The greater rapidity of effects on CD4 and CD8 might be related to the additional binding of ConA to CD2 and the TCR. The only notable longer-term effect with ConA was that as with PHA, the surface CD8 expression increased at 4 to 8 days. The similar effects of PHA and ConA on CD8 suggest that the second mechanism cited above, in relation to PHA, may be the more likely.

In summary, using this approach, we found that αCD3 had delayed effects on CD4 and CD8; PHA had delayed effects on CD8 only and in a direction opposite to that of αCD3; and ConA had very rapid effects on CD3, CD4, and CD8, as well as a delayed effect on surface CD8. These effects involve both surface and cytoplasmic antigen expression and are more consistent with degradation or retention, rather than with shedding or increased production. They have not been reported by others and may reflect direct interactions between CD4 or CD8 and CD3 and/or interregulation of CD3 expression and that of these coreceptor molecules.

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