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Differential Modulation of Surface and Intracellular Protein Expression by T Cells after Stimulation in the Presence of Monensin or Brefeldin A

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Intracellular cytokine staining is an increasingly popular analytical tool that can be used to define the profile of cytokines in various disease states. One important requirement for this assay is the inclusion of a protein transport inhibitor in stimulated cell cultures to trap the cytokine, thus allowing a brighter signal. Two compounds commonly used for this purpose are brefeldin A (BFA) and monensin (MN). Flow cytometry was used to assess the differential effects of BFA and MN on surface CD3, -4, -8, and -69 expression and the intracellular expression of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) following stimulation with phorbol myristate acetate and ionomycin. We found that BFA blocked the majority of $CD3^+$ cells from expressing surface CD69, but BFA did not inhibit intracellular CD69 expression. MN did not significantly inhibit surface CD69 expression. With regard to lymphocyte marker expression following activation, surface CD4 expression was significantly downregulated; however, less downregulation was observed with BFA treatment than with MN treatment. Analyzing intracellular cytokine expression, BFA trapped a greater percentage of TNF-α inside activated cells than MN. An analysis of the cytokine concentration in culture supernatants indicated that cells treated with MN released TNF-α and IFN-γ from the cells, while the BFA-treated cells released IFN-γ only. With prolonged (18-h) stimulation, the cells treated with MN were less viable than those treated with BFA. We conclude that the choice of a protein transport inhibitor is an important variable in this assay. When developing this method as a tool for clinical immunology laboratory analysis, investigators should consider the differential effects of BFA and MN on results.

Methods for intracellular cytokine staining are becoming widely accepted as an analytical tool in the immunology laboratory. This staining technique allows the delineation of distinct cytokine-producing leukocyte subsets within a mixed cell population. The methodology involves the fixation and permeabilization of the target cells for detection of intracellular cytokines by flow cytometry. This method was first proposed by Jung et al. (9) as a modification of a protocol that was originally designed for analysis using a fluorescence microscope (21). Further publications on this subject have resulted in reports addressing the kinetic aspects of cytokine expression (11) and the development of a whole-blood method (23). Using this methodology, a number of groups have detected imbalances in cytokine production in different disease states (4, 6, 26, 27).

A key aspect of intracellular cytokine detection is trapping the cytokine within the cell. Generally, unstimulated cells produce undetectable amounts of cytokine. Therefore, the cells must be stimulated; a popular choice for stimulation is the combination of phorbol myristate acetate (PMA) and ionomycin (ION), which induces rapid induction of many cytokines. Thus, early quantification (i.e., at 2 to 4 h) of the number of cells expressing a cytokine and determination of the relative amount of cytokine per cell can be made. A protein transport inhibitor is added to the cultures to prevent the release of cytokines from the cells. Two commonly used compounds are monensin (MN) and brefeldin A (BFA). MN is derived from *Streptomyces cinnamonensis* and is an Na^+ ionophore that disrupts intracellular Na^+ and H^+ gradients, exerting its greatest effects on the regions of the Golgi apparatus that are associated with the final stages of secretory vesicle maturation (13; E. Chu, J. Elia, D. Sehy, D. Ernst, and C. Shih, Hotlines [Pharmingen] **3:**9–10, 1997). BFA is a macrocyclic lactone that is produced by a variety of fungi and is synthesized from palmitate. BFA was originally isolated from *Penicillium brefeldianum* as described by Dinter and Berger (7) and appears to inhibit protein secretion early in a pre-Golgi compartment (between the endoplasmic reticulum and Golgi). The mechanism of this action is complicated and is best explained by Dinter and Berger in their review of Golgi-disturbing agents (7). In a review of the literature addressing intracellular staining, we found MN to be the most common choice for the protein transport inhibitor. Differences related to the choice of protein transport inhibitor used for intracellular cytokine staining have been previously reported for the measurement of intracellular cytokine production by $CD4^+$ mouse splenocytes after either anti-CD3–anti-CD28 or PMA-ION stimulation (Chu et al., Hotlines [Pharmingen], 1997). Nylander and Kalies (14) assessed the effects of BFA and MN on viability, intracellular IFN-γ expression, and surface CD69 expression in $CD4^+$ mouse splenocytes and reported BFA both to have lower cytotoxicity than MN and to be more effective in blocking CD69 surface expression. Recently, it was also shown that the expression of specific cytokines in cultures of isolated monocytes could be altered depending on the protein transport inhibitor used (22). The present report addresses differences observed

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between MN and BFA treatments when intracellular cytokine (e.g., tumor necrosis factor alpha [TNF-α] and gamma interferon [IFN-γ]) production, lymphocyte surface marker (CD3, -4, and -8) expression, and expression of the activation marker CD69 were analyzed following PMA-ION stimulation in a human whole-blood culture system. The report addresses the suggested basis for these differences.

MATERIALS AND METHODS

Specimen collection. Blood samples were obtained from healthy volunteers who gave informed consent. The number of donors totaled 12; 8 were female (average age, 46 years; range, 23 to 57 years), and 4 were male (average age, 50 years; range, 40 to 61 years). Not all specimens were used in all analyses. This study was approved under New York State Department of Health IRB Study 98-1-08.

Materials. The antibodies used for surface and intracellular staining were obtained either from BD Pharmingen (San Diego, Calif.) as fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC) conjugates or from BD Immunocytometry Systems (San Jose, Calif.) as a peridinin chlorophyll protein (PerCP) conjugate. The FACSLyse (commercial fixative solution) used to fix cells after surface staining was obtained from BD Immunocytometry Systems. The lysing buffer used for viability assessment (PharmLyse) and the reagents used in the intracellular staining protocol for cell fixation (Cytofix/Cytoperm) and permeabilization (PermWash) were obtained from BD Pharmingen. Sodium heparin Vacutainers used for blood collection were obtained from Becton Dickinson (Franklin, N.J.). MN, BFA, PMA, ION, and propidium iodide (PI) were obtained from Sigma (St. Louis, Mo.). Medium (RPMI 1640) was obtained from BioWhittaker. The OptEIA enzyme-linked immunosorbent assay (ELISA) kits used to determine cytokine concentrations were obtained from BD Pharmingen.

Cell culture. A whole-blood culture system was used as previously described (23). Briefly, whole, heparinized (sodium heparin) blood was diluted 1:2 with RPMI 1640 (1 part blood to 2 parts medium), and aliquots (2 ml) were aseptically pipetted into 13-ml tubes. A total of six tubes were required per individual assayed; three tubes were left unstimulated, and the remaining three tubes were stimulated with PMA (50 ng/ml) and ION (1 μM). Of the three stimulated tubes, one was treated with BFA (10 μg/ml, or approximately 35.7 μM), one was treated with MN (2 μM), and one was left untreated. Surface CD69 expression, cytokine amounts in the culture supernatants, and viability were assessed after 2, 4, and 18 h of culture. The intracellular expression of CD69, IFN-γ, and TNF-α and the surface expression of lymphocyte markers (CD3, -4, and -8) were assessed following 4 h of culture. All cell cultures were incubated for the prescribed periods at 37°C, 5% CO_2, with and without protein transport inhibitors.

Viability analysis. The viability of lymphocytes was assessed by PI incorporation ($n = 5$). From each culture tube, 300 μl of blood-medium was transferred to a Falcon tube (12 by 75 mm; BD Falcon, Bedford, Mass.). Two milliliters of a 1× PharmLyse solution was added to each tube, and the red blood cells were lysed for 10 min. The cells were centrifuged for 10 min at 400 × *g*, washed with phosphate-buffered saline (PBS), and resuspended in 0.5 ml of PBS; 10 μl of a 0.5-mg/ml solution of PI was added to the cells. The cells were incubated for 5 min at room temperature and then kept on ice. Specimens were analyzed using the flow cytometer within 20 min of staining.

Intracellular cytokine staining. Following 4 h of PMA-ION stimulation, cell aliquots ($n = 7$) were surface stained with a combination of FITC-conjugated CD3, PerCP-conjugated CD4, and APC-conjugated CD8, fixed with 2 ml of a 1× FACSLyse solution, and stored overnight in fixative at 4°C. The following day, the fixed cells were centrifuged at 500 × *g* for 10 min, washed with 1 ml of staining buffer (3% heat-inactivated FBS in PBS with 0.1% sodium azide), and resuspended in 600 μl of staining buffer; 200-μl aliquots were then dispensed in triplicate into a 96-well round-bottom plate. The plates were centrifuged as described earlier, and intracellular staining for cytokines was performed via a slightly modified version of the protocol supplied by BD Pharmingen (supplied in their intracellular cytokine staining kit). Briefly, cells were treated with Cytofix/Cytoperm Buffer (for permeabilization) and washed with a 1× PermWash solution following each step in the procedure. For intracellular cytokine staining, one of the following antibodies was added per well for each set of triplicate wells: mouse immunoglobulin G1 (IgG1) isotype control (clone MOPC-21), mouse anti-IFN-γ (clone 4S.B3), or mouse anti-TNF-α (clone MAb11).

CD69 staining. For assessing surface CD69 expression ($n = 5$), cells were stained with CD3-PerCP and either FITC-conjugated mouse IgG1 (isotype control) or CD69-FITC and then fixed and stored overnight as previously described. Specimens not used to assess intracellular CD69 (from the 2- and 18-h time points) were washed once and resuspended in staining buffer prior to analysis. Specimens that were evaluated for intracellular CD69 (4-h time point; $n = 5$) were permeabilized, split into two aliquots, and stained intracellularly with either PE-conjugated mouse IgG1 (isotype control) or CD69-PE. All staining was performed as described earlier for the intracellular cytokines; following staining, the cells were resuspended in 0.5 ml of staining buffer and analyzed.

Flow cytometric analysis. The specimens were analyzed on a BD FACSCalibur using CellQuest software. Intracellular and surface CD69 was analyzed by gating on the $CD3^+$ lymphocytes in a CD3-versus-side scatter (SSC) dot plot. One-dimensional histograms of surface and intracellular CD69 expression were based on the $CD3^+$ gate. Histogram markers were set based on the isotype controls in conjunction with the unstimulated controls.

Surface marker expression was analyzed by first drawing a gate around the lymphocytes in a forward scatter-versus-SSC dot plot; a dot plot was drawn of CD3 versus SSC based on the lymph gate. A second gate was drawn around the $CD3^+$ lymphocytes in the CD3-versus-SSC plot. To analyze CD3 expression, a histogram was created gated on the lymph gate; to analyze CD4 and CD8 expression, histograms were gated on the $CD3^+$ gate. Markers used to assess a shift in fluorescence of the surface markers were set based on the unstimulated (control) cultures.

To analyze intracellular cytokine expression by $CD4^+$ and $CD8^+$ lymphocytes, a gate was first drawn around the lymphocytes in a dot plot of forward scatter versus SSC. A dot plot of CD3 versus SSC was drawn based on the lymph gate, and a second gate was drawn around the $CD3^+$ cells. Dot plots based on the $CD3^+$ gate were created to analyze either CD4 or CD8 versus the PE-conjugated antibody (either the isotype control, IFN-γ, or TNF-α). Quadrant markers were set according to both the unstimulated control cells and the isotype control. The percent of cytokine expressed by the lymphocyte subsets was computed using the following formula based on the percentage of gated values given for the double positive cells: (stimulated − stimulated isotype control) − (unstimulated − unstimulated isotype control).

Quantification of cytokines by ELISA. Culture supernatants ($n = 5$) from all treatment groups were saved following 2, 4, and 18 h of incubation, frozen at −80°C until analysis, and then analyzed by ELISA. The culture supernatants were analyzed for IFN-γ and TNF-α according to kit instructions. Standard curves for the cytokines employed 300, 150, 75, 37, 18, 9, and 5 pg/ml for IFN-γ and 500, 250, 125, 63, 31, 15, and 7 pg/ml for TNF-α. The plates were analyzed using a BIO-TEK series UV900C plate reader, and data reduction was performed with KC jr. software (BIO-TEK).

Statistical analysis of results. The results from the flow cytometric data were analyzed for statistically significant differences using SigmaStat software (version 2.0). Surface expression of CD3, CD4, CD8, and CD69 among the treatment groups (unstimulated [UNS]; stimulated, [STM]; stimulated, brefeldin A-treated [STM-BFA]; and stimulated, monensin-treated [STM-MN]) were analyzed using one-way analysis of variance. Statistically significant results were identified by a P value of <0.05. The expression of intracellular cytokines was analyzed using Student's t test; BFA- and MN-treated groups were compared to ascertain whether the observed differences in cytokine expression were significant.

RESULTS

Analysis of CD69 expression. The results for the surface and intracellular expression of CD69, an early activation marker, were used to assess any differential effects between MN and BFA. However, prior to analysis of the expression and translocation of CD69 after activation, we evaluated the effects of fixation and permeabilization on the assessment of CD69. The same anti-CD69 (FITC conjugate) was used to ensure there were no differences based on antibody specificity. Activated cells were treated in three different ways: cells were surface stained for CD69 and CD3 (PerCP conjugate) and fixed; cells were fixed and then stained with anti-CD69 and anti-CD3; and cells were surface-stained with anti-CD3 and anti-CD69, fixed, permeabilized, and stained intracellularly for CD69. All treated cells were analyzed by flow cytometry. Following activation, the cells had increased surface expression of CD69, but no significant changes in CD3 expression. Overnight fixation

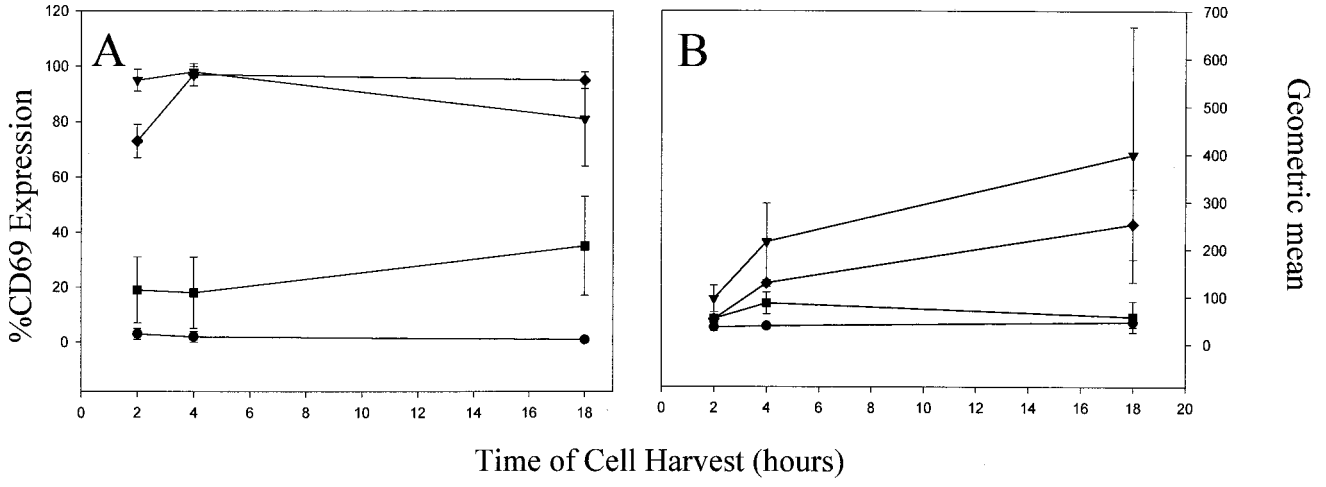

FIG. 1. The kinetics of surface CD69 expression by $CD3^+$ cells following PMA-ION stimulation for the percentage of cells expressing CD69 (A) and the fluorescence intensity of the $CD69^+$ cells (B) in UNS (●), STM (▼), STM-BFA (■), or STM-MN (◆) cultures. The values reported here are representative of five separate analyses (error bars, standard deviations).

prior to surface staining eliminated anti-CD69 but not anti-CD3 binding; however, CD3 antibody has been known to be able to bind to epitopes on fixed cells (BD Biosciences Application Note, Detecting intracellular cytokines in activated lymphocytes, 1999). Cells surface stained with anti-CD69 and then fixed had no change in either the percentage of cells positive for surface CD69 or the geometric mean fluorescent intensity (GM) between the stained, nonpermeabilized (positive, 93% ± 8%; GM = 74 ± 9) and the stained, permeabilized (positive = 93% ± 9%; GM = 77 ± 9) cells (unless otherwise noted, values are presented as means ± standard deviations).

The kinetic analysis of CD69 expression on the surface of $CD3^+$ cells showed that cells from both the STM and STM-MN groups had an early, high percentage of CD69 expression; this high percentage of expression was observed at all measured time points and was equivalent between the two groups (Fig. 1A). However, this level of expression was not observed for the STM-BFA cell group; the percentage of CD69 seen on the surface of the STM-BFA cells was only one-third of the percentage of CD69 on the surface of STM-MN cells following 18 h of incubation (35% ± 18% versus 95% ± 3%; $P < 0.05$). In contrast to the percentages, the GMs for surface CD69 expression showed that the amount of CD69 on the cells of the STM group differed significantly ($P < 0.05$) from all other groups with the exception of the STM-MN group at the 18-h time point. The GM values of the STM-BFA and STM-MN cell groups were not significantly different at any time point, even though differences between the values at 18 h appear large (58 ± 33 versus 253 ± 74 [Fig. 1B]). The GM of the cells in the STM-MN group differed from those in the UNS group at the 18-h time point only, while the GMs of the UNS and STM-BFA groups did not differ at any time point.

To evaluate the ratio of surface to intracellular expression of CD69, dual analysis of surface and intracellular CD69 following 4 h of activation was performed. In Fig. 2, it can be seen that both the percentage and amount of CD69 per cell were expressed to a greater extent on the cell surface for the STM and STM-MN cell groups (Fig. 2D and F) than were expressed intracellularly (Fig. 2A and C), while the opposite result was true for the STM-BFA cell group (Fig. 2B and E). Pairwise comparisons of the stimulated groups showed that while the percentages of the STM and STM-MN groups were significantly different ($P < 0.05$) from the STM-BFA group for both surface and intracellular expression, they did not differ from each other. However, the degree of expression per cell (GM) was significantly different ($P < 0.05$) in pairwise comparisons of all groups.

Effect of protein transport inhibitors on lymphocyte surface markers. Lymphocyte activation with PMA-ION has been reported to result in the downregulation of lymphocyte surface marker expression on both mouse and human T cells, particularly for CD4 expression (2, 18). As expected, in our analyses CD4 expression did decrease following activation. The expression of CD3 and CD8 decreased as well, although not to the degree observed with CD4. To detect differences in surface marker expression among the different treatment groups, one-way analysis of variance analysis was performed; for all significant differences, P values were <0.05.

Surface CD4 expression in the STM-BFA and STM-MN cell groups (Fig. 3E and F) was downregulated less than that in the STM group (Fig. 3D). The percentage of cells expressing CD4 in the UNS group (63% ± 8%) did not differ from that in the STM-BFA group (65% ± 5%), while the percentages in the STM-MN group (39% ± 10%) and the STM group (20% ± 17%) were significantly different from those of both the UNS and STM-BFA groups, as well as each other. However, the GMs of the STM (34 ± 10), STM-BFA (62 ± 18), and STM-MN (42 ± 8) cell groups, while significantly different from those of the UNS group (129 ± 41), did not differ from each other. Percentages of the CD8 marker did not differ among the different groups (Fig. 3G to I), while pairwise comparisons of the GMs showed significant differences between the UNS (2,013 ± 255) and the STM-BFA (1,684 ± 214) groups and between the UNS and STM-MN (1,615 ± 152) groups but not between the UNS and STM (1,756 ± 215)

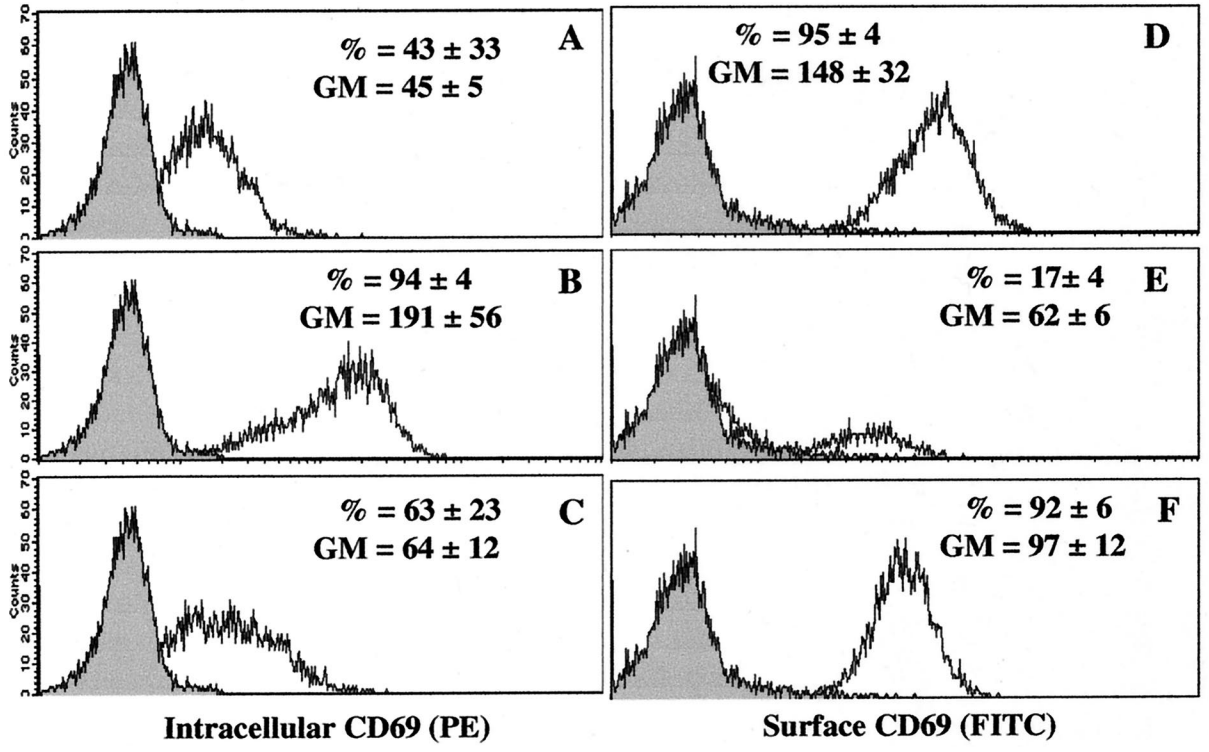

FIG. 2. Intracellular (A to C) and extracellular or surface (D to F) CD69 expression by $CD3^+$ cells after 4 h of stimulation with PMA-ION. Cells were treated three ways: STM (A and D), STM-BFA (B and E), and STM-MN (C and F). For each histogram, % is the percentage of cells expressing CD69, and GM is the geometric mean of CD69 expression (fluorescence intensity). The histograms show results for one individual that are representative of all analyses performed ($n = 5$), and results are reported as the mean ± standard deviation. Shaded areas represent the unstimulated control; unshaded areas represent the activated cultures.

groups. The GMs of the stimulated groups did not differ significantly in comparison to each other. For CD3 expression (Fig. 3A to C), the percentages expressed by the STM-BFA cells (62% ± 7%) and the STM cells (56% ± 3%) differed significantly from that expressed by the UNS cells (72% ± 5%), while CD3 expression in the STM-MN cells (67% ± 4%) did not. Expression by the STM-MN cell group also differed significantly from the STM cells but not from the STM-BFA cells. Analyzing GMs for CD3 expression, again the STM-BFA (128 ± 28) and the STM (101 ± 33) cell groups differed from the UNS cells (194 ±36), while the STM-MN cells (132 ± 35) did not. The GMs of the stimulated cell groups did not differ when compared among each other.

Cytokine expression by lymphocyte subsets. The difficulty encountered by researchers analyzing intracellular cytokine expression by the CD4 subset is a direct result of the degree to which this marker is downregulated by PMA-ION stimulation. To circumvent this problem, various approaches have been suggested, including the analysis of $CD8^-$ cells instead of $CD4^+$ cells (19), using a purified CD4 population (16) or using a specific CD4 monoclonal antibody (8). To obtain accurate values that would allow us to fairly compare the results of intracellular cytokine expression between the STM-BFA and STM-MN cell groups, we were required to use the $CD8^-$ subset rather than the $CD4^+$ subset because of the difficulty encountered in trying to separate $CD4^-$ cells from $CD4^+$ cells. This difficulty is depicted in the plots shown in Fig. 4; while there are two peaks representing the $CD4^+$ and $CD4^-$ subsets in the STM-BFA plot (Fig. 4A), only one peak is observed in the STM-MN plot (Fig. 4B). Originally, we analyzed intracellular cytokine expression by the CD3 subsets following 2 h of incubation; the differences observed between TNF-α and IFN-γ expression were not significant (data not shown). However, 4 h of PMA-ION stimulation, a common activation period when assessing intracellular cytokine expression, resulted in significant differences in both the percentage and amount of intracellular TNF-α expression, but not IFN-γ expression, between the STM-MN and the STM-BFA cell groups (Table 1). For all significantly different results, the expression of cytokine in the STM-BFA cells was greater than that in the STM-MN cells ($n = 7$).

To investigate possible reasons for the observed difference in cytokine expression between the cell groups, we assessed the amount of cytokine in supernatants collected from the various treatment groups. Supernatants were collected following 2, 4, and 18 h of culture. As shown in Table 2, TNF-α could not be detected in the supernatants from the STM-BFA group at any time point, while both the STM and STM-MN groups contained detectable amounts. A detectable amount of IFN-γ was measured in some supernatants from the STM-BFA group, but only after 18 h.

Viability analysis. The suggestion has been made that BFA is less toxic to cells than MN is (14). We analyzed the uptake of PI, a DNA intercalating dye which binds to the DNA of cells

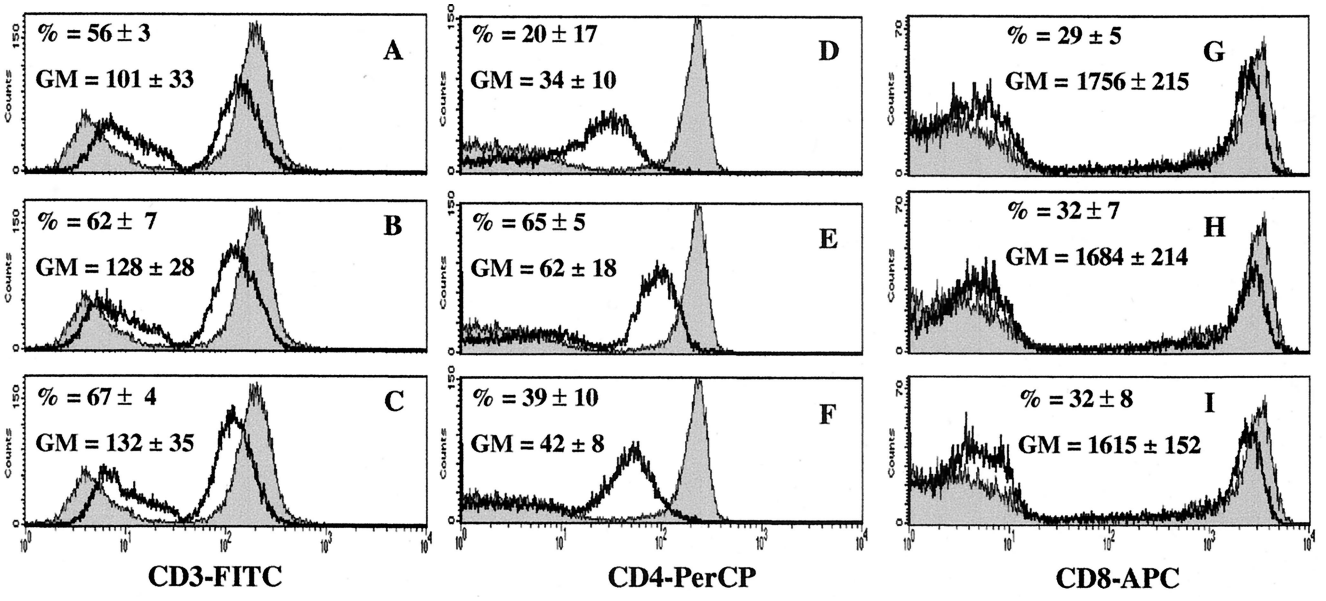

FIG. 3. Analysis of lymphocyte surface markers. Whole-blood cell cultures were stimulated with PMA-ION alone (A, D, and G), with BFA (B, E, and H), or with MN (C, F, and I) and then surface stained with CD3-FITC, CD4-PerCP, and CD8-APC. The shaded areas represent the unstimulated (control) cells. The histograms are from one individual and are representative of all analyses performed. The results are for the percentage of cells positive for a surface marker and the geometric mean of fluorescence intensity of the positive cells, reported as the mean ± standard deviation ($n = 7$).

whose membranes no longer provide an impermeable barrier ($n = 5$); results are shown in Table 3. After prolonged (18-h) stimulation, the viability of the STM-MN cell group differed significantly ($P < 0.05$) from the UNS (control) group, while that of the STM-BFA cell group did not. A visual inspection of the cultures showed a greater degree of red blood cell lysis evident in the STM-MN group than in the STM-BFA group.

DISCUSSION

In this report, two protein transport inhibitors commonly used in intracellular cytokine staining (BFA and MN) were compared with regard to expression of CD69 (an activation marker), CD3, CD4, and CD8 (lymphocyte surface markers), and intracellular expression of cytokines (IFN-γ and TNF-α). Unstimulated cells and cells stimulated with PMA and ION with or without treatment with either BFA or MN were assayed by flow cytometry. Significant differences among groups were obtained with the different protein transport inhibitors.

Both protein transport inhibitors aided in the retention of cytokines within the cell; however, there were differences in the amount of cytokine retained between groups treated with either BFA or MN. Previous publications have reported differences in CD69 and cytokine expression between cultures treated with BFA or MN [14, 22; Chu et al., Hotlines (Pharmingen), 1997], but these results were obtained using cultures of isolated $CD4^+$ mouse splenocytes or human monocytes. Herein, we investigated whether these observations applied to whole-blood cultures containing multiple human lymphocyte subsets as well as whether there were differential effects on the expression of surface markers and cytokines by $CD4^+$ and $CD8^+$ cells.

The effect of protein transport inhibitors on CD69 expression. Although MN and BFA differentially blocked the expression of surface CD69 after cell activation, neither BFA nor MN inhibited protein synthesis based on intracellular CD69 expression. However, it is possible there could be differential effects on the expression of other proteins. Dual staining of lymphocytes with CD69 labeled with two fluors (FITC and PE) dem-

TABLE 1. Cytokine expression by T cells following PMA-ION stimulation[a]

Cytokine	T-cell subset	% Cytokine-positive cells (mean ± SD)		P^c	GM (mean ± SD)[b]		P
		STM-BFA	STM-MN		STM-BFA	STM-MN	
TNF-α	$CD8^-$	15 ± 5	8 ± 5	0.025	66 ± 27	28 ± 12	0.004
	$CD8^+$	13 ± 4	5 ± 2	<0.001	130 ± 44	24 ± 14	<0.001
IFN-γ	$CD8^-$	8 ± 4	7 ± 4	NS	120 ± 73	123 ± 59	NS
	$CD8^+$	14 ± 5	15 ± 8	NS	114 ± 67	111 ± 45	NS

[a] Cell-associated cytokine expression was quantified by flow cytometry assay ($n = 7$) as described in Materials and Methods.
[b] GM of fluorescence intensity.
[c] The statistical significance between the STM-BFA and the STM-MN groups. NS, not significant.

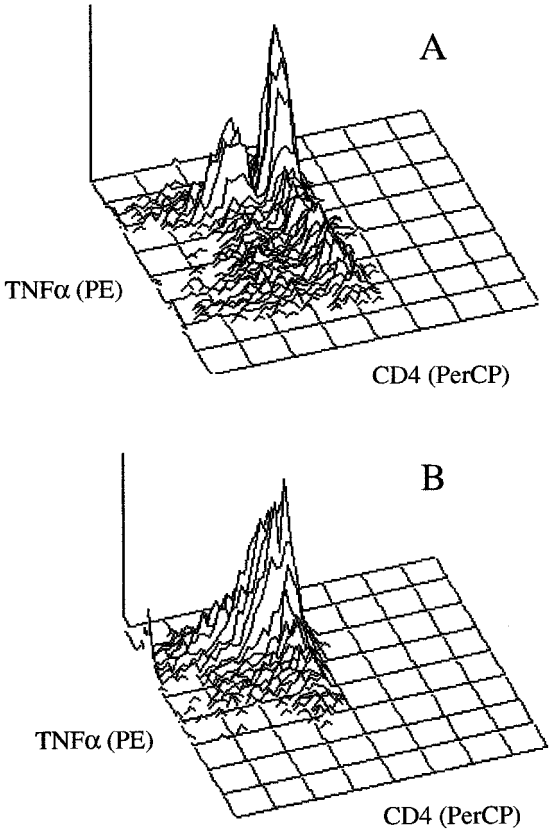

FIG. 4. TNF-α expression in the CD4 T-lymphocyte subset for BFA-treated (A) or MN-treated (B) cells following 4 h of PMA-ION stimulation depicted by three-dimensional dot plots. The dot plots are from one individual and are representative of all analyses performed ($n = 7$).

onstrated BFA produced a more effective blockage of surface CD69 expression than MN; there was a greater amount of CD69 intracellularly in the BFA-treated cells than on the surface of the cells, while the opposite was true for the STM-MN cell group (Fig. 2).

Analysis of CD69 kinetics indicated that while the percentages of surface $CD69^+$ cells for the STM and STM-MN cells were equivalent, the amounts of surface CD69 expressed on the STM-MN-treated cells were significantly lower than those on the STM cells. Two conclusions can be drawn from these results: either MN-treated cells have a lesser amount of surface CD69 because of an overall inhibitory effect on the transport of proteins to the surface, or the CD69 is being surface expressed in an altered nonimmunoreactive form. Because CD69 is a type II transmembrane glycoprotein, evidence for these possibilities can be drawn from other experiments using MN to block protein secretion. Kubo and Pigeon (10) demonstrated that in the presence of MN there were alterations in the intracellular processing of proteins in Daudi cells. Specifically, the terminal glycosylation of surface IgM was affected. However, this alteration did not affect the surface expression of the altered form of IgM. Another study (15) analyzed the effect of MN on the glycosylation of α_1-protease inhibitor in cultured rat hepatocytes. The researchers found that the conversion of α_1-protease inhibitor to its final form was blocked in the presence of MN and that there was evidence for the existence of an intermediately processed form of the protein.

The results of the kinetic experiment showed that the STM-BFA cells expressed greater percentages of surface CD69 with increasing incubation time. The experimental results corroborated previously published work demonstrating that BFA induces a delay in the expression of glycosylated proteins. Using cultured rat hepatocytes to examine the glycosylation of α_1-protease inhibitor, Misumi et al. (12) reported the glycosylation process to be delayed by BFA; however, unlike the altered form of α_1-protease inhibitor produced in the presence of MN, a fully glycosylated, unaltered protein was eventually expressed in the presence of BFA.

Our data, in conjunction with previous data, suggest the observed difference between STM and STM-MN surface CD69 expression after activation may be due to the expression of two structurally different forms of the same protein. MN may alter the glycosylation of the CD69 protein; however, unlike the effects of fixation, the glycosylation differences did not substantially inhibit recognition by the antibody. The expression of surface CD69 differed in the STM-BFA cells because of a delay in the processing of the glycosylated protein, a suggestion supported by the data shown in Fig. 2. For the STM-BFA cell group, the amount of intracellular CD69 was threefold greater than the amount on the surface of the cells, while the opposite was true for the STM cultures. Adding together the intracellular and surface amounts of CD69 for these two cell groups, the total amount (GM) of CD69 in the two treatment groups was equivalent (STM-BFA, 253 ± 60; STM, 194 ± 35) differing only in the distribution of the protein.

The effect of protein transport inhibitors on lymphocyte surface marker expression. The activation of lymphocytes by phorbol esters has been shown to downregulate the expression of CD3, CD4, and CD8 on the cell surface (2, 18, 20). Our data

TABLE 2. Analysis of cytokine levels in cell culture supernatants[a]

Time (h)	Cytokine level (pg/ml) in cultures treated as indicated					
	TNF-α			IFN-γ		
	STM	STM-BFA	STM-MN	STM	STM-BFA	STM-MN
2	340 ± 447	ND[b]	220 ± 291	56 ± 96	ND	12 ± 15
4	395 ± 506	ND	374 ± 488	180 ± 189	ND	157 ± 195
18	1425 ± 635	ND	482 ± 369	282 ± 292	24 ± 33	206 ± 214

[a] The amount of TNF-α and IFN-γ was assessed ($n = 5$) following 2, 4, and 18 h of PMA-ION stimulation in STM, STM-BFA, and STM-MN cultures; cytokine levels in unstimulated control cultures were not detectable.
[b] ND, not detectable.

show similar results, with CD4 being the most severely downregulated marker (Fig. 3). The downregulation of surface CD4 can be initiated by PMA, which activates protein kinase C (PKC); this activation involves the translocation of PKC from the cytoplasm to the plasma membrane and leads to the phosphorylation of serine residues in the cytoplasmic tail of the CD4 molecule; the dissociation of the CD4-tyrosine kinase p56 *lck* complex; and an increase in the association of CD4 with clathrin-coated pits, which results in the downregulation of CD4 via endocytosis (17). Therefore, while the PMA-ION combination is a popular choice for inducing intracellular cytokine expression, analyzing whether the CD4 or the CD8 T-cell subset produces a particular cytokine is difficult due to the loss of the CD4 marker. In our study, we observed that the inclusion of a protein transport inhibitor during PMA-ION stimulation lessened the downregulation of CD4 to different degrees, dependent on the inhibitor used.

The results in Fig. 3 show the order of the degree of CD4 downregulation to be (from most to least) STM, STM-MN, and STM-BFA. The difference in CD4 expression between the BFA and MN groups, while not significant, affected the visual data: the $CD4^+$ and $CD4^-$ groups were distinguishable from each other in the STM-BFA plots, but not in the STM-MN plots (Fig. 4). Reasons for the differential effect of the inhibitors on CD4 expression likely relate to the mechanisms by which CD4 is downregulated. In the case of MN, Takeuchi et al. (25) have reported that MN accelerates the proteolytic degradation of PKC in HL60 cells treated with tetradecanoyl phorbol acetate (PMA), an inducer of PKC translocation leading to downregulation; the downregulation of PKC refers to the proteolytic degradation of PKC following its translocation from the cytosol to the plasma membrane. Takeuchi et al. (25) presented evidence showing the addition of MN to cells stimulated with PMA expedited PKC degradation. This increased rate of PKC degradation could result in the incomplete phosphorylation of the serine residues in the cytoplasmic tail, a necessary step for the downregulation of CD4. In our results, less phosphorylation in the STM-MN cells compared to that in the STM cells could account for the lesser degree of CD4 downregulation in the STM-MN cells compared to the STM cells.

Following the dissociation of the CD4-p56 *lck* complex, CD4 associates with clathrin-coated pits prior to endocytosis (17). Studying the association of GLUT-4 with endocytic clathrin-coated vesicles, Chakrabarti et al. (5) stated that BFA caused complete disassembly of clathrin lattices at the *trans*-Golgi network in 3T3-L1 adipocytes and led to a decrease in the amount of clathrin-coated vesicles that could be purified from the treated cells. Chakrabarti et al. (5) also found BFA treatment did not affect the number of clathrin-coated vesicles in the plasma membrane. The disruption of clathrin lattices in the *trans*-Golgi network could explain the decrease in CD4 downregulation observed in the STM-BFA cells compared to the STM cells; some downregulation of CD4 is posited to occur because of clathrin-coated vesicles available in the plasma membrane, but the degree of endocytosis normally seen in PMA-ION-treated lymphocytes may have been impaired by treatment with BFA because the formation of additional clathrin-coated vesicles is impeded.

TABLE 3. Viability of lymphocytes following PMA-ION stimulation[a]

Time (h)	% Viable cells			
	UNS	STM	STM-BFA	STM-MN
2	88 ± 3	84 ± 9	85 ± 9	74 ± 7[b]
4	77 ± 10	69 ± 7	77 ± 7	64 ± 14
18	77 ± 7	84 ± 5	69 ± 7[c]	56 ± 10[b,c]

[a] Viability is the percentage of cells not incorporating propidium iodide as described in Materials and Methods; results are expressed as the mean ± SD (n = 5). All significant results are defined by a P of <0.05.
[b] Result is significantly different from the UNS group.
[c] Result is significantly different from the STM group.

The effect of protein transport inhibitors on intracellular cytokine staining. Significant differences in the intracellular expression of TNF-α were detected between the STM-BFA and STM-MN groups after 4 h of stimulation. After assessing the amount of secreted cytokine in the supernatants from the various treatment groups by ELISA, detectable amounts of both IFN-γ and TNF-α were present in supernatants from the STM and STM-MN (but not the UNS) groups. IFN-γ could be detected in some supernatants from the STM-BFA cells, but only after 18 h of incubation. Thus, the lower levels of intracellular cytokine in the STM-MN cells may be due, at least in part, to increased cytokine secretion compared to the STM-BFA cells.

The reason behind the differences in secretion rates may relate to the intrinsic nature of these cytokines. Human TNF-α is a nonglycosylated type II transmembrane protein of 233 amino acids (aa). After transport to the plasma membrane, the transmembrane form is proteolytically cleaved at the plasma membrane by the action of TNF-α-converting enzyme (TACE), a glycosylated membrane-bound metalloproteinase that cleaves the membrane-bound 233-aa form into a soluble 157-aa form (1). Because this proteolytic cleavage must take place prior to release of the soluble form of TNF-α, a possible reason for the observed difference may be associated with the enzyme TACE. Solomon et al. (24) studied the conversion of pro-TNF-α (233 aa) to soluble TNF-α (157 aa) using LPS stimulation of human monocytes and found that the addition of a hydroxamic acid-based metalloprotease inhibitor led to an almost complete inhibition of TNF-α release by the monocytes. If the glycosylation of TACE, or its insertion into the membrane, is delayed by the mechanism proposed for the delay observed with CD69 expression in STM-BFA cells, the membrane-bound form of TNF-α could not be cleaved. With regard to IFN-γ, the human form is a heterogeneously glycosylated protein (3). Although the amounts detected inside the cells of the STM-BFA and STM-MN groups are equivalent, the amount secreted into the supernatant from the STM-BFA cells is 10% of that detected from the STM-MN cells. This again may be attributed to a delay in processing, specifically in glycosylation, of the protein in the STM-BFA cells, as opposed to processing in the STM-MN cells.

The data presented here confirm previous reports [14, 22; Chu et al., Hotlines (Pharmingen) **3**:9–10, 1997] that show that differences in lymphocyte marker and intracellular cytokine expression may be dependent on the choice of protein transport inhibitor used in conjunction with intracellular staining. Our data also underscore the fact that although there is great

interest in using this technique as a tool for the clinical immunology laboratory, unexpected factors such as those explored here indicate that it may be necessary to further evaluate reagent choices to ensure some measure of reproducibility among laboratories. When considering all the factors that were analyzed, it appears that BFA may be a better choice than MN for detecting intracellular cytokines. From our results, we observed that the advantages of using BFA rather than MN are greater lymphocyte viability following prolonged stimulation, easier analysis of the CD4 subset, and more-efficient intracellular trapping of specific proteins. However, the mechanisms by which BFA and MN affect the measurement of other proteins analyzed with this assay must be further assessed before firm recommendations as to the choice of a protein transport inhibitor can be conclusively stated.

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