Explore what's possible with innovative research tools

Attaining high-dimensional scientific insights can be overwhelming but with BD you're never alone.

Built on over 45 years of flow cytometry expertise, our line of innovative research tools and services offers you a complete cell analysis solution to streamline your research and support you on the path to experimental success. Our advanced flow cytometers, informatics and reagents combined with multiomics instrumentation can help you harness the power of high-dimensional biology to rapidly expand your understanding of complex biological systems.

So, go beyond your research limitations and explore with confidence. Discover the difference.

bdbiosciences.com/expand

BD

Original Articles

Whole Blood Fixation and Permeabilization Protocol with Red Blood Cell Lysis for Flow Cytometry of Intracellular Phosphorylated Epitopes in Leukocyte Subpopulations

Sue Chow,[1] David Hedley,[1,2] Patricia Grom,[3] Robert Magari,[4] James W. Jacobberger,[5] and T. Vincent Shankey[3]*

[1]Department of Pathology, Princess Margaret Hospital, Toronto, Ontario, Canada
[2]Department of Medical Oncology and Hematology, Princess Margaret Hospital, Toronto, Ontario, Canada
[3]Advanced Technology Center, Beckman Coulter, Inc., Miami, Florida
[4]Cellular Analysis Center, Beckman Coulter, Inc., Miami, Florida
[5]Case Comprehensive Cancer Center, Cleveland, Ohio

Received 20 August 2004; Revision Receievd 7 March 2005; Accepted 20 April 2005

Background: Previous studies of intracellular expression of phospho-epitopes in human leukocytes using flow cytometry have used erythrocyte removal or lysis before fixation. Because many of the phospho-epitopes of interest are part of signaling networks that respond to the environment and turn over rapidly, the interval and manipulations used to eliminate erythrocytes from samples have the potential to introduce artifacts. We report a procedure to fix samples containing red blood cells with formaldehyde and then remove erythrocytes by lysis. Detection of phospho-Thr 202/Tyr 204-p44/42 extracellular-regulated kinase (ERK) after phorbol ester acetate (PMA) stimulation was used as a model to measure phospho-epitopes in leukocyte populations in whole blood.

Methods: Normal blood samples were activated with PMA followed by formaldehyde fixation and subsequent treatments with detergents and protein denaturants. The effects of each treatment were monitored by light scatter, selected CD expression intensity, and phosphorylated ERK (pERK) expression.

Results: Red cells could be lysed using 0.1% Triton X-100 after brief fixation of whole blood with 2% or 4% formaldehyde. Light scatter improved as a function of formaldehyde concentration and inversely with MeOH concentration. CD3 signal intensity increased when MeOH concentration was reduced. The ratio of pERK immunofluorescence in PMA-stimulated versus nonstimulated (control) samples was highest with high MeOH (90%) and lowest without MeOH treatment. This pattern is consistent with epitope unmasking by alcohol. The pERK epitope could also be unmasked by treatment with high salt, urea, acid, or heat, but none of these produced the level of unmasking of MeOH and each of these was associated with degradation of light scatter and CD3 staining intensity. The final procedure employed 4% formaldehyde, 0.1% Triton X-100, followed by 50% methanol denaturation. Samples prepared in this way demonstrated good preservation of light scatter and surface immunophenotypic patterns, similar to those obtained using a commercial whole blood/red blood cell lysing system (Q-Prep) and an acceptable PMA-stimulated pERK signal (essentially 100% of $CD3^+$ cells that are pERK positive).

Conclusions: Brief fixation of whole blood in 4% formaldehyde followed by treatment with Triton X-100 results in erythrocyte lysis and leukocyte light scatter and immunophenotypic features equivalent to those of other commercial lysis reagents. Intracellular pERK staining is significantly improved by treatment with methanol, but levels of MeOH above 50% degrade light scatter and CD3 expression. This protocol (formaldehyde/Triton X-100/MeOH) circumvents potential artifactual changes in phospho-epitopes due to removal of erythrocytes or erythrocyte lysis followed by fixation, and results in a pERK signal that resolves positive from negative cell populations. © 2005 Wiley-Liss, Inc.

Key terms: signal transduction; phosphospecific antibody; flow cytometry; Fisher distance; light scatter; cell surface markers

Cytometry is a well-developed technology for the characterization of hematologic malignancies based on correlated measurement of multiple surface immunophenotypic markers and, less frequently, internal lineage related

*Correspondence to: T. Vincent Shankey, Advanced Technology Center, Beckman Coulter, Inc., 11800 SW 148th Avenue, Miami, FL 33196.
E-mail: vincent.shankey@coulter.com

Published online 3 August 2005 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cyto.a.20167

antigens. Most of these antigens are long-lived with low turnover and are relatively unaffected by sample preparation. Thus, different anticoagulants, overnight storage and shipment, whole blood staining, and several erythrocyte lysis techniques result in reproducible, convenient, and flexible assays. Recently, interest has been stimulated in potentially less robust assays of cell signaling components based on the relatively recent availability of phosphorylation state-specific antibodies (1–6). Because of the quantitative and correlative natures of cytometry, this represents a novel and potentially powerful approach to classification of hematologic malignancies including the selection of patients for molecular targeted therapies and monitoring drug effects in individual patients (1,6–8).

Analysis of signal transduction pathways by flow cytometry presents technical problems that are not currently encountered in routine clinical applications (1–5). The phosphorylation states of individual signaling elements change rapidly in response to stimuli and therefore may be subject to changes due to sample collection, storage, preparation, and staining that may obscure the signaling state of the condition under study. In a previous publication (1) we used flow cytometry to detect stimulated changes in phosphorylated extracellular-regulated kinase (pERK) that were correlated with western blot analysis. This measurement has been viewed as a surrogate marker for signals originating upstream and traveling through ERK, as an integrating node in studies of drugs that target upstream signaling pathways. However, because the original protocol employed hypotonic lysis before fixation, which could potentially underestimate pharmacodynamic effects due to drug dissociation and reversal of the effect on signaling before fixation, we explored the possibility of immediate fixation followed by erythrocyte lysis or removal.

A second issue with the original procedure was loss of light scatter information due the second step, treatment with 90% MeOH, which was necessary to unmask the pERK epitope. Each of these problems was addressed by increasing the formaldehyde concentration and decreasing the MeOH concentration. We report an improved technique that employs immediate formaldehyde fixation to stop cellular processes followed by erythrocyte lysis followed by denaturation with MeOH. This procedure results in a detectable and acceptable pERK signal, preserves light scatter better than the original protocol, and results in better preservation of cell surface epitopes. Although we report results for ERK phosphorylation, we have since detected robust and specific signals for phospho-S473-AKT, phospho-S235/236-S6 ribosomal protein, and pERK simultaneously by using this protocol (manuscript in preparation). Therefore, we believe that this procedure will be more widely applicable to the study of phospho-epitope expression in whole blood samples.

MATERIALS AND METHODS

Sample Acquisition

Whole blood samples were obtained from normal donors (ages 26 to 63 years) according to institutionally approved protocols. Whole blood was collected into Vacutainer tubes (BD Vacutainer Systems, Franklin Lakes, NJ, USA) that contained ethylenediaminetetraacetic acid and used within 24 h of venipuncture. In some cases, complete blood cell count (CBC) values for samples were obtained using a Coulter LH-750 analyzer (Beckman Coulter, Inc., Miami, FL, USA).

Reagents and Solutions

Phorbol myristate acetate (PMA; Sigma Chemical Corp., St. Louis, MO, USA) was prepared as a 40-μM working solution in 100% anhydrous ethanol. EM-grade, methanol-free formaldehyde was purchased as a 10% solution from Polysciences (Warrington, PA, USA). Formaldehyde was stored at room temperature in the dark and used within 6 months of purchase. Non-ionic detergents were purchased from Pierce Biotechnology (Rockford, IL, USA). These included 10% aqueous solution of Triton X-100, Triton X-114, Tween-20, Tween-80, Nonidet P-40, Brij-35, and Brij-58 and powders octyl-B-glucoside, octyl-B-thioglucopyaranoside, and CHAPS (3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). Powdered detergents were dissolved in phosphate buffered saline (PBS) to make 10% stock solutions. All detergents were diluted with PBS immediately before use. PBS was free of calcium and magnesium. Wash buffer consisted of PBS with 4% fetal bovine serum (FBS) or 2% bovine serum albumin (Sigma Chemical Corp.). Freezing medium consisted of 10% glycerol and 20% FBS in RPMI 1640 and was kept at −20°C.

Cell Fixation, Red Blood Cell Lysis, and Permeabilization

Method A. One hundred microliters of blood was stimulated with 400 nM PMA for 10 min at 37°C, and then erythrocytes were lysed by addition of 2 ml deionized water for 30 s, followed immediately by the addition of 222 μl 10× PBS as previously described (1). Samples were centrifuged (18,000*g* for 3 min) and resuspended in 100 μl 2% formaldehyde in PBS for 10 min at room temperature. Samples were subsequently permeabilized by the addition of 1 ml 100% methanol (stored at −20°C). This method has been used previously to measure phospho-ERK1/2 (pERK) expression in PMA-stimulated lymphocytes (1).

Method A.′ To decrease the amount of time between hypotonic lysis and fixation, a variation was introduced into method A. Concentrated formaldehyde solution (10%) was added directly to the sample to obtain a 2% or 4% final concentration for 30 s, followed by the addition of deionized water, with subsequent return to isotonicity using 10× PBS. The sample was then incubated for 10 min at room temperature before centrifugation (18,000*g*) and subsequent treatment with 1 ml 100% methanol.

Formaldehyde-Triton X-100-Methanol: (F/TX/MeOH). This is the final version of an improved method reported here. Sixty-five microliters of 10% formaldehyde was added to 100 μl blood (final concentration 4%) and incubated for 10 min at room temperature, followed by the addition of 1 ml Triton X-100 (diluted in PBS) to obtain 0.1% final

concentration. After 30 min of incubation at room temperature, 1 ml cold wash buffer was added. Samples were centrifuged, resuspended in 1 ml 50% MeOH diluted in PBS (stock stored at −20°C), and incubated at 4°C for a minimum of 10 min before further processing.

Antibodies

A monoclonal antibody to the activation state epitopes, phospho-Thr 202/Tyr 204, on ERK1/2 (p44/42 MAPK) was kindly provided by Dr. Bradley Smith (clone E10, Cell Signaling Technologies, Beverly, MA, USA). The antibody was conjugated with Alexa Fluor 488 (A488, Molecular Probes, Eugene, OR, USA) according to the manufacturer's directions. Conjugated anti-pERK had a dye-to-protein ratio between 4.3 and 6.3 and was used at 0.2 μg/10^6 cells in 100 μl, which was at the signal to noise optimum determined by titration. Surface markers CD3-phycoerythrin (PE; clone UCHT-1), CD45-PE (clone J.33), CD19-PE (clone J4.119), CD13-PE (clone SJ/D1), CD14-PE (clone RMD52), CD33-PE (clone D3HL60.251), and anti-tubulin-fluorescein isothiocyanate (clone TB1A337.7) were obtained from Beckman Coulter, Inc.

Immunofluorescence Staining

Surface staining with CD markers was performed for 100 μl whole blood samples using three different preparation techniques (Q-Prep, F/TX, and F/TX/MeOH); Q-Prep was used according to the manufacturer's recommended protocol that was provided in the product insert (Beckman Coulter, Inc.). Q-Prep (which lyses red blood cells [RBCs] with dilute formic acid, returns the solution to neutral pH, and then fixes the remaining cells with 0.1% paraformaldehyde, final concentration) was used as a representative whole blood preparative technique that would allow subsequent analysis of intracellular phosphoepitopes without significantly altering cell surface CDs or light scatter profiles of white blood cell (WBC) populations. Antibody incubations were done for 30 min at room temperature, followed by two washes with 2 ml wash buffer (645*g* for 4 min). Final pellets were resuspended in 1 ml wash buffer.

For intracellular staining, fixed and permeabilized cells (without or with methanol treatment, F/TX or F/TX/MeOH, respectively) were washed once (1,000*g* for 3 min) with 2 ml cold wash buffer. Antibodies were added to cell pellets (100 μl final volume) and incubated at room temperature for 15 to 30 min. Stained samples were then resuspended in 2 ml wash buffer, filtered through 35-μm nylon mesh, centrifuged at 1,000*g*, and resuspended in 150 to 300 μl wash buffer.

Flow Cytometry

For experiments measuring changes in phospho-specific epitopes, flow cytometric measurements were performed with an Epics Elite flow cytometer (Beckman Coulter, Inc.) using 20-mW 488-nm illumination. Fluorescein isothiocyanate or Alexa Fluor 488 fluorescence was collected through a 525 ± 10-nm bandpass filter, and PE was collected through a 575 ± 20-nm bandpass filter. Two thousand to 10,000 positive events (generally CD3 positive) were acquired and saved as listmode files. Data analysis was performed with Elite software to calculate mean fluorescence intensity (MFI) and percentage of positive events.

In experiments comparing the relative resolution (Fisher distances, see below) of lymphocytes, monocytes, and granulocytes using forward angle light scatter (FS) and side scatter (SS) measurements and measurements of the relative fluorescence intensity of selected surface markers in whole blood samples prepared using the three different techniques (Q-Prep, F/TX, and F/TX/MeOH), an FC-500 flow cytometer (Beckman Coulter, Inc.) was used in the standard configuration provided by the manufacturer. Illumination was 488 nm. Data acquisition was gated on FS versus SS and 45,000 cells were collected. For samples prepared using F/TX or F/TX/MeOH, identical settings for gain and high voltage were used for FS and SS detectors. For samples prepared using Q-Prep, a lower voltage (3.3 times) was used for both scatter detectors to enable resolution of the three leukocyte populations.

Data Analyses

Fisher distances. As illustrated in Figure 1, to determine the effect of different fixation/permeabilization protocols on the ability to identify and quantify major leukocyte populations (lymphocytes, monocytes, and granulocytes), we calculated Fisher distances between the leukocyte clusters defined by FS and right angle SS (9). The calculation was performed with the following equation:

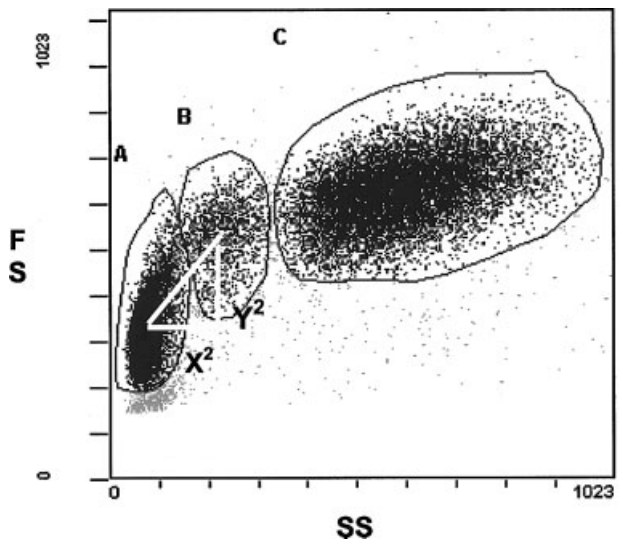

FIG. 1. Method used to calculate Fisher distances. Histograms of FS versus SS were used to measure the distance between lymphocyte (A), monocyte (B), and granulocyte (C) populations. The distance between the centers of two populations (such as lymphocytes and monocytes, shown here) was determined for X and Y axes as the hypotenuse completing the right triangle. The Fisher distance represents the measured distance divided by the sum of the standard deviations of the two populations. This sample was prepared using Q-Prep.

$$r\ (\text{distance}) = \sqrt{X^2 + Y^2} \quad (1)$$

where X represents SS and Y represents FS, and

$$\text{Fisher Distance} = \frac{r}{SD_A SD_B} \quad (2)$$

where SD_i represents the average of standard deviations of FS and SS distributions for population i and i represents A or B. In Figure 1, the calculation of r is illustrated where population A represents the lymphocyte cluster and population B represents the monocyte cluster. The distribution centers were calculations of the mean. Means and standard deviations were obtained from instrument software after gating each cluster subjectively.

Leukocyte quantification. Lymphocyte, monocyte, and granulocyte enumerations were obtained by flow cytometry by using FS and SS gates to calculate fractional values. These values were multiplied by the WBC count.

Precision. To assess protocol-related reproducibility, means, standard deviations, and coefficients of variation were calculated on replicates for subpopulations identified with lineage-specific surface markers.

Method comparisons. Methods were compared by Fisher distances, CBC parameters, and MFIs of surface markers. Analysis of variance and Tukey-Kramer statistical tests were used to compare Fisher distances between different sample preparation methods. Agreement between methods for each surface marker using MFI and CBC was evaluated with Bland-Altman plots (10). Methods agree with each other when there are no significant differences between them. The "true" differences between two methods are referred to as total bias. These biases may be coming from different sources (11,12). Total bias is not equal to the observed differences between methods because the latter contains an error term that is mainly associated with the imprecision but is not related to biases. We estimated total bias from the data and plotted these estimates instead of the observed differences between two methods as originally described by Bland and Altman (10). The estimates of total bias were obtained from the statistical model, $D = TB + E$, where D is the observed difference between two methods for each sample, TB is the total bias, and E is a random error that is mainly associated with the imprecision of a measurement. Details on the estimation of TB can be found in Magari (13). Tolerance limits for 95% confidence and 99% coverage were calculated based on the estimates of standard error. SAS analysis software (SAS Institute, Cary, NC, USA) was used for all statistical analyses.

RESULTS

Figure 2 shows that fixation by adding formaldehyde directly to whole blood followed by a wash with detergent (lower panels) resulted in efficient erythrocyte lysis and equivalent detection of tubulin but insufficient detection of pERK when compared with the original method by Chow et al. (1) (Method A). This effect, noted before for other antigens, is a hallmark of epitope masking that can be unmasked by protein denaturing treatment (14–16). However, denaturing with 90% MeOH (Method A) resulted in reduced CD3 staining intensity and an alteration of the percentage CD3-positive events (Fig. 2 and data not shown). Further, comparison of denaturing and nondenaturing conditions (Fig. 2) illustrated that right-angle light scatter was compromised by 90% MeOH. This has been noted previously (16) and repeated in this study comparing Method A with commercial immunophenotyping preparations using Q-Prep and IntraPrep (data not shown.).

Effect of Detergents on RBC Lysis

Several detergents have been reported to affect detection of surface and internal antigens of blood cells; see references in Jacobberger (16,17). Therefore, we investigated the impact of different detergents and detergent concentrations on RBC lysis and on WBC resolution using light scatter. We tested 10 non-ionic or zwitterionic detergents. Some of these have been variously described as more or less "gentle" in the literature. Only three of the detergents, TX-100, NP-40, and Brij-58, showed significant RBC lysis after 2% formaldehyde fixation for 10 min at room temperature. These detergents had a deleterious effect on WBC light scatter at detergent concentrations above 0.2%, whereas concentrations below 0.1% resulted in incomplete RBC lysis. Thirty minutes of incubation at room temperature with 0.1% detergent was optimal (data not shown).

Formaldehyde Concentration and Incubation Temperature

We examined the effects on RBC lysis and light scatter by testing formaldehyde concentrations of 2%, 4%, 6%, and 8% at room temperature and at 37°C for 10 min. We found that 2% or 4% formaldehyde treatment at room temperature allowed lysis of RBCs, whereas fixation at 37°C resulted in cell clumping and incomplete RBC lysis. Light scatter patterns also improved as a function of formaldehyde concentration.

Other Denaturants

Phospho-ERK levels after PMA stimulation were 2 to 4 times above unstimulated controls when denaturation was not used (Fig. 2B). In contrast, 90% MeOH treatment variously resulted in a 15- to 30-fold increase (e.g., Fig. 2A). Therefore, we investigated whether other denaturants including high salt, urea, low pH, or heat would unmask pERK without detrimental effect on WBC resolution.

After fixation of whole blood with 2% formaldehyde for 10 min at room temperature and treatment with 0.1% detergent for 30 min, samples were made 1 M or 2 M in NaCl or urea by direct addition to samples. For samples exposed to low pH, detergent was removed by centrifugation, and cells were resuspended in 1 ml 1 M sodium acetate buffer (pH 5.0). All samples were treated for 30 min at room temperature, centrifuged, and washed. Temperature-treated samples were incubated at 70°C for 10 min

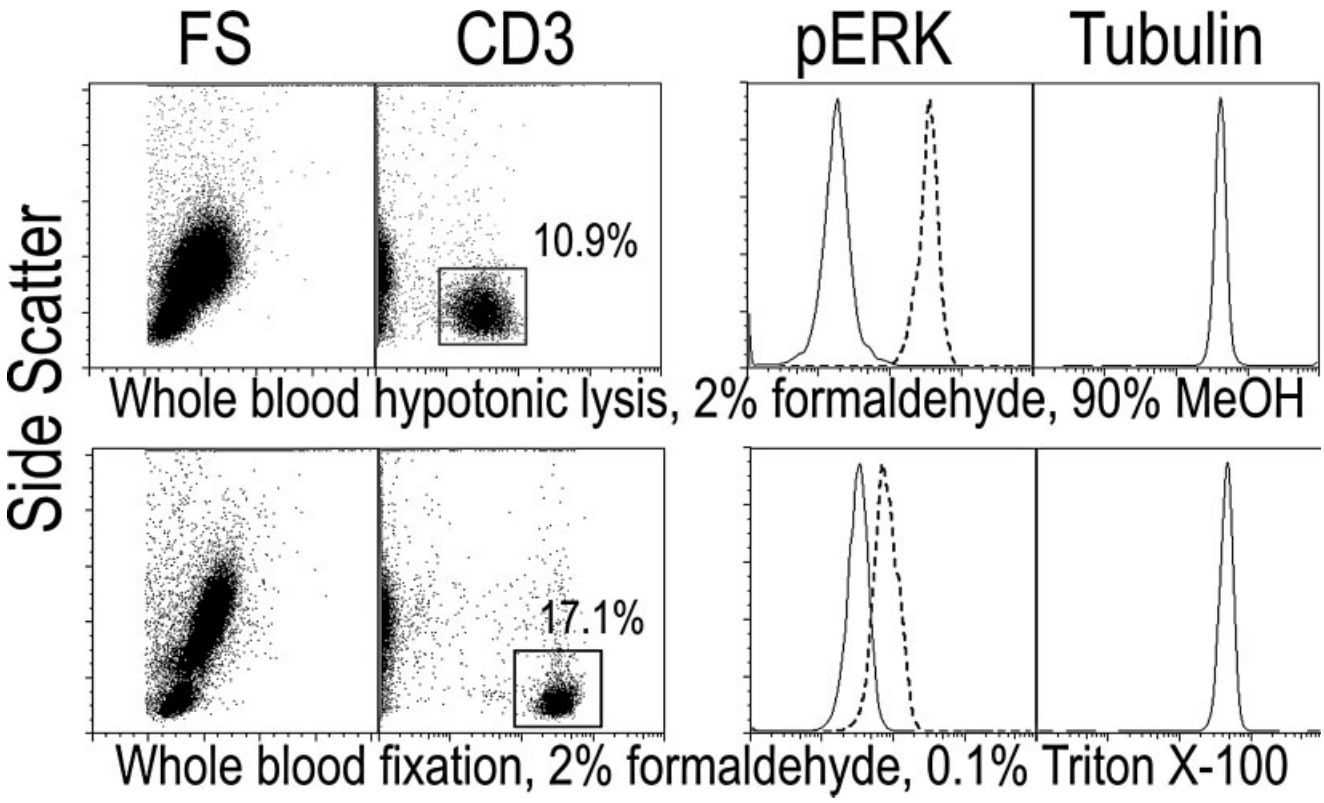

FIG. 2. Comparison of the results of whole blood hypotonic lysis method (Method A, upper panels) with whole blood fixation with 2% formaldehyde and detergent permeabilization/RBC lysis method (lower panels). Results demonstrate improved resolution of WBC populations and greater intensity of CD3 staining (numbers in CD3 vs. SS histograms indicate percentage of CD3-positive events as a total of all WBCs), identical staining for anti-tubulin, but inferior pERK staining of $CD3^+$ cells after formaldehyde/Triton X-100 treatment. Histograms showing pERK staining include PMA-stimulated (dashed lines) and unstimulated control (solid lines) samples.

after fixation and permeabilization. For all conditions, samples were subsequently washed and then stained for CD3 and pERK. Results are summarized in Table 1. None of the denaturing treatments compared well with 90% MeOH concentration. In addition, all of these treatments resulted in degradation in light scatter and poor resolution of WBC populations, similar to that seen using 90% MeOH. High-temperature treatment gave an 11-fold increase of pERK over background, but CD3 staining intensity decreased more than 10-fold. Thus, the case for epitope masking (as opposed to antigen extraction) was supported, but we found no denaturing conditions that provided signal-to-background ratios that were equal to high MeOH concentration.

Alcohol Concentrations

To evaluate pERK unmasking with alcohols, experiments were undertaken to determine whether the level of the pERK signal was dependent on alcohol concentration. After 30-min exposure to 0.1% Triton X-100 and addition of 1 ml wash buffer, 4% formaldehyde-fixed whole blood samples were centrifuged and resuspended in a series of methanol or ethanol (EtOH) concentrations and incubated at 4°C for 15 min or longer. Samples were then washed and stained for CD3 and pERK as above. At alcohol concentrations of 60% or greater, there was degradation of the light scatter pattern associated with a decrease in CD3 staining intensity similar to the pattern resulting from 90% MeOH. This effect was more pronounced with EtOH. Using 50% or lower alcohol, light scatter improved considerably, associated with increased CD3 staining intensity. Figure 3 shows an example of the effect of different alco-

Table 1
Impact of Denaturation Conditions on pERK Staining

	pERK immunofluorescence ratio[a]		
Method A	26.0	26.0	34.4
Treatment	TX-100[e]	Brij 58[e]	NP-40[f]
F/Det[b] (no alcohol)	2.7	2.7	3.2
F/Det + 1N NaCl[c]	4.8	3.1	3.7
F/Det + 1M Urea[c]	4.2	3.9	3.9
F/Det + 1M Acid pH5	7.0	6.9	7.6
F/Det + Heat 70 deg C	11.5	ND[d]	ND[d]

[a]Ratio of pERK immunofluorescence for PMA-treated versus nontreated whole blood gated on CD3-positive cells.
[b]F/Det: Treatment with 2% formaldehyde for 10 min at room temperature followed by 0.1% detergent for 30 min at room temperature.
[c]2 M after Triton X-100 treatment.
[d]Not determined.
[e]Donor A.
[f]Donor B.

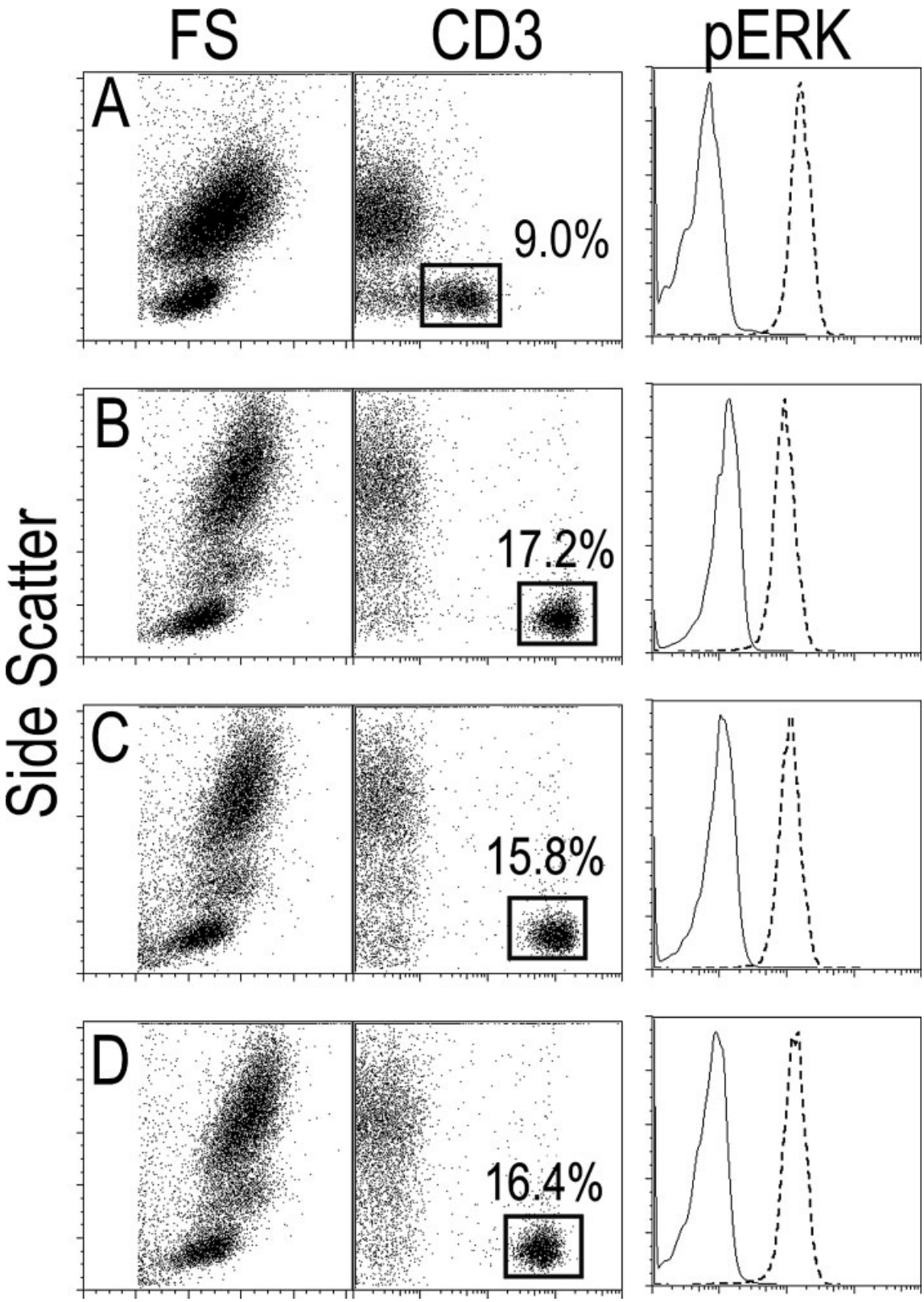

FIG. 3. Comparison of the effects of alcohol treatments on resolution of WBC populations by light scatter, CD3 expression (numbers in CD3 vs. SS histograms indicate percentage of CD3-positive events as a total of all WBCs), and pERK expression. **A:** Hypotonic lysis followed by 2% formaldehyde and fixation with 100% methanol (Method A′), showing loss of light scatter resolution and CD3 expression, but highest signal/background for pERK expression. The other three samples show whole blood processed using 4% formaldehyde/0.1% Triton X-100 technique, (**B**) no alcohol treatment, (**C**) 50% methanol, and (**D**) 50% ethanol. Histograms showing pERK staining include PMA-stimulated (dashed lines) and unstimulated control (solid lines) samples.

Table 2
Effects of Formaldehyde Concentration and Alcohols on pERK Signal using the F/TX Method

		pERK[a]			$CD3^+$ %		$CD3^+$ MFI		Autofluorescence[b]	
		CTRL	PMA	PMA/CTRL	CTRL	PMA	CTRL	PMA	CTRL	PMA
2% Formaldehyde										
Donor 1	F/TX	3.6	5.6	1.6	30.1	28.6	98.4	97.2	0.33	0.33
	+50% MeOH	3.2	7.1	2.2	29.9	30.7	84.4	80.2	0.3	0.33
	+50% EtOH	2.2	10.0	4.6	29.5	28.5	43	42.6	0.26	0.28
Donor 2	F/TX	3.5	6.5	1.8	12.7	11.7	101	99.1	0.29	0.28
	+50% MeOH	3.3	6.6	2	12.2	13.9	92	81.7	0.26	0.28
	+50% EtOH	2.6	8.9	3.4	12	13.3	55.9	46.2	0.28	0.25
4% Formaldehyde										
Donor 1	F/TX	3.2	14.9	4.7	30.4	30.5	80.6	87.1	0.36	0.33
	+50% MeOH	3.0	16	5.3	30.6	29.6	73	69.8	0.33	0.32
	+50% EtOH	2.1	16.7	8.1	28.8	29.2	44.3	50.9	0.3	0.28
Donor 2	F/TX	2.9	14.8	5.1	14.1	15	83.7	89.7	0.28	0.27
	+50% MeOH	2.7	16.0	5.9	13.4	13.9	75.7	86.3	0.29	0.29
	+50% EtOH	2.5	19.0	7.5	13	14.2	62.2	59.8	0.26	0.27

[a]Gated on CD3-PE positives; MFI of pERK-Alex Fluor 488. CTRL, control.
[b]Gated on unstained lymphocytes; autofluorescence in the Alexa Fluor 488 channel.

hol treatments: staining for pERK following PMA treatment was 12-fold greater with 50% MeOH than that of the unstimulated control cells, compared with 29-fold for 90% MeOH and 8-fold for 4% formaldehyde/Triton X-100 without alcohol treatment. This improvement in the signal-to-background ratio resulted from decreased background staining and increased staining of the PMA-stimulated cells, and was more pronounced with EtOH. Samples treated with 50% EtOH gave a stronger pERK signal; however, these samples degraded rapidly as a function of time. Samples denatured with 50% MeOH could be stored overnight at −20°C. These findings indicate that MeOH and EtOH are not interchangeable.

Formaldehyde Concentration Revisited

When 2% and 4% formaldehyde concentrations were tested with 50% MeOH denaturation, not only did 4% formaldehyde preserve the scatter resolution better, but the ratio of the pERK signal of PMA treated to unstimulated control increased from 2- to 4-fold to 4- to 8-fold. Results presented in Table 2 show that high formaldehyde concentration improved pERK antigen detection by improving antigen retention (compare 2% with 4%) and by an unmasking effect.

Comparison of Whole Blood Fix-Lyse and Whole Blood Lyse-Fix Protocols

We compared the original Method A with the F/TX/MeOH method by light scatter pattern, CD3 intensity and fraction, and pERK ratio (stimulated to unstimulated). Figure 4 shows representative data. The upper two panels show that there is no significant temperature dependence for the fixation step. The original method of Chow et al. (1) was done at 37°C. Comparing these data to the results obtained with the F/TX/MeOH method (Fig. 4, bottom panels), it is evident that this whole blood fix-lyse technique gives a greatly enhanced forward versus orthogonal light scatter signal, with the monocytes being particularly well resolved, and improved CD3 surface immunofluorescence. However, the pERK signal is attenuated (compared with the original, Method A), presumably due to incomplete protein denaturation.

Sample Storage Revisited

With the original lyse-fix method A (1), PMA-activated peripheral blood samples could be stored in 90% MeOH at −20°C for several months with insignificant loss of pERK signal, similar to results subsequently reported by Krutzik and Nolan (3). However, detection of pERK was much less stable with storage in 50% methanol. Therefore, we compared the pERK signal in samples held for 1 week in 50% MeOH with storage (after F/TX treatment) in cell freezing medium (RPMI 1640 with 10% glycerol and 20% FBS). Samples in freezing medium were thawed and washed once with 2 ml wash buffer and then treated with 50% MeOH before staining. As shown in Figure 5, overnight storage in 50% MeOH did not significantly affect the pERK signal. After 1 week in 50% MeOH, the pERK ratio (stimulated to unstimulated) was reduced (Fig. 5D). Storage of cells in freezing medium for 1 week appeared to be better but was reduced compared with the control (Fig. 5E vs 5A). Although the pERK ratio (stimulated to unstimulated) deteriorated with 1 week of storage, the percentage of positive cells was not significantly affected.

Fix-Lyse Impact on Light Scatter

To quantify the effect of the F/TX/MeOH method on light scatter patterns, we compared results for whole blood samples prepared using the Q-Prep system (Beckman Coulter, Inc.) with those obtained after treatment with 4% formaldehyde/Triton X-100 (F/TX) or F/TX/MeOH. The choice of the Q-Prep system was arbitrary as a

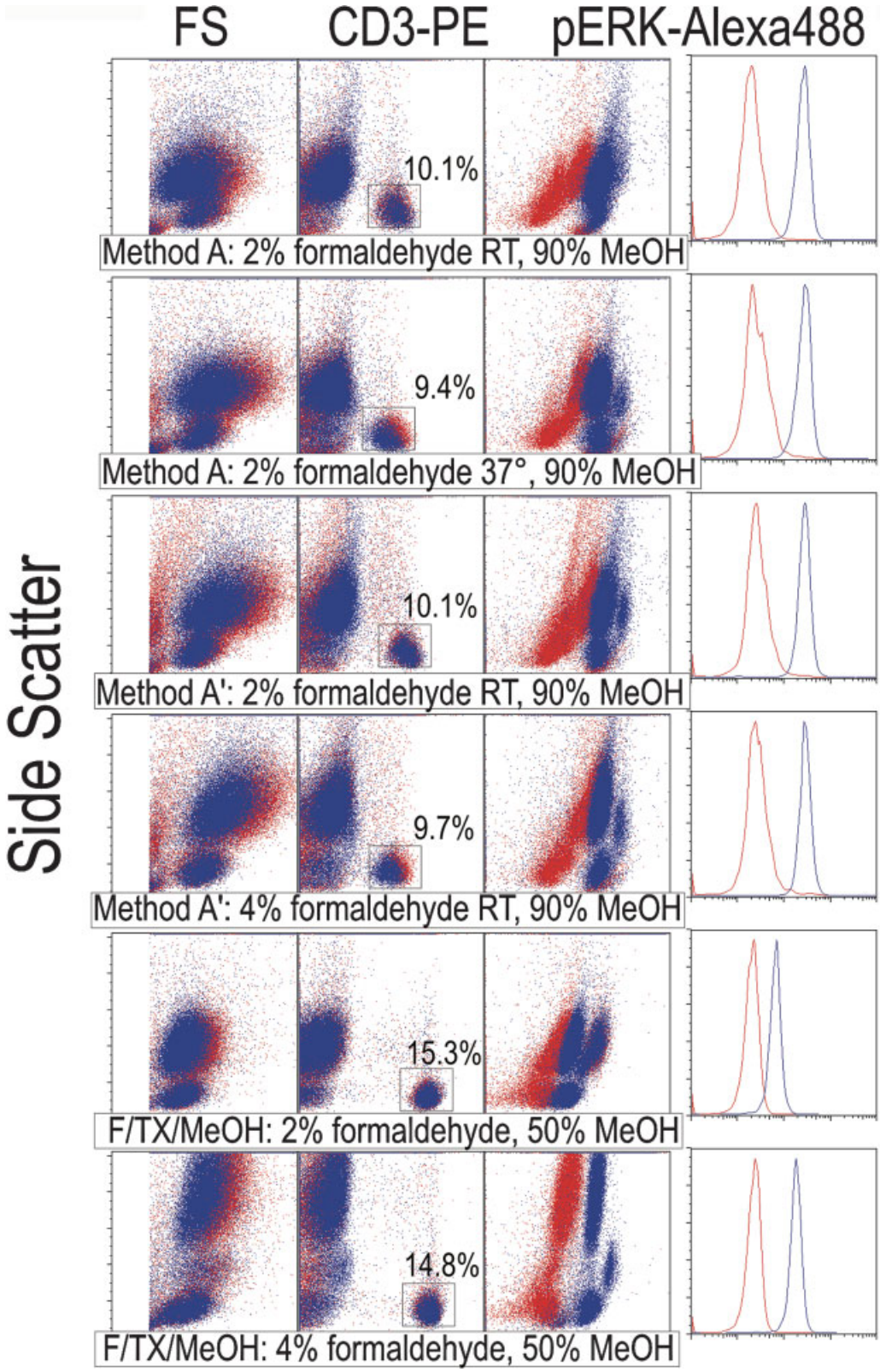

FIG. 4. Comparison of light scatter, CD3, and pERK staining obtained using different protocols for measuring PMA activation of whole blood. Numbers in CD3 versus SS histograms indicate percentage of CD3-positive events as a total of all WBCs. The top four panels show minimal differences for fixation temperature or formaldehyde concentration using the hypotonic lysis techniques (Method A or A′). In contrast, whole blood fixation shows significant enhancement of light scatter and pERK resolution using 4% versus 2% formaldehyde (bottom panels). Note the sequential improvements obtained by increasing fixation conditions and the addition of 50% methanol compared with the preliminary work shown in Figure 2, bottom panels. Blue indicates PMA-stimulated samples and red areas indicate unstimulated samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

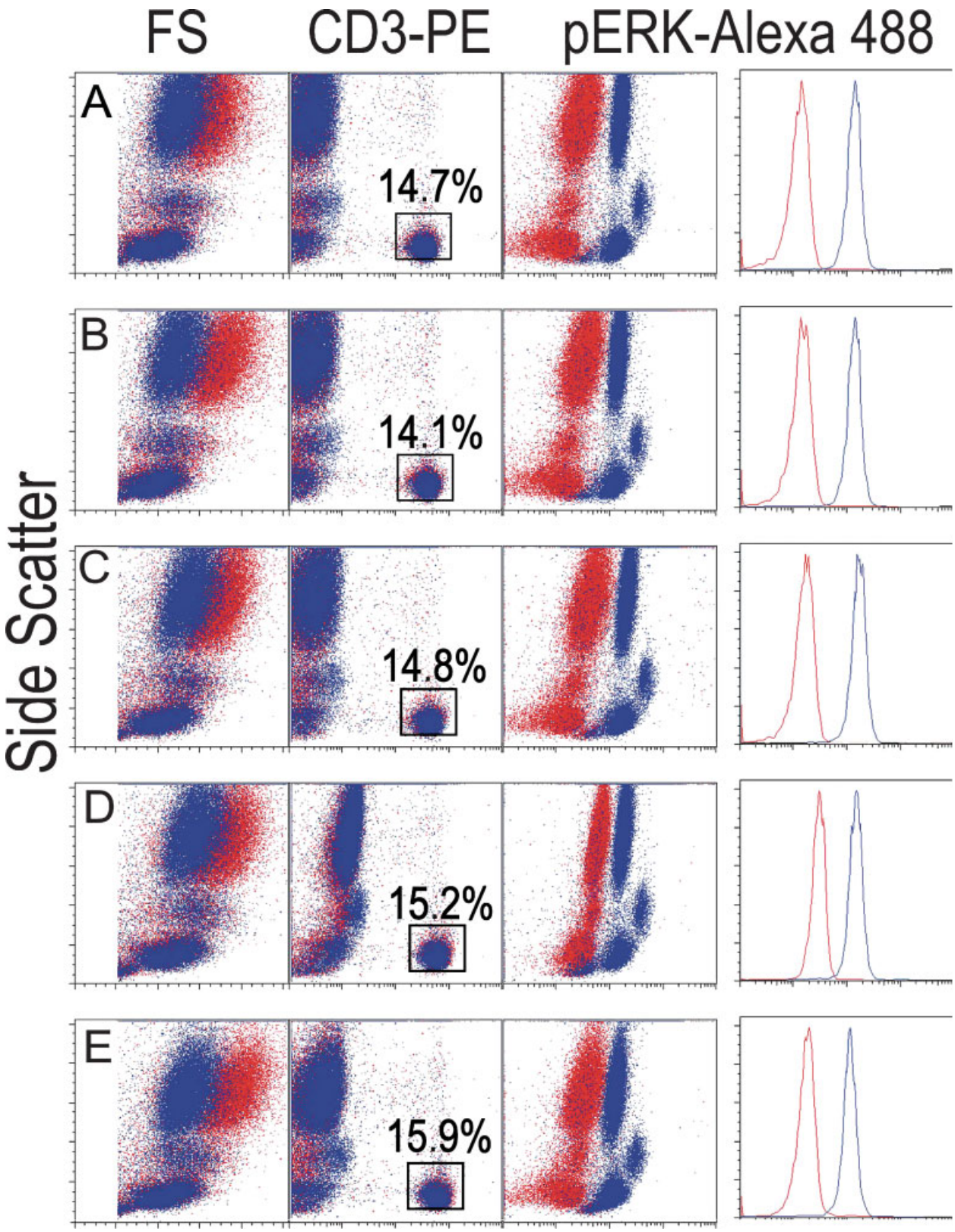

FIG. 5. Effects of storage conditions on light scatter, CD3, and pERK staining. Numbers in CD3 versus SS histograms indicate percentage of CD3-positive events as a total of all WBCs. Samples were prepared as in Figure 4, bottom panels, and then stained and analyzed after storage under the following conditions: (**A**) control for 50% methanol storage stained and run immediately; (**B**) control for freezing medium storage, processed as for sample A; (**C**) overnight storage in 50% methanol; (**D**) 1-week storage in 50% methanol; (**E**) 1-week storage in freezing medium. Samples for C–E were stored at −20°C. Blue indicates PMA-stimulated samples and red indicates unstimulated samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 3
Summary of Data Analysis for Fisher Distance Calculations

Method	Difference[a]	SE[b]	DF[c]	*P*	Tukey-Kramer *P*
Lymphocytes to monocytes					
Q-Prep vs. F/TX	−0.435	0.01612	408	<0.0001	<.0001
Q-Prep vs. F/TX/MeOH	−0.4553	0.01612	408	<0.0001	<.0001
F/TX vs. F/TX/MeOH	0.0203	0.01612	408	0.2092	0.42
Monocytes to granulocytes					
Q-Prep vs. F/TX	−0.4002	0.01478	408	<0.0001	<.0001
Q-Prep vs. F/TX/MeOH	−0.6053	0.01478	408	<0.0001	<.0001
F/TX vs. F/TX/MeOH	0.2051	0.01478	408	<0.0001	<.0001

[a]Difference in measured Fisher distance between two whole blood preparation techniques indicated.
[b]Standard error.
[c]Degrees of freedom.

reference method but has an advantage in reproducibility compared with manual preparations. Table 3 summarizes the results of 24 normal donors treated with these three different techniques comparing the relative separation of lymphocytes, monocytes, and granulocytes using measurements of the Fisher distances between light scatter populations (Fig. 1).

Fisher distances between lymphocytes and monocytes were 2.20 for samples prepared using Q-Prep, 1.76 for F/TX, and 1.74 for F/TX/MeOH. As indicated in Table 3, the difference between Q-Prep and the other methods were significant, but the difference between the two fix-lyse methods was not. Fisher distances between monocytes and granulocytes were 2.57 for samples prepared by Q-Prep, 2.17 for F/TX, and 1.97 for F/TX/MeOH. One way of interpreting these data is that there is about a 30% degradation of the light scatter resolution when comparing the fix-lyse procedures with the lyse-fix procedure (2.2/1.76 = 1.26 and 2.57/1.97 = 1.30). Further, there appears to be a slight (10%) effect of the MeOH step on granulocytes (2.17/1.97 = 1.10), but much of the effect on light scatter can be attributed to fixation with formaldehyde.

Aliquots of whole blood samples prepared by all three techniques were measured using an LH-750 (Beckman Coulter, Inc.) analyzer to determine whether any blood cell population was decreased (lost) as a consequence of sample preparation. Analysis was performed using bias plots (Fig. 6) to determine whether there was a significant difference in recoveries of different WBC populations. Lymphocyte populations (Fig. 6, top panel) were consistently overestimated by all three whole blood preparation techniques as compared with the CBC determination, with no significant difference in lymphocyte determinations when comparing the three whole blood techniques. In all likelihood, this difference between the three flow cytometric lymphocyte determinations and the CBC was the result of including events with low scatter (debris, platelets) in the lymphocyte gate. Comparisons of the recoveries of monocytes (Fig. 6, center panel) and granulocytes (Fig. 6, bottom panel) showed no significant variations for any of the whole blood techniques for monocytes and a small but insignificant decrease in the recoveries of granulocytes for all three whole blood techniques compared with the CBC.

Fix-Lyse Impact on Surface Markers

We compared signal intensity of markers routinely used to identify lymphocytes (CD3, 19), monocytes (CD13, 14), and granulocytes (CD13, 33) for samples prepared with Q-Prep, F/TX, and F/TX/MeOH. After preparation and washing, samples were incubated with a single surface marker antibody and analyzed by flow cytometry to determine the percentage of positive cells and MFI.

The results of CD marker determinations on 24 individual donors are presented in Table 4 and Figure 7. As shown in Table 4, although there was some variation in the MFI for any one marker in comparing the three different whole blood preparation techniques, the only significant decrease in staining intensity was seen for CD19 in whole blood samples treated with F/TX/MeOH. As shown in the bias plot for CD19 expression (Fig. 7A, middle panel), there was considerable variability for expression (MFI) of this marker in samples prepared using F/TX with or without MeOH, suggesting differential sensitivity of this epitope to F/TX (and methanol) treatment in individual blood donors. Because we did not repeat these measurements using multiple sampling of the same donor, we cannot rule out experimental variability rather than donor variability. In either case, CD19-positive cells could be readily detected in all donors, regardless of the whole blood preparation technique used. Although other markers showed some increase or decrease in staining intensity (MFI) in comparing different methods, in all cases there was sufficient staining intensity to readily differentiate positive versus negative cell populations.

DISCUSSION

The purpose of this study was to develop a "fix-lyse" method to measure intracellular phospho-epitopes in samples of whole blood, bone marrow, or other fluids containing RBCs. Previous studies by our laboratories (1,5) and by Krutzik et al. (3,4) have demonstrated the utility of using brief formaldehyde fixation followed by alcohol for tissue culture cells, human peripheral blood mononuclear cells, or mouse splenocytes. In these studies, samples containing RBCs were first depleted of erythrocytes by lysis. Two problems were evident. First, removal of interfering RBCs (density gradient separation or RBC lysis) allows time and environments during which artifactual changes

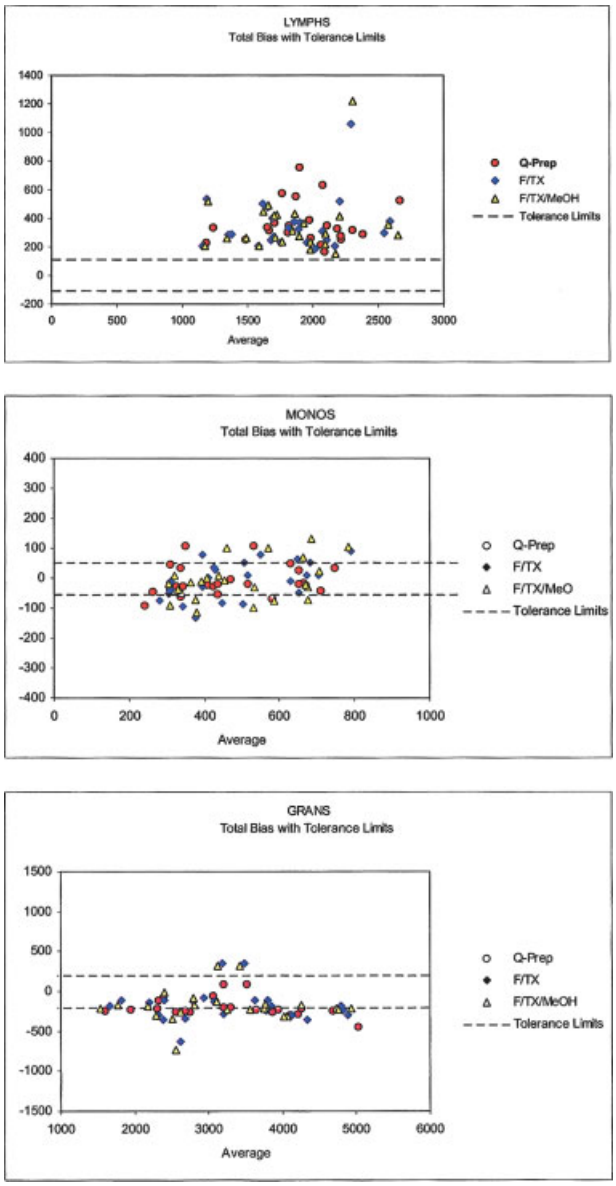

FIG. 6. Estimates of total bias for WBC populations (top: lymphocytes; middle: monocytes; bottom: granulocytes) determined by flow cytometry compared with CBC values determined by LH750. Approximate tolerance limits (dashed lines) determined by the CBC are plotted against the determinations for lymphocytes (top graph), monocytes (middle graph) or granulocytes (lower graph) using determinations of WBC populations from individual samples prepared using Q-Prep (red circles), F/TX (blue diamonds), or F/TX/MeOH (yellow triangles). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in phosphorylation may occur. Second, detection of some phospho-specific epitopes (e.g., pERK, pSTAT1, pSTAT5) was significantly improved by protein denaturation with alcohol (3,4), but alcohol fixation resulted in degradation of the light scatter, a property that has been noted previously. Further, some surface makers, important for subset analysis of leukocytes, were compromised.

To address the first problem, we examined the impact of initial fixation of whole blood samples with formaldehyde followed by detergent treatment on RBC lysis. Fixation of whole blood was initiated to provide the least amount of time between cessation of experimental treatments and fixation of phospho-epitopes in the sample. There have been several studies aimed at whole blood fixation and permeabilization in which light scatter was considered important. One of these is a study by Francis and Connelly (18) describing the properties of the commercial fix and permeabilizing reagent, PermeaFix (Ortho Diagnostic Systems, Raritan, NJ, USA). This single-step reagent produced light scatter resolution of lymphocytes and monocytes equivalent to ammonium chloride lysed samples of whole blood. In addition, leukocyte surface staining was preserved, cells were permeable, and intracellular antigens could be stained. This reagent is now sold by InVirion (Frankfort, MI, USA). We tested this reagent for pERK staining and, like other methods that leave proteins in a native state, the pERK signal was low (Chow and Hedley, unpublished data). Macey et al. (19) tested several other commercial fixation/permeabilization/lysis reagents for whole blood for the ability to preserve light scatter. Results were variable; all procedures affected light scatter but several produced good patterns in which the three main leukocyte subpopulations could be resolved. Neither of these studies indicated what level of formaldehyde was in these commercial reagents. In the present study, we have shown that we can fix whole blood with high concentrations of formaldehyde (2–4%) for brief periods (10 min), and that this will result in preparations in which the erythrocytes can be completely lysed by incubation in non-ionic detergent solutions at low concentration. Although we tested several different detergents, we did not observe an improved effect when compared with Triton X-100. Thus, we have a method in which blood can be treated with a known amount of formaldehyde and RBCs can be lysed after fixation, thus eliminating leukocyte purification steps. In addition, we have

Table 4
Intensity of CD Marker Expression on Different WBC Populations using Different Whole Blood Preparation Techniques

	Fluorescence intensity					
	Q-Prep		F/TX		F/TX/MeOH	
Marker	MFI	SD[b]	MFI	SD	MFI	SD
Lymphocytes[a]						
CD45	353.6	110.7	201.7	76.0	275.6	56.9
CD3	123.1	29.7	126.6	20.5	124.9	18.7
CD19	48.3	12.0	36.6	84.2	6.3[c]	1.9
Monocytes[a]						
CD45	224.9	66.8	260	108.8	351.3	62.7
CD13	84.1	45.6	41.5	19.2	41.9	18.3
CD14	81.4	28.9	103.7	21.6	86.4	15.7
CD33	44.0	20.9	18.9	10.7	20.1	9.4
Granulocytes[a]						
CD45	69.9	20.9	127.0	56.9	185.7	37.8
CD13	53.3	20.5	51.4	12.0	53.0	6.0
CD33	12.1	2.7	12.2	7.6	12.0	7.9

[a]WBC populations determined by light scatter (SS vs. FS).
[b]Standard deviation.
[c]Significant decrease in level of CD expression compared with Q-Prep.

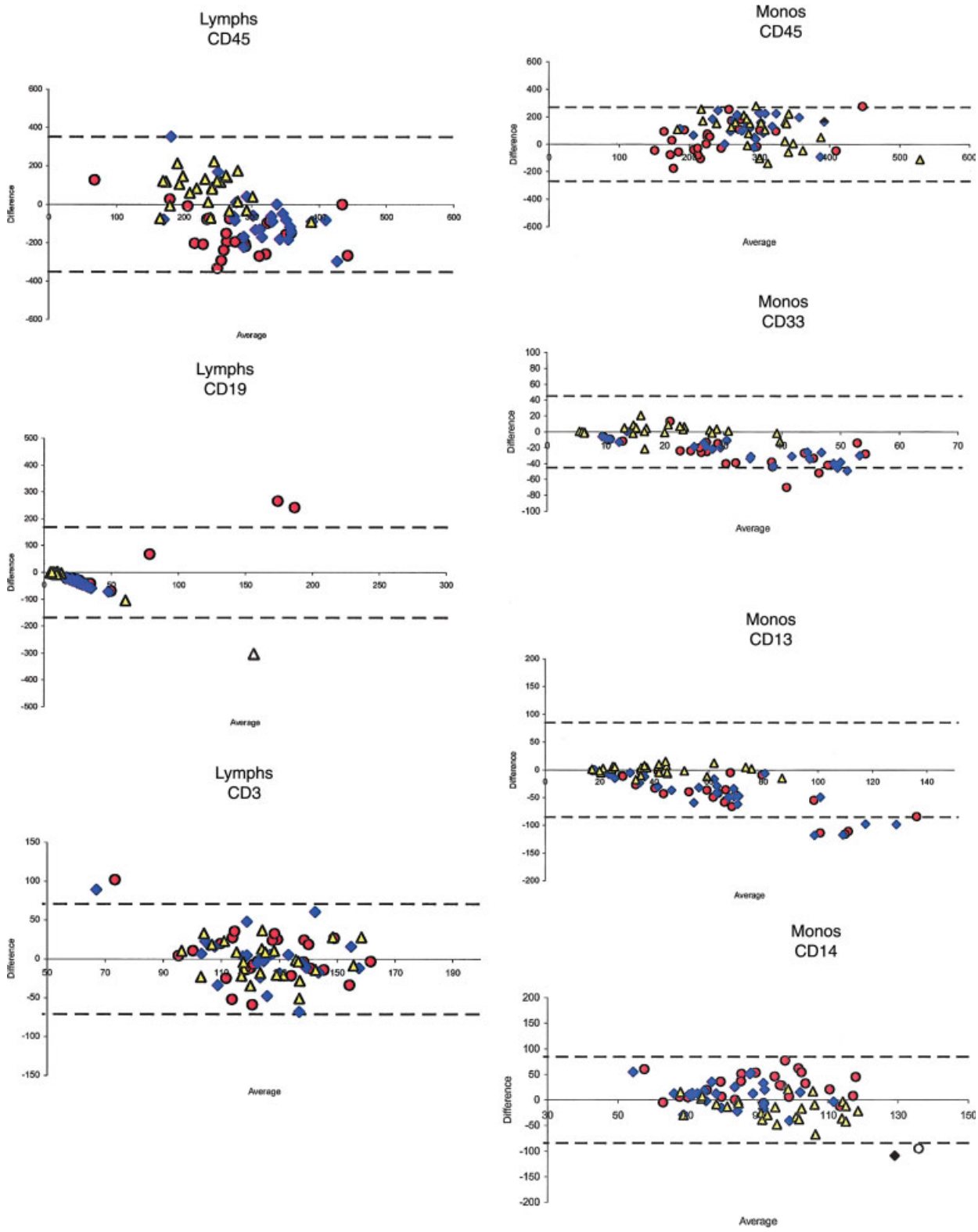

FIG. 7. Estimates of total bias for MFI of WBC populations (**A:** lymphocytes; **B:** monocytes; **C:** granulocytes) for whole blood samples prepared by three different techniques (Q-Prep, F/TX, or F/TX/MeOH). Approximate tolerance limits (dashed lines) are plotted against the average for two of the methods used for preparing individual whole blood samples: Q-Prep versus F/TX (yellow triangles), Q-Prep versus F/TX/MeOH (red circles), or F/TX versus F/TX/MeOH (blue diamonds). Individual graphs represent comparison of the results seen for each surface marker (CD45, CD3, CD19, CD13, CD14, and CD33); some of these markers are expressed on more than one WBC population. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

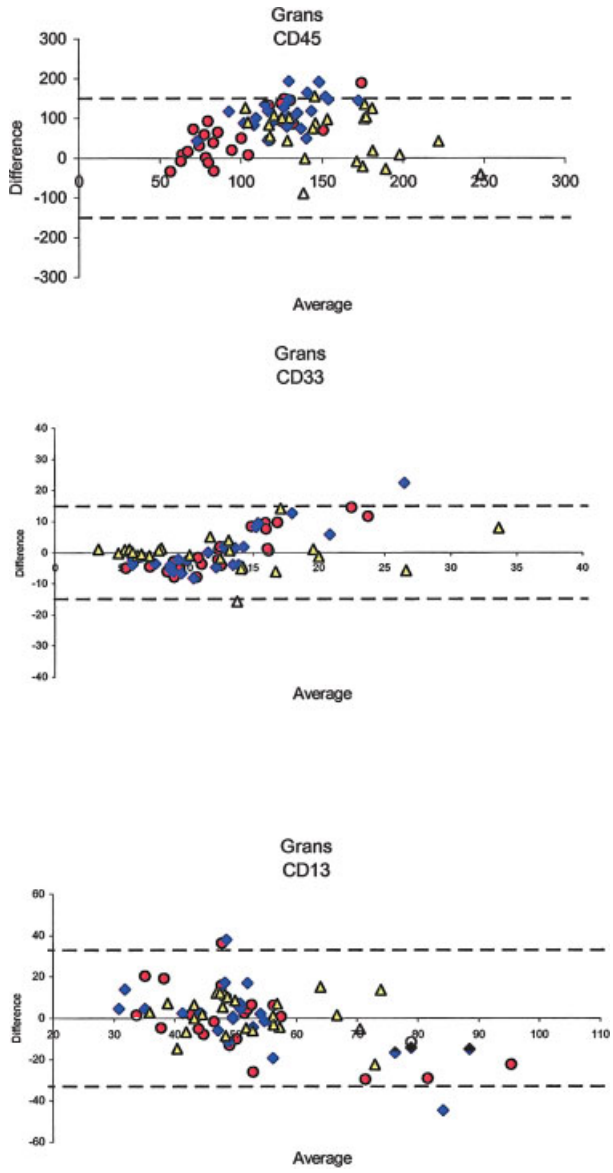

FIG. 7. Continued.

demonstrated that this formaldehyde fixation/detergent permeabilization technique, a fix-lyse procedure, maintains light scatter properties of WBC populations sufficient to allow discrimination of all three leukocyte groups and preserve expression of several common leukocyte subset surface markers.

To address the loss of pERK signal, we tested other means of protein denaturation. Among these were high salt, urea, heat, and acid. Although each of these treatments increased the level of pERK detection, none of these produced cell preparations in which the pERK signal was as high as that achieved with 90% MeOH. Previously, it had been shown that light scatter signals of leukocytes could be retained at lower concentrations of alcohol fixation (20). Therefore, we examined this and confirmed and demonstrated that the loss of light scatter, CD3 intensity, and pERK intensity were dependent on alcohol concentration. By lowering the alcohol concentration to 50%, we added a denaturation step and arrived at a whole blood fixation procedure that provided good light scatter patterns and high CD3 intensity and a significantly unmasked pERK signal. Although our original lyse-fix technique (Method A) resulted in a higher pERK signal, poor resolution of WBC populations, and low CD3 intensity, this new approach resulted in an intermediate pERK signal, good resolution of WBC populations, and high CD3 intensity. Because unmasking is likely to be the result of protein denaturation and extraction, this intermediate pERK signal may be the result of increased retention of molecules that could be extracted by the alcohol step, but are retained by the additional cross-linking at higher formaldehyde concentration. For pERK and other highly expressed epitopes, this is not a severe limitation. The fraction of pERK-positive cells remains the same. Detecting less than the total number of epitopes in the cells becomes important only when the total number of epitopes is low to begin with, or in the hypothetical case that detection is not random with respect to other variables, e.g. epitope localization, and that non-randomness effects detectability. For an epitope assay system that yields a 30% coefficient of variation (normal or log-normal distributions), if the stimulated state is 5 to 10 times the unstimulated state, and the new method imparts a 66% reduction in the stimulated signal, the fractional increase and the MFI shift are completely resolvable by standard cytometric analyses of immunofluorescence as reported by Sladek and Jacobberger (21). We recognize that this may not be sufficient for some epitopes. In that case, the level of alcohol can be increased with a compromise on light scatter and level of expression of some surface markers.

At present, we do not have data that would indicate whether the fix-lyse (F/TX/MeOH) approach could provide a signal that is biologically equivalent to that of Method A (high MeOH). However, although we do not have evidence that our original technique provides biologically relevant measurements, we do know that the signal is equivalent to western blot analysis. In general, our experience indicates that fluorescence-based cytometric measurements are more precise but less sensitive than western blots (5,16). Further, by comparing western blotting with cytometry for samples that have been extracted with various solutions (Frisa and Jacobberger, unpublished data), we have come to the expected conclusion that we detect only a fraction of the intracellular epitope by cytometry, i.e., a significant portion of the epitope is not available for staining after alcohol fixation/permeabilization and staining at antibody saturation. The effect of this situation is that the amplitude of response in an experiment is lower compared with western blotting, which is likely to be lower compared with the native state of the cell. Thus, we would argue that our improved method (F/TX/MeOH) is sufficient for the majority of studies in which we wish to preserve light scatter and surface immunophenotype (yet wish to rapidly stop a whole blood

reaction), and in which our expected signal is sufficiently large above an appropriate background signal.

One benefit to alcohol treatment (as in the original method, or in F/TX/MeOH method from this study) is that it allows storage of fixed samples at −20°C for prolonged periods. Although our studies have only measured the impact of short-term storage (24 h with minimal change in pERK expression), a previously published study has suggested a 20% to 30% decrease in the measured levels of several different phospho-epitopes (as measured by flow cytometry) after as much as 5 months storage of samples in alcohol at −20°C (3). Frisa and Jacobberger (unpublished observations) measured decreases in each of several non–phospho epitopes in cells stored in 90% MeOH within a 6-month period. However, the loss of detectability is a complex process that appears to be a balance of chemical changes in the epitope under study and other masking elements; therefore, each epitope should be investigated individually before long-term storage.

In summary, we have reported the development of a protocol that allows processing of whole blood samples using an initial fixation step, followed by a detergent treatment step to permeabilize WBCs and lyse RBCs. A final methanol treatment step (which can be omitted, depending on specific assay requirements) improves the signal-to-noise ratio for pERK expression (and other phospho-epitopes, such as p-AKT and p-S6, manuscript in preparation) and can provide a medium for short-term sample storage at −20° C. The significant advantages of this protocol over previously reported techniques for the analysis of intracellular phospho-epitopes (1–8) include the initial fixation step, which maximizes the likelihood of phospho-epitope retention, the ability to use samples containing RBCs, and preservation of light scatter and representative cell surface (CD) determinants needed for clinical cytometry, while providing a detectable and acceptable pERK signal that is significantly above background levels.

LITERATURE CITED

1. Chow S, Patel H, Hedley DW. Measurement of MAP kinase activation by flow cytometry using phospho-specific antibodies to MEK and ERK: potential for pharmacodynamic monitoring of signal transduction inhibitors. Cytometry 2001;46:72–78.
2. Perez OD, Nolan GP. Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry. Nat Biotechnol 2002;20:155–162.
3. Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. Cytometry 2003;55A:61–70.
4. Krutzik PO, Iish JM, Nolan GP, Perez OD. Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical application. Clin Immunol 2004;110:206–221.
5. Jacobberger JW, Sramkoski RM, Frisa PS, Ye PP, Gottlieb MA, Hedley DW, Shankey TV, Smith BL, Paniagua M, Goolsby CL. Immunoreactivity of Stat5 phosphorylated on tyrosine as a cell-based measure of Bcr/Abl kinase activity. Cytometry 2003;54A:75–88.
6. Pallis M, Seedhouse C, Grundy M, Russell N. Flow cytometric measurement of phosphorylated STAT5 in AML: Lack of specific association with FLT3 internal tandem duplications. Leuk Res 2003;27:803–805.
7. Irish JM, Hovland R, Krutzik PO, Perez OD, Bruserud O, Gjertsen BT, Nolan GP. Single cell profiling of potentiated phospho-protein networks in cancer cells. Cell 2004;118:217–228.
8. Desplat V, Lagarde V, Belloc F, Chollet F, Leguay T, Pasquet J-M, Praloran V, Mahon F-X. Rapid detection of phosphotyrosine proteins by flow cytometric analysis in Bcr-Abl–positive cells. Cytometry 2004;62A:35–45.
9. Riley JS. Statistical analysis and optimal classification of blood cell populations using Gaussian distributions (PhD dissertation). Miami: Florida International University; 2003.
10. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;1:307–310.
11. Westgard JO, Hunt MR. Use and interpretation of common statistical tests in method-comparison studies. Clin Chem 1973;19:49–57.
12. Krouwer JS. Setting performance goals and evaluating total analytical error for diagnostic assays. Clin Chem 2002;48:919–927.
13. Magari RT. Bias estimation in method comparison studies. J Biopharm Stat 2004;14:881–892.
14. Schimenti JK, Jacobberger JW. Fixation of mammalian cells for flow cytometric evaluation of DNA content and nuclear immunofluorescence. Cytometry 1992;13:48–59.
15. Bauer KD, Jacobberger JW. Analysis of intracellular proteins. Methods Cell Biol 1994;41:351–376.
16. Jacobberger JW. Flow cytometric analysis of intracellular protein epitopes. In: Stewart C, Nicholson J, editors. Immunophenotyping.New York: Wiley-Liss; 2000. p 361–405.
17. Jacobberger JW. Intracellular antigen staining: quantitative immunofluorescence. Methods Enzymol 1991;2:207–218.
18. Francis C, Connelly MC. Rapid single-step method for flow cytometric detection of surface and intracellular antigens using whole blood. Cytometry 1996;25:58–70.
19. Macey MC, McCarthy DA, Milne T, Cavenagh JD, Newland AC. Comparative study of five commercial reagents for preparing normal and leukaemic lymphocytes for immunophenotypic analysis by flow cytometry. Cytometry 1999;38:153–160.
20. Carbonari M, Tedesco T, Fiorille MA. A unified procedure for conservative (morphology) and integral (DNA and immunophenotyping) cell staining for flow cytometry. Cytometry 2001;44:120–125.
21. Sladek TT, Jacobberger JW. Flow cytometric titration of retroviral expression vectors: comparison of methods for analysis of immunofluorescence histograms derived from cells expressing low antigen levels. Cytometry 1993;14:23–31.