

## A Whole-Blood Assay for Qualitative and Semiquantitative Measurements of CD69 Surface Expression on CD4 and CD8 T Lymphocytes Using Flow Cytometry

LONY C. L. LIM, MICHELLE N. FIORDALISI, JANET L. MANTELL, JOHN L. SCHMITZ,\*  
AND JAMES D. FOLDS

*McLendon Clinical Laboratories, Clinical Immunology Laboratory, University of North Carolina Hospitals, Chapel Hill, North Carolina 27514*

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**A whole-blood flow cytometry-based assay was utilized to assess CD4 and CD8 T-lymphocyte activation in response to phytohemagglutinin (PHA) stimulation. T-lymphocyte activation was assessed by qualitative (percent CD69) and semiquantitative (anti-CD69 antibody binding capacity) measurements of CD69 surface expression. Whole-blood samples from 21 healthy and 21 human immunodeficiency virus (HIV)-infected (<500 absolute CD4 counts per mm<sup>3</sup>) individuals were stimulated with 20 µg of PHA per ml for 18 to 24 h. The proportions of activated CD4 and CD8 T lymphocytes expressing CD69 (percent CD69) and the levels of CD69 expression on each T-lymphocyte subset (anti-CD69 antibody binding capacity) were measured. By using this assay system, T-lymphocyte activation was impaired in both CD4 and CD8 T-lymphocyte subsets of HIV-infected individuals. The proportions of CD69-positive CD4 and CD8 T lymphocytes were 43 and 27% lower, respectively, in samples from HIV-infected individuals compared to samples from healthy individuals. Similarly, the levels of CD69 expression on each activated CD4 and CD8 T-lymphocyte subset were 48 and 51% lower, respectively. These results suggest that both qualitative and semiquantitative measurements of CD69 surface expression by flow cytometry can be used to assess T-lymphocyte activation.**

CD69 is one of the earliest activation antigens that is expressed on the surface of activated peripheral blood T lymphocytes upon in vitro stimulation with provocative stimuli (1–4, 9, 12, 16, 17, 22, 27). It is usually not expressed on resting T lymphocytes. CD69 surface expression precedes DNA synthesis during cell cycle kinetics and CD25, CD71, and HLA-DR activation antigen expression (1, 2). Induction of CD69 surface expression occurs within 1 to 2 h after triggering of the protein kinase C activation pathway and calcium mobilization by engagement of the T-cell receptor/CD3 complex or stimulation with phorbol esters (3, 4, 22, 27). CD69 expression peaks between 16 and 24 h and then gradually declines (1, 2, 27). Engagement of other receptors like CD2/CD28 (16, 17, 19) and CD5/CD28 (29) also induces CD69 expression. In addition, mitogens, superantigens, alloantigens, and specific antigens all induce CD69 surface expression (1–3, 6, 9, 12, 15–17, 19, 25). However, the proportion of T lymphocytes that express CD69 varies with the different stimulants.

Several investigators have demonstrated the efficacy of measuring CD69 surface expression on T lymphocytes as a method to assess T-lymphocyte activation and function (1, 2, 10, 15–17, 19, 21, 25). The clinical laboratory utility of assessing T-lymphocyte activation has been studied in human immunodeficiency virus (HIV)-infected individuals (15, 19, 21) and individuals with type I diabetes (10) and systemic lupus erythematosus (20). A significantly lower proportion of CD69-activated T lymphocytes were detected in peripheral blood mononuclear cells (PBMC) from HIV-infected individuals stimulated with phytohemagglutinin (PHA) (15) and anti-CD3

monoclonal antibodies (19, 21), in comparison to PBMC from healthy individuals. Similarly, when PBMC from individuals with type I diabetes and systemic lupus erythematosus were stimulated with PHA, lower proportions of CD69-activated T lymphocytes were detected (10, 20).

The methodologies employed in the above studies measured the proportion of T lymphocytes expressing CD69 but did not measure the levels of CD69 expression (10, 15, 19, 21). We utilized a modified whole-blood flow cytometry-based assay (16) to assess T-lymphocyte activation by the qualitative (percent CD69) and semiquantitative (anti-CD69 antibody binding capacity [ABC]) measurements of CD69 surface expression. Whole-blood samples from healthy and HIV-infected (<500 absolute CD4 counts per mm<sup>3</sup>) individuals were stimulated with PHA, and the proportion of activated CD4 and CD8 T-lymphocyte subsets expressing CD69 (percent CD69) and the level of CD69 expression on each activated T-lymphocyte subset (anti-CD69 ABC) were measured.

### MATERIALS AND METHODS

**Whole-blood samples.** Whole-blood samples were obtained from healthy and HIV-infected (<500 absolute CD4 counts per mm<sup>3</sup>) individuals. Samples were collected in sterile green-top Vacutainer tubes containing sodium heparin anticoagulant (Becton Dickinson Systems, Franklin Lakes, N.J.) and processed within 6 h. Informed consent was obtained from all volunteers according to the policies of the University of North Carolina internal review board.

**Monoclonal antibodies.** Mouse anti-human CD3 fluorescein isothiocyanate (FITC), CD5 FITC, CD8 PerCP, and CD69 phycoerythrin (PE)-conjugated monoclonal antibodies were obtained from Becton Dickinson Immunocytometry Systems (San Jose, Calif.). Mouse anti-human CD4 CyC and CD45 PE-conjugated monoclonal antibodies were obtained from PharMingen (San Diego, Calif.). Appropriate immunoglobulin G1 and IgG2a fluorochrome-conjugated antibodies used as isotype controls were also obtained from both commercial vendors.

**Whole-blood activation assay.** Briefly, 125-µl samples of whole blood were added to 125 µl of RPMI 1640 culture medium (Sigma, St. Louis, Mo.) with and without PHA (Sigma) in round-bottom wells of a 96-well microtiter plate (Corning Glass Works, Corning, N.Y.). The microtiter plate was incubated at 37°C, 5%

\* Corresponding author. Mailing address: McLendon Clinical Laboratories, Clinical Immunology Laboratory, University of North Carolina Hospital, CB#7600, 1035 East Wing, 101 Manning Dr., Chapel Hill, NC 27514. Phone: (919) 966-8453. Fax: (919) 966-0486. E-mail: jschmitz.dh11@mail.unch.unc.edu.

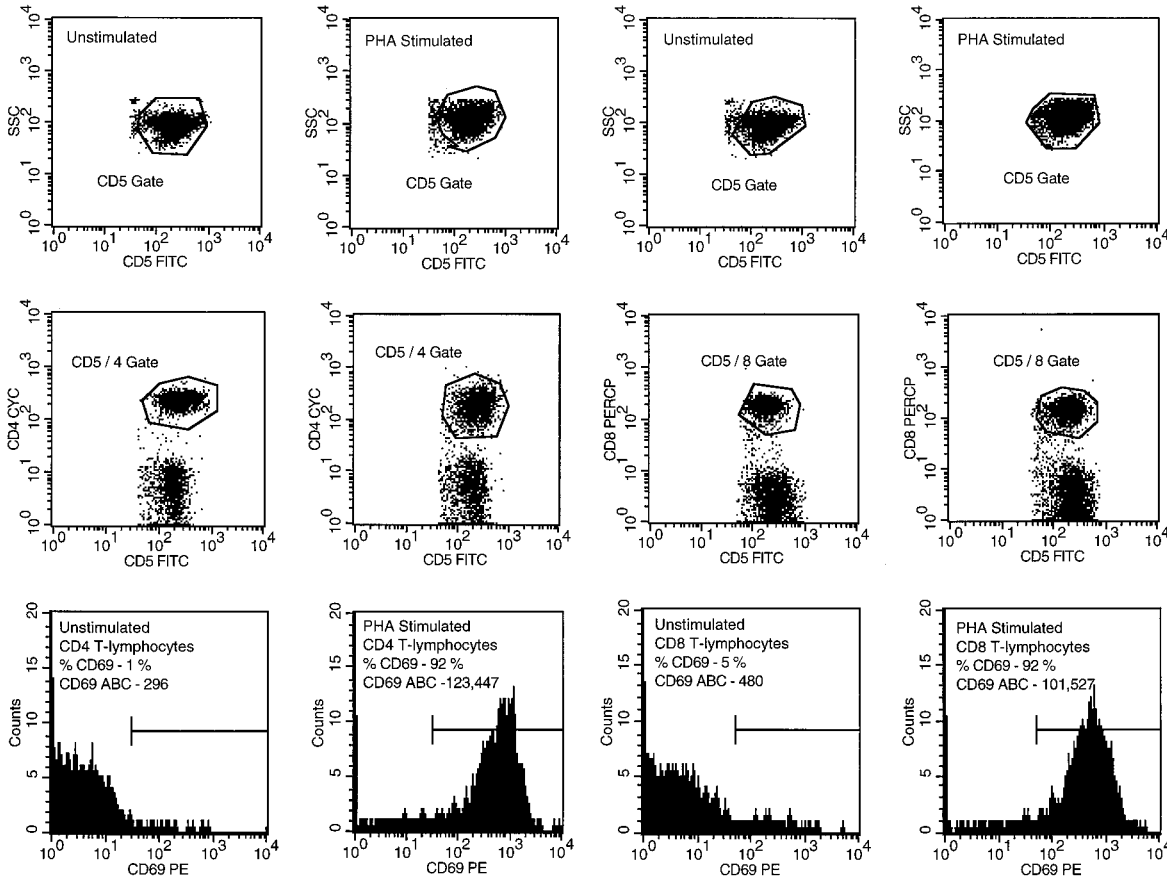


FIG. 1. Flow cytometric gating strategy used to identify CD69 surface expression on CD4 (left paired columns) and CD8 (right paired columns) T lymphocytes. Dot and histogram plots of unstimulated and PHA (20 µg per ml) stimulated whole-blood samples from a representative normal healthy individual. Initially, live CD5 gates (top row) were established to identify and differentiate CD5-positive lymphocytes. From these gates, CD5/4 and CD5/8 subgates (middle row) were derived for double positive CD5/4 and CD5/8 T lymphocytes, respectively. CD69 expression (bottom row) on each CD4 and CD8 T lymphocyte subset was then determined from the CD5/4 and CD5/8 gates, respectively. Percent CD69 and anti-CD69 ABC values are also shown.

CO<sub>2</sub> for 18 to 24 h. The final concentration of PHA used ranged from 2.5 to 20 µg per ml.

**Lyse-no-wash sample preparation for flow cytometry.** After 18 to 24 h, 100-µl samples of whole blood incubated with and without PHA were added to 12- by 75-mm tubes (Becton Dickinson Labware, Lincoln Park, N.J.) containing 5 µl each of CD5 FITC, CD69 PE, and CD4 CyC or CD8 PerCP. Samples were then vortexed and incubated at room temperature for 15 min. After incubation, 500 µl of fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson Immunocytometry Systems) was added to each tube to lyse erythrocytes. The tubes were vortexed and incubated at room temperature for at least 30 min before flow cytometry data acquisition. Appropriate isotype controls were also prepared and processed in a similar manner. In other experiments, whole-blood samples incubated with and without PHA were stained with 5 µl each of CD45 PE and CD3 FITC or CD5 FITC and processed as described above.

**Qualitative and semiquantitative measurement of CD69 surface expression by using flow cytometry.** All samples were analyzed with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems) with Cell Quest software (Becton Dickinson Immunocytometry Systems) for data acquisition and analysis.

For qualitative measurements of CD69 surface expression on CD4 and CD8 T lymphocytes, the proportions of CD4 and CD8 T lymphocytes expressing CD69 (percent CD69) in PHA-stimulated and unstimulated samples were determined by using three-color flow cytometry acquisition and analysis. Briefly, by using live and logical gating strategies, CD4 and CD8 T lymphocytes were detected and differentiated by using forward and side scatter, CD5 FITC, CD4 CyC, and CD8 PerCP fluorescence parameters (Fig. 1, top and middle). Data for a minimum of 2,000 CD5 FITC/CD4 CyC and CD5 FITC/CD8 PerCP events were acquired and analyzed. Histograms of CD69 PE fluorescence were then established from CD5 FITC/CD4 CyC and CD5 FITC/CD8 PerCP gating parameters (Fig. 1, middle and bottom). Cursors were set to differentiate CD69-negative and CD69-positive cells in unstimulated samples. Using these cursor settings, the net proportions of CD4 and CD8 T lymphocytes expressing CD69 was determined by

subtracting the percentage of unstimulated CD69 positive cells from the percentage of PHA-stimulated CD69-positive cells in respective histograms. The use of different fluorochrome conjugated anti-CD4 CyC and anti-CD8 PerCP antibodies in combination with CD5 FITC did not cause any difficulties in identifying double positive CD5 FITC/CD4 CyC or CD5 FITC/CD8 PerCP T lymphocytes or affect compensation parameters of the FACScan flow cytometer. All experimental procedures were optimized prior to evaluating study samples.

The semiquantitative measurement of CD69 surface expression on CD4 and CD8 T-lymphocyte subsets was determined by measuring the levels of CD69 expression on each subset (anti-CD69 ABC) by using the Quantum Simply Cellular (QSC) kit (Flow Cytometry Standards Corp., San Juan, Puerto Rico). Briefly, the QSC kit contained a mixture of five sets of microbeads that were covalently coated with different quantities of polyclonal goat anti-mouse antibodies. Four sets of microbeads were coated with known calibrated quantities of goat anti-mouse antibody, and a fifth set of microbeads remained unbound. To determine the anti-CD69 ABC, 5 µl of CD69 PE was added to a 12- by 75-mm tube (Becton Dickinson Labware) containing a suspension of 50 µl of QSC microbeads and 60 µl of PBS buffer. The tube was then vortexed and incubated at room temperature for 15 min. After incubation, 500 µl of FACS lysing solution (Becton Dickinson Immunocytometry Systems) was added, and the tube was vortexed and incubated at room temperature for at least 30 min prior to flow cytometry data acquisition. By using forward scatter, side scatter, and CD69 PE fluorescence gating strategies, the microbead sets were detected and a total of 10,000 data events were acquired and analyzed. Histogram profiles of CD69 PE fluorescence were used to differentiate the microbead sets. The median channel fluorescence (MCF) of each set of microbead spheres was obtained and used with QuickCal software (Flow Cytometry Standards Corp.) to generate a standard curve calibration plot of anti-CD69 PE ABC according to the manufacturer's instructions. Using the calibration plot, anti-CD69 PE ABC of PHA-stimulated and -unstimulated CD4 and CD8 T-lymphocyte subsets were obtained by extrapolating MCF values from CD69 PE histogram plots of CD4 and CD8 T

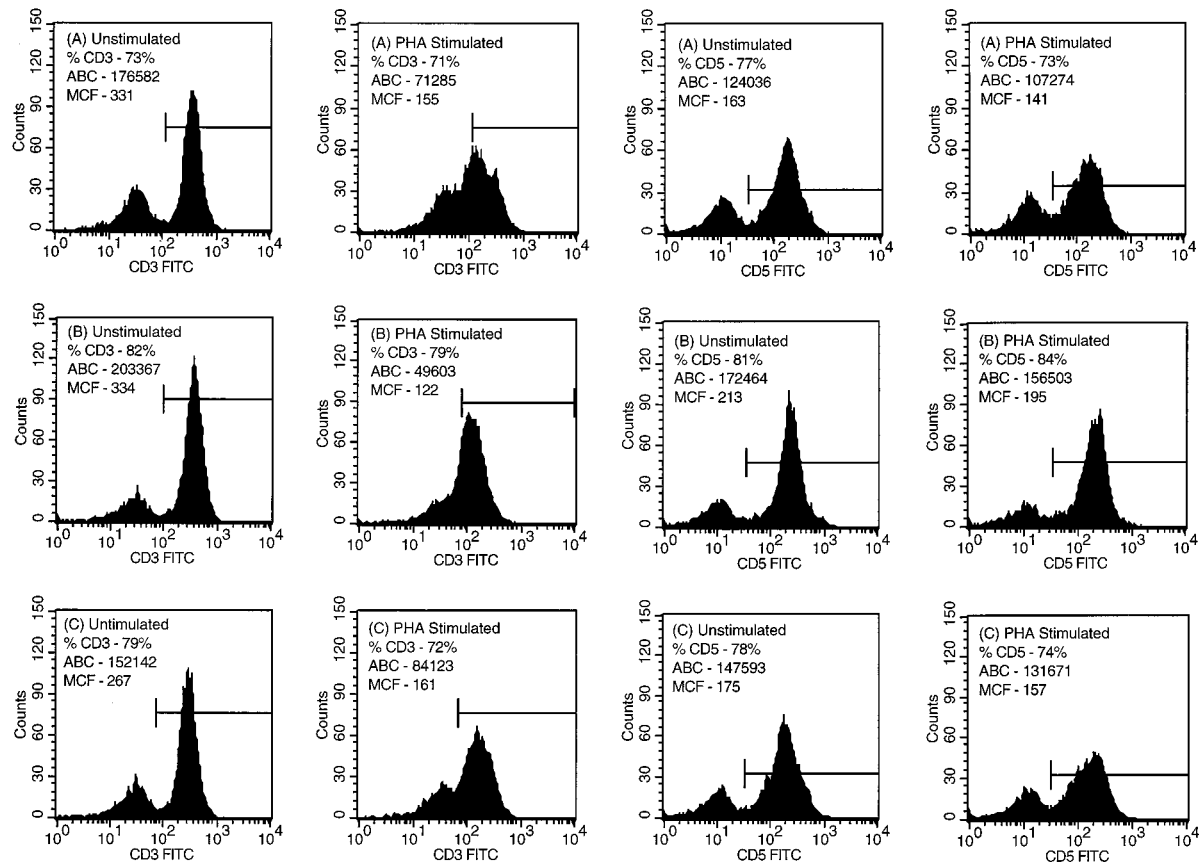


FIG. 2. Flow cytometric histogram plots of lymphocytes expressing CD3 FITC (left paired columns) and CD5 FITC (right paired columns) in whole-blood samples of healthy individuals stimulated with PHA (20  $\mu$ g per ml) and unstimulated. Histogram plots were derived from a lymphocyte gate using SSC versus CD45 gating strategy. Three representative samples from 10 healthy individuals are shown, (A) top, (B) middle, and (C) bottom rows. Percent CD3 and CD5, ABC, and MCF values are also shown.

lymphocytes expressing CD69. In other experiments, anti-CD3 FITC and anti-CD5 FITC ABCs were also obtained for PHA-stimulated and unstimulated lymphocytes.

**Semiquantitative measurement of CD3 and CD5 surface expression on PHA-stimulated lymphocytes.** The semiquantitative measurements of CD3 and CD5 expression on lymphocytes in PHA-stimulated and unstimulated whole-blood samples were determined by anti-CD3 FITC ABC and CD3 FITC MCF and anti-CD5 FITC ABC and CD5 FITC MCF, respectively. Using live and logical gating strategies, CD45-positive lymphocytes in whole-blood samples stimulated with PHA or unstimulated were detected and differentiated from other cell types by using forward scatter, side scatter, and CD45 PE fluorescence parameters. The anti-CD3 FITC and anti-CD5 FITC ABC, and CD3 FITC and CD4 FITC MCF values were then determined using histogram plots derived from CD45-positive lymphocyte gates. Data for a minimum of 5,000 CD3 FITC and CD5 FITC events were acquired and analyzed. Semiquantitative CD3 and CD5 ABC values were determined by using QSC beads (Flow Cytometry Standards Corp.) as described above.

**Statistics.** Experimental values obtained were subjected to analysis by using the Mann-Whitney U test to determine statistical significance. Values of  $P$  that were  $<0.05$  were considered to be statistically significant.

## RESULTS

**Decreased CD3 surface expression on lymphocytes after PHA stimulation.** The levels of CD3 and CD5 surface expression on lymphocytes in whole-blood samples from 10 healthy individuals stimulated with PHA and unstimulated were measured by using flow cytometry. By comparing CD3 FITC and CD5 FITC fluorescence parameters after 18 to 24 h of whole-blood stimulation with PHA (Fig. 2), there were no significant

changes in the percentages of CD3- and CD5-positive lymphocytes in both PHA-stimulated (mean CD3,  $71\% \pm 6\%$ ; mean CD5,  $75\% \pm 9\%$ ) and unstimulated (mean CD3,  $73\% \pm 10\%$ ; mean CD5,  $74\% \pm 9\%$ ) samples. However, significant decreases in anti-CD3 FITC ABC (57%;  $P < 0.0001$ ) and CD3 FITC MCF (49%;  $P < 0.0001$ ) were measured in PHA-stimulated lymphocytes compared with unstimulated lymphocytes. By comparison, there were no significant decreases in anti-CD5 FITC ABC (7%;  $P = 0.2611$ ) and CD5 FITC MCF (7%;  $P = 0.3150$ ) in PHA-stimulated lymphocytes compared with unstimulated lymphocytes (Fig. 2). These results suggest that the use of CD3 as a pan-T-lymphocyte marker could prove problematic for identifying PHA-stimulated T lymphocytes correctly because of poor resolution of CD3-positive and CD3-negative cells. The decrease in CD3 modulation may result in the loss of CD3 cells from data analysis. Mardiney et al. (17) had previously reported similar decreased CD3 levels on PHA-stimulated lymphocytes. Due to the significant decrease in the level of CD3 following PHA stimulation, the pan-T-lymphocyte CD5 marker was used in combination with CD4 and CD8 markers to identify CD5/4 and CD5/8 T-lymphocyte subsets throughout this study.

**Titration of PHA measuring CD69 surface expression on activated CD4 and CD8 T lymphocytes.** CD4 and CD8 T lymphocytes in whole-blood samples from 12 healthy individuals (six females and six males) stimulated with 2.5, 5, 10, and 20  $\mu$ g

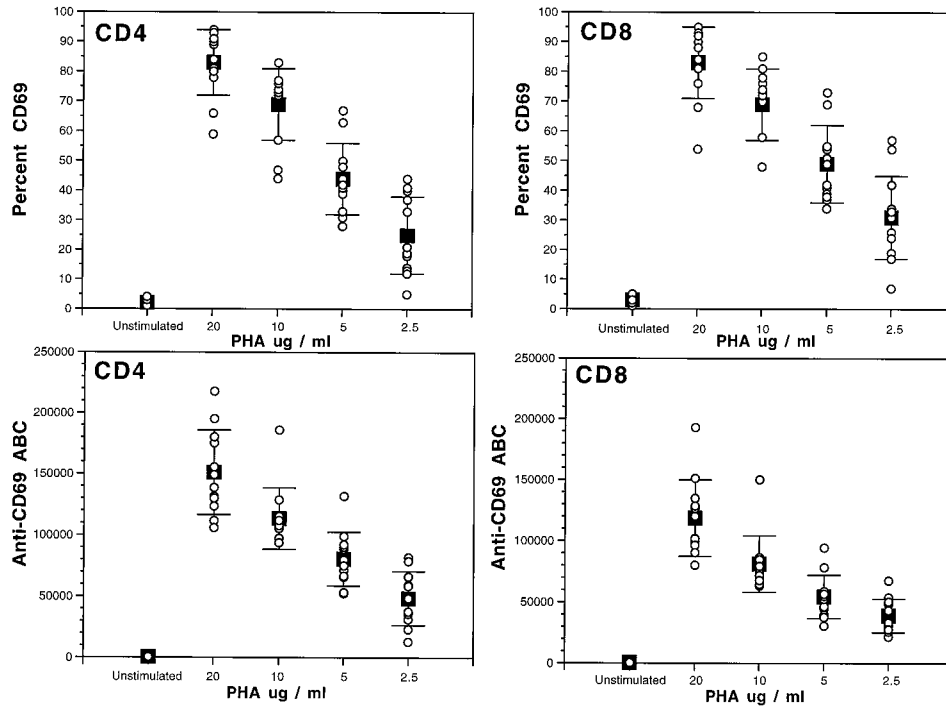


FIG. 3. The percent CD69 (top panels) and anti-CD69 antibody binding capacity (bottom panels) measurements of CD69 expression on CD4 T lymphocytes (left panel) and CD8 T lymphocytes (right panel) in whole-blood samples of 12 healthy individuals (open circles) stimulated with 2.5, 5, 10, and 20  $\mu\text{g}$  of PHA per ml and unstimulated. Mean values (filled squares) plus or minus a single standard deviation of all samples measured at each PHA concentration are shown.

of PHA per ml for 18 to 24 h were activated and expressed increasing amounts of CD69 with increasing concentrations of PHA as measured by flow cytometry analysis (Fig. 3). The concentration of 20  $\mu\text{g}$  of PHA per ml was optimal in stimulating the largest proportion of CD69-positive CD4 T lymphocytes (mean, 83%  $\pm$  11%) by comparison to unstimulated CD4 T-lymphocytes (mean 2%  $\pm$  1%). Similarly, at this PHA concentration, the largest proportion of CD69-positive CD8 T lymphocytes (mean, 83%) was also detected by comparison to unstimulated CD8 T lymphocytes (mean, 3%). Although the proportions of PHA-stimulated CD4 and CD8 T lymphocytes expressing CD69 were equal, the levels of CD69 expression were higher in the PHA-stimulated CD4 T-lymphocyte subset (mean, 151,179  $\pm$  34,650 ABC) than in the CD8 T-lymphocyte subset (mean, 118,658  $\pm$  31,223 ABC) compared to the unstimulated CD4 (mean, 359  $\pm$  132 ABC) and CD8 (mean, 449  $\pm$  242 ABC) T-lymphocyte subsets, respectively. Concentrations of PHA above 20  $\mu\text{g}$  per ml did not significantly increase the proportions of CD69-positive CD4 and CD8 T lymphocytes or the levels of CD69 expression on either T-lymphocyte subset (data not shown).

**CD69 surface expression on activated CD4 and CD8 T lymphocytes in whole-blood samples of healthy and HIV-infected individuals stimulated with PHA.** Whole-blood samples from 21 healthy and 21 HIV-infected (<500 absolute CD4 counts per  $\text{mm}^3$ ) individuals were stimulated with 20  $\mu\text{g}$  of PHA per ml for 18 to 24 h. By measuring the proportions of CD4 and CD8 T lymphocytes expressing CD69, significantly lower proportions of CD69-positive CD4 and CD8 T lymphocytes were detected in samples from HIV-infected individuals by comparison to samples from healthy individuals ( $P < 0.0001$ ) (Fig. 4 and Table 1). The proportions of CD69-positive CD4 and CD8 T lymphocytes in samples from HIV-infected individuals

were 43 and 27% lower, respectively, compared to samples from healthy individuals. Similarly, the levels of CD69 expression on activated CD4 and CD8 T-lymphocyte subsets were also significantly lower in samples from HIV-infected individuals ( $P < 0.0001$ ) (Fig. 4 and Table 1). The levels of CD69 expression on each activated CD4 and CD8 T lymphocyte subset in samples from HIV-infected individuals were 48 and 51% lower, respectively, compared to samples from healthy individuals.

**DISCUSSION**

T-lymphocyte function can be assessed by measurement of cell proliferation in the presence of different stimulants, such as mitogens, alloantigens, and specific antigens (7, 13). Traditionally, cell proliferation is determined in vitro by incubating purified PBMC with the provocative T-lymphocyte stimulant over several days and measuring the amount of newly synthesized DNA by using radiolabeled thymidine incorporation (7). By using similar in vitro stimulation procedures, several investigators have demonstrated the efficacy of measuring T-lymphocyte activation as a measure of T-lymphocyte function by determining the proportion of activated CD69 T lymphocytes by using flow cytometry (2, 10, 15-17, 19, 21, 25). Efficacy studies measuring peripheral blood T-lymphocyte responses in healthy individuals to in vitro stimulation with PHA (2, 10, 15, 17), concanavalin A (17), staphylococcal enterotoxin B (2, 25), and soluble anti-CD2/CD2R (16, 17) and anti-CD3 (21, 25) monoclonal antibodies have demonstrated the use of CD69 measurement to parallel and predict cell proliferation measurements that use radiolabeled thymidine incorporation. Furthermore, comparisons of CD69 measurement and radiolabeled thymidine incorporation have also been made in HIV-

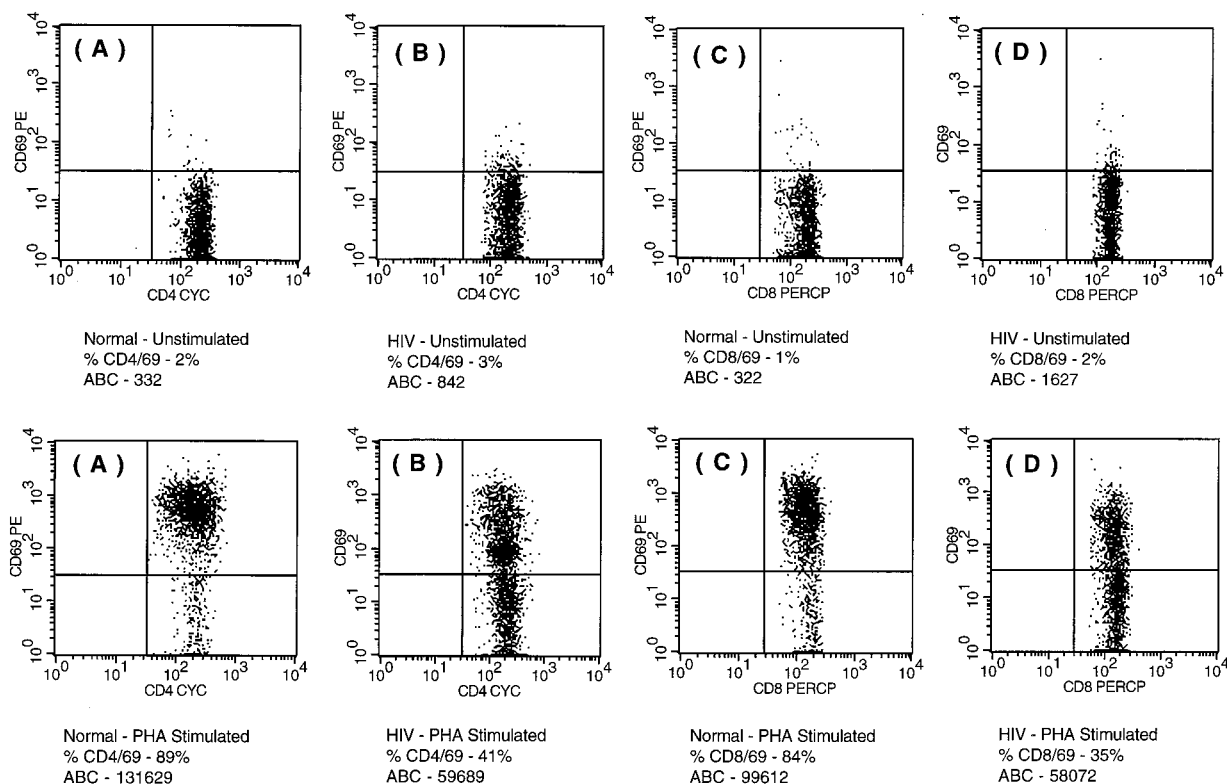


FIG. 4. Flow cytometric dot plots of CD69 expression on unstimulated (top row) and PHA stimulated (bottom row) CD4 (left paired columns) and CD8 (right paired columns) T lymphocytes from a representative healthy individual (A and C) and HIV-infected individual (B, D). Percent CD69 and anti-CD69 ABC values are also shown.

infected individuals (15, 19, 21) and individuals with type I diabetes (10). CD69 measurements correctly predicted radiolabeled thymidine incorporation measurements as an assessment of T-lymphocyte function (10, 15, 19, 21). A significantly lower proportion of CD69-positive T lymphocytes and lower levels of radiolabeled thymidine incorporation were detected in PBMC from HIV-infected individuals stimulated with PHA (15) and anti-CD3 monoclonal antibodies (19, 21) than in PBMC from healthy individuals. Similarly, when PBMC from individuals with type I diabetes were stimulated with PHA, a lower proportion of CD69-positive T lymphocytes and lower levels of radiolabeled thymidine incorporation were detected (10). These studies have demonstrated the use of CD69 measurement to assess T-lymphocyte function in immunodeficiency states.

We adapted and modified a whole-blood flow cytometry-based assay to assess T-lymphocyte function by CD69 measurement of activated T lymphocytes (16). Specifically, whole-blood samples were incubated with PHA for 18 to 24 h, and the degree of T-lymphocyte activation was determined qualitatively by the proportion of activated CD4 and CD8 T lymphocytes expressing CD69 (percent CD69) and semiquantitatively by measuring the level of CD69 expression (anti-CD69 ABC) on each activated T-lymphocyte subset.

In our assay system, whole-blood samples were used in place of purified PBMC in cell preparations stimulated with PHA. Maino et al. (16) previously showed that there were no significant differences in the proportion of CD69-positive T lymphocytes when whole-blood samples or PBMC were stimulated with mitogen. The presence of erythrocytes and granulocytes in

whole-blood samples did not affect CD69 expression. The use of whole-blood samples also provided a physiologically suitable medium for stimulation (presence of hormones and cytokines) and reduced the necessity of cell separation, counting, and washing (16). Our assay system also required a small volume of less than 1 ml of whole blood to perform. This is advantageous for assessment of immunodeficiency in T-lymphocyte activation, especially in infants.

We chose PHA as the T-lymphocyte stimulant from a variety of T-lymphocyte polyclonal stimulants. In initial experiments with whole-blood samples from healthy individuals, PHA stimulated and activated the largest proportion of T lymphocytes expressing CD69. The proportion of T lymphocytes that were activated upon in vitro stimulation and expressed CD69 was dependent on the concentration of the PHA used. PHA stimulation induced, on average, greater than 75% of T lymphocytes from healthy individuals to express CD69. Concanavalin A, pokeweed mitogen and staphylococcal enterotoxin B stimulated smaller proportions (between 10 and 50%) of T lymphocytes to express CD69 (data not shown). Other investigators have also reported similar results (2, 9, 15–17, 25). This may be attributed to the different T-lymphocyte specificity of each stimulant. We also evaluated the use of soluble anti-CD2/CD2R and anti-CD3/CD28 paired monoclonal antibody combinations as polyclonal T-lymphocyte stimulants. When used in a soluble format, anti-CD2/2R and anti-CD3/28 monoclonal antibody combinations induced a heterogeneous response in healthy individuals. A range of 10 to 80% of CD69-positive T lymphocytes was detected in different individuals (data not shown). The heterogeneous response may be attributed in part

TABLE 1. Decreased CD69 surface expression on activated CD4 and CD8 T lymphocytes in HIV-infected individuals

Sample	Healthy individuals				HIV-infected individuals			
	CD4 T lymphocytes		CD8 T lymphocytes		CD4 T lymphocytes		CD8 T lymphocytes	
	% CD69	Anti-CD69 ABC	% CD69	Anti-CD69 ABC	% CD69	Anti-CD69 ABC	% CD69	Anti-CD69 ABC
1	88	131,297	83	99,290	60	89,491	58	31,717
2	90	174,974	88	119,622	58	80,220	65	36,920
3	80	129,739	79	101,659	34	103,272	40	73,386
4	83	179,810	84	126,102	52	86,713	53	52,663
5	89	123,151	88	101,247	62	80,672	84	49,743
6	92	217,498	90	192,705	65	56,915	73	32,491
7	89	138,260	89	134,424	53	65,335	67	47,303
8	65	155,115	64	95,592	36	58,847	33	56,445
9	75	110,962	73	79,103	69	77,992	84	60,495
10	88	148,939	87	128,282	50	86,761	72	76,680
11	76	105,419	81	90,048	54	75,389	63	52,768
12	61	198,440	58	129,427	43	59,642	52	46,621
13	63	178,781	62	136,935	49	130,214	63	86,031
14	64	154,313	66	113,011	26	83,877	37	52,170
15	64	114,491	60	77,498	73	134,893	64	64,984
16	90	137,511	89	99,994	24	34,041	51	28,244
17	84	123,089	77	121,650	43	105,406	63	78,218
18	85	169,599	87	162,257	29	56,384	40	77,197
19	94	125,879	92	116,976	67	94,874	74	66,515
20	84	232,378	87	147,327	27	47,479	45	70,725
21	71	136,666	70	106,801	7	38,019	38	65,755
Mean $\pm$ SD	80 $\pm$ 11 <sup>a</sup>	151,729 $\pm$ 34,106 <sup>b</sup>	75 $\pm$ 18 <sup>c</sup>	118,093 $\pm$ 26,865 <sup>d</sup>	47 $\pm$ 17 <sup>a</sup>	78,402 $\pm$ 26,014 <sup>b</sup>	58 $\pm$ 15 <sup>c</sup>	57,480 $\pm$ 16,284 <sup>d</sup>
% Decrease					41 <sup>e</sup>	48 <sup>e</sup>	23 <sup>e</sup>	51 <sup>e</sup>

<sup>a</sup> CD4 T lymphocytes, percent CD69, normal healthy individuals versus HIV-infected individuals,  $P < 0.0001$ .

<sup>b</sup> CD4 T lymphocytes, anti-CD69, ABC normal healthy individuals versus HIV-infected individuals,  $P < 0.0001$ .

<sup>c</sup> CD8 T lymphocytes, percent CD69, normal healthy individuals versus HIV-infected individuals,  $P < 0.0001$ .

<sup>d</sup> CD8 T lymphocytes, anti-CD69 ABC, normal healthy individuals versus HIV-infected individuals,  $P < 0.0001$ .

<sup>e</sup> Percent decrease = [(mean for normal healthy individuals - mean for HIV-infected individuals)/mean for normal healthy individuals]  $\times$  100.

to the responder and nonresponder effects due to the isotype of the monoclonal antibodies used (IgG1 versus IgG2A) and the presence of specific accessory cells that are required for binding and cross-linking the antibodies (14, 26). As a result of these observations, PHA was chosen as the T-lymphocyte stimulant because of its broad range of polyclonal T-lymphocyte stimulation and activation.

Following a dose response study of PHA concentrations (2.5 to 20  $\mu$ g per ml) used to stimulate T-lymphocytes, the optimal concentration for induction of CD69 expression was found to be 20  $\mu$ g per ml (Fig. 2). Concentrations above this did not significantly increase the proportions of CD69-positive CD4 and CD8 T-lymphocytes or the levels of CD69 expression on each T-lymphocyte subset (data not shown). When whole-blood samples from healthy individuals were stimulated with PHA (20  $\mu$ g per ml), equal proportions of CD4 and CD8 T lymphocytes expressing CD69 were activated, but the levels of CD69 expression on each activated CD4 and CD8 T-lymphocyte subset were different (Fig. 3 and 4 and Table 1). Higher levels of CD69 were expressed on the CD4 T-lymphocyte subset than on the CD8 T-lymphocyte subset. The reason for this difference is not known, and further studies are needed to confirm this finding. However, these results provide a baseline quantitation of CD69 expression on normal healthy PHA-activated CD4 and CD8 T-lymphocyte subsets. The measurement of CD69 levels was performed by using calibrated QSC beads that quantitated the amount of anti-CD69 antibody bound to CD69 antigens on each T-lymphocyte subset. It was not a direct measure of the number of CD69 antigens present on each T-lymphocyte subset population but a measurement of the antibody binding capacity of activated T lymphocytes for

anti-CD69 antibodies. This measurement provided a reliable assessment of CD69 levels of expression (24).

By using both qualitative (percent CD69) and semiquantitative (anti-CD69 ABC) baseline measurements of CD69 expression on PHA-activated CD4 and CD8 T-lymphocyte subsets of normal healthy individuals, impaired CD4 and CD8 T-lymphocyte activation responses were detected in whole-blood samples of HIV-infected individuals stimulated with PHA (Fig. 4 and Table 1). Impaired T-lymphocyte responses to PHA stimulation in HIV-infected individuals have been reported (5, 18, 23). Our results demonstrate that both qualitative and semiquantitative measurements of CD69 can be used to assess CD4 and CD8 T-lymphocyte activation to polyclonal stimulation with PHA. Within the CD4 T-lymphocyte subset, the proportion of PHA-activated CD4 T lymphocytes expressing CD69 in samples from HIV-infected individuals was 43% lower than that from healthy individuals. A 48% decrease in CD69 levels was also measured in PHA-activated CD4 T lymphocytes in samples from HIV-infected individuals. Similarly, the proportion of PHA-activated CD8 T lymphocytes expressing CD69 in samples from HIV-infected individuals was 26% lower, and the levels of CD69 expression decreased by 51%. The observed differences between CD4 and CD8 T-lymphocyte responses to PHA stimulation in HIV-infected individuals may be attributed to the immunopathogenesis of HIV resulting in different levels of T-lymphocyte subset dysfunction (11).

Qualitative CD69 measurements have been shown to parallel and predict cell proliferation measurements by using radio-labeled thymidine incorporation in immunodeficiency states (10, 15, 19, 21). Our finding that semiquantitative measure-

ment of CD69 levels paralleled the qualitative measurements provides a rationale for further study to determine if semi-quantitative measurements might be useful indicators of T-lymphocyte function in immunodeficiency states. Quantitative measurements of CD38 and HLA-DR antigen levels on CD8 T lymphocytes in HIV infection have been useful for disease staging and prognosis (11). Can measurements of CD69 levels predict whether activated T lymphocytes will continue into the cell cycle and proliferate? Because CD69 is one of the earliest activation antigens expressed on T lymphocytes upon in vitro stimulation, CD69 may serve as a costimulatory receptor to receive additional activation signals that will commit the cell towards proliferation. Other investigators have demonstrated that the engagement of the CD69 antigen induced cell proliferation (3, 8, 28). Further studies are needed to determine if a threshold level of CD69 expression can be measured to predict cell proliferation.

In summary, we have demonstrated that both qualitative and semiquantitative measurements of CD69 can be used to assess CD4 and CD8 T-lymphocyte activation upon polyclonal stimulation with PHA. Studies are under way to determine if both forms of CD69 measurement can accurately predict T-lymphocyte immune restoration in HIV-infected individuals treated with highly active antiretroviral therapy.

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