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Utility of Flow Cytometric Detection of CD69 Expression as a Rapid Method for Determining Poly- and Oligoclonal Lymphocyte Activation

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CD69 is a lymphoid activation antigen whose rapid expression (≤2 h postactivation) makes it amenable for the early detection of T-cell activation and for subset activation analyses. In the present study we evaluated the utility of flow cytometric detection of CD69 expression by T cells activated with polyclonal stimuli (anti-CD3 and staphylococcal enterotoxin B [SEB]) and oligoclonal stimuli (tetanus toxoid and allogeneic cells) using flow cytometry. Following activation of T cells with anti-CD3 or SEB, CD69 is detectable at ≤4 h following activation, with anti-CD3 peaks at 18 to 48 h. Dose titration experiments indicated that CD69 expression largely paralleled that in [^{3}H]thymidine incorporation assays, although the former offered a more sensitive measure of T-cell activation at limiting doses of activator than [^{3}H]thymidine incorporation when cells were activated with either anti-CD3 or SEB. However, activation of T cells with either tetanus toxoid or allogeneic stimulator cells failed to induce detectable CD69 expression at up to 7 days of culture. Subset analyses of anti-CD3- and SEB-activated T cells indicated that populations other than T cells can express CD69 following stimulation with T-cell-specific stimuli, indicating that CD69 can be induced indirectly in non-T cells present in the population. These findings indicate that CD69 is a useful marker for quantifying T-cell and T-cell subset activation in mixed populations but that its utility might be restricted to potent stimuli that are characterized by their ability to activate large numbers of cells with rapid kinetics.

The measurement of lymphocyte function and activation can provide useful information for assessing the level of immune reactivity in research or clinical applications or in cases in which immune competency could be important. Historically, in vitro assessments of lymphocyte activation have relied on the detection of lymphocyte DNA synthesis as an index of proliferation, although these approaches generally require extended incubation times and do not allow assessments of preferential activation of lymphoid subsets. The early activation antigen CD69 has recently generated interest as a possible marker for use in flow cytometry-based assays for cellular activation (11). CD69 is a member of the natural killer cell gene complex family that is expressed by T cells within 30 min of T-cell receptor ligation (6, 9, 11). CD69 is also expressed by natural killer cells activated by exposure to interleukin 2 or interleukin 12, as well as by all monocytes and by polymorphonuclear leukocytes (4, 5, 15). The fact that CD69 is rapidly expressed upon T-cell activation and is readily amenable to detection by immunofluorescence and flow cytometry suggests its possible utility as a marker for the rapid assessment of T-cell activation (6, 13). Although flow cytometry-based assays offer a number of advantages over radionuclide incorporation techniques, including their ease of performance and ability to be used in subset-specific analyses, it is not clear whether CD69 induction is a generally useful activation marker, nor is the extent to which it compares with thymidine incorporation assays clear.

In the present study we evaluated the induction of CD69 expression as a parameter for detecting T-cell activation following activation by various stimuli and compared it with assessments obtained by thymidine incorporation. The results from the study demonstrate the utility and sensitivity of CD69 detection for the activation of T cells by polyclonal stimuli but indicate that it is of limited usefulness for detecting stimulation by specific antigens or alloantigens.

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MATERIALS AND METHODS

Antibodies and reagents. Fluorochrome-conjugated anti-CD69 (phycoerythrin anti-Leu 23), anti-CD5 (fluorescein isothiocyanate [FITC]–anti-Leu 1), anti-CD4 (FITC–anti-Leu 3), and anti-CD8 (FITC–anti-Leu 2) were obtained from Becton Dickinson, San Jose, Calif. FITC-conjugated goat anti-mouse immunoglobulin G1 was obtained from Southern Biotech (Birmingham, Ala.), and OKT3 (anti-CD3) was obtained from Ortho Pharmaceuticals (Raritan, N.J.). Staphylococcal enterotoxin B (SEB), purified MOPC 21 (immunoglobulin G1), and propidium iodide were obtained from Sigma Chemical Co. (St. Louis, Mo.). Tetanus toxoid was obtained from the Massachusetts Department of Public Health (Boston, Mass.).

Healthy volunteers. A total of eight healthy volunteers (three females and five males) participated in the present institutional review board-approved study after providing informed consent. Six of the volunteers were Caucasian; two males were Asian. Volunteers had median ages of 39 years (age range, 37 to 41 years) for males and 28 years (age range, 27 to 37 years) for females.

Normal cells and cell lines. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by using Ficoll-Hypaque density gradients (12). Lymphoblastoid cell lines for use as allogeneic stimulator cells in mixed lymphocyte reactions were generated by culturing normal PBMCs with Epstein-Barr virus-containing culture supernatants collected from the MCU-V5 marmoset cell line.

Activation of PBMCs. PBMCs (10^6/ml) were cultured with the designated stimuli in either flat-bottom 24-well plates (phenotyping) or 96-well plates (thymidine incorporation assay) (Costar). Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum in the presence or absence of activators that included anti-CD3, SEB, or tetanus toxoid at the designated concentrations. For mixed lymphocyte reactions, cultures were established in RPMI 1640 supplemented with 15% autologous human serum containing 1×10^5 responder PBMCs and 5×10^4 irradiated stimulator cells. Stimulator cells were irradiated with a Gammacell irradiator at a dose of 2,500 R. For measurement of proliferation by the thymidine incorporation assay, cultures were pulsed with 1 μCi of [^{3}H]thymidine for the final 8 h of culture and the cells were harvested with a PHD cell harvester (Cambridge Technology). Incorporated radiolabel was quantified by beta scintillation counting. All comparisons of CD69 expression and thymidine incorporation were performed on parallel activation cultures.

Cell surface immunofluorescence. Cell surface immunofluorescence was car-

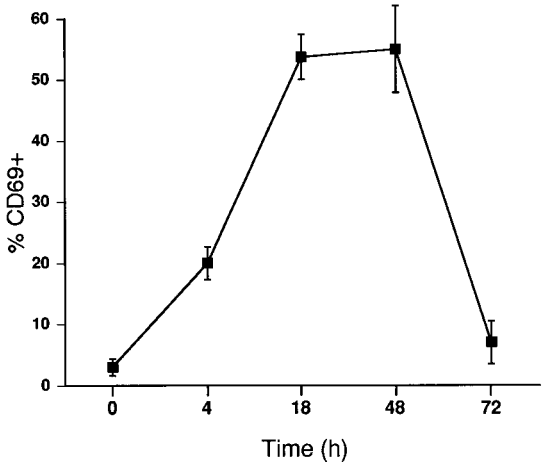

FIG. 1. Time course of CD69 expression on T cells following activation of PBMCs with 50 ng of anti-CD3 per ml. The results represent the means ± standard errors of the means of four independent experiments with four different donors.

ried out either with directly conjugated antibodies or, in some cases of CD69 detection, by indirect immunofluorescence. In either case, the cells were incubated for 30 min on ice with saturating amounts of the primary antibody and were washed three times with Hanks balanced salts solution (without phenol red) containing 5% newborn calf serum. For indirect immunofluorescence, the cells were subsequently incubated with an optimal concentration of FITC-goat anti-mouse immunoglobulin G1 on ice and were washed as described above for the primary antibody step. The cells were analyzed with a Becton Dickinson FACStar Plus instrument, and nonviable cells were excluded from the analysis by their ability to exclude the vital dye propidium iodide (50 μg/ml). A total of 10,000 events were analyzed for each sample.

RESULTS

Time course of CD69 induction. Initial experiments were performed to assess the time course of CD69 expression by PBMCs when they were stimulated with 50 ng of anti-CD3, a polyclonal T-cell activator, per ml. Consistent with previously published observations (11, 13), CD69 is rapidly expressed (≤4 h), and peak expression, measured in terms of percent $CD69^+$ cells, occurs at 18 to 48 h (Fig. 1). By 72 h of activation, the percentage of CD69-expressing cells is reduced to near background levels. Similar kinetics were observed following stimulation with 5 μg of SEB per ml (data not shown). The observation that the maximum percentage of $CD69^+$ cells in all cases (mean, 55% ± 7%) was less than the mean percentage of $CD3^+$ cells in PBMC preparations (mean, 67% ± 4%) suggested either that not all T cells express CD69 upon activation or that at the time of peak expression some of the T cells have already reverted to a $CD69^-$ phenotype.

Subsequent experiments were performed to test the sensitivity of the CD69 detection assay compared with that of the [^{3}H]thymidine incorporation assay. PBMCs were activated with decreasing concentrations of anti-CD3 antibody and were assayed for CD69 expression after 18 h of stimulation or were assayed for thymidine incorporation after 72 h of stimulation. The results, presented as a percentage of the maximal response achieved for each parameter (Fig. 2), showed a correlation between CD69 expression and thymidine incorporation by regression analysis ($r = 0.91$; $P < 0.05$). Furthermore, the data indicated that CD69 expression provides a more sensitive means of detection of activation than thymidine incorporation. Although the responses at the lower doses of anti-CD3 (0.05 and 0.1 ng/ml) varied among individuals and the lower doses yielded higher standard errors, for all individuals tested, the levels of CD69 expression were nevertheless discernible from control levels at each of these concentrations, whereas thymidine incorporation levels were indistinguishable from background levels. These results indicate that the induction of CD69 expression provides a more sensitive and earlier detection parameter for T-cell activation than thymidine incorporation when using suboptimal concentrations of a polyclonal stimulus.

Similar studies were performed with SEB, which does not polyclonally activate T cells but, rather, activates only those subsets that express the Vβ3, 12, 14, 15, 17, and 20 T-cell receptor families (2, 8). By using SEB, we attempted to determine whether the sensitivity of CD69 detection was significantly reduced relative to that of thymidine incorporation when a smaller percentage of T cells was activated. The results (Fig. 3) demonstrated a correlation between the relative numbers of $CD69^+$ cells and thymidine incorporation by regression analysis ($r = 0.98$; $P < 0.01$). The results also indicated that a higher percentage of maximal response is observed at limiting concentrations of SEB when CD69 is used as the activation parameter compared with that observed by thymidine incorporation. Thus, even under conditions in which smaller percentages of T cells are activated, CD69 expression offers a sensitive and early measure of in vitro T-cell activation. Moreover, for each activator, the CD69 dose-response curve largely paralleled that observed for thymidine incorporation, supporting the utility of CD69 expression as a semiquantitative assessment of activation.

Using two-color immunofluorescence, we explored the subset composition of the $CD69^+$ T cells that were activated by SEB and anti-CD3. Following T-cell activation with allogeneic cells and tetanus toxoid, the frequencies of $CD69^+$ cells ranged from 6 to 13% and 0 to 4%, respectively (measurements were made on days 3 to 6 and the range of daily mean values are given). The levels of proliferation ([^{3}H]thymidine incorporation) following T-cell activation with allogeneic cells and tetanus toxoid were 23,052 ± 4,750 and 5,462 ± 495 cpm, respectively. These results were obtained from three independent experiments with a total of three donors. Both anti-CD3 and

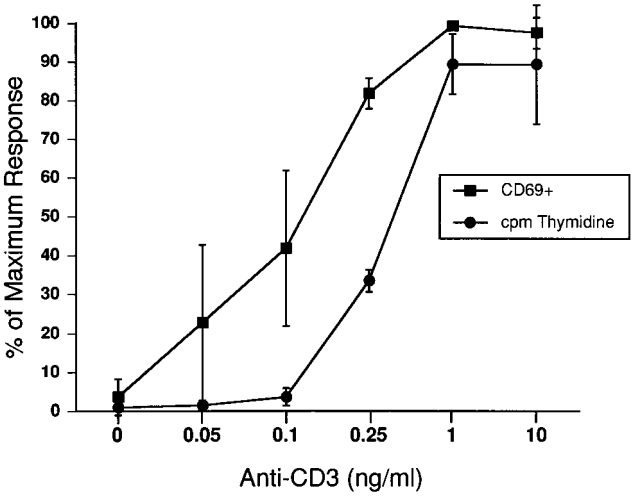

FIG. 2. Titration of anti-CD3 stimulatory effects on CD69 expression and [^{3}H]thymidine incorporation. CD69 expression and thymidine incorporation were assessed in parallel cultures at 24 and 72 h after the initiation of the respective cultures by using PBMCs collected from three different donors. Results are presented as the percentage of maximal values ± standard errors of the means.

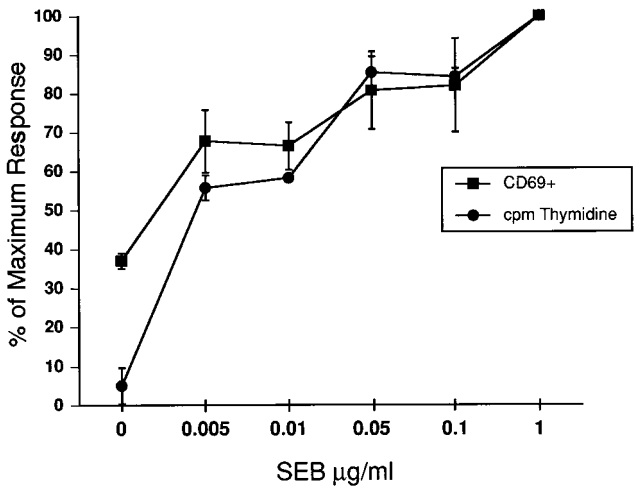

FIG. 3. Titration of SEB stimulatory effects on CD69 expression and [^{3}H]thymidine incorporation. CD69 expression and thymidine incorporation were assessed in parallel cultures at 24 and 72 h after the initiation of the respective cultures by using PBMCs collected from three different donors. Results are presented as the percentage of maximal values obtained for three independent experiments ± standard errors of the means.

SEB activated the induction of CD69 expression on both CD4 and CD8$^+$ T cells (Table 1). In the case of anti-CD3, the relative percentages of CD4 and CD8 cells coexpressing CD69 mimicked their relative percentages prior to activation, supporting the notion that anti-CD3 activates both populations equally. Furthermore, CD69$^+$ cells induced by anti-CD3 were entirely CD4$^+$ or CD8$^+$, indicating that the expression was restricted to T cells. Even though SEB is known to preferentially activate CD4$^+$ T-cell proliferation (7), immunofluorescence analysis revealed that the CD69$^+$ population comprised both CD4$^+$ and CD8$^+$ cells at ratios comparable to those found in the starting populations (approximately 2:1). Furthermore, a small but reproducible percentage of CD69$^+$ cells induced by SEB did not express either CD4 or CD8, and subsequent analyses suggested that these cells comprised B cells and monocytes (data not shown). These results indicate that CD69 was induced on cells that were not directly activated to proliferation by antigen receptor engagement but might have been activated to CD69 expression through the indirect effects of soluble factors released by activated T cells.

Subsequent experiments were performed to evaluate the level of expression of CD69 following antigen-specific activation in order to determine whether CD69 expression is detectable and quantitative under more stringent conditions of activation. Toward this end we activated PBMCs with alloantigenic stimulator cells or with the soluble antigen, tetanus toxoid. Both stimuli induced significant proliferative responses when the responses were measured 6 days following culture initiation. Despite significant proliferation induced by either stimulus, CD69 expression was not significantly increased above background levels at any time point at which values were obtained following culture initiation (days 3 to 6). Thus, any induction of CD69 expression that might occur following activation by either of the specific activators occurs below the threshold of useful detectability by the techniques applied in the present studies.

DISCUSSION

CD69 is an activation marker expressed by T cells and other cell types early following exposure to a stimulus. In the case of T cells, CD69 expression is inducible within 3 h and is expressed in the membrane at levels that allow easy detection by flow cytometry. These characteristics suggest that CD69 may serve as a useful marker for the sensitive and rapid detection of T-cell activation by flow cytometric-based assays very early following activation.

In the present study we examined the parameters of CD69 expression and detection under various conditions of T-cell activation and compared CD69 detection with a more standard measure of T-cell activation represented by the incorporation of tritiated thymidine. As a radionuclide-based assay, tritiated thymidine incorporation can be regarded as a highly sensitive method of detecting DNA synthesis. Despite this, CD69 expression was more readily detectable than thymidine incorporation following activation with suboptimal concentrations of anti-CD3 or SEB. In this regard, CD69 expression allows for the more sensitive detection of activation under conditions at concentrations of activator that do not induce measurable proliferative responses. Moreover, CD69 expression is detectable at earlier time points than thymidine incorporation. CD69 expression also appears to provide a reasonable semiquantitative measure of activation on the basis of the observation that the dose-response curve of CD69 expression parallels closely the dose-response curve observed for thymidine incorporation. Thus, it should be possible to use CD69 to provide a simple and semiquantitative measure of T-cell activation.

The ability of CD69 expression to more sensitively detect activation at lower concentrations of activator than are required for detection by thymidine incorporation is consistent with the possibility that these parameters measure qualitatively distinct properties of activated cells. That is, the signaling requirements for the induction of cellular proliferation might be more stringent than those required for the induction of CD69 expression. In this way, cells that are "partially" activated (e.g., by cytokines alone or receptor engagement alone) might express CD69 but fail to go on to proliferate. Although previous reports indicate that for T-cell expression of CD69 receptor engagement must occur, CD69 can also be induced on other cells, such as natural killer cells, by soluble factors alone (1, 3, 9, 14). This latter observation supports the concept that not all CD69-expressing cells in a population, although activated, go on to full proliferation without additional signals. Furthermore, the ability of cells such as natural killer cells to express CD69 following cytokine exposure alone might explain the observation that not all CD69-expressing cells in activated PBMCs are T cells (9). The ability of soluble factors to amplify CD69 expression and detectability may explain the greater sensitivity of CD69 expression compared with that of thymidine incorporation in detecting activation by suboptimal concentrations of polyclonal activation, although it also suggests

TABLE 1. CD4 and CD8 subset composition of CD69$^+$ cells following activation with anti-CD3 or SEB[a]

Stimulus	% CD69$^+$ cells	% of CD69$^+$ cells coexpressing the following:	
		CD4	CD8
None	4 ± 1	ND[b]	ND
Anti-CD3 (50 ng/ml)	57 ± 3	73 ± 5	31 ± 5
SEB (0.1 µg/ml)	56 ± 7	56 ± 11	27 ± 3

[a] Paired results obtained with cells from three donors stimulated with either anti-CD3 or SEB in the same experiment.
[b] ND, not determined.

that induction of CD69 expression differs functionally from thymidine incorporation assays.

CD69 expression for assessments of T-cell activation appears to be best suited for assessing activation induced by polyclonal activators. Activation by specific recall antigens or alloantigens either failed to induce CD69 expression or induced levels of CD69 that were too low to allow meaningful analysis of activation levels. The inability to detect CD69 expression under such circumstances might reflect the fact that such responses involve fewer cells and exhibit more prolonged kinetics of induction. In this way the numbers of CD69-expressing cells at any one time point might be below the threshold of detection by flow cytometry.

In contrast to these findings, other investigators reported the induction of CD69 expression on 4.4% of T cells following a 4-h exposure of whole blood to the recall antigen *Candida albicans* (11). The reason for the discrepancy with these earlier findings and our present inability to detect CD69 following stimulation with tetanus toxoid or allogeneic cells is unclear, but it may be due in part to the highly unorthodox and unique response engendered by *C. albicans*. Specifically, this response involved an abnormally higher fraction (1:25) of activated T cells than would be expected for recall antigen-specific T-cell clones (10). Furthermore, the fact that the response was restricted to $CD4^-$ T cells is difficult to reconcile with the expectation that $CD4^+$ T cells would be activated under such conditions and are likely required for activation of the $CD4^-$ subset. Thus, it is likely that the expression of CD69 in response to *C. albicans* may result from other, nonspecific processes such as the effects of antigen-antibody complexes or direct activation of $\delta\gamma$ T cells or by superantigen effects.

Although CD69 expression is a consequence of T-cell activation, the functional significance of CD69 expression remains unknown. The fact that a large percentage of $CD4^-$ cells express CD69 after activation with SEB (which preferentially activates $CD4^+$ T cells) suggests the possibility that cells other than those directly activated by stimuli might express CD69. This possibility is further supported by the observation that a $CD3^-$ subset expresses CD69 following activation with anti-CD3. Thus, CD69 expression may not completely overlap with cells that undergo DNA synthesis following antigen receptor engagement. Although the immunologic consequences of lymphocyte clonal expansion are well established and critical to the expression of immunity, the relevance of CD69 expression to this process is less well understood. Consequently, despite its utility as a marker of activation, CD69 expression does not yet provide a definable insight into the functional significance and/or consequences of CD69 expression; this point should be considered when attempting to interpret the results of CD69 expression experiments.

Despite these theoretical limitations, the induction of CD69 expression following T-cell activation represents a sensitive, rapid, and semiquantitative method for detecting activation. In view of the difficulties inherent in the use of radionuclide-based proliferation assays, CD69 expression provides an attractive alternative methodology, with the added capability of allowing analysis of activation of individual T-cell subsets.

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