

# Dithiothreitol has a dose-response effect on cell surface antigen expression

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**Background:** The use of Dithiothreitol (DTT) to improve cell dispersion is an integral step in induced sputum examination, which has become an important noninvasive method of assessing airway inflammation. Several studies have shown that sputum treatment with DTT does not affect cell morphology, differential cell counts, and cytokine levels in the supernatant. However, the effect of DTT on cell surface marker expression has not been systematically studied.

**Objective:** We have investigated the effect of different DTT concentrations on antigen expression on peripheral blood cells compared with antigen expression on PBS-treated cells.

**Methods:** Peripheral blood from different healthy donors was incubated with either DTT or PBS, washed, and then incubated with different fluorescence-labeled antibodies. Analysis was performed after lysis of erythrocytes on a calibrated flow cytometer. Respective cell populations were identified, and the mean fluorescence intensity of surface-marker expression for each cell population was compared between DTT- and PBS-treated cells.

**Results:** We found that DTT decreased the expression of CD11a and CD49d on lymphocytes and eosinophils. The expression of CD11a on neutrophils was also decreased after DTT treatment. DTT increased CD11b expression on lymphocytes, neutrophils, and eosinophils. DTT might also have a mild effect on cell activation. It decreased the expression of CD2 on lymphocytes and variably affected the expression of EG2 in eosinophils, although it had no significant effect on HLA-DR expression on lymphocytes.

**Conclusion:** Our findings show that DTT can affect antigen expression on lymphocytes, neutrophils, and eosinophils and suggest the need for further investigation of similar consequences on induced sputum analysis. (*J Allergy Clin Immunol* 1999;103:873-6.)

**Key words:** Dithiothreitol, induced sputum, CD11a, CD11b, very late antigen-4, inflammatory cells

Induced sputum examination has become an important noninvasive method of assessing airway inflammation.<sup>1</sup> To disperse cells from sputum efficiently, dithiothreitol (DTT) has been widely used for inflammatory cell examination since 1978.<sup>2</sup> Several studies have shown that sputum treatment with DTT does not affect cell morphology and differential cell counts.<sup>2,3</sup>

## Abbreviations used

|        |                             |
|--------|-----------------------------|
| BAL:   | Bronchoalveolar lavage      |
| DTT:   | Dithiothreitol              |
| ECP:   | Eosinophil cationic protein |
| PE:    | Phycoerythrin               |
| VLA-4: | Very late antigen-4         |

In a recent study Efthimiadis et al<sup>4</sup> found that sputum treated with DTT compared with PBS has lower proportions of viable cells, but the proportion of cell counts, fluid-phase fibrinogen, IL-5, and IL-8 are unaffected by DTT treatment. In fact, DTT-treated sputum has higher total cell counts and levels of eosinophil cationic protein (ECP) because of better cell dispersion.<sup>4</sup> However, the effect of DTT on cell surface marker expression has not been systematically studied. Using dithioerythritol, a compound structurally almost identical to DTT, Hansel et al<sup>5</sup> briefly noted that both CD11a and CD11b expression on dithioerythritol-treated eosinophils are significantly decreased compared with that found on PBS-treated cells. However, the effects of DTT on other cell types and on the expression of other cell markers have not been reported.

In a previous study<sup>6</sup> we found that the expression of sputum eosinophil surface marker, very late antigen-4 (VLA-4), is reduced significantly compared with that found on blood eosinophils of asthmatic subjects.<sup>6</sup> These results differ from the findings of Georas et al,<sup>7</sup> who found that there is little or no change in the expression of VLA-4 on eosinophils harvested from bronchoalveolar lavage (BAL) fluid compared with eosinophils harvested from blood. We speculated that the difference between VLA-4 expression on eosinophils from sputum and BAL fluid could be due to differences in the techniques of sample processing. Therefore in this study we investigated the effect of different DTT concentrations on antigen expression on peripheral blood cells.

## METHODS

### Subjects

Five-milliliter samples of venous blood taken from normal healthy subjects (8 subjects for EG2 stain and 5 subjects for all other markers) were collected into tubes containing EDTA. The study was approved by the hospital ethical review committee.

### Study design

Each blood sample was divided into 5 equal aliquots: 4 were pre-treated with different concentrations of DTT (0.1%, 0.25%, 0.5%, and 1%) and 1 with PBS. mAbs CD11a, CD11b, and CD49d were

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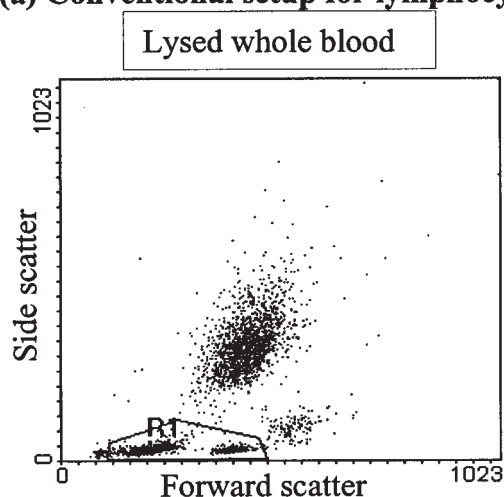
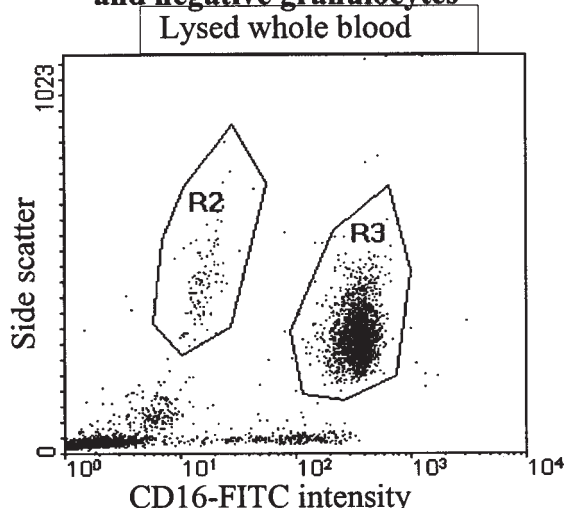
**(a) Conventional setup for lymphocytes****(b) New setup for CD16 positive and negative granulocytes**

**FIG 1.** Gating out different cell populations from lysed whole blood by flow cytometry. R1 represents lymphocytes (a), and R2 and R3 represent eosinophils and neutrophils, respectively (b).

chosen for detecting cell surface adhesion molecule lymphocyte function-associated antigen-1, macrophage antigen-1, and VLA-4, respectively. CD2, a marker for T cells; HLA-DR, a marker for activated T cells; and EG2, a marker for activated eosinophils were also selected for the study. All the treated samples were processed simultaneously for mAb staining and analyzed by flow cytometry.

### Study methods

Samples were processed according to the method of Efthimiadis et al.<sup>4</sup> Peripheral blood (5 mL) was first collected in an EDTA tube, subdivided into 1 mL per tube, and treated with 8 mL of PBS (pH 7.4) or 4 mL of different concentrations (0.1%, 0.25%, 0.5%, and 1%) of DTT (D-0632, Sigma) for 15 minutes at 37°C with constant shaking. Subsequently, 4 mL of PBS was added to the tubes with DTT. Then all the tubes were centrifuged at 300g for 5 minutes and

washed once with 10 mL of PBS. The cell pellets were resuspended in 1 mL of PBS for antibody staining. The contents of each tube were further subdivided into 7 tubes of 100  $\mu$ L each. Five of the 7 tubes were stained directly with the following fluorescence-conjugated mAbs: isotopic control, CD11a/CD16, CD11b/CD16, CD49d/CD16, and HLA-DR/CD2, respectively. The tubes were incubated for 30 minutes at 4°C. Red blood cells were lysed by FACS lysing solution (Becton Dickinson) after the incubation. Cells were then spun, washed, and resuspended in 0.5 mL of 1% paraformaldehyde PBS for flow-cytometric detection.

Two of the 7 tubes were first treated to lyse the red blood cells, permeabilized with FACS permeabilizing solution (Becton Dickinson), and then incubated with EG2 mAb and mouse IgG1 for 30 minutes at 4°C. The cells were then spun and washed twice with PBS. The cell pellet was resuspended in 100  $\mu$ L of PBS and stained with goat anti-mouse IgG1-phycoerythrin (PE) mAb for 30 minutes at 4°C. The indirectly stained cells were washed, spun, and resuspended in 0.5 mL of 1% paraformaldehyde PBS for detection by flow cytometry.

### Flow cytometry

The mAbs used were as follows: fluorescent mAb CD11a-PE and CD11b-PE from PharMingen (San Diego, Calif); EG2 mAb from Pharmacia (Sweden); and mAbs CD2-FITC, CD49d-PE, HLA-DR-PE, CD16-FITC, goat anti-mouse-IgG1-PE, and isotopic control (mouse IgG1-PE, IgG2a-PE and IgG1-FITC) from Becton Dickinson. Flow cytometry was performed by using Coulter Epics Elite ESP.

Fig 1 shows 2 setups for identifying lymphocytes, eosinophils, and neutrophils from lysed whole blood by flow cytometry. The first dot plot (Fig 1, A) shows the conventional forward-sideward scatter dot plot in which lymphocytes (R1) were identified according to their size and 90° light scatter (granularity).<sup>8</sup> The second dot plot (Fig 1, B) shows a new setup based on CD16-positive and CD16-negative staining<sup>9</sup> to identify neutrophils and eosinophils, respectively. The sideward and FITC fluorescence dot plot shows 2 clear groups in which the CD16-negative staining group is composed of eosinophils (R2), and the CD16-positive group is composed of neutrophils (R3). At least 10,000 cells and a minimum of 200 gated eosinophils were recorded. The cells in the R2 region were sorted by Coulter Epics Elite ESP. Cytospin and Giemsa staining showed that 98% of the sorted cells (R2) were eosinophils.

### Data analysis

Immunofluorescence histograms were analyzed by WinMDI software (Amherst, Mass). The mean fluorescence intensity of different surface markers on different cell types pretreated with DTT and PBS are listed in Table I. The differences between different groups were compared by 2-way ANOVA with Tukey's HSD test; significance was assumed at *P* values less than .05.

## RESULTS

### DTT effect on CD16 expression on neutrophils

CD16 expression on neutrophils showed no statistically significant difference in the PBS- and DTT-treated groups (Table I). The eosinophil population was not influenced by different concentrations of DTT treatment.

### DTT effect on CD11a and CD11b expression

DTT pretreatment significantly reduced CD11a expression on lymphocytes, neutrophils, and eosinophils compared with PBS treatment, and the effect was dose

**TABLE I.** Mean fluorescence intensity (mean  $\pm$  SD) of histograms for different surface markers on different cell types pretreated with DTT or PBS

|                    | No. of patients | PBS               | 0.1% DTT           | 0.25% DTT          | 0.5% DTT           | 1% DTT             |
|--------------------|-----------------|-------------------|--------------------|--------------------|--------------------|--------------------|
| <b>Lymphocytes</b> |                 |                   |                    |                    |                    |                    |
| CD11a              | 5               | 616.0 $\pm$ 170.3 | 532.2 $\pm$ 184.1* | 448.4 $\pm$ 175.3* | 372.8 $\pm$ 172.5* | 308.0 $\pm$ 164.1* |
| CD11b              | 5               | 56.3 $\pm$ 7.8    | 80.7 $\pm$ 12.7*   | 73.4 $\pm$ 10.5*   | 75.5 $\pm$ 8.9*    | 74.8 $\pm$ 12.0*   |
| CD49d              | 5               | 129.4 $\pm$ 20.9  | 45.5 $\pm$ 8.3*    | 32.4 $\pm$ 6.6*    | 21.2 $\pm$ 4.6*    | 20.8 $\pm$ 5.4*    |
| CD2                | 5               | 580.9 $\pm$ 169.0 | 314.1 $\pm$ 108.1* | 267.7 $\pm$ 89.9*  | 220.4 $\pm$ 88.7*  | 174.9 $\pm$ 62.7*  |
| DR                 | 5               | 123.2 $\pm$ 17.5  | 128.6 $\pm$ 39.6   | 132.4 $\pm$ 37.9   | 129.5 $\pm$ 31.7   | 128.7 $\pm$ 31.2   |
| <b>Neutrophils</b> |                 |                   |                    |                    |                    |                    |
| CD11a              | 5               | 482.1 $\pm$ 88.0  | 373.4 $\pm$ 120.9* | 318.5 $\pm$ 100.9* | 227.5 $\pm$ 99.5*  | 234.6 $\pm$ 90.7*  |
| CD11b              | 5               | 346.0 $\pm$ 167.9 | 598.7 $\pm$ 275.5* | 585.3 $\pm$ 254.0* | 588.7 $\pm$ 258.4* | 590.9 $\pm$ 248.7* |
| CD49d              | 5               | 10.1 $\pm$ 1.5    | 7.8 $\pm$ 1.3      | 7.5 $\pm$ 1.1      | 6.8 $\pm$ 0.7      | 7.0 $\pm$ 0.6      |
| CD16               | 5               | 315.5 $\pm$ 68.7  | 321.1 $\pm$ 61.1   | 317.2 $\pm$ 69.0   | 314.4 $\pm$ 68.7   | 313.0 $\pm$ 67.1   |
| <b>Eosinophils</b> |                 |                   |                    |                    |                    |                    |
| CD11a              | 5               | 697.1 $\pm$ 177.1 | 562.2 $\pm$ 160.9* | 477.3 $\pm$ 174.5* | 403.9 $\pm$ 170.5* | 342.7 $\pm$ 162.0* |
| CD11b              | 5               | 392.1 $\pm$ 167.2 | 578.0 $\pm$ 232.8* | 570.8 $\pm$ 240.6* | 568.2 $\pm$ 243.7* | 554.7 $\pm$ 252.5* |
| CD49d              | 5               | 227.7 $\pm$ 61.3  | 67.7 $\pm$ 13.9*   | 38.1 $\pm$ 11.6*   | 25.1 $\pm$ 7.1*    | 23.3 $\pm$ 7.0*    |
| EG2                | 8               | 561.7 $\pm$ 218.0 | 728.3 $\pm$ 211.5* | 499.1 $\pm$ 184.8* | 512.3 $\pm$ 218.4* | 468.5 $\pm$ 202.2* |

\*Statistically significant difference compared with the PBS-treated group.

dependent (Table I). DTT significantly increased the CD11b expression on lymphocytes, neutrophils, and eosinophils, but the effect was not dose dependent.

#### DTT effect on VLA-4 (CD49d) expression

DTT markedly decreased, in a dose-dependent pattern, the CD49d expression on lymphocytes and eosinophils, and the effect was statistically significant. DTT had no significant influence on neutrophil CD49d expression (Table I).

#### DTT effect on HLA-DR and CD2 expression of lymphocytes

DTT did not have a significant effect on HLA-DR expression on lymphocytes, but it significantly reduced the CD2 expression on lymphocytes, and the effect was dose dependent.

#### EG2 expression of eosinophils

The effect of DTT on EG2 expression in eosinophils was not consistent (Table I). EG2 expression was slightly but significantly increased in eosinophils treated with 0.1% DTT compared with eosinophils treated with PBS, whereas at other DTT concentrations there were slight but significant reductions in EG2 expression compared with that for PBS.

### DISCUSSION

This study shows that DTT affects cell surface antigen expression. It decreases the expression of CD11a and CD49d on lymphocytes and eosinophils, and the expression of CD11a on neutrophils is also decreased after DTT treatment. DTT increases the CD11b expression on lymphocytes, neutrophils, and eosinophils. DTT might also have a mild effect on cell activation. It does not influence HLA-DR expression, but it decreases the expression of CD2 on lymphocytes. DTT also influences the expres-

sion of EG2 on eosinophils. The significance of these findings is relevant to studies that use induced sputum because DTT is widely used in the preliminary processing of sputum for the further study of cells and supernatant in sputum.

The effect of DTT on cell adhesion molecule expression had been noted earlier by Hansel et al.<sup>5</sup> They found that isolated eosinophils treated with DTT had significantly reduced expression of CD11a and CD11b compared with those treated with PBS. Our findings confirm that the presence of DTT reduces CD11a expression on eosinophils but that it increases the expression of CD11b on eosinophils, as well as on lymphocytes and neutrophils. The different results could be due to the different techniques in the sample processing. Hansel et al.<sup>5</sup> studied granulocytes isolated with Dextran and Ficoll-Hypaque, whereas in this study DTT was added directly into the blood sample. Furthermore, a previous study has shown that Ficoll-Hypaque can activate monocytes in vitro by means of altered expression of CD16/CD18 and CD62L.<sup>10</sup>

The findings of this study could offer an explanation for some of the findings ascribed to physiologic differences between blood, bronchial secretions, and sputum. In an interesting study Walker et al.<sup>11</sup> observed that there were significant elevations of CD11b expression on eosinophils from sputum and BAL fluid compared with that found on blood eosinophils and that CD11b expression on sputum eosinophils was much higher than that on eosinophils from BAL fluid (mean = 224 vs 144.6). Because sputum and BAL fluid are airway secretions, the big increase in CD11b expression on eosinophils from sputum compared with eosinophils from BAL fluid could very likely be due to the DTT treatment of sputum samples, rather than to any mechanistic differences of airway inflammation between the 2 airway fluids.

In an earlier study<sup>6</sup> we found that the expression of CD49d on sputum eosinophils was decreased compared

with that on blood eosinophils from the same asthmatic subjects, although Georas et al<sup>7</sup> found that there was little or no change in CD49d expression on eosinophils from BAL fluid compared with those from blood. Because this study showed that DTT dramatically decreased CD49d expression on lymphocytes and eosinophils, the differences of CD49d expression between sputum and BAL eosinophils in those previous studies could again be explained by the use of DTT in the processing of sputum.

We have shown that DTT at a widely used concentration (0.1%) can upregulate the EG2 expression on eosinophils. Because EG2 only binds to the secreting form of ECP,<sup>12</sup> this finding suggests that eosinophils are activated during the processing with DTT.

Popov et al<sup>3</sup> found that DTT at the 0.1% concentration slightly decreased the immunohistochemical staining of EG2. The results from our study were somewhat ambiguous. It showed that although DTT at the usual concentration of 0.1% did not significantly affect the percentage of eosinophils (CD16-negative granulocytes detected by flow cytometry), it significantly increased the fluorescence intensity of EG2 staining compared with PBS-treated cells by flow cytometry. However, at higher concentrations (0.25%, 0.5%, and 1%) DTT was found to decrease the intensity of EG2 fluorescence staining. The reason for this observation is unclear. Whether higher concentrations of DTT could cause a certain degree of degranulation with subsequent influence on intracellular staining of ECP in eosinophils is still a question and needs to be further investigated.

In this study we did not find a statistically significant difference in CD16 expression on neutrophils pretreated with DTT compared with that on neutrophils pretreated with PBS. This is different from the results of Loppow et al.<sup>13</sup> In the article by Loppow et al, CD16 staining was increased after DTT treatment, although DTT did not result in changes of the cellular proportions within one sample. It is conceivable that differences in the sample preparation (eg, DTT concentration, processing time, and temperature) could potentially influence the results on the cell surface staining. However, it is difficult to compare our results to those of Loppow et al<sup>13</sup> because they did not indicate the details of the method in their abstract.

In summary, this study has shown that DTT can influence cell adhesion molecule expression, as well as some

important surface markers (eg, CD2 expression). It might also have a minor influence on cell activation. Because DTT is routinely used for sputum cell dispersion, the results shown in this study suggest the need for further investigation of similar consequences on induced sputum analysis.

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