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SULFHYDRYL REDUCING AGENTS PROMOTE NEUTROPHIL ADHERENCE WITHOUT INCREASING SURFACE EXPRESSION OF CD11b/CD18 (Mac-1, Mo1)

Barbara R. Schwartz and John M. Harlan

Department of Medicine, University of Washington, Seattle, WA 98195

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SUMMARY: The disulfide reducing agents dithioerythreitol and dithiothreitol, but not oxidized dithiothreitol, induced polymorphonuclear neutrophils to adhere to endothelial cells or to plastic. Adherence was inhibited by monoclonal antibodies 60.1 and 60.3, which are directed to functional epitopes on the CD11b and CD18 polypeptides of the neutrophil membrane adhesion complex (Mac-1, Mo1). The increased adherence induced by the sulfhydryl reducing agents was not accompanied by increased expression of CD11b/CD18. These studies demonstrate that a qualitative alteration in CD11b/CD18 is sufficient to promote neutrophil adherence.

Stimulated neutrophil adherence to endothelium *in vitro* (1,2) and *in vivo* (3) is mediated by the neutrophil membrane heterodimer CD11b/CD18 (Mac-1, Mo1) (4). The mechanism whereby CD11b/CD18 increases neutrophil adhesiveness is uncertain. Neutrophil activation by a variety of agents increases surface expression of CD11b/CD18 (5) by promoting translocation of cytoplasmic granules to plasma membrane (6). Since increased surface expression of CD11b/CD18 is temporally associated with increased adhesiveness, it has been postulated that stimulated neutrophil adherence to endothelium is mediated by the newly translocated heterodimer. Recent studies, however, indicate that increased surface expression of CD11b/CD18 is neither necessary nor sufficient for neutrophil adhesion to endothelial cells *in vitro* (7). We now demonstrate that exposure of neutrophils to sulfhydryl reducing agents promotes neutrophil adherence to endothelium and to

Abbreviations used in this paper: BSA: bovine serum albumin; DTE: dithioerythritol; DTT: dithiothreitol; FITC: fluorescein isothiocyanate; fMLP: formyl-methionylleucylphenylalanine; MAb: monoclonal antibody; PMA: phorbol 12-myristate-13-acetate; PMN: polymorphonuclear neutrophils.

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plastic without increasing surface expression of CD11b/CD18. These results support the hypothesis that qualitative alterations in the heterodimer are sufficient for the increase in neutrophil adhesiveness.

METHODS

Endothelial cells.

Bovine aortic endothelial cells and human umbilical vein endothelial cells were isolated by collagenase treatment of vessels (8,9). Cells were maintained in RPMI 1640 (Whittaker M.A. Bioproducts, Walkersville, MD) with 10% (for bovine) or 20% (for human) fetal bovine serum (Hyclone Laboratory, Logan, UT).

Preparation of Neutrophils.

Blood was drawn from normal donors with informed consent into syringes containing heparin (10 U/ml). PMN were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient centrifugation and dextran sedimentation with hypotonic lysis of contaminating red cells and washed with cold phosphate buffered saline without calcium and magnesium (10). Neutrophils were labeled with (^{51}Cr) (as sodium chromate, 200-500 Ci/g, New England Nuclear, Boston, MA) as previously described (11). Prior to assay, neutrophils were resuspended in RPMI medium at $2x10^6$/ml.

Adherence assays.

Endothelial cells were grown in 48- or 96-well tissue culture clusters (Costar, Cambridge, MA) and washed. For experiments without endothelial cells, plates were incubated at 37°C for two hours with fetal bovine serum, or with 1% BSA that had been heated at 80°C for three minutes, and washed. Neutrophils were added to wells immediately before or after reagents and incubated at 37°C for 15 to 30 minutes.

^{51}Cr assay. After incubation, the nonadherent cells from each well were removed and combined with one wash. The adherent cells were lysed with 1N NH_4OH and combined with one wash. Radioactivity was determined with a Micromedic Gamma Spectrophotometer (Horsham, PA). Percent adherence was determined as the fraction of adherent cpm (adherent cpm divided by total recovered cpm (nonadherent + adherent from each well)) x 100%.

Crystal violet assay. For crystal violet staining, nonadherent cells were removed by inversion and the plates washed with medium. Plates were stained with crystal violet (0.5% in 20% methanol) for 15 minutes at room temperature and washed extensively with running water. The stain was extracted by the addition of 0.1 M sodium citrate pH 4.2 in 50% ethanol and the absorbance at 570 nanometers (A_{570}) read on a Behring ELISA Processor II (Behring, Marburg, FRG). Adherence was determined as the fold-increase in A_{570} compared to PMN without treatment. In experiments testing PMN adherence to endothelium, the A_{570} of stained endothelial cells alone was subtracted from that of wells containing both cell types.

Immunofluorescence Flow Cytometry.

For immunofluorescence flow cytometry PMN were treated with test agent at room temperature for 10 minutes, chilled and treated with heat-inactivated adult bovine serum to block nonspecific immunoglobulin binding before incubation with MAbs directed to members of the CD11b/CD18 leukocyte membrane glycoprotein complex. MAb 60.1 recognizes a functional epitope on the CD11b polypeptide

(2). MAb 60.3 recognizes a functional epitope on the CD18 polypeptide (or quaternery structure formed by the CD11/CD18 heterodimer) (12).The bound MAb was stained with FITC-goat anti-mouse IgG (TAGO, Burlingame, CA). The samples were fixed with freshly diluted 1% paraformaldehyde. Analysis was performed using a FACS 440 (Becton Dickinson and Co., Sunnyvale, CA) with quantitative determination of peak fluorescence intensity. Results are expressed as fold-increase in peak fluorescence compared to untreated cells.

Reagents.
DTT, oxidized DTT (trans-4,5-dihydroxy-1,2-dithiane), DTE, and crystal violet were purchased from Sigma Chemical Co. (St. Louis,MO). BSA was obtained from Irvine Scientific (Santa Ana, CA). MAb 60.1 was a gift of Dr. Patrick Beatty, Puget Sound Blood Center, Seattle, WA.

RESULTS

DTT and DTE induce PMN adherence. Dithiothreitol and DTE induced PMN to adhere to bovine endothelial cells (Figure 1) or to human endothelial cells (data not shown) in a dose-dependent manner. Oxidized DTT did not induce PMN adherence to bovine endothelial cells (Figure 1) or to human endothelial cells (data not shown). Dithiothreitol induced PMN adherence to BSA-blocked plastic to the same extent as to endothelium. Incubation with DTT (2 mM) increased adherence to BSA-blocked plastic by 7.2 ± 0.2-fold vs. 7.3 ± 0.8-fold to endothelial cells (means ± 1 SD of 4 replicates). In

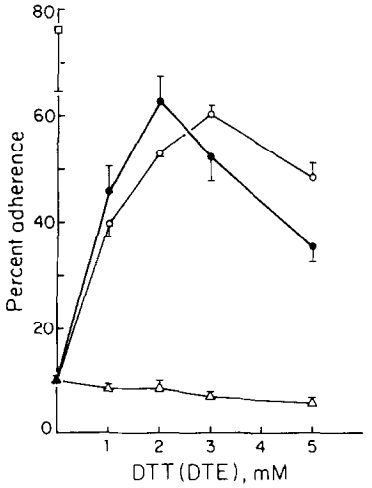

Figure 1. *DTT and DTE, but not oxidized DTT, induce neutrophils to adhere to endothelial cells.* ^{51}Cr-labeled PMN were incubated with DTT (closed circles), DTE (open circles) or oxidized DTT (triangles) at room temperature for 10 min and tested for adherence to bovine endothelial cells. PMA (50 ng/ml)-stimulated adherence (square) is included for comparison. *Abscissa*: Concentration (mM) of DTT, DTE, oxidized DTT. *Ordinate*: Percent adherence. Means ± 1 SD of 4 replicates are shown.

TABLE 1. Competing disulfides prevent DTT-induced PMN adherence

Neutrophils[a]	% Adherence[b]
Basal	2.4 ± 0.3
DTT	40.9 ± 23.9
DTT + reduced sulfhydryl	36.6 ± 23.0
DTT + disulfide	9.3 ± 4.6

[a]DTT and equimolar concentrations of homocysteine (cysteine) or homocystine (cystine) were mixed and tested for ability to induce adherence of PMN to bovine endothelial cells.

[b]Means ± SEM of 3 experiments.

contrast, oxidized DTT caused only 1.1 ± 0.1 and 1.8 ± 0.6 fold-increases in adherence to plastic and to endothelial cells, respectively. If compounds containing competing disulfide groups such as homocystine or cystine were present, the effect of DTT on PMN adherence was markedly reduced (Table 1). The same compounds with reduced sulfhydryls, homocysteine or cysteine, did not compete with PMN for the DTT (Table 1).

Surface expression of CD11b and CD18. Neutrophil adherence induced by DTT was inhibited by MAb 60.1 (anti-CD11b) and MAb 60.3 (anti-CD18). MAb 60.1 inhibited DTT-induced PMN adherence by 90 ± 15% and MAb 60.3 by 100 ± 5% (means ± SEM of three or four experiments, respectively). The binding of MAb 60.1 and 60.3 to DTT-treated neutrophils was studied by immunofluorescence flow cytometry and the same PMN preparations were tested for adherence to endothelial cells. Binding of MAb 60.1 and 60.3 to DTT- or DTE-treated neutrophils was not increased compared to untreated cells, whereas binding to PMA-treated cells was markedly increased. Nevertheless, adherence of DTT- or DTE-treated neutrophils was comparable to adherence of PMA-stimulated cells. Results obtained with MAb 60.1 are presented in Table 2. Results with MAb 60.3 were similar. In addition, DTT pretreatment of neutrophils followed by washing did not prevent PMA-induced increased expression of CD11b or CD18. CD11b expression increased 12-fold and CD18 expression seven-fold following stimulation of DTT-pretreated PMN by PMA (data not shown).

DISCUSSION

In this study we report that treatment of PMN with disulfide-reducing agents DTT and DTE increased adherence to both endothelial

TABLE 2. DTT and DTE induce neutrophil adherence without increasing surface expression of CD11b[a]

Treatment	Fold-increase in CD11b Expression	Fold-increase in Adherence
	Experiment 1	
Unstimulated	1.0	1.0
DTT	0.9	9.4 ± 0.4
Oxidized DTT	1.5	1.5 ± 0.2
DTE	1.4	9.9 ± 0.8
PMA	7.0	9.8 ± 0.9
	Experiment 2	
Unstimulated	1.0	1.0
DTT	1.2	17.8 ± 1.6
Oxidized DTT	1.0	1.3 ± 0.4
DTE	1.0	16.8 ± 1.6
PMA	3.7	20.1 ± 0.7

[a]Neutrophils were incubated with reagents in polypropylene tubes for 10 min at 37° before processing for immunofluorescence flow cytometry, or incubated with reagents in microtitre plates for 20 min at 37° before staining of adherent cells with crystal violet. Results are presented as the fold-increase in MAb binding or adherence compared to unstimulated PMN. Surface expression of CD11b was quantitated by binding of MAb 60.1. Adherence was quantitated by determining the A_{570} of crystal violet-stained cells. A_{570} for unstimulated neutrophils was 0.08±0.02 (Expt 1) and 0.05±0.01 (Expt. 2). Values represent the means ± SD of 4 replicate wells in each experiment. The concentrations of reagents were: DTT 5 mM, oxidized DTT 5 mM, DTE 5 mM and PMA 20 ng/ml.

cells and plastic. Dithiothreitol- or DTE-induced neutrophil adherence was similar to that induced by other stimuli, e.g. PMA, calcium ionophore and fMLP, in that it was inhibited by anti-CD11b or anti-CD18 MAbs (MAbs 60.1 and 60.3, respectively) (1). Since DTT induced PMN adherence to BSA-blocked plastic to the same extent as to endothelium, we conclude that the DTT and DTE act on the PMN, not on the endothelial cells. Two observations demonstrate that neutrophil adherence induced by DTT and DTE was due to their activity as disulfide reducing agents. First, oxidized DTT was not effective. Second, the effect of DTT and DTE was inhibited by coincubation with competing disulfide reagents.

Although DTT and DTE induced PMN adherence, these agents did not increase surface expression of CD11b/CD18. This result is consistent with previous studies demonstrating that PMN increase adherence to endothelial cells when stimulated by fMLP, PMA and calcium ionophore A23187, even when increased surface expression of CD11b/CD18 is prevented by an anion channel blocker (7). These

observations suggest that increased adherence is due to qualitative rather than quantitative alterations in CD11b/CD18.

Dithiothreitol has also been reported to induce platelet aggregation (13). Although we have no direct evidence, it seems likely that the relevant disulfide groups affected by DTT and DTE are associated with extracellular aspects of the platelet or PMN plasma membrane. We speculate that the critical disulfides are in fact located within the relevant integrin receptors, CD11b/CD18 and GP IIb/GP IIIa. Both beta-chain polypeptides, CD18 (14) and GP IIIa (15), contain disulfide-rich regions in their extracellular domains. It is possible that reduction of disulfide bonds in these molecules alters the conformation of the heterodimers, thereby exposing adherence-reactive sites.

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REFERENCES

1. Harlan, J.M., Killen, P.D, Senecal, F.M., Schwartz, B.R., Yee, E.K., Taylor, R.F., Beatty, P.G., Price, T.H., and Ochs, H.D. (1985) Blood 66:167-178.

2. Wallis, W.J., Hickstein, D.D., Schwartz, B.R., June, C.H., Ochs, H.D., Beatty, P.G., Klebanoff, S.J., and Harlan, J.M. (1986) Blood 67: 1007-1013.

3. Arfors , K.E., Lundberg, C., Lindbom, L., Lundberg, K., Beatty, P.G., and Harlan, J.M. (1987) Blood 69:338-340.

4. Anderson, D.C., Miller, L.J., Schmalstieg, F.C., Rothlein, R. and Springer, T.A. (1986) J. Immunol. 137: 15-27.

5. Arnaout, M.A., Spits, H., Terhorst, C., Pitt, J., and Todd, R.F.III (1984) J. Clin. Invest. 74:1291-1300.

6. Bainton, D.F., Miller, L.J., Kishimoto, T.K., and Springer, T.A. (1987) J. Exp. Med. 166:1641-1653.

7. Vedder, N.B. and Harlan, J.M. (1988) J. Clin. Invest. 81:676-682.

8. Schwartz, S.M. (1978) In Vitro 14:966-980.

9. Jaffe, E.A., Nachman, R.L., Becker, C.G., and Minick, C.R. (1973) J. Clin Invest. 52:2745-2756.

10. Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21 (Suppl. 97):77-89.

11. Gallin, J.I., Clark, R.A., and Kimball, H.R. (1973) J. Immunol. 110:233-240.

12. Beatty, P,G., Ledbetter, J.A., Martin, P.J., Price, T.H., and Hansen, J.A. (1983) J. Immunol. 131:2913-2918.

13. Zucker, M.B. and Masiello, N.C. (1984) Thromb. Haemostas. 51:119-124.

14. Kishimoto, T.K., O'Connor, K., Lee, A., Roberts, T.M., and Springer, T.A. (1987) Cell 48:681-690.

15. Fitzgerald, L.A., Steiner, B., Rall, S.C. Jr., Lo, S.-s., and Phillips, D.R. (1987) J. Biol. Chem. 262:3936-3939.