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Effects of Thiols on Sheep Erythrocytes Resulting in Enhanced Rosette Formation with Human T Lymphocytes (39489)

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Sheep red cells (SRBC) adhere specifically to human T lymphocytes (T-L) to form rosettes (1, 2). Since the cellular interaction is weak, marked variance in the number of rosette forming cells (RFC) in normal human peripheral blood has been reported by different investigators (3-5). Recently we described an improved rosetting technique using SRBC treated with the thiol *S*,2-aminoethylisothiouronium bromide (AET) (6). Interaction of AET-treated SRBC (SRBC-A) with T-L results in prompt formation of large rosettes resistant to mechanical disruption so that higher, reproducible percentages of RFC are detected in isolated peripheral blood lymphocytes (PBL). Since a number of mechanisms could account for this enhanced cellular interaction, studies were undertaken to determine the effects of AET and other compounds on SRBC membranes.

Materials and methods. Chemical modification of SRBC. Sterile sheep blood anticoagulated in Alsever's solution was purchased from Grand Island Biological Co. or obtained from a sheep housed at the Minneapolis Veterans Administration Hospital; blood was used within 2 weeks of bleeding unless otherwise specified. AET and 1,1 dimethylguanidine hydrochloride (DMG) were purchased from Aldrich Biochemicals. Bis(2-guanidinoethyl) disulfide (GED) was synthesized by air oxidation of a slightly alkaline, aqueous AET solution. The melting point (198–199°) and infrared spectrum of the product were identical to values reported for GED (7). Dithiothreitol (DTT) was obtained from Calbiochem, 2-mercaptoethanol (2-ME) from Eastman Biochemicals, and iodoacetamide (IA), N-ethylmaleimide (NEM), and dithiodinicotinic acid (DTDNA) from Sigma Chemical Co. All reagent solutions were freshly prepared just before use. AET and GED solutions ≥ 0.15 *M* were prepared in 0.01 *M*, pH 9.0 Tris–HCl buffer (Tris); lesser concentrations of these compounds and all DTT, 2-ME, and DMG solutions were made up in Tris-buffered saline (TBS). IA, NEM, and DTDNA were dissolved in phosphate-buffered (0.01 *M*, pH 7.4) saline (PBS). One volume of packed, washed SRBC was incubated (30 min, 37°) with 4 vol of AET, DMG, GED, DTT, or 2-ME at concentrations ranging from 1.5×10^{-5} to 3.0×10^{-1} *M* and washed thoroughly in RPMI 1640 (RPMI). Aliquots of packed SRBC and SRBC–A were incubated (1 hr, 37°) with equal volumes of IA, NEM, or DTDNA at concentrations of 1.5×10^{-5} to 1.5×10^{-2} *M* and thrice washed in RPMI. Trypsinization of SRBC and SRBC–A was performed by incubating (37°, 1 hr) a 2 mg/ml solution of recrystallized trypsin (Worthington Biochemicals) with equal volumes of 5% SRBC or SRBC–A suspensions in RPMI. Soybean trypsin inhibitor (Sigma Chemical Co.) was added and the cells were washed repeatedly in saline and once in RPMI. If trypsinized SRBC were to be treated with AET, they were washed only once before addition of AET.

Isolation and rosetting of normal PBL. Human PBL were isolated and rosetted with native and chemically modified SRBC as previously described (6). Rosetting enhancement was calculated as the percentage of PBL rosetting with modified SRBC minus the percentage of rosetting with control SRBC (SRBC incubated in buffer).

Solubilization and electrophoresis of SRBC and SRBC–A stroma. Hemoglobin-depleted SRBC and SRBC–A membranes were prepared by the method of Dodge *et*

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al. (8), solubilized in 1% sodium dodecyl sulfate (SDS), and electrophoresed in 5.6% polyacrylamide gels containing SDS (SDS–PAGE) (9). Gels were stained with Coomassie blue or PAS and scanned at 530 and 560 nm, respectively.

Analyses of stromal components. Solubilized membrane protein was measured by a modified Lowry method (10). Membrane sialic acid was measured as described by Warren (11). Phospholipid phosphorus was determined after chloroform: methanol extraction of SRBC and SRBC–A membranes (12) and ashing of the solubilized lipids (13, 14). Reactive (free) and totally solubilized membrane sulfhydryls (SH) were measured with 5,5′-dithiobis-(2-nitrobenzoic) acid (Sigma Chemical Co.) (15, 16). All SH measurements were performed within 2 days of membrane preparation.

Electron spin resonance (ESR) measurements. These studies were performed in collaboration with Dr. Ronald Mason. All labeling reagents were purchased from Syva Corp. SRBC and SRBC–A were labeled with 5-nitroxide stearic acid (5NSA) by adding 1 μl of 0.1 *M* 5NSA in ethanol to 0.5 ml of a 50% washed erythrocyte suspension. SH-labeled cells were prepared by incubating washed red cells with 5×10^{-4} *M* *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) iodoacetamide (ISL) or with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide (MSL) in PBS. ESR spectra were measured at room temperature.

Results. Effects of various reagents on SRBC. Treatment of SRBC with 0.143 *M* AET, pH 9.0, did not alter the protein content nor the sialic acid:protein or phospholipid:protein ratios of SRBC–A compared with SRBC (TAble I), but markedly enhanced the rosetting activity of these cells (Fig. 1). As reported by others (17), the sialic acid content of SRBC (and of SRBC–A) diminished with storage of blood at 4° (Table I). Compared with SRBC, SRBC–A exhibited modest (~10%), but statistically significant, decrements in both total ($P < 0.01$) and reactive ($P < 0.001$) membrane SH groups.

Since rosetting enhancement by AET could be due to neutralization of the negative surface charge of SRBC membranes by

TABLE I. COMPARISON OF MEMBRANE COMPOSITIONS OF SRBC AND SRBC–A

		Membrane constituents									
		Protein × 100		SH (10^{-8} *M*/mg) protein				Sialic Acid (10^{-8} *M*/mg of protein)		Phospholipid (μ*M*/mg of protein)	
		dry wt of stroma		Total		Reactive					
Sheep blood	Storage days at 4°	SRBC	SRBC–A	SRBC	SRBC–A	SRBC	SRBC–A	SRBC	SRBC–A	SRBC	SRBC–A
VA	1	53.3	52.1	5.96 ± 0.41[a]	5.86 ± 0.39	2.77 ± 0.21	2.43 ± 0.15	7.77 ± 0.32	8.24 ± 0.35	0.87 ± 0.05	0.74 ± 0.02
GIBCO	1									0.77 ± 0.1	0.80 ± 0.1
GIBCO	6									0.78 ± 0.3	0.80 ± 0.03
GIBCO	10	50.4	49.8	6.20 ± 0.14	5.88 ± 0.19	2.77 ± 0.09	2.49 ± 0.07	7.05 ± 0.25	7.15 ± 0.28	0.77 ± 0.03	0.82 ± 0.03
VA	20	54.7	54.4	6.16 ± 0.16	5.82 ± 0.2	2.79 ± 0.14	2.44 ± 0.11	6.60 ± 0.26	6.64 ± 0.3	0.85 ± 0.08	0.84 ± 0.04
GIBCO	>50	54.2		6.07 ± 0.3	5.65	2.66 ± 0.16	2.39	6.00 ± 0.17	5.79		

[a] One standard deviation from the mean of four to five individual observations.

strongly cationic guanidino groups of AET, SRBC were exposed to GED and DMG, positively charged, guanidino-containing compounds that lack an active SH group present in AET at pH 9.0. SRBC were also treated with 2-ME and DTT, uncharged thiols, to observe their effects on rosetting. The results (Fig. 1) show that GED and DMG were inactive, but appropriate concentrations of DTT and 2-ME clearly facilitated rosetting. This suggested that SRBC membrane SH groups were required for rosetting. However, treatment of SRBC and SRBC-A with the SH-alkylating agents IA, NEM, and DTDNA at high concentrations (1.5×10^{-2} *M*), before or after thiol treatment, did not impair rosetting; in contrast, trypsinization of native or thiol-treated SRBC totally inhibited rosetting (Table II).

Page-SDS and ESR studies. No discernible differences between SRBC and SRBC-A membranes were detectable by SDS-PAGE or by ESR.

Discussion. The physicochemical forces responsible for adherence of SRBC to T-L have not been elucidated. It has been proposed that coulombic forces or hydrogen bonding between lymphocyte membrane NH_2 groups and negative sites on SRBC are involved (18). Others have suggested that

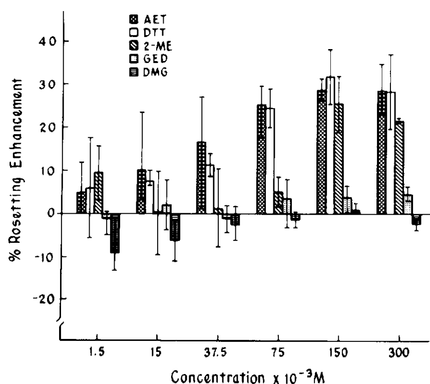

FIG. 1. Dose-response relationship between SRBC treated with various thiols and their analogs to enhancement of rosetting. The mean and absolute variations observed are plotted for each concentration of each compound. Rosetting enhancement is calculated as the percentage of rosetted lymphocytes after exposure to treated SRBC − the percentage of rosetted lymphocytes after incubation with SRBC exposed to buffer alone.

TABLE II. EFFECTS OF SULFHYDRYL INHIBITORS AND TRYPSIN ON SRBC AND SRBC-A ROSETTING

Treatment of SRBC		Percentage of rosetted lymphocytes
Initial	Final	
PBS	PBS	45.8 ± 5.8[a]
PBS	TBS	38.0 ± 1.0
IA	TBS	40.6 ± 1.0
IA	AET	75.5 ± 4.5
AET	PBS	70.0 ± 4.0
AET	IA	70.8 ± 4.0
NEM	TBS	37.3 ± 4.0
NEM	AET	68.5 ± 1.5
DTDNA	TBS	32.7 ± 4.1
DTDNA	AET	71.5 ± 4.0
Trypsin	TBS	0
Trypsin	AET	0
AET	Trypsin	0

[a] One standard deviation from the mean of four to five individual observations.

T-L rosetting is mediated by SRBC surface glycopeptides having the oligosaccharide sequence sialic acid → galactose → *N*-acetylglucosamine linked to a mannose:*N*-acetylglucosamine core (19). Since trypsinization removes much of the surface glycoprotein from red cells (17) and abolishes rosetting of SRBC with T-L (1, 2), this supports the concept that SRBC surface glycoprotein serves as the T-L receptor; however, these observations shed no light on the mechanism of thiol-enhanced facilitation of rosetting.

At pH 9.0, AET is converted to 2-mercaptoethylguanidine (20), which contains one free SH group. This thiol readily forms mixed disulfides (S-S) with free SH groups of SRBC membranes. Although many activities of intact erythrocytes cease after they are exposed to SH-active agents, Godin and Schrier (21) have shown that the effects of these compounds may not result simply from membrane SH modification, but rather from structural changes induced in red cell membranes (e.g., solubilization of membrane components or disruption of membrane S-S bridges).

The data (Tables I and II) indicate that free membrane SH groups are not directly involved in rosetting and that thiol-enhanced rosetting is not mediated by mechanisms involving solubilization or removal of SRBC membrane components such as sialic acid, as previously described by Weiner and

associates (22). It appears likely that a major effect of AET and other thiols on SRBC is to disrupt S–S bridges, resulting in important conformational changes in the SRBC membrane. Thiol treatment is known to induce striking immunologic alterations in normal human red cells similar to those observed in paroxysmal nocturnal hemoglobinuria (PNH) erythrocytes (23–25). Several investigators have speculated that cleavage of red cell membrane S–S bonds induces the PNH-like lesion (24, 26–28). We propose that analogous changes in thiol-treated SRBC membranes are responsible for their increased adherence to T-L. However, neither naturally occurring PNH red cells nor normal human red cells treated with AET rosette with T-L (unpublished observations); consequently, thiol treatment of red cells does not appear to create membrane sites that interact nonspecifically with T-L.

If changes in membrane configuration are responsible for thiol-enhanced rosetting, three possibilities must be considered: Thiols may (1) not change the number of reactive sites on SRBC but may stabilize the interaction of exposed sites with T-L; (2) uncover cryptic additional sites identical to those readily accessible on SRBC; and (3) create different, possibly charge-dependent, sites on SRBC membranes. Number 3 is unlikely since various thiols having different charge characteristics are effective in facilitating rosetting of SRBC but not of human red cells; moreover, trypsinization destroys the reactive moiety of thiol-treated, as well as of unmodified, SRBC. Number 2 is improbable since trypsinization destroys all T-L receptor sites on intact SRBC despite subsequent thiol treatment. If buried, potentially reactive sites identical to those accessible on unaltered SRBC membranes exist and are exposed by thiol treatment, it is unlikely that a large molecule such as trypsin (mol wt ~ 20,000) (29) would have access to and destroy such moieties in intact SRBC. The most plausible explanation for thiol-enhanced rosetting is an alteration in SRBC membrane configuration that stabilizes the interaction of surface-reactive groups (probably glycoproteins) with T-L. Unfortunately, electrophoretic and ESR studies did not detect the postulated configurational changes. Possibly other techniques, such as fluorescence polarization, may reveal such alterations and, thereby, provide valuable insights into red cell membrane structure and its modification by thiols.

Summary. The mechanism of enhanced interaction of T-L with SRBC–A has been explored and the following results obtained: (1) Compared with SRBC, SRBC–A contain identical quantities of protein, phospholipid, and sialic acid, but have slightly reduced SH content; (2) treatment of SRBC with thiols having charge properties different from AET also facilitates rosetting; (3) exposure of SRBC to various SH-alkylating reagents, either before or after thiol treatment, does not diminish thiol-enhanced rosetting; (4) trypsinization of SRBC and of SRBC–A completely abolishes the ability of these cells to rosette; and (5) no differences could be detected between intact SRBC and SRBC–A, or in their solubilized membrane components, by SDS–PAGE and by ESR. These results indicate that free SH groups of SRBC membranes are not directly involved in the rosetting process. Thiol-enhanced rosetting probably results from cleavage of membrane S–S bridges resulting in configurational changes within the SRBC membrane. The interaction of an SRBC membrane receptor, probably glycoprotein in nature, with T-L is thereby facilitated.

1. Wybran, J., and Fudenberg, H. H., Trans. Assoc. Amer. Physicians **84,** 239 (1971).
2. Jondal, M., Holm, G., and Wigzell, H., J. Exp. Med. **136,** 207 (1972).
3. Wybran, J., Carr, M. C., and Fudenberg, H. H., J. Clin. Invest. **51,** 2537 (1972).
4. Bentwich, Z., Douglas, S. D., Siegal, F. P., and Kunkel, H. G., Clin. Immunol. Immunopathol. **1,** 511 (1973).
5. Mendes, N. F., Tolnai, M. E. A., Silveira, N. P. A., Gilbertsen, R. B., and Metzgar, R. S., J. Immunol. **111,** 860 (1973).
6. Kaplan, M. E., and Clark, C., J. Immunol. Meth. **5,** 131 (1974).
7. Hino, T., Tana-ami, K., Yamada, K., and Akaboshi, S., Chem. Pharm. Bull. (Tokyo) **14,** 1193 (1966).
8. Dodge, J. T., Mitchell, C., and Hanahan, D. J., Arch. Biochem. Biophys. **100,** 119 (1963).
9. Fairbanks, G., Steck, T. U., and Wallach, D. F. H., Biochemistry **10,** 2606 (1971).

10. Ji, T. H., Anal. Biochem. **52,** 517 (1973).
11. Warren, L., J. Biol. Chem. **234,** 1971 (1959).
12. Nelson, G. J., Biochem. Biophys. Acta **144,** 221 (1967).
13. Ames, B. N., *in* "Methods in Enzymology" (E. F. Neufeld and V. Ginsburg, eds.), Vol. VIII, p. 115. Academic Press, New York (1966).
14. Chen, P. S., Jr., Toribara, T. Y., and Warner, H., Anal. Chem. **28,** 1756 (1956).
15. Ellman, G. L., Arch. Biochem. Biophys. **82,** 70 (1959).
16. Boyne, A. F., and Ellman, G. L., Anal. Biochem. **46,** 639 (1972).
17. Cook, G. M. W., Heard, D. H., and Seaman, G. V. F., Nature (London) **188,** 1011 (1960).
18. Lalezari, P., Nehlsen, S. L., Novodoff, J., and Lalezari, I., Proc. Natl. Acad. Sci. USA **72,** 697 (1975).
19. Boldt, D. H., and Armstrong, J. P., Blood **44,** 922 (1974) (Abstr.).
20. Khym, J. X., Shapiro, R., and Doherty, D. G., J. Amer. Chem. Soc. **79,** 5663 (1957).
21. Godin, D. V., and Schrier, S. L., J. Membrane Biol. **7,** 285 (1972).
22. Weiner, M. S., Bianco, C., and Nussenzweig, V., Blood **42,** 939 (1973).
23. Sirchia, G., Ferrone, S., and Mercuriali, F., Blood **25,** 502 (1965).
24. Kann, H. E., Mengel, C. E., Meriwether, W. D., and Ebbert, L., Blood **32,** 49 (1968).
25. Ferrone, S., Merubini, E., Mercuriali, F., and Sirchia, G., Brit. J. Haematol. **23,** 5 (1972).
26. DeSandre, G., Cortesi, S., Corrocher, R., Falezza, G., and Perona, G., Brit. J. Haematol. **18,** 551 (1970).
27. Sirchia, G., Zanella, A., Perrella, M., Mercuriali, F., and Ferrone, S., Experientia **28,** 191 (1972).
28. Goldstein, B. D., Brit. J. Haematol. **26,** 49 (1974).
29. National Academy of Science, "Specifications and Criteria for Biochemical Compounds," 3rd ed., p. 116. National Academy of Science, Washington, D. C. (1972).

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