Vol. 182, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1992 Pages 151-157

FORMATION OF NITRIC OXIDE HEMOGLOBIN IN ERYTHROCYTES CO-CULTURED WITH ALVEOLAR MACROPHAGES TAKEN FROM BLEOMYCIN TREATED RATS

A.E. Huot [1,2], H. Kruszyna [3], R. Kruszyna [3], R.P. Smith [3] and M.P. Hacker [1,4]

[1]Vermont Regional Cancer Center and the Departments of [2]Medical Technology and [4] Pharmacology, University of Vermont, Burlington, VT 05405

[3]Department of Pharmacology and Toxicology , Dartmouth Medical School, Hanover, NH 03756

Received October 31, 1991

Summary. Alveolar macrophages, taken from rats treated with a single intratracheal dose of bleomycin, release reactive nitrogen intermediates in the form of nitric oxide which are cytostatic to murine leukemia L1210 cells. When cultured in the presence of erythrocytes the cytostatic activity of alveolar macrophages was inhibited which corresponded with an increase in nitrosylated hemoglobin content when compared with erythrocytes cultured alone. These results suggest that erythrocytes inhibit alveolar macrophage cytostatic activity by preventing reactive nitrogen intermediates from reaching target cells because the hemoglobin serves as a sink for reactive nitrogen intermediates in the form of nitric oxide.

Diffuse pulmonary alveolitis culminating in chronic fibrosis has been reported following exposure to a variety of agents which include paraquat, BLM , organic dusts and hyperoxia (1-4). Irrespective of the nature of the initial damaging agent, one of the first observations in the lungs is increased vascular permeability (5). Various secretory products of activated AM have been implicated as possible mediators of pulmonary damage including oxygen radicals (6), tumor necrosis factor (7-9), arginase and neutral proteases (10,11), and RNI (12-15).

Abbreviations: bleomycin **BLM**; alveolar macrophages **AM**; reactive nitrogen intermediates **RNI**; N^G-monomethyl-arginine **NMA**; erythrocytes **RBC**; AM taken from BLM treated animals **BLM-AM**; intratracheal **i.t.**; phosphate buffered saline **PBS** (2.5 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl and 8.1 mM Na_2HPO_4, pH 7.4); endothelium derived relaxing factor **EDRF**; nitric oxide **NO**; electron paramagnetic resonance spectroscopy **EPRS**; donor horse serum **DHS**; minimal essential medium **MEM**; bronchoalveolar lavage fluid cells **BAL**; standard error of the mean **SEM**.

0006-291X/92 $1.50

Previously, we reported that AM activated by BLM induced lung damage caused cytostasis in co-cultured murine leukemia L1210 cells (16,17). The consequences of BLM-AM induced cytostasis included inhibition of both DNA synthesis and aconitase activity as have been described for other target cells exposed to activated macrophages (18-20). We established that the production of RNI, measured as nitrite, by BLM-AM correlated with cytostatic activity (17). Further, we demonstrated that the RNI were the result of an L-arginine dependent effector mechanism which could be inhibited by the substrate specific analogue NMA. In addition, RBC in the co-culture inhibited the cytostatic activity of BLM-AM and reduced the amount of measurable nitrite.

In a related system, investigators have shown that the action of EDRF is inhibitable by both free oxyhemoglobin and to a lesser extent by methemoglobin (21), and that EDRF and the RNI, NO, have very similar properties (22-23). The purpose of this current investigation is to determine whether intracellular hemoglobin was nitrosylated by the RNI produced by BLM-AM thus preventing RNI damage to the L1210 target cells by trapping the NO. We addressed this using visible absorption spectrophotometry and EPRS of hemoglobin isolated from RBC cultured in the presence and absence of BLM-AM.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were obtained from the National Institutes of Health, Bethesda, MD. The animals were maintained on a 12 h dark, 12 h light cycle in a temperature and humidity controlled environment. They were housed in facilities approved by the Association for Accreditation of Laboratory Animal Care at the University of Vermont, in cages covered with filter tops for a minimum of 1 week prior to entry into the study. Purina Rat Chow and water were available ad libitum.

Cells. Murine leukemia L1210 cells were routinely grown at 37°C, in a humidified environment of 90% air and 10% CO_2, in antibiotic-free McCoys 5A medium supplemented with 5% DHS. For experimental purposes, MEM with 5% DHS, 1% glutamine, 1% MEM non-essential amino acids, 1% MEM amino acids and an antibiotic-antimycotic containing 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 ug/ml amphotericin B was used. To avoid potential interference with the spectrophotometric assays for nitrite and hemoglobin this medium did not contain phenol red. Both media, the glutamine and the amino acid supplements were obtained from Gibco, Grand Island, NY, the DHS was purchased from JRH Biosciences and the antibiotics were purchased from Sigma Chemical Company, St. Louis, MO. Cells were negative for Mycoplasma (Cell Shipper DNA Fluorochrome stain, Bionique Laboratories).

Induction of Pulmonary Inflammation and Collection of AM and RBC. Rats (200-250 gm) received a single fibrogenic dose of BLM (Blenoxane, a generous

gift from Bristol-Myers Co.), 3.6 mg/kg, given i.t. while under light anesthesia induced by intraperitoneally administered chloral hydrate (15). Control animals received a comparable volume of 0.9% sterile saline (NaCl) i.t.. Twenty-four hours later animals were sacrificed by a pneumothorax following i.p. sodium pentobarbital (Anthony Products Co., Arcadia, CA). The lungs were removed and lavaged with a total of 50 ml of ice-cold $Ca^{++}Mg^{++}$ free PBS. RBC obtained by cardiac puncture and BAL were isolated as previously described (16,17).

Assay for Macrophage Mediated Cytostasis. L1210 and AM were co-cultured in 96 well microtiter plates (Corning) for 24 h. Each well contained 5 x 10^4 effector cells and 5 x 10^3 L1210 target cells in 200 ul medium. The co-cultures were pulsed with [^{3}H]-thymidine (ICN Radiochemicals; specific activity 6.7 Ci/mmol), 0.5 uCi, for 2 h before being harvested onto glass filters with a PHD harvester (Cambridge Technology Inc.). In some experiments, 250 uM NMA (Sigma Chemical Co., St. Louis MO) final concentration, 2 x 10^6 RBC, or medium was added to the co-cultures.

Nitrite Assay. AM, 3 x 10^5, were cultured in MEM in 10% CO_2 at 37°C for 18 to 24 h. Supernate (100 ul) was tested for nitrite as previously described (17,18). In some experiments, 250 uM NMA final concentration, varying concentrations of RBC, or MEM was added to the cultures. Results are expressed as the nanomoles of nitrite/3 x 10^5 AM. Chemicals were purchased from Sigma Chemical Company, St. Louis, MO.

Hemoglobin Assay. The spectral characteristics of the hemoglobin in the RBC cultures established for the nitrite assay were determined. Specifically, RBC were harvested from the 96 well plates and suspended in 0.9 ml of PBS containing 0.1% Triton X-100. Samples were vortexed and allowed to stand at room temperature for 5 minutes before being scanned on the spectrophotometer. Results are expressed as the change in absorbance at three wavelengths (540 nm, 576 nm, and 620 nm) between RBC incubated alone and RBC incubated with BLM-AM.

EPRS. 10^6 AM or BLM-AM were cultured in the presence or absence of 10^8 RBC for 24 h as described above. At the end of the incubation, the RBC were harvested, washed once with PBS and suspended in 0.4ml of PBS. They were then frozen at -70° C until analyzed for nitrosylated hemoglobin (HbNO) as previously described (24,25). In some experiments 1mM NMA was added to the co-cultures. In all experiments, RBC cultured alone were used as a baseline which was subtracted from all the test samples. Results are expressed as the micromoles of HbNO/10^8 RBC.

RESULTS. Initially we determined the spectral characteristics of RBC lysate following co-culture with BLM-AM for 24 h. The absorption spectrum of this lysate demonstrated a decreased absorbance at 540 and 576 nm, and increased absorbance at 620-630 nm, and 470-490 nm (Figure 1). These changes are similar to those observed by Smith and Kruszyna (26) for the trapping of NO by extracellular hemoglobin. Further, nitrite causes the oxidation of hemoglobin resulting in the formation of methemoglobin (27). Consistent with this hypothesis,

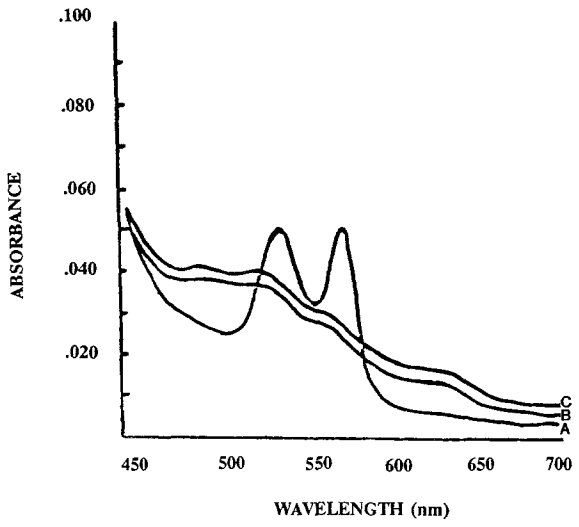

Figure 1. **Spectral changes in hemoglobin exposed to BLM-AM.** Rats were given a single, i.t. dose of BLM and sacrificed 24 h later. AM were harvested and co-cultures were established as described in Material and Methods. After 48 h, RBC were collected from the co-cultures, lysed and scanned spectrophotometrically over several wavelengths. The scans depicted are of a typical experiment. (A) represents 3 x 10^6 RBC in the absence of BLM-AM. (B) and (C) represent equivalent numbers of RBC in the presence of 3 x 10^5 BLM-AM from two different rats.

at low ratios of RBC:BLM-AM (10:1), the pigment of the RBC becomes brown which is the characteristic color of methemoglobin.

To determine if a stoichiometric relationship existed between RNI production by BLM-AM and hemoglobin concentration, varying amounts of RBC were co-cultured with a constant number of BLM-AM. After 48 h, we assayed the supernate for nitrite concentration and quantitated the spectral changes of the hemoglobin as described. As the concentration of RBC increased, the amount of measurable nitrite in the medium decreased (Table 1). A significant negative correlation existed between increased absorbance at 620 nm and decreased nitrite concentration.

To confirm that this spectral shift was due to HbNO formation, we assayed the RBC using EPRS. Table 2 demonstrates that RBC co-cultured with BLM-AM have increased HbNO when compared with RBC co-cultured with AM taken from saline treated controls. Further, when the arginine analog NMA was added to BLM-AM:RBC co-cultures, the RBC had no measurable HbNO. This corresponds with the observation that BLM-AM lack cytostatic activity when cultured in the presence of NMA and supports our hypothesis that NO is an important mediator of cytostasis in this model.

Table 1. **Correlation of nitrite concentration and change in absorbance characteristics of hemoglobin.** BLM-AM were co-cultured in the presence and absence of increasing concentrations of RBC. After 48 h the RBC were harvested, lysed and scanned spectrophotometrically. Results are reported as the mean change in absorbance ± the SEM. Concurrently, 100 µl of supernate was assayed for nitrite concentration as described in Materials and Methods. Results are expressed as the nanomoles of nitrite per 300,000 BLM-AM ± the SEM (n=14 for each condition). Linear regression analysis was performed using a software package developed at the University of Vermont. There is a significant negative correlation between increasing absorbance at 620 nm and decreasing measurable nitrite in the medium (correlation coefficient = -0.9976).

RBC x 10^6	delta Absorbance			[Nitrite]
	540 nm	576 nm	620 nm	
0	---	---	---	7.16 ± 1.11
3	-.013 ± .001	-.028 ± .002	+.012 ± .0004	5.82 ± 0.68
6	-.034 ± .001	-.056 ± .002	+.018 ± .0006	3.61 ± 0.62
9	-.049 ± .005	-.075 ± .007	+.023 ± .0013	1.63 ± 0.48
12	-.074 ± .008	-.102 ± .011	+.041 ± .0165	0.84 ± 0.33

DISCUSSION. The role for RNI has been established as an important effector mechanism in macrophage mediated cytostasis (12-15). We have previously shown that BLM-AM spontaneously release RNI, through an L-arginine dependent effector mechanism, and that RNI are responsible for inhibition of both DNA synthesis and aconitase activity in L1210 cells (17). Further, BLM-AM cytostatic activity was inhibited by the presence of RBC in the co-culture (16). We now report that the hemoglobin isolated from RBC co-cultured with BLM-

Table 2. **Changes in HbNO concentration when RBC are co-cultured with BLM-AM.** Rats were given a single i.t. dose of BLM or 0.9% saline and sacrificed 24 hours later. AM were harvested and co-cultures were established as described in Materials and Methods. Twenty four hours later, RBC were harvested and assayed by EPRS for HbNO formation as described. Results are expressed as micromoles of HbNO/10^8 RBC/10^6 AM ± the SEM (n = 12 for each condition). Analysis of variance was performed using a software package developed at the University of Vermont. * indicates $p < 0.05$.

Co-Culture Conditions	[HbNO]
Control AM + RBC	0.2 ± 0.1
BLM-AM + RBC	4.0 ± 1.5*
BLM-AM + RBC + NMA	0

AM has a significant shift in spectral characteristics suggestive of hemoglobin nitrosylation. This observation is substantiated by EPRS spectra characteristic of HbNO. These results indicate that the mechanism of RBC protection against BLM-AM cytostasis is through Hb absorption of NO.

NO, a short lived molecule which has also been described as the product of endothelial cells, EDRF, is a potent vasodilator (22), and it has recently been identified in rats and mice in endotoxin shock (28). The similarities between EDRF and RNI or NO produced by activated macrophages is striking and raises many interesting questions about the link between inflammation and edema. It has long been known that EDRF is inhibitable by oxyhemoglobin and to a lesser extent by methemoglobin (29-31), thus the role of hemoglobin in preventing vasodilation by EDRF could be important physiologically during hemorrhage. Similarly, there may be a role for hemoglobin in limiting damage to surrounding tissue during episodes of inflammation. One consequence of hemorrhage into an area of inflammation may be down regulation of activated macrophages and subsequent prevention of extensive tissue damage. Further, the resolution of the acute inflammatory response may be modified by the presence of blood in the area. Taken together, we hypothesize that the down regulation of RNI production by BLM-AM permits up-regulation of another process in the AM which is important in the fibrotic phase which follows.

Acknowledgments: The authors wish to thank Bristol-Myers Company for the generous donation of Blenoxane and R. Mary Gundel for editing this manuscript. This work was supported in part by grants from the Lake Champlain Cancer Research Organization (AEH), the American Cancer Society CH-380 (MPH) and NIH grant HL 14127 from the National Heart, Lung and Blood Institute (RPS). MPH is a recipient of an RCDA from the National Cancer Institute. The Bruker ESP-300 spectrophotometer was purchased with funding from NSF (Grant CHE-8701406).

REFERENCES

1. Adamson, I.Y.R. (1984) Environ. Health Perspect. **55**, 25-36.
2. Barry, B.E. and Crapo, J.D. (1985) Am. Rev. Respir. Dis. **132**, 548-555.
3. Hampson, E.C.G.M. and Pond, S.M. (1988) Br. J. Exp. Pathol. **69**, 57-68.
4. Cooper, J.A.D., Jr., Zitnik, R.J. and Matthay, R.A.. (1988) Ann. Rev. Med. **39**, 395-404.
5. Gerberick, G.F., Jaffe, H.A., Willoughby, J.B. and Willoughby, W.F. (1986) J. Immunol. **137**, 114-121.

6. Conkey, N.S., Yarbro, J.W., Ferrari, H.A. and Zeidler, R.B. (1986) Mol. Pharm. **30**, 48-52.
7. Beutler, B. and Cerami, A. (1988) Ann. Rev. Biochem. **57**, 505-518.
8. Lee, J. and Vilcek, J. (1987) Lab. Invest. **56**, 234-248.
9. Piguet, P.F., Collart, M.A., Grau, G.E., Kapanci, Y. and Vassalli, P. (1989) J. Exp. Med. **170**, 655-663.
10. Currie, G.A. (1978) Nature **273**, 758-759.
11. Adams, D.O., Kao, K-J, Farb, R. and Pizzo, S.V. (1980) J. Immunol. **124**, 293-300.
12. Hibbs, J.B., Jr., Taintor, R.R. and Vavrin, Z. (1988) Science **235**, 473-476.
13. Ding, A.H., Nathan, C.F. and Stuehr, D.J. (1988) J. Immunol. **141**, 2407-2412.
14. Amber, I.J., Hibbs, J.B., Jr., Taintor, R.R. and Vavrin, Z. (1988) J. Leukocyte Biol. **43**, 187-192.
15. Stuehr, D.J. and Nathan, C.F. (1989) J. Exp. Med. **169**, 1543-1555.
16. Huot, A.E., Gundel, R.M. and Hacker, M.P. (1990) Cancer Res. **50**, 2351-2355.
17. Huot, A.E. and Hacker, M.P. (1990) Cancer Res. **50**, 7863-7866.
18. Zwilling, B.S. and Campolito, L.B. (1977) J. Immunol. **119**, 838-841.
19. Granger, D.L. and Lehninger, A.L. (1982) J. Cell Biol. **95**, 527-535.
20. Granger, D.L., Taintor, R.R., Cook, J.L. and Hibbs, J.B., Jr. (1980) J. Clin. Invest. **65**, 357-370.
21. Stuehr, D.J., Gross, S.S., Sakuma, I., Levi, R. and Nathan, C.F. (1989) J. Exp. Med. **169**, 1011-1020.
22. Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1988) Hypertension **12**, 365-372.
23. Sakuma, I., Stuehr, D.J., Gross, S.S., Nathan, C.F. and Levi, R. (1988) Proc. Natl. Acad. Sci., USA **85**, 8644-8667.
24. Kruszyna, H., Kruszyna, R., Smith, R.P., and Wilcox, D.E. (1987) Toxicol. Appl. Pharmacol. **91**, 429-438.
25. Wilcox, D.E., Kruszyna, H., Kruszyna, R. and Smith R.P. (1990) Chem. Res. Toxicol. **3**, 71-76.
26. Smith, R.P. and Krusynza, H. (1979) J.Pharmacol. Exp. Ther. **191**, 557-563.
27. Spagnuolo, C., Rinelli, P., Coletta, M., Vecchini, P., Chiancone, E. and Ascoli, F. (1988) Biochim. et Biophys. Acta **956**, 119-126.
28. Wang, Q., Jacobs, J., Peleo, J., Kruszyna, H., Kruszyna, R., Smith, R. and Wilcox, D. (1991) Life Sci, Pharmacol LeH. in press.
29. Hogan, J.C., Lewis, M.J. and Henderson, A.H. (1988) Br. J. Pharmacol. **94**, 1020-1022.
30. Wellum, G.R., Irvine, T.W., Jr., and Zervas, N.T. (1980) J. Neurosurg. **53**, 486-490.
31. Martin, W., Smith, J.A. and White, D.G. (1986) Br. J. Pharmacol. **89**, 563-571.