MACROLIDE ANTIBIOTICS IMPROVE PHAGOCYTIC CAPACITY AND REDUCE INFLAMMATION IN SULFUR MUSTARD-EXPOSED MONOCYTES

Xiugong Gao[1]*, Radharaman Ray[2], Yan Xiao[3], Keiko Ishida[1], and Prabhati Ray[1]

[1]Walter Reed Army Institute of Research
Silver Spring, MD 20910

[2]United States Army Medical Research Institute of Chemical Defense
Aberdeen Proving Ground, MD 21010

[3]National Institute of Standards and Technology
Gaithersburg, MD 20899

ABSTRACT

Sulfur mustard (SM) inhalation causes apoptosis and death of airway epithelial cells as well as inflammation in the airway. Efficient clearance of the cell debris by alveolar macrophages (AMs) is necessitated to reduce the inflammation. Macrolide antibiotics have been reported to have anti-inflammatory properties by modulating the production of proinflammatory cytokines and mediators, and by improving macrophage functions. The present study investigated the effects of four FDA-approved macrolide antibiotics, namely azithromycin, clarithromycin, erythromycin, and roxithromycin, on macrophage chemotactic and phagocytotic function and on inflammatory cytokines/mediators production *in vitro* using SM-exposed monocyte THP-1 cells.

Using flow cytometry we found that chemotaxis and phagocytosis of the monocytes reduced upon exposure to 10 μM SM (8.1% and 17.5%, respectively) were restored by treatment with 10 μM of any of the four macrolides. Cytokine measurements using real-time RT-PCR and ELISA revealed that overexpression of proinflammatory cytokines following SM exposure was decreased by 50%–70% with macrolide treatment. Similarly, immunocytochemical detection of inducible nitric oxide synthase (iNOS) showed that exaggerated expression of iNOS induced by SM exposure was totally inhibited by treatment with macrolides.

Together, these data demonstrate that macrolide antibiotics were effective in improving the degenerated chemotactic and phagocytotic functions of macrophages following SM exposure, and in reducing SM-induced overproduction of proinflammatory cytokines and mediators. These effects may lead to improved clearance of apoptotic material in the airway and ultimately result in reduced airway inflammation and injury caused by SM inhalation. Our results suggest that macrolide antibiotics may serve as potential vesicant respiratory therapeutics.

1. INTRODUCTION

Sulfur mustard (SM), a vesicant with potential for application as both warfare and terrorism agents, causes blistering of the skin as well as damage to the eyes and the respiratory tract. Starting from World War I, SM has been used numerous times in the last century, causing injuries and deaths to hundreds of thousands of individuals (Szinicz, 2005; Kehe and Szinicz, 2005). Being the major chemical agent during World War II, SM was produced and stockpiled by many countries and is probably still the most distributed chemical warfare agent in the world (Szinicz, 2005). Therefore, SM remains a significant military and civilian threat.

Alveolar macrophages (AMs) have direct contact with inhaled vapor and could react directly with SM (Amir et al., 2000). Besides acting as the protective cells by phagocytosing microbes and particulate matters invading the lungs, macrophages also serve a very important function of stimulating and maintaining humoral immunity via production of cytokines and activation of T-cells. Therefore macrophages are considered the first line of defense against inhaled chemicals and participate in the immune reaction to lung injury by the release of oxidants and inflammatory cytokines. Damage to macrophages by SM is thus detrimental to not only their phagocytic activity but also their immune protection against infection following SM inhalation injury.

Previous studies have shown that SM inhalation causes apoptosis and death of airway epithelial cells as well as inflammation in the airway (Gao et al., 2007; Ray et al., 2008). If these cells are not efficiently cleared by neighboring AMs, an accumulation of apoptotic and necrotic fragments may ensue, promoting the ongoing inflammation and loss of structural integrity, which are hallmarks of SM inhalation exposure (Eisenmenger et al., 1991). This may result, at least partially, from an over-

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
DEC 2008	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Macrolide Antibiotics Improve Phagocytic Capacity And Reduce Inflammation In Sulfur Mustard-Exposed Monocytes	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Walter Reed Army Institute of Research Silver Spring, MD 20910**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM002187. Proceedings of the Army Science Conference (26th) Held in Orlando, Florida on 1-4 December 2008, The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**8**	

whelming of the clearance capacity of AMs, either by excess apoptosis of airway epithelial cells, or by a functional degeneration in the AMs after SM exposure.

An increasing number of studies have suggested that macrolide antibiotics have anti-inflammatory properties by modulating the production of proinflammatory cytokines (Rubin, 2004). In addition, a few studies have reported improved macrophage functions in the presence of these antibiotics (Xu et al., 1996; Hodge et al., 2006). Our current research has focused on therapeutic treatment strategies that aim specifically at improving SM inhalation injuries. In this study, the effects of four FDA-approved macrolide antibiotics, *i.e.*, azithromycin, clarithromycin, erythromycin, and roxithromycin, on macrophage chemotactic and phagocytotic function, and on the expression of proinflammatory cytokines and mediators, were tested *in vitro* using SM-exposed monocyte THP-1 cells.

2. MATERIALS AND METHODS

2.1 Reagents

Sulfur mustard (2,2'-dichlorodiethyl sulfide; 4 mM) was acquired from the US Army Edgewood Research, Development and Engineering Center (Aberdeen Proving Ground, MD). Erythromycin and roxithromycin were obtained from Sigma (St. Louis, MO). Azithromycin and clarithromycin were kindly provided by William Ellis of the Department of Chemical Information, WRAIR (Silver Spring, MD). All the antibiotics were dissolved in ethanol at a concentration of 10 mM and then diluted to the desired concentrations using culture medium. Yellow-green fluorescent latex beads (L0905, ø2.0 µm, amine-modified, 2.5% solids) were obtained from Sigma.

2.2 Cell Culture

Human monocyte THP-1 cells were obtained from ATCC (Manassas, VA). Cells were grown as suspension in the optimized media as formulated by the manufacturer and cultured at 37 °C under humidified 5% CO_2. Cells were maintained and subcultured following instructions from the manufacturer.

2.3 SM Exposure and Macrolide Antibiotic Treatment

Before exposure, the medium was replaced with fresh medium and cell density was adjusted to ~10^6 cells/ml. Macrolides were added to the medium immediately before exposure. Aliquots of frozen SM stock solution (4 mM) were thawed in a safety chemical fume hood at room temperature, vortexed for 1 min, and then diluted in fresh medium and added to the cell culture at the final concentrations indicated. The treated culture flasks were maintained at room temperature in the safety hood for 1 hr to allow venting of volatile SM, then transferred to an incubator of 37°C with 5% CO_2 and incubated for 23 hr. For subsequent chemotaxis and phagocytosis analyses, cells were washed in fresh medium and spin down at 130 *g* for 7 min (repeated 3 times) to remove dead cells (debris). The remaining cells (>98% alive) were then diluted to the desired densities with fresh medium.

2.4 Cell Viability Assay

Cell viability was measured by the MTS assay (Cory et al., 1991) using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit from Promega (Madison, WI) and instructions from the manufacturer were followed. Briefly, 100 µl of cell suspension at ~2.0×10^5 cells/ml was added into wells of a 96-well plate, cells were then incubated with macrolide antibiotic solutions with or without SM exposure. The plate was kept at 37 °C with 5% CO_2 for 24 h. Afterwards 20 µl of assay reagent was added to each well that contained 100 µl cell suspension, the plate was incubated for additional 3 h, and the resultant absorbance was recorded at 490 nm using a 96-well plate reader. Each experiment was performed with eight independent replicates and repeated three times.

2.5 Chemotaxis to Chemotactic Peptide

Chemotactic ability was tested using the Migratest chemotaxis kit from Orpegen Pharma (Heidelberg, Germany), following a protocol adapted from the manufacturer's instructions. Briefly, 350 µl of 50 nM chemotactic peptide *n*-formyl-Met-Leu-Phe (fMLP) solution was added into wells of a 24-well plate, a cell culture insert was then inserted into each well and 100 µl of cell suspension at ~5.0×10^5 cells/ml was added subsequently into the insert. Cells were allowed to migrate through the membrane (pore size of 3.0 µm) of the insert into the wells by incubation at 37 °C for 30 min without shaking. Precisely at the end of incubation the inserts were removed, and the cell suspension in the plate wells were mixed with counting beads. The number of migrated cells was then determined by flow cytometric analysis on a FACSCalibur analyzer (BD Biosciences; San Jose, CA). Two regions were defined for monocytes and counting beads respectively. Data acquisition was ended after acquiring exactly 2,000 events in the second region (beads), and the number of events in the first region (monocytes) was then counted and compared.

2.6 Phagocytosis of Latex Beads

One ml of monocyte suspension at ~1.0×10^6 cells/ml was mixed with 50 µl of fluorescent latex beads suspension diluted to ~1.0×10^9 beads/ml in phosphate-buffered saline (PBS). The mixture was incubated at 37 °C for 30 min without shaking for 1 h. Then cells were

washed with 1 ml PBS and spin down at 130 *g* for 7 min (while beads remained in suspension at this speed). The wash-spin cycle was repeated for totally three times to completely remove un-phagocytosed beads. The cells were then fixed in 70% (v/v) ethanol for 30 min and resuspended in 1 ml of PBS. Intracellular fluorescence was subsequently analyzed by flow cytometry.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA kits from BD Biosciences (San Diego, CA) were used for the quantification of IL-1β, IL-6, IL-8, and TNF in the culture medium following the manufacturer's instructions. The results are expressed as a percentage of the control (cells unexposed to SM) and represent the mean ± SE of three experiments performed in triplicate.

2.8 Real-Time RT-PCR

Total RNA was isolated from ~1.0×10^7 cells of each sample, using the RNAqueous-4PCR kit from Ambion (Austin, TX) following the manufacturer's instructions and with the optional DNase I treatment step to avoid contamination with genomic DNA. Reverse transcription of mRNA was carried out using the High-Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA), using 1 µg of total RNA in a 100 µl final volume. Real-time PCR was carried out on the 7500 Fast Real-Time PCR system of Applied Biosystems using TaqMan Gene Expression Assays primer/probe sets and the standard thermal-cycling conditions for relative quantification designed by the manufacturer. Results were analyzed with the SDS software v1.3.0 on the system using the $2^{-\Delta\Delta C_T}$ method. β-actin was used as an endogenous control to correct for variations in input RNA amount and in cDNA amplification of different samples.

2.9 Immunocytochemical Analysis

A thin layer of cells on slide was prepared using Shandon Cytospin 4 cytocentrifuge (Thermo Scientific; Waltham, MA). The cells were rinsed with 10 mM Tris, 150 mM NaCl, pH 7.4 (TBS) and fixed in 10% zinc formalin solution. The slides were then processed robotically on the BenchMark XT IHC/ISH workstation of Ventana (Tucson, AZ) to react with the primary and secondary antibodies. The primary antibody used was mouse anti-iNOS MAb from BD Biosciences (San Jose, CA), and was diluted 1:1000 in TBS + 0.5% (w/v) Triton X-100 (TBST). The secondary antibody was pre-diluted biotinylated rabbit anti-mouse IgG from Ventana. iNOS signals were detected by quantum dot-streptavidin conjugates (streptavidin-Qdot655) from Invitrogen (Carlsbad, CA), which was diluted 1:200 with the Qdots incubation buffer (Invitrogen). Fluorescence signals were visualized, captured and processed as reported previously (Xiao and Barker, 2004; Xiao et al., 2005).

2.10 Data Analysis

For comparative studies, Student's *t*-test (unpaired) or one-way ANOVA tests (with Bonferroni post test if $p < 0.05$) were used for statistical analysis. Differences were considered statistically significant if a p value of < 0.05 was achieved.

3. RESULTS

3.1 Cell Viability

MTS assay of cell viability 24 h post-exposure showed that SM exerted cytotoxicity in a concentration-dependent manner in monocyte THP-1 cells (Fig. 1A). At concentrations below 1.0 µM, no significant decrease in cell viability was observed. At 100 µM, however, SM caused severe toxicity with only 4.2% of the cell population survived. At 10 µM, SM caused 39.0% of cell death. Roxithromycin did not show significant protective effect on cell viability in the concentration range of 0.1–50 µM (data not shown). At higher concentrations (> 50 µM), roxithromycin alone caused reduction in the viability of THP-1 cells (Fig. 1B). Similar results were observed for other macrolides (data now shown). Based on these observations, the following experiments were performed with 10 µM of SM and/or 10 µM of macrolide antibiotics. This concentration of macrolides is clinically achievable (Jain and Danziger, 2004).

3.2 Chemotaxis

Chemotactic ability of monocyte THP-1 cells was tested by allowing the cells to migrate through a membrane with a pore size of 3.0 µm towards a concentration gradient of the chemotactic peptide fMLP. The number of migrated cells was then determined by flow cytometric analysis (Fig. 2). For cells exposed to 10 µM of SM, chemotaxis of the surviving cells decreased 41.4% compared with normal cells not exposed to SM ($p < 0.001$). Treatment with 10 µM of macrolide antibiotic increased the number of chemotactic cells by 33.2%–41.2% for normal cells and 124.4%–133.1% for SM-exposed cells ($p < 0.001$), with no significant differences observed among the four different macrolides (Fig. 3). It is apparent that macrolides restored chemotaxis ability of the surviving SM-exposed cells on a par with similarly treated normal cells.

3.3 Phagocytosis

Phagocytotic capacity of monocytes was tested using fluorescent latex beads and analyzed by flow cytometry (Steinkamp et al., 1982). Representative histograms showing fluorescence distribution in the cells are seen in Fig. 4, and Fig. 5 is a bar graph showing the statistic

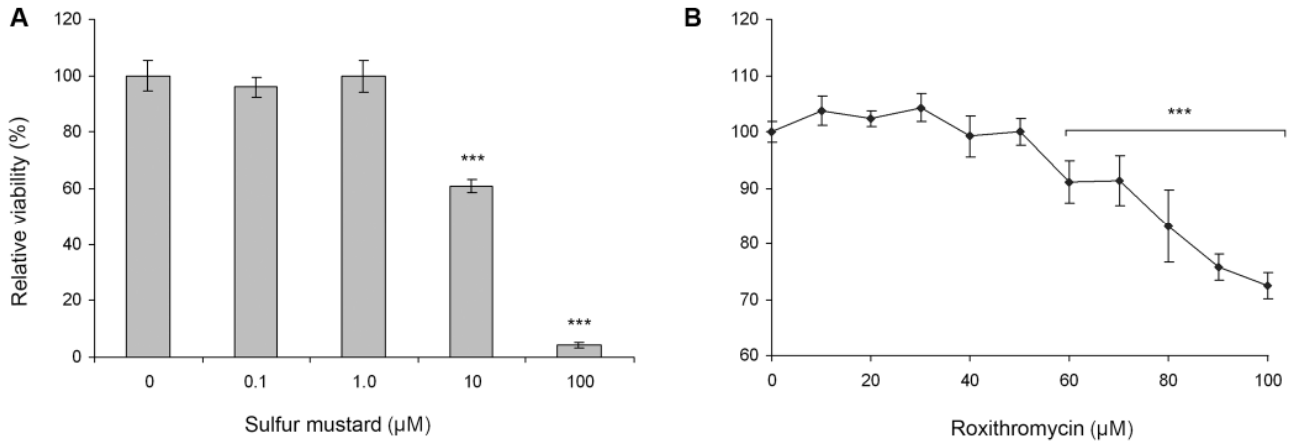

Figure 1. Effect of (A) sulfur mustard (SM) and (B) roxithromycin on the viability of monocyte THP-1 cells. Cell viability was measured using the MTS assay 24 h after SM exposure or after incubation with roxithromycin, and is expressed as a percentage of the control (0 μM). ***$p < 0.001$ versus control.

results. Normal cells not incubated with fluorescent beads (Fig. 4, top panel) produced low baseline fluorescence, with only ~2.9% cells having intensity over 20 (a.u.). After incubation with latex beads, 41.2% of the cell population has phagocytosed beads within the cell. When cells were exposed to 10 μM of SM, phagocytosis of surviving cells decreased comparing with cells not exposed to SM, with the percentage of cells having phagocytosed beads decreased to 26.4% ($p < 0.001$). Treatment with 10 μM of azithromycin, clarithromycin, erythromycin, or roxithromycin increased phagocytotic cells to 64.5%, 63.4%, 62.0%, and 65.3% respectively for normal cells, and to 62.5%, 61.4%, 61.3% and 62.6% respectively for SM-exposed cells (Fig. 5). For all the four macrolide antibiotics, the differences between cells treated and untreated by these drugs were significant ($p < 0.001$); whereas the differences between the various macrolides were not significant. Therefore, macrolides improved phagocytosis of surviving cells to a level that is comparable with that of similarly treated normal cells.

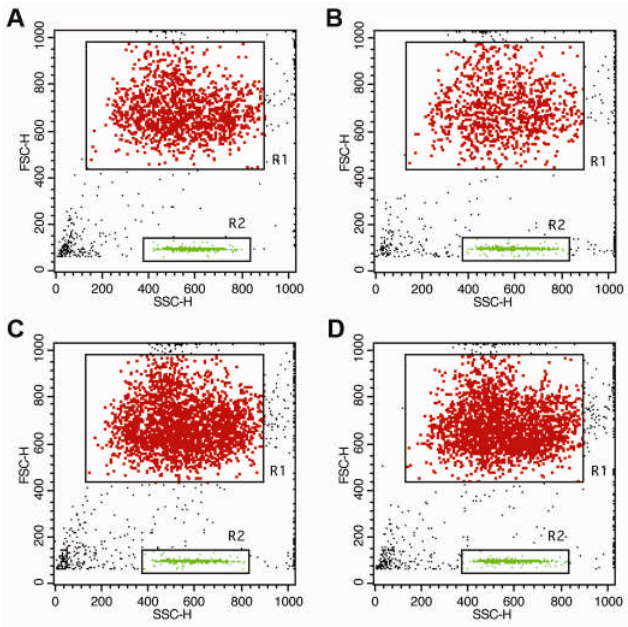

Figure 2. Representative dot plots showing the effect of sulfur mustard (SM) and roxithromycin on chemotaxis of monocyte THP-1 cells. R1 represents cells migrated through the membrane barrier, R2 represents counting beads. (A) normal cells; (B) cells survived after exposure to 10 μM SM; (C) normal cells treated with 10 μM roxithromycin; and (D) surviving cells treated with 10 μM roxithromycin. In all cases the number of events in R2 (beads) is exactly 2,000. Events in R1 are highlighted using the Hilite Dots feature of the BD CellQuest Pro software.

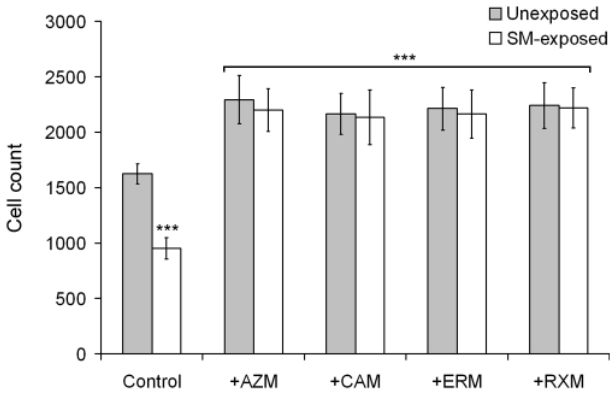

Figure 3. Effect of macrolide antibiotics on chemotaxis of monocyte THP-1 cells. The number of events (cells) in the first region (R1) of Fig. 2 was counted and subtracted by that of a control experiment where no chemoattractant was added, and the mean values with standard deviation are shown. "+" indicates the macrolide antibiotic added after SM exposure. AZM, azithromycin; CAM, clarithromycin; ERM, erythromycin; and RXM, roxithromycin. ***$p < 0.001$ between the two control cells (unexposed and SM-exposed) and cells treated with macrolides versus their respective controls.

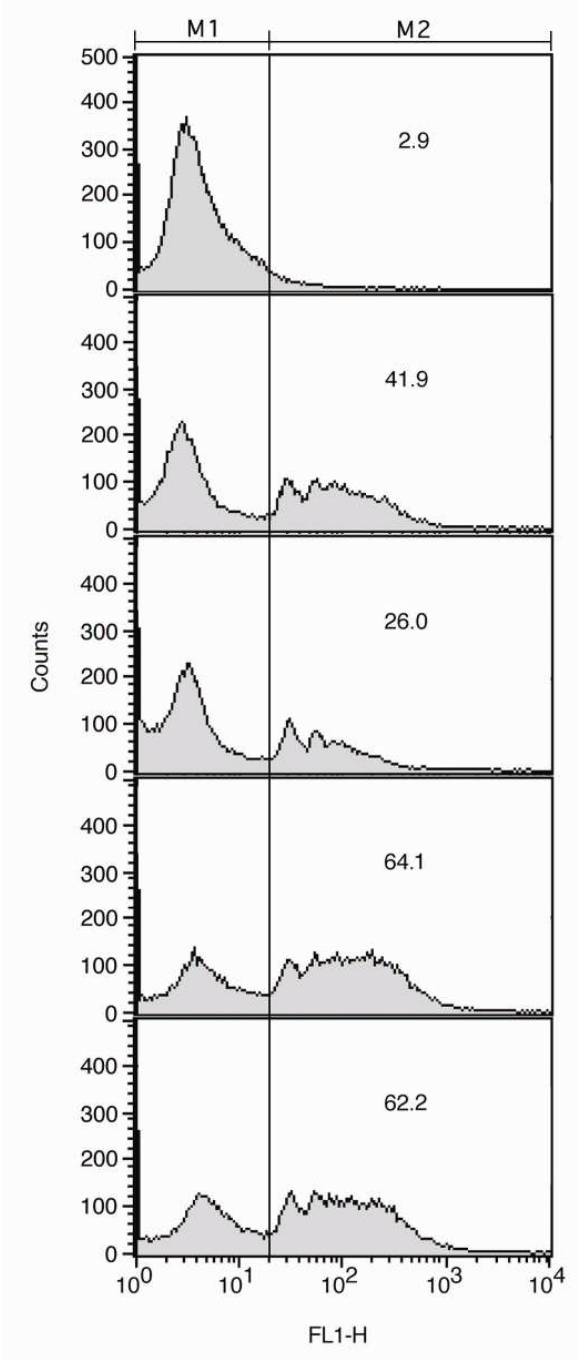

Figure 4. Representative histograms showing the effect of sulfur mustard (SM) and roxithromycin on phagocytosis of fluorescence latex beads by monocyte THP-1 cells. The histogram is divided by two markers, with M1 (1–20) represents cells without phagocytosis and M2 (20–9910) cells with phagocytosed beads. The five panels in the figure represent respectively (from top to bottom): 1) normal cells not incubated with beads; 2) normal cells; 3) cells surviving SM (10 μM) exposure; 4) normal cells treated with 10 μM roxithromycin; 5) cells surviving SM (10 μM) exposure and treated with 10 μM roxithromycin. The percentages of phagocytic cells in the figure are from a single experiment as shown in the histograms, while those in the text are mean values of three experiments.

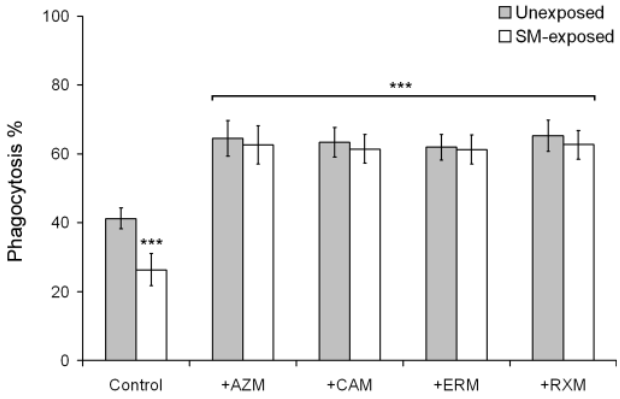

Figure 5. Effect of macrolide antibiotics on phagocytosis of monocyte THP-1 cells. The percentage of phagocytic cells is shown with standard deviation. "+" indicates the macrolide antibiotic added after SM exposure. AZM, azithromycin; CAM, clarithromycin; ERM, erythromycin; and RXM, roxithromycin. ***$p < 0.001$ between the two control cells (unexposed and SM-exposed) and cells treated with macrolides versus their respective controls.

3.4 Cytokine Expression and Release

ELISA experiments measuring proinflammatory cytokines in the culture media (Fig. 6A) showed that exposure to 10 μM SM increased release of interleukin (IL)-8 and tumor necrosis factor (TNF) substantially and that of IL-6 slightly. The increases reached to 9.2-, 5.7- and 1.8-fold, respectively. No significant change was observed for the release of IL-1β. With 10 μM roxithromycin, release of the overexpressed cytokines was significantly inhibited, by 64.2%, 65.0%, and 32.6% respectively. Other macrolides showed similar results.

The mRNA expression level of the proinflammatory cytokines, as obtained from the real-time RT-PCR experiments, agreed with their protein release patterns (Fig. 6B). Exposure to 10 μM SM increased the mRNA expression to 16.3-, 7.5- and 2.7-fold, respectively for IL-8, TNF, and IL-6. Treatment with 10 μM roxithromycin reduced mRNA expression by 68.0%, 62.4%, and 53.6% respectively. The mRNA level of IL-1β did not change significantly. No significant differences between different macrolides were observed.

3.5 iNOS Expression

iNOS expression in THP-1 cells was measured by immunocytochemistry using quantum dots as fluorophore. Shown in Fig. 7A–F are two-dimensional (2D) images showing the effect of the macrolides on iNOS expression in SM-exposed THP-1 cells. SM at 10 μM caused overexpression of iNOS, and this effect was inhibited by the four macrolides. Results from the quantitative analysis of the fluorescence signals are summarized in Fig. 7G.

Exposure to 10 µM SM dramatically increased iNOS expression (8.8-fold). However, when 10 µM macrolide antibiotics were added to the culture medium, iNOS expression was brought down to approximately the basal level. Differences among various macrolide were not statistically significant.

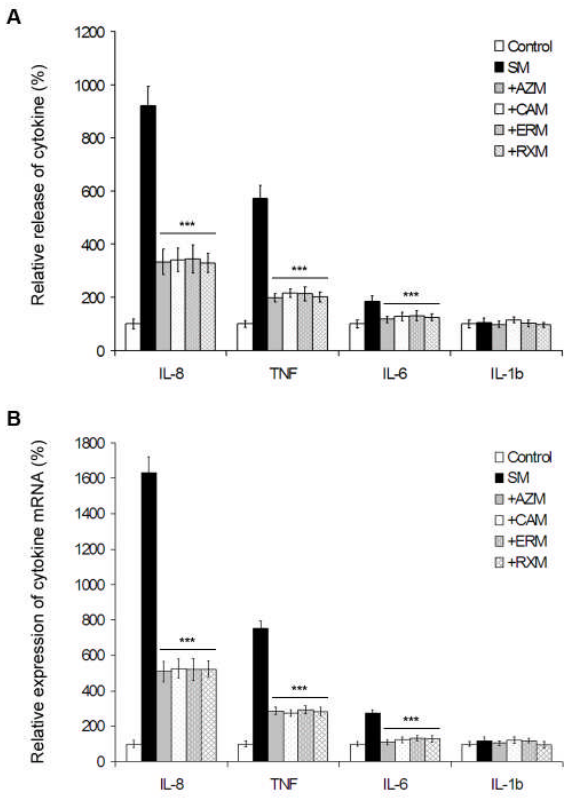

Figure 6. Inhibition by macrolide antibiotics of pro-inflammatory cytokine (A) protein release and (B) mRNA expression from SM-exposed monocyte THP-1 cells. Ten (10) µM of the four macrolide antibiotics were added to cells exposed to 10 µM SM. Cytokine release and mRNA expression were measured 24 h after exposure by ELISA and real-time RT-PCR, respectively. Results are expressed as a percentage of the control (unexposed) cells. "+" indicates the macrolide antibiotic added after SM exposure. AZM, azithromycin; CAM, clarithromycin; ERM, erythromycin; and RXM, roxithromycin. ***p < 0.001 versus cells exposed to SM only ("SM").

4. DISCUSSION

In contrast to the protective effect seen in airway epithelial cells (Gao et al., 2007), the macrolides did not increase cell viability in macrophages. On the contrary, cell viability decreased significantly when macrolide concentration exceeded 50 µM. The reason for this effect remains to be determined; however, it was likely a result of the high intracellular concentration of macrolides in macrophages. It is well known that macrophages concentrate macrolide antibiotics with an intracellular/extracellular concentration ratio (C/E) reaching several hundreds (Labro, 2002). High extracellular concentrations (>50 µM) of macrolide antibiotics may result in very high intracellular concentration that eventually become toxic to the cells.

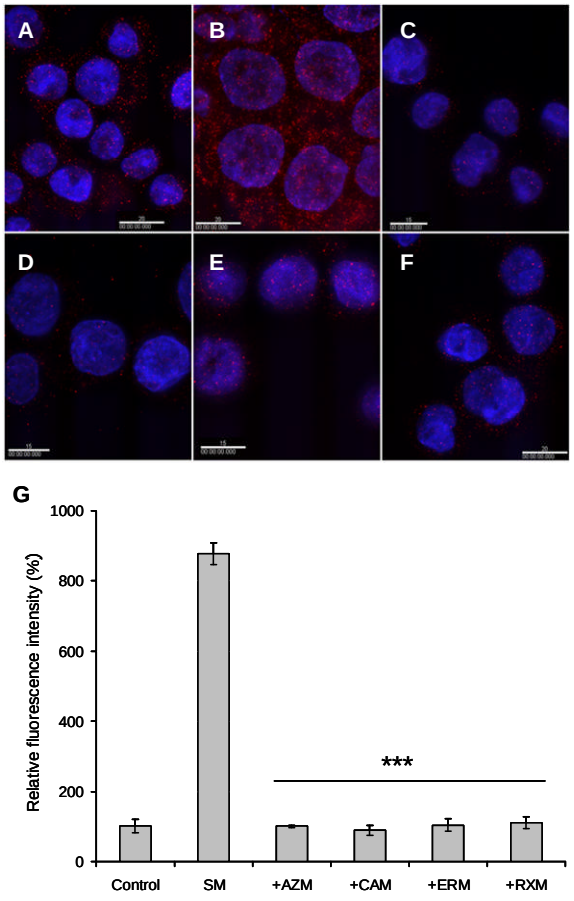

Figure 7. Effect of macrolide antibiotics on iNOS expression in SM-exposed monocyte THP-1 cells. (A) control; (B) cells exposed to 10 µM SM; (C–F) cells exposed to 10 µM SM followed by treatment with 10 µM azithromycin, clarithromycin, erythromycin and roxithromycin, respectively. Representative 2D images from each sample are compared. The bars represent 20 µm in A, B and F, and 15 µm in C, D and E. (G) Relative fluorescence intensity showing quantitatively the effect of macrolide antibiotics on iNOS expression in SM-exposed monocyte THP-1 cells. Average fluorescence intensities were computed from cells ($n \geq 10$) in each sample and are expressed as relative to the control (100%). The concentrations for SM and various macrolides were all 10 µM. "+" indicates the macrolide antibiotic added after SM exposure. AZM, azithromycin; CAM, clarithromycin; ERM, erythromycin; RXM, roxithromycin. *** p <0.001 versus cells exposed to SM only ("SM").

Nevertheless, macrolide antibiotics significantly improved macrophage chemotactic and phagocytic activities. Following SM exposure, the chemotactic and phagocytic capacity of the surviving cells was impaired by 41.4% and 26.4%, respectively. Treatment with

macrolides (10 μM) restored these functions on a par with similarly-treated normal (unexposed) cells. It is worth to note that chemotaxis and phagocytosis of normal cells increased significantly (≥33.2% and ≥50.3%, respectively) after treatment with 10 μM macrolides. The improved chemotactic and phagocytic ability of macrophages may have clinical significance with regard to new therapy for SM inhalation toxicity, where there is inflammation associated with ineffective repair of the injured epithelium and loss of structural integrity. This inflammatory condition is associated with increased apoptosis and death of airway epithelial cells (Gao et al., 2007; Ray et al., 2008). In addition, the results presented here show that impaired capacity for phagocytic clearance of apoptotic bronchial epithelial cells is also likely to be important in SM inhalation toxicity. It has been demonstrated that macrophages play a key role in the resolution of inflammatory responses (Knapps et al., 2003).

In addition to the phagocytotic function, AMs also participate in host defense against SM-induced airway injury through the release of proinflammatory cytokines and oxidant mediators (Amir et al., 2000). Pro-inflammatory cytokines and mediators play a central role in initiating and propagating the inflammatory responses in both acute and chronic inflammatory processes. However, dysregulated production of these cytokines and mediators, particularly when in excess, may become pathogenic. There is compelling evidence that macrolide antibiotics exerted anti-inflammatory effects through modulation of the inflammatory cascade which is composed of a network of cytokines and chemokines (Tamaoki et al., 2004). Proinflammatory cytokines majorly include IL-1β, IL-6, IL-8, and TNF-α. Previous studies demonstrated that exposure of airway epithelial cells to SM *in vitro* resulted in a rapid increase in the mRNA expression and the protein release for all of the four cytokines. The increase was several fold for IL-1β and TNF, and 10- to 20-fold in the cases of IL-6 and IL-8 (Gao et al., 2007). Treatment with roxithromycin reduced the exaggerated expression of these cytokines by ~70%. The findings in the current study were very similar.

iNOS has been suggested to be an important biomarker of inflammation, as its overexpression leads to excessive production of NO, which contributes to the pathophysiology of inflammation and the resultant tissue damage (Gaston et al., 1994). NO is involved in several types of acute and chronic inflammation (Ialenti et al., 1992). Overproduction of NO by type II NOS or iNOS is associated with the development of airway inflammation (Kharitonov et al., 1995). Similar to our previous findings with airway epithelial cells (Gao et al., 2007; Gao et al., 2008), the current study demonstrated that macrolide antibiotics suppressed SM-exaggerated iNOS expression to near normal level in AMs.

The reduced overexpression of proinflammatory cytokines and iNOS by macrolide antibiotics in SM-exposed AMs is in agreement with previous *in vivo* and *in vitro* studies (Tsai and Standiford, 2004), and further supports the contention that macrolides can inhibit the production of proinflammatory mediators. This suppressive effect of macrolides may not be accounted for by their antimicrobial properties but rather by their anti-inflammatory actions, as evidenced by the fact that other antibiotics such as amoxicillin and cefaclor, the penicillin and cephalosporin antibiotics respectively, had no such effects even at high concentration (data not shown). Thus the immunomodulatory effects of antibiotics appear to be specific to macrolides. Kohri et al. (2000) made similar findings.

Some studies have reported differences in the potency of different macrolides in suppressing inflammation. For example, Ianaro et al. (2000) found that roxithromycin appears more effective than erythromycin and clarithromycin, whereas azithromycin only slightly affect the inflammatory reaction in a rat model of acute inflammation. In our study, no significant difference was found among the four macrolide, suggesting that these drugs are equally effective regardless the differences in the structure of the lactone ring. Kohri et al. (2000) also found similar results.

CONCLUSIONS

The current findings suggest that macrolides were effective in improving the degenerated chemotactic and phagocytotic functions of macrophages following SM exposure, as well as in reducing SM-induced over-production of proinflammatory cytokines and mediators. These effects may lead to improved clearance of apoptotic material in the airway and ultimately result in reduced airway inflammation and injury caused by SM inhalation. Thus, macrolide antibiotics can exert therapeutic effects independent of their anti-bacterial activity, suggesting that they may serve as potential vesicant respiratory therapeutics.

ACKNOWLEDGEMENTS

The opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army or the Department of Defense. This research was supported by the Defense Threat Reduction Agency - Joint Science and Technology Office, Medical S&T Division. Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedures. Such identification does not imply recommendation or endorsement by the National Institute of Standards and

Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

REFERENCES

Amir, A., Chapman, S., Kadar, T., Gozes, Y., Sahar, R., and Allon, N., 2000: Sulfur mustard toxicity in macrophages: effect of dexamethasone. *J. Appl. Toxicol.*, **20**, S51–S58.

Cory, A.H., Owen, T.C., Barltrop, J.A., and Cory, J.G., 1991: Use of an aqueous soluble tetrazolium/ formazan assay for cell growth assays in culture. *Cancer Commun.*, **3**, 207–212.

Eisenmenger, W., Drasch, G., von Clarmann, M., Kretschmer, E., and Roider, G., 1991: Clinical and morphological findings on mustard gas [bis(2-chloroethyl)sulfide] poisoning. *J. Forensic Sci.*, **36**, 1688–1698.

Gao, X., Ray, R., Xiao, Y., Barker, P.E., and Ray, P., 2007: Inhibition of sulfur mustard-induced cytotoxicity and inflammation by the macrolide antibiotic roxithromycin in human respiratory epithelial cells. *BMC Cell Biol.*, **8**, 17.

Gao, X., Ray, R., Xiao, Y., and Ray, P., 2008: Suppression of inducible nitric oxide synthase expression and nitric oxide production by macrolide antibiotics in sulfur mustard-exposed airway epithelial cells. *Basic Clin. Pharmacol. Toxicol.*, **103**, 255–261.

Gaston, B., Drazen, J.M., Loscalzo, J., and Stamler, J.A., 1994: The biology of nitrogen oxides in the airways. *Am. J. Respir. Crit. Care Med.*, **149**, 538–551.

Hodge, S., Hodge, G., Brozyna, S., Jersmann, H., Holmes, M., and Reynolds, P.N., 2006: Azithromycin increases phagocytosis of apoptotic bronchial epithelial cells by alveolar macrophages. *Eur. Respir. J.*, **28**, 486–495.

Ialenti, A., Ianaro, A., Moncada, S., and Di Rosa, M., 1992: Modulation of acute inflammation by endogeneous nitric oxide. *Eur. J. Pharmacol.*, **211**, 177–182.

Ianaro, A., Ialenti, A., Maffia, P., Sautebin, L., Rombolà, L., Carnuccio, R., Iuvone, T., D'Acquisto, F., and Di Rosa, M., 2000: Anti-inflammatory activity of macrolide antibiotics. *J. Pharmacol. Exp. Ther.*, **292**, 156–163.

Jain, R. and Danziger, L.H., 2004: The macrolide antibiotics: a pharmacokinetic and pharmacodynamic overview. *Curr. Pharm. Des.*, **10**, 3045–3053.

Kehe, K. and Szinicz, L., 2005: Medical aspects of sulphur mustard poisoning. *Toxicology*, **214**, 198–209.

Kharitonov, S.A., Wells, A.U., O'Connor, B.J., Cole, P.J., Hansell, D.M., Logan-Sinclair, R.B., and Barnes, P.J., 1995: Elevated levels of exhaled nitric oxide in bronchiectasis. *Am. J. Respir. Crit. Care Med.*, **151**, 1889–1893.

Kohri, K., Tamaoki, J., Kondo, M., Aoshiba, K., Tagaya, E., and Nagai, A., 2000: Macrolide antibiotics inhibit nitric oxide generation by rat pulmonary alveolar macrophages. *Eur. Respir. J.*, **16**, 62–67.

Knapp, S., Leemans, J.C., Florquin, S., Branger, J., Maris, N.A., Pater, J., van Rooijen, N., and van der Poll, T., 2003: Alveolar macrophages have a protective antiinflammatory role during murine pneumococcal pneumonia. *Am. J. Respir. Crit. Care Med.*, **167**, 171–179.

Labro, M.T., 2002: Cellular accumulation of macrolide antibiotics. Intracellular bioactivity. In *Macrolide Antibiotics.* Edited by Schönfeld W, Kirst H. Basel: Birkhäuser Berlag AG; pp37–52.

Ray, R., Keyser, B., Benton, B., Daher, A., Simbulan-Rosenthal, C.M., and Rosenthal, D.S., 2008: Sulfur mustard induces apoptosis in cultured normal human airway epithelial cells: evidence of a dominant caspase-8-mediated pathway and differential cellular responses. *Drug Chem. Toxicol.*, **31**, 137–148.

Rubin, B.K., 2004: Immunomodulatory properties of macrolides: overview and historical perspective. *Am. J. Med.*, **117**, 2S–4S.

Steinkamp, J.A., Wilson, J.S., Saunders, G.C., and Stewart, C.C., 1982: Phagocytosis: flow cytometric quantitation with fluorescent microspheres. *Science*, **215**, 64–66.

Szinicz, L., 2005: History of chemical and biological warfare agents. *Toxicology*, **214**, 167–181.

Tamaoki, J., Kadota, J., and Takizawa, H., 2004: Clinical implications of the immunomodulatory effects of macrolides. *Am. J. Med.*, 117, 5S–11S.

Tsai, W.C. and Standiford, T.J., 2004: Immunomodulatory effects of macrolides in the lung: lessons from in-vitro and in-vivo models. *Curr. Pharm. Des.*, **10**, 3081–3093.

Xiao, Y. and Barker, P.E., 2004: Semiconductor nanocrystal probes for human metaphase chromosomes. *Nucleic Acids Res.*, **32**, e28.

Xiao, Y., Telford, W.G., Ball, J.C., Locascio, L.E., and Barker, P.E.: Semiconductor nanocrystal conjugates, FISH and pH. *Nat. Methods*, **2**, 723.

Xu, G., Fujita, J., Negayama, K., Yuube, K., Hojo, S., Yamaji, Y., Kawanishi, K., and Takahara, J., 1996: Effect of macrolide antibiotics on macrophage functions. *Microbiol. Immunol.*, **40**, 473–479.