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Effects of sodium methyldithiocarbamate on selected parameters of innate immunity and clearance of bacteria in a mouse model of sepsis

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Abstract

Aims—Sodium methyldithiocarbamate (SMD), the third most widely used conventional pesticide in the United States, has been reported to inhibit several parameters associated with inflammation and to decrease resistance to infection. In a previous study, survival time was markedly decreased when mice were treated orally with SMD shortly before challenge with a high dose of *Escherichia coli* (*E. coli*) that was lethal to most of the control mice. In the present study, we evaluated selected parameters of the innate immune system using a lower challenge dose of *E. coli*, to determine which (if any) of these parameters reflected continued changes through 24 h.

Main methods—Bacterial clearance from the peritoneal cavity, production of chemokines and cytokines, and body temperature were measured.

Key findings—All these parameters were reduced by SMD up to 12 h after bacterial challenge, but the concentration of the anti-inflammatory cytokine IL-10 was increased. Even so, mice in the control and SMD-treated groups cleared most bacteria by 24 h. Other parameters (cytokine concentrations and body temperature) were also normal or near normal by 24 h. The same dosage of SMD administered intranasally also did not significantly decrease survival. Hypothermia from 16 to 28 h correlated with lethal outcome, but SMD significantly increased hypothermia only at 2 and 4 h after challenge.

Significance—In spite of substantial early inhibition by SMD of parameters known to be important for resistance to infection, bacterial clearance and survival were not altered, suggesting immunological reserve and/or rapid recovery after transient effects of SMD.

1. Introduction

Sepsis with multi-organ dysfunction remains a major cause of mortality in the United States. Patients who suffer from septic peritonitis experience an even higher mortality rate [5]. Sodium methyldithiocarbamate (SMD) is one of the most widely used conventional pesticides in the U.S. [6]. An accidental spill in 1991 caused medical symptoms including

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Conflict of interest statement

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irritation of the eyes and respiratory tract and induction or exacerbation of asthma [1,4]. Our previous studies indicated that SMD suppresses innate immunity in mice in part by increasing IL-10 production and decreasing IL-12 concentrations at early stages of infection [16]. In a previous paper, calculations are shown indicating that exposure of a human to approximately 2.3 ml of concentrated SMD solution is slightly greater than the exposure used in mice in the present study [11], if one expresses the dosages as mg/m² of body surface area, which generally provides better concordance of biological effects than expressing dosages as mg/kg of body surface area [17]. Thus, exposure levels in the present study are designed to be relevant to occupational exposure in humans.

Pro-inflammatory cytokines, such as TNF- α , IL-1 β and IL-12, are important in innate immune responses and that peritoneal macrophages are necessary for resistance to *Escherichia coli* peritonitis and sepsis [18,23,26]. Cytokines and chemokines play important roles in enhancing antimicrobial capacity of phagocytes and recruiting other innate leukocyte populations to the sites of infection [22]. In our previous study, a high dose of *E. coli* was used to challenge the innate immune system beyond its capacity to ultimately allow survival. This was done to provide the maximum possible sensitivity in detecting innate immune dysfunction caused by SMD [16]. It was anticipated that even a small degree of innate immune system dysfunction would decrease survival times to a greater extent, because the innate immune system was already challenged beyond its ability to effectively respond and prevent death. The purpose of the present study was to evaluate the effects of SMD on resistance to *E. coli* sepsis using a lower challenge dose to determine if decreased innate immune function could also be detected under less rigorous (and potentially more relevant) conditions. An additional purpose of this study was to measure a series of holistic innate immune parameters over time to determine if SMD affected these parameters in a manner that was consistent with its effects on bacterial clearance. Although such associations would not conclusively demonstrate a cause–effect relationship between SMD-induced innate immune changes and decreased bacterial clearance, it would provide an indication of parameters worth further investigation to determine particular cause–effect relationships.

It is hypothesized that sepsis often leads to an improperly controlled and excessive inflammatory response with substantially elevated levels of inflammatory mediators such as cytokines and chemokines [7,20]. Some of these cytokines and chemokines are vasoactive and cause or contribute to dilation and leakiness of blood vessels, which can produce shock [2,24]. Relationships between bacterial clearance, body temperature, and concentration of inflammatory mediators in response to different levels of treatments that modify survival in sepsis have not been systematically investigated. One of the criticisms of mouse models of sepsis is that they almost always employ only healthy young adult animals, whereas most humans with sepsis are children and elderly or have other health problems [12]. In the study described here, we used an anti-inflammatory environmental toxicant to alter parameters that influence resistance to sepsis in an effort both to determine the properties of this toxicant and to learn more about the interactions between bacterial clearance, body temperature, and inflammatory mediators in sepsis in a mouse model designed to represent the situation with human sepsis patients, who often have immune system dysfunctions.

Because the most likely route of exposure to SMD is dermal or inhalation, and we have not previously evaluated the effect of SMD administered by intranasal instillation, later experiments in this project were designed to determine if SMD by this route decreased survival in our sepsis model.

2. Materials and methods

2.1. Animals

Female C57Bl/6 $\times$ C3H F1 mice were purchased through the National Cancer Institute's Animal Program and used in experiments at 8 to 12 weeks of age after being given at least 2 weeks to recover from shipping stress and become acclimated to our animal facility. Mice were housed in groups of five in a temperature and humidity controlled environment. Mice were kept on a 12 h light/dark cycle and given access to food (Purina Lab Chow) and water ad libitum. All the experiments were consistent with the NIH Guide and Mississippi State University regulations and approved by the Institutional Animal Care and Use Committee of University. Animals were housed and procedures were conducted in a laboratory animal facility accredited by the American Association of Accreditation of Laboratory Animal Care (AAALAC).

2.2. Survival assay

Mice treated with *E. coli* (2×10^8 per mouse by intraperitoneal injection in a volume of 0.2 ml of phosphate buffered saline) were observed every 6 h for 72 h, and the time of death or the time at which the mice were observed to be moribund (indicated by signs such as loss of righting reflex, substantially decreased spontaneous and induced locomotion, decreasing grooming behavior, and decreased food and water intake) was recorded. Moribund mice were immediately euthanized using inhalation of carbon dioxide. In previous studies, we have not observed recovery of mice that were moribund. The time to death and the time to moribund status and euthanasia were both recorded as time to death for purposes of statistical analysis. The *E. coli* strain used in this study, its isolation from one of the mice in our colony, and the rationale for using this model of sepsis instead of the widely used cecal ligation and puncture method are described in our previous study [15]. However, as explained in the Introduction of the present paper, a lower challenge dose of *E. coli* was used here (2×10^8 per mouse) than in our previous study (2×10^9 per mouse) to allow detection of more subtle relationships between immunological parameters and bacterial clearance.

2.3. Administration of chemical and the mouse model of sepsis

Sodium methylthiocarbamate (SMD) (ChemService, West Chester, PA) was dissolved in sterile water for oral administration using an 18 gauge gavage needle. The indicated dosages were administered in a volume of 0.4 ml or less and an equivalent volume of vehicle (water) was given to mice in the control group. For intranasal administration, SMD was dissolved in sterile physiological saline (Sigma Chemical Co, St. Louis, MO). Mice were treated with SMD by intranasal instillation (in a volume of 50 μ l) under halothane anesthesia [27]. We confirmed the results reported by Ebino et al. that this method leads to uniform distribution of a hydrophilic compound (such as methylene blue) throughout both lungs (results not shown). Administration of SMD by either route was followed 15 min later by intraperitoneal

injection of non-pathogenic *E. coli* ($2 \times 10^8/200 \mu\text{l}$) as described previously [3,14]. Body temperature was monitored using an anal thermistor probe and an electronic digital thermometer for 72 h and the number of survivors was counted.

2.4. Collection of serum and peritoneal lavage fluid

Blood samples were obtained retroorbitally while the animal was under isoflurane anesthesia. Peritoneal lavage fluid was obtained by injection of 1 ml of PBS with 10% fetal bovine serum, followed by vigorous massage to mix the contents of the peritoneal cavity. Fluid was collected with a syringe. Samples (typically 0.7 ml) were centrifuged to separate cells. The small volume wash was done first to minimize dilution of the sample and to thereby increase the sensitivity of cytokine detection. The remainder of the cells in the peritoneal cavity were harvested by injecting 7 ml of PBS with 10% fetal bovine serum, withdrawing as much fluid as possible (using a 25 g needle and syringe), and this lavage fluid was used to resuspend cells from the previous 1 ml injection. This yielded about 6 ml of cell suspension per mouse. After centrifugation, the cells were resuspended in PBS with 1% fetal bovine serum, cell counts were performed and cytospin slides were prepared. The slides were stained with standard Wright Giemsa stain, and manual differential cell counts were performed at 600 $\times$ magnification to allow observation of bacteria as well as mammalian cells. To enumerate bacteria, 1:10 serial dilutions of peritoneal lavage fluid were plated in triplicate on LB agar. Colonies were counted after overnight incubation at 37 °C and the dilution yielding 30–300 colonies per plate was counted and the mean value of the triplicate plates was used in subsequent calculations to determine bacteria per peritoneal cavity for each mouse. Blood samples were obtained just prior to peritoneal lavage by retroorbital bleeding under halothane anesthesia, blood was allowed to clot, and serum was harvested after centrifugation and stored frozen (–80 °C) until needed for cytokine and chemokine analysis.

2.5. Cytokine and chemokine assays

Cytokines were quantified using ELISA kits from BD Pharmingen (San Diego, CA), as described previously [28]. The kit used for IL-12 in these studies was specific for the p40 subunit.

2.6. Statistical analysis

The results were analyzed by the Prism 6.0 software (GraphPad Software, La Jolla, CA). Control and SMD-treated groups were compared using a t-test with the Holm–Sidak correction for multiple comparisons. In the time course experiment, the overall changes due to treatment (SMD) and time were analyzed by two-way ANOVA for effects of treatment, time, and the interaction between treatment and time. Values of $p < 0.05$ were considered statistically significant. Survival analysis was done using the Mantel–Cox log rank test, which tests for difference in survival time or pattern, not just differences in the final survival rate.

3. Results

3.1. Effect of sodium methyldithiocarbamate (SMD) on bacterial clearance from the peritoneal cavity 2–24 h after *E. coli* challenge

In this study, several parameters thought to be involved in resistance to sepsis were measured. We have previously reported that oral administration of SMD at 200 or 300 mg/kg significantly decreases survival in sepsis [16]. However, in that study, a very high challenge dose of *E. coli* was used (2×10^9 viable bacteria per mouse) with the rationale that this would be beyond the capacity of the innate immune system to protect most mice and that detection of even more rapid disease progression in SMD treated mice would be a sensitive measure of the effects of SMD. However, in the present study a lower challenge dose of *E. coli* was used, which would allow clearance of bacteria by most or all control mice and provide a greater distinction in the responses between treated and control animals through the first 24 h. This was done in an effort to determine if differences in key parameters related to inflammation could indicate the relative importance of these parameters in lethal outcomes in sepsis. Acute administration of SMD at 200 mg/kg by gavage significantly increased the number of viable bacteria isolated from the peritoneal cavity from 2 to 12 h after challenge (Fig. 1). However, this was followed by a return to values nearer control values by 24 h (Fig. 1). As expected, the number of bacteria in the peritoneal cavity decreased rapidly and progressively in control mice, but bacterial clearance was delayed in mice treated with SMD 15 min before bacterial challenge.

3.2. The percentage of leukocyte types in the peritoneal cavity and the percentage of leukocytes associated with bacteria 2–24 h after challenge

As reported in our previous study [16], the total number of leukocytes in the peritoneal cavity did not significantly change during the course of *E. coli* sepsis (data not shown). Results from a differential count of cells in the peritoneal cavity (Fig. 2) showed that macrophages were the predominant cell type in the peritoneal cavity 2 h after challenge. However, neutrophils were predominant 24 h after *E. coli* challenge. There was a small but significant decrease in the percentage of neutrophils in SMD-treated mice at 4 and 12 h, but not at 24 h. The number of bacteria within neutrophils or macrophages cannot be definitively determined by microscopic observation because of the difficulty in distinguishing bacteria adhering to the surface of the phagocyte and bacteria within the phagocyte. However, clearance of bacteria yields fewer phagocytic cells with associated bacteria, and this parameter was used here as in our previous study [14] to estimate the effectiveness of killing and degradation of bacteria. The percentage of macrophages with 3 or more associated bacteria was increased by SMD at 4 and 12 h, indicating that less clearance of bacteria from macrophages occurred in SMD treated mice. Decreased clearance of bacteria by neutrophils was indicated by a lower percentage of neutrophils with 3 or fewer associated *E. coli* in SMD-treated mice as compared to control mice (Fig. 2). Thus, clearance of bacteria by both neutrophils and macrophages was diminished by SMD. The number of bacteria per phagocytic cell was not quantified, but the overall number of bacteria decreased from 12 to 24 h (Fig. 1), indicating that bacteria were being killed in both control and SMD-treated mice, but that the clearance was not as rapid in SMD-treated mice.

3.3. Effect of SMD on the concentration of selected cytokines and chemokines in the peritoneal cavity 2–24 h after *E. coli* challenge

Most pro-inflammatory cytokines and chemokines (IL-1 β , IL-12, CCL3, and CCL4) were suppressed in mice treated with SMD at 200 mg/kg at 2, 4, or 12 h in peritoneal lavage fluid, whereas the anti-inflammatory cytokine IL-10 was increased at early times (Fig. 3). However, IL-6 was not suppressed by SMD at 200 mg/kg. All cytokines and chemokines, except IL-6, were at or near control values by 24 h. However, IL-6 was significantly increased at 24 h, possibly as a result of the earlier elevation of the number of bacteria in SMD-treated groups (Fig. 2). Statistical analysis by 2-way ANOVA revealed that both time and treatment (SMD) significantly affected the results for most cytokines and chemokines and that there were significant interactions between time and treatment for some of them, indicating that SMD altered the pattern of change in cytokine/chemokine concentration over time in response to *E. coli*.

3.4. Effect of SMD on body temperature 4–24 h after *E. coli* challenge

Body temperature was significantly decreased by *E. coli* in both control and SMD-treated mice (Fig. 4), but at 4 h it was significantly lower in mice treated with SMD than in mice treated with *E. coli* only. Body temperatures of control mice and mice treated with SMD (200 mg/kg) had returned to nearly normal values and were not significantly different by the end of the 24 h observation period. This is consistent with results shown in Figs. 1 and 3 indicating that substantial effects of SMD at 2, 4, or 12 h were minimal by 24 h.

3.5. Does administration of SMD by intranasal instillation (instead of gavage as in the other experiments in this study) cause mortality suggestive of greater effects than oral administration?

Because it seemed that most mice treated with SMD by gavage cleared bacteria effectively and were likely to survive and because dermal and inhalation are probably the most common routes of human exposure to SMD, an additional experiment was performed using intranasal instillation of SMD. Results shown in Fig. 5 upper panel indicate that mice treated with SMD at 200 or 250 mg/kg by intranasal instillation did not have survival patterns significantly different from those of vehicle-treated mice (using a Mantel–Cox log rank test). Thus, at comparable dosages as used in most of the experiments in this paper, intranasal SMD administration had minimal effects on survival, even though we have previously shown that intranasal instillation significantly inhibits systemic cytokine production [13]. However, the difference between *E. coli* and *E. coli* plus SMD (250 mg/kg) groups in Fig. 5 lower panel was almost significant ($p = 0.0710$), suggesting that additional studies are needed before concluding with confidence that this compound does not affect innate immunity when delivered by the respiratory route.

3.6. Is there an association between hypothermia induced by *E. coli*, SMD, or both and survival?

To determine if there was an association between the degree of hypothermia and survival, mice that were dosed by gavage (as in Figs. 1–4) that ultimately survived and mice that ultimately died (or became moribund and were euthanized), and body temperatures over

time are reported in Fig. 6. The results demonstrated that body temperature was still significantly decreased (from a pre-treatment value of about 36.5 °C) at 16 h after *E. coli* administration. This was followed by a linear increase in temperature over time in both survivors and non-survivors, through 24 h. However, the regression line for non-survivors remained lower than for survivors throughout the time period measured. This was determined using the linear regression routine in Prism 6.0, which can compare the slope and elevation (intercept) of two lines. The results of that analysis indicated that the slopes were not significantly different for survivors and non-survivors, but elevation was significantly different. The results suggest that non-survivors in both the control and treatment groups have more severe hypothermia than mice that ultimately survive.

4. Discussion

The occurrence and severity of hypothermia and decreased production of pro-inflammatory cytokines from 0 to 12 h, corresponded well with decreased clearance of bacteria caused by SMD in this study. The observed decrease in bacterial clearance through 12 h could reflect the decrease in cytokines that act in an autocrine or paracrine manner to increase the ability of phagocytes to kill bacteria. The kinetics of cytokine changes are consistent with this idea. At times during or somewhat later than the suppression of pro-inflammatory cytokines or the enhancement of IL-10 (an anti-inflammatory cytokine), bacterial counts were higher in SMD-treated mice. Most cytokines returned to near normal values by 24 h and this was associated with bacterial clearance that was similar in SMD-treated and control mice. Thus, bacterial clearance recovered to near normal values as did most cytokines and chemokines by 24 h in SMD-treated animals.

The potential role of hypothermia in decreased bacterial killing should be considered. The enzymes that generate reactive oxygen and nitrogen species would be expected to exhibit a temperature optimum at normal or slightly elevated body temperature. However, *E. coli* causes hypothermia in mice, which is exacerbated by SMD. Based on fundamental thermodynamic principles of enzyme action, it would be expected that lower body temperatures could significantly decrease the enzyme catalyzed reaction rates for production of reactive oxygen and nitrogen species. Experimental evidence in support of this expectation has been reported in the form of inhibition of TNF- α and nitric oxide production in hypothermic rats [9]. Thus, direct temperature-dependent decreases in activity of enzymes that produce antimicrobial agents could explain a portion of the effects of SMD on delayed clearance of bacteria noted in the present study. However, it should also be noted that hypothermia could decrease the growth rate of bacteria, and could diminish tissue damage associated with septic shock and multiple organ failure. The relative importance of all of the effects of hypothermia on survival in sepsis remains unknown and should be the topic of future studies. However, results shown here (Fig. 6) are consistent with reports in humans indicating that more severe hypothermia is associated with increased risk of death [8,25]. Thus, the adverse effects seem to be more important than the beneficial effects, or hypothermia could be an epiphenomenon which correlates with the actual (unknown) causes for increased risk of lethal outcome.

The role of pro- and anti-inflammatory cytokines and chemokines in microbial clearance and in lethality in sepsis is complex and somewhat controversial. It is generally thought that very high concentrations of cytokines in the blood late in the course of sepsis contribute to septic shock and are associated with a poor outcome. However, early production of key cytokines can enhance clearance of bacteria and survival in animal models [19,21]. The present study indicated that SMD decreased production of several pro-inflammatory cytokines and chemokines and increased production of IL-10 early in the course of sepsis. However, mice treated with SMD at 200 mg/kg still cleared most bacteria by 24 h. This indicates that the early inhibition only delayed clearance but did not ultimately prevent clearance from occurring and that the effects of SMD were transient. This contrasts with the effects of SMD at the same dosage in mice given a 10-fold greater challenge dose of *E. coli* than in the present study [16]. In that study, the early inhibition of cytokines and chemokines induced by *E. coli* lipopolysaccharide was associated with poor clearance of bacteria that led to overgrowth of *E. coli* and more rapid death than was observed in control animals.

Although results from the present study indicated a decreased ability of macrophages and neutrophils to clear bacteria from 2 to 12 h after bacterial challenge in SMD-treated mice, the percentage of neutrophils in the peritoneal cavity was only slightly less than in control animals (Fig. 2). This indicates that decreased chemotaxis associated with decreased chemokine production is not likely the major cause of decreased bacterial clearance. In addition, clearance of bacteria by both macrophages and neutrophils was inhibited through 12 h by SMD (Fig. 2), suggesting the possibility that a common mechanism inhibiting microbial killing explains the effects observed in this study.

In addition to further understanding the effects of SMD on the immune system, a goal of this study was to determine the relative importance of cytokine and chemokine levels, body temperature changes, or altered chemotaxis of leukocytes into the peritoneal cavity in bacterial clearance in sepsis. The results indicate that innate clearance of or resistance to *E. coli* under the conditions in the present study was delayed but not substantially decreased by SMD at 200 mg/kg administered either orally or by intranasal instillation. However, it should be noted that using a higher *E. coli* challenge dose by gavage, we reported that SMD at 200 or 300 mg/kg significantly decreased survival time [16]. These results highlight the importance of challenge conditions on the effect of toxicants on the outcome of infectious diseases. In considering our previous results and the results shown here, it seems likely that mice were able to recover from initial suppression of innate immune mechanisms, because the challenge dose of bacteria did not quickly overwhelm the animal's defense mechanisms as was the case when a higher challenge dose was used in our previous study. It should be noted that the dosage of SMD used in this study is relevant with regard to occupational exposure, but higher than anticipated due to environmental exposure [11]. Considering that there was a trend toward decreased survival in mice treated with SMD intranasally in this study, it is possible that a higher dose of SMD, such as 300 mg/kg, which we have used in previous studies by gavage and by dermal exposure, would have significantly decreased survival. However, the results demonstrate that SMD is not more toxic when administered by inhalation as compared to administration by gavage, so our previous estimates of risk would seem to apply to both routes of administration. Thus, it remains possible that high level exposure to SMD that may occur occupationally could, under some circumstances,

delay or diminish resistance to infectious diseases. Epidemiological studies of persons involved in the application, manufacture, or transport of SMD will be needed to determine if increased incidence or severity of infectious diseases is observed.

Little is known of the quantitative relationships between changes in immunological parameters and the amount of change in resistance to infection caused by toxicants. We reported a method to analyze such relationships using machine learning techniques [10]. In the present report, we have documented that quantitatively significant changes in production of chemokines and cytokines, body temperature, and movement of neutrophils to the site of infection (peritoneal cavity) are caused by SMD. Nevertheless, bacterial clearance and survival were not significantly altered by 24 h after SMD treatment. This demonstrates that substantial (but transient) inhibition of important mediators of innate immunity can occur without ultimately affecting host resistance to lethality in sepsis. This suggests the possible existence of immunological reserve capacity. However, our previous study using a larger challenge dose of bacteria [16] suggests that this functional reserve is conditional and may not exist if individuals are exposed to high doses of infectious agents. Whether suppressed immunological functions in the absence of decreased host resistance constitute an adverse effect has been discussed for some time. One position that is actually supported by our results is that substantial suppression of immunological parameters known to be important in resistance to infection will be found to inhibit resistance to infection if the proper model is examined. Although we did not find inhibition of host resistance in this study, we noted that SMD at the same dosage used here significantly decreased host resistance in an *E. coli* sepsis model induced by a larger challenge dose of bacteria [13].

5. Conclusion

Sodium methyldithiocarbamate altered innate immunological parameters thought to be important in resistance to sepsis and decreased bacterial clearance through 12 h, but these effects were transient and did not significantly alter bacterial clearance or survival at 24 h or later. These results demonstrate that animals can recover from significant transient decreases in pro-inflammatory cytokines and chemokines and in body temperature, whereas such transient changes were associated with decreased survival time when a higher challenge dose of bacteria was used. These results highlight the importance of the conditions under which resistance to infection is evaluated when determining the effects of drugs or chemicals on resistance to infection. They also demonstrate that the quantitative relationships between the bacterial challenge dose, amount of suppression of innate immune parameters, duration of suppression, and outcome are poorly understood and that methods are needed to predict outcomes based on microbial load and status of the immune system.

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Fig. 1.

Decreased clearance of *E. coli* from the peritoneal cavity in sodium methyldithiocarbamate (SMD)-treated mice from 2 to 12 h after bacterial challenge. Groups of 5 mice were treated with SMD or vehicle (water) 15 min before challenge with *Escherichia coli* (*E. coli*) (2×10^8 per mouse, intraperitoneally). At the indicated times after challenge a treatment group and a control group were euthanized, and peritoneal lavage was performed. Viable bacteria were enumerated by serially diluting lavage fluid and performing plate counts. Statistical values determined by Student's t test at each time point comparing control and SMD-treated groups were as follows: 2 h ($p = 0.6987$, $t = 0.4013$, $df = 8$); 4 h ($p = 0.0484$, $t = 2.471$, $df = 6$); 12 h ($p = 0.0046$, $t = 3.331$, $df = 15$); and 24 h ($p = 0.0433$, $t = 2.258$, $df = 12$). All differences with p values less than 0.05 are indicated by *.

Fig. 2.

Effect of SMD on the percentage of macrophages and neutrophils in the peritoneal cavity over time and effect on elimination of bacteria over time. Mice ($n = 5$ per group for 2 h and 4 h and $n = 10$ per group for 12 h and 24 h) were treated with SMD by gavage 15 min before intraperitoneal challenge with *E. coli*. The percentage of macrophages and neutrophils per total cells was determined by manual differential counts of cytopspin preparations. In the bottom panels, the percentage of macrophages with 3 or more associated bacteria and the percentage of neutrophils with three or less associated bacteria are shown. These values provide an indication of the effectiveness of bacterial clearance over time. Statistical values obtained using Student's t-test comparing values between control and SMD-treated groups at each time point were as follows: 2 h ($p = 0.2337$, $t = 3.505$, $df = 8$); 4 h ($p = 0.0484$, $t = 2.471$, $df = 6$); 12 h ($p = 0.0046$, $t = 3.331$, $df = 15$); and 24 h ($p = 0.0433$, $t = 2.258$, $df = 12$). All differences with p values less than 0.05 are indicated by *.

Fig. 3.

SMD at 200 mg/kg by gavage alters the amount or timing of the response of most cytokines and chemokines in the peritoneal fluid following *E. coli* challenge. The results shown represent means $\pm$ SEM ($n = 5-10$ mice per group). Values for which there was a significant difference between the control and treated groups at a particular time are indicated by * ($p < 0.05$, as determined by Student's *t* test). Results were also analyzed by two-way analysis of variance (ANOVA), and the analysis indicated the following: for IL-6, the effect of time was $p < 0.0001$, the effect of treatment was $p = 0.0252$, and the effect of the interaction was $p = 0.1298$; the *F* value for time was $F(3, 43) = 28.66$, *F* value for treatment was $F(1, 43) = 5.376$, and the *F* value for the interaction was $F(3, 43) = 1.989$; for IL-10, the effect of time was <0.0001 , the effect of treatment was 0.2283 , and the interaction between time and temperature was $.0056$; the *F* value for IL-10 for interaction was $F(3,43) = 4.810$, for

treatment $F(3,43) = 1.494$, and for time, $F(3,43) = 11.05$; for IL-12, the effects of time, treatment, and interaction were all <0.0001 , and the F value for IL-12 for interaction was $F(3, 47) = 10.28$, for time $F(3, 47) = 30.33$, and for treatment $F(1, 47) = 86.55$; for IL-1 β , the effect of time was <0.001 , the effect of treatment was 0.0086, and the effect of the interaction was 0.0035; the F value for time was $F(2, 40) = 48.39$, for treatment $F(1, 40) = 7.6279$, and for the interaction $F(2, 40) = 7.627$; for CCL-3 the effect of time was 0.0039, the effect of treatment was 0.8324, and the interaction effects was 0.3105; the F value for time was $F(2, 43) = 1.202$, for treatment $F(1, 43) = 0.04535$, and for the interaction $F(2, 43) = 1.202$; for CCL-4, the effect of time was <0.0001 , the effect of treatment was 0.0029, and the effect of the interaction was 0.0003; the F value for time was $F(2, 43) = 10.10$, the F value for treatment was $F(2, 43) = 48.99$, and for the interaction $F(2, 43) = 10.01$.

Fig. 4.

SMD at 200 mg/kg by gavage significantly decreased or delayed recovery of body temperature in mice treated with *E. coli*. Mean body temperature $\pm$ SEM is shown ($n = 10$ mice per group). Values for which there was a significant difference between the control and treated groups at a particular time are indicated by * ($p < 0.05$, as determined by Student's t test). Results were also analyzed by two-way analysis of variance (ANOVA), and the analysis indicated the following: for body temperature, the effect of time was $p = 0.0037$ ($F(3, 70) = 4.917$), the effect of treatment was $p < 0.0001$ ($F(1, 70) = 17.42$), and the interaction between time and temperature was not significant ($p = 0.9276$) ($F(3, 70) = 0.1528$).

Fig. 5.

Effects of intranasal instillation of SMD on survival of mice challenged with *E. coli*. Mice were observed every 6 h for 72 h, and time of death or moribund status was recorded at each of the observation times ($n = 10$ mice per group). Mice that were moribund upon observation were immediately removed from the study and euthanized as recommended by our Institutional Animal Care and Use Committee. Results were analyzed statistically using the Mantel log rank test as implemented by Prism 6.0 software (GraphPad, San Diego, CA). The two groups in the upper panel (control and SMD at 200 mg/kg) were not significantly different ($p = 0.97$ by the Mantel–Cox log rank test). Results in the lower panel are from an experiment with the same design in which mice were treated with SMD at 250 mg/kg.

Results in this experiment were not quite statistically significant ($p = 0.0707$ by the Mantel–Cox log rank test).

Fig. 6. Effect of SMD at 200 mg/kg by gavage on body temperature in mice treated with *E. coli*. Mice were treated with SMD 15 min before *E. coli* challenge and times shown are times after *E. coli* challenge. Results are shown comparing body temperature in mice that ultimately survived (n = 14) or did not survive (or became moribund and were euthanized, n = 6). Values shown are means $\pm$ SEM and the regression lines are shown along with the 95% confidence intervals (dashed lines). Analysis by Prism 6.0 software indicated that the slopes of the two lines were not significantly different ($p = 0.8848$, $F = 0.0211487$, $DF_n = 1$ $DF_d = 74$). The elevations of the lines were, however, significantly different ($p = 0.002$, $F = 15.821$, $DF_n = 1$ $DF_d = 75$).