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Protection of Mice from Tularemia Infection with Ultra-Low, Serial Agitated Dilutions Prepared from *Francisella tularensis*-Infected Tissue[1]

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Abstract—Reports of immunomodulation with serial agitated dilutions (SADs) of cytokines, hormones, minerals, and whole tissue led to this inquiry as to whether exposure to a complex SAD preparation produced from *Francisella tularensis*-infected mice could alter the immune response and the effects of subsequent challenge with this pathogen *in vivo*. Six SAD preparations of reticuloendothelial tissue from *F. tularensis*-infected C3H/HeN mice were produced through a process of serial $\log_{10}$ and $\log_{100}$ dilutions in 70% ethanol interspersed with 30-second agitation. SAD preparations were analyzed for protein content and for contamination with ^{1}H-NMR spectroscopy. Three preparations contained detectable protein by Lowry and NMR analysis, and three were diluted beyond detection of protein. These preparations were administered orally for 1 month to 147 animals randomly assigned to SAD or dilutent control groups. All animals were then challenged with a lethal dose (LD_{50} or LD_{75}) of *F. tularensis* and evaluated for time to death and total mortality. In a series of 15 trials, the SAD preparations consistently produced increased mean times to death (MTD; MTD SAD = 18.6 days [range, 12.9–25.6]; MTD controls = 13.7 days [range, 11.6–15.6]), and decreased mortality (SAD: 53%; control: 75%) when compared with matched control groups given the dilutent only. Protection was not related to the level of dilution, the number of times vortexed, or the presence or absence of original substance from the tissue. Active and inactive solutions could be distinguished from one another using ^{1}H NMR-spectroscopy. Two preparations induced specific anti-*tularemia* IgG antibody production before challenge. This anomalous finding needs independent repetition and further investigation.

Keywords: homeopathy — nosodes — tularemia — animal model — immunology — vaccination — ultra-low dilutions — SADs

Introduction

Dose-dependent reverse effects (DDREs), or hormesis, refers to the observation that when living organisms are exposed to very low doses of infectious or toxic agents, their reaction is often the opposite of that observed after exposure to high doses (Calabrese, McCarthy, and Kenyon, 1987; Luckey, 1980). Biological and physical agents of all types (including potent toxins, essential nutrients, pesticides, and penicillin) demonstrate DDREs on an equally broad range of living systems from single-cell bacteria and fungi to multicellular organisms and across a variety of phyla and species, including humans (Calabrese and Baldwin, 1997; Furst, 1987; Neafsey, 1990; Stebbing, 1987).

These reverse effects are assumed to occur because low-level exposures to otherwise damaging agents induce protective and reparative responses in cells (such as induction of heat shock proteins) without causing cellular damage (Boxenbaum, Neafsey, and Fourier, 1988; Stebbing, 1982; van Wijk and Wiegant, 1997). Recent studies report that under certain conditions, however, these effects occur with solutions diluted beyond the point when sufficient molecules remain to provide any specific molecular stimulus (Bastide, 1997; Endler and Schulte, 1994; Schulte and Endler, 1998). The majority of these studies report that vortexing the solution in a serial fashion during the dilution process can enhance or preserve this activity. Diverse effects from these serial agitated dilutions (SADs) have been reported in clinical studies (usually in reference to homeopathy; Boissel *et al.*, 1996; Kleijnen, Knipschild, and ter Riet, 1991; Linde *et al.*, 1997) in toxicology (Anderson *et al.*, 1990; Bascands *et al.*, 1987; Cazin *et al.*, 1987; Fisher and Capel, 1982; Fisher *et al.*, 1987; Larue *et al.*, 1985; Linde *et al.*, 1994; Wagner, Kreher, and Jurcic, 1988), and in immunology (Bastide *et al.*, 1987; Bastide, Doucet-Jaboeuf, and Daurat, 1985; Benveniste *et al.*, 1991; Carriere, Dorfman, and Bastide, 1988; Daurat *et al.*, 1986; Daurat, Dorfman, and Bastide, 1988; Davenas *et al.*, 1988; Harisch and Kretschmer, 1988; Maddox, Randi, and Stewart, 1988; Poitevin, Davenas, and Benveniste, 1988; Sainte-Laudy and Belon, 1996b). Immunomodulating effects include increased cytotoxic and antibody-secreting cell production with SAD preparations of cytokines (Bastide, Doucet-Jaboef, and Daurat, 1985; Carriere, Dorfman, and Bastide, 1988; Daurat *et al.*, 1986; Daurat, Dorfman, and Bastide, 1988), NK cell stimulation with hormones (Bastide *et al.*, 1987; Daurat *et al.*, 1986), histamine release, basophil degranulation and CD subset shifting with histamine and anti-IgE antibodies (Benveniste *et al.*, 1991; Davenas *et al.*, 1988; Harisch, Kretschmer, and von-Kries, 1987; Poitevin, Davenas, and Benveniste, 1988; Sainte-Laudy and Belon, 1996a, 1996b; others

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have reported no such effect from anti-IgE SADs, however: Hirst *et al.*, 1993; Ovelgonne *et al.*, 1992), and macrophage and mast cell activation with SAD preparations from minerals (Davenas, Poitevin, and Venveniste, 1987; Harisch and Kretschmer, 1988; Harisch and Kretschmer, 1989), nonspecific adjuvants (Poitevin, Davenas, and Benveniste, 1988), and whole tissue homogenates (Sainte-Laudy, Haynes, and Gershwin, 1986). Jacques Benveniste, a prominent immunologist and one of the foremost proponents of this ultra-high SAD phenomenon, also claims that biologically active signals from SAD preparations can be captured and transmitted electronically to biological systems (Benveniste *et al.*, 1997; Hadji, Arnoux, and Benveniste, 1991).

Interestingly, homeopathic physicians since the 18th century have claimed that administration of SAD preparations of infectious tissue (called "nosodes" in that literature) can prevent the spread of epidemic diseases (Boenninghausen, 1887; Campbell, 1909; Castro and Nogueira, 1975; Davis, 1904; English, 1987; Fox, 1987; Gibson, 1958; Krishnamurty, 1970; Linn, 1904; Morgan, 1899; Rastogi and Sharma, 1992; Taylor-Smith, 1950). In addition, studies done in the first half of this century reported that SADs of infected tissue could alter immune reactions as evidenced by alteration of the Schick test (a now outdated method of screening for diphtheria immunity; Chavanon, 1932; Paterson and Boyd, 1941; Shephard, 1967). With rare exceptions, these studies have major flaws that make it impossible to judge the efficacy of this approach despite persistent claims.

There are numerous speculative hypotheses as to how such information might be captured and stored in SAD preparations, if this indeed occurs. These hypotheses include differences in minority oxygen singlets (Berezin, 1990), water cluster formation in solvents (Anagnostatos, Pissis, and Viras, 1995), electromagnetic signal transfer (Endler *et al.*, 1997), informational content of solutions (Bastide and Lagache, 1997), the interaction of "systemic memory" with complex feedback dynamics of living systems, and others (see Jonas and Jacobs, 1996, pp. 85–91 for a short summary). One current testable hypothesis is that it occurs through the arrangement of minority oxygen singlets in water or alcohol (Berezin, 1990). Consistent with this hypothesis are reports that the action of SAD-enhanced preparations are reduced or eliminated by extremely high or low temperatures, after exposure to electromagnetic radiation and when prepared in the absence of oxygen (Benveniste *et al.*, 1991; Cazin *et al.*, 1991). In addition, broadening in the hydroxyl region and other changes are said to occur in the nuclear magnetic resonance (NMR) spectra of these preparations (Demangeat, Gries, and Poitevin, 1997; Weingartner, 1989).

In the present study, we analyzed whether a SAD preparation of infected tissue when given to mice can induce protection against an infectious challenge by the same organism. We chose murine tularemia (*Francisella tularensis*, live vaccine strain [LVS]) as the model because infection with this organism has been characterized in our lab and is rapidly lethal for C3H/HeN mice, providing a clear endpoint—death and days to death, as outcome measures (Fortier *et al.*, 1991). We tested the null hypothesis that groups of animals given SAD

preparations of tissue from *F. tularensis*-infected mice would respond no differently than would control mice given only dilutent to a subsequent challenge with a lethal dose of *F. tularensis*.

Methods

SAD Preparations

Two specific pathogen free, 6-week old, male C3H/HeN mice (Harlan Sprague Dawley, Indianapolis, IN) were infected intranasally with a sublethal dose (10^3 colony forming units [CFUs]) of *F. tularensis*, LVS (ATCC, Rockville, MD). On Day 7 postinfection, the mice were sacrificed, and target organs for the infection (lungs, liver, and spleen) were removed and processed aseptically. These organs were placed in 15 ml gel/saline media and homogenized in a sterile Ten Broek homogenizer. Tissue homogenates were serially diluted in phosphate-buffered saline (PBS) and plated on cystine heart agar plates with Isovitalex (Difco, Detroit, MI) and Fe phosphate (Sigma, St. Louis, MO) in triplicate. Plates were incubated at 37°C for 72 hours and colonies counted to assess bacterial numbers in infected tissues. Tissue from two additional LVS-infected mice was homogenized in a 500 ml Wedgewood mortar with 60 mg of Lactose, U.S.P. (Pfanstiehl Lab., Waukegan, IL), for 1 hour and mixed with the liquid portion of tissue homogenate.

Six SAD preparations were made from the mixed tissue homogenate in the following manner:

1. 0.1 ml of infected tissue homogenate was mixed with 0.9 ml of 70% ethanol in sterile, glass, 5 ml tubes. This mixture was agitated (as described below) for 30 seconds. This tube was labeled Ft/SAD-1d (*F. tularensis*/agitated ultra-high dilution - 1st $\log_{10}$ [decimal] dilution).
2. 0.1 ml of Ft/SAD-1d then was mixed with 0.9 ml of 70% ethanol in a sterile 5 ml glass tube. This mixture was agitated again for 30 seconds. This tube was labeled Ft/SAD-2d.
3. The above procedure was repeated until serial agitated dilutions were prepared at the 3d, 7d, and 14d levels, each number representing the $\log_{10}$ dilution of the original preparation.
4. Three more preparations were made using a homogenate-to-solute ratio of 1:100. This was done by mixing 0.01 ml of infected tissue homogenate with 0.99 ml of 70% ethanol in sterile, glass, 5-ml tubes and agitating for 30 seconds. This tube was labeled Ft/SAD-1c (*F. tularensis*/agitated ultra-high dilution—1st $\log_{100}$ [centesimal] dilution). This procedure then was repeated as in the $\log_{10}$ preparations until the 30c, 200c, and 1000c levels were obtained. Each number in this series represents the $\log_{100}$ dilution of the original preparation. Immunomodulation has been reported with these solutions *in vitro, in vivo,* and in clinical trials (Bastide, Doucet-Jaboeuf, and Daurat, 1985; Bastide *et al.*, 1987; Benveniste *et al.*, 1991; Carriere, Dorfman, and Bastide, 1988; Daurat *et*

al., 1986; Daurat, Dorfman, and Bastide, 1988; Davenas *et al.*, 1988; Ferley *et al.*, 1989; Harisch and Kretschmer, 1988; Linde *et al.*, 1997; Papp *et al.*, 1998; Poitevin, Davenas, and Benveniste, 1988; Reilly *et al.*, 1994; Sainte-Laudy and Belon, 1996b).

All agitation was performed manually (except the 1000c preparation) by vertically shaking the solution vial vigorously onto a firm surface 60 times at the rate of two succusions per second. The 1000c preparation was prepared commercially using the 3c as a starting dilution (Quinn Pharmaceuticals, Berkeley, CA). Control groups received 70% alcohol agitated 60 times in an identical fashion.

Analysis of SAD and Control Preparations

Each SAD preparation was divided into three portions for analysis of bacterial count, protein content, and Proton-Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy.

Bacterial count. We determined bacterial count (CFU) on the original homogenate as described under SAD Preparation. Each of the subsequent dilutions made with 70% alcohol had no viable bacteria on culture. Estimated nonviable bacterial concentration was made from the original homogenate.

Protein analysis. Protein concentration was estimated by the Lowry spectrophotometric analysis method (Markwell *et al.*, 1978). The sensitivity of this method is 0.01 mg/ml of protein.

^{1}H NMR-spectroscopy. Samples were analyzed for ^{1}H NMR-spectral differences in an attempt to distinguish between active and control solutions and to look for possible paramagnetic contaminants. NMR Spectroscopy was done at the Department of Chemistry of the U.S. Naval Academy, Annapolis, Maryland. Three cleaned and dried NMR sample tubes (Wilmad 528-PP, 5mm, Wilmad Glass Co., Buena, NJ) were filled with 0.8 ml of the test SAD preparation or control alcohol solution. The alcohol analyzed was the same 70% ethanol solution used to prepare the SADs. A single, clean, coaxial inner cell (Wilmad WGS-5BL) was filled with 0.2 ml of deuterium oxide (Isotec, Inc., Miamisburg, OH) and inserted into the sample tubes to provide the locking solvent for external referencing with each spectra. Recording was done with a General Electric NMR QE-300 spectrometer. Sample tubes were washed three times between each analysis with the solution to be evaluated. The central peak of CH_3 was defined at 1.5 parts per million (ppm). The spectral areas of interest for this study were the H_2O and OH signals between 4.5 and 6 ppm, where evidence of increased proton exchange in the hydroxyl region of the solution was detected (Cooper, 1980). Spectral broadening was quantified by calculating the ratio of OH curve height to width at midaxis for controls and by subtracting the same ratio from each sample.

Animal handling. All experiments were conducted at the barrier animal facility of the Department of Cellular Immunology, Walter Reed Army Institute of Research, Rockville, Maryland. The barrier facility is designed to inhibit

spread of infectious agents from one animal cage to another, as well as from investigators to animals and from animals to personnel. Animal handling was performed in a laminar flow hood to ensure safety and prevent contamination of the animals. All animals received uniform handling, identical feed, water and environmentally controlled conditions, including constant temperature, humidity, and a 12-hour light–dark cycle.

Randomization and matching. The animals were age-matched, 5- to 6-week-old, male, C3H/HeN mice (Harlan Sprague Dawley). Seven SAD preparations were tested in a total of 15 trials using 8–12 mice per trial. For each trial, animals were randomly divided by an animal caretaker into two groups of 4–6 mice each and placed in separate cages. These cages then were randomly assigned to either the experimental (SAD) or control (70% ethanol) group. All animals were treated in a uniform way receiving the preparation (SAD or ethanol) at a dose of 0.03 ml per mouse given orally through a sterile pipette. All preparations (including controls) were shaken for 10 seconds before administered.

Blinding. Expectation control of the experiment was done over 2 months by having research assistants who were completely blind to the nature of the experiment and to the hypothesized "active" and "inactive" preparations administer the SADs. Blinding of the tularemia challenge (the only maneuver theoretically possible to influence the outcome) was done by having a third party cover all cage labels for the animals to be challenged and having a single individual (W.J.) deliver all challenges in a uniform way on randomly selected cages. In this way, both topical and group blinding was maintained for the critical elements of the experiment.

Prophylaxis of animals. The prechallenge dosage regime was one dose (0.03 ml), twice per day for 3 days followed by one dose every 3 days for 10 doses. After challenge, mice were given the SAD or control preparation twice a day for 20 days. Total number of doses was approximately 16 per mouse delivered over the 30 days prior to challenge and 40 doses delivered in the 20 days after challenge. The animals readily took this amount orally, obviating the need for direct gastric delivery, anesthesia, or multiple injections.

Challenge procedure. On Day 30 from the start of prophylaxis (3 days after the previous SAD or control dose), all mice were challenged intranasally (i.n.) with either a LD_{50} or LD_{75} dose of LVS (10^3 or 10^4 CFU, respectively). This dose will kill 50 to 75%, of untreated mice within 20 days. Animals were anesthetized with 0.06 mg of Telazol (Robbins, Richmond, VA), i.m., and then inoculated i.n. with LVS diluted in 0.05 ml PBS to the desired concentration. Bacterial counts were done on the challenge solution in duplicate both pre- and postchallenge. Two trials were conducted for each SAD preparation (three for Ft/SAD-14d) using a high (LD_{75}) and low (LD_{50}) challenge dose. In addition, two trials were conducted in mice given a mixed SAD prophylaxis regime that dosed animals with ascending dilutions (Ft/SAD-14d, 30c, 200c, 1000c each given twice for 1 week). These groups were called the ascending groups.

Outcome Measures

Antibody screening. Before starting SAD prophylaxis and again before infectious challenge, blood was drawn from each group through the lateral tail vein, pooled, centrifuged, and the serum frozen at –20°C. Serum was analyzed using enzyme-linked immunosorbent assay (ELISA) for antibody titers to whole organism LVS using the following procedure. Immulon plates were coated with 5×10^6 organisms and incubated overnight at 4°C. Plates then were washed three times with saline-Tween solution, blocked with 200 ul of 10% fetal bovine serum and rewashed. Each serum batch was screened for the presence of antibodies by adding serial 10-fold dilutions of serum (100 μl/well) and incubated for 90 minutes at 37°C and washed. One tenth μg/well of peroxidase labeled goat antimouse Ig antibody was added and incubated for 90 minutes at 37°C. Substrate (KPL solution A and B, 1:1) was then added at 100 μl per well and color read at 410 nm in 30 minutes. A reading was considered positive if optical density of the test serum was equal to or greater than positive control serum produced by LVS vaccinated mice.

Mortality and time to death. The two primary outcome measures were time to death and mortality. Animals were checked twice daily after infectious challenge and the death day for each animal recorded. The experiment was terminated 30 days postchallenge.

Data analysis. Data were entered into a spreadsheet format (Microsoft Excel, MicroSoft Corp., Redmond, WA) and analyzed by standard statistical software packages (StatView II, SuperAnova; Abacus Concepts, Berkeley, CA; and JMP, SAS Institute, Cary, NC). To control for the influence of censured data from mice that survived challenge, both parametric and nonparametric methods were used. The difference between harmonic mean death day (the mean reciprocal death time, MRDT) of matched groups was evaluated using a two-tailed, paired *t* test and the Wilcoxon sign rank test. The MRDT was the sum of one over each death day divided by the total number of mice in the group. This calculation gave an estimate of resistance to infection in the group based on time to death and overall mortality while controlling for censured and outlying data from surviving animals. The value obtained was between 0 and 1, in which 0 = *total resistance* and 1 = *death of all animals on the first day*.

Death-day data were also analyzed without this transformation using the Mann–Whitney *U* nonparametric test. Mortality ratios were analyzed with standard chi-square methods, and stratified mortality was evaluated using the Mantel-Haenszel rate ratio method. One-way analysis of variance (ANOVA) was used to evaluate the influence of SAD treatment effect while controlling for challenge dose with both death day and MRDT as outcome measures.

Results

Bacterial Count

Bacterial counts done on the stock homogenate showed these tissues harbored 10^7 CFU of LVS. No viable bacteria were found in any of the SAD preparations nor in the two alcohol control preparations that were cultured. Estimates of the number of nonviable bacteria in the SADs indicated that only the Ft/SAD-3d and 7d preparations contained bacteria: 10^4 and 1 organism, respectively (Table 1, column 2).

Protein Analysis

Protein concentration was 0.55 mg/ml in the initial homogenate, 0.098 mg/ml in the Ft/SAD-3d preparation and less than 0.001 mg/ml in all other SAD preparations and the two controls tested. Estimated protein content for the Ft/SAD-7d and 14d preparations were 1 ng/ml and 0.1 fg/ml, respectively (Table 1, column 3). The calculated amount of protein given to each mouse over the 45-day experiment was 16.5 micrograms, 1.68 nanograms, and 0.17 fentograms for the 3d, 7d, and 14d levels, respectively (Table 1, column 4). Preparations in the centesimal series had no remaining protein, either detectable or theoretical.

NMR Spectroscopy

The ^{1}H NMR-spectra of the SAD groups were easily distinguished from control groups regardless of the dilution level (Table 2). All SAD preparations had a moderate to large amount of OH spectral broadening compared with control groups. The average height to width ratio of SAD preparations was 3.4 (range 1.8–4.8) compared with control preparations where the height-to-width

TABLE 1
Analysis of SAD Preparations

Preparation	CFU count	Protein	Protein/mouse
Homogenate	10^7	550 (g/ml)	NA
3d	(10^4)	9.8 (g/ml)	16.5 (g)
7d	(1)	(1 ng/ml)	(1.68 ng)
14d	0	(0.1 fg/ml)	(0.17 fg)
30c	0	0	0
200c	0	0	0
1000c		0	0
Control 1	0	0	0
Control 2	0	0	0

Note: CFU = colony forming units. Analysis is of CFU, protein concentration, and total protein received per mouse during prophylaxis. Numbers in parenthesis are calculations based on analysis of the stock homogenate.

TABLE 2
NMR Spectra of SAD Preparations

Preparation	Height = width ratio	OH curve[a] broadening
3d	1.8	9.2
7d	3.3	7.7
14d	3.9	7.1
30c	4.8	6.2
200c	3.0	8.0
1000c	ND	ND
Mean	3.4	7.6
Control	11	0

Note: Results of NMR spectroscopy evaluating OH spectral curve broadening between 4.5 and 6 ppm.
[a] Height=width ratio of control preparation minus the height=width ratio of SAD preparation. Higher numbers indicate greater broadening.

ratio was 11. The 30c and 200c preparations had height to width ratios of 4.8 and 3.0, respectively. NMR spectroscopy was not done on the commercial SAD preparation because the alcohol concentration of this preparation was not identical to other SADs (50% vs 70% ETOH).

Evaluation of Prophylaxis

Antibody screening. Serum from the 30c- and 200c-treated mice was positive for anti-*tularensis* antibodies after Week 4 of SAD treatment. Anti-*tularensis* antibodies were not found in other SAD-treated groups. Titer endpoints were 1:100 or less before prophylaxis and greater than 1:10,000 just before challenge in the 30c- and 200c-treated mice and in the LVS-vaccinated

TABLE 3
Anti LVS Antibodies in SAD Groups

Preparation	Pre = AUHD treatment	Pre = LVS challenge
3d	ND	1:100
7d	ND	1:100
14d	ND	1:100
30c	1:100[a]	$1:10^4$
200c	1:100	$1:10^4$
1000c	1:100	1:100
Ascending	1:100	1:100[b]
Positive control	ND	$1:10^4$
Negative Control	1:100	1:100

Note: LVS = live virus strain; AUHD = agitated ultra-high dilutions; ND = Not done. Endpoint anti-*tularensis* antibody titers of pooled serum from mice before SAD treatments and just prior to LVS challenge.
[a] This sample was drawn 3 days after start of SAD treatment.
[b] This sample was drawn 15 days before LVS challenge.

mice (positive controls). Titer endpoints were always 1:100 or less in the 3d, 7d, 14d, 1000c, and ascending groups and in the normal (untreated) mice (Table 3).

MRDT. The MRDT was calculated for SAD-treated and control mice for each matched trial. MRDT ranged from 0 to 0.16 for all trials and, as expected, was higher in the trials with a higher challenge dose. SAD-treated mice consistently showed reduced MRDT (increased resistance to infection) over their

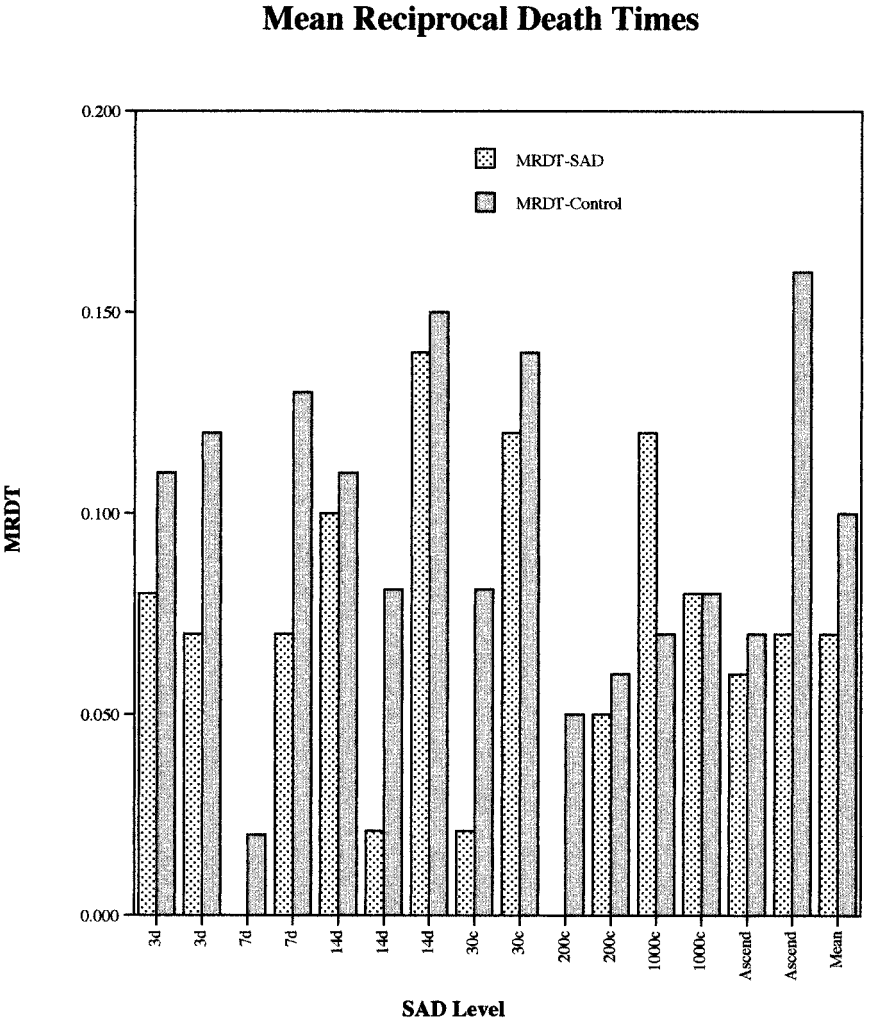

Fig. 1. Mean reciprocal death times (MRDT) of SAD-treated mice (first columns) and their matched control groups (second columns) over 15 trials. Lower MRDT scores indicate increased resistance to infection. First trial listed for each SAD level is the LD_{50} challenge. Second and third trial listed is the LD_{75} challenge.

TABLE 4
Analysis of Mortality After SAD Treatments

Preparation	No. in group	Mortality		Relative risk[a]
		SAD	Control	
3d	20	6	9	9
7d	18	2	5	2.5
14d	34	11	14	1.27
30c	22	6	9	1.5
200c	20	2	7	3.5
1000c	20	8	6	0.75
Ascending	20	6	8	1.33
Dilutions(3d-14d)	70	18	27	1.36
Solutions(30-As)	82	22	30	1.5
All Groups	152	40	57	1.89[b]

Note: Mortality of mice treated with each SAD preparation and matched control group.
[a] Relative risk after stratification.
[b] Mantel-Haenszel chi-square for all groups (95%CI 1.23–2.92, $p < .005$).

matched control group (mean difference = .03). All but two of the 15 trials showed a reduced MRDT in the SAD group (Wilcoxon sign rank test, $z = 3.408$, $p = .0007$, paired t test, $t = 7.518$, $p = .0001$, two-tailed). There was no relationship between level of dilution in the SAD preparations and protection. All preparations except Ft/SAD-1000c demonstrated protection (Figure 1).

These results were checked directly against actual death day using the raw data for all SAD groups with Day 30 as the cut off. Mean time to death for SAD-treated mice was 18.6 (range 12.9–25.6) and for controls was 13.7 (Range: 11.6–15.6). The Mann Whitney U test using death day for all groups was 3.087, ($p = .002$), consistent with the MRDT evaluation. SAD treatment delayed death by approximately 5 days. One way ANOVA showed that SAD treatment had a significant impact on death day ($F = 11.51$, $p = .0009$) and MRDT ($F = 4.96$, $p = .037$) even after controlling for challenge dose (Table 5).

Mortality. Each group was monitored daily for deaths up to 30 days postchallenge. Relative risk was above 1 in all groups except Ft/SAD-1000c. The Mantel-Haenszel rate ratio (after stratification into treatment groups) was not different than the crude rate ratio for all groups, indicating that no individual preparation disproportionally influenced the overall result (M-H RR 1.89; 95%CI 1.23-2.92, two-tailed $p = .0037$; Table 4). Overall mortality was 53% in the SAD group and 75% in the matched control group with little difference between solution or dilution groups. Chi-square analysis on the proportion of deaths in each group was 8.41 ($p = .0037$). SAD treatment prevented 22% of deaths. The number of organisms required to kill 50% of the animals (LD_{50}) in the control group was 6,000 CFU compared with 20,000 CFU in the SAD-treated group (Table 5).

TABLE 5
Summary Evaluation of SAD Effects

Outcome	SAD	Control	Evaluation	*p* value
MDT (days)	18.6	13.7	$z = 3.08$	.002[a]
MRDT	0.07	0.10	$t = 7.518$	.0001[b]
By nonparametric test			$z = 3.41$	.0007[c]
Controlled for challenge dose			$F = 4.96$	.037[d]
Mortality	53%	75%	$\chi^2 = 8.41$	.0037
Prevented fraction of deaths by SAD preparations = 22%				
Challenge dose (CFU)	7300	7300		
LD_{50} (CFU)	20,000	6000		

Note: CFU = colony forming units; MDT = mean time to death; MRDT = mean reciprocal death time. Summary evaluations of mortality (MDT) and MRDT in all groups.
[a] Mann-Whitney *U* test.
[b] Students two-tailed *t*-test.
[c] Wilcoxon sign rank test.
[d] One way ANOVA on mean time to death after controlling for challenge dose.

Conclusion

The results in this series of experiments did not confirm the null hypothesis that ultra-low, SAD dilutions prepared from infected tissue acted like chemically identical controls in their ability to protect against subsequent bacterial challenge by the same organism. SADs reduced overall mortality by 22%, delayed death by 5 days, and tripled the number of organisms required to kill 50% of the animals. Two SADs (30c and 200c) stimulated the production of specific anti-*tularensis* antibodies in animals after 1 month of exposure. No protein was detected in SAD preparations, but they could be distinguished from control solutions with ^{1}H NMR-spectroscopy.

All SAD preparations, except the 1000c, consistently imparted some protective effect. Environmental exposures while in transit or differences in the method of preparation of the commercial SAD may have altered the 1000c solution, rendering it different than those produced in our lab. Data from studies using the SADs made at our lab suggest protection was not related to level of dilution, number of times vortexed, or absence of bacteria or protein in the preparations.

The lack of relationship between response and dilution level is probably a reflection of the wide gap between dilutions selected for testing. *In vitro* work on SADs in which multiple serial, 10-fold dilutions are examined usually report a quadratic or sinusoidal pattern (rather than a linear or logarithmic) dose-response relationship (Boxenbaum, Neafsey, and Fournier, 1988; Davenas, Poitevin, and Benveniste, 1987; Davenas *et al.*, 1988; Poitevin, Davenas, and Benveniste, 1998; Schulte and Endler, 1998). Peaks and valleys of effect are found when multiple and closely spaced dilutions are tested. We tested only six widely spaced dilutions and so may have missed an existent dose-response relationship.

SAD solutions could be distinguished from control solutions using ^{1}H NMR-spectroscopy; however, the relevance of these findings in unclear. Broadened hydroxyl band width can occur from a variety of causes, including contamination with paramagnetic particles or increased proton exchange in the OH bonding region. This study was not designed to control for these possibilities, and therefore, no conclusions can be made about this finding.

Reports in the literature testing the SAD prophylaxis hypothesis in laboratory models or in veterinary populations have yielded mixed results. Taylor reported no prophylactic effect from a SAD preparation derived from the husk of the lungworm *Dictyocaulus vivparus* in experimental lung infection in cows after there were promising reports from veterinarians (Taylor, Mallon, and Green, 1989). The experiment used the 30c preparation, dosed the animals four times with the SAD over a month, and gave no doses after challenge. Study power was low. Oberbaum, Weismann, and Bentwich (1989) reported on a study on a retroviral infection with a SAD preparation. This study used SAD preparations (range 6d–30d) derived from C type B-trophic retrovirus (LP-BM5 MuLV), which produces a murine AIDS-like syndrome (MAIDS). Groups of mice were treated either orally or by injection (i.p.) three times a week, but, in contrast to Taylor, SAD preparations were given only after the infectious challenge. Animals given the 12d SAD prepared from stock containing less than 5 infectious units of virus had significant reductions in spleen weights (the primary marker of the disease) compared with saline–alcohol treated animals. No other level of SAD demonstrated an effect (Oberbaum, Weismann, and Bentwich, 1989). Day reported that a nosode prepared from parvovirus stopped an epidemic of kennel cough in dogs (Day, 1987). No control group was included, however, and therefore, no comparison with the natural course of the epidemic could be made. Apparently, the use of nosodes and other SAD and homeopathic preparations are popular among European animal farmers for the control of epidemic outbreaks. Several reports of SAD use for control of bovine mastitis have been reported (Day, 1986; Merck, Sonnenwald, and Rollwage, 1989). Langer reported on a series of field tests and controlled experiments on bovine mastitis using various mixtures of nosodes, SADs, and homeopathic remedies that were both protective and therapeutic (Langer, 1990).

What is the mechanism of SAD-induced protection? Besides artifact or experimental error, the most likely explanation is that SAD preparations of LVS-infected tissue contain sufficient immunogenic signals to stimulate an immune response in recipients. Protection does not seem dependent on humoral immunity because anti-*tularemia* antibodies were detected only in serum from 30c- and 200c-treated mice (Table 3). This is not unusual in tularemia infection, however, in which the primary protective mechanism is thought to be cellular rather than humoral (Tärnvik, 1989). Specific antibody induced by SAD treatment, then, cannot account for protection seen in all groups treated with SAD treatment. Induction of specific antibody response to SAD treatments on other

systems is also variable. Davies reported no antibody production 2 weeks after three doses of a SAD preparation from influenza A2 (Davies, 1971). Weisman and colleagues, on the other hand, have reported highly significant modulation of specific antibody generation in mice using SAD preparations of K-lipid hemocyanin when given three times a week for 8 weeks (Weisman *et al.*, 1997). Neither of these investigators included cytokines, tissue protein, or other adjuvants reported *in vitro* to modulate immune function when prepared as SADs. If ultra-low dose SADs do have specific effects, one can hardly expect those effects to be large or induced after only a few doses over a short time period. No research has been done to systematically investigate these questions.

What is the active agent in the final SAD solutions? Of the many speculative hypotheses previously discussed, one is that stable isotopic positional correlations (IPCs) of minority oxygen singlets are produced around molecules in the stock solutions and subsequently "locked in" during agitation by the polaronic self-stabilization properties of nonlinear media (Berezin, 1990). IPCs, then, might mimic the original molecules through interaction with receptors and enzyme systems on or in the cell. This hypothesis takes into consideration current information about SAD action and is testable by manipulating the concentration of oxygen isotopes in the solution as it is prepared. In addition, the molecular organization of these solutions could be further evaluated using techniques such as thin layer crystallography, NMR, infrared, Raman, or other spectrographic methods (del Giudice, Preparata, and Vitiello, 1988; Popp, Li, and Mei, 1989). One currently popular hypothesis is that water can hold and deliver specific electromagnetic signals (with coherent wavelengths similar to a laser) to which biological systems respond (Benveniste *et al.*, 1997). Others claim these observed effects are all attributable to experimental error and artifact (Maddox, 1988; Metzger, 1988; Plasterek, 1988; Seagrave, 1988). We currently are attempting to replicate these findings under more stringent conditions (*e.g.,* total blinding and using uniform aerosol dosing methods, additional control groups, *etc.*)

Whatever the mechanism, protection produced by SAD preparations in this study was unexpected yet consistent over 15 trials. If these effects represent a real and general phenomenon, SAD preparations might provide a simple and rapid method of protecting against infectious agents for which we do not yet have adequate prophylactic or therapeutic regimes or to agents with emerging resistance to current treatments. This is especially important as the world becomes increasingly mobile and the chance for rapid spread of unusual or resistant infectious pathogens increases. Further investigation into this phenomenon is warranted.

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