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DECONTAMINATION OF COMBAT WOUNDS IN THE INJURED SOLDIER.(U)
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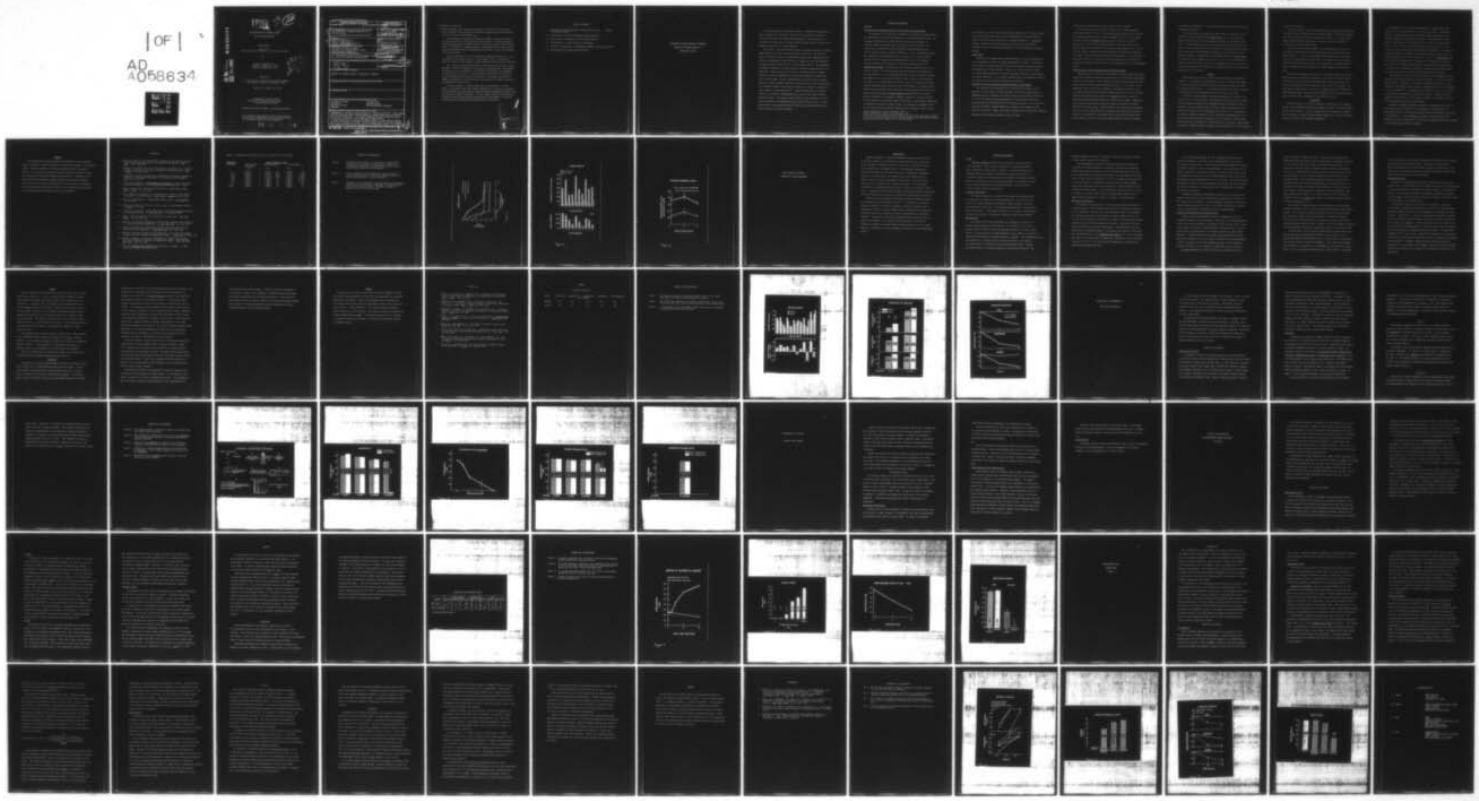

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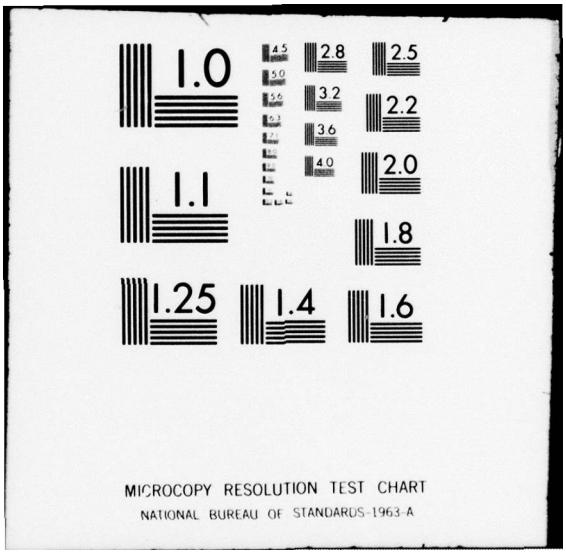
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DECONTAMINATION OF COMBAT WOUNDS IN THE INJURED SOLDIER

Annual Report

June 1978
(for the period of January 1, 1977 to May 31, 1978)

by

Milton T. Edgerton, M.D.
Richard F. Edlich, M.D., Ph.D.
George T. Rodeheaver, Ph.D.

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Supported by

U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

Contract No. DADA 17-72-C-2153

Department of Plastic Surgery
University of Virginia Medical Center
Charlottesville, Virginia

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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle)	5. TYPE OF REPORT & PERIOD COVERED
Decontamination of Combat Wounds in the Injured Soldier,	Annual Report 1 January 1977-31 May 1978
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s)	8. CONTRACT OR GRANT NUMBER(s)
Milton T. Edgerton, M. D. Richard F. Edlich, M.D., Ph. D. George T. Rodeheaver, Ph. D.	DADA 17-72-C-2153
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
Department of Plastic Surgery University of Virginia Medical Center Charlottesville, Virginia	61102A 3A161102B71R, 01.036 62772A 3S762772A814, 00.036
11. CONTROLLING OFFICE NAME AND ADDRESS	12. REPORT DATE
U.S. Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701	1 June 1978
	13. NUMBER OF PAGES 83 pages
14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*	15. SECURITY CLASS. *(of this report)* Unclassified
12 79 p.	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

proteolytic enzymes
antibiotics
anaerobic bacteria
iodophors
Pluronic F-68
montmorillonite
fire extinguisher chemicals

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Management of traumatic injuries should be rapid and effective. This therapy should be directed towards reestablishment of normal tissue functions without any further debilitation. This report summarizes several studies on how infection may be reduced in the contaminated traumatic wound.

Topical application of proteolytic enzyme solution to open wounds enhances the action of antibiotic therapy. Topical enzymes remove the surface coagulum which protects bacteria from the action of antimicrobial agents. By dissolving the coagulum the bacteria are exposed and more effectively eliminated. The

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20. Abstract (continued)

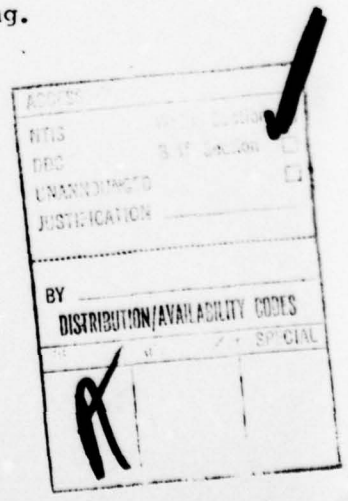
enzyme treatment also allows more antibiotic to reach the site of injury. This treatment of open wounds with proteolytic enzymes does not affect wound healing or normal tissue defenses.

The quantitative relationship between the number of anaerobic bacteria and the development of infection has not been defined. We have developed an anaerobic quantitation procedure that will allow us to evaluate this relationship. The procedure is patterned after our published aerobic bacterial quantitation procedure except that modifications were made in order that anaerobiosis was maintained.

Pluronic Polyol F-68 is the only detergent approved by the FDA as a safe and effective skin wound cleanser. Using Pluronic F-68 as a solubilizer agent for iodine we have prepared a stable iodophor that has no toxic additives.

Soil contains infection potentiating factors which totally inhibit tissue defenses. Effective elimination of bacterial contamination must be performed if infection is to be prevented. The activity of antibiotics is influenced by the presence of clay soil. Acid and neutral antibiotics like penicillin are not inhibited by the acidic clay particles. In contrast, the basic or amphoteric antibiotics like gentamicin or tetracycline are rendered ineffective by complexation with the soil.

In many situations, a burning human must be extinguished. In these cases it is imperative that the chemical employed be as non-toxic as possible. The multipurpose fire extinguisher containing ammonium dihydrogen phosphate was the least toxic material evaluated. The more common chemicals like potassium bicarbonate and sodium bicarbonate potentiated the development of infection, inhibited leukocyte function and retarded normal wound healing.



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MECHANISMS BY WHICH PROTEOLYTIC ENZYMES PROLONG THE GOLDEN PERIOD OF ANTIBIOTIC ACTION

The "golden" period of antibiotic action in contaminated open wounds is short, lasting only a few hours. During this time interval, topical or systemic antibiotics can prevent the development of wound infection. If treatment with antibiotics is delayed beyond this short period of time, their therapeutic benefit is no longer apparent.

The length of time this "golden" period of antibiotic action is inversely proportional to the time in which the wound is left open. The effective period of antibiotic action in contaminated wounds closed immediately after injury is significantly longer than that in wounds left open and subjected to delayed primary closure three to 24 hours after injury.[1] The exaggerated inflammatory response of the open wound has provided an explanation for this difference. When the wound is left open, its vessels undergo a dramatic increase in vascular permeability, allowing extravasation of protein-rich fluid into the wound. We have suggested that this fluid forms a coagulum around the surface bacteria, preventing them from contact with the antibiotic. The therapeutic benefit of proteolytic enzymes as an adjunct to antibiotics lends credence to this hypothesis.[2,3] Topical application of an aqueous solution of proteolytic enzyme dramatically prolongs the golden period of antibiotic action. The degree to which enzymes enhance antibiotic action is directly related to the magnitude of their fibrinolytic activity. In this investigation, the mechanisms by which proteolytic enzymes potentiate antibiotic activity are further examined.

Materials and Methods

In vitro

Potentiation of Antibiotic Action in the Presence of Clotted Blood

The purpose of this study was to determine if proteolytic enzymes enhance the antibacterial activity of antibiotics in clotted contaminated blood. The in vitro contaminated clot model employed in this study simulates the fibrinous exudate that develops on the surface of the open wound. This coagulum which surrounds the wound contaminant has been implicated as a barrier against topical or systemic antibiotics. Fresh, citrated whole blood from volunteers was equilibrated to 37° C before use. A series of tubes containing 0.8 ml of blood was contaminated with 0.1 ml of 0.9% sodium chloride containing 10^3 organisms of a penicillin sensitive strain of Staphylococcus aureus. To two-thirds of the tubes was added 0.1 ml of 0.1 M calcium chloride. The presence of calcium ions initiated the formation of a whole blood clot. To the remaining tubes which acted as clot-free controls was added 0.1 ml of 0.9% sodium chloride. After 1 hour at 37° C each tube received 3.5 ml of trypticase soy broth+and 0.25 ml of a solution containing various concentrations of penicillin from 0-100 units/ml. All tubes without blood clots and one-half of the tubes with clots then received 0.25 ml of 0.9% sodium chloride containing Bacillus subtilis protease which is the active protease contained in the commercial product Travase.R* The final activity of this enzyme in the tube was 20,000 PC units**/ml, a concentration sufficient to hydrolyze the blood clot within 1 hour. All tubes were incubated at 37° C for 5 hours with gentle mixing by inversion every 90 minutes. After 5 hours

+DIFCO Laboratories, Detroit, Michigan 48210
*Flint Laboratories, Morton Grove, Illinois 60053
**A PC unit is that amount of enzyme required to produce the same optical density at 275 nm as that of a solution of 1.5 mcg tyrosine/ml after the enzyme has been incubated with 35 mg of casin at 37° C for one minute

the penicillin in each tube was inactivated by adding 0.04 ml of penicillinase.[+] The contents within each tube were then homogenized for 30 seconds and the number of viable bacteria quantitated by standard techniques. A comparison between the number of bacteria remaining in the tubes of the different treatment groups will identify the magnitude of their antibacterial activity.

In vivo

Animal Model

Adult male New Zealand albino rabbits weighing 2-3 kg were anesthetized by an intravenous injection of sodium pentobarbital (30 mg/kg). The back of each animal was clipped, depilated with Surgex,[R] washed thoroughly with water and swabbed with 70% ethyl alcohol. Using a No. 15 stainless steel knife, six standardized paravertebral incisions were made in the skin of each rabbit. The wounds, measuring 3 cm. in length, extended down to the panniculus carnosus. The wounds were left open until treatment was initiated.

Fibrinolytic Activity of Topical Enzyme Solutions on Open Wounds

The purpose of this experiment was to quantitate the degree of clot hydrolysis in open wounds following treatment with topical enzyme solution. Immediately prior to wounding, 10 animals received an intravenous injection of 1.5 ml of 0.9% sodium chloride containing 95% clottable human fibrinogen. The fibrinogen was labelled with ^{125} I* and had an activity of 32.5 uCi/1.5 ml. Following injection, standard wounds were created on each animal and left open for three hours. During this period serum proteins, including the labelled

*Amersham Searle, Arlington Heights, Illinois 60005

fibrinogen, will extravasate into the wound to form a coagulum.

At the end of this time interval, one half of the wounds received a topical application (0.1 ml) of 0.9% sodium chloride serving as the controls. The remaining wounds received a topical treatment (0.1 ml) of 0.9% sodium chloride containing B.subtilis protease with an activity of 12,000 PC units/0.1 ml. Each topical solution remained on the wound surface for 10 minutes before being blotted dry with a sterile gauze sponge (0.7 x 3.0 cm). The gauze sponge was removed from the wound and its radioactivity measured by a Sample Changer University 601 Deep Well gamma counter. The efficiency of the topical solution in removing the fibrinous exudate was ascertained by comparing the levels of radioactivity in the sponges used to blot the different wounds.

Wound Antibiotic Levels Following Topical Enzyme Treatment

The purpose of this study was to ascertain the degree topical proteolytic enzyme wound treatment enhanced the concentration of systemically administered antibiotic in the wound. After wounding, the rabbits were divided into three groups which were left untreated respectively for 0, 3, or 6 hours. At the appropriate time, one half of the wounds in each group received 0.1 ml of 0.9% sodium chloride and the remaining wounds received 0.1 ml of 0.9% sodium chloride containing 12,000 PC units of B. subtilis protease. After ten minutes each wound was blotted dry with a sterile gauze sponge. Immediately after blotting, each animal received an intravenous injection of benzyl-penicillin (100,000 unit/kg). Twenty minutes later each wound was subjected to 0.1 ml of 0.9% sodium chloride to solubilize the extravascular penicillin present at the wound site. The animals were sacrificed ten minutes later and an aliquot (0.02 ml) of fluid was removed from each wound for analysis

of antibiotic concentration. A blood sample was also taken for assay of the serum penicillin level.

The concentration of antibiotic in each aliquot was determined by the method of Grove and Randall[4] using Sarcina lutea as the test organism. Appropriate dilutions of each fluid sample were made in a phosphate buffer (pH 6). Filter paper discs (1/4 inch) were wetted with the test fluids and placed on plates seeded with the test organism. The agar plates were prepared with a base layer of Difco Antibiotic Medium 2 and a seed layer of Difco Antibiotic Medium 1 inoculated with a stock broth culture of Sarcina lutea. The agar plates were incubated for 18 hours at 37° C. The concentration of antibiotic in the fluid was ascertained by comparing the size of the zone of inhibition around the filter paper disc to that around discs containing known concentrations of penicillin.

Results

The antibacterial action of penicillin is inhibited by clotted blood. (Figure 1). At antibiotic concentrations less than 1.0 units/ml, the number of bacteria surviving in clotted blood is significantly greater than that in unclotted blood (Table 1). At concentrations above this level, the action of penicillin was not influenced by the clot utilized in this test.

Proteolytic enzyme treatment enhanced the antibacterial action of the penicillin at all concentrations. The magnitude of this potentiating effect can be estimated by examining the minimum inhibitory concentration (MIC) of antibiotic in each treatment group. The MIC is that concentration of antibiotic which inhibits the proliferation of the bacteria. In this system, the initial inoculum of bacteria is 10^3 and thus the concentration of antibiotic that limits the level of bacteria to 10^3 is the MIC. The MIC value for penicillin in the presence of clotted blood is 1.0 units/ml. When enzyme is used as an adjunct the MIC is reduced to 0.06 units/ml or a 16 fold increase

in penicillin activity.

All wounds treated topically for ten minutes with enzyme solution resulted in significantly more hydrolysis and subsequent removal of fibrinous coagulum than similar wounds treated with normal saline (Figure 2). In sixty paired wounds, enzyme treatment resulted in an average of 535% more removal of fibrin than the saline treated wounds.

Following topical enzyme treatment, the concentration of antibiotic at the surface of the wound was significantly higher than that for the control wounds (Figure 3). In the group of wounds treated immediately with enzyme solution, the concentration of antibiotic in the wound was 223% greater than that for wounds subjected to saline. This enhancement of wound antibiotic levels was apparent even when proteolytic enzyme treatment was delayed six hours.

The elevated antibiotic concentration in the wounds treated with topical enzyme solution was remarkably similar to that found in the serum. During this study, the serum levels of benzylpenicillin, thirty minutes after injection, ranged from 40-100 units/ml with a mean value of 73 units/ml. The wide range in serum values is the result of rapid elimination of penicillin from the blood stream. Baker and Hunter[5] reported that an initial penicillin level of 2000 units/ml decreased to 10 units/ml in one hour.

Discussion

Following incision of tissue, a fibrinous clot develops on the wounded surface. This fibrinous material gains immediate access to the wound through the cut ends of vessels. Once the divided ends of the vessels are plugged by clot formation, further fibrinous deposit develops as a result of extravasation from vessels with increased permeability.

The presence of a clot in the wound serves as a double edged sword. Blood loss is obviously reduced by clotting in the vessels. Furthermore, the clot surrounds bacteria and other foreign agents and prevents their systemic dissemination through the lymphatics. These beneficial effects must be weighed against the nefarious role of the clot in potentiating infection. May et al[6] and Conolly and Golovsley[7] reported that up to 30% of clinical wound infections were associated with hematomas. The infection potentiating effect of hematoma in soft tissue wounds was clearly shown in the experimental study of Krizek and Davis.[8] When 5×10^7 Escherichia coli were injected subcutaneously in the absence of a hematoma, the organisms were effectively contained and eventually eliminated without complications. In contrast, when a hematoma was present, the bacteria proliferated beyond the capabilities of the local defenses and 80% of the animals died.

The presence of a blood clot not only inhibits local defenses, but also inhibits the beneficial effects of antibiotic therapy. O'Connel[9] has shown that penicillin penetration into clots is inversely proportional to their thickness. The degree to which penicillin penetrates the clot is also related to its concentration.[10] At therapeutic blood levels of 0.5 to 1.0 units/ml, penicillin fails to penetrate a 3 mm layer of fibrin. Increasing the concentration of penicillin in the blood will enhance clot penetration, but may also be associated with serious side effects.

Proteolytic enzymes were found to enhance the antibacterial action of all concentrations of antibiotic in the presence of clotted blood. By hydrolyzing the clot, the bacteria were more effectively exposed to the action of the antibiotic. This enhancement of antibiotic action may also prove beneficial in other conditions [6,7,11-15] where a contaminated fibrin loci appears to be refractory to antibiotic treatment.

Summary

These experimental studies provide an explanation for the therapeutic value of proteolytic enzymes as adjuncts to antibiotic treatment of contaminated wounds. Following wounding, a fibrinous coagulum develops on the wound surface. This coagulum surrounds the bacteria and protects them from contact with systemically or topically administered antibiotics. Treatment of the wound surface with proteolytic enzymes disrupts the coagulum and exposes the bacteria to the action of the antibiotic. The topical use of enzymes is also associated with significant increases in the concentration of antibiotic at the wound and thus a decrease in the rate of infection.

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Table 1: Potentiation of Antibiotic Action in Presence of Clotted Blood

PENICILLIN (units/ml)	VIABLE BACETERIAL COUNT					
	Clotted Blood With Enzyme (log)	p	Unclotted Blood (log)	p	Clotted Blood (log)	p
0	6.4±0.3	-	6.0±0.1	ns	6.6±0.0	ns
0.01	6.1±0.2	-	4.9±0.4	0.025	6.7±0.1	0.025
0.02	5.5±0.2	-	4.1±0.3	0.005	6.5±0.1	0.005
0.04	3.8±0.3	-	2.7±0.1	0.005	6.2±0.1	< 0.001
0.08	1.3±1.0	-	2.4±0.2	ns	5.6±0.1	< 0.001
0.10	1.3±0.9	-	2.5±0.1	ns	5.4±0.0	< 0.001
1.0	1.3±0.9	-	2.3±0.0	ns	2.8±0.2	ns
10.0	1.3±0.9	-	2.4±0.0	ns	2.5±0.2	ns
100.0	1.2±0.9	-	1.8±0.1	ns	2.5±0.2	ns

LEGEND FOR ILLUSTRATIONS

Figure 1 — The antibacterial efficacy of penicillin is significantly inhibited in the presence of clotted blood. Addition of a proteolytic enzyme with the antibiotic dissolves the clot and potentiates antibiotic effectiveness.

Figure 2 — Topical treatment of open wounds with enzyme solution for a short period results in significantly enhanced removal of wound coagulum compared to saline treatment.

Figure 3 — Treatment of open wounds with a topical solution of proteolytic enzyme results in significant enhancement of wound antibiotic levels after systemic therapy. The enzyme treatment is beneficial even in 6 hour old wounds.

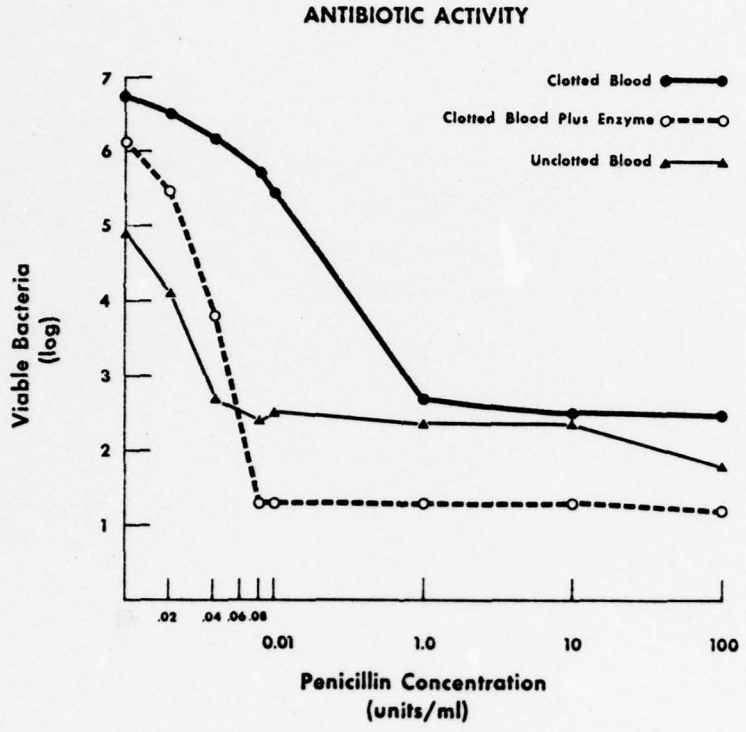
ANTIBIOTIC ACTIVITY
Clotted Blood
Clotted Blood Plus Enzyme
Unclotted Blood
Viable Bacteria (log)
7
6
5
4
3
2
1
.02
.04
.06
.08
0.01
1.0
10
100
Penicillin Concentration (units/ml)

Figure 1

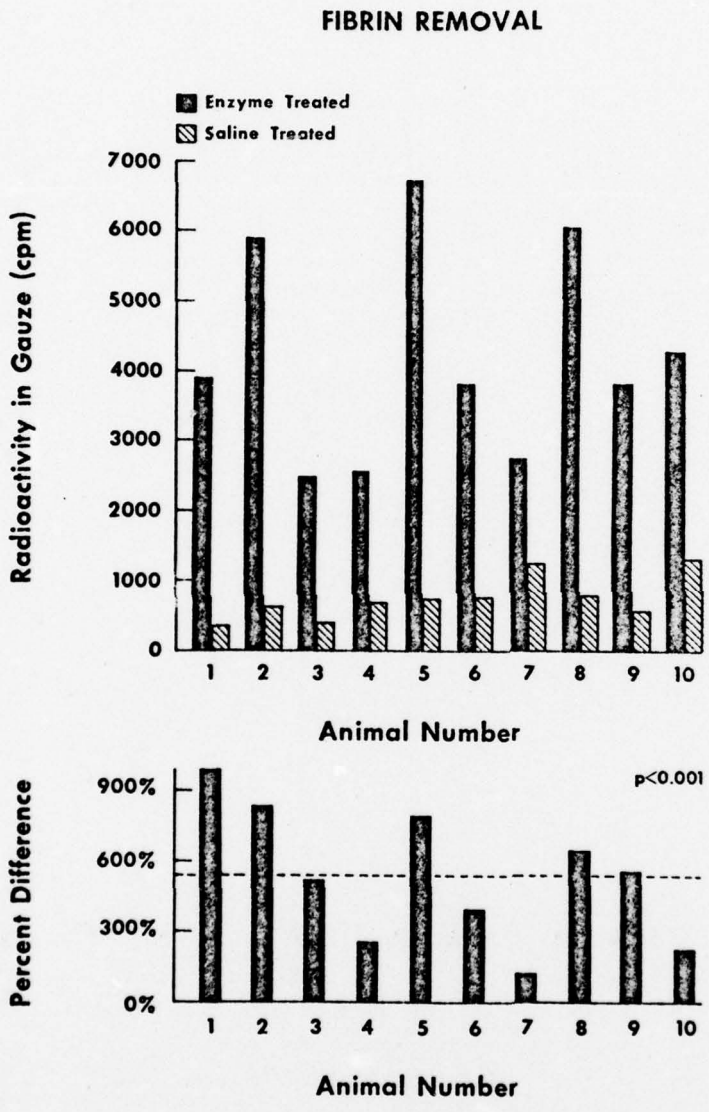
FIBRIN REMOVAL
Enzyme Treated
Saline Treated
Radioactivity in Gauze (cpm)
7000
6000
5000
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8 9 10
Animal Number
Percent Difference
900%
600%
300%
0%
p<0.001
1 2 3 4 5 6 7 8 9 10
Animal Number

Figure 2

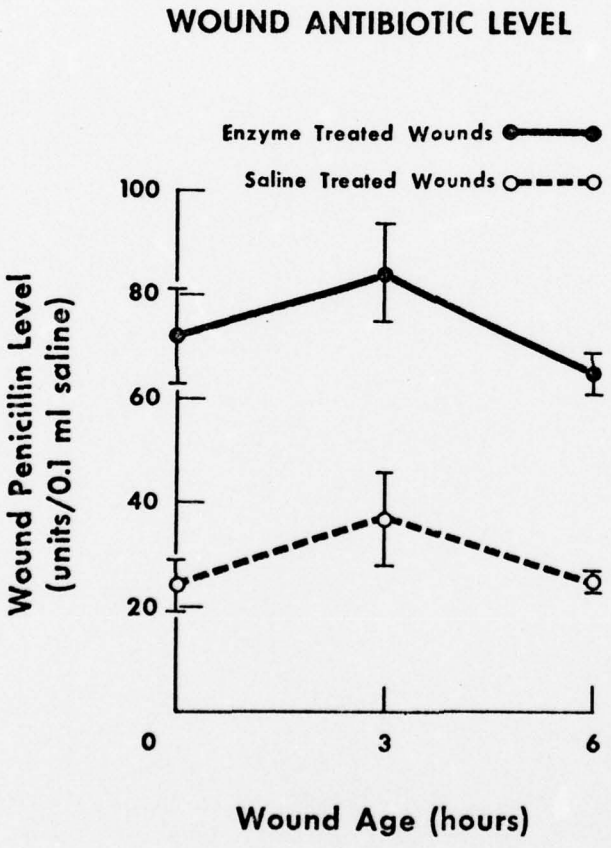
WOUND ANTIBIOTIC LEVEL
Enzyme Treated Wounds
Saline Treated Wounds
Wound Penicillin Level
(units/0.1 ml saline)
100
80
60
40
20
0
3
6
Wound Age (hours)

Figure 3

SIDE EFFECTS OF TOPICAL PROTEOLYTIC ENZYME TREATMENT

INTRODUCTION

Immediate treatment of heavily contaminated wounds with either topical or systemic antibiotics can reduce their wound infection rate. When antibiotic treatment is delayed for three or more hours, the benefits of antibiotic treatment are considerably less.[1] This developing resistance to antimicrobial therapy has been correlated with an exaggerated inflammatory response in the open wound. The vessels within these wounds exhibit a marked increase in vascular permeability with a protein rich fluid extravasating into the wound to form a fibrinous coagulum. As the coagulum accumulates, the contaminated wound becomes refractory to antibiotic treatment. It appears that the fibrinous coagulum surrounds the bacteria and shields them from contacting the topically or systemically administered antibiotics.

Hydrolysis of this coagulum by proteolytic enzymes considerably prolongs the effective period of antibiotic action.[2,3] The efficacy of proteolytic enzymes as adjuncts to antibiotic treatment is directly related to their fibrinolytic activity. Topical treatment with an enzyme that has substantial fibrinolytic activity can enhance the wound's susceptibility to antibiotic treatment. This benefit of proteolytic enzymes must be weighed against any possible side effects. As a result of their fibrinolytic activity, the enzymes may interfere with wound healing or damage the host or local tissue defenses. The validity of these hypotheses are examined in these experimental studies.

MATERIALS AND METHODS

Enzyme

A Bacillus subtilus protease was the proteolytic enzyme employed in these experiments. This enzyme was supplied as a lyophilized powder by Flint Laboratories, (Morton Grove, Illinois). In previous studies, this protease exhibited considerable fibrinolytic activity and proved to be an effective adjunct to delayed antibiotic treatment of contaminated wounds. Before each experiment, the enzyme was solubilized in 0.9% sodium chloride to the desired concentration and filtered through a 0.22 μ Millipore^{R} filter to remove bacterial contaminants.

Standard Animal Model

Male, Hartley guinea pigs weighing 300-350 grams were anesthetized by an intraperitoneal injection of sodium pentobarbital. The back of each animal was clipped, depilated with Surgex^{R}, washed thoroughly with water, and swabbed with 70% alcohol. Two standardized paravertebral incisions were made in each animal. The wounds, measuring 3 cm in length, extended down to the panniculus carnosus. Bleeding was stopped by sterile gauze pressure.

Wound Healing

The purpose of this first experiment was to examine the influence of topical enzyme treatment on wound healing. In 12 animals, 0.1 ml of the enzyme solution (12,000 PC units) was applied topically to one wound in each animal. The concentration of enzyme employed in these studies is sufficient to enhance the effectiveness of delayed antibiotic treatment. The contralateral wound was subjected to a similar volume of 0.9% saline serving as the control. Ten minutes later all wounds were closed with microporous tape. Fourteen days post-wounding, the breaking strength of each wound was assessed. The

breaking strength of a wound is a measure of the force required to disrupt a wound without regard to its dimensions.

This measurement was initiated by making two parallel incisions on each side of the healing wound at a distance of 6 mm from the wound edge. Through these parallel incisions, clips were firmly attached to each side of the healing wound 3 mm from the wound edge. One clip remained stationary while the other clip was attached by a wire to a continuous drive motor with a screw gear advance. Attachment to this drive motor was made through a strain gauge which was electrically connected to a Hewlitt-Packard recorder through an amplifier system. As the drive motor pulled the clip at a rate of 8.9 cm/min., the strain gauge recorder system provided a measure of the applied tension (gram-force) necessary to disrupt the healing wound.

Resistance to Infection

The purpose of this phase of the study was to determine the effect of topical proteolytic enzyme treatment on the tissue's resistance to infection. In this experiment, 28 guinea pigs with standardized wounds were evaluated. One wound on each animal was subjected to a topical treatment with 0.1 ml of 0.9% saline containing 12,000 PC units of enzyme. The contralateral wound received 0.1 ml of saline and served as a control. Ten minutes later each wound was blotted with a sterile gauze sponge. The animals were then subdivided into two treatment groups. In one group, both wounds in each animal were contaminated by 3.3×10^5 Staphylococcus aureus in 0.1 ml of 0.9% saline. The wounds in the other group of animals were subjected to a larger bacterial inoculum (1.1×10^6). Five minutes after contamination, the edges of the wounds were approximated by tape.

On the fourth postoperative day, the inflammatory responses of the wounds were measured. The width of the indurated margin of each wound was recorded in millimeters. Each wound was opened and examined for evidence of purulent discharge. After visual assessment, a relative estimate of the number of bacteria in each wound was made. The entire length of each wound was swabbed three times with a cotton-tipped applicator after which time the contaminated swab was immersed into 5 ml of 0.9% saline. The tube containing the bacterial suspension was then agitated on a Vortex^{R} mixer for one minute. The number of bacteria in the suspension was then quantitated by standard serial dilution technics.

A comparison between the inflammatory responses of the enzyme treated wounds to those of the control wounds provides insight into the effect of the enzyme on tissue defenses. If the inflammatory responses of the enzyme treated wounds are greater than that of the control wounds, this indicates that this enzyme exerts a deleterious effect on tissue defenses..

Leukocyte Phagocytosis and Intracellular Kill

Traumatic injury and subsequent bacterial contamination elicit an inflammatory response against infection. Alteration in the microvasculature occurs that allows extravasation of phagocytic cells into the tissue. These cells possess the capacity to ingest and kill invading bacteria. Once the microorganism is phagocytized, it is normally destroyed by intracellular digestion. Microbial death does not always follow phagocytosis, however, and certain bacteria will survive within the leukocyte for prolonged periods of time. This phase of the study examined the effect of the proteolytic enzyme on the ability of the leukocyte to phagocytize and kill bacteria.

In vitro measurement of leukocyte function was patterned after the

technic described by Mandell and Vest.[4] Fresh venous blood collected in heparinized tubes was combined with an equal volume of 3% Dextran and placed at a 45° angle for one hour in order to sediment the erythrocytes. The supernatant containing plasma, leukocytes, and platelets was centrifuged at 280 g for 12 minutes and the resulting button of white blood cells (10^5) was resuspended in 3.4 ml of Hank's balanced salt solution and 0.4 ml of autologous serum. The fresh autologous serum contains the opsonins and complement system that facilitates phagocytosis of foreign particles. To one half of the white cell suspensions was added 4 mg (12,000 PC units) of enzyme powder. The remaining cell suspensions were not subjected to the enzyme and served as controls. All tubes received 0.2 ml of a bacterial suspension (10^7) of S. aureus (ATCC 12,600). The tubes were then rotated at 12 rpm and maintained at 37° C.

Upon bacterial inoculation and at one and two hours post inoculation, a measured aliquot (0.1 ml.) of fluid was removed from both test and control samples. This aliquot was diluted in sterile water (9.9 ml) and vortexed for 2 minutes in order to lyse the leukocytes. The number of bacteria within the water represents both the free bacteria as well as those phagocytized but viable within the cells. Knowledge of the total number of bacteria over time revealed the phagocytic bactericidal capacity of the white cells.

A second aliquot (0.5 ml) was also removed upon inoculation and at one and two hours post inoculation and diluted in sterile saline (4.5 ml). Through differential centrifugation (280 g), the free bacteria (supernatant) were separated from the leukocytes (sediment). After sampling the sediment and supernatant, the leukocytes in the sediment were then lysed in sterile water in order to release any phagocytized yet viable bacteria. The number

of free extracellular and intracellular bacteria was measured using standard serial dilution and plating technique. Quantitation of extracellular bacteria (supernatant) over time provides a measure of phagocytosis, while quantitation of intracellular bacteria (sediment) provides an index of the actual bactericidal action of the leukocytes.

Antibiotic Activity

Treatment of contaminated wounds with an antibiotic can reduce their infection rate. The adjunctive use of an enzyme solution may be contraindicated if the enzyme degrades the antibiotic and thereby reduces its antibacterial activity. This potential side effect of proteolytic enzymes was examined in this *in vitro* study which employed the mean inhibitory concentration (MIC) of the antibiotic as the standard test.

The following antibiotics were selected for testing: penicillin (Pen G-Squibb); cephalothin (Keflin-Lilly); tetracycline (Tetracyn-Pfizer); gentamicin (Garamycin-Schering); and chloramphenicol (Chloromycetin sodium succinate - Parke Davis). A series of test tubes were prepared to contain 4.5 ml of trypticase soy broth (Difco) with 12,000 PC units of enzyme and 10^5 organisms of *S. aureus*. To triplicate sets of tubes were added 0.5 ml of various doses of the antibiotics. A similar series of tubes was prepared which did not contain the enzyme serving as the controls. All tubes were incubated at 37° C. for 24 hours. The MIC of the antibiotic was designated as the lowest concentration of antibiotic necessary to prevent the development of turbid growth. A comparison between the mean inhibitory antibiotic concentration in the presence of the enzyme to that of the control provided insight into the effect of the enzyme on the antibacterial activity of the antibiotics.

Results

Topical treatment of the wound with a proteolytic enzyme did not impair wound healing (Figure 1). In fact, the mean breaking strength of enzyme-treated wounds was higher than control wounds but the difference was not significant. Similarly, short term topical enzyme treatment had no significant damaging effect on the wound's resisteance to infection (Figure 2). For the animals receiving 3.3×10^5 bacteria per wound the enzyme treated wounds were associated with an elevated bacterial count compared to that in control wounds. The bacterial counts of the saline and enzyme treated wounds receiving 1.1×10^6 bacteria were similar. All enzyme-treated wounds had significantly wider margins of induration when compared to saline-treated controls.

The *in vivo* results of enzyme activity in wounds were in sharp contrast to the deleterious effects observed for enzymes on *in vitro* leukocyte function. In the presence of enzyme, leukocytes were not able to phagocytize bacteria (Figure 3). This phenomenon is indicated by no decrease in bacterial count in the supernatant of the centrifuged test solutions.

Finally, the enzyme did not inactivate or reduce the activity of any antibiotics tested as judged by their MIC values (Table 1).

Discussion

The benefits of topical enzyme treatment of wounds as an adjunct to antibiotic therapy can be accomplished with negligible side effects. A short term treatment with aqueous enzyme solutions which lasts only 10 minutes does not interfere with healing or wound defenses *in vivo*. In our experimental study, the breaking strength of enzyme treated wounds did not differ

significantly from that of the control wounds subjected to 0.9% saline. This observation is consistent with the finding of Harris and Reinhart[5] who measured the influence of the Bacillus subtilus protease on the contraction and epithelial repair of skin wounds in rabbits. In their study, enzyme ointment was applied twice daily to skin defects on the backs and ears of rabbits. The proteolytic enzyme ointment did not interfere with the repair of these wounds as compared to that of untreated control wounds. Additional studies by these investigators indicated that the bacterial flora of these skin wounds was not modified by treatment with the enzyme. The proteolytic enzyme did not alter the level of bacterial contamination of the wound and the same organisms were present in the control and enzyme treated wounds. Apparently, this prolonged treatment with enzyme did not significantly interfere with the development of the fibrin lattice necessary for the securement and growth of fibroblasts as the wound heals.[6]

Prolonged treatment of large surface areas of the body with proteolytic enzyme appears to have a damaging effect on systemic defenses. Hummel et al[7] reported that the topical enzyme treatment of the burn wound was associated with the development of sepsis. Enzymatic agents dissolved the eschar rapidly leaving soft pablum for bacteria and opened vascular channels for bacterial invasion. Dimick[8] later reported that this potential for systemic sepsis was minimized by the concomitant use of an antimicrobial with the topical enzyme treatment.

The deleterious effects of this proteolytic enzyme on leukocytes may contribute to the development of systemic sepsis. In the presence of the enzyme, leukocytes are not able to phagocytize bacteria. The mechanism by which this enzyme interferes with phagocytosis is not explained by this

study and must await future studies. A direct injury to the membrane of the leukocyte or damage to the complement or opsonin system by the enzyme are possible explanations for its deleterious effects. Restricting the wound contact time of the aqueous enzyme to ten minutes considerably reduces the likelihood of the adverse systemic reactions encountered following prolonged treatment with the enzyme ointment.

Summary

The results of this study indicate that short term treatment of wounds with topical enzymes appears to be safe and is accompanied by no clinically significant side effects. Topical wound treatment with this enzyme did not interfere either with healing of experimental wounds or the activity of the antibiotics. Wounds subjected to this enzyme treatment exhibited a normal capacity to resist infection, even though this enzyme interfered with leukocyte phagocytosis *in vitro*. The potential benefits of this treatment in experimental wounds as an adjunct to antibiotics considerably outweigh its damaging effects.

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3. Rodeheaver, G., Marsh, D., Edgerton, M.T., and Edlich, R.F.: Proteolytic enzymes as adjuncts to antimicrobial prophylaxis of contaminated wounds. Amer. J. Surg. 129: 537, 1975.

4. Mandell, G.L., and Vest, T.K.: Killing of intraleukocytic Staphylococcus aureus by rifampin; in vitro and in vivo studies. J. Infect. Dis. 125: 486, 1972.

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8. Dimick A.R.: Experience with the use of proteolytic enzyme (Travase) in burn patients. J. Trauma 17: 948-955, 1977.

(TABLE)

Antibiotic Activity

Group	Penicillin	Cephalothin	Tetracycline	Gentamicin	Chloramphenicol
			MIC (ug/ml)		
Control	1.0	1.0	1.0	1.0	100
Enzyme	1.0	1.0	1.0	1.0	100

LEGENDS FOR ILLUSTRATIONS

Figure 1 The breaking strength of the enzyme treated wounds did not differ from that of the control wounds subjected to saline.

Figure 2 The local tissue defenses of the enzyme treated wounds, as measured by the infection rate, did not differ from that of the control wounds.

Figure 3 In the presence of the proteolytic enzyme, the ability of leukocytes to phagocytize bacteria was impaired.

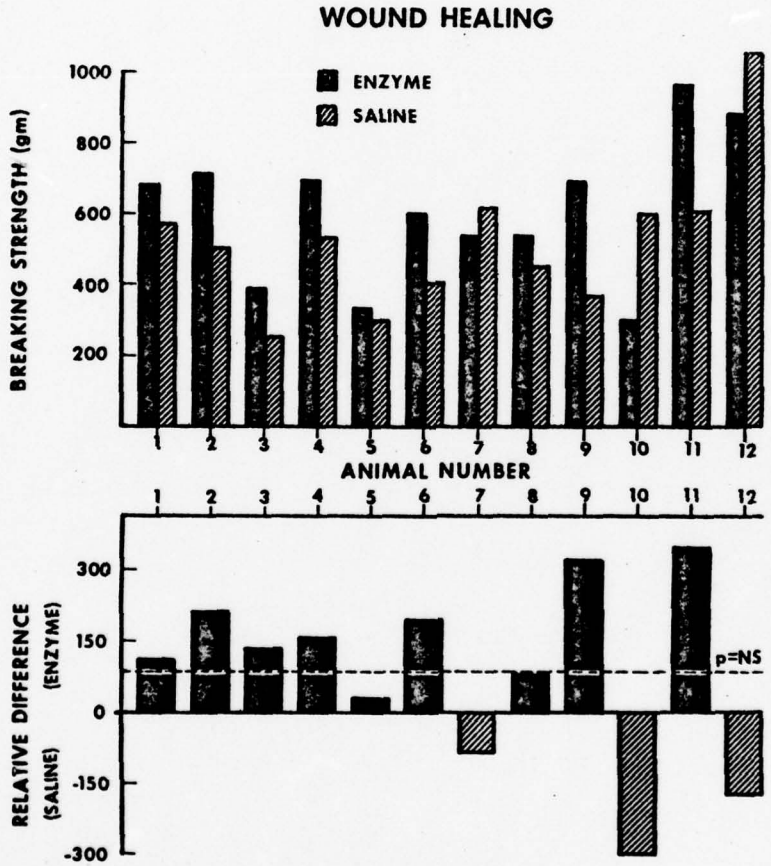

WOUND HEALING
ENZYME
SALINE
BREAKING STRENGTH (gm)
1000
800
600
400
200
1 2 3 4 5 6 7 8 9 10 11 12
ANIMAL NUMBER
1 2 3 4 5 6 7 8 9 10 11 12
RELATIVE DIFFERENCE (ENZYME) (SALINE)
300
150
0
-150
-300
p=NS

Figure 1

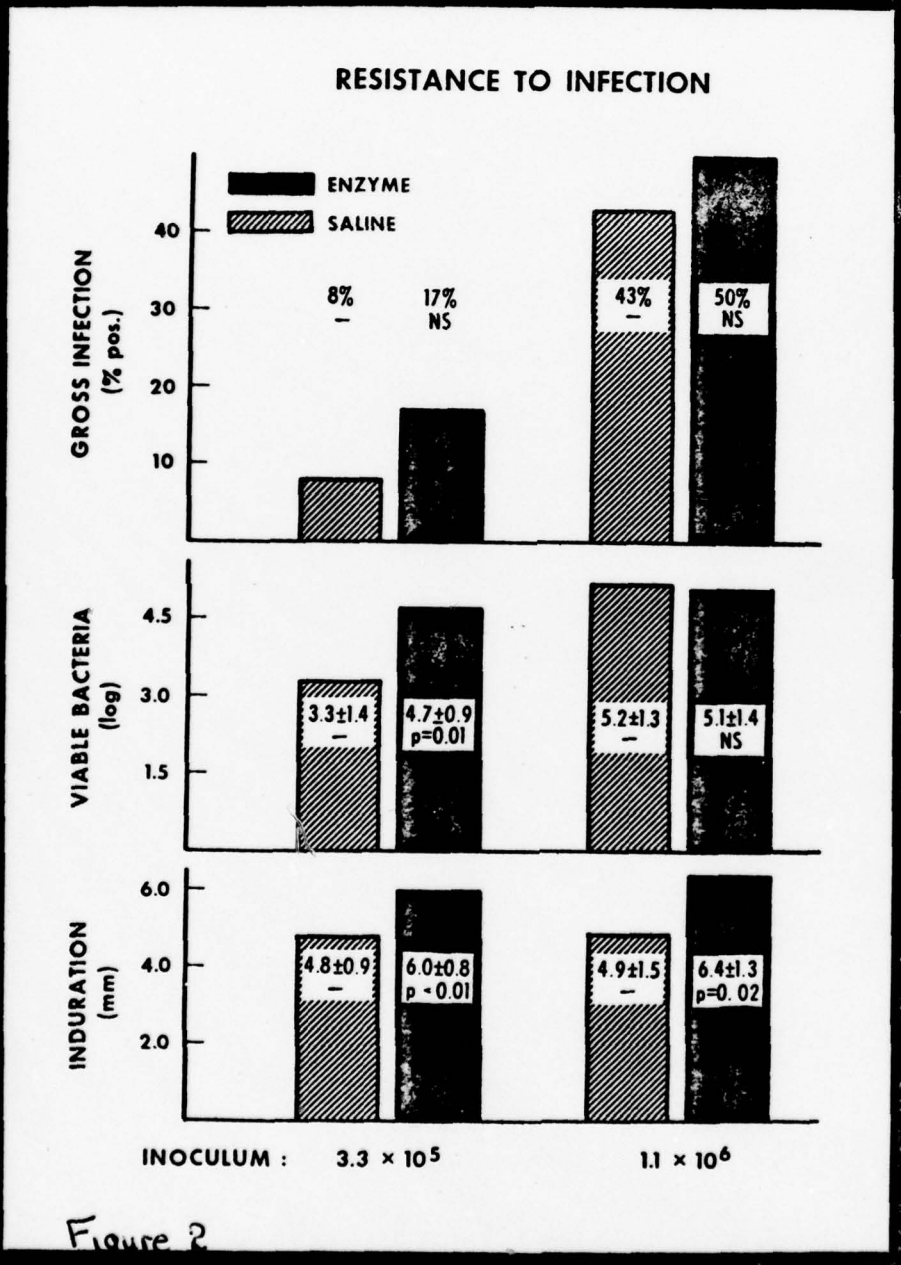

RESISTANCE TO INFECTION
ENZYME
SALINE
GROSS INFECTION (% pos.)
40
30
20
10
8%
–
17%
NS
43%
–
50%
NS
VIABLE BACTERIA (log)
4.5
3.0
1.5
3.3±1.4
–
4.7±0.9
p=0.01
5.2±1.3
–
5.1±1.4
NS
INDURATION (mm)
6.0
4.0
2.0
4.8±0.9
–
6.0±0.8
p < 0.01
4.9±1.5
–
6.4±1.3
p=0. 02
INOCULUM : 3.3 × 10^5
1.1 × 10^6

Figure 2

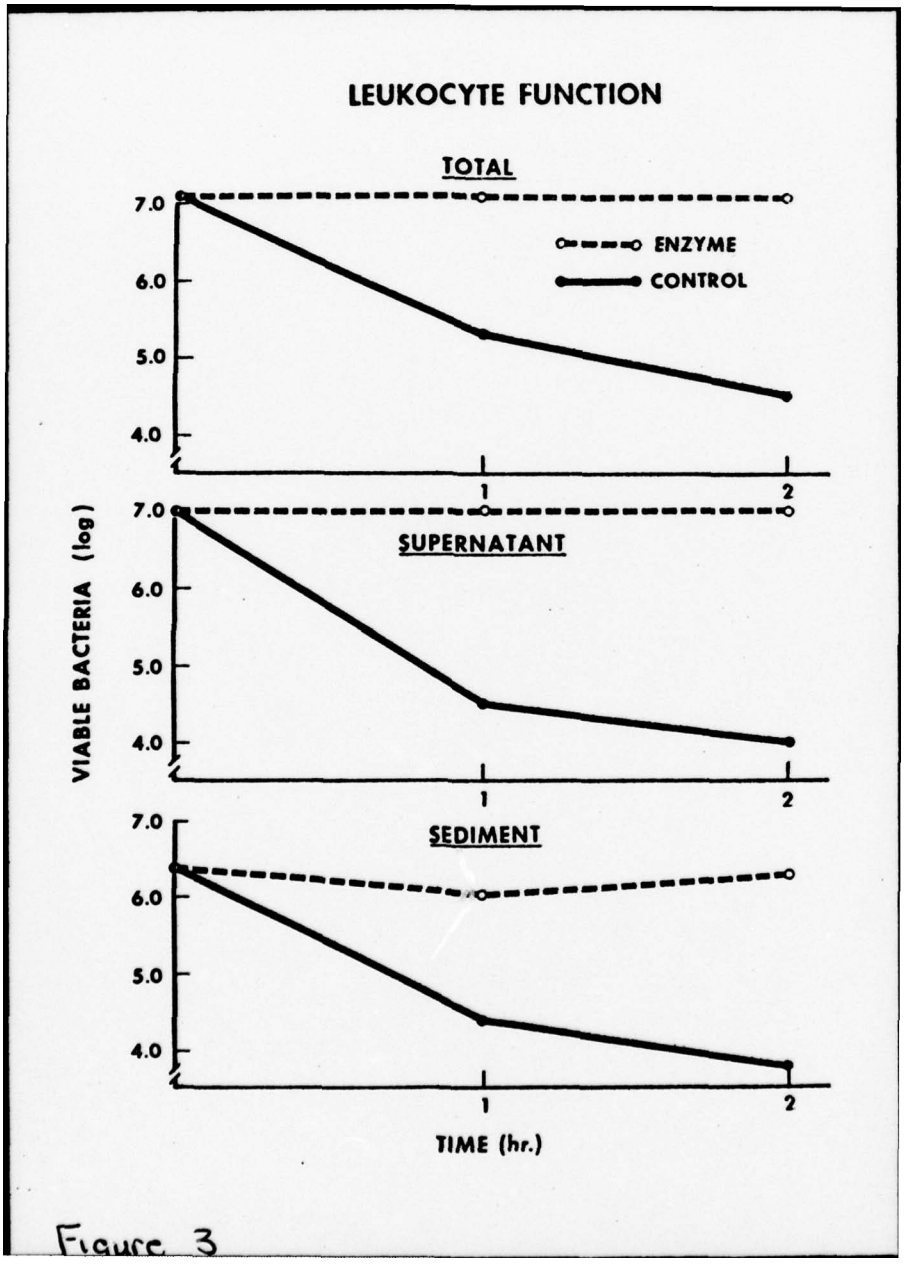

LEUKOCYTE FUNCTION
TOTAL
ENZYME
CONTROL
SUPERNATANT
SEDIMENT
VIABLE BACTERIA (log)
7.0
6.0
5.0
4.0
1
2
TIME (hr.)

Figure 3

QUANTITATION OF ANAEROBES IN SOFT TISSUE INFECTIONS

A critical number of bacteria appears to be necessary to elicit infection in soft tissue wounds. The infective dose of aerobic bacteria in wounds in healthy tissue is 10^6 bacteria or greater. When aerobic bacterial counts are below this level, the wound will heal consistently without infection. This remarkable resistance to infection has been identified in all soft tissue tested.

The quantitative relationship between the number of anaerobic bacteria and the development of infection has not been defined. Heretofore, quantitative bacteriologic techniques have been performed under aerobic conditions which do not permit measurement of obligate anaerobic organisms. The purpose of this study was to establish a simple and reproducible technique which could be utilized by any clinical laboratory to quantitate the number of obligate anaerobic bacteria in tissues.

Materials and Methods

Quantitation Technique

Our approach to anaerobic bacterial quantitation was patterned after our published aerobic bacterial quantitation procedure except that anaerobiosis was maintained throughout the procedure (Fig. 1). The technique is initiated by placing a weighed tissue sample into a Sorvall^R 50 ml modified homogenization chamber containing 15 ml of pre-reduced salt solution. Carbon dioxide is continually introduced from the bottom of the chamber through stainless steel tubing (inner diameter 2 mm). Using a Virginia Polytechnic Institute

Anaerobic System (Bellco Glass Inc.) the homogenate is subjected to 3 serial 1:10 dilutions in pre-reduced salt solution. An aliquot (0.1 ml) from the homogenate and each serial dilution is placed in molten (45-50^{o} C) Brain Heart Infusion Roll tubes (Carr/Scarborough). After gassing each tube with carbon dioxide, it is rotated along its long axis until the agar cools in a thin film along the walls. The sealed tubes are then incubated at 37^{o} C for 24 to 96 hours. The number of colonies in the tube containing between 30 and 300 colonies is counted. Assuming that each colony is the product of a single organism, the result is reported as the number of bacteria per gram of tissue. All tubes are observed for an additional two weeks to determine if any further growth develops.

Evaluation

A quantitative procedure for obligate anaerobic bacteria is judged to be successful if it permits recovery of the least aerotolerant organism. The purpose of the first part of the study was to identify the aerotolerance of anaerobic pathogens that result in clinical disease in humans. The organisms included in this study were Bacteroides fragilis, Fusobacterium neucleatum, Fusobacterium varium, and Peptostreptococcus anaerobius. Prior to each experiment, the test organism was transferred to pre-reduced brain heart infusion broth and incubated at 37^{o} C. During the log phase of growth, an aliquot of bacteria was added to pre-reduced salt solution which had been oxidized by exposure to air. After an exposure of 6 hours, the number of viable organisms remaining in the tubes were quantitated.

In the second part of the study, the aerotolerance of the organism

was compared to its survival during homogenization under varying environmental conditions. In this series of experiments, a suspension of organisms in oxidized fluid was homogenized for one minute in air. This homogenization procedure was repeated under anaerobic conditions using the same test organisms. Following homogenization, the number of viable bacteria remaining in the homogenate was quantitated.

Results

Surprisingly, suspensions of three of the four obligate anaerobic bacteria were not affected by six hours of exposure to oxidized fluid (Fig. 2). Ps. anaerobius was extremely sensitive to air with no viable organisms recovered after six hours. This loss of viable organisms occurred rapidly in an aerobic environment with a significant decrease in viable bacteria within 30 minutes (Fig. 3).

As expected, the most aerotolerant organisms survived homogenization in an aerobic environment. In contrast, the average number of organisms of the least aerotolerant Ps. anaerobius was influenced by homogenization in air (Fig. 4). The deleterious effects of aerobic homogenization on the recovery of this organism was eliminated by performing the procedure under aerobic conditions (Fig. 5).

Discussion

Quantitation of aerobic organisms in clinical specimens has been useful in predicting safety of wound closure (both primary and delayed primary), in determining graft bed receptivity, and in diagnosing the onset of burn

wound sepsis. Heretofore, the quantitative techniques employed did not permit recovery of obligate anaerobes. This technical shortcoming has limited our understanding of the importance of obligate anaerobes as pathogens. A technique is reported herein which permits quantitation of obligate anaerobic bacteria in tissue. This technique can easily be reproduced in any clinical laboratory. Based on the results of this study, we are employing this new technique routinely in our medical center.

LEGENDS FOR ILLUSTRATIONS

Figure 1: The proposed anaerobic quantitation scheme is patterned after our routine aerobic procedure.

Figure 2: After exposure to oxidized fluid for 6 hours, Ps. anaerobius was the only obligate anaerobic organism tested which was adversely affected.

Figure 3: Exposure of Ps. anaerobius to oxidized fluid resulted in significant reductions in the number of viable organisms.

Figure 4: Sensitivity to aerobic homogenization was correlated with aerotolerance. Loss of viable organisms occurred only with Ps. anaerobius.

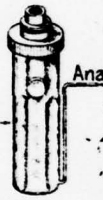
Figure 5: Quantitation of Ps. anaerobius under anaerobic conditions resulted in total recovery.

Anaerobic Quantitation Technique

Anaerobic Transport Tube

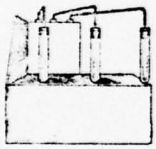
① Weigh Sample

② Transfer to Modified Anaerobic Homogenation Chamber



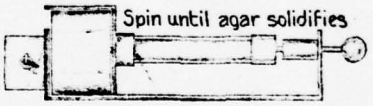
Anaerobic Gas (CO_2)

③ Homogenize Anaerobically for 1 minute

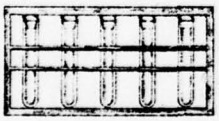


④ (3) Serial Dilutions (1:10) in pre-reduced salt solutions

⑤ Plate 0.1 ml. Aliquot of each dilution and homogenate in molten agar tube, 50°C



⑥ Spin until agar solidifies



⑦ Incubate 24-96 hours; count appropriate dilution (30-300*)

⑧

* TOO NUMEROUS TO COUNT

⑨ Calculate # of organisms / gm of Sample

Figure 1

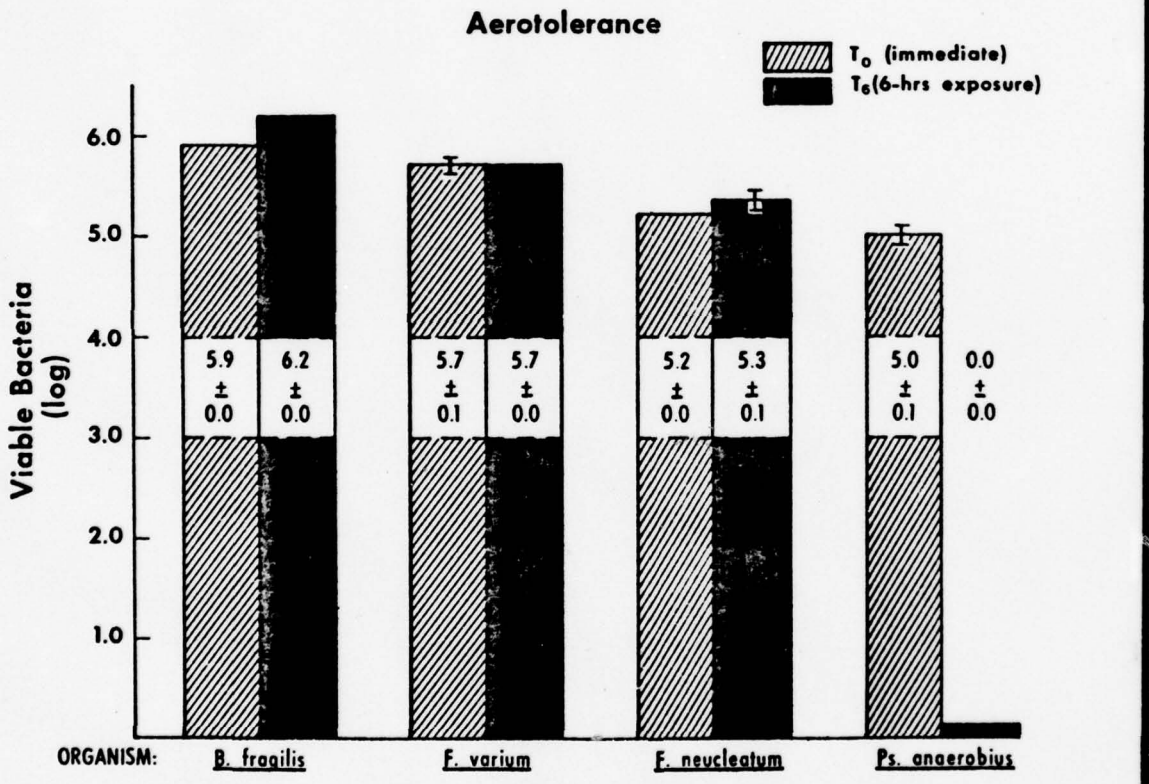

Aerotolerance
T_0 (immediate)
T_6(6-hrs exposure)
Viable Bacteria (log)
6.0
5.0
4.0
3.0
2.0
1.0
5.9 ± 0.0
6.2 ± 0.0
5.7 ± 0.1
5.7 ± 0.0
5.2 ± 0.0
5.3 ± 0.1
5.0 ± 0.1
0.0 ± 0.0
ORGANISM:
B. fragilis
F. varium
F. neucleatum
Ps. anaerobius

Figure 2

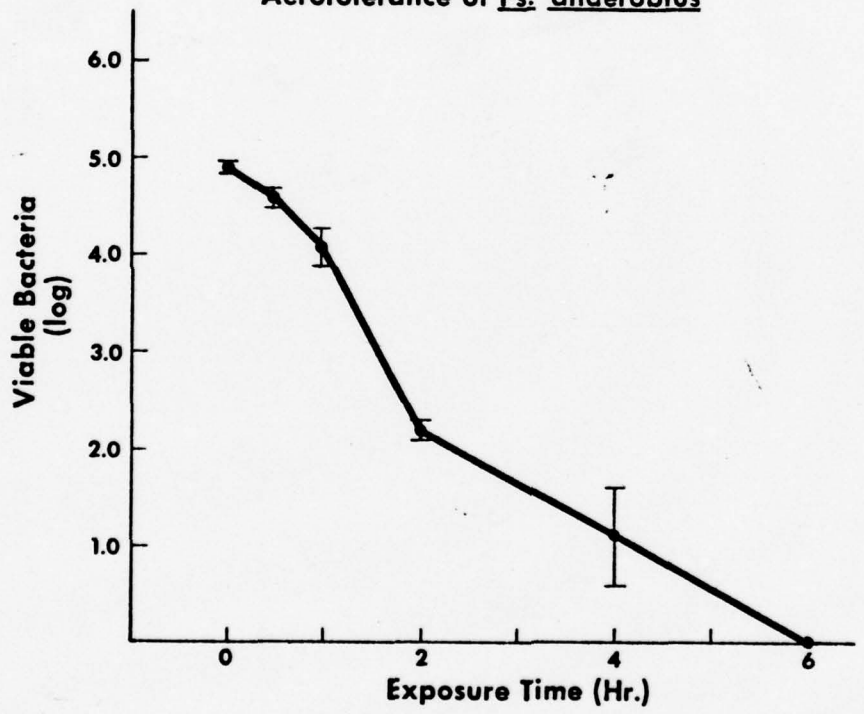

Aerotolerance of Ps. anaerobius
Viable Bacteria (log)
6.0
5.0
4.0
3.0
2.0
1.0
0
2
4
6
Exposure Time (Hr.)

Figure 3

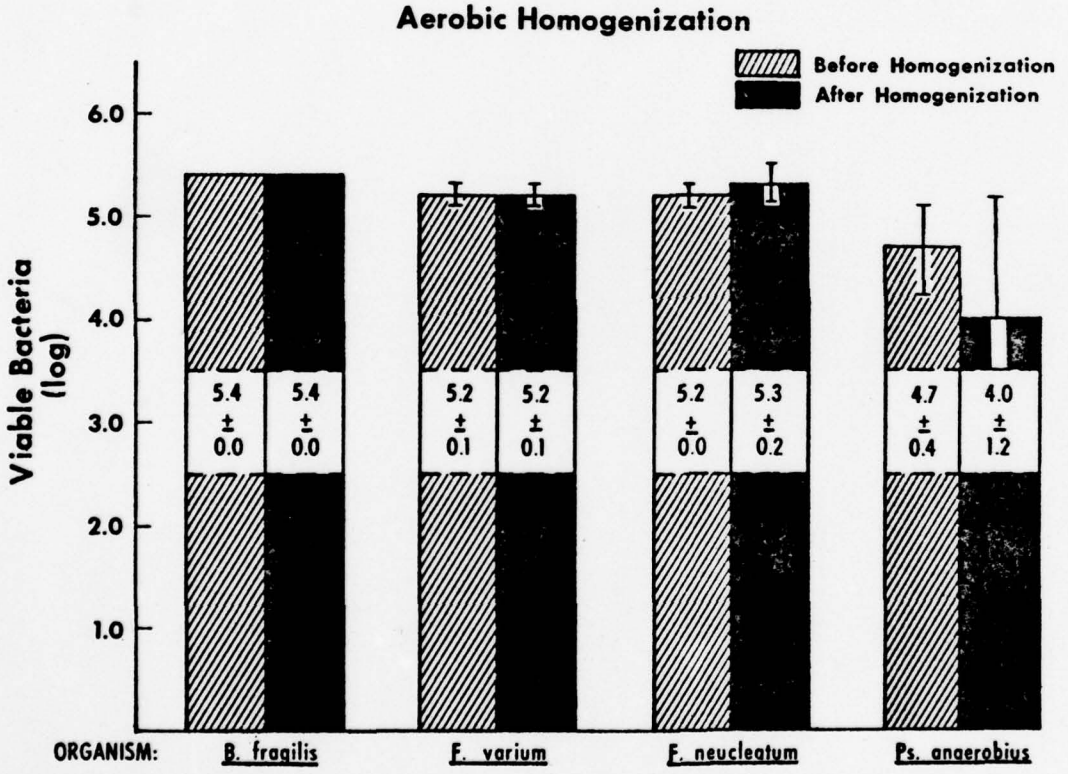

Aerobic Homogenization
Before Homogenization
After Homogenization
Viable Bacteria (log)
6.0
5.0
4.0
3.0
2.0
1.0
5.4 ± 0.0
5.4 ± 0.0
5.2 ± 0.1
5.2 ± 0.1
5.2 ± 0.0
5.3 ± 0.2
4.7 ± 0.4
4.0 ± 1.2
ORGANISM:
B. fragilis
F. varium
F. neucleatum
Ps. anaerobius

Figure 4

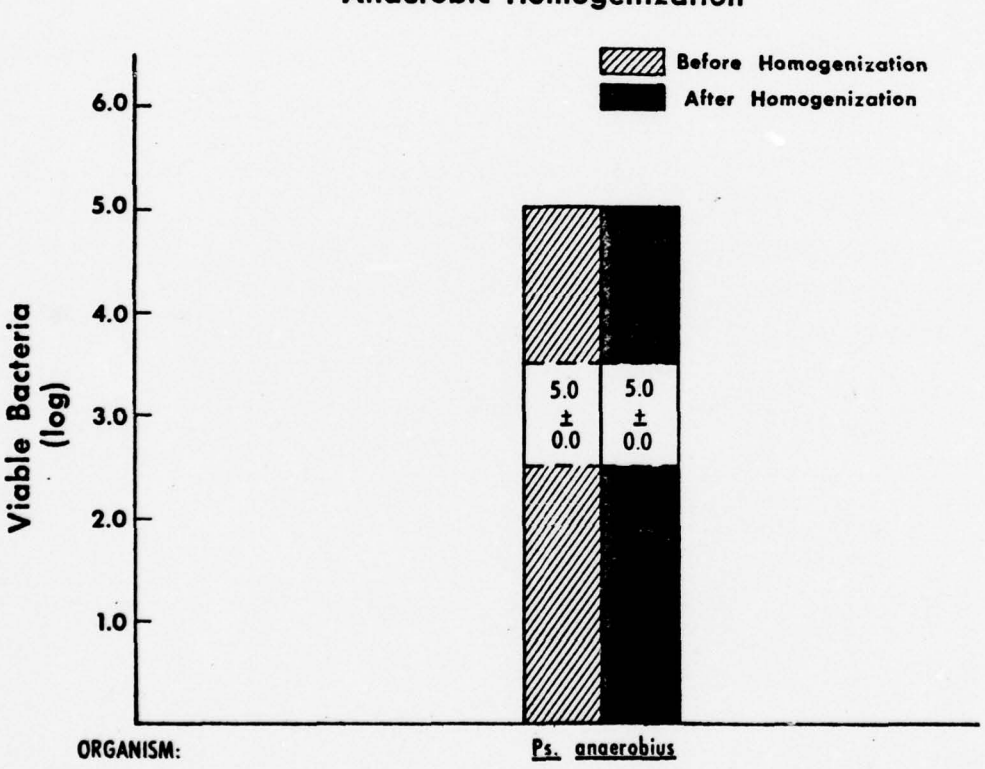

Anaerobic Homogenization
Before Homogenization
After Homogenization
Viable Bacteria (log)
6.0
5.0
4.0
3.0
2.0
1.0
5.0 ± 0.0
5.0 ± 0.0
ORGANISM:
Ps. anaerobius

Figure 5

Development of a Stable

Pluronic F-68 Iodophor

Previous studies have identified a surfactant that is safe for human use! The surfactant, Pluronic F-68, was the only detergent approved by the FDA as a safe and effective skin wound cleanser. To enhance its therapeutic potential we have used the Pluronic F-68 to solubilize iodine. This iodine-Pluronic F-68 complex was shown to be more effective in reducing bacterial levels on skin and wounds than non-complexed iodine or other iodophors such as Betadine.

During the initiation of toxicity studies we discovered that dilutions of the iodophor with water led to instability and the generation of a brown precipitate. The chemical configuration of this precipitate has not been fully characterized and may therefore, be a source of toxicity. Consequently, it has been our goal to formulate a more stable iodophor.

Experimental Design

The original iodophor was prepared by dissolving 10 gm of Pluronic F-68 in 100 ml of water followed by 1 gm of iodine and 1 gm of sodium iodide. This mixture yielded a dark iodophor solution containing 0.97 $\pm$ 0.03 % available iodine at a pH of 3.8-4.0. A tenfold dilution of this solution in water yielded a brown precipitate after 1 hour. In the next series of experiments, we attempted to stabilize the iodophor by altering one of the following parameters: surfactant concentration, pH, iodide concentration and composition.

Surfactant Concentration

Pluronic F-68 is a block copolymer of ethylene oxide and propylene oxide. This particular polymer consists of a hydrophobic core (20%) of polypropylene and hydrophilic ends (80%) of ethylene oxide. In solution the Pluronic

forms micelles with the hydrophobic core solubilizing the iodine.

The first thought was to increase the concentration of Pluronic in the solution to help stabilize the iodine. Increasing the concentration of Pluronic F-68 to 30% stabilized the diluted solution for 24 hours after which time the precipitate developed.

pH

Once the precipitate was formed the addition of acid would lead to resolubilization. Pluronic F-68 iodophors with a pH of 1 or 2 were stable following dilution. Solubility in this case was achieved by the formation of oxonium ions. However, solutions with such a low pH are considered unacceptable, even though the commercial iodophor Acu-dyne has a pH in this range.

Iodide Composition and Concentration

Excess iodide is known to stabilize iodine systems. However, our studies seem to indicate that the associated cation also had a pronounced effect on the stability of the Pluronic-iodine complex. For example, the presence of potassium iodide prevents the complexation of iodine with Pluronic F-68. In our initial iodophor, sodium iodide led to solubilization but instability upon dilution. Therefore, we postulated that the use of lithium iodide would give a more stable solution. The use of 1% lithium iodide was better than 1% sodium iodide but the resulting solution was still unstable at dilutions greater than 1:10 after 24 hours. The use of hydrogen iodide permitted the addition of iodide without the associated alkali metal cation resulting in a stable iodophor. However, since hydrogen iodide is a strong acid, the pH was lowered to less than 1.

Addition of base was necessary to reduce the acidity. The hydrogen iodide solution was titrated with lithium phosphate which gave an iodophor which was infinitely stable on dilution and had an acceptable pH value of 4.5.

Final Iodophor

The stable iodophor consists of 20% Pluronic F-68, 1% iodine, 2% hydrogen iodide and 2% lithium phosphate. With the development of this stable iodophor, we are proceeding with our toxicity studies.

ACTIVITY OF ANTIBIOTICS IN CONTAMINATED WOUNDS CONTAINING CLAY SOIL

Most traumatic wounds are contaminated to some degree by soil and run a high risk of infection. Studies in our laboratory have identified the specific components of soil which potentiate the development of infection. These components called infection potentiating fractions (IPFs) are located in the clay and organic fractions of soil and are characterized by their large surface area and high chemical reactivity. Further studies have examined the mechanisms by which soil enhances infection. The presence of the IPFs in wounds interferes with the natural tissue defenses which include phagocytosis and serum bactericidal capacity.

The experimental studies reported herein suggest another explanation for the refractoriness to therapy of soil containing wounds. In vitro and in vivo studies clearly demonstrate that soil IPFs limit the antibacterial effect of specific antibiotics. This inactivation appears to be the result of a chemical reaction between the charged antibiotic and the soil IPFs. In this paper, the results of these experiments are reported and their clinical implications are discussed.

Materials and Methods

Experimental Design

This investigation consists of in vitro and in vivo studies designed to examine the influence of soil IPFs on the activity of antibiotics. The soil IPF employed in these studies was montmorillonite clay (<0.2 microns) which was saturated homoionically with magnesium ions through repeated equilibration and washing with 0.5 normal magnesium choloride. Excess salt was removed by repeated washings with deionized water, as evidenced by a negative

indication of chloride using the silver nitrate test. The sample was dried by desiccation under vacuum over silica gel. The clay was sterilized by exposing it to 12 per cent ethylene oxide at 130 degrees F in a closed system for one and three-quarters hours followed by an 18 hour aeration at 120 degrees F. Dryness was maintained by desiccation over diphosphorus pentoxide.

A penicillin-sensitive strain of Staphylococcus aureus (CDC #2801), maintained on tryptose blood agar plates, was employed in these studies. Eighteen hours prior to each experiment, this bacteria was transferred by sterile loop to 25 ml of trypticase soy culture broth. The broth culture was agitated at 37° C for 18 hours before centrifugation. The bacterial suspension was washed twice with 0.9 percent saline solution. The bacteria were then serially diluted in 0.9% saline until the suspension contained a designated number of bacteria for inoculation.

The following antibiotics were used in these studies: penicillin-G (Sigma Chemical Corporation, St. Louis, Mo.), cephalothin sodium (Keflin^{R}, Eli Lily and Co., Indianapolis, Ind.), chloramphenicol sodium succinate (Chloromycetin^{R}, Parke Davis & Co., Detroit, Mich.), tetracycline hydrochloride (Tetracyn^{R}, Pfizer Inc., New York, N.Y.), gentamicin (Garamycin^{R}, Schering Pharmaceutical Corporation, Kenilworth, N.J.), and neomycin sulfate (The Upjohn Company Kalamazoo, Mich.). These antibiotics were obtained as either a lyophilized powder or injectable solution.

In Vitro

The purpose of these experiments was to examine the effect of the soil IPF on the activity of the antibiotics in vitro. The activity of the antibiotics was judged by the minimal antibiotic concentration necessary to kill bacteria. The minimal bactericidal concentration (MBC) of antibiotic was determined by the following standard technique. A series of tubes were arranged to contain 4.0 ml of trypticase soy broth, 0.5 ml of 0.9% sodium chloride containing 10^6 S. aureus and 0.5 ml of varying concentrations of the test antibiotic (0-1000.0 ug/ml). The tubes were divided into two treatment groups. Each tube in one group received 20 mg of montmorillonite while the other set of tubes received no soil serving as the control. All tubes were then rotated at 6 revolutions per minute at 37^{o} C for 24 hours. At the end of this time period a sterile cotton swab was used to transfer a sample of each test solution onto an agar plate which in turn was incubated for 18 hours at 37^{o} C. The lowest antibiotic concentration which killed all viable bacteria was judged to be the MBC of the antibiotic. The influence of soil on the antibiotic activity was ascertained by comparing the MBC in the presence of soil to that in the controls without soil.

In Vivo

The purpose of this series of in vivo experiments was to determine the effect of the soil IPFs on the therapeutic efficacy of antibiotics in the treatment of a standardized contaminated wound. Male, Hartley guinea pigs (300-350 gm) were anesthetized with sodium pentobarbital and their dorsal hair removed with electric clippers. Further hair removal was accomplished with a depilatory (Surgex^{R}). After washing their backs with water, their skin was swabbed with 70% alcohol. Two standardized incisions, parallel

and equidistant from the vertebral column, were made on each guinea pig. Each incision was 3 cm in length extending down to the panniculus carnosus. All wounds were then inoculated with 0.1 ml of 0.9% sodium chloride containing a designated number of bacteria. Ten minutes later, one wound on each animal received 20 mg of soil IPF while the contralateral wound served as a control. The wound edges of each wound was approximated with microporous tape. The animals were then divided into treatment groups. Five days following wounding, the animals were sacrificed and the wound bacterial count was determined using standard microbiologic techniques.

Treatment Groups

The purpose of the first series of experiments was to examine the influence of soil IPFs on the growth of bacteria in wounds. In wounds contaminated by clay, the inoculum was 2.5×10^2 organisms. At three, six, twelve, and 24 hours after wounding, the wound bacterial counts were quantitated.

In the second set of experiments, the efficacy of immediate and delayed penicillin treatments on the level of contamination in clay treated wounds was examined. At specified (0,6,12,18 and 24 hours) times after contamination of the clay treated wounds with 2.5×10^2 organisms, specified groups of animals received an intraperitoneal injection of benzylpenicillin (100,000 units) which was repeated every 12 hours for four days.

In the third series of experiments, the therapeutic value of varying dosages of gentamicin in control wounds contaminated with 3×10^6 S. aureus was ascertained. Separate groups of animals received an intraperitoneal injection of varying dosages of gentamicin (0,1,8,16 mg) which were repeated every 12 hours for four days. The optimal dose of gentamicin was employed immediately to treat wounds containing a combination of clay and S. aureus (2.5×10^2).

Results

The influence of soil IPFs on the activity of antibiotic was predicted by the chemical composition of the antimicrobial agent (Table 1). The antibacterial activity of neutral or acidic antibiotics was not influenced by soil IPFs. In contrast, montmorillonite inactivated basic antibiotics to the greatest degree followed by the amphoteric group.

When wounds were contaminated with 10^2 S. aureus, the tissue defenses actively maintained the bacterial levels. In contrast, when the wounds contained montmorillonite, the bacteria proliferated to 10^7 organisms within 18 hours (Figure 1). The acidic antibiotics which were unaffected by montmorillonite in vitro resulted in a dramatic decrease in wound bacterial counts in clay treated wounds (Figure 2). The efficacy of penicillin was reduced by delaying the onset of treatment. During the time in which antibiotic treatment was postponed, bacteria proliferated rapidly reaching a level which was not susceptible to antibiotic treatment. Basic antibiotics like gentamicin have little benefit in contaminated clay treated wounds (Fig. 3,4). In the absence of clay, gentamicin eliminated the wound bacteria.

DISCUSSION

Wounds contaminated by soil IPFs run a high risk of infection. In the presence of clay, as few as 100 bacteria are necessary to elicit infection. This infective dose of bacteria is nearly 10,000 fold less than that needed to infect clean wounds without soil IPF. This infection potentiating effect of soil reflects its deleterious effect on tissue defenses.

Meticulous debridement and cleansing by high pressure irrigation are mandatory in wounds contaminated by IPFs. In wounds that are not susceptible

to extensive debridement, like hand injuries, traces of IPF may remain in the wound encouraging the growth of bacteria. In these cases, antibiotic treatment of the residual bacteria is essential to limit the development of infection. The efficacy of the antibiotic in the presence of clay is predicted by the chemical composition of the antibiotic. The silicate surface of the soil IPF yields a highly negative surface which complexes with the basic and amphoteric antibiotics limiting their activity both *in vivo* and *in vitro*. The acidic antibiotics like cephalosporin and penicillin do not bind with clay and exert their antibacterial effect in wounds contaminated with the soil IPFs. Treatment with these antibiotics should be initiated immediately before the bacteria proliferate to levels which are refractory to antimicrobial therapy.

EFFECT OF CLAY ON ANTIBIOTIC ACTIVITY

	Acidic or Neutral			Amphoteric	Basic		
Antibiotic	Penicillin	Cephalothin	Chloramphenicol	Tetracycline	Gentamicin	Neomycin	Streptomycin
MBC* (μg/ml)	0.1	1.0	1000	10	10	10	20
MBC + Clay (μg/ml)	0.1	1.0	1000	100	1000	1000	100

* Minimum Bactericidal Concentration

Table 1

LEGENDS FOR ILLUSTRATIONS

Figure 1: In wounds containing clay, low levels of bacterial contamination rapidly proliferate to infective levels.

Figure 2: The acidic antibiotic penicillin is not inhibited by the presence of clay in the wound. Penicillin therapy is most beneficial if begun within the first 6 hours following injury.

Figure 3: In contaminated wounds without clay, the efficacy of gentamicin therapy was directly related to the dose.

Figure 4: Gentamicin therapy has no effect on bacterial proliferation in wounds containing clay.

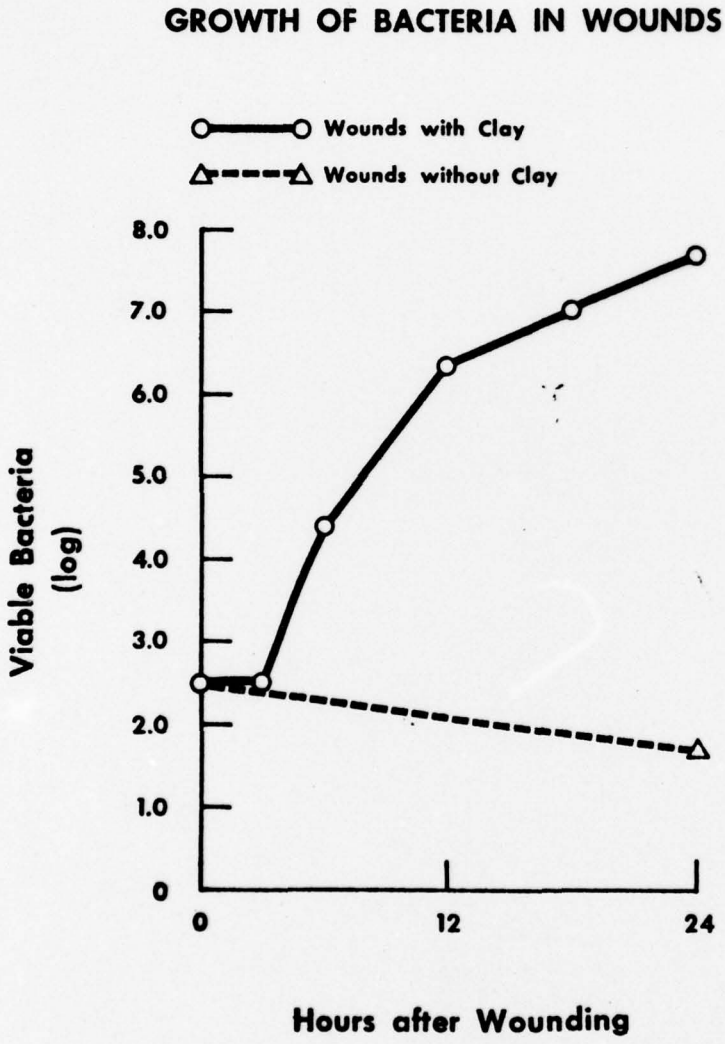

GROWTH OF BACTERIA IN WOUNDS
Wounds with Clay
Wounds without Clay
Viable Bacteria (log)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0
12
24
Hours after Wounding

Figure 1

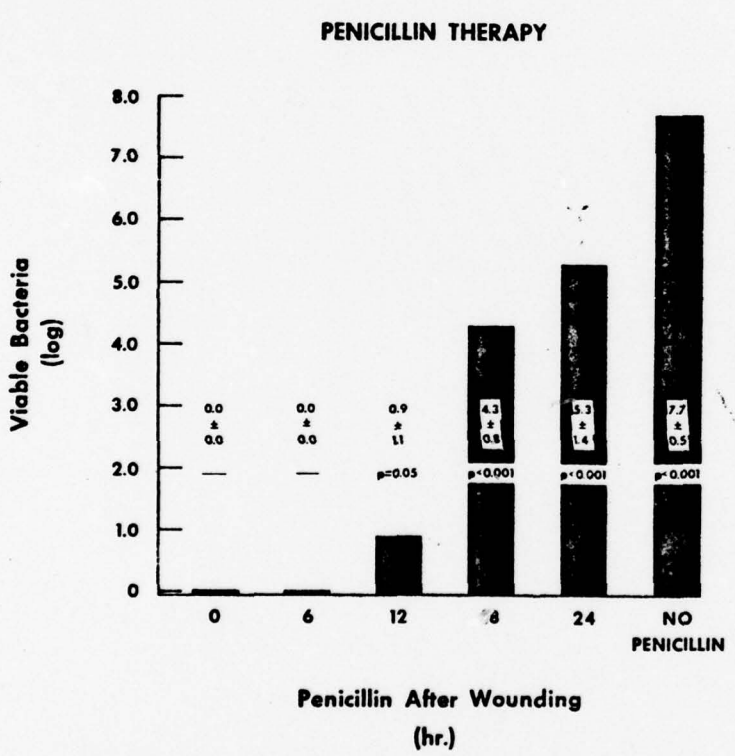

PENICILLIN THERAPY
Viable Bacteria (log)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0.0 ± 0.0
0.0 ± 0.0
0.9 ± 1.1
4.3 ± 0.8
5.3 ± 1.4
7.7 ± 0.5
p=0.05
p<0.001
p<0.001
p<0.001
0
6
12
18
24
NO PENICILLIN
Penicillin After Wounding (hr.)

Figure 2

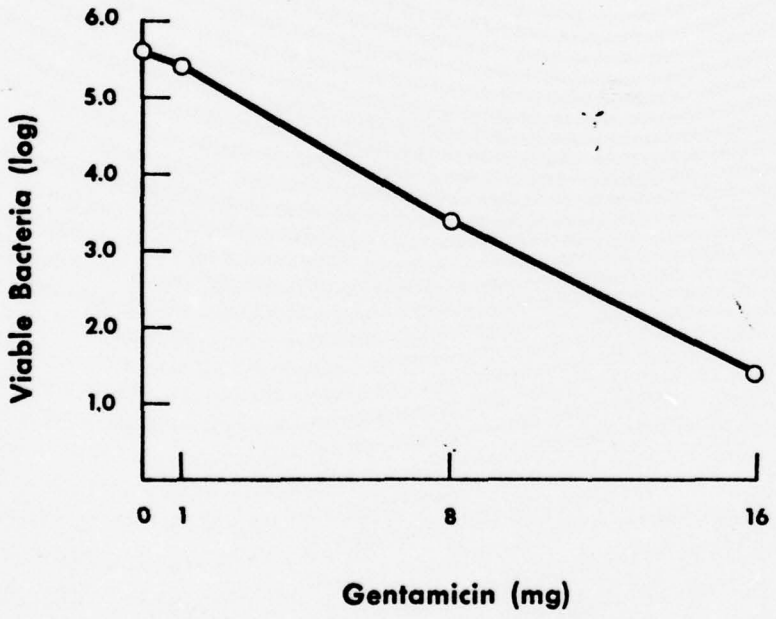

DOSE RESPONSE CURVE OF GEN MICIN
Viable Bacteria (log)
6.0
5.0
4.0
3.0
2.0
1.0
0
1
8
16
Gentamicin (mg)

Figure 3

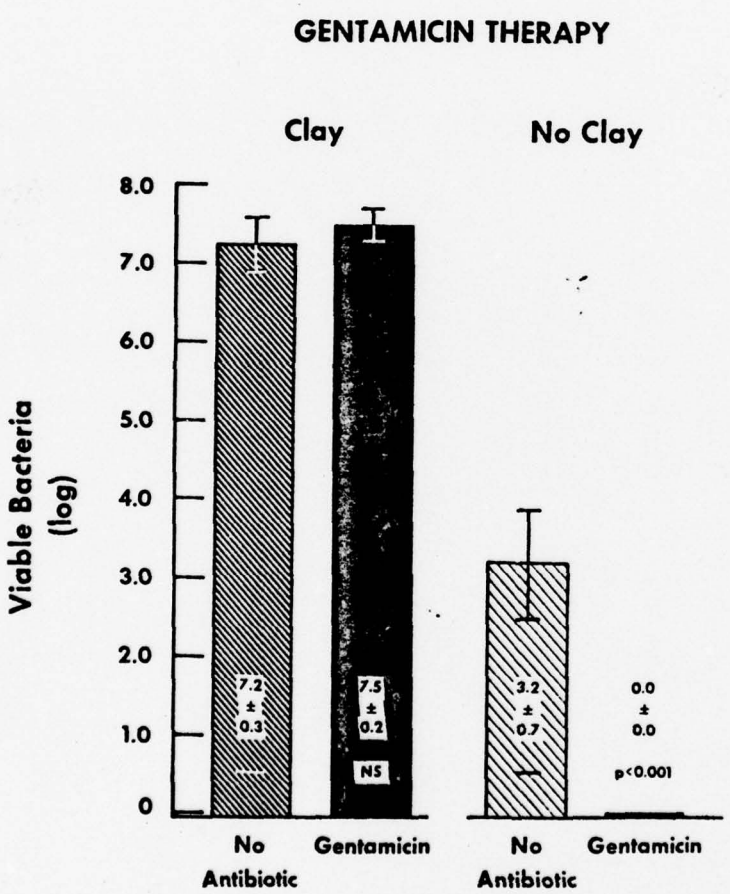

GENTAMICIN THERAPY
Clay
No Clay
Viable Bacteria (log)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
7.2 ± 0.3
7.5 ± 0.2
NS
3.2 ± 0.7
0.0 ± 0.0
p<0.001
No Antibiotic
Gentamicin
No Antibiotic
Gentamicin

Figure 4

EXTINGUISHING THE FLAMING BURN VICTIM

Introduction

When confronted by a burning human, the rescuer is trained to roll the flaming victim on the ground. In other circumstances, the burn victim is not susceptible to movement. Victims of self-emolation who forcefully resist the rescuers efforts are cases in point. Burning victims trapped in enclosed spaces, like an automobile, can not be rescued until the flames are extinguished. The sleeping adult or child whose bed is engulfed in flames also can not be moved without considerable risk to the rescuer. In these cases, the portable fire extinguisher is the simplest and safest method of extinguishing the flames.

The selection of a fire extinguisher must be based on the nature of the burning material as well as the potential toxic manifestations of the ingredients of the extinguisher on the victim. The multipurpose dry chemical extinguishers possess an unusual efficiency to extinguish many classes A,B,C) of fire. This obvious benefit has been weighed against hypothetical toxic manifestations of the dry chemical following contact with soft tissue. Heretofore, the validity of these proposed side effects have never been the subject of experimental inquiry. The results of our study demonstrate negligible toxicity of the multipurpose dry chemicals following contact with soft tissue demonstrating that they are safe for extinguishing the flaming clothes of human burn victims.

Materials and Methods

Dry Chemicals

The dry chemicals employed in this study were the industrial grade materials used in portable fire extinguishers. Sodium bicarbonate dry chemical which was one of the first agents developed, is recommended for only class B and class C fires (flammable liquids and electrical equipment). Potassium bicarbonate dry chemical (Purple K) is also only for class B and

class C fires. Ammonium dihydrogen phosphate is a multipurpose dry chemical that extinguishes class A fires (ordinary combustibles) as well as class B and C fires.

Experimental Design

The purpose of this study was to identify the dry chemical which would be safest for extinguishing the burning human. The safety of each dry chemical was assessed by examining its effect on wound healing and tissue defenses against infection. These parameters are easily susceptible to reproducible experimental measurements using standardized models.[1,2,3]

Resistance to Infection: Twelve, New Zealand rabbits (2-3 kg) were anesthetized with intravenous sodium pentobarbital (33 mg/kg) and their dorsal hair clipped with electrical shears. Following depilation with Surgex, their backs were thoroughly washed with water and surgically prepared with an iodophor, followed by a 70% alcohol wash. Using aseptic technique, eight incisions were made through the paravertebral skin. Each incision was 3 cm. in length and extended down to the panniculus carnosus. When bleeding occurred, hemostasis was accomplished by direct pressure using a sterile gauze sponge.

The experimental animals were divided into three test groups. All wounds were contaminated with 0.02 ml of 0.9% saline containing a designated number (10^4, 10^5, or 10^6) of Staphylococcus aureus (CDC #2801). Five minutes later, a measured amount (10 mg) of each dry chemical was added separately to two standardized wounds in each animal. The remaining two wounds served as controls and did not receive any dry chemical. The wound edges were then approximated by microporous tape after which the wounds were covered by gauze bandages.

On the fourth postoperative day, the animals were sacrificed and their wounds examined for the presence of infection. After removal of the bandage and closure tapes, the skin margins of the wounds were treated with an iodophor preparation followed by 70% alcohol. Each wound was then opened and examined for the presence of purulent exudate. The wound was considered infected when pus was evident. Each wound was then excised and homogenized in 5 ml of 0.9% saline. Using standard serial dilution and plating techniques, the number of bacteria present in each wound was determined. The infection rate and bacterial counts of the treated wounds were compared to that of the controls. A significant elevation in bacterial counts and/or infection in the treated wounds is interpreted as damage to the tissue defenses by the dry chemical.

Leukocyte Function

One of the host's primary defense mechanisms is the phagocytosis of bacteria by leukocytes. The effect of the dry chemicals on in vitro leukocyte function was assessed in this study. Fresh venous blood collected in heparinized tubes was combined with an equal volume of 3% Dextran and placed at a 45^{o} angle for one hour in order to sediment the erythrocytes. The supernatant containing plasma, leukocytes,and platelets was centrifuged at 280 g for 12 minutes and the resulting button of white cells (10^{5}) was resuspended in 3.4 ml of Hank's balanced salt solution and 0.4 ml of autologous serum. The fresh autologous serum contains the opsonins and complement system that facilitate phagocytosis of foreign particles. The tubes of white cell suspensions were treated with a measured amount (20 mg) of dry chemical. One set of white cell suspensions did not receive any chemicals

serving as controls. After 30 minutes a measured aliquot (0.2 ml) of 0.9% saline containing 10^7 S. aureus was added to all tubes which were rotated at 12 rpm and maintained at 37^{o} C.

Upon bacterial inoculation and two hours later, a measured aliquot (0.1 ml) of fluid was removed from both test and control white cell suspensions. Each aliquot was then diluted in sterile water (9.9 ml) in order to lyze the leukocytes. The lyzed white cell suspension was agitated in a Vortex^{R} mixer for two minutes. The total number of bacteria within the water was quantitated by standard serial dilution and plating technics and represents those bacteria which were ingested but remained viable within the cell. The change in bacterial count during the two hour period of study provides a measure of the bactericidal capacity of leukocytes. This result was reported as the log percent inhibition of bacterial kill and calculated by the following formula:

$$\text{Log \% inhibition} = 100\% - 100 \frac{(\log \text{bacteria}_{0\ hr} - \log \text{bacteria}_{2\ hr})_{\text{TEST}}}{(\log \text{bacteria}_{0\ hr} - \log \text{bacteria}_{2\ hr})_{\text{CONTROL}}}$$

A second series of experiments were undertaken to provide further insight into the mechanisms by which the dry chemical, potassium phosphate, interfered with leukocyte phagocytosis. White cell suspensions were divided into two groups. One group was subjected to 20 mg of potassium bicarbonate while the other did not receive dry chemical serving as control. All treated and control suspensions were inoculated with S. aureus. Upon bacterial inoculation and at one and two hours later, a measured aliquot (0.5ml) of fluid was removed from each suspension and added to 4.5 ml of sterile, 0.9% saline. Through differential centrifugation (280 g), the free bacteria

(supernatant) was separated from the leukocytes (sediment). After separating the supernatant from the sediment, the leukocytes in the sediment were then lyzed in sterile water to release any phagocytized yet viable bacteria. The number of bacteria in each fraction was quantitated using standard serial dilution and plating technics. Quantitation of extracellular bacteria (supernatant) over time provides a measure of phagocytosis while quantitation of intracellular bacteria (sediment) provides an index of the bactericidal capacity within the leukocyte.

Wound Healing

Using the technique described above, standardized experimental paravertebral incisions were made in eight rabbits. A measured amount (50 mg) of each dry chemical was added separately to two wounds in each animal. The two remaining wounds in each animal were not treated with the dry chemicals serving as the controls. The wound edges were then approximated by tape and the wounds were covered by bandages.

On the fourteenth postoperative day, the animals were sacrificed and the breaking strength of the intact wounds was measured. Two incisions were made parallel to each healing wound at a distance of 6 mm from the wound edges. Clips were then firmly attached to each side of the wound at a distance of 3 mm from the wound edge. While one clip remained stationary, the contralateral clip was retracted at a constant rate of extension by attaching it to a strain gauge which was electrically connected to a calibrated recorder through an amplifier system. A constant rate of extension (8.8 mm/sec) was obtained using a continuous drive motor with a screw gear advance. The force necessary to disrupt the healing wound was printed on a chart recorder and expressed in grams.

Results

The infection potentiating effect of ammonium dihydrogen phosphate was considerably less than the other dry chemicals (Fig. 1). In wounds containing 10^5 bacteria the presence of either postassium or sodium bicarbonate potentiated significantly higher infection rates than wounds containing ammonium dihydrogen phosphate. In heavily contaminated wounds (10^6) the presence of ammonium dihydrogen phosphate was associated with significantly more infection than control wounds not containing a foreign material. The presence of purulent discharge in the standardized wound was associated with a elevated bacterial count. The level of bacterial contamination was highest in the presence of potassium bicarbonate followed by sodium bicarbonate and then ammonium dihydrogen phosphate.

The damage to the tissue resistance by the dry chemicals can be correlated with its deleterious effect on leukocyte function (Fig. 2). The ability of leukocytes to kill bacteria is significantly altered by each dry chemical. Potassium and sodium bicarbonate inhibited leukocyte function to a significantly greater degree than did ammonium dihydrogen phosphate.

The inhibition of leukocyte function by potassium bicarbonate is due to impairment of phagocytosis as well as intracellular kill (Fig. 3). The presence of increased numbers of bacteria in the supernatant of centrifuged suspension of leukocytes exposed to the dry chemical indicates inhibition of leukocyte phagocytosis. The elevated bacterial counts of the treated leukocyte sediment after lysis is consistent with an impairment in leukocyte kill of the phagocytized bacteria by the dry chemical.

With the exception of potassium bicarbonate, the dry chemical did not damage wound healing (Fig. 4). The breaking strength of healing wounds treated with either ammonium dihydrogen phosphate or sodium bicarbonate did not differ from that of the control wound. In potassium bicarbonate treated wounds, the breaking strength was significantly lower than that of the controls.

DISCUSSION

Extinguishers are designed for emergency use and should be thought of as vital tools to protect life and safety. The best approach to extinguishing the flaming burn victim is to employ an extinguisher that extinguishes as many classes of fire as possible.[4] The water type fire extinguisher can react adversely on a class B fire (flammable liquids) by making it flame up and spread. If any water extinguisher is used on a fire in or near live electrical equipment (Class C fire), the conductivity of the water stream has the potential of transmitting a fatal shock to the operator (Class C fire). The CO_2 extinguisher which is best suited for the type B and C fires is only one half as effective on a weight basis in extinguishing fires as is the dry chemical. Multipurpose dry chemical extinguishing agents like ammonium dihydrogen phosphate, are recognized for their unusual efficiency in extinguishing class A (wood, clothing, paper), B and C fires.

It is indeed fortunate that the efficient multipurpose dry chemical (ABC) is relatively inocuous to human tissues. Topical treatment of wounds with relatively large amounts of this dry chemical does not interfere with healing.

Tissues contaminated with this agent exhibit a remarkable ability to resist infection, especially at moderate levels of contamination. These minimal toxic manifestations to the multipurpose chemical are in sharp contrast to the damaging effects of the potassium and sodium bicarbonate dry chemicals. It is a fortuitous coincidence that the toxic bicarbonate dry chemical has a considerably more limited range of activity, especially against class A fires, than does the multipurpose dry chemical.

In hospital settings, the size and weight of the fire extinguisher is an important factor which must also be considered. The current "standard" water extinguisher is the 2 1/2 gallon size weighing 30 lbs. The carbon dioxide and dry chemical extinguishers weigh approximately one third the weight of the water extinguisher and are easily carried by all members of the emergency health care team.

In the hospital, it is essential that the extinguisher is located quickly and put into use as soon as possible after clothing ignition. The location of column or wall mounted extinguishers becomes visible by painting a red rectangle or band above the extinguisher at a height of approximately 8 to 10 feet. Your extinguisher should be mounted so that the top is not more than 3 1/2 to 5 feet from the floor. Extinguishers that are installed in recessed cabinets or wall recesses are generally more difficult to locate and gain access to.

It is important for the hospital and prehospital medical team to realize that no time can be wasted in practicing on how to use the extinguisher. Once activated, the 10 lb. multipurpose extinguisher has a discharge time of approximately 8 to 12 seconds. The multipurpose extinguisher should be held upright and discharged at a distance of 6 to 10 freet from the flaming

victim. The dry chemical should be discharged just under the flames, using a side to side motion sweeping the entire width of the fire.

After extinguishing the fire, the victim should be removed from the area in which the extinguisher was discharged. Although the multipurpose chemicals can be regarded as non-toxic materials, they are irritating if breathed in high concentrations. Once the patient is evacuated, remove all non-adherent burnt clothing from the victim which continues to be a heat source even when the flames are extinguished.

Despite the advantages of the multipurpose dry chemical extinguisher, many hospitals hesitate to employ it in this setting because of the potential damage to electrical equipment. Medical equipment with delicate electrical contacts are rendered inoperative following contact with the dry chemical. These dry chemicals are highly corrosive and difficult to remove from medical equipment after fire extinction. However, the salvage of medical equipment at the expense of the burn patient provides little consolation to the victim.

SUMMARY

The multipurpose dry chemical (ABC) fire extinguishers possess an unusual efficiency to extinguish many classes of fire (A, ordinary combustible; B, flammable liquids; C, electrical equipment). This exemplary performance of multipurpose extinguishers is associated with minimal toxic effects to humans. Following contact of soft tissue with the multipurpose dry chemical, the tissue exhibits a remarkable ability to resist infection and a normal capacity to heal following injury. This negligible toxicity of the multipurpose dry chemical makes them safe for extinguishing the flaming clothes of human burn victims.

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LEGENDS FOR ILLUSTRATIONS

Fig. 1 The infection potentiating effect of ammonium dihydrogen phosphate was less than the other dry chemicals.

Fig. 2 Ammonium dihydrogen phosphate exerted the least damaging effect on leukocyte bactericidal capacity of all dry chemicals tested.

Fig. 3 The inhibition of leukocyte function by potassium bicarbonate is due, in part, to impairment of phagocytosis as well as intracellular kill.

Fig. 4 With the exception of potassium bicarbonate, the dry chemicals did not damage wound healing.

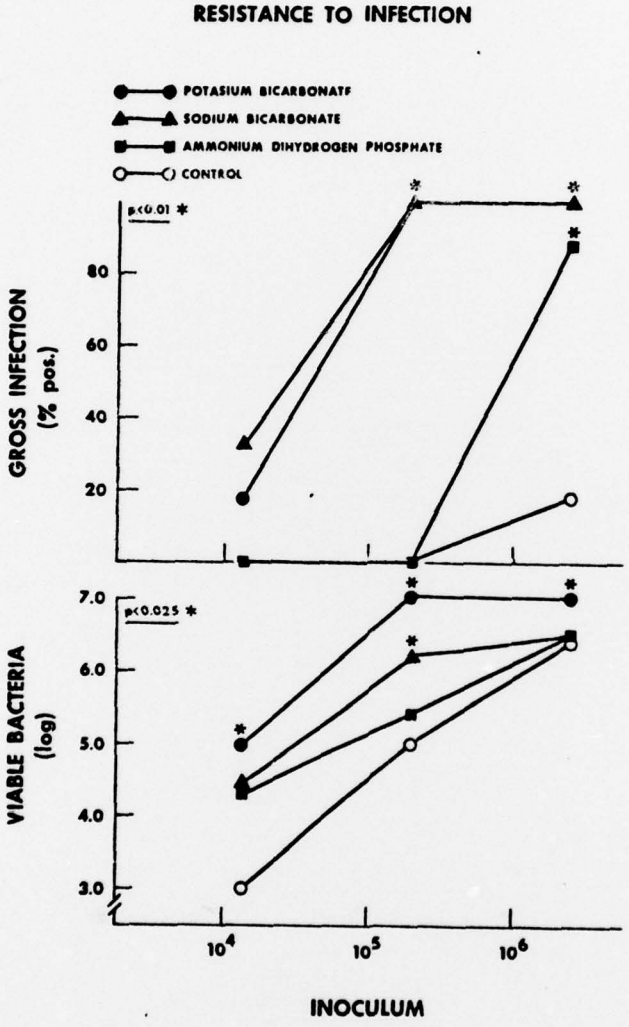
RESISTANCE TO INFECTION
POTASIUM BICARBONATE
SODIUM BICARBONATE
AMMONIUM DIHYDROGEN PHOSPHATE
CONTROL
p<0.01 *
GROSS INFECTION
(% pos.)
80
60
40
20
p<0.025 *
VIABLE BACTERIA
(log)
7.0
6.0
5.0
4.0
3.0
10^4
10^5
10^6
INOCULUM

Figure 1

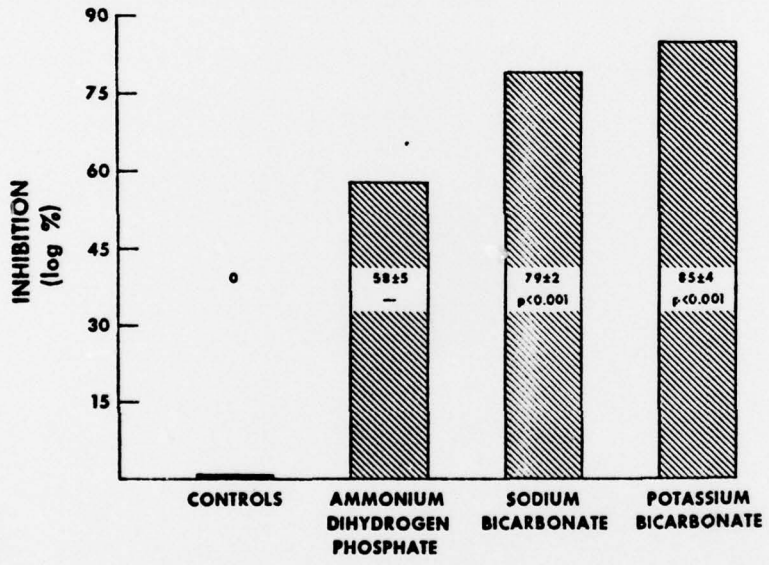
LEUKOCYTE BACTERICIDAL CAPACITY
INHIBITION (log %)
90
75
60
45
30
15
0
58±5
—
79±2
p<0.001
85±4
p<0.001
CONTROLS
AMMONIUM DIHYDROGEN PHOSPHATE
SODIUM BICARBONATE
POTASSIUM BICARBONATE

Figure 2

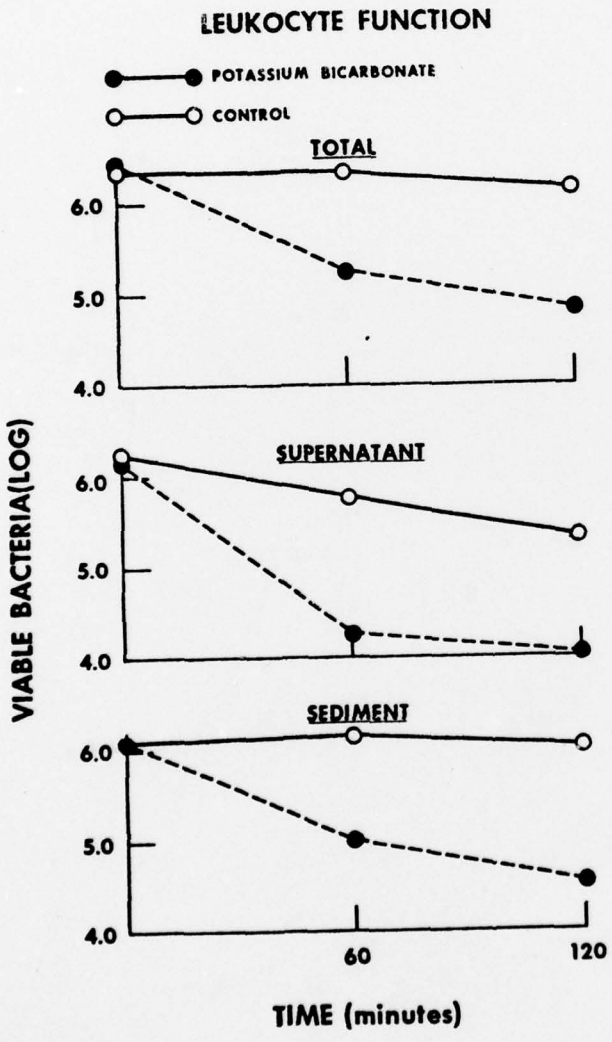
LEUKOCYTE FUNCTION
POTASSIUM BICARBONATE
CONTROL
TOTAL
SUPERNATANT
SEDIMENT
6.0
5.0
4.0
VIABLE BACTERIA(LOG)
60
120
TIME (minutes)

Figure 3

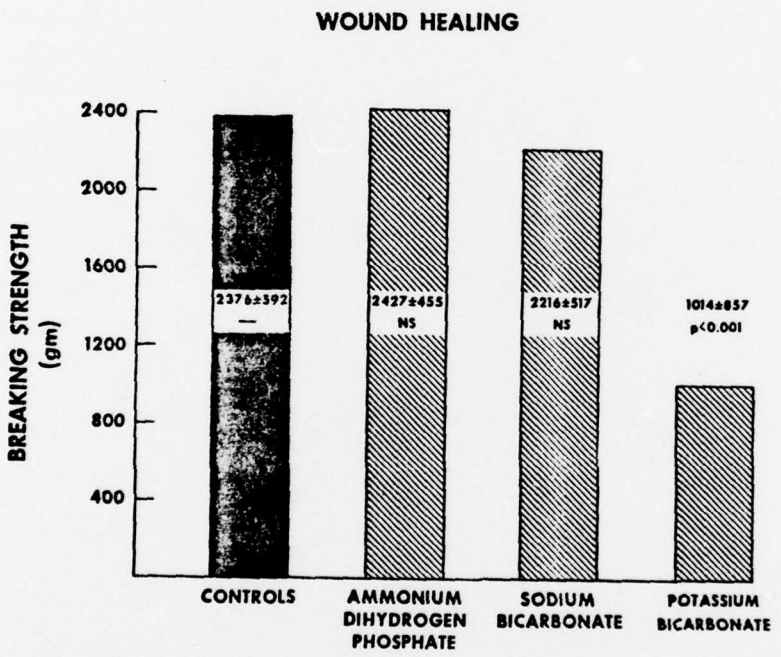
WOUND HEALING
BREAKING STRENGTH (gm)
2400
2000
1600
1200
800
400
2376±392
—
2427±455
NS
2216±517
NS
1014±857
p<0.001
CONTROLS
AMMONIUM DIHYDROGEN PHOSPHATE
SODIUM BICARBONATE
POTASSIUM BICARBONATE

Figure 4

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