PUBLIC HEALTH SIGNIFICANCE OF STAPHYLOCOCCUS HYICUS AND INTRACELLULAR STAPHYLOCOCCAL ENTEROTOXINS IN FOODS

A Thesis

Submitted to the Faculty of the Graduate

School of the University of Minnesota

BY

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IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

June, 1981

DEDICATION

To my loving and understanding parents,

Hon. Mr. Justice and Chief (Mrs.) J.M. Adesiyun.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my advisor, Dr. S.R. Tatini for his interest, guidance and understanding throughout the course of this investigation and for his constructive criticism and suggestions in the preparation of this thesis.

I am very grateful to Dr. R.K. Anderson, my co-advisor, for his outstanding guidance and fatherly warmth throughout my graduate studies in the U.S.A.

My appreciation to the Kwara State Government of Nigeria for the scholarship award that enabled me to pursue my studies; to Ahmadu Bello University, Zaria, Nigeria, for the study fellowship award, thus providing financial support and to the Food and Drug Administration for providing funds for part of this research.

Special thanks to members of my family for their love, support, and understanding.

Finally, I am indebted to my fiancée, Yemi, for her love, concern, and perseverance throughout my stay away from home.

TABLE OF CONTENTS

PAGE

INTRODUCTION 1

LITERATURE REVIEW 5

TO THE READER 11

PART A

EXPERIMENT 1: 13

Incidence of Ketamine-induced emesis in cynomologus monkeys used in Staphylococcal enterotoxin bioassay.

a) Abstract 13

b) Introduction 13

c) Materials and Method 15

d) Results and Discussion 22

e) References 26

EXPERIMENT 2: 28

Suitability of cynomologus monkeys for Staphylococcal enterotoxin bioassay

a) Abstract 28

b) Introduction 28

c) Materials and Methods 29

d) Results and Discussion 31

e) References 37

PAGE

EXPERIMENT 3: 38

Production of Enterotoxin(s) by Staphylococcus hyicus

a) Abstract 38

b) Introduction 38

c) Materials and Methods 40

d) Results and Discussion 44

e) References 51

EXPERIMENT 4 ; 54

Production of neutralizing antibodies for enterotoxin produced by Staphylococcus hyicus (VII 76)

a) Abstract 54

b) Introduction 54

c) Materials and Methods 55

d) Results and Discussion 59

e) References 64

PART B

EXPERIMENT 1: 65

Biological activity of intracellular Staphylococcal enterotoxin A

a) Abstract 65

b) Introduction 65

c) Materials and Methods 66

d) Results and Discussion 68

e) References 74

EXPERIMENT 2: 75

Influence of environmental factors on release of

intracellular Staphylococcal enterotoxin A

a) Abstract 75

b) Introduction 76

c) Materials and Methods 77

d) Results and Discussion 80

e) References 88

EXPERIMENT 3: 90

Influence of Certain gastro-intestinal enzymes on

release of intracellular Staphylococcal enterotoxin A

a) Abstract 90

b) Introduction 90

c) Materials and Methods 91

d) Results and Discussion 94

e) References 97

DISCUSSION AND SUMMARY 102

BIBLIOGRAPHY 105

INTRODUCTION

Staphylococcal enterotoxins are the leading cause of bacterial foodpoisoning in Canada and the United States (Todd, 1978). Nearly 30% of all reported foodborne disease outbreaks of bacterial origin annually are attributed to staphylococci (Bergdoll, 1979). The common vehicles of staphylococcal foodborne intoxication are meat and meat products, dairy products, bakery products, custard and cream-filled pastries, egg and egg-products and fish. Although food items prepared in food service establishments or homes are the major source of staphylococcal intoxications, processed and ready-to-eat food items have also been implicated, especially in larger outbreaks due to their wider distribution.

The efforts of both regulatory and food industry personnel to demonstrate the actual presence of enterotoxins in food implicated in foodborne disease outbreaks and/or to project the potential presence of staphylococcal enterotoxins in foods for routine quality control, have been hampered seriously by the lack of specific, sensitive and practical laboratory tests.

Though significant progress has been made in the past two decades in identifying and establishing five serological types of staphylococcal enterotoxins A through E (Casman et. al., 1960, 1967; Bergdoll et al., 1959, 1965, 1971), this problem is far from being solved as it is known that unidentified enterotoxins still exist (Bergdoll, 1970).

Prior to the development of the in vitro serological assays for detection of staphylococcal enterotoxins in foods, the clinical syndrome (rapid onset, 1-7 hours; nausea, vomiting, abdominal cramps, and diarrhea), epidemiological findings and the isolation of coagulase-positive Staphylococcus aureus from implicated foods formed the basis for diagnosis. The basic assumption has been that all enterotoxigenic staphylococci are coagulase-positive and therefore are S. aureus (Buchanan and Gibbons, 1974). This assumption was supported by the surveys conducted on enterotoxigenicity of coagulase-positive staphylococci from various sources including foods implicated in foodpoisoning (Casman et. al., 1967; Burbianka, 1971; Payne and Wood, 1974; Wieneke, 1974). These surveys indicated that 50% or more of coagulase-positive staphylococci produce one or more of the five known enterotoxins. However, such intensive studies have not been conducted on coagulase-negative staphylococci or other non-S. aureus species.

Nevertheless, there are reports of involvement of coagulase-negative staphylococci in foodborne outbreaks (Omori and Kato, 1959; Breckenridge and Bergdoll, 1971). Studies conducted on these coagulase-negative staphylococci suggested that they are variants or mutants of S. aureus because coagulase-positive organisms were isolated from them after several consecutive transfers (Lotter and Genigeorgis, 1975, 1977). These authors did not conclusively prove that these coagulase-positive revertants and coagulase-negative parents were indeed S. aureus by DNA:DNA homology studies with the reference strains. Species designation on the basis of a

single criterion like coagulase test and its presumed high correlation with enterotoxin production may lead to erroneous hazard potential evaluation of these organisms because coagulase-negative enterotoxigenic strains would be considered non-hazardous. Danielssen and Hellberg (1977) showed that 7% of coagulase-negative staphylococci examined produced enterotoxins A, B, or C.

Another complicating factor with respect to growth and enterotoxin production is the fact that growth of enterotoxigenic strains of staphylococci to high populations may not always result in detectable amounts of enterotoxins. This is especially true for growth which occurs under non-optimial or stress conditions like low pH, water activity (a_w), temperature and oxygen tension (Barber and Deibel, 1972; Tatini, 1973; CDC, 1979). This led to the belief that if S. aureus grows under adverse conditions, enterotoxin may not be produced in detectable levels. Staphylococcal enterotoxins being exotoxins are released from cells into the growth medium but such release may be inhibited by the adverse conditions leading to false-negative test results when assays included extracellular enterotoxins. This aspect has not received much attention and may in part explain why enterotoxins are not always detected in foods which contained high counts of enterotoxigenic S. aureus and caused foodpoisoning. The presently used test for enterotoxins in food detects only the extracellular enterotoxins and not intracellular enterotoxins despite the fact that when foods are contaminated by enterotoxigenic strains of staphylococci, humans consume

enterotoxins (if present) and staphylococcal cells with foods.

In view of the fact that unidentified enterotoxins exist and detection of enterotoxins may not always be successful, public health officials have now resolved to using the following criteria to establish staphylococcal enterotoxin as the etiological agent in foodpoisoning: the presence of $\geq 10^5$ coagulase-positive S. aureus per gm or ml of food, the presence of the same S. aureus phage-type (when typable) in the vomitus and/or feces of the patient and the incriminated food and/or the actual demonstration of detectable amounts of any of the five known staphylococcal enterotoxins.

Since enumeration of coagulase-positive staphylocci in foods is simple and practical, food processors have utilized this criterion for routine quality control in foods to assess the enterotoxin hazard potential prior to release for human consumption especially in fermented and dried food products. This approach however has three major limitations: 1) the assumption that only coagulase-positive S. aureus produce enterotoxins and other staphylococci do not; 2) the assumption that the maximum growth of staphylococci in foods is always assessable; staphylococci may die out in fermented and cooked foods; 3) the assumption that enterotoxins are always detectable in foods; intracellular enterotoxins are not tested for in foods by the present scheme. It is therefore obvious that there are limitations in the present laboratory analysis of foods in proving staphylococcal enterotoxin as the etiologic agent of foodborne intoxication.

This investigation was initiated primarily to improve laboratory investig-

ations and interpretations of staphylococcal foodborne intoxications by seeking answers to the following questions:

1) Do coagulase-negative staphylococci and non- S. aureus species produce any enterotoxins (known or unknown)?
2) Is intracellular staphylococcal enterotoxin A present in S. aureus cells biologically active to cause foodpoisoning?
3) What conditions promote in vitro release of intracellular staphylococcal enterotoxin A?

REVIEW OF LITERATURE

Staphylococci have been implicated in foodborne illnesses even before the characterization of the species and genus (Dack, 1956). It was Barber in 1914 who provided direct evidence that Staphylococcus albus present in raw milk from a mastitic cow produced a toxic factor which caused gastroenteritis in human volunteers. He showed that the toxic factor was the result of multiplication of these organisms in cream or milk. Dack (1956) later reproduced the symptoms of staphylococcal poisoning by feeding culture filtrates of enterotoxigenic strain to human volunteers, thus proving that a metabolite released by growing staphylococcal cells was responsible for intoxication. Staphylococcal food- poisoning is therefore attributed to the consumption of pre-formed staphylococcal enterotoxins with symptoms of rapid onset, 1-7 hours, nausea, vomiting, retching, abdominal cramping and diarrhea (Bergdoll, 1979). Though intoxication is a rule rather than the exception,

certain instances of infectio-toxicosis have been reported where the staphylococci grew and multiplied in the host's intestine with ultimate release of enterotoxin(s) (Casman, 1965; Kienitze, 1966; Hallander et. al., 1967).

Staphylococci have been known to cause a variety of infections in humans and animals and their ability to produce coagulase has been shown to correlate highly with pathogenicity (Gemmell et. al., 1975; Evans et. al., 1950; Bergdoll, 1972). The species that produces coagulase is S. aureus and since there were no practical in vitro tests for detection of enterotoxins which cause intoxication in humans, public health officials have utilized the presence of coagulase-positive staphylococci as evidence of potential presence of enterotoxin as well. Consequently, coagulase-negative staphylococci have been ignored.

To date, five immunologically different enterotoxins, A through E, have been identified through use of bioassays in cats and monkeys (Bergdoll, 1970; Dolman et. al., 1940; Denny et. al., 1963; Surgalla et. al., 1953; Davison et. al. 1938). Cat bioassay requires prior exposure of culture filtrates to either heat (Dolman and Wilson, 1940), anti-hemolysin sera (Dolman, 1943) or proteolytic enzymes (Denny and Bohrer, 1963; Casman and Bennett, 1963) to eliminate non-specific responses from hemolysins. The feeding of culture filtrates to young monkeys is presently considered the most reliable bioassay for staphylococcal enterotoxins since only enterotoxin is known to elicit emesis in them (Bergdoll, 1970; Surgalla et. al., 1953; Schantz, 1965). Purification of enterotoxins, A (Schantz, et. al., 1972), B (Schantz et. al.,

1965), C (Borja and Bergdoll, 1967; Avena and Bergdoll, 1967) D(Chang and Bergdoll, 1979) and E (Borja et. al., 1972) and production of specific antisera in rabbits (Bergdoll et. al., 1959; Casman and Bennett, 1964), provided reliable in vitro serological tests for detection of these enterotoxins in foods.

Through the use of serological tests, it was shown that 50% or more of the coagulase-positive staphylococci produced one or more of the five known enterotoxins with some indication of existence of unidentified biologically active enterotoxins (Bergdoll, 1970). Consequently, laboratory investigations of staphylococcal intoxications focused upon isolation and enumeration of coagulase-positive (S. aureus) staphylococci and/or the demonstration of one of the known enterotoxins in food (Baer et. al., 1976). However, coagulase-negative staphylococci have been isolated from foods implicated in foodborne intoxications (Omori and Kato, 1959; Breckenridge and Bergdoll, 1971; Thatcher and Simon, 1959) and these were shown to produce enterotoxins (Lotter and Genigeorgis, 1975). These authors in a later study (Lotter and Genigeorgis, 1977) concluded that the enterotoxigenic coagulase-negative staphylococci were mutants or variants of S. aureus and suggested that the ability to produce enterotoxins should be considered a taxonomic criterion for S. aureus. Thus, they reaffirmed to themselves that only S. aureus strains are capable of producing enterotoxins and are of public health concern. However, these authors did not conclusively prove that they were dealing with S. aureus species through DNA: DNA homology studies.

Studies conducted on characterization of staphylococci from poultry, swine and cattle showed that coagulase production is not unique to S. aureus (Devriese et. al., 1975, 1978, 1979). They established a new species named S. hyicus (Sompolinsky) comb nov containing two subspecies, S. hyicus subsp. hyicus which are coagulase positive or negative and S. hyicus subsp. chromogenes which are coagulase negative. Therefore, speciation on on the basis of coagulase production alone as is presently done (Buchanan and Gibbons, 1974; Baer, et. al., 1976) results in improper designation of the organism.

Investigations on the correlation between enterotoxigenicity and other physiological traits did not show 100% correlation with any trait (Genigeorgis, 1963; Bergdoll, 1970). However, production of coagulase and thermonuclease showed good correlation with enterotoxigenicity (Tatini, 1976; Bergdoll, 1970, 1972) and these are presently used or recommended for use in routine quality control. Since enterotoxigenicity of coagulase negative strains is not well established, it is not known if these are enterotoxigenic and should be included in laboratory investigations of outbreaks or routine quality control. A recent study by Danielssen and Hellberg (1977) showed that 7% of coagulase-negative strains of staphylococci produced enterotoxins A, B, or C. These investigators did not test for enterotoxins D and E.

It is obvious from the above that the ability to produce enterotoxins (known or unknown) relative to species of Staphylococcus is not well established. While the use of coagulase and/or thermonuclease may be

useful (without regard to speciation on this basis) in projecting hazard potential of foods, such projections may be erroneous if coagulase and thermonuclease-negative strains of staphylococci produce enterotoxins.

The laboratory confirmation of etiologic agent of staphylococcal food-borne intoxication may fail if staphylococci are killed after growing to high levels and produced only trace amounts of known enterotoxins (possibly some unknown) that may not be detected by the presently used serological assay system. This situation may be pertinent to fermented and heated foods where cells die out (Barber and Deibel, 1972; Bergdoll, 1976).

All the previous research conducted on the influence of food systems and environmental conditions on growth and enterotoxin production by S. aureus indicated that while growth might occur under a variety of conditions, toxin production was restricted to a narrower range of conditions (Miller and Fung, 1975; Tatini, 1976; Genigeorgis and Sadler, 1966; Jarvis et. al., 1973; McLeane et. al., 1968, Friedman, 1968). Through the use of non-growing cells, factors that affect cell growth and toxin synthesis have been delineated and studied independently (Miller and Fung, 1975, 1976, 1977; Markus and Silverman, 1968, 1969, 1970; Jarvis et. al., 1975). While growth of staphylococci have been demonstrated to occur under non-optimal conditions as low temperature of 7-10°C, pH of 4-5, a_w of 0.86 - 0 90 and presence or absence of oxygen (Genigeorgis et. al., 1969; Barber and Deibel, 1972; Genigeorgis et. al., 1971; Scott, 1953; Troller, 1971, 1972) the level of enterotoxin has been found to be substantially inhibited under these

conditions.

The existence of intracellular staphylococcal enterotoxin B (ISEB) has been demonstrated in S. aureus cells (Miller and Fung, 1976, 1977; Haines and Harmon, 1973) with limited available information on its accumulation and release (Markus and Silverman, 1968, 1969, 1970). Lee (1978) showed the presence of intracellular staphylococcal enterotoxin A (ISEA) and its accumulation in S. aureus at low a_w (0.90 - 0.95). He detected 4.14 ug of ISEA per 10^{12} cells grown in a medium of a_w 0.90 (adjusted with glycerol) and only 0.70 ug when grown in a medium of Aw 0.99 and no glycerol. He also demonstrated that the a_w inhibited release of ISEA when it was below 0.95.

Therefore, if staphylococci grow in foods of low a_w, there may be a significant accumulation of intracellular enterotoxin with only trace amounts of extracellular enterotoxin. Likewise, other non-optimal conditions as low temperature, pH, etc might also result in higher levels of intracellular enterotoxin.

To date, information is only available on the biological activity of extracellular enterotoxin (Raj. et. al., 1969; Dack et. al., 1931; Bergdoll, 1976; Dangerfield cited by Bergdoll, 1970). However, there is no information on the biological activity of intracellular enterotoxin as to whether some enzymatic activation is required prior to or after its release into growth medium by staphylococcal enzymes. If intracellular enterotoxin is biologically active, then the method used to detect enterotoxins in foods should include its detection which is presently not done.

TO THE READER

This thesis is divided into two sections, namely, Section A and B consisting of 4 and 3 experiments respectively. These manuscripts have been prepared for submittal to scientific journals and are presented here in the respective journal style.

SECTION A (Pages 13 - 64)

Contains information on the enterotoxigenicity of Staphylococcus hyicus and its public health significance.

Experiment 1 (Pages 13 - 27)

This investigation was conducted to eliminate the possibility of Ketamine-induced (used as tranquilizer) emesis in cynomologus monkeys which would result in false-positive results for enterotoxigenicity.

Experiment 2 (Pages 28 - 37)

Since cynomologus monkeys (Macaca fascicularis) have not been routinely used for staphylococcal enterotoxin bioassay, their sensitivity and specificity to staphylococcal enterotoxin was established prior to using them for testing S. hyicus strains.

Experiment 3 (Pages 38 - 53)

The enterotoxigencity of S. hyicus strains was established using cynomologus monkeys known to respond specifically (emesis) to staphylococcal enterotoxin.

Experiment 4: (Pages 54 - 64)

Neutralizing antibodies to the enterotoxic factor secreted by S. hyicus (VII 76) was produced in rabbits indicating that it is antigenic.

SECTION B (Pages 65 - 101)

Contains information on the biological activity of intracellular staphylococcal enterotoxin A (SEA) and its in vitro and in vivo release.

Experiment 1 (Pages 65 - 74)

This experiment demonstrates in vivo release of intracellular SEA by staphylococcal cells fed to cynomologus monkeys. The biological activities of intracellular and extracellular SEA were compared.

Experiment 2: (Pages 75 - 89)

This experiment shows the influence of environmental factors (pH, temperature and water activity) on in vitro release of intracellular SEA.

Experiment 3 (Pages 90 - 101)

The investigation shows the influence of gastro-intestinal enzymes on release of intracellular SEA.

SECTION A

EXPERIMENT 1

Incidence of Ketamine-induced emesis in cynomologus monkeys (*Macaca fascicularis*) used for Staphylococcal enterotoxin bioassay.

ABSTRACT

Ten (24%) of forty-one cynomologus monkeys (Macaca fascicularis) showed emetic response to specific levels of Ketamine injected intramuscularly. Reduction of the level of Ketamine to one-half or less of the emetic level, resulted in faster recovery from sedation yet provided adequate time for safe intubation and subsequent intragastric feeding of Staphylococcal enterotoxin (SE) in only six of the ten monkeys without emesis. The onset of the first emetic episode with Ketamine was similar to that induced by Staphylococcal enterotoxin A (SEA). Cynomologus monkeys showing emetic response to ketamine could still be used for SE bioassay if experimentally determined non-emetic dose for individual monkeys is employed for sedation.

INTRODUCTION

Staphylococci produce enterotoxins which cause gastroenteritis in humans and monkeys. Emesis is the most consistent symptom associated with staphylococcal intoxication. While the production of the presently known staphylococcal enterotoxins (Types A, B, C, D, and E) is established on the

basis of in vitro serological assays (2), detection of new staphylococcal enterotoxins (SE) is based on bioassays utilizing monkeys or cats (2,3). Monkey bioassay is the most reliable method used to establish enterotoxigenicity of staphylococci (2, 4). Feeding six young rhesus monkeys with test culture growth and observing for emetic response within 5 hours is the standard procedure. Emesis in two or more of the six is considered positive for SE.

Physical restraint is often used for intragastric feeding of test material but this approach is not safe for human personnel or the monkeys. Melling (5) used ketamine to sedate rhesus monkeys (Macaca mulatta) for safe insertion of stomach tube and intragastric feeding of Bacillus cereus culture filtrates. The monkeys maintained their swallowing reflexes and did not vomit in response to ketamine during sedation or within 5 hours after recovery from sedation. The author then recommended the use of ketamine to sedate monkeys prior to feeding intragastrically.

Ketamine is a dissociative anaesthetic which produces an anaesthetic state wherein the pupillary, corneal, pharyngeal, patellar and pedal reflexes are retained with lack of response to painful stimuli (6). Though the mechanism of action of ketamine is not fully understood (11, 12, 13), it has been successfully utilized as a sedative in subhuman primates as rhesus and pigtail monkeys (7, 8, 9). However, there are reports of complications and side effects such as hallucinations, screaming, salivation, reduced breathing, and even emesis (10, 14, 15). But, Melling (5) did not observe any

side effects of Ketamine as causing emesis or influencing emetic dose-50 (ED_{50}) for SEB in rhesus monkeys.

Cynomologus monkeys have been used for SE bioassay to a limited extent (16) without ketamine sedation. However, it is not known if cynomologus monkeys would respond to ketamine in a manner similar (no effect on ED_{50} and not inducing emesis) to that observed with rhesus monkeys used for SE bioassay (5). This communication presents data on the incidence of ketamine-induced emesis in cynomologus monkeys.

MATERIALS AND METHOD

Animals

The study was conducted on 41 young cynomologus monkeys, 20 males and 21 females, weighing 1.85 - 5.5 kg. The animals were obtained from Primate Imports.[1] Following air transit and receipt, the monkeys were quarantined for 30 days in individual cages with automatic self-feed water supply. They were certified healthy and tuberculosis-free by the resident veterinarian before use.

Anaesthesia

Ketamine HCl[2] was administered by the intramuscular (im) route into

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[1] Primate Imports, 34, Munson St., Port Washington, Long Island, N.Y. 11050.

the thighs at doses ranging from 2.5 - 20 mg/kg. Varying doses of Ketamine were injected to individual monkeys to determine the dose that allowed adequate sedation without eliciting emesis. Atropine Sulfate[3] at doses of 0.05 and 0.10 mg/kg was administered by the im route 30 min prior to Ketamine injection in an attempt to possibly eliminate ketamine-induced emesis.

Staphylococcal enterotoxin A (SEA)

Crude SEA prepared by the method described by Bergdoll (2) was used. The SEA concentration was determined by the microslide gel double diffusion and single gel diffusion in tube (17, 18). Enterotoxin A and Antiserum A were supplied by R.W. Bennett[4] and M.S. Bergdoll[5] respectively.

Antiserum Neutralization of SEA

To demonstrate specific emetic response to SEA, pre-determined levels of millipore-filter[6] sterilized crude SEA were mixed with 5 mls of antiserum A (titer of 1:640). The mixture was incubated at 37 C for 1 hour prior to feeding the monkeys.

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2 Ketaset, Bristol Lab., Myers Co., Syracuse, N.Y.

3 Atropine Sulfate, Pitman-Moore, Inc., Washington Crossing, N.J. 08560

4 Division of Microbiology, F.D.A., Washington, D.C.

5 Food Research Institute, University of Wisconsin, Madison, Wisconsin.

6 Millipore, Millipore Corp., Bedford, MA. 01730.

Oral feeding of Test Material

Monkeys were fasted for 24 hours or longer prior to im injection of Ketamine. During sedation, a lubricated catheter[7] was inserted intragastrically via the nose and throat. The test material (50 mls) was introduced into the stomach via the catheter using a 60 ml plastic syringe.[8] The test material consisted of 50 ml of NZ Amine NAK broth[9] (growth medium) or NZ Amine NAK plus SEA or NZ Amine NAK plus SEA plus Antiserum A. In some instances, no test material was fed to the monkeys following Ketamine sedation. The monkeys were continuously observed for emetic response during and 5 hours after recovery from sedation.

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7 Sovereign Polypropylene catheter, Size 8F.

8 Monoject, Sherwood, Medical Industry, Inc., Delano, Florida 32720.

9 NZ Amine, Type NAK, Humko Sheffield Chemical, an operation of Kraftco Corp., Norwich, N.Y.

Table 1. Incidence of Ketamine induced emesis in cynomologus monkeys.

Dose of Ketamine (mg/kg.)	Number of Monkeys Tested*	Monkeys Vomiting**					
		Male		Female			
		Number	Weight (kg.)	Number	Weight (kg.)	Total	Percent Vomiting
< 5.0	41	3	4.4, 4.7, 4.7	0	0	3	7.3
5.0 - 9.9	41	6	2.7, 4.4, 4.5, 4.7, 4.7, 5.5	1	3.1	7	17.1
10.0 - 14.9	41	6	2.7, 4.4, 4.5, 4.7, 4.7, 5.5	3	2.8, 3.1	9	22.0
> 15.0	41	6	2.7, 4.4, 4.5, 4.7, 4.7, 5.5	4	2.0	10	24.4

* 20 males, 21 females weighing 1.85 - 5.5 kg.

** Emesis occurred within 5 hours following administration of Ketamine.

Table 2. Cynomologus monkeys* found unsuitable for SEA bioassay after Ketamine Sedation

Identification of Monkey	Weight (Kg)	50 ml. of Sterile NZ Amine NAK**		None		
		Dose of Ketamine Hcl (mg/ Kg.)	First Emetic Episode (Min.)	Dose of Ketamine Hcl (mg/ Kg)	First Emetic Episode (Min.)	Observation
02T	4.5	6.4	137(1)	3.2	---	Not adequately sedated for safe intubation
02X	4.4	6.4	142(1)	3.2	92(2)	Just barely sedated
02S	4.7	6.4	118(1)	3.2	105(2)	Just barely sedated
02W	4.7	6.4	109(3)	3.2	85(1)	Just barely sedated

* Four of the ten monkeys that vomited in response to ketamine HCl shown in Table 1.

** Growth medium used for production of SEA.

( ) Frequency of emesis.

--- Absence of emetic response during a 5 hour-observation period.

Table 3. Emetic and Nonemetic Levels of Ketamine HCl in Cynomologus Monkeys*

Identification of Monkey	Weight (kg)	Level of Ketamine HCL (mg/kg)	Material fed **					
			50 ml of Sterile NZ-Amine			None		
			Emesis	First emetic episode (min.)	Frequency of emesis	Emesis	First emetic episode (min.)	Frequency of emesis
88D	5.5	2.7	---	--	---	---	---	---
		7.3	+	206	3	+	193	3
97G	2.0	10.0	-	---	-	-	-	-
		20.0	+	159	1	+	164	3
02I	2.7	3.7	-	-	-	-	-	-
		5.6	+	129	1	-	123	1
02F	3.1	4.8	-	-	-	-	-	-
		7.3	+	200	2	+	136	1
02L	3.1	4.8	-	-	-	-	-	-
		11.1	+	228	1	+	138	2
02C	2.8	5.5	-	-	-	-	-	-
		11.2	+	122	4	+	124	3

* Six of the ten monkeys that vomited in response to Ketamine HCl in Table 1.

** Data shown are averages from duplicate trials.

+ Emesis
- No emesis
} During a 5 hour - observation period

Table 4. Response of Cynomologus Monkeys Sedated with Nonemetic Level of Ketamine to Staphylococcal enterotoxin A

Identification of Monkey	Level of Ketamine HCl (mg/kg)	Amount of SEA fed (ug)	Response		
			Emesis	Time of First first emetic episode (min.)	Frequency of emesis
88D	2.7	12.0	+	90	1
		12.0	+	190	1
97G*	10.0	16.0	+	125	2
		13.2	+	156	3
02I	3.7	18.0	-	-	0
		10.8	-	-	0
02F*	4.8	10.8	+	100	10
		4.8	+	111	3
02L*	4.8	10.8	+	128	5
		4.8	+	84	6
02C	5.5	18.0	-	-	0
		10.8	-	-	0

* Specificity of emetic response to SEA was subsequently confirmed by antiserum neutralization of SEA

+ emesis
- no emesis
} During a 5 hour - observation period

RESULTS AND DISCUSSION

As can be seen from Table 1, weight of the monkeys did not appear to have any significant effect on the dose of Ketamine required to induce emesis as both small and large monkeys vomited at dose ranges of 5.0 - 9.9, 10 - 14.9 and >15 mg Ketamine/Kg. However, sex of the monkeys seems to have some influence on the emetic dose of Ketamine. Males appear to be more sensitive than the females. Of the responding monkeys, 6 were males and 4 females. In other words, 6 (30%) of 20 males and 4 (19%) of 21 females tested showed Ketamine-induced emesis. Since the sample size is small, it is not possible to draw definite conclusions relative to the effect of sex on emetic dose of Ketamine. The data however, indicate that there is a high incidence of Ketamine-induced emesis among cynomologus monkeys and that for each monkey, there is a specific emetic dose of Ketamine.
Since all the monkeys were fasted for 24 hours or longer prior to injecting Ketamine, gastric contents could be ruled out as the cause of vomition as found by Sears (10) with nonfasted individuals.

At this stage, it would appear likely that these monkeys would be eliminated from SE bioassays if resources permit. If resources do not allow, physical restraint might be utilized for intragastric feeding of SE. On the other hand, it is possible that there is a specific emetic level and a lower non-emetic level of Ketamine for each monkey. If the latter dose provides adequate sedation for safe intubation and subsequent intragastric feeding, one can use this level rather than discard such monkeys from SE bioassay.

As shown in Table 2, even lower levels of Ketamine, 6.4 mg/kg to 3.2 mg/kg were found to be emetic to 3 of the 10 monkeys. Even though the level of Ketamine was non-emetic in one monkey, 02T, it did not provide adequate sedation for safe intubation with stomach tube.

However, (in Table 3), 6 of the 10 monkeys did not vomit at lower levels of Ketamine than that which caused emesis (Table 1). Therefore, these six monkeys could be used for SE bioassays with use of non-emetic level of Ketamine for sedation. Data (not shown here) using the non-emetic level of Ketamine were consistent because each of these monkeys was subsequently used for SE bioassay of at least six staphylococcal culture filtrates of unknown enterotoxigenicity with no emesis. Although the sedation period was shorter with the non-emetic level of Ketamine for each monkey, there was ample time (about 10 minutes) for safe handling, intubation and feeding.

Data presented in Table 4 show that two of the six monkeys were resistant to 18 ug of SEA, reaffirming the fact that the dose of Ketamine found non-emetic (Table 3) was indeed non-emetic. The four other monkeys responded to SEA by vomiting. Since crude SEA was used, it is possible that the emetic responses in these monkeys might have been induced by some interactive effect(s) of ketamine and staphylococcal metabolites other than SEA. Antiserum neutralization of SEA prior to feeding was used to demonstrate the specificity of emetic response to SEA. Emesis caused by SEA was eliminated by Antiserum A neutralization of SEA prior to feeding and using non-emetic level of Ketamine (Table 4). Only 3 of the 4 monkeys that responded to

SEA were used in the Antiserum A neutralization due to limited availability of Antiserum A. The remaining 31 monkeys that did not show emetic response to Ketamine at the dose range used,were subsequently found to respond specifically to SEA and their sensitivities determined (another study).

As shown in Table 4, the onset of first emetic episode with SEA was similar to that observed with Ketamine (Table 3). Therefore, one could not differentiate Ketamine- and SEA-induced emesis based on the onset of first emetic episode. Likewise, distinguishing on the basis of frequency of emesis is inappropriate because this depends on the sensitivity of the individual monkey and the dose administered. SEA and Ketamine showed similar frequency of emesis (Tables 3 and 4, with Monkey 97G).

MacCathy et. al. (11) using Ketamine at a dose level of 5 mg/kg in dogs concluded that it stimulated the cardiovascular system (CVS) by affecting the sympathetic nervous system. Traber et. al. (12, 13) however, showed that Ketamine was devoid of a direct sympathomimetic influence and successfully completely blocked its positive chronotropic effect (raising the cardiac output and blood pressure) with atropine sulfate. Information is not available on the possible influence of atropine sulfate on Ketamine-induced emesis. In this study, administration of 0.05 - 0.1 mg/kg Atropine sulfate prior to injecting Ketamine did not eliminate emesis (data not provided).

If the monkeys used in this study had been randomly selected from the source (Primate Imports), based on a 95% confidence interval, Ketamine at doses of 2.7 - 20 mg/kg could induce emesis in 11 - 38% of cynomologus

monkeys.

This communication is intended to alert researchers planning to use cynomologus monkeys for SE bioassay of the possibility of false-positive results in ketamine sedated monkeys.

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EXPERIMENT 2

Suitability of Cynomologus Monkeys (Macaca fascicularis) for Staphylococcal Enterotoxin Bioassay.

ABSTRACT

Cynomologus monkeys (Macaca fascicularis) were tested for their sensitivity and specificity to staphylococcal enterotoxin A (SEA). Thirty-two of 38 monkeys vomited within 5 hours in response to intragastric feeding of 4.8 - 18 µg of crude SEA. Twenty-four of these 32 responding monkeys were subjected to specificity study by feeding crude SEA which was neutralized with specific Antiserum A. Twenty-two (92%) of the 24 demonstrated specificity by not vomiting when fed neutralized crude SEA. The two (8%) remaining monkeys showed specificity only with purified SEA neutralized with the Antiserum. The emetic dose -50 for crude SEA appeared to be 6.5 µg per monkey. These data suggest that cynomologus monkeys are suitable for SE bioassay.

INTRODUCTION

New staphylococcal enterotoxins (SE) are identified on the basis of bioassays using cats and monkeys (Bergdoll, 1970, 1979; Denny and Bohrer, 1966). These animals are also used for bioassays during purification of the presently identified SE (Schantz et. al., 1965, 1972). While both species of animals show emetic response to SE, monkeys are considered more reliable

because they do not vomit in response to staphylococcal metabolites other than SE as sometimes reported in cats (Surgalla et. al., 1953; Bergdoll, 1979; Dolman and Wilson, 1940). Young rhesus monkeys (Macaca mulatta) have been used for SE identification and emesis in at least two of six monkeys within five hours following feeding of culture filtrate is considered a positive response for presence of SE (Bergdoll, 1970). However, with restriction on exportation of rhesus monkeys by governments of foreign sources, these have become scarce and very expensive ($850 per monkey).

Cynomologus monkeys (Macaca fascicularis) have been used to a limited extent in SE bioassay (Robern et. a., 1975) despite their ready availability and reasonable expense ($250 per monkey). There are no published data relative to the suitability of this species of monkeys for SE bioassay. This communication presents data which show that cynomologus monkeys are suitable for SE bioassay.

MATERIALS AND METHODS

Production of Crude SEA

Crude SEA was prepared by growing Staphylococcus aureus (F_{265}) in 4% NZ Amine (Humko Sheffield, Memphis, TN 38101) in a 14 liter fermentor (New Brunswick Scientific Co., Inc., N.J. 08903) at 37°C for 24 hours, with filtered air supplied at a flow rate of 3-4 liters per minute. The culture growth was spun down in a centrifuge (Sorvall , Dupont Instruments, Newtown, CT. 06470) at 10,000 rpm for 15 min and the culture

supernatant was millipore-filter (Millipore , Millipore Corp., Bedford, MA 01730) sterilized and used as crude SEA.

Determination of SEA level

The amount of SEA in the crude supernatant was estimated by the single diffusion tube assay of Weirether et. al. (1966) and the double diffusion microslide assay of Casman et. al. (1969). The specific Antiserum A and reference enterotoxin A were from R.W. Bennett of the F.D.A., Washington, D.C.

Antiserum A Neutralization of SEA

Antiserum A with a 1:640 titer by the microslide test, was used to neutralize crude SEA. Five milliliters of millipore-filtered undiluted antiserum was mixed with predetermined amounts of SEA and incubated at 37°C for 1 hour prior to bioassay.

Animals

Thirty-eight cynomologus monkeys weighing 2-5 kg were obtained from Primate Imports (34 Munson Street, Port Washington, Long Island, N.Y. 11050). Upon receipt, they were quarantined for 30 days prior to use and certified healthy by the resident veterinarian at the Research Animal Resources of the University of Minnesota.

Oral Feeding

A specific level of Ketamine HCl (Ketaset , Veterinary Products, Bristol Lab., Syracuse, N.Y. 13201) , found non-emetic (Experiment 1) was injected intramuscularly to sedate the monkeys. Sedated monkeys were intubated with lubricated catheter (Sovereign Propylene, Size 8F) and the test material was introduced with a 60 ml syringe as described by Melling (1977). Following recovery from Ketamine sedation (usually 20-30 minutes), the monkeys were observed for emesis continuously for 5 hours.

RESULTS AND DISCUSSION

Data shown in Table 1 indicate that none of the 38 monkeys vomited when injected with Ketamine at doses of 2.7 - 6.0 mg/kg without any test material fed. Also, growth media and saline did not cause any emesis. Thirty-two (84%) of 38 monkeys vomited within 5 hours, between 2-3 hours, in response to feeding crude SEA in amounts ranging from 4.8 - 18.0 µg per monkey. Individual monkeys have been reported to vary in their response to SE (Bergdoll, 1970). Due to limitations on availability of specific Antiserum A, only 24 of the 32 responding monkeys were tested for specific emetic response to SEA. As can be seen, 22 (92%) of these monkeys showed specificity in their response to SEA by not vomiting when neutralized (with Antiserum A) SEA was fed. When these monkeys were fed the same level of SEA without neutralization after 10 days, they did vomit (Data not provided). This suggests that the lack of emetic response to neutralized SEA was not due to

Table 1. Emetic response of Cynomologus Monkeys to Staphylococcal enterotoxin A (SEA) and Growth Media

Test Material	Amount Fed Intragastrically	Number of Monkeys Fed	Number of Monkeys Vomiting	Percent Responding
Ketamine HCl 2.7 - 6.0 mg/kg injected intra-muscularly	None	38	0	0
Brain Heart Infusion Broth	50 ml	38	0	0
NZ Amine NAK Broth	50 ml	38	0	0
Saline (0.85% NaCl)	50 ml	38	0	0
Crude SEA	4.8 - 18.0 ug	38	32	84.2
SEA plus Anti-serum A	4.8 - 18.0 ug plus 5 ml Antiserum A	24	2*	8.3

* With two of the monkeys, crude SEA could not be neutralized with Anti-A but it was accomplished with purified SEA (CA2E) using same batch of Antiserum A in same amount

Table 2. Dose-response of Cynomologus Monkeys to Crude Staphylococcal Enterotoxin A (SEA)

Amount of SEA (µg)	Number of Monkeys		Percent Vomiting
	Fed	Vomiting	
0*	16	0	0
4.8	16	5	31.3
7.2	16	9	56.3
10.8	16	9	56.3
13.2	16	14	87.5
18.0	16	14	87.5

* Each monkey fed 50 mls of sterile NZAmine NAK.

Table 3. Emetic Dose-50 (ED_{50}) of Cynomologus Monkeys to Crude Staphylococcal Enterotoxin A

Identification of Group of Monkeys	Number of Monkeys Used	Range of Weight (kg)	Sex		ED_{50} (μg)
			Male	Female	
02	16	2.5 - 3.0	1	15	6.5*
88	6	4.4 - 5.6	6	0	7.0
93	6	2.2 - 2.9	3	3	7.9
97	5	2.0 - 2.8	2	3	11.0*
All Groups	33	2.0 - 5.6	12	21	6.5 - 11.0

* Determined graphically by plotting the log of dose against probit values.

immunity and that their emetic response was to SEA specifically. Neutralization of crude SEA did not eliminate emesis in 2 of the 24 monkeys. However, when these two monkeys were given purified SEA (CA2E) of the same level as crude SEA, neutralized with the same antiserum A, they did not vomit. One of the monkeys was extremely sensitive to SEA (vomiting in response to 4.8 ug of crude or purified SEA). It is possible that these two monkeys were sensitive to some staphylococcal metabolite other than SE or being a crude preparation, a second SE (unidentified) might have been present in the culture filtrate. This latter suggestion is unlikely because at least some of the other 22 monkeys should have responded if another SE were present. In any event, the data show that a large percentage (92%) of these monkeys showed emesis within 5 hours as a specific response to SEA. Thus, cynomologus monkeys could be used for identification of new SE or for bioassays during purification studies or known enterotoxins.

The emetic dose -50 (ED_{50}) for SEA, B or C in rhesus monkeys has been reported to be 5 ug per animal and 0.02 - 0.03 µg per kg by intragastric and intravenous routes, respectively (Bergdoll, 1970). Cynomologus monkeys were shown to respond to 0.04 µg/kg of SEC by the intravenous route (Robern et. al., 1975). There are no reports of oral feeding of SE for cynomologus monkeys. Table 2 shows data on the dose-response of 16 cynomologus monkeys to crude SEA. These monkeys have not been used for any bioassay prior to this determination. As the dose of SEA increased, the number of monkeys vomiting increased, 5 (31.3%) and 14 (87.5%) of 16

monkeys vomiting when fed 4.8 and 18.0 µg, respectively.

As shown in Table 3, this group of 16 monkeys showed an ED_{50} value of 6.5 µg per monkey as determined graphically using the method described by Hoffman (1963). The dose is comparable to what has been reported for rhesus monkeys (5 µg per 2-3 kg body weight) by Bergdoll (1979). Ideally, purified SEA should have been used to determine the ED_{50} in this species of monkeys but at the time of the study, adequate supply of purified SEA was not available. Table 3 also shows the ED_{50} value for 3 other groups of cynomologus monkeys that have been used to test for enterotoxigenicity of several staphylococcal cultures prior to feeding the various doses of crude SEA. For all monkeys tested, the ED_{50} showed a range of 6.5 - 11.0 µg/monkey.

It would appear as though rhesus monkeys are somewhat more sensitive than the cynomologus monkeys tested in this study, 5 µg versus 6.5 µg per 2-3 kg body weight. This difference could be a reflection of the small sample size utilized in this study. Nevertheless, the data shown here relative to sensitivity and specificity of response to SEA do indicate that cynomologus monkeys are suitable for identification of new SE and also for purification studies with known enterotoxins.

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EXPERIMENT 3

Production of Enterotoxin(s) by *Staphylococcus hyicus*

ABSTRACT

Five strains of *Staphylococcus hyicus* (3 of subspecies *hyicus* and 2 of *chromogenes*) were tested for their ability to produce known enterotoxins serologically and unknown enterotoxins(s) through use of monkey bioassay. None of the five strains produced any detectable level of known enterotoxins A-E. However, all five produced emetic responses in 2 or more of 6 cynomologus monkeys when culture growth was fed intragastrically. One of the five strains (*S. hyicus* subspecies *hyicus*, VII 76) produced emetic responses in 3 of 6 monkeys with 50 ml of culture growth. The other four strains required 1,000 ml of culture growth to produce emetic response in 2 of 6 monkeys. Enterotoxin production is therefore not unique to *S. aureus* species. Since some of these organisms are coagulase and thermonuclease negative, they would be ignored in food hazard potential evaluations.

INTRODUCTION

Staphylococci produce enterotoxins which are the leading cause of bacterial foodpoisoning in many countries (Todd, 1978). The ability to produce one or more of the five known enterotoxins (A-E) is considered

unique to Staphylococcus aureus species (Lotter, and Genigeorgis, 1975, 1977). Therefore, laboratory investigations involved in establishing the cause of bacterial foodborne outbreaks and/or routine quality control procedures utilize detection and enumeration of S. aureus species in foods as an indicator of potential presence of enterotoxins. Coagulase production is the most commonly used criterion for confirming the presence of S. aureus in foods (Baer et. al., 1976). A high correlation between coagulase production and enterotoxigenicity has been reported (Lachica et. al., 1969; Payne and Wood, 1974; Wieneke, 1974; Burbianka, 1971). However, coagulase negative staphylococci have been associated infrequently with foodpoisoning outbreaks (Omori et. al., 1959; Breckinridge and Bergdoll, 1971; Thatcher and Simon, 1956). Danielsson and Hellberg (1977) reported that as much as 7% of coagulase-negative strains tested produced one of the known enterotoxins (A, B, C).

Lotter and Genigeorgis (1975, 1977) concluded that coagulase-negative enterotoxigenic strains of S. aureus were variants or mutants of S. aureus because they were able to isolate coagulase positive organisms from the parent culture after repeated transfers. These authors even suggested that the ability to produce enterotoxins should be used as a taxonomic criterion in the classification of S. aureus. These authors never established the species identity of these organisms with reference strains using DNA:DNA homology.

Devriese et. al. (1978) recently established a new species called S. hyicus containing two subspecies, hyicus and chromogenes. This new species shares two characteristics namely coagulase and thermonuclease production, with S. aureus. Strains that belong to the former subspecies are coagulase variable and thermonuclease positive while the latter are coagulase and thermonuclease negative. These organisms seem to constitute normal flora of food animals (Devriese et. al., 1977; 1978, 1979) and thus, are likely to present problems in identification of potential enterotoxigenic strains in food on the basis of coagulase and thermonuclease tests. It is not known if these organisms produce any known or unknown enterotoxins. The objective of this study was to determine if S. hyicus strains produce known or unknown enterotoxins using in vitro serological assays and bioassays, respectively.

MATERIALS AND METHODS

Organisms

Staphylococcus hyicus was obtained from Dr. L.A. Devriese of Belgium. S. aureus (F_{265}) was obtained from R.W. Bennett of F.D.A., Washington, D.C. Reference strains for S. aureus, S. epidermidis and S. saprophyticus were from American Type Culture Collection (ATCC). These cultures were stored on beads at -20°C and when needed the cultures were revived by placing two beads in Brain Heart Infusion (BHI) or 4% NZ Amine, NAK (NZA) (Humko-Sheffield, Norwick, N.Y.) broth and incubated at 37°C for 24

hours. The broth cultures were transferred twice into fresh broth and incubated for 24 hours prior to use as the inoculum for toxin production.

Culture growth conditions:

Sac culture growth was achieved by the method of Donnelly et. al. (1967) by placing 60 mls double strength NZA (6%) inside 1.4" x 20" dialysis tubing (Union Carbide Corp., Chicago) placed in a 300 ml Erlenmeyer flask. Fifteen milliliters of 0.02 M Phosphate buffer-saline was placed outside the tubing and the unit sterilized at 121°C for 15 minutes. Each flask was inoculated with 0.2 ml of revived 24 hour culture. The flasks were incubated at 37°C for 48 hours on a gyratory shaker at 190 rpm. The culture growth was centrifuged (Sorvall RC-5 Centrifuge, DuPont Co., Newton) at 10,000 rpm for 10 minutes. The supernatant was filter-sterilized and used for bioassay in monkeys and serological assay.

Regular culture growth was produced by a modification of the procedure described by Bergdoll (1970). One-liter flask containing 400 mls of growth medium was inoculated with culture and incubated for 72 hours at 37°C on a shaker (280 rpm). Cell harvest was handled in the same way described above.

The culture growth was concentrated (20-fold) by osmodialysis in polyethylene glycol (Union Carbide Corp., Chicago) at 4°C overnight, when 1,000 ml volume of growth was fed to monkeys.

Test for Cultural Characteristics

Coagulase test was performed on 24 hour culture growth using rabbit plasma (Difco) and Porcine plasma (Hormel Co., Minn.). Test and inter-

pretation of results were done as described by Baer et. al. (1976) and Sperber and Tatini (1975).

Thermonuclease was tested for by the method of Lachica et. al. (1972) with incubation of Toluidine-Blue DNA Agar plates at 37°C for 4 hours.

Hemolysin production was tested for on human and sheep blood agar. Culture filtrates were put in 2 mm diameter wells in agar plates. The plates were incubated at 37°C for 24-48 hours. Presence of any clearing or hemolysis after incuabation was considered positive.

Protein A determination in cell wall was determined on cell pellets from 500 mls of 24 hour culture growth (in BHI) obtained by centrifugation at 10,000 x g for 20 minutes. Cell pellet was resuspended in 30 mls of TSC buffer (0.05 M Tris-HCl, 0.145 M NaCl, 0.015M Citrate, pH 7.4). Fifty units of lysostaphin was added and incubated for 2 hours at 37°C. The pH of pooled supernatant was adjusted to 3.5 and centrifuged. The pH of supernatant was adjusted to 7.0, dialysed against phosphate buffer (0.05M, pH 6.8) for 24 hours and later concentrated in PEG at 4°C overnight. Concentrate was spun down at 3,500 x g for 5 minutes and the supernatant assayed for protein A using human gamma globulin (IgG, Cappel Labs) and purified protein A (Pharmacia Co., Sweden) by the microslide technique of Casman and Bennett (1965).

Establishing species Identity of Staphylococci:

Culture identity was established by conducting DNA:DNA hybridization on DNA s' of unknown strains attached to nitrocellulose filters and exposing

these to ^{3}H-labelled DNA from reference ATCC strains under restrictive conditions (using formamide) at 60°C for 40 hours. The procedure of Meyer and Schleifer (1978), a modification of earlier techniques of Gillespie and Spiegelman (1965) and Denhardt (1965), was used in the study.

Testing for Enterotoxins:

The known enterotoxins A-E were tested for in culture supernatant by the double gel diffusion (microslide) technique. The antisera for types A-E and reference toxins were obtained from R.W. Bennett of F.D.A., Washington, D.C. Culture filtrates of unknown enterotoxigenicity and negative serologically for types A-E, were assayed in cynomologus monkeys.

Bioassay in Monkeys:

Cynomologus monkeys weighing 2-5 kg which showed specific emetic response (Experiment 2) to staphylococcal enterotoxin A (SEA) were used in the study. Ketamine sedated monkeys were intragastrically fed 50 ml volume of culture growth or 20-fold concentrate of 1,000 ml growth by the procedure described by Melling (1977). The monkeys were continuously observed for emetic response for 5 hours following recovery from sedation (20-30 minutes).

RESULTS AND DISCUSSION

The S. hyicus cultures used in this study were isolated from animals (Table 1). The initial characteristics of these organisms were reported by Devriese et. al. (1975). These authors established this species as S. hyicus subspecies hyicus and chromogenes by conducting DNA:DNA hybridization with reference strains (Devriese et. al., 1978). Strains of the former subspecies are coagulase variable while of the latter are coagulase-negative. Therefore, these organisms may not be detected as hazardous organisms on the basis of coagulase test as is presently done.

As can be seen from Table 2, all the S. hyicus cultures were coagulase negative in rabbit plasma following 6 hours incubation. Culture VII 131 was however, positive in pig plasma after 6 hours under the same test conditions. The reference S. aureus was positive and S. epidermidis was negative. Culture VII 76 showed variable reactions in plasmas over repeated testings sometimes yielding 3+ to 4+ reactions in 2 hours with one source of rabbit plasma and not the other. All three S. hyicus subspecies hyicus were thermonuclease positive and two (VII 76 and VII 131) of the three were cell wall protein A negative. The two strains, B04 and VA 608, from the subspecies chromogenes were thermonuclease negative. All strains tested were hemolysin negative except S. aureus (F_{265}). Most S. aureus strains are considered positive for coagulase, thermonuclease and cell wall protein A (Buchanan and Gibbons, 1974). Based on these criteria, these S. hyicus cultures may represent variants

Table 1. Sources of Strains of S. hyicus Used in This Study

Designation of S. hyicus strain	Subspecies	Source of Isolation (Devriese et. al., 1978)
VII 76	hyicus	Nares and Skin of Belgian Poultry (Healthy)
VII 131	hyicus	Nares and Skin of Belgian Poultry (Healthy)
VA 519	hyicus	Nares and Skin of Belgian Swine (Healthy)
VA 608	chromogenes	Belgian Swine (Healthy)
B 04	chromogenes	Lesions in Belgian Cows

Table 2. Characteristics of Staphylococcal Cultures Used in This Study.

Culture Identification	Thermonuclease Production	Hemolysin Production	Cell Wall Protein A	Coagulase Production				Status
				Difco Rabbit Serum Hours		Porcine Rabbit Serum Hours		
				2	6	2	6	
S. hyicus VII 76	+	--	--	--	--	--	1+	**Variable**
S. hyicus VII 131	+	--	--	--	--	2+	3+	Variable
S. hyicus VA 519	+	--	+	--	--	--	--	Negative
S. hyicus VA 608	--	--	ND	--	--	--	--	Negative
S. hyicus B04	--	--	ND	--	--	--	--	Negative
S. aureus F_{265}*	+	+	+	3+	4+	3+	4+	Positive
S. epidermidis ATCC 14990**	--	--	--	--	--	--	--	Negative

+ Positive -- Negative

* Positive
** Negative } Controls for coagulase test

ND Not Done

Table 3. DNA Homology Values for S. hyicus Cultures with ATCC Reference Strains

DNA filter	S. aureus 12600	S. epidermidis 14990	S. saprophyticus 15305	S. hyicus VII 131
S. aureus 12600	100	13	13	9
S. epidermidis 14990	8	100	10	4
S. saprophyticus 15305	13	11	100	13
Calf thymus	6	12	12	3
S. hyicus VII 76	17	21	21	92
S. hyicus VII 131	9	14	16	100
S. hyicus VA 519	3	10	23	28

Table 4. Response of Cynomologus Monkeys to Intragastric Feeding of S. hyicus Culture Growth

Strains of S. hyicus	Sac Culture[a]			Broth Culture				Enterotoxin[b] A - E
				1,000 mls.		50 mls.		
	Volume (mls.)	No Vomiting No. Fed	First Emetic Episode (min.)	No. Vomiting No. Fed	First emetic Episode (min.)	No. Vomiting No. Fed	First Emetic Episode (min.)	
VII 76	60	5/6	166(2), 115(6), 95(5), 125(6), 23(2)	ND	0	3/6	132(4), 102(3), 128(3)	---
VII 131	40	2/6	123(1), 104(1)	2/6	112(1), 197(2)	1/6	73(4)	---
VA 519	80	3/6	234(5), 193(3)	2/6	158(3), 171(2)	1/6	103(4)	---
VA 608	ND	ND	0	3/6	199(1), 136(1), 94(1)	0/6		---
B04	ND	ND	0	2/6	75(1), 122(2)	0/6	---	---

--- Negative

[a] Sac culture technique of Donnelly et. al. (1967)

[b] Double gel diffusion (microslide) technique of Casman et. al. (1965).

ND Not done.

( ) Frequency of emesis.

or mutant of S. aureus (Lotter and Genigeorgis, 1975, 1977). But as shown in Table 3, these S. hyicus cultures are not S. aureus as has earlier been shown by Devriese et. al. (1978). The data confirm them to be distinctly a new species. DNA homology values were 3, 9, and 17% with S. aureus for S. hyicus strains VA 519, VII 131, and VII 76, respectively. A high homology value of 92% was obtained for cultures VII 76 and VII 131. Culture VA 519 with a 28% homology might represent another subspecies of hyicus (Devriese et. al., 1978).

Data in Table 4 show that all five strains of S. hyicus reported were positive for enterotoxin in the basis of vomiting response in cynomologus monkeys. Emesis within 5 hours in at least two of six monkeys following feeding of culture filtrates is considered a positive response to staphylococcal enterotoxin (Bergdoll, 1970; Surgalla et. al., 1953). These cultures were negative serologically for the five known enterotoxins. This suggests that these cultures produce new enterotoxin(s) and therefore would be a public health hazard. One of these cultures, VII 76 is in the same category as the known enterotoxin-producers in that 50 ml culture growth was positive for enterotoxin (3 of 6 monkeys vomited within 5 hours). The other four cultures would be considered weak enterotoxin-producers as they required 1,000 mls culture growth to elicit emesis in two of six monkeys. The data presented here show that Staphylococcus species other than S. aureus produce biologically active new enterotoxin(s) and that these organisms would normally be ignored as non-hazardous on the basis of routinely used

coagulase test with rabbit plasma. It is not known if these organisms produce the same or different enterotoxin(s). Studies are underway to establish the new enterotoxin produced by culture VII 76 as a distinct serological entity as has been done with known enterotoxins.

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EXPERIMENT 4

Production of Neutralizing Antibodies for Enterotoxin Produced by Staphylococcus hyicus (VII-76)

ABSTRACT

Partially purified enterotoxin(s) from S. hyicus (VII 76) was injected into a New Zealand White rabbit over a 6-week period. The serum obtained from the rabbit neutralized emetic response in 2 of 2 cats and 2 of 2 cynomologus monkeys administered by the intraperitoneal and intragastric routes, respectively. This indicates that the biologically active enterotoxin(s) produced by S. hyicus (VII 76) is antigenic.

INTRODUCTION

The production of new biologically active enterotoxin(s) by Staphylococcus hyicus has been demonstrated (Experiment 3). Purification and production of neutralizing antibodies for enterotoxin is needed to establish this as a new enterotoxin. This communication presents evidence for this new enterotoxin by partial purification and production of neutralizing antibodies.

MATERIALS AND METHODS

Organism:

S. hyicus supspecies hyicus (VII 76) was obtained from Dr. L.A. Devriese of Belgium and was identified to be enterotoxigenic by monkey feeding test.

Growth of Organism:

Forty-liters of culture growth was produced using 400 ml of NZ Amine NAK (Humko Sheffield, Norwich, N.Y. 13815) containing 0.2% filter-sterilized yeast extract in 1-liter Erlenmeyer flasks. Incubation was at 37°C for 48 hours on a gyratory shaker at 280 rpm. Culture supernatant obtained after centrifugation (Sorvall Dupont Inst., Newtown, CT 06470) at 10,000 rpm for 5 minutes was placed in dialysis tubing and concentrated by osmodialysis against 50% polyethylene glycol at 4°C overnight. The concentrate was lyophilized and stored at room temperature.

Partial Purification of Enterotoxin(s):

The enterotoxin purification methods of Borja and Bergdoll (1967) and Schantz et. al. (1972) were used with slight modifications. The procedures used were as follows: Lyophilized culture growth was dissolved in 800 mls of 0.01M sodium phosphate buffer of pH 5.5. The reconstituted culture was spun down at 15,000 rpm for 20 minutes in a refrigerated centrifuge and the supernatant saved. Resulting pellet was redissolved in 200 mls of

same buffer and the same procedure repeated. The pH of the pooled reconstituted supernatants was adjusted to 5.5 with 0.01M H_3PO_4 and kept at 4 C.

Pre-treatment of Carboxymethylcellulose (CMC):

The CMC used was Selectacel ion-exchange, no. 77, type 20 (Carl Schleicher and Schuell, Co.). A procedure similar to that described by Borja and Bergdoll (1967) and Peterson et. al. (1956) was used. One-hundred and sixty grams of CMC was added to 1 liter of distilled water and the paste formed was thoroughly mixed. Seven hundred milliliters of 0.1N NaOH was added to the CMC paste in a beaker prior to placing in an ice-water bath for 30 minutes (with occasional stirring). The CMC was washed several times with distilled water until the pH was 7.6. Later, 700 mls of 0.1N HCl was added, mixed thoroughly and washed several times till the pH was 5.3. Finally, the CMC was resuspended in 0.01M sodium phosphate buffer of pH 5.5 and washed several times till the pH remained constant at 5.5 (overnight at 4 C).

Loading of CMC Column:

A glass column with an inside diameter of 2.5 cm and 45 cm long (Pharmacia Fine Chem., Uppsala, Sweden) was used. The equilibrated CMC 0.01M, pH 5.5) was introduced into the column and packed with liquid nitrogen. One hundred milliliters of 0.01M sodium phosphate buffer of pH 5.5 was passed through the column at a rate of 40 mls/hour. Four hundred milliliters of reconstituted supernatant (representing 20 liter growth) was

percolated through the column at a flow rate of 18 mls/hour.

Elution From CMC Column:

Sodium phosphate buffer of 0.01 M and pH 5.0. was passed through the column at 38 mls/minute until the "breakthrough" peak containing unabsorbed material returned to the baseline as indicated by the U-V analyzer (LKB Unicord II 8300, S-16125 Bromma I, Sweden) connected to the column. Elution was done by stepwise increase in molarity and pH of sodium phosphate buffer: 0.02M, pH 6.0; 0.06M, pH 6.4; 0.1M, pH 6.8; and 0.2M, pH 7.5 at a flow rate of 38 mls/hour and eluate collected in 10 ml. fractions. The optical density at 277 nm and pH of fractions representing the peaks were determined and were pooled. Fractions representing each peak were pooled, dialysed against distilled water for 12 hours at room temperature and assayed for emesis in cynomologus monkeys (Macaca fascicularis).

Protein Determination:

The procedure described by Lowry et. al. (1951) was used.

Antiserum Production:

A female, New Zealand White rabbit weighing 4.3 kg was used to produce antibody to concentrated, pooled fractions of partially purified S. hyicus (VII 76). A modification of the protocol described by Casman and Bennett (1964) was used.

Five milliliters of pre-immunization serum was obtained from the central ear vein. Blood was allowed to clot at room temperature for 30 minutes in centrifuge tubes, fibrin clots were dislodged from tube wall with a glass rod. The clotted blood was spun down by centrifugation (GLC-2 Refrigerated Sorvall Centrifuge, Newton, CT. 06470) at 1,500 rpm for 10 minutes. The supernatant (serum) was stored frozen.

The antiserum to partially purified toxin from S. hyicus (VII 76) was prepared by injecting the filter-sterilized toxic factor mixed in a ratio of 1:1 with Freund's complete adjuvant (Difco Lab., Detroit, Michigan) subcutaneously at several sites in a 6 by 15 cm shaved area on either side of the spine (area easily accessible in the restrainer). The protocol of injection was 11.8, 17.8, 35.6, 89.0, and 178 ug (Protein) of partially purified S. hyicus (VII 76) concentrate at weeks 0, 1, 3, 4, and 5, respectively. Thirty milliliters of bloood was collected through the ear vein after the sixth week and serum was prepared as described earlier.

Serological Assay:

The microslide double gel diffusion technique of Casman and Bennett (1963) was used. Serial dilutions of antiserum to S. hyicus (VII 76) growth were assayed against crude and partially purified culture growth from the same strain (VII 76). The pre-immunization serum was also tested in a similar manner.

Antiserum Neutralization of Enterotoxin(s):

The procedure used was similar to that described by Bergdoll et. al. (1965). The antigen-antiserum mixture was incubated for 1 hour at 37°C prior to intraperitoneal injection of cats and intragastric feeding of cynomologus monkeys using the procedure described earlier (Experiment 2).

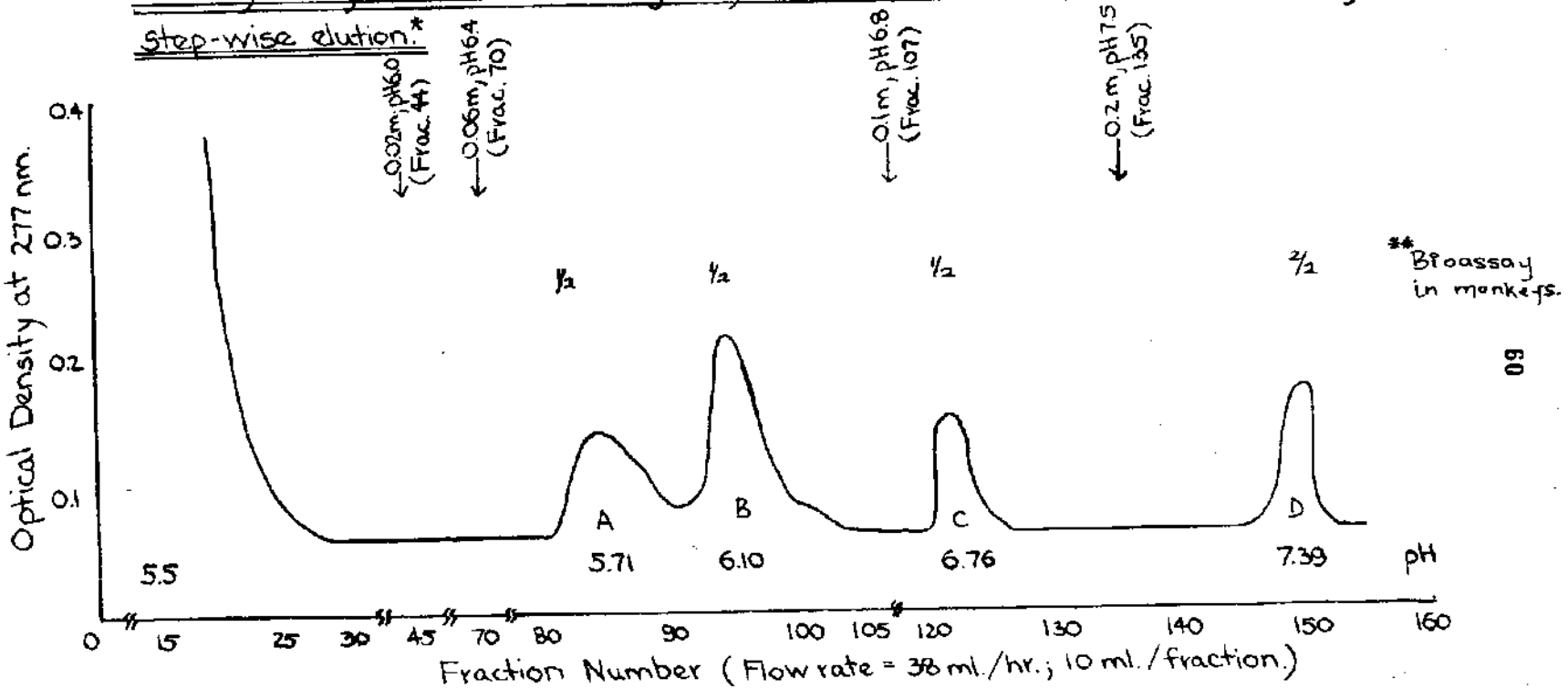
RESULTS AND DISCUSSION

During preliminary trials with S. hyicus (VII 76) it was found that the 48 hour culture growth showed the highest optical density at 650 nm , viable cell count and also 50% of the cynomologus monkeys fed the culture filrates, vomited within 5 hours. Culture growth after 56 hours was found more toxic, eliciting emesis in 80% of the monkeys fed. Since 56 hour growth might have more extraneous proteins, 48 hour culture growth was employed for purification. CMC adsorption and desorption of enterotoxins under specific selective conditions have been used for purification of known enterotoxins (Schantz et. al. 1965, 1972; Avena and Bergdoll, 1967; Chang and Bergdoll, 1979; Borja et. al., 1972).

As shown in Figure 1, there were four major peaks following stepwise elution from CMC. Based on the optical density values at 277 nm, it was obvious that the amount of protein present in these fractions was small. When 15 ml of pooled fractions representing each of the four peaks (A, B, C and D) was fed to each of two monkeys, emesis was noted within 5 hours in one of two monkeys for each of peaks A, B, and C, and two of two monkeys

Figure 1

Carboxymethylcellulose chromatography of concentrated S bykus (VII 76) using step-wise elution.*



** Bioassay in monkeys.

09

* Sodium phosphate buffer.
Fractions with peaks were pooled and assayed in cynomologus monkeys.

** Number of monkeys vomiting/Number of monkeys fed.

Table 1. Influence of Anti-Strain VII 76 Serum on Biological Activity of Partially Purified and Crude S. hyicus (VII 76).

Animal Used	Route of Administration	Material fed or injected and response: Before Serum Neutralization — Material Administered*	Number of Animals Used	Number of Animals Vomiting	After Serum Neutralization — Material Administered	Number of Animals Used	Number of Animals Vomiting
Cats	Intra-peritoneal	0.70 mls. of CMC-eluate of S. hyicus (VII 76)	2	2	0.70 ml. of CMC eluate plus Anti-serum**	2	0
Monkeys	**Intragastric**	50 mls. of crude S. hyicus (VII76) growth	**2**	**2**	50 mls. of crude S. hyicus (VII76) filtrate plus antiserum	**2**	**0**

* Per cat or monkey.

** 1.5 and 2.5 mls of Antiserum were used for neutralization of enterotoxin with each cat and monkey, respectively.

for peak D. Due to the overall low OD_{277}, the peaks were pooled and used as partially purified toxin to inject into the rabbit. A great deal of biochemical manipulations are needed to purify enterotoxins when present in small quantities in the presence of large amounts of other protein metabolites (Change and Bergdoll, 1979).

Data shown in Table 1 demonstrate the presence of enterotoxin-neutralizing antibody in the serum produced against partially purified S. hyicus (VII 76) growth. Two of two cats injected partially purified S. hyicus (VII 76) vomited but none of the two showed any response when injected the same amount of enterotoxin(s) neutralized with antiserum. To eliminate build-up of resistance being responsible for the lack of response to neutralized material, the same level of partially purified enterotoxin was injected 2 days later and emesis resulted in the two cats (date not provided). Two of two monkeys that vomited in response to 50 mls. of crude culture filtrate of S. hyicus (VII 76), did not vomit when fed antiserum neutralized culture growth. This confirms that enterotoxin-neutralizing antibody was indeed present in the antiserum.

An attempt was also made to identify the enterotoxin antigen in the microslide technique for possible application for in vitro serological assay. Dilutions of pre- and post-immunization sera tested against undiluted crude and partially purified S. hyicus (VII 76) indicated the presence of a number of antigen-antibody precipitin lines (data not shown). Pre-immunication serum showed a lower antibody titer (1:8) than the post-immunization serum

(1:90) in terms of appearance of precipitin lines. Antigen-antibody precipitin lines were also detected when tested with culture growth from other S. hyicus or S. aureus cultures against the 1:90 dilution of immune serum. This indicates that the antigen(s) used was of low purity and a greater degree of purification is needed to produce good quality antiserum that will permit establishment of in vitro serological assay. Efforts are underway to accomplish this.

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SECTION B

EXPERIMENT 1

Biological Activity of Intracellular Staphylococcal Enterotoxin A

ABSTRACT

Intracellular staphylococcal enterotoxin A released by lysostaphin treatment of Staphylococcus aureus cells was found to be biologically active in cynomologus monkeys. The activity was comparable to that of extracellular enterotoxin A; 6 of 6 monkeys vomited within 5 hours with extracellular enterotoxin and 4 of 6 also vomited in response to the same serological level (4.8 µg per monkey) of intracellular enterotoxin. One other monkey responded to a slightly higher level (6 µg) of intracellular enterotoxin. Feeding S. aureus cells containing intracellular enterotoxin A to cynomologus monkeys resulted in emesis in 3 of 5 monkeys within 3 hours. This suggests that consumption of S. aureus cells could lead to staphylococcal food poisoning.

INTRODUCTION

Growth of enterotoxigenic staphylococci in foods could result in production of extracellular enterotoxins and cause foodpoisoning (Bergdoll, 1970, 1979). Such growth may also result in staphylococcal cells containing intracellular enterotoxins (Lee, 1978; Miller and Fung, 1976, 1977). Certain bacterial toxins require activation of their biological activity by some

enzymes before or after release (Boroff and Dasgupta, 1971). A correlation has been established between the serological and biological activity of extracellular staphylococcal enterotoxins (Raj and Bergdoll, 1969; Bergdoll, 1970) but information is not available on the biological activity of intracellular staphylococcal enterotoxin.

The purpose of this study is to investigate if intracellular staphylococcal enterotoxin is biologically active and if its in vivo release from cells could cause intoxication in monkeys.

MATERIALS AND METHODS

Organism:

Staphylococcus aureus (F_{265}) an enterotoxin A producer which was obtained from R.W. Bennette of F.D.A., Washington, D.C. was used in the study.

Growth of Organism:

The organism was grown in 4% NZ Amine broth (Sheffield Products, Memphis, TN 38101) supplemented with 0.2% yeast extract, for 24 hours at 37°C in a 14 liter fermentor (New Brunswick Scientific Co., N.J.) filter-sterilized air supply at a flow rate of 3-4 liters per minute and a stirrer speed of 180 rpm. Cells were harvested by centrifuging culture growth at 17,000 X G for 10 minutes in a centrifuge (Sorvall, DuPont Instrument, Newtown, CT 06470). The supernatant served as extracellular Staphylococcal enterotoxin A (ESEA).

Intracellular Enterotoxin A

Cell pellets were washed 4 times with 0.05 M Tris (hydroxymethy-aminomethane) buffer of pH 7.4. Supernatant from the last wash was concentrated 50-fold by osmodialysis against 50% polyethylene glycol (PEG) at 4°C. The concentrate was assayed for SEA by the microslide technique of Casman and Bennett (1965).

Lysis of Cells:

Washed cells were suspended in Tris buffer at about 5.0×10^9 cfu/ml and lysed with 15 units of lysostaphin per ml (Sigma Chem., Missouri) at 37°C for 2 hours on a gyratory shaker at 280 rpm. DNAse and RNAse (Sigma Chem., Missouri) were added to reduce viscosity of the lysate. Degree of lysis was estimated by viable cell count on Baird Parker Agar (BPA) and optical density at 650 nm.

Detection of SEA:

Extracellular SEA concentration was determined by the microslide gel diffusion technique of Casman and Bennett (1965) and single gel diffusion in tube (Weirether, 1966). Only the microslide technique was used for ISEA detection following lysostaphin lysis of cells because of accompanying cloudiness.

Preparation of Viable Cells for in vivo Release of Enterotoxin A:

Cell pellets from fermentor growth were resuspended and washed 4 times

with 0.05M Tris buffer of pH 7.4. Cell pellets from the last wash were stored frozen at -20°C. Prior to use, cell pellets were placed in a refrigerator for 16-18 hours for defrosting. Liquid obtained from this cell paste after centrifugation was assayed for presence of SEA by the microslide technique. The defrosted cells were resuspended in 50 ml of sterile saline for feeding of the monkeys. An aliquot of supernatant from the cell suspension fed to the monkeys was also assayed for SEA by the microslide assay.

Bioassay with Cynomologus Monkeys:

Lysostaphin-released ISEA, ESEA and viable cells (containing ISEA) were assayed in cynomologus monkeys that showed specific responses to staphylococcal enterotoxin (Section A, Experiment 2). The procedure of Melling (1977) was used for feeding the material to the monkeys.

When cells were fed, fresh fecal samples were taken at 0, 8, 24, 30, 48, and 72 hours. One gram of feces was added to 9 mls of phosphate buffer, was thoroughly mixed on a vortex mixer before plating on BPA after serial dilution.

RESULTS AND DISCUSSION

Data presented in Table 1 show the response of cynomologus monkeys to intragastric feeding of crude extracellular and intracellular enterotoxins (ESEA and ISEA respectively). Five of six monkeys vomited in response to 4.8 µg of ESEA each and the sixth monkey (97G) required 16 µg. Four of six

Table 1. Biological Activity of Extracellular and Intracellular[a] Staphylococcal Enterotoxin A in Cynomologus Monkeys*

	Extracellular Enterotoxin A			Intracellular Enterotoxin A		
Monkey Identification	Amount of toxin (ug)	Time of 1st emetic episode (min)	No. of times of emesis	Amount of toxin (ug)	Time of 1st emetic episode (min)	No. of times of emesis
02B	4.8	99	3	4.8	92	9
02F	4.8	111	3	4.8	103	6
02L	4.8	84	6	4.8	98	2
02D	4.8	96	8	4.8	90	3
02K	4.8	126	3	4.8	--	0
				6.0	173	2
97G	16.0	125	2	15.0	---	0

* Specificity of responses by monkeys to staphylococcal enterotoxin A was ascertained by serum neutralization test.

a Enterotoxin A released by lysostaphin treatment of S. aureus cells.

-- No emesis observed.

Table 2. Response of Cynomologus Monkeys to Feeding Viable *S. aureus* Cells*

Monkey Identification	Emetic response Times** of Vomition	Other observations
02B	1 hour 23 minutes 1 hour 27 minutes 2 hours 9 minutes 2 hours 38 minutes 31 hours 37 minutes 31 hours 57 minutes	There was evidence of diarrhea after about 1 hour 24 minutes. Exhibited great distress after about 14 hours.
02D	2 hours 39 minutes 3 hours 3 minutes	Did not begin eating food until after 14 hours.
02L	1 hour 35 minutes	Showed great distress before vomition.
02F	No emesis	Began eating after 3 hours.
02K	No emesis	Very quiet and did not eat until after 14 hours.

* Each monkey was fed 2.5×10^{13} *S. aureus* cells intragastrically; the level of intracellular enterotoxin was 6 μg.

** Elapsed time after feeding cells.

Table 3. Faecal Excretion of S. aureus (F_{265}) by Cynomologus Monkeys Fed Live Organisms*

Identification of Monkey	Viable Cell Count on Biard-Parker Agar (cfu/ml) after*				
	8 hours.	24 hours	30 hours	48 hours	72 hours
02B	1.8×10^{10}	7.0×10^{8}	9.3×10^{6}	3.2×10^{6}	2.6×10^{5}
02D	2.2×10^{10}	7.4×10^{8}	1.6×10^{7}	1.5×10^{6}	2.5×10^{5}
02L	7.0×10^{6}	4.5×10^{7}	4.2×10^{7}	4.9×10^{6}	1.2×10^{4}
02F	1.6×10^{10}	5.5×10^{7}	9.0×10^{6}	6.8×10^{4}	2.5×10^{2}
02K	1.6×10^{10}	2.2×10^{7}	1.5×10^{7}	4.3×10^{5}	4.0×10^{3}

* 2.5×10^{13} viable cells.

monkeys given 4.8 µg of ISEA, also responded by vomiting within an incubation period similar to that of ESEA. One of the monkeys (02K) vomited when given a higher level (6.0 µg) of ISEA, suggesting development of immunity in this monkey between feedings. ESEA was first fed to monkeys and same dose of ISEA given later. Resistance build-up by monkeys to staphylococcal enterotoxins has been reported (Surgalla et. al., 1953). These data indicate that the ESEA and ISEA are comparable in their toxicity to cynomologus monkeys. They also demonstrate that no activation is required after release as reported for type E botulinum neurotoxin (Boroff and Dasgupta, 1971). Although lysostaphin was employed for release, it is unlikely that this enzyme has any effect since feeding of viable staphylococcal cells (not exposed to lysostaphin) elicited emesis (Table 2).

When humans consume foods containing S. aureus cells and ESEA, they show foodpoisoning symptoms within 2-6 hours (Bergdoll, 1970). While the ESEA might cause rapid symptoms, the ISEA present in the S. aureus cells may cause delayed intoxication because release of such toxin may require some time. To verify this, cynomologus monkeys were intragastrically fed viable S. aureus cells containing ISEA sufficient to cause intoxication (as seen in Table 1). As shown in Table 2, three of five monkeys vomited within 2.80 hours after being fed the cells. This suggests that ISEA is released rapidly in the gastro-intestinal tract (GIT) to cause intoxication within the time normally associated with typical staphylococcal foodpoisoning. Two (02F and 02K) did not vomit indicating the lack of release of sufficient levels of

ISEA in the appropriate area of the gastro-intestinal tract. One of the monkeys, 02B, which appeared to be the most sensitive in this group (Table 1), also showed delayed vomition (after 30 hours).

It is possible that the viable S. aureus fed might have multiplied in the gastro-intestinal tract and produced SEA causing delayed vomiting. It does not appear to be the case because data shown in Table 3 do not support this. The responding and non-responding monkeys to SEA showed a steady decline in fecal S. aureus count over a 72 hour period. Delayed emetic responses were noted in humans suffering from staphylococcal foodpoisoning when emesis occurred after 2 and 3 days in two patients respectively (Efferson et. al, 1975). However, the data presented here are inadequate to draw definite conclusions regarding delayed intoxication by ISEA.

The information provided here do indicate that ISEA is biologically active and is released in vivo to cause staphylococcal intoxication. This might explain at least in part, the reasons for lack of demonstrable amounts of enterotoxins in foods which caused typical staphylococcal foodpoisoning in humans as only extracellular enterotoxin is tested for.

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EXPERIMENT 2

Influence of Environmental Factors on Release of Intracellular Staphylococcal Enterotoxin A

ABSTRACT

The influences of temperature, water activity (a_w) and pH on release of intracellular enterotoxins A and D from from Staphylococcus aureus (Strain 237) cells grown at low a_w (0.90) were evaluated by determining optical density at 650 nm, viable cell count on Baird-Parker Agar and presence of enterotoxins A and D by the microslide immunodiffusion assay before and after exposure to the test experimental conditions. Cells grown at low a_w (0.90) did not release any detectable levels of enterotoxin A or D when resuspended in Tris buffer of the same a_w (0.90). However, when cells grown at 0.90 a_w were resuspended in Tris buffer of 0.99 a_w, there was release of enterotoxin A and D which was temperature and pH dependent. Enterotoxin A was released at a faster rate at 45°C and at pH 8.5 than at 4°C, 21°C , or 37°C and pH 5.0. The release of enterotoxins under these conditions did not result from lysis of S. aureus cells suggesting the involvement of an active transport of enterotoxins which is optimal at 45°C and pH 8.5.

INTRODUCTION

Staphylococci are unique among foodborne pathogens in that they are able to grow and produce enterotoxins under conditions that adversely affect others. They grow in the range of 0.83 - 0.99 water activity (a_w), of pH 4-10 and presence or absence of oxygen (1, 5, 6, 14). While growth of staphylococci occurs under these conditions of low a_w, pH and oxygen tension, the level of enterotoxin produced is considerably reduced (1, 2, 7, 12, 19). Troller (17, 18) and Troller and Stinson (19) demonstrated that extremely small amounts of enterotoxins A or B were produced in foods of low a_w adjusted with glycerol despite good growth ($>10^8$ cfu/ml). Lee (8) investigated the influence of a_w and humectant on synthesis of staphylococcal enterotoxin A (SEA) and found that while enterotoxin was synthesized at reduced rates at low a_w it's release was restricted when Aw was decreased below 0.95. This situation would result in accumulation of intracellular staphylococcal enterotoxin A (ISEA) with trace amounts of extracellular staphylococcal enterotoxin A (ESEA). Niskanen (11) using less sensitive extraction procedures concluded that there was no detectable intracellular enterotoxin in S. aureus cells grown in a dialysis sac under aerobic conditions. On the other hand, Miller and Fung (9, 10) demonstrated the existence of SEB using radioimmunoassay. Such intracellular staphylococcal in foods could be a public health hazard if biologically active (demonstrated in Experiment 5) and released in vitro or in vivo.

The purpose of this investigation was to evaluate release of ISEA in vitro under conditions of increased a_w, various temperatures (4 - 100°C) and pH of 5, 7, and 8.5.

MATERIALS AND METHODS

Bacterial Culture:

Staphylococcus aureus Strain 237 (an enterotoxin A and D producer) was obtained from R.W. Bennett of the Food and Drug Administration, Washington, D.C. The stock culture was stored at -20°C on beads.

Growth Conditions:

The growth medium with an a_w of 0.90 was prepared with 112.99g of Glycerol (Fisher Scientific Company, N.J. 07410), 303.2 ml of distilled water and 20g of NZ Amine NAK (Humko-Sheffield, Norwick, N.Y.). The pH was adjusted to 7.0 with 2N NaOH prior to sterilization at 121°C for 15 minutes. After cooling at room temperature, 0.2% of filter-sterilized yeast extract (BBL, Division of Becton, MD 21030) was added. Medium of a_w 0.99 was made with 4% NZ Amine supplemented with yeast extract as mentioned above with no glycerol.

Water activity measurements were made on a Sina model EZFBA-Hx humidity sensor (Beckman Instrument Inc., Cedar Grove, N.J.) connected to a model SMT hygroline recorder of the same manufacturer.

Working culture of S. aureus (237) was obtained by transferring two beads aseptically into sterile NZ Amine broth and incubated for 18 hours at 37°C and used as the inoculum. Inoculated media in one-litre Erlenmeyer were incubated on a gyratory shaker (280 rpm) for 24 hours and 48 hours for a_w 0.99 and 0.90 media, respectively.

Conditions for Non-growing Cells:

Cells were harvested from culture growth by centrifugation (Sorvall, DuPont Co., Newton, CT 06470) for 10 min at 8,000 rpm. Cell pellets from low (a_w) media were washed four times with the corresponding medium, each time using 100 mls. The supernatant from the last washing was concentrated 50-fold in dialysis tubing (Union Carbide Corp., Chicago) submerged in 50% polythelene glycol (PEG) overnight at 4°C and later assayed for staphylococcal enterotoxin A (SEA).

Exposure of Washed Cells to Various Conditions

Water Activity:

Washed cells from media of a_w 0.90 were suspended in 10 mls of Tris buffer of a_w 0.99 to a final cell concentration of about 10^{11} cells/ml. For assays with no change in a_w, washed cells from a_w 0.90 and 0.99 media were suspended in Tris buffer plus NaCl (a_w 0.90) and Tris buffer, (0.99) respectively.

pH:

Three different pH's, 5.0, 7.0, and 8.5 were used to investigate the effect of pH on release of intracellular enterotoxin. Filter-sterilized 2N NaOH and HCl were used for pH adjustment and the volume in each assay tube was kept at a constant volume of 10 mls.

Exposure Temperatures:

Tubes containing 10 mls cell suspensions were kept in water baths of 21°, 37°, 45°, 60°and 98-100°C. A refrigerator was used for the 4°C incubation. Cell suspensions were kept under static conditions (no shaking) during exposure to various temperatures.

Assay Methods:

Prior to exposure of the 10 ml aliquots to any condition, 1.0, 0.5 and 0.1 ml portions were removed for viable cell counts on Baird Parker Agar (BPA), optical densities at 280 nm and OD_{650} readings respectively. For OD_{280} determinations, 0.50 ml portion was spun down at 15,000 rpm for 20 minutes and the supernatant was used for assay. All spectrophotometric determinations were done on a Hitachi Spectrophotometer model (Hitachi, Ltd., Tokyo, Japan).

Enterotoxin assay was done using 5 mls of the lyophilized supernatant rehydrated in 0.25 ml. (20-fold concentration) of Brain Heart Infusion plus saline for the microslide technique described by Casman and Bennett(3).

Amount of enterotoxin A present was estimated by finding the end point in the microslide using serial two-fold dilutions and multiplying this titer with the sensitivity (0.06 ug/ml) and the volume of rehydrate.

Chemicals:

Chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) was used at a dosage of 100 ug/ml of cell suspension. Lysostaphin (Sigma Chemical Co., St. Louis, Mo.) was used at a dosage of 500 µg/ml aliquot of cell suspension. Enterotoxin A and Antiserum A were obtained from R.W. Bennett of the F.D.A., Washington, D.C.

RESULTS AND DISCUSSION

Data presented in Table 1 show that intracellular staphylococcal enterotoxin A (ISEA) was released within the temperature range of 21-60°C when cells grown at 0.90 a_w were resuspended at 0.99 and not at 0.90 a_w. ISEA was detected after 48 hours at 4°C and not after 15 hours as shown. ISEA was rapidly released within 15 minutes at 60°C. It would appear that cells grown at low a_w of 0.90 would need to be exposed to a higher Aw of 0.99 to bring about release of ISEA.

Data presented in Table 2 show that there was no significant change in the viable S. aureus population and/or optical density at temperatures of 21-45°C suggesting that the release of enterotoxin was not due to death or lysis of S. aureus cells under these conditions.

Table 1 Influence of Aw and Temperature on Release of SEA

Temperature °C	Duration of Exposure	Water Activity of Growth Medium*	Water Activity of Suspending Medium**	Enterotoxin A
4	15	0.90	0.90	ND
		0.90	0.99[a]	ND
21	4	0.90	0.90	ND
		0.90	0.99	+
37	2	0.90	0.90	ND
		0.90	0.99	+
45	2	0.90	0.90	ND
		0.90	0.99	+
60	0.25	0.90	0.90	ND
		0.90	0.99	+
100	0.25	0.90	0.90	ND
		0.90	0.99	ND

a SEA was detected after 48 hours.

ND Not detected.

\+ SEA detection in 20-fold concentrate.

* NZ Amine NAK plus glycerol.

** Tris buffer plus NaCl or Tris buffer without NaCl.

Table 2. Cell Viable, Direct Microscopic Count and Optical Density of S. aureus Exposed to Various Temperatures*

Time-Temperature Conditions	Viable Cell Count on BPA (cfu/ml.) Before x 10^{11}	After x 10^{11}	Direct Microscopic Count (cells/ml.) Before x 10^{11}	After x 10^{11}	Optical Density at 650 nm Before x 1,000	After x 1000	Increase in OD_{280}
48 hours at 4°C	1.45	0.90	1.62	1.43	0.20	0.19	4.0
2 hours at 21°C	1.21	1.09	1.49	1.39	0.19	0.18	1.0
4 hours at 21°C	1.09	1.04	1.24	1.20	0.19	0.18	2.0
2 hours at 37°C	1.19	1.14	1.52	1.22	0.19	0.18	6.0
2 hours at 45°C	1.14	0.81	1.47	1.41	0.19	0.17	9.0
0.25 hr. at 60°C	1.90	0.43	2.07	1.62	0.20	0.18	12.0
500 µg Lysostaphin 2 hrs. at 37°C	1.85	0.000013	2.10	0.000071	0.20	0.002	63.0

* All assays performed at pH 7.0 and SEA was detected in 20-fold concentrate.

Table 3. Loss of Serological Activity of SEA Following Exposure to Various Time-Temperature Conditions at pH 7.0

Time-Temperature Exposure*	Concentration of Crude SEA (μg/ml.)	Percent Loss in Serological Activity**
Control	0.96	0
0.25 hr at 60°C	0.54	43.8
1 hr at 60°C.	0.42	56.3
0.25 hr at 100°C	0.48	50.0
0.50 hr at 100°C	0.30	68.8

* Assays performed simultaneously with those of intracellular SEA release in appropriate waterbaths.

** Levels determined in triplicate slides by the microslide test technique.

Table 4. Release of Intracellular Enterotoxin* in the Presence or Absence of Chloramphenicol at 21 and 37 C

Time-Tempera-Condition	Chloramph-enicol **	Viable Cell Count		Optical Density at 650 nm		SEA
		Before x 10^{11}	After x 10^{11}	Before x 1000	After x 1000	
4 hours at 21°C	Present	1.30	1.30	0.18	0.18	+
4 hours at 21°C	Absent	1.50	1.35	0.18	0.18	+
2 hours at 37°C	Present	1.75	1.25	0.18	0.16	+
2 hours at 37°C	Absent	1.40	1.15	0.16	0.15	+

* From cells grown in a medium of Aw of 0.90 and resuspended in Tris buffer of Aw of 0.99 and pH 7.4.

** 100 µg/ml. of cell suspension.

+ SEA qualitatively detected in 20-fold concentrate.

Table 5. *Influence of pH and Temperature on Release of ISRA*

Time-Temperature Condition	pH*	Viable Cell Count on BPA (cfu/ml.) x 10^{11}		Optical Density at 650 nm x 1000		SEA Released per 10^{12} cells** (µg)
		Before	After	Before	After	
4 Hours at 21°C	5.0	1.28	0.73	0.12	0.12	0.17
	7.0	1.28	1.13	0.13	0.13	0.19[a]
	8.5	1.27	1.05	0.13	0.13	0.23
2 Hours at 37°C	5.0	1.12	0.49	0.12	0.12	0.19
	7.0	1.19	0.78	0.12	0.12	0.27
	8.5	1.06	0.79	0.12	0.11	0.27
2 Hours at 45°C	5.0	1.16	0.35	0.11	0.11	0.26
	7.0	1.32	0.93	0.12	0.11	0.39
	8.5	1.14	0.80	0.12	0.11	0.63

a Exposure for 2 hours at 21°C at pH 7.0 released 0.07 µg/10^{12} cells.

* The three pH's (5.0, 7.0 and 8.5) were similarly used at 4°C for 15 hours, 60°C for 0.25 hour and 100°C for 0.25 hour with no detectable effects.

** Based on Direct microscopic counts.

As shown in Table 3, there was loss of serological activity at 60° and 100°C which increased with time, 43.8 and 56.3% losses after 15 minutes and 1 hour at 60°C, respectively. After 15 minutes at 100°C, a 50% loss of activity was observed. This explains in part the reason for lack of detectable level of enterotoxin at 100°C (Table 1), as toxin was rapidly inactivated.

Data shown in Table 4 indicate that ISEA released from S. aureus cells was not the result of de novo synthesis. Release occurred both in the presence or absence of chloramphenicol which inhibits protein synthesis.

Data presented thus far, seem to suggest that an increase in the a_w of the system causes some activation of enzymes associated with transport of ISEA to the outside environment because growing cells at low a_w (0.90) do indeed excrete SEA. Washing and resuspending of cells in SEA-free medium also creates an SEA gradient causing some passive diffusion as observed with cells kept at 4°C for 48 hours. Therefore, both passive and active diffusion of ISEA might be occurring under these conditions. Aeration and presence of sources of nitrogen and glucose have been shown to enhance excretion of SEB by Miller and Fung (9, 10). It is known that conditions that support optimal growth of S. aureus were not optimal for enterotoxin synthesis and release (4, 15, 10). Also, temperatures higher than 37°C and pH greater than 8.0 were reported optimal for enterotoxin production (16, 20).

To further evaluate if active enzyme transport was involved in the more rapid release of ISEA observed at higher temperatures of 37°and 45°C, cells were resuspended in a buffer of higher a_w (0.99) and three different pH's

(5.0, 7.0, and 8.5), under similar conditions and ISEA released was quantitated. As can be seen from data in Table 5, at each temperature, there was a higher level of ISEA released at pH 8.5 than 5.0. Though there was no difference in the amount of released ISEA at pH 7.0 and 8.5 at 37°C, there was a significant difference between pH 7.0 and 8.5 at 45°C, 0.39 and 0.63 $\mu g/10^{12}$ cells respectively even though there was no lysis of cells. The amount of ISEA released at each pH was also shown to be temperature-dependent, for example, at pH 8.5, 0.27 and 0.63 $\mu g/10^{12}$ cells of ISEA were released at 37° and 45°C respectively. These data suggest selective activation of enzymes associated with transport of ISEA. Thus the information provided confirms the previously published data (13, 20) which indicates the optimum temperature for SEA and SEB production were between 40 - 45°C.

Though these data were obtained with static cultural conditions, it would appear feasible that exposure of reconstituted foods (increasing a_w) to 45°C and pH 8.5 with shaking might enhance release of ISEA for detection in foods. Such an application will however be hampered if large numbers of dead cells are contained in contaminated food unless they autolyse rapidly.

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EXPERIMENT 3

Influence of Certain Gastrointestinal Enzymes on Release of Intracellular Staphylococcal Enterotoxin A

ABSTRACT

Staphylococcus aureus (F_{265}) cells containing intracellular enterotoxin A (ISEA) were exposed to various gastrointestinal enzymes at 4°, 25°, and 37°C. Of the enzymes (Pancreatin, Pepsin, Trypsin, α-amylase, Protease, RNAse, DNase, peptidase, lipase and esterase) tested, only pancreatin released detectable ISEA within 30 minutes without death or lysis of S. aureus cells. Pancreatin might be useful in releasing ISEA from staphylococcal cells in foods for its detection.

INTRODUCTION

When staphylococci grow in foods, the enterotoxigenic strains synthesize and excrete enterotoxins into food and could cause foodborne intoxication if consumed (Bergdoll, 1970). Spahl (1981) demonstrated that when staphylococci grew in foods at low water activity (A_w) and pH, the cells accumulated some of the enterotoxin as intracellular staphylococcal enterotoxin (ISE). Such ISE may be released in foods (in vitro) or in the gastro-intestinal tract (in vivo) to cause intoxication.

Lysostaphin, is an endopeptidase (glycinase) which specifically lyses S. aureus cells releasing ISE and other cell components (Iversen and Grov, 1973). This enzyme has been widely used for in vitro release of ISE and for protoplasting cells (Miller and Fung, 1977; Schuhardt et. al., 1969). Since in vivo release of intracellular staphylococcal enterotoxin A (ISEA) caused emesis in cynomologus monkeys (Section B, Experiment 1), a variety of proteolytic, amylolytic, lipolytic enzymes and perhaps some other enzymes found in the gastrointestinal tract of humans might lyse and/or release ISEA from S. aureus.

The purpose of this investigation was to evaluate the in vitro release of ISEA from S. aureus under the influence of certain gastrointestinal enzymes.

MATERIALS AND METHODS

Organism and Growth Conditions

Staphylococcus aureus (F_{265}) was from R.W. Bennett of the F.D.A., Washington, D.C. Cells were grown in Brain Heart Infusion (BBL Cockeysville, Maryland 21030) supplemented with 0.2% yeast extract in a 14 liter fermentor (New Brunswick Scientific Co., N.J. 08903) at 37°C for 48 hours with an air flow of 3-4 liters and agitated at 180 rpm. Cells were harvested by centrifugation (Sorvall , DuPont Co., Newton, CT 06470) at 8000 rpm for 10 minutes. The pH, OD_{650} and viable cell count on Baird Parker Agar (BPA) and Trypticase Soy Agar (TSA) were det-

ermined prior to harvesting of cells.

Preparation of Cells for Enzyme Treatment

Cell pellets were resuspended in 100 ml portion of 0.05 M Tris buffer of pH 7.4 and were thoroughly resuspended in the buffer using a vortex mixer (Scientific Products, Evanston, Illinois), then spun down at 8,000 rpm for 15 minutes. Cell washing was repeated 3 times and the last supernatant saved for enterotoxin assay. Washed cells with no detectable enterotoxin in a 50-fold concentrate of last washing, were finally resuspended in 10 mls of Tris buffer to contain approximately 10^{11} cells. The pH's of cell suspensions were approximately adjusted using filter-sterilized 2N HCl and NaOH.

Enzyme Treatment

Pancreatin, Trypsin, Pepsin, α-amylase, Protease, Ribonuclease, Deoxyribonuclease, Peptidase, lipase, Esterase and lysostaphin (Sigma Chemical Co., St. Louis, Mo. 63178) were used. Pancreatin, Trypsin and Pepsin each at levels of 2, 4 and 6 mg respectively and 6 mg level of each of lipase, esterase, peptidase, α-amylase, protease, deoxyribonuclease, ribonuclease, were added to 5 mls of Tris buffer. Lysostaphin at a level of 500 μg was added to 5 mls of same buffer. These enzyme solutions were millipore-filter sterilized and added to 10 mls of S. aureus cell suspension. Cell suspension in Tris buffer (5 mls) without added

enzymes served as controls. Portion of cell-enzyme mixture was analyzed immediately for OD_{650}, pH and count on BPA. Tubes containing cell-enzyme mixture were later incubated at the respective desired incubated temperature with occasional mixing of cell suspension.

Enterotoxin Assay

Enzyme-treated cells were spun down at 8,000 rpm for 10 minutes and 10 ml of resulting supernatant lyophilized and rehydrated with BHI in saline. The microslide immunodiffusion procedure of Casman and Bennett (1965) was used to detect staphylococcal enterotoxin A (SEA) No attempt was made to quantitate the level of SEA.

Electron Microscopy

Enzyme-treated cells were fixed in 3% glutaraldehyde in 0.1 M Phosphate buffer and postfixed in 1% osmium tetroxide. Fixed cells were dehydrated successively in 10, 50, 75, and 95% acetone. Cells were cured in Epon Araldite at 60°C for 24 hours and silver sections were made with an ultratome III using a diamond knife. Staining was achieved using Uranyl and lead acetate and electron micrographs were made on Zeiss 10A electron microscope.

RESULTS AND DISCUSSION

Data presented in Table 1 show the effect of exposure of S. aureus (F_{265}) cells to different gastrointestinal enzymes. Control cells and cells exposed to pepsin, trypsin did not release any detectable level of SEA (in a 30-fold concentrate) at levels of 2 and 4 mg during 3 and 2 hour respectively at 37°C. Pancreatin, however, at levels of 2, 4 and 6 mg released detectable amounts of SEA at the same concentrating factor after 3, 2, and 0.5 hour exposure at 37°C, respectively but not after 15 minutes.

Table 2 shows the effects of Pepsin, Pancreatin and Trypsin on the viability, optical densities and release of intracellular staphylococcal enterotoxin A (ISEA) from S. aureus (F_{265}) cells. Trypsin and pancreatin did not result in any significant changes in the optical density (OD_{650}) and viability on BPA plates. Pepsin treatment at pH 2 caused death of S. aureus (four-logcycle decrease) in plate count after exposure. There was no appreciable change in the optical density. This suggests that the adverse pH of 2.0 used for pepsin resulted in loss of viability with no accompanying lysis of cells as has been reported with lysostaphin (Miller and Fung, 1977; Iversen and Grov, 1973).

Table 3 shows the effect of temperature on the activity of Pancreatin on release of ISEA from S. aureus cells. No ISEA release was observed with the control following incubation at 4°, 25°and 37°C. Pancreatin released ISEA at 25°and 37°C but not at 4°C. As in Table 1, there was no

Table 1. Effect of Enzyme Concentration and Length of Incubation at 37 C on Release of Enterotoxin A from S. aureus (F_{265})

Treatment	Level of Enzyme (mg.)	Duration of Exposure (hrs.)	Enterotoxin A 30 x conc.
Control	2	3	-
Pepsin			-
Trypsin			-
Pancreatin			+
Control	4	2	-
Pepsin			-
Trypsin			-
Pancreatin			+
Control	0	0.50	-
Pancreatin	6	0.50	+
Control	0	0.25	-
Pancreatin	6	0.25	-

Table 2. Effect of Certain Gastrointestinal Enzymes on Release* of Enterotoxin A From S. aureus (F_{265})

Treatment	Level of Enzyme (mg)	pH	Before Incubation		After Incubation		Enterotoxin A in 20 x conc.
			OD650 1:100	BPA X10^{10}cfu/ml	OD650 1:100	BPA 10^{10}cfu/ml	
Control	0	7.6	1.0	3.2	1.01	3.2	---
Pepsin	6	2.0	1.0	3.0	0.89	0.0001	---
Trypsin	6	7.6	1.0	3.2	0.90	3.4	---
Pancreatin	6	7.6	1.0	3.2	1.01	3.2	+

* Incubation was at 37°C for 30 minutes; data based on the average of two trials.

BPA = plate count on Biard-Parker agar.

Table 3. Effect of Temperature on Release of Enterotoxin A From S. aureus (F_{265}) Cells by Pancreatin*

Treatment	Level of Enzyme (mg)	Temp °C	Before Incubation OD_{650} 1:100	Before Incubation BPA cfu/ml x 10^{10}	After Incubation OD_{650} 1:100	After Incubation BPA cfu/ml x 10^{10}	Enterotoxin A in 30x Conc.
		4	0.31	3.0	0.30	2.9	--
Control	0	25	0.31	4.0	0.30	3.9	--
(Cells Alone)		37	0.32	3.2	0.30	3.0	--
		4	0.32	2.8	0.31	2.7	--
Pancreatin	6	25	0.31	3.6	0.31	3.9	+
		37	0.32	4.3	0.31	4.1	+
Lysostaphin	500 µg	37	0.31	3.1	0.09	0.00006	+

* Duration of exposure was 30 minutes.

--- No SEA detected.

\+ SEA detected.

Table 4. Effect of Certain Pancreatic Enzymes on Release* of Enterotoxins A From S. aureus (F_{265}) cells.

Treatment	Before Exposure		After Exposure		Enterotoxin A
	OD_{650} x 100	BPA cfu/ml x 10^9	OD_{650} x 100	BPA cfu/ml x 10^9	in 30 x Conc.
Control	0.11	2.5	0.11	2.6	---
Lipase	0.11	2.4	0.09	1.5	---
Esterase	0.13	1.4	0.10	1.4	---
Peptidase	0.13	2.0	0.12	2.0	---
∝- Amylase	0.10	3.0	0.09	3.5	---
Protease	0.11	2.1	0.09	1.6	---
RNase	0.12	2.2	0.10	1.7	---
DNase	0.12	1.5	0.11	1.3	---
Pancreatin	0.11	3.0	0.09	2.9	+

* Incubation was at 37°C for 30 minutes.

--- No SEA detected.

significant change in the OD_{650} and BPA counts after exposure. Lysostaphin at a level of 500 µg released detectable SEA with a significant decrease in OD_{650} and BPA counts due to lysis of S. aureus cells. The fact that Pancreatin at 4°C failed to release ISEA but did at 25°and 37°C strongly suggests that toxin release was a direct activity of the enzyme.

The absence of a significant change in OD_{650} and BPA count after exposure to pancreatin compared to the significant demonstrable decrease caused by lysostaphin suggest that they possess different mechanisms of release of ISEA. Lysostaphin possesses an endopeptidase (glycinase) activity which breaks the glycyl-glycyl bridges in the peptidoglycan of the cell wall causing lysis (Iversen and Grov, 1973). However, no information is available on the mechanism of action of pancreatin in releasing ISEA.

In order to elucidate the possible mechanism of ISEA release by pancreatin, electron microscopic studies were conducted on enzyme-treated cells and control cells (without enzymes). Unfortunately, electron micrographs did not show any definite lesions created in the cell walls of pancreatin-treated cells to permit release of toxin.

Table 4 shows the effects of certain component enzymes of Pancreatin on S. aureus cells. It was only Pancreatin that released detectable ISEA. The possible explanations for this finding are: 1) only some and not all component enzymes were tested; 2) these individual enzymes released very minute levels of ISEA not detectable at the con-

centrating factor used; 3) there may be a necessity for sequential activities of these component enzymes, possibly at different sites of the cell wall, to accomplish release of ISEA. Additional detailed studies need to be done with pancreatin to identify the component enzyme(s) and the mechanism of release of ISEA.

In view of the fact that in vivo release of ISEA occurs in cynomologus monkeys fed viable cells (Section B, Experiment 1) with subsequent emesis, it is possible that pancreatin, a digestive enzyme (usually in the gastrointestinal tract) plays a role in release. Pancreatin could be added to foods implicated in intoxication but containing no detectable staphylococcal enterotoxins (known), to facilitate in vitro release of ISEA and subsequent detection in foods.

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DISCUSSION AND SUMMARY

Studies on cynomologus monkeys showed that Ketamine caused emesis in 24% of the 41 monkeys intramuscularly injected at dose levels of 2.7-20 mg/kg. Of relevance was the finding that both ketamine and staphylococcal enterotoxin A (SEA)-induced emesis could not be distinguished based on time of first emetic episode and/or frequency of emesis. A protocol was developed which involved screening all monkeys acquired for staphylococcal enterotoxin in bioassay (where emesis is a cardinal sign in diagnosis) for ketamine-induced emesis and to determine the non-emetic but sedative level for each monkey. By so doing, ketamine-induced emesis and accompanying false-positive response were eliminated while maintaining safety for both monkeys and human handlers.

Studies on cynomologus monkeys using SEA showed them to be specific and are of comparable sensitivity (ED-50 of 6.5 ug per monkey; Section A, Experiment 2, Tables 2 and 3) to rhesus monkeys. Cynomologus monkeys are therefore, suitable for staphylococcal enterotoxin (SE) bioassay.

Coagulase positive staphylococci (*S. aureus* strains) are considered to be enterotoxigenic because of the high degree of correlation reported between coagulase and enterotoxin production. Consequently, coagulase test is used to screen foods in quality control and as basis for diagnosis for foodborne outbreaks. This thesis established that enterotoxigenicity is not unique to *S. aureus* as five strains of *S. hyicus* (coagulase negative or variable) of animal origin, produced enterotoxin(s) as demonstrated by the monkey feed-

ing test (Section A, Experiment 3, Table 4). In addition, the enterotoxin(s) produced was not any of the presently known serological types (A-E). One of the strains S. hyicus (VII76) was found to be of comparable enterotoxigenicity to the known enterotoxin producers (as 50 mls of culture filtrate elicited emesis in 3 of 6 monkeys). Enterotoxin-neutralizing antibody was produced against the enterotoxic factor produced by S. hyicus (VII76). Although the incidence of enterotoxigenicity among S. hyicus is unknown, the fact that 5 strains were found to be enterotoxigenic and that some were coagulase and thermonuclease negative, points to the need to include these organisms in hazard potential evaluations. This also suggests the need for purification and development of antibodies for in vitro serological tests which will permit establishment of incidence of these types of organisms and their enterotoxin(s) in foods and their response to food processing parameters.

Intracellular staphylococcal enterotoxin A (ISEA) was demonstrated to be biologically active in cynomologus monkeys (Section B, Experiment 1, Table 1). The activity was comparable to that of extracellular staphylococcal enterotoxin A (ESEA). Correlation between serological and biological activities of ESEA reported by others was also demonstrated for ISEA in this thesis. The fact that viable staphylococcal cells (containing ISEA) fed to monkeys elicited emesis suggests that in vivo release of ISEA occurred. Although fecal and/or vomitus were not assayed to conclusively prove release of ISEA, the fact that pancreatin (a gastrointestinal enzyme) released ISEA in vitro from the same staphylococcal cells (Section B, Experiment 3, Tables

1 and 2) supports this view. Other digestive enzymes not assayed in vitro may have also played some role in the release. Staphylococcal foodpoisoning has long been known to be an intoxication and this thesis shows another aspect of its intoxication namely, the release of ISEA in the gastrointestinal tract and resultant intoxication.

Studies on the influence of environmental factors on release of ISEA by staphylococcal cells grown at low water activity (a_w) show that an increase in a_w (0.90 to 0.99) is required for release. Under these conditions, the rate of release of ISEA was found to be pH and temperature-dependent (in the range of 4-45°C). Release of ISEA was faster at pH 8.5 than at pH 5.0, and also faster at 45°C than at 21°C at same pH. This finding of optimal release conditions suggest the involvement of active transport of SEA.

The fact that intracellular enterotoxin was biologically active and cells containing ISEA caused intoxication in monkeys suggests that methods for detection of ISEA in foods especially low moisture foods, should be developed and included in laboratory investigations. Pancreatin and/or exposure to 45°C for 2 hours at pH 8.5 could be utilized to enhance detection of intracellular enterotoxins in foods.

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