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A. Pope, ... , I. T. Nathanson, A. L. Nutt

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VI. THE TOXIC INFLUENCE OF THE BACTERIAL FLORA,
PARTICULARLY *CLOSTRIDIUM WELCHII*, IN EXU-
DATES OF ISCHEMIC MUSCLE¹

By A. POPE, P. C. ZAMECNIK, J. C. AUB, A. M. BRUES, R. J. DUBOS,
I. T. NATHANSON, AND A. L. NUTT

(From the Medical Laboratories of the Collis P. Huntington Memorial Hospital of
Harvard University at the Massachusetts General Hospital, and the
Department of Comparative Pathology and Tropical Medicine,
Harvard Medical School, Boston, Massachusetts)

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As has been shown in previous reports from this laboratory (10, 25, 75), toxic effects resembling traumatic shock have been observed following the intravenous injection of fluids exuding from dog muscles after prolonged ischemia.

The fact that 28 per cent of these fluids were highly toxic, whereas the remainder seemed harmless, strongly suggested that their toxic effects were not due to the presence of intracellular substances lost as the result of cell damage (70). For the same reason, it appeared doubtful that the occasional toxic effects were caused by breakdown products of muscle cell constituents resulting from ischemia, since the experimental conditions were all approximately identical. Attention has been directed, therefore, to the one constituent of these fluids which was obviously highly variable, namely, the bacterial flora.

Another bit of indirect evidence pointed in this direction. On several occasions, dog muscle tissue was excised and minced with sterile precautions and incubated in sterile dog plasma at 37° C., for 5 hours. The plasma was then separated by centrifugation and injected intravenously into recipient dogs. Such material was invariably toxic, rapidly producing a shock-like state and death, and it was always infected with micro-organisms which on direct smear were seen to be large gram-positive rods. However, if a mixture of blended dog muscle and plasma were sterilized by passage through a Seitz filter before incubation,

it never exhibited toxic properties when administered to a recipient animal.

EXPERIMENTAL

Our method of muscle ligation and muscle exudate collection has been described (14). All operative procedures were carried out under sodium pentobarbital anesthesia and with the customary aseptic precautions.

Amounts of fluid exuding from the 2 muscle groups in a 5-hour period after release of ligatures varied in these experiments from 2 to 17 ml. per kgm. body weight of the donor dog. The composition of this fluid and its shock-producing effect when administered intravenously have been described in detail (70, 75).

Practically all of the muscle exudates contained bacteria as demonstrated by direct gram stain. The amount of bacterial contamination in most of these fluids was determined by quantitative culture methods. Five-tenths ml. of the fluid to be tested were mixed with 4.5 ml. of beef broth, and 3 successive 1:10 dilutions in broth were made. Two blood agar plates were then divided into quadrants and a loopful (approximately 1/μl.) of each dilution was streaked onto 1 quadrant of each plate. One of these was incubated aerobically and the other in an atmosphere of 5 per cent CO₂ in nitrogen. After 36 hours of incubation at 37° C., differential colony counts were made and identification of the bacterial species present was undertaken. Colony counts were multiplied by the appropriate factors to give numbers of viable bacteria per ml. of original fluid, and these were checked qualitatively with observations on direct smears. Clostridia were isolated and cultivated from various fluids.

In an attempt to reproduce at will the picture of toxicity following fluid injection, 2 strains of *Clostridia perfringens* thus isolated were used in 3 experiments. In these experiments, at the time of muscle ligation, 2 to 5 ml. of a broth culture of these organisms were injected into each of the 2 muscle groups; after the period of occlusion of the blood supply the ligatures were released as usual and fluid collected. These have been termed "reinforced" fluids. Bacterial counts were made on the first fluid obtained from each leg, on a final sample drain-

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ing off at the end of a 6-hour period, and from the pooled fluid.

A series of dog muscle biopsies have been done in an effort to discover whether clostridia can be grown from samples of dog muscle obtained under aseptic precautions. After preparing the skin in the same way as described for the operative ligation of muscles, the skin was incised and the edges retracted. The subcutaneous fascia was then incised and retracted with a fresh set of sterile instruments, and samples of muscle (about 1 gram) were excised, using a third set of instruments. In other cases, the skin was seared either by a hot scalpel or electrocautery. The remainder of the procedure was accomplished by means of electro-surgical technique. Samples of rectus and sartorius muscle were taken by both these methods. Biopsies of skin were also removed after the usual preparation including a very strong solution of iodine. All biopsy specimens were placed at once into meat infusion broth and incubated in an atmosphere of 5 per cent CO₂ in nitrogen.

RESULTS

In spite of the aseptic precautions, the fluid exudates collected after muscle ischemia have all contained bacteria. In most wounds, the body takes care of such contaminations, but traumatized anoxic tissues offer excellent media for growth and toxin formation. It appears, therefore, likely that traumatizing experiments on dogs must in general be complicated by contamination with bacteria which, in most observations on shock, would have time to exert a toxic influence.

Organisms cultured from fluids. Aerobic cultures were made in 30 instances. One of these cultures was made from a pool of 2 fluids used for injection and another from a pool of 9 fluids, so that 39 exudates are represented. Anaerobic cultures were made of 19 separate fluids, all of which are also represented in the figures given for aerobic flora. Table I shows the results of these cultures, and it will be noted that staphylo-

cocci and clostridia are the most common contaminants, each having been cultivated from the great majority of fluids. Where clostridia were cultured to determine type, as well as morphology, *Cl. perfringens* was obtained.

Bacteria in relation to toxicity. On 19 separate fluids, quantitative bacterial counts were made by both aerobic and anaerobic culture, and the toxicity of 18 of these fluids was determined by intravenous infusion into recipient dogs. In 5 cases, 2 fluids were pooled for assay purposes.

TABLE II
Muscle exudates: correlation of properties with number of Clostridia

Donor dog number	10 ⁶ × Clost./ml.	10 ⁶ × Staph./ml.	Fluid output ml./kgm.	Recipient dog number *	Toxicity
166	0.001	1.0	7	167	0
217-218	0.01	2.8-3.0	8-6	219	0
221-222	0.03	0-0	5-4	223, 224	0
226-227	0.14	0.06-0	5-3	228, 233	0
214-215	0.17	0.09-0	3-10	216	0
186-187	2.7	0.5-6.0	15-6	188	0
177	2.8	0.8	14	179	0
168	3.0	0.6	17	169	0
181	5.0	2.4	16	183	+
171	35.0	+	17	174, 175	0
176	160.0	28	8	178	0
180	500.0	40	16	182	+
172	750.0	+	13	173	+

* The average fluid output of this series of animals is approximately 9 ml. per kgm. In a larger series the average output was 12 ml. per kgm.

** Staphylococci present but not quantitated.

Table II shows the results. It can be seen that the fluids with relatively low bacterial counts were always non-toxic; the 3 toxic fluids were included in the 5 highest clostridial counts. Staphylococcal counts correlated slightly less well with toxicity, but there was considerable correlation between the 2 organisms. There also appears to be some correlation between the bacterial counts and the amounts of fluid produced.

Growth and toxin production "in vivo." In 7 instances, data are available showing the increase in bacterial content of fluid obtained during the 6-hour period after release of ligatures. Cultures and colony counts were made on the first fluid and again on fluid accumulating at the end of the period. The results, as shown in Table III, demonstrate the magnitude of the increase. Since many of the initial counts were too low to be established quantitatively under the conditions

TABLE I
Micro-organisms identified in muscle exudates

Organism	Number of times identified	Number of cultures made	Percentage of fluids present
Clostridia	13	19	72
<i>Staph. albus</i>	23	30	78
<i>Staph. aureus</i>	8	30	27
<i>B. subtilis</i>	9	30	30
Coliform bacilli	7	30	23
<i>M. tetragenus</i>	4	30	13
Streptococci	2	30	7
Unidentified	2	30	7

used (*i.e.* below 10,000 per ml.), they have been taken as 10^4 per ml. for the purpose of obtaining a figure representing the average of the increments. The calculated mean increment is, therefore, a minimum value. It is seen that there is greater than 1000-fold increase in the concentration of viable clostridia and staphylococci during the 6-hour period following release of the constricting band. Assuming a generation time of approximately 30 minutes, this rapid increase indicates that multiplication of clostridia must have started immediately, as soon as ischemia provided satisfactory anaerobic conditions for growth.

TABLE III
Increase of bacterial counts from initial to terminal fluids

Dog	Clostridia		Staphylococci	
	Initial	Terminal	Initial	Terminal
176	$<10^4$	1.6×10^8	$<10^4$	2.8×10^7
177	4×10^4	2.8×10^8	$<10^4$	2.5×10^8
180	2.5×10^5	1.0×10^9	2.2×10^8	1.0×10^8
181	$<10^4$	3.0×10^7	$<10^4$	1.0×10^7
186			6×10^4	2.5×10^8
187			$<10^4$	2.5×10^7
222	10^4	6×10^4		
Average	6.4×10^4	2.4×10^8		
Factor of difference:				
(By average of increments)		4×10^3	11×10^3	
(By increment of averages)		4×10^3	5×10^3	

Reinforced fluids. In the case of fluids from muscles which, at the time of ligation, received injections of cultures of bacteria (*Cl. perfringens*) isolated from earlier experiments, extremely toxic exudates were obtained in comparison with those collected in routine experiments. The 3 fluids in this series, when injected intravenously, uniformly produced a shock-like picture terminating in death in 8 instances. In these cases, 5 ml. per kgm. (or approximately one-third of the dose used in the earlier experiments) were given intravenously, and in all cases marked hemolysis was observed in the bloods of the recipients at the time of death. In 3 of the 8 experiments, bacteria had been removed by filtration through a Chamberland candle in 1 case and by centrifugation in the other 2. Washed bacteria obtained by centrifugation were used in 2 experiments and produced no obvious effect except

hyperthermia (41° and 42° C., respectively). In 1 further experiment, a lethal amount of reinforced fluid was injected along with 5 ml. polyvalent gas gangrene antitoxin (Lederle's) with complete protection against the effects of the fluid. Thus, the effects of the reinforced fluids are due to the presence of an exotoxin which is neutralized by gas gangrene antitoxin.

Increases in bacterial content and toxin concentration during the collection period were noted. Tests were made on the first fluid, the last fluid obtained at the end of 6 hours, and the pool of the total fluid obtained. In the case of 1 of these experiments, in addition to bacterial counts, toxicity was determined as the volume of fluid containing 1 mouse m.l.d. There was a 300-fold increase in bacterial count and at least a 10-fold increase in the toxin concentration during the 6 hours after release of ligatures. When a 10 ml. culture was injected into the muscles at the time of ligation, the injected culture contained in all 100 mouse m.l.d. (L.D. 100) of toxin; following release of the muscle ligature, 270 ml. of fluid exuded from the muscle containing 1350 m.l.d. of toxin. It may be assumed that additional toxin was present in the muscles at the end of this period. Thus, the anoxic muscle *in vivo* has been shown to be a good medium for multiplication and toxin production by toxigenic clostridia.

Anaerobic cultures of biopsy specimens. In 11 of 13 intact dogs from which muscle biopsies were obtained, clostridia were grown from at least 1 muscle specimen, and they were cultivated in 13 instances out of a total of 25 such specimens (0.5 to 1 gram of tissue). Thus, clostridia were obtained from 85 per cent of the dogs whose muscles were biopsied. In addition, 36 per cent of the muscle specimens yielded coliform organisms and 8 per cent of them staphylococci.

In 2 dogs, all of 5 cultures of surgically prepared skin were positive for clostridia, and 1 from each dog showed the presence of staphylococci, coliform organisms, and diphtheroids. These data suggest that organisms of the gas gangrene group are common inhabitants of dog skin, where they presumably exist as spores, and are exceedingly readily introduced into the underlying tissues during operative procedures. The possibility that they are normal inhabitants of dog muscle in some instances is not ruled out.

Anaerobic cultures were also made of muscle specimens obtained from 7 rats; 1 of these cultures yielded clostridia, another yielded a culture of gram-positive cocci, and the remainder were sterile.

Eighteen cultures were made of specimens of human muscle obtained from various elective operative procedures.¹ In no case were clostridia grown from these specimens although staphylococci were obtained in 10 cases.

Identity of organisms. Clostridia obtained from muscle exudates and from dog biopsy specimens were identified as *Cl. perfringens* in the following way.

Anaerobic cultures (in broth and on agar media) of muscle extracts and from dog biopsy specimens yielded large numbers of organisms which were identified as *Cl. welchii* (*perfringens*) on the following grounds.

The colonies and broth cultures were found to consist of rods approximately 1μ in width and varying from 3 to 10μ in length. Very young organisms were frankly gram-positive but became gram-negative within 24 to 36 hours, all intermediate degrees of intensity of staining being observed in 1 given culture according to its age.

Spores were very rarely encountered and in particular could not be detected in sugar media.

The stormy fermentation of litmus milk and other fermentation reactions were typical of *Cl. welchii*. Gelatin was liquified but no digestion of serum was observed.

The colonies were either smooth or rough, hemolytic or non-hemolytic (β hemolysis). The fact that these different colonial types could all be derived from 1 single colony isolated upon aging of the culture indicates that they were due to bacterial dissociation rather than being representatives of different bacterial species.

The cultures grown in ordinary glucose broth were only a little toxic for mice (MLD 0.1 ml.). Toxicity could be much increased by growing the organism in meat media containing 0.5 per cent glucose or in media specially devised for the production of Welch toxin (MLD 0.001 ml.).² The toxin could be neutralized by Welch antitoxin.

¹ We are indebted to Dr. Carroll B. Larson for the bulk of these specimens.

² This toxin was supplied by Dr. A. Pappenheimer, Jr.

DISCUSSION

The presence of bacteria of the gas gangrene group in normal dog muscle has been reported (87, 88), and both our studies and the confirmatory one of Cope and Langohr (unpublished data) have indicated that such organisms may be normal inhabitants of dog muscle; but it is more likely that they are introduced during operative manipulations, since they appear to be such frequent contaminants of dog skin. In all probability, they exist in skin as spores resistant to the usual methods of surgical preparation. In the dog, therefore, gas bacilli are potential contaminants of artificially-traumatized or surgically-treated tissues, and all experimental procedures which tend to result in ischemia and anoxia would produce conditions extremely favorable for their multiplication and toxin formation.

It should be emphasized, also, that from both biopsy material and "sterile" collected exudates from ischemic muscles, other microorganisms than clostridia, especially staphylococci and bacilli of the coliform group, have been frequently cultivated. The creation of damaged and relatively ischemic tissue tends to promote the growth of many types of pathogens, especially anaerobes, and among the commonly contaminating organisms are included several species capable of producing powerful exotoxins (89, 90). The ready accessibility of clostridia, the fact that conditions for their growth and toxin production are optimal, and the potency of their characteristic toxins make it important to investigate the rôle which such bacteria might play in the sequence of changes that constitute the syndrome of traumatic shock.

The toxins of several species of bacteria have been shown to have specific pharmacologic effects on the circulation with certain features in common with the classical changes of shock. Notable examples of these are the exotoxins of gram-negative bacilli (89, 90). The production of hemolysis and death in rabbits by the intravenous administration of Welch bacillus toxin has been reported (91), and the action of this toxin has been further described (92). Experiments in this laboratory with purified *Cl. perfringens* and *Cl. oedematiens* toxins have confirmed these results in dogs and demonstrated both local and generalized toxic effects of these agents on the circulatory system.

These facts make it clear that the element of bacterial infection and its consequences are factors that must be taken into consideration in physiologic experiments which continue for longer than a few hours. Particularly in experiments on traumatic shock in which large areas of damaged and ischemic tissue are produced, the rôle of bacterial products in contributing towards the clinical outcome cannot be ignored. Interpretation of the results of experiments in which parenteral administration of various extracts of normal and traumatized tissues is made should involve consideration of the bacterial factor (93). This has been recognized and it has been shown that bacteria-free extracts of these types are without physiologic effects (65).

The possible rôle of micro-organisms in the genesis of traumatic shock following the injury of men in battle remains to be evaluated. Although human muscle biopsy specimens have been, in our experience, generally sterile, wounds sustained under battle conditions are usually contaminated with many species of bacteria, and the gas bacillus ranks high in its incidence in war wounds (94, 95). Certainly in many of the latter, conditions must be very favorable for the multiplication of such contaminants, and it is conceivable that sub-clinical amounts of highly toxigenic strains of bacteria, especially of the gas gangrene group, might produce sufficient quantities of toxin to be a factor in the development of circulatory failure, either by promoting loss of vascular fluid at the site of trauma or by acting generally on the cardio-vascular system. That the former may be a not insignificant effect in the case of the Welch bacillus toxin has been suggested (96). The irreversibility of the shock-like state produced by the intravenous administration of *Cl. perfringens* and *Cl. oedematiens* toxins (unpublished data) indicates that the production of toxins of these types in contaminated wounds can play a significant rôle in subsequent development of "irreversible" shock.

These considerations and especially the urgency of elucidating the pathogenesis of irreversible peripheral circulatory failure make it important to study human cases of traumatic shock from the point of view of the bacteriology of their wounds in order to determine what counterpart the findings reported in this paper have in clinical shock.

SUMMARY

The bacterial flora of ischemic canine muscle exudates has been investigated. *Clostridium perfringens* and *Staphylococcus albus* have been recovered from most of these exudates in widely varying concentrations. The clostridia have been shown to multiply and produce toxin in ischemic muscle, the multiplication starting as soon as ischemia provided satisfactory anaerobic conditions for growth. They are present in many biopsy specimens of normal dog muscle obtained with the use of rigid surgical techniques of skin sterilization and sterile handling of specimens. Clostridia are not present in normal human muscle.

Evidence has been obtained indicating a correlation between the toxicity of the fluids as administered to recipient dogs and their bacterial content. We think this bacterial contamination is the source of a "toxic factor" in experimental shock. Bacterial infection and its consequences must be taken into consideration in physiological experiments, particularly in those involving injury to tissue, even where performed by surgical methods.

The possible relation of clostridial infection to human wound shock remains to be elucidated. The frequent contamination of war wounds with these organisms indicates the importance of such an evaluation.

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