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'IN VIVO' ROLE OF 'PSEUDOMONAS AERUGINOSA' TOXINS AND HOST RESPONSE

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13. ABSTRACT

Current studies indicated that mice receiving either sub-cutaneous or intradermal injections of viable *P. aeruginosa* cells exhibited systemic infections after several days. The primary target organs appeared to be the kidneys and lungs. Administration of an equal number of dead cells did not kill the mice although approximately 30% of the animals exhibited black necrotic lesions similar in appearance to ecthyma gangrenosum.

Attempts to establish pulmonary infections in mice receiving per-oral administraiton of *P. aeruginosa* directly into the lungs was unsuccessful. Pretreatment of mice with either mucin, methotrexate, or elipten phosphate did not predispose the mice to pulmonary infection. However, per-oral administraitdon of viable cells into the stomach of mice resulted in fatal systemic infections.

Chronic systemic infections of rabbits were established in order to study the sequence of events leading to severe kidney damage. Renal lesions were detected by the 5th to 7th day post-infection along with progressive azotemia. No difference in susceptibility to infection was observed with rabbits surgically induced unilateral ureteral obstruction and non-ligated rabbits.

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"*In Vivo* Role of *Pseudomonas aeruginosa* Toxins

and Host Response"

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SUMMARY

A protease from *P. aeruginosa* having lethal and dermonecrotic properties, was capable of hydrolyzing native collagen, was purified 1,568-fold with a recovery of 24% by use of chemical and chromatographic technics. The enzyme preparation appeared to be homogeneous when subjected to chromatographic, electrophoretic, ultracentrifugational, and amino acid analyses. A standard state sedimentation coefficient of 2.10 S was calculated and further analyses indicated that the enzyme had a molecular weight of 17,500 and dimerizes under certain conditions to yield an apparent molecular weight of 34,000. In addition to native collagen, the enzyme catalyzed the hydrolysis of congocoll, azocoll, soluble collagen and casein, but did not attack orcein-elastin, azoalbumin, p-toluene sulfonyl-L-arginine methyl ester, benzoyl-L-tyrosine ethyl ester, and the hexapeptide N-Cbz-glycyl-L-prolyl-glycyl-glycyl-L-prolyl-L-alanine. Enzymatic activity against congocoll was six-fold greater at pH 7.5 in Tris-HCl than in phosphate buffer at the same ionic strength. Cobalt, and to a lesser extent, zinc ions appeared to activate the enzyme, especially in phosphate buffer. Sodium cyanide and PCMB did not appreciably inhibit enzyme activity, while ammonium sulfate, EDTA and cystine displayed a significant inhibitory effect under certain conditions.

Several new areas of *in vivo* investigation using live cells of *P. aeruginosa* have been initiated. Preliminary studies indicate that mice receiving subcutaneous injections of the organism exhibit systemic infections after several days. The primary target organs appear to be the kidneys and lungs, with some pathology noted in other organs as well. Administration of dead cells produced black necrotic lesions similar

in appearance to ecthyma gangrenosum. Organisms administered into the lungs appeared to be rapidly cleared by the mice and death did not ensue unless the animal was previously debilitated by various chemical and physical treatments. However, per-oral administration of live organisms into the stomach resulted in an LD_{50} of 5×10^6 organisms. Other preliminary results with rabbits indicate that viable *P. aeruginosa* cells can induce disseminated intravascular coagulation (DIC), while dead cells or their endotoxin did not.

RESULTS

Purification and characterization of a *P. aeruginosa* protease exhibiting collagenase activity were initiated. Values for a typical purification are summarized in Table 1. For convenience, all fractions were monitored for enzyme activity by use of the congocoll assay during the purification procedure. A 1568-fold increase in specific activity with a 24% recovery was obtained. The purified enzyme obtained from the post-sephadex G-150 column was difficult to characterize due to its lability. The enzyme could not be stored in the refrigerator for prolonged periods without substantial loss in activity. In addition, lyophilization, or freezing of freshly purified preparations at -60°, resulted in an almost total loss of activity.

This final fraction demonstrated a single band when electrophoresed on SDS-polyacrylamide gels. However, electrophoresis in Tris-glycine buffer, pH 8, according to the method of Davis, resulted in one major and one minor band. Both bands were capable of congocoll hydrolysis and when the area of the major band was cut from an unstained gel, macerated in buffer, and the eluting material reelectrophoresed, both the major and minor bands were again detected.

SDS Acrylamide Gel Electrophoresis. Mobilities of the standard proteins and of *Pseudomonas* collagenase were determined and a semilogarithmic plot of these values indicate a molecular weight for *Pseudomonas* collagenase of 34,000 ± 2,000 Daltons.

Molecular Weight Determination by Sephadex G-150. The void volume of the column (V_o) was determined by blue dextran to be 140 ml. The column bed was 85 cm high and 2.5 cm in diameter which correspond to a total volume (V_t) of 1,688 ml. A semi-logarithmic plot of K_{av} against the logarithm of

molecular weight for each standard was determined. The K_{av} calculated for *Pseudomonas* collagenase corresponded to a molecular weight of 17,500 $\pm$ 500 Daltons.

Ultracentrifugation. The ultracentrifugation patterns of purified collagenase indicated a single, homogenous peak without shoulders. From the data, a value for the time rate of change of the logarithm of the boundary position of 1.527×10^{-4} was calculated, resulting in an observed sedimentation coefficient of 2.35×10^{-13}.

A partial specific volume of 20^{0} of 0.749 was assumed for this protein, which resulted in a standard state sedimentation coefficient ($s_{20,w}$) of 2.10 S.

Amino Acid Analyses. The results of amino acid analyses are summarized in Table 2, which shows the average of values from analyses at 22 and 118 hr of hydrolysis. Data for serine, threonine, and phenylalanine were extrapolated to zero time. Amide nitrogen and tryptophan were not determined. The number of residues is based upon one cysteine per molecule. This corresponds to a minimum molecular weight of 9,900. If two cysteine residues per molecule are assumed, a molecular weight of 18,900 results.

Substrate Specificity. The relative degrees of hydrolysis of several common substrates are presented in Table 3. The reaction temperature and time of incubation vary among the assay systems according to the established convention for each substrate. For comparative purposes, units of enzymatic activity are expressed in the manner employed by the originators of each assay.

The results of the viscometric assay of tropocollagen hydrolysis by clostridial and *Pseudomonas* collagenase were compared. Both reaction mixtures contained 100 ug of enzyme protein which resulted in values of

152.8 units/mg for *Pseudomonas* collagenase and 276 units/mg for clostridial collagenase when calculated by the method of Seifter and Gallop.

Inhibitor-Activator Studies. The effects of potential inhibitors and activators of *Pseudomonas* collagenase are presented in Table 4. After exposing the purified enzyme to the various compounds at 25° for 1 hr, the mixtures were assayed by congocoll hydrolysis in both Tris-HCl and phosphate buffers. The final concentration of these compounds in the reaction mixture was 5×10^{-4}M. The values listed are the percent activity of enzyme solutions calculated after subtracting the absorbance of control blanks containing no enzyme.

Table 1

Summary of Purification of *Pseudomonas* Collagenase

Fraction	Volume (ml)	Congocoll Units/ml	mg Protein (ml)	Units/mg	Total Units	% Recovery	Fold Purification
Crude	3000	31	9	34	93,000	100	--
Ethanol	20	4200	3.8	1105	84,000	90	325
Post QAE	10	3650	1.5	2433	36,500	39	715
Post G-150	6	3750	0.70	5333	22,500	24	1568

Table 2

AMINO ACID ANALYSIS OF PURIFIED COLLAGENASE

22 hr Hydrolysis

AMINO ACID	CORRECTED MICROGRAMS	CORRECTED MICROMOLES	RESIDUES	CORRECTED RESIDUES
Aspartic acid	7.100	0.0563	11.7	12
Threonine	2.676	0.0242	5.0	5
Serine	3.167	0.0322	6.9	7
Glutamic acid	4.197	0.0297	6.2	6
Proline	2.011	0.0189	3.9	4
Glycine	3.311	0.0530	11.0	11
Alanine	2.785	0.0358	7.4	8
Valine	2.509	0.0231	4.8	5
Cysteine	0.547	0.0048	1.0	1
Methionine	1.126	0.0078	1.6	2
Isoleucine	1.[illegible]29	0.0107	2.2	3
Leucine	2.053	0.0166	3.4	4
Tyrosine	4.768	0.0267	5.6	6
Phenylalanine	2.553	0.0158	3.3	5
Lysine	2.471	0.0176	3.7	4
Histidine	1.409	0.0094	1.9	3
Arginine	3.212	0.0188	3.9	4

Table 3

SUBSTRATE SPECIFICITY OF *PSEUDOMONAS* COLLAGENASE

Substrate*	Temperature (°C)	Time (min)	Activity
Congocoll	37	15	7,000 units/mg
Azure Hide Powder	37	15	1,630 units/mg
Azocoll	37	15	720 units/mg
Azoalbumin	37	15	0
Orcein Elastin	37	15	0
Native Collagen	20	1080	31 μmoles leu/mg
Soluble Collagen	20	30	152.8 units/mg
Hexapeptide	37	30	0
Casein	37	20	35 μg protein/mg
TAME	37	30	0
BTEE	37	30	0

*See Materials and Methods section.

Table 4

EFFECT OF VARIOUS COMPOUNDS ON THE HYDROLYSIS OF CONGOCOLL BY PURIFIED *PSEUDOMONAS* COLLAGENASE

Compound*	Activity Remaining Phosphate Buffer	Activity Remaining Tris-HCl Buffer
EDTA	27%	21%
Cystine	8%	0%
NaCN	98%	92%
PCMB	95%	99%
$MgCl_2$	78%	96%
$CaCl_2$	90%	102%
$FeCl_2$	6%	93%
$MnCl_2$	77%	88%
$CoCl_2$	150%	119%
$ZnCl_2$	117%	100%

*Final concentration in the reaction mixture was 5×10^{-4} M.

The following *in vivo* studies are currently being completed. Peroral administration of *P. aeruginosa* into the stomach of female Swiss-Webster mice resulted in an LD_{50} value of 4.5×10^6. Mice which had been pre-treated with 2.5 mg of elipten phosphate (inhibitor of steroid synthesis) were not pre-disposed to infection. Consequently, the LD_{50} values for untreated and elipten phosphate-treated mice were the same. Histopathology studies using sub-lethal doses of 8×10^4 to 8×10^5 viable cells showed no signed of necrosis within the stomach and intestinal tract over a 15 day holding period. However, acute congestion of the pulmonary tissues were observed. Most animals had venous congestion but focal areas of a chronic interstitial pneumonia was also present. Animals receiving lethal infective doses showed much more pathological severity than those receiving sub-lethal doses. However, no lesions were detected in the gastro-intestinal tract; however, the lungs exhibited mild to moderate hemorrhage with additional edema. The gross inflammatory process was of mixed infiltrates and the consolidation was consistent of bronchial pneumonia. Hemorrhage was generally seen throughout the lungs with the capillaries markedly distended with erythrocytes, although the alveolar sacs were free of erythrocytes. Renal histopathology was apparent and indicated the presence of acute tubular necrosis. The glomeruli were diffusely involved in proliferative changes probably mesangial, but possibly endothelial in nature. In many cases, Bowsman's space was obliterated and the parietal epithelium appeared hyperplastic. The renal blood vessels were similar to the lung pathology, in that a mononuclear infiltration accumulated which obliterated some of the small vessels. In some cases, vasculitis along with focal necrosis in the vessel walls was seen.

The administration of *Pseudomonas aeruginosa* ATCC 19660 per-orally into the lungs of Swiss-Webster female mice in concentrations as high as 2.75 x 10^9 did not result in an LD_{50}. Even the pretreatment of these animals with Elipten phosphate (2.5 mg/animal) did not lower their resistance to pulmonary infection. The lungs of these animals were involved in an interstitial pneumonia. Those animals receiving lower doses of *P. aeruginosa* (10^7) had foci of pneumonitis with hyperemia causing congestion of the lungs. Higher concentrations of cells produced an expected, more severe pneumonitis. The bronchi of these animals were congested causing atelactisis. There was much interstitial hemorrhage throughout the lungs as well as marked venous congestion. The inflammation was patchy with mixed cellular infiltrates. One animal showed well-defined nodular abscesses composed of primarily monocytes with PMN's along the periphery. The central core of these abscesses were of the hemorrhage coagulative, necrotic type with numerous bunches of bacteria. There was early fibrosis around the abscesses. The bacterium were numerous throughout the lung - intraalveolar, parenchymal, intrabronchial, peribronchial, and perivascular. The histopathologic picture seen in the liver appeared toxic in nature. No cellular infiltrates were present at all concentrations of inoculum and no bacterium were visible either. The liver had a moderate venous congestion but in some cases the parenchyma appeared anemic with hepatocytes undergoing necrobiosis. The lobular architecture was disturbed but the sinusoids seemed spared of any toxic degeneration. Toxic changes were also present in the kidneys of these animals. Again, as in the liver no bacteria were detectable in the kidneys. There was some narrowing of the cortex and a degenerative pattern was also seen with toxic nephrons and also many hypercellular glomeruli were present. No

significant infiltrates were present in any of the animals and some venous congestion was found in animals receiving high doses of organisms (2.75×10^9). The cloudy swelling seen within the kidneys could possibly be due to toxic products elicited by the organism resulting from a septicemia. No impressive pathology was found in the large intestines, the spleen, or adrenal glands which were within normal limits. The group of mice which were pretreated with elipten phosphate had no pathologic changes identifiable only in this group. Thus, no significant difference could be seen between the normal animals and the pretreated animals receiving the per-oral administrations of *P. aeruginosa* into their lungs.

SUMMARY OF PATHOLOGY OBTAINED BY I.V., I.P. AND S.C. INFECTIONS

1. I.V. Injections

With dilutions above the LD_{50} of 10^8, death usually occurred within 24 hr. The mice just before death showed ruffled fur, and their eyes usually became encrusted. Upon autopsy, the lungs were very hemorrhagic and the liver appeared anemic. Microscopically, liver hepatocytes showed widespread degeneration and a great deal of interstitial hemorrhage was found in the lungs.

In animals which survived longer than 24 hr, the formation of white focal abscesses were observed in both the lungs and liver. The kidneys showed petechial hemorrhage with the formation of a few localized abscesses. Also after 48 hr, the spleen was slightly enlarged.

Histopathologically, typical interstitial pneumonia was found in the lungs with a few focal abscesses found in the periphery. The lungs showed a great deal of interstitial hemorrhage with a mixed cellular infiltration made up mainly of mononuclear cells. The liver maintained its basic architecture, but widespread degeneration of hepatocytes was noted along with central venous congestion and some thrombosis. The liver also typically contained a few classical bacterial abscesses walled off by fibroblasts. Both polynuclear cells and mononuclear cells were present. In the kidneys the normal number of glomeruli were usually present, but they were hypercellular with a mixed infiltrate. Interstitial hemorrhage was present in both the cortical and medullary areas. The tubules appeared dilated and septic thrombi were present in the tubules and veins.

2. I.P. Injections

The pathology was the same as for '. Injections, except both the

large and small intestines showed severe hemorrhage, and a lot of fluid was present in the peritoneum. Microscopically the intestines showed a great deal of lymphoid development. Also the tips at the pili were necrosed.

3. Subcutaneous and/or Intradermal inoculations.

In the 1st 24 hr the mice showed a great deal of swelling at the site of inoculation giving a humped back appearance, with ruffled fur. Also, their eyes tended to become encrusted. Animals which died in the first 24-48 hr showed very little gross pathology, outside of very hemorrhagic lungs and hemorrhagic spots on the liver. The histopathology appeared the same as the I.V. inoculated mice which died in the first 24 hr.

Animals inoculated with dilutious at or near the LD_{50} of 10^8 cells, and survived, developed large hemorrhagic, necrotic, purulent lesions. Upon autopsy, both the liver and lungs of these animals were hemorrhagic and contained many large white necrotic foci. The kidneys showed petechial hemorrhage and occasionally renal abscesses were noted. The spleens of these animals were always enlarged to about three times their normal size (splenomegaly). Viable organisms were cultured from the liver, lungs, peritoneum and skin.

Microscopically, frank, widespread interstitial pneumonia with walled off focal abscesses, located in the perifery, were found in the lung. Atelectasis was prominent with a great deal of hemorrhage and a mixed cellular infiltrate. In the liver, ballooning degeneration of hepatocytes was evident along with the development of many large focal abscesses. Coagulative necrosis with fibroblast activity and deposition was seen in these abscesses. A mixed cell infiltrate was also present.

Later in the course of infection, frank interstitial nephritis developed in the kidneys with early ascending abscesses. The greatly

enlarged spleen appeared normal except for an increase in the number of PMN's and megakarocytes.

The skin lesions were typical of an acute bacterial inflammation, with a mixed cellular infiltrate. Most of the dermis was necrosied away, and the necrosis extended deep into the connective tissue and muscle layers.