INFECTION AND IMMUNITY, Mar. 1972, p. 363-369
Vol. 5, No. 3

Vascular Permeability Factor of *Pseudomonas aeruginosa*

HIDEO KUSAMA AND ROBERT H. SUSS

Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201

Received for publication 3 November 1971

The production of vascular permeability factor (PF) by certain strains of *Pseudomonas aeruginosa* has been demonstrated in rabbits injected intradermally with culture filtrates followed by intravenous injection with Pontamine Sky Blue 6BX. The dose-response curve was found to be rectilinear when lesion diameters, within the range of 10 to 20 mm, were plotted against log dose. Thus, PF in test filtrates can be measured with reasonable accuracy by the concomitant testing of a reference PF. In contrast to the titers of PF obtained with *Vibrio cholerae* cultures, those with strains of *P. aeruginosa* were rather low. Thus far, PF has been demonstrated only in shallow still cultures of *P. aeruginosa* and not in shake cultures. A variety of commercial media were tested for the production of PF, but none was satisfactory. A synthetic medium that gave more reproducible and higher yields of PF was developed. Cultivation at 30 C generally gave higher yields of PF than at 37 C. PF was destroyed by heating at 60 C for 30 min or by digesting with trypsin or Pronase. Strains producing larger amounts of PF appeared to have greater virulence when inoculated onto the surface of burns in mice than those yielding little or no PF.

Investigations during the past decade have established that *Vibrio cholerae* produces an enterotoxin both in vivo and in vitro and that the toxin causes increased capillary permeability when injected into the skin of experimental animals. Many similarities between *V. cholerae* and *Pseudomonas aeruginosa* led us to investigate the possibility that *P. aeruginosa* may produce a similar substance. Under rather limited cultural conditions, about 90% of the strains tested were found to produce various amounts of the vascular permeability factor (PF) as an extracellular product. This paper deals with the method of preparation and general characteristics of PF.

Some strains of *P. aeruginosa* have been known to be highly virulent for experimental animals when inoculated onto the burned skin, whereas others are quite avirulent. While working with PF, we found that some of the greater PF producers were those that had been used as virulent strains in burned mice (17, 18, 22). Therefore, the study has been extended to the nature of pseudomonad infection in burned animals to determine any possible role of PF in the pathogenesis. A significant correlation found between the ability of *P. aeruginosa* organisms to produce PF in vitro and the degree of virulence in burned mice will be discussed.

MATERIALS AND METHODS

Bacteria. Strains of *P. aeruginosa* maintained in this laboratory were used, including prototype strains of Verder and Evans' classification (25) originally obtained from the National Institute of Allergy and Infectious Diseases, Bethesda, Md., and representative strains of Habs' classification (6) received from various parts of Europe. Strains isolated from patients at Albany Medical Center Hospital and two strains virulent for burned mice generously provided by R. J. Jones, Birmingham Accident Hospital, Birmingham, England, were also included.

Culture filtrates. For most experiments, a medium was dispensed into 8-oz Owens oval bottles in 15-ml amounts. One of the bottles was inoculated with a few drops of stock suspension that had been frozen until use and incubated in the horizontal position for 6 hr. One drop of the resulting culture was inoculated into a new bottle, which was incubated in the horizontal position with the cap loosened. This condition provided a surface-volume ratio of approximately 4 cm^2/ml. After 18 to 20 hr, the culture was centrifuged, and the supernatant fluid was filtered through a 450-nm membrane filter (Millipore Corp., Bedford, Mass.).

Animals. Female NYLAR A mice (18 to 20 g), supplied by the Griffin Laboratory of this Division, were used for testing the virulence of *P. aeruginosa* organisms both in burned and normal mice. New Zealand White rabbits, weighing approximately 3 kg each, were

generally used for measuring the PF activity of culture filtrates.

Infecting organisms. Trypticase Soy Broth (BBL) was inoculated with *P. aeruginosa* organisms and kept at 37 C for 18 hr. The culture was centrifuged, and the supernatant fluid was discarded. The cells were suspended and diluted with a diluent to give 100 Klett units in a Klett-Summerson colorimeter with a no. 54 filter. The suspension thus prepared contained approximately 6×10^8 organisms per ml. Isotonic buffered saline at *p*H 7.2 [(BS) (NaCl, 6.8 g; Na_2HPO_4, 1.48 g; KH_2PO_4, 0.43 g per liter)] was used as the diluent for burned mice experiments, and a further 1:30 dilution was prepared for surface inoculation. For intraperitoneal inoculation of normal mice, the initial suspension was made in Trypticase Soy Broth.

Infection of burned mice. The technique used by Rosenthal (22) and by Markley and Smallman (17) was followed. Mice were anesthetized with diethyl ether, and the tail was immersed in water at 70 C for 5 sec. Five hours after the burn, the tail was dipped into a suspension containing approximately 2×10^7 organisms per ml. Groups of 10 mice in plastic cages were observed for 21 days. Usually 20 mice were used for each *Pseudomonas* strain, and mortality was recorded daily. Burned mice without inoculation did not die during the observation period. All dead mice among infected groups were autopsied, and cultural examination was made of heart blood, liver, spleen, kidney, and fluid in the small intestine.

LD_{50} in normal mice. Mice were inoculated intraperitoneally with 0.5 ml of serial 10-fold dilutions of bacterial suspension with either broth or 5% hog gastric mucin (granular mucin, type 1701-W, Wilson Laboratories, Chicago, Ill.). Ten mice were used for each dilution. Portions (0.1 ml) of appropriate dilutions were spread on agar plates for counting viable units. The animals were observed for 1 week. Midean lethal dose (LD_{50}) was calculated by the method of Reed and Muench (21).

Assay of PF. Most of the techniques used for determining PF potency of culture filtrates were the same as described previously for cholera PF (2, 3). An arbitrary potency was assigned to a reference preparation which was included in all titrations. Serial threefold dilutions of both the reference and test filtrates were made with BS, and 0.1 ml of each dilution was injected in duplicate into the skin of two to four rabbits. Eighteen to 20 hr after injection, a 5% solution of Pontamine Sky Blue 6XB (1.2 ml/kg) was injected intravenously. An hour later, diameters of blue lesions were measured and then plotted against the logarithm of dose. As will be presented in detail in the next section, an important difference from the procedures used for cholera PF was that the potency of a test sample relative to the reference was determined by the parallel line assay within the range of 10 to 20 mm of lesion diameter.

Assay of elastase. Elastase in culture filtrates was measured by the method of Oakley and Banerjee (19) with some modifications. Elastin (Mann Research Laboratories, New York, N.Y.) was treated with alkali (19). A solution containing 0.2% agar (a preparation of a high gel strength, supplied by S. Arima, National Institute of Health, Tokyo, Japan), 0.05% elastin, and 1:10,000 thimerosal in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer at *p*H 8.0 was pipetted into 7 by 100 mm tubes to form columns 40 mm high. Serial twofold dilutions of filtrates were made with 0.05 M Tris-hydrochloride buffer at *p*H 8.0; 0.1 ml of each dilution was layered on an elastin column and kept at 37 C. The titer of elastase was expressed as the reciprocal of the highest dilution that gave definite elastin digestion at 48 hr.

Chemicals. Amino acids for preparing media were purchased from General Biochemicals, Chagrin Falls, Ohio. For enzymatic treatment of filtrates, twice crystallized, lyophilized trypsin (Worthington Biochemical Corp., Freehold, N.J.) and Pronase, (B grade; Calbiochem, Los Angeles, Calif.) were used.

RESULTS

Media for PF production. Various commercial media, including Brain Heart Infusion (BBL), Trypticase Soy Broth (BBL), heart infusion broth (Difco), and Brain Heart Infusion (Difco), were tested for production of PF under various conditions, such as shaken and unshaken cultures, at various surface to volume ratios and at different temperatures. PF was not demonstrable in any of these media except in Brain Heart Infusion (BBL), in which a few strains showed the definite presence of PF. However, another batch of the same medium failed to produce PF in detectable amounts. Casamino Acids-yeast extract-glucose medium (11) and 2% peptone water (3), which were successfully used for PF production of *V. cholerae*, also gave negative results. Finally, synthetic medium I (Table 1) was developed by modifying the syncase medium of Finkelstein et al. (4) so that it could support good growth of *P. aeruginosa*. Many strains produced definite amounts of PF in this medium, and the results were quite reproducible. If yeast extract was added to this medium, PF was not detectable. Liu (12) employed three amino acids for producing extracellular toxins of *P. aeruginosa* in his synthetic medium. In synthetic medium II in Table 1, therefore, certified Casamino Acids (Difco) was replaced with 0.3% L-α-alanine, 0.3% L-aspartic acid, and 0.3% L-glutamic acid. It was also found that the amount of ferric chloride in medium I could be reduced to one-tenth. For the sake of simplicity, 1 ml of a salt mixture (8.9% Na_2SO_4, 4.2% $MgCl_2 \cdot 6H_2O$, 0.4% $MnCl_2 \cdot 4H_2O$, and 0.05% $FeCl_3 \cdot 6H_2O$ in 1% HCl) was added per liter of medium, instead of a solution of each salt. *p*H was adjusted with 10% NaOH, and the medium was sterilized by passing through a 220-nm membrane filter (Millipore Corp.). Medium II gave results quite comparable with medium I, and the experiments reported here-

TABLE 1. *Composition of media used for permeability factor (PF) production*

Ingredient	Syncase medium[a]	Synthetic medium (g/liter)	
	(g/liter)	I	II
Na_2HPO_4	5.0		
K_2HPO_4	5.0		
KH_2PO_4		1.0	1.0
Sucrose	5.0		
Glucose		10.0	10.0
NH_4Cl	1.18	1.18	1.18
Na_2SO_4	0.089	0.089	0.089
$MgCl_2 \cdot 6H_2O$	0.042	0.042	0.042
$MnCl_2 \cdot 4H_2O$	0.004	0.004	0.004
$FeCl_3 \cdot 6H_2O$	0.005	0.005	0.0005
Casamino Acids, certified (Difco)	10.0	10.0	
L-α-Alanine			3.0
L-Aspartic acid			3.0
L-Glutamic acid			3.0
*p*H		7.0	7.0

[a] R. A. Finkelstein et al. (4).

after were carried out by using medium II exclusively.

Changing the phosphate concentration did not improve the yield of PF. Liu and Hsieh (14) reported that the incorporation of ammonium salts into the medium could inhibit the production of protease of *P. aeruginosa* and thereby enhance the yield of the lethal toxin. Therefore, we added 1 to 4% ammonium sulfate to medium II to suppress the protease production. However, no increase in the yield of PF was observed.

With the various media so far tested, PF has never been demonstrated with shake cultures, but only in such shallow still cultures as described above. Cultivation at 30 C generally gave yields three to five times those obtained at 37 C.

Dose-response curve of PF. The titer of PF in filtrates was usually low, giving a definite skin reaction at 1:10 dilution but little or no reaction at 1:100 dilution. A large batch of filtrate was therefore prepared with strain Mills and passed through a PM30 ultrafiltration membrane (Amicon Corp., Lexington, Mass.) under 50 psi of nitrogen at 4 C. Most PF activity was found in the retained material, which was then passed through a 450-nm membrane filter (Millipore). This concentrated filtrate was chosen as a reference PF that should be included in all titrations of unknown samples. Part of the reference was diluted with an equal volume of BS containing 0.2% crystalline bovine albumin (Nutritional Biochemicals Corp., Cleveland, Ohio) and 1:5,000 thimerosal. A portion of the diluted

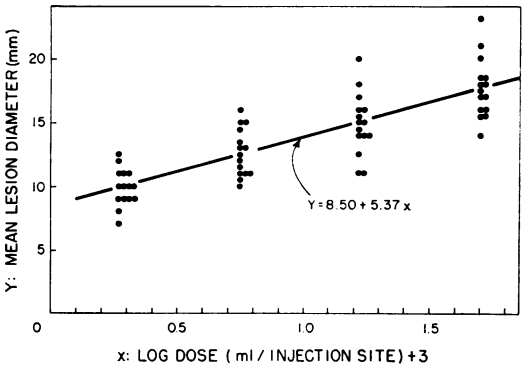

FIG. 1. *Skin response to graded doses of a reference PF of P. aeruginosa. Each dilution (0.1 ml) was injected in duplicate into the skin of New Zealand White rabbits. One spot represents the mean value of two lesions in a rabbit.*

reference was stored at 4 C, and another portion was lyophilized. Serial three-fold dilutions of this diluted reference were made with BS and injected in duplicate into the skin of rabbits. At 18 to 20 hr, erythema and circumscribed dome-shaped induration developed at the site of injection. However, the extent of induration appeared to be less prominent than that with *V. cholerae* PF. With lower dilutions, the central area often became necrotic, and remained free from the dye injected. Further study is required to determine whether the necrosis is evoked by PF itself or is a result of another contaminating substance. The blue lesion did not necessarily correspond to the area of induration. Very often the former was smaller than the latter.

Figure 1 illustrates lesion diameters in 15 rabbits plotted against the logarithm of the dose of the reference. The dose-response curve is rectilinear within the range of 10 to 20 mm of lesion diameter. The slope of the regression line was found to be five (or an increase of 5 mm in the lesion diameter would be expected if the amount of filtrate injected is increased 10-fold), a result similar to that obtained with other skin-reacting bacterial toxins (7). Arbitrarily, one skin test dose (STD) was assigned to the smallest dose in Fig. 1 which can still create definite and observable reactions within the range of the rectilinear portion. This dose was 0.1 ml of 1:50 dilution of the reference. The undiluted reference should, therefore, contain 500 STD of PF per ml. PF titer of a twofold dilution of the reference did not change significantly at 4 C for 2 months, but a gradual decrease in titer was observed upon further storage. The lyophilized reference exhibited its original activity upon reconstitution.

For routine assay of PF, the relative potency

of a test sample and its fiducial limits can be calculated according to the method of Finney (5). With four rabbits, the fiducial limit of 50% can be expected at the 5% level of probability.

Examination of filtrates from about 50 strains showed that the amount of PF varied. With some strains, PF was as high as 100 STD per ml, whereas with about 10% of the strains tested PF was not detectable even by injecting undiluted filtrates. PF values of a portion of the filtrates tested are listed (Table 2). Each of these values was determined with four rabbits.

Some characteristics of PF. After filtrates were heated at 60 C for 30 min, PF became undetectable at the lowest dilution tested. Trypsin solution in BS was added to filtrates at a final concentration of 1 mg/ml, and the mixtures were kept at 37 C for 1 hr. The treated samples showed no PF activity at the lowest dilution tested. A similar result was obtained by treating filtrates with Pronase.

The major obstacle in processing filtrates was their highly viscous nature, which made centrifugation, filtration, and concentration very difficult. Addition of streptomycin sulfate to a concentration of 0.5% helped to remove a part of the viscous material, presumably nucleic acid. Preliminary study indicated that most of the PF was found in the material retained when culture filtrates were passed through a PM30 membrane with a model 202 Diaflo apparatus (Amicon Corp., Lexington, Mass.) under 50 psi of nitrogen. When a small batch of filtrate was passed through an XM100A membrane, most of the PF activity was found in the effluent. However, prolonged filtration of a large batch resulted in clogging of the membrane and a change in the cut-off level. Thus, PF began to remain in the retentate.

Pseudomonad infection in burned mice. When the burned tails of mice were exposed to *Pseudomonas* organisms, the organisms invaded through the injured area and reached the blood stream. If highly virulent strains, such as strain 180 or 2243 (Fig. 2) were used, bacteria continued to multiply in the blood until the host succumbed (primary septicemia). Thus, most mice died within a few days after inoculation.

If strains with moderate virulence, such as B1024, B744, or Mills (Fig. 2), were used, a small portion of mice died of primary septicemia, but most of them survived. The bacteria disappeared from the blood stream but were disseminated to various organs where they started to colonize. In mice surviving longer than 4 to 5 days, typical findings were superficial multiple abscesses in the kidneys already described by Jones et al. (8) and by Millican et al. (18).

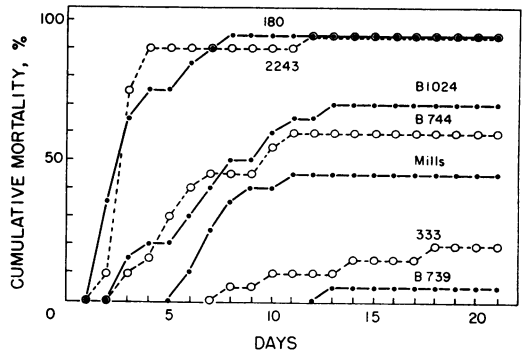

FIG. 2. *Mortality of burned mice infected with P. aeruginosa strains of various degrees of virulence. After the tail was burned at 70 C for 5 sec, it was dipped in a suspension containing approximtaely 2 × 10^7 organisms per ml. Twenty mice were used for each strain.*

Accumulation of a large amount of fluid in the upper part of the small intestine was a pathological finding seen at this stage although it was also observable at primary septicemia. Sometimes, the intestine was so distended that the intestinal wall looked like a thin membrane and the adjacent parietal peritoneum was bile-stained. A pure culture of the infecting *Pseudomonas* strain was usually obtained from the intestinal fluid. In these mice, the bacteria that had multiplied in various organs eventually invaded the blood stream again, and the animals died of secondary septicemia. Strains with low virulence, such as 333 or B739 in Fig. 2, caused no deaths from primary septicemia, but some mice died later from secondary septicemia. Several strains caused no death during 21 days of observation (Table 2).

In general, with most of the strains, except with some highly virulent ones, the mortality of burned mice resulted mainly from secondary septicemia. In contrast, when normal mice were infected intraperitoneally, deaths occurred within a few days from primary septicemia. Deaths later than 5 days were rather rare. If they occurred, however, the same autopsy findings as those in burned mice with secondary septicemia were observed.

Correlation of mouse virulence and PF production. Eighteen strains which have been studied more extensively are listed in Table 2 in the order of their virulence for burned mice. LD_{50} values for normal mice measured by intraperitoneal inoculation of the organisms in either broth or mucin are also tabulated. Generally speaking, there was a correlation between the virulence determined in burned mice and that in

TABLE 2. *Virulence in mice and permeability factor (PF) titer in culture filtrate strains of P. aeruginosa*

Strain	Type		Source[a]	Mortality in burned mice (%)	LD_{50} in normal mice		PF in filtrate (STD[b]/ml)	Elastase titer in filtrate
	Verder and Evans	Habs			In broth	In mucin		
2243	1		N.I.H.	95	1.4×10^6	10	120	0
180	1		ATCC 19660	95	7.0×10^6	5.9×10^2	90	8
B1024		3	A.M.C.	70	1.5×10^6	<4	80	0
B4			R. J. Jones	70	2.5×10^5	<5	70	0
329		1	O. Sandvik	65			40	16
B744		11	A.M.C.	60	4.3×10^6	2.6×10^2	100	0
Mills	4		N.I.H.	45	1.9×10^7	7.2×10^3	110	16
P14			R. J. Jones	35	1.6×10^6	<5	60	0
5937		5	M. Véron	30			60	0
333		5	R. Wokatsch	20	$>2.9 \times 10^7$	$>3.6 \times 10^4$	40	0
5933		1	M. Véron	15	7.0×10^7	7.7×10^4	10	0
2915	7		N.I.H.	15	7.5×10^7	1.8×10^3	<10	16
B739		13	A.M.C.	5	2.1×10^7	$>2.6 \times 10^5$	10	0
330		2	R. Wokatsch	0	1.3×10^6	<3	20	8
121		11	R. Wokatsch	0	2.9×10^6	<4	<10	4
5936		4	M. Véron	0	3.4×10^7	1.2×10^5	10	4
1M	5		N.I.H.	0	$>5.7 \times 10^8$	$>2.2 \times 10^5$	40	16
5938		7	M. Véron	0	1.4×10^7	8.0×10^4	10	0

[a] N.I.H., National Institutes of Health; A.M.C., Albany Medical Center Hospital.
[b] Skin test doses.

normal mice. However, such strains as 330 and 121 were avirulent for burned mice but were highly virulent for normal mice, particularly when suspended in mucin. This may indicate that the ability of organisms to penetrate the injured skin is one of the factors that determine the virulence in this particular experimental model.

A scatter diagram made by plotting cumulative mortalities in burned mice against PF titers in filtrates of the corresponding strains is shown in Fig. 3. Although the number of strains tested was small, the correlation of the two properties is apparent, the coefficient of correlation being 0.83.

Elastase has been considered to be one of the extracellular products of *P. aeruginosa* that may play a role in the pathogenesis. For the sake of comparison, elastase titers in filtrates were also included in Table 2. No definite relationship can be seen between the virulence in mice and elastase titer. In fact, synthetic medium II used exclusively in this study was well suited for PF production but very poor for elastase production.

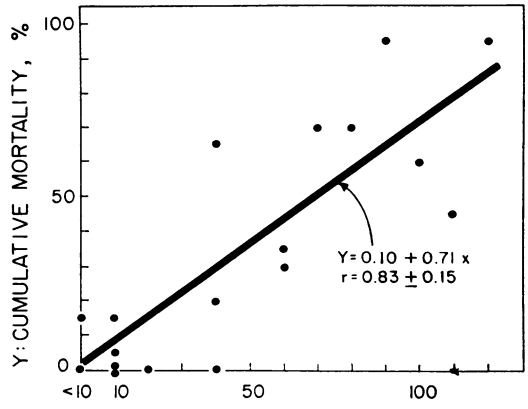

FIG. 3. *Relationship of virulence in burned mice and PF titer in culture filtrate of P. aeruginosa. After the tail was burned at 70 C for 5 sec, it was dipped in a suspension containing approximately 2×10^7 organisms per ml. The virulence is expressed as the cumulative mortality at 21 days. PF in filtrate was measured in the skin of four rabbits, with concomitant testing of the reference PF; the titer of the test filtrate was expressed in terms of the assigned potency of the reference.*

DISCUSSION

PF of *P. aeruginosa* was demonstrated in culture filtrates only under carefully defined cultural conditions. Moreover, the titers of PF were low as compared with those obtained with *V. cholerae*. Enrichment of media with yeast extract or vigorous shaking during cultivation had a beneficial effect on PF production of *V. cholerae* but exerted an adverse effect on PF of *P. aeruginosa*. Kusama and Craig (11) reported that PF titers of a protease-producing

strain of *V. cholerae* were lower and less stable than those obtained with a protease nonproducer. They postulated the presence of a PF-destroying substance. Liu and Hsieh (14) have shown a similar phenomenon with *P. aeruginosa*; the suppression of protease by the incorporation of ammonium salts into the medium resulted in increased yields of the lethal toxin. Although we failed to increase the yield of PF by adding ammonium sulfate to our synthetic medium II, there is still the possibility that a PF-destroying substance exists in culture filtrates of *P. aeruginosa*. The difficulty in demonstrating PF under usual cultural conditions may be the result of such a substance. The synthetic medium II we developed may be providing conditions that keep the PF-destroying substance at a minimum so that PF becomes demonstrable as the balance of two factors, whereas shaking or the addition of yeast extract may enhance the production of the destroying factor.

The dose-response curve of PF of *P. aeruginosa* is rectilinear within the range of 10 to 20 mm. The slope of the regression line is steep enough to perform the skin test with reasonable accuracy. As with some other skin-reacting bacterial toxins, lesions with diameters less than 10 mm are often less intense and difficult to measure precisely, and they are less reproducible than larger lesions. Also, the dose-response curve is not rectilinear for lesions with diameters of less than 10 mm.

For the quantitative assay of cholera PF, one "blueing dose" was defined by Craig (2, 3) as the amount of PF that produced a mean diameter of blueing of 8 mm in guinea pigs or 7 mm in rabbits. This definition has been widely used. However, there is no absolute amount of PF which evokes a 7- or 8-mm lesion; the size of the lesion depends upon the sensitivity of the individual animal. Our definition of STD is entirely arbitrary. The size of the lesion produced by one STD is not important. Our approach is to test two preparations in the same animals and to try to express the potency of one preparation in terms of the potency of the other by the parallel line assay.

The importance of the type of animal used for testing PF should be carefully noted. Unlike cholera PF, guinea pigs (Hartley strain, supplied from the Griffin Laboratory) were found to be insensitive to PF of *P. aeruginosa*. Griffin Laboratory rabbits (hybrid of Flemish Giant and Chinchilla) were tried, but rarely found to be good reactors. Finally, New Zealand White rabbits purchased from a local dealer were utilized. Most of these were found to be sensitive and gave clear-cut skin reactions.

Many investigators (1, 23, 24, 26, 27) have studied the diphasic increase in capillary permeability found in inflammation induced by thermal injury, due apparently to the activation of endogenous mechanisms. Furthermore, the extreme susceptibility to *P. aeruginosa* infection of burned rats and mice has been well documented (8, 9, 15–18, 22). McRipley and Garrison (15, 16) have suggested that the increased permeability that permits *Pseudomonas* to reach remote tissues may be one of the factors contributing to the increased susceptibility of burned animals. It seems likely that if the organisms themselves produce PF, while multiplying in the burned tissue, a greater increase in capillary permeability may be expected from the additive effect of thermal injury and a bacterial product. This might then facilitate bacterial dissemination in the host. To understand the role of PF in the pathogenesis of *P. aeruginosa* infection, the isolation, purification, and characterization of PF should be undertaken as the next step.

Cholera enterotoxin, even in its purified form, has been shown to affect differently the function of various tissues (20). For example, it induces fluid outpouring when introduced into the intestinal lumen, but acts as PF when injected into the skin. However, there is no evidence to indicate that the toxin alters the permeability of mucosal capillaries in the intestine (20). This suggests that PF of *P. aeruginosa* might evoke different responses in different tissues, and a given specific response might be of importance in the pathogenesis of the infection.

PF of *P. aeruginosa* differs from that of *V. cholerae* in many respects. Studies should be made to determine whether any antigenic relationship exists. Recently, Kubota and Liu (10), utilizing response in the ligated rabbit intestine as the study method, reported the existence of an enterotoxin of *P. aeruginosa*. As with *V. cholerae*, it may be that the substance responsible for the enterotoxicity and that for the PF activity are identical or closely related.

Twofold dilutions of a filtrate with the PF potency of 80 STD/ml were tested for toxicity in mice by intraperitoneal injection. Some mice died within a few days, and LD_{50} appeared to be 0.06 ml. Further studies are needed to determine whether this toxicity is a result of the PF itself or the presence of the lethal toxin described by Liu (13).

ACKNOWLEDGMENT

This investigation was supported by Public Health Service research grant 5 S01 RR05649-05 from the National Institutes of Health.

LITERATURE CITED

1. Allison, F., Jr., and M. G. Lancaster. 1959. Studies on the pathogenesis of acute inflammation. I. Changes of endothelial permeability in rabbit ear-chambers injured by heat. Brit. J. Exp. Pathol. **40**:324–337.
2. Craig, J. P. 1965. A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. Nature (London) **207**:614–616.
3. Craig, J. P. 1966. Preparation of vascular permeability factor of *Vibrio cholerae*. J. Bacteriol. **92**:793–795.
4. Finkelstein, R. A., P. Atthasampunna, M. Chulasamaya, and P. Charumethee. 1966. Pathogenesis of experimental cholera: biologic activities of purified Procholeragen A. J. Immunol. **96**:440–449.
5. Finney, D. J. 1964. Statistical method in biological assay, 2nd ed., p. 113–117. Hafner Publishing Co., New York.
6. Habs, I. 1957. Untersuchungen über die O-Antigene von *Pseudomonas aeruginosa*. Z. Hyg. **144**:218–228.
7. Jerne, N. K. 1951. A study of avidity based on rabbit skin responses to diphtheria toxin-antitoxin mixtures. Acta Pathol. Microbiol. Scand. Suppl. **87**:3–183.
8. Jones, R. J., D. M. Jackson, and E. J. L. Lowbury. 1966. Antiserum and antibiotic in the prophylaxis of burns against *Pseudomonas aeruginosa*. Brit. J. Plast. Surg. **19**: 43–57.
9. Jones, R. J. 1970. Passive immunization against gram-negative bacilli in burns. Brit. J. Exp. Pathol. **51**:53–58.
10. Kubota, Y., and P. V. Liu. 1971. An enterotoxin of *Pseudomonas aeruginosa*. J. Infect. Dis. **123**:97–98.
11. Kusama, H., and J. P. Craig. 1970. Production of biologically active substances by two strains of *Vibrio cholerae*. Infect. Immunity **1**:80–87.
12. Liu, P. V. 1964. Factors that influence toxigenicity of *Pseudomonas aeruginosa*. J. Bacteriol. **88**:1421–1427.
13. Liu, P. V. 1966. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. III. Identity of the lethal toxins produced *in vitro* and *in vivo*. J. Infect. Dis. **116**:481–489.
14. Liu, P. V., and H. Hsieh. 1969. Inhibition of protease production of various bacteria by ammonium salts: its effect on toxin production and virulence. J. Bacteriol. **99**:406–413.
15. McRipley, R. J., and D. W. Garrison. 1964. Increased susceptibility of burned rats to *Pseudomonas aeruginosa*. Proc. Soc. Exp. Biol. Med. **115**:336–338.
16. McRipley, R. J., and D. W. Garrison. 1965. Effect of burns in rats on defense mechanisms against *Pseudomonas aeruginosa*. J. Infect. Dis. **115**:159–170.
17. Markley, K., and E. Smallman. 1968. Protection by vaccination against *Pseudomonas* infection after thermal injury. J. Bacteriol. **96**:867–874.
18. Millican, R. C., G. Evans, and K. Markley. 1966. Susceptibility of burned mice to *Pseudomonas aeruginosa* and protection by vaccination. Ann. Surg. **163**:603–610.
19. Oakley, C. L., and N. G. Banerjee. 1963. Bacterial elastases. J. Pathol. Bacteriol. **85**:489–506.
20. Pierce, N. F., W. B. Greenough III, and C. C. J. Carpenter, Jr. 1971. *Vibrio cholerae* enterotoxin and its mode of action. Bacteriol. Rev. **35**:1–13.
21. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Amer. J. Hyg. **27**:493–497.
22. Rosenthal, S. M. 1966. A procedure for measurement of wound healing, with special reference to burns. Proc. Soc. Exp. Biol. Med. **123**:347–349.
23. Sevitt, S. 1958. Early and delayed oedema and increase in capillary permeability after burns of the skin. J. Pathol. Bacteriol. **75**:27–37.
24. Spector, W. G., and D. A. Willoughby. 1959. Experimental suppression of the acute inflammatory changes of thermal injury. J. Pathol. Bacteriol. **78**:121–132.
25. Verder, E., and J. Evans. 1961. A proposed antigenic schema for the identification of strains of *Pseudomonas aeruginosa*. J. Infect. Dis. **109**:183–193.
26. Wells, F. R., and A. A. Miles. 1963. Site of the vascular response to thermal injury. Nature (London) **200**:1015–1016.
27. Wilhelm, D. L., and B. Mason. 1960. Vascular permeability changes in inflammation: the role of endogenous permeability factors in mild thermal injury. Brit. J. Exp. Pathol. **41**:487–506.